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EDITORIAL

RNA interference and antiviral therapy

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Abstract

RNA interference (RNAi) is an evolutionally conserved gene silencing mechanism present in a variety of eukaryotic species. RNAi uses short double-stranded RNA (dsRNA) to trigger degradation or translation repression of homologous RNA targets in a sequence-specific manner. This system can be induced effectively *in vitro* and *in vivo* by direct application of small interfering RNAs (siRNAs), or by expression of short hairpin RNA (shRNA) with non-viral and viral vectors. To date, RNAi has been extensively used as a novel and effective tool for functional genomic studies, and has displayed great potential in treating human diseases, including human genetic and acquired disorders such as cancer and viral infections. In the present review, we focus on the recent development in the use of RNAi in the prevention and treatment of viral infections. The mechanisms, strategies, hurdles and prospects of employing RNAi in the pharmaceutical industry are also discussed.

Key words: RNA interference; Short hairpin RNA; Micro RNA; Antiviral therapy; Viral infection; Human immunodeficiency virus; Hepatitis C virus; Hepatitis B virus; SARS-coronavirus

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INTRODUCTION

RNA interference (RNAi), a highly conserved gene silencing mechanism plays an important role in the regulation of gene expression. This system was examined in a broad variety of species including plants, fungi, yeasts, nematodes, flies and mammals. In fact, RNAi serves as a safeguard for the preservation of genomic integrity. It protects the host from viral infections and invasion by mobile genetic elements by degrading the exogenous genomic material (e.g., viral RNAs).

RNAi is triggered by small double-stranded RNA (dsRNA) and functions at all levels, including transcription[1], post-transcription[2] and translation[3]. The first reports on RNA-induced post-transcriptional gene silencing (PTGS) phenomena were published in the early 90s, when Napoli[4] and Van der Krol[5] described the co-suppression of both viral transgenes and their homologous endogenous genes in transgenic plants. Similar inactivation of gene expression called "Quelling" was observed in *Neurospora crassa* by transformation with homologous sequences[6]. In 1995, sense RNA was demonstrated to be as effective as antisense RNA in disrupting the expression of *par-1* in *Caenorhabditis elegans*[7]. The mechanism of action remained enigmatic until 1998, when Fire and Mello discovered that dsRNA, instead of the single-stranded sense or antisense RNA, mediated gene silencing by degrading endogenous mRNAs in a sequence-specific manner[8]. They also challenged a previous report published in 1995 claiming it to be an artificial effect of dsRNA contamination. Further studies have revealed that RNAi can occur at both the transcription and post-transcription levels. Transcriptional gene silencing involves histone H3 methylation and the formation of heterochromatin[9-11]. Post-transcriptional gene silencing includes small interfering RNA (siRNA) that mediates sequence-specific target RNA degradation, and micro RNA (miRNA) which promotes blockage of protein translation at the 3'-untranslated region (3'UTR)[12].

In recent years, RNAi has become a powerful tool to probe gene functions and to rationalize drug design. It has been employed as a prophylactic and therapeutic agent for combating a wide range of disorders, including infectious diseases, tumors and metabolic disorders. Several lethal viruses, including human immunodeficiency virus (HIV), the hepatitis C and B viruses (HCV & HBV), coronavirus, influenza A virus (IAV), human papillomavirus (HPV), have been shown to be inhibited or eliminated by RNAi. These findings have emphasized the potential of RNAi in clinical applications. In the present review, we discuss the mechanism of RNAi, and its role in the prevention and the treatment of viral infections.

Mechanisms of RNAi

Biochemical and genetic studies have revealed the detailed

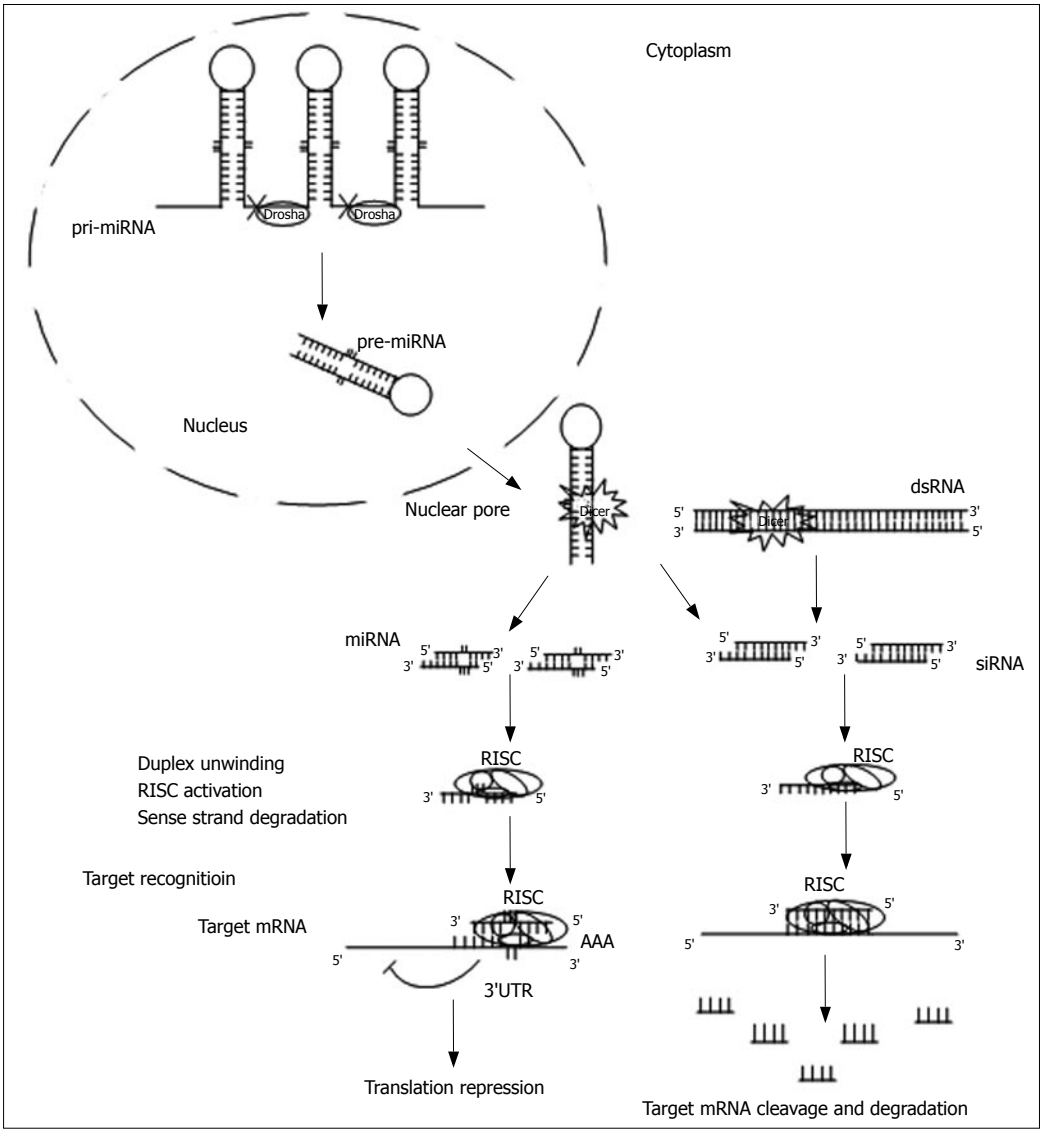

Figure 1 The RNA interference pathways.

mechanism by which dsRNA-mediated gene silencing takes place. In general, the mechanism includes two major steps: the initiator step and the effector step (Figure 1).

In the initiator step, long dsRNAs, which are produced by endogenous genes, invading viruses, transposons or experimental transgenes, are initially recognized by a dsRNA-binding protein, RDE-4/R2D2[13,14]. They are then submitted to and cleaved by the RNase Ⅲ-like nuclease Dicer[15], which generates 21-23 nucleotide duplex RNAs with overhanging 3' ends[16], called small interfering RNAs (siRNAs). The presence of highly conserved Dicer in yeast[11], plants[17,18], *C. elegans*[19], *Drosophila*[15], mice[20] and humans[21,22], suggests that the RNAi pathways share similar basic mechanisms in these organisms.

In the effector step, siRNAs are incorporated into a multicomponent nuclease complex, the RNA-induced silencing complex (RISC)[23]. The antisense strand of the duplex directs RISC to recognize and to cleave cognated target RNAs, which undergoes specific base pairing and endonucleolytic cleavage. This leads to the degradation of the unprotected and single-stranded target RNA. To date, several components of the RISC have been identified, including some conserved argonaute proteins that share the PAZ domain with Dicer family proteins[24].

The Dicer also cleaves the 60-70nt long precursor miRNA (pre-miRNAs) into miRNAs, which are of similar size as siRNAs. This pathway is referred to as miRNA-dependent gene silencing. The pre-miRNAs, whose structures are imperfectly complementary to each strand, are generated from endogenous stem loop precursors or hairpins, named primary-miRNA (pri-miRNA). The pri-miRNAs are first cleaved by Drosha RNase Ⅲ in the nucleus[25]. The resulting pre-miRNAs are then exported into the cytoplasm for further processing by Dicer. The complex of the activated RISC and miRNA binds the 3'UTR of specific mRNAs, which triggers cleavage by perfect base-pairing, or translational repression by partial base-pairing recognition[26-29].

Strategies for RNA interference

In order to study the functional genomics and biology of RNA interference, much effort has gone into the study of

artificial RNAi-inducing gene silencing. Strategies for delivery of RNAi reagents into mammalian cells can be divided into two types, the transient RNAi and the stable/inducible RNAi.

The methods commonly used in producing siRNA extraneously include chemical synthesis, *in vitro* transcription, and recombinant human Dicer/*E. coli* RNase Ⅲ digestion of long dsRNAs. These siRNAs can be transiently transfected into target cells. Alternatively, the short hairpin RNAs (shRNAs) are expressed endogenously from plasmids and viral vectors. The shRNA expression cassettes can be stably integrated into the genome of target cells, transcribed intranuclearly and processed into siRNAs by Dicer in the cytosol. In general, RNA Pol Ⅲ promoters (i.e., U6, H1 and tRNA promoters) are commonly used to drive shRNA expression in the RNAi studies. The viral vectors including retro-[30-33], lenti-[34-36], adeno- and adeno-associated viral vectors[37-39] have been demonstrated to feature high-efficiency gene delivery and can overcome the obstacles of cell-type-dependent transfection. The development of an inducible RNAi system has certainly enhanced our understanding of candidate genes' functions, as it provides an invaluable genetic switcher that allows the inducible and reversible control of specific gene's expression *in vitro*[40-42] and *in vivo*[43-45].

RNAi applications to combat viral infection

Viral infection is a serious public health, social and economic problem. More effective approaches are urgently needed to prevent viral propagation. Several studies have shown that RNAi technology has potential advantages over traditional measures such as the use of anti-viral drugs and vaccines, because of its ease of use, rapidity of action, high efficiency and specificity of activity when applied to the different stages of virus-host interactions[46]. In this section we will focus on the prospective use of RNAi in several common human pathogens such as HIV, HCV, HBV, SARS-coronavirus and influenza virus.

Human immunodeficiency virus

Human immunodeficiency virus type 1 (HIV-1) is the first primate virus shown to be inhibited by RNAi. HIV is a retrovirus that has been categorized into the subgroup of lentiviruses. Upon infection, the positive strand of the HIV's RNA genome is reversely transcripted into a linear dsDNA soon after the virus enters the host cells by receptor recognition and cell adhesion. The linear dsDNA becomes circularized, is then transported into the nucleus and integrated into the host chromosome as a provirus. By utilizing the host enzymes, HIV provirus converts viral genes into mRNAs, which are used as blueprints for the subsequent expression of viral structure proteins and enzymes. It has been suggested that the genomic RNA or the newly transcribed viral mRNAs are good targets for siRNAs intervention.

It is unclear whether RNAi can target RNA genome of HIV-1 infectious particles. Jacque reported siRNA-mediated inhibition of the early and late steps of HIV-1 replication, by targeting various regions of the HIV-1 genome and by preventing the formation of viral complementary-DNA intermediates[47]. Other workers have suggested that the incoming HIV-1 RNA genome may not be accessible to siRNAs[48,49]. To date, several viral target sequences have been identified. These include the structure proteins, Gag[48,50-52] and Env[52,53]; the reverse transcriptase Pol[48]; the regulatory proteins, Tat[54,55] and Rev[54,56], and the two accessory proteins Nef[47,57,58] and Vif[47] (Table 1). The long terminal repeats that the integrase employs to insert HIV's DNA genome into host DNA, have also been targeted[47,51].

Several studies have demonstrated that HIV may be able to escape RNAi target by mutations[58-60]. To overcome this problem, lentiviral vectors incorporated with different shRNA-expressing-cassettes, which can simultaneously target multiple sequences including conserved sequences of the HIV genome, have been constructed[61,62]. Another proposed strategy using RNAi application is the targeting of host genes. Some host genes are essential for viral replication but have a much slower mutation rate than the viral genes. These genes have been targeted by RNAi, and the results are very encouraging[63-69] (Table 1). Down-regulation of the cell surface CD4 receptor and/or one of the co-receptors CCR5 and CXCR4 by RNAi has led to dramatic reduction of viral entry into cells[34,70,71]. Compared with CD4 and CXCR4, CCR5 has been found to be a preferential target, since no immune defects or host mortality was observed on its deletion[72,73]. Therefore, careful selection of host immutable co-factors that are important for viral replication, but not for host survival, is of prime importance in the development of anti-HIV strategies. Furthermore, simultaneous targeting by RNAi of both the virus and host factors[50,74] has been shown to be more effective in inhibiting HIV-1 replication than the targeting of either virus or host factors alone.

Hepatitis C virus

Hepatitis C virus infection is a major cause of chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC). The estimated number of infected individuals are about 170 million worldwide[75], which accounts for nearly 3% of the world's population. The World Health Organization (WHO) has recognized HCV infection as a global health problem.

HCV is a small, enveloped RNA virus that belongs to the Flaviviridae family. The cytoplasmic replicating virus contains a 9.6 kb RNA genome that functions as the messenger RNA and replication template. The development of anti-HCV drugs has accelerated since the replicon-based culture system was established a few years ago[76,77]. Several regions of the HCV's RNA genome, including 5'UTR and the coding sequences of Core, NS3, NS4B and NS5B, are sensitive to the action of siRNA[78-83] (Table 2). The therapeutic potential of RNAi was further emphasized by *in vivo* studies[84,85]. The administration of siRNA and shRNA to target cell surface receptor FAS[86], caspase 8[87] and NS5B[84], has resulted in the destruction of cognate mRNAs and protection of mice from liver failure. The use of multiple siRNAs against highly conserved HCV sequences with and without host cell cofactors may limit the emergence of resistant viruses as has been demonstrated in several studies[88-92] (Table 2).

Table 1 Strategies designed to inhibit HIV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Cell type	Delivery methods	Inhib. of virus prod. (fold)	Reference
Viral Gene							
LTR, Vif, Nef	siRNA (21 bp)/ shRNA (19 bp)[1]	T7	Plasmid	Magi, PBLs	Transfection	> 20	[47]
Gag, Pol	siRNA (21 bp)	-	-	HOS.T4.CXCR4	Transfection	> 10	[48]
Gag, LTR	siRNA (23 bp)/ dsRNA (21nt)[2]	-	-	U87-CD4$^+$-CCR5$^+$/CXCR4$^+$, PBMC	Transfection	4	[51]
Gag, Env	dsRNA (441-531nt)[3]	-	-	COS, Hela-CD4$^+$, PBMC, ACH2	Transfection	70	[52]
Tat + Rev	siRNA (21 bp)	-	-	293T, Jurkat, PBMC	Transfection	> 15	[54]
Rev (Tat)	siRNA (21 bp)[5]	Dual U6	Plasmid	293/EcR	Transfection	10000	[56]
Nef	dsRNA (556nt)[3]	-	-	MT4-T, U937	Transfection	2.5	[57]
Env	siRNA (20 bp) /shRNA (20 bp)[4]	U6	Plasmid, Lentivirus	COS, MT-4	Transfection / Transduction	> 10	[53]
Nef	shRNA (21 bp)	H1	Retrovirus	SupT1	Transduction	> 10	[58]
Gag, Pol, Int, Vpu	shRNA (21 bp)	H1	Lentivirus	293T, Magi, GHOST hi5, CEM-A, Molt-4, PBMC	Transduction	> 20	[61]
Cellular gene							
Tsg101	siRNA (21 bp)	-	-	293T	Transfection	10-20	[63]
LEDGF/p75	siRNA (21 bp)	-	-	Hela	Transfection	NR	[64]
P-TEFb (CDK9/CyclinT1)	siRNA (21 bp)	-	-	Hela, Magi	Transfection	3-5	[65]
hRIP	siRNA (21 bp)	-	-	Hela, Jurkat, Macrophages	Transfection	-100	[66]
Emerin	siRNA (21 bp)	-	-	Hela, Macrophages	Transfection	> 10	[67]
LEDGF/p75, HRP2	siRNA (21 bp)	-	-	Hela-P4	Transfection	2-3	[68]
CXCR4	siRNA (21 bp)	-	-	HOS-CD4$^+$, HOS-CD4$^+$-CXCR4$^+$/CCR5$^+$	Transfection	3-5	[70]
Importin 7	siRNA (21 bp)	-	-	Hela, Macrophages	Transfection	-10	[69]
CXCR^{4+} CD4, CXCR^{4+} CCR5	shRNA (19/21 bp)[6]	-	-	Magi-CXCR4/CCR5, PBMC	Transfection	> 15	[71]
CCR5	shRNA (19 bp)	U6	Lentivirus	Magi-CCR5, PBLs	Transduction	3-7	[34]
Combination of viral and cellular genes							
Gag, CD4	siRNA (21 bp)	-	-	Magi-CCR5, Hela-CD4	Transfection	4-25	[50]
Tat, RT, NF-κB (p65)	siRNA (21 bp)	-	-	Magi, Jurkat	Transfection	5-500	[74]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA tested in a specific cell model. All siRNAs were prepared by chemical synthesis unless indicated otherwise. LTR: Long terminal repeat; PBLs: Peripheral blood lymphocytes; PBMC: Peripheral blood mononuclear cell; Pol: Polymerase; Env: Envelope; Tsg101: Tumor susceptibility gene 101; LEDGF/P75: Lens epithelium-derived growth factor/transcription co-activator p75; NR: Not reported; P-TEF: Positive transcription elongation factors; hRIP: Human Rev-interacting protein; HRP2: Hepatoma-derived growth factor related protein 2; RT: Reverse transcriptase; NF-κB: Nuclear factor-NF-κB. [1]shRNA expressed from a transfected plasmid under the control of a T7 promoter. [2]dsRNA produced by *in vitro* T7 promoter-mediated transcription. [3]dsRNA produced by *in vitro* SP6/T7 promoter-mediated transcription. [4]shRNA and siRNA expressed from transfected plasmids under the control of one and two U6 promoters respectively, shRNA further stably expressed from a recombinant lentiviral vector driven by a U6 promoter. [5]siRNA expressed from a transfected plasmid under the control of two U6 tandem promoters that drive the synthesis of each of the siRNA strand. [6]shRNA produced by *in vitro* T7 promoter-mediated transcription.

Hepatitis B virus

Hepatitis B virus infection is a major public health problem. It is estimated that, approximately 2 billion people are infected with HBV worldwide, and about 400 million are HBV chronic carriers[93]. HBV infection is highly prevalent in Asia and South Africa and results in over one million deaths worldwide annually.

Although the clinical symptoms caused by HBV and HCV infection are very similar, the viruses are completely unrelated[94]. HBV, the prototypical member of the Hepadnaviridae family, is one of the smallest DNA viruses (-3.2 kb), which can undergo reverse-transcription for viral replication. The HBV genome contains four overlapping open reading frames: P (polymerase-reverse transcriptase), C (core structure protein), S (surface glycoprotein) and X (HBx protein). After the uncoated nucleocapsids enter the nucleus, the HBV genome is repaired to form a covalently closed circular DNA (cccDNA), which is a template for messenger RNA transcription. The RNA intermediates-pregenomic and subgenomic RNAs, coding for viral multifunctional proteins, are transported into the cytoplasm where translation is initiated. After the pregenomic transcript is packaged into virion core particle, it is reversely transcribed by viral reverse transcriptase, thus producing a single stranded (-) DNA. Based on the structure of the (-) stranded DNA, a complementary (+) DNA strand is synthesized. Due to the lack of proofreading function of its polymerase, HBV undergoes rapid mutagenesis, with the creation of a large number of drug-resistant variants. These drug-resistant variants are further amplified under selective pressure during antiviral treatment, resulting in the elimination of the anti-viral effect and virus rebound during treatment. In severe cases, this can lead to death, even after cessation of treatment. Because of this challenge, new drugs with different targets or drug metabolism mechanisms are urgently required for better treatment outcome.

Several sites of the HBV genome including the *P, Pre*

Table 2 Strategies designed to inhibit HCV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
In vitro studies							
Viral gene							
5′-UTR	siRNA (21 bp)	-	-	5-2 cells (Huh-7)	Transfection	-6	[79]
	siRNA (21 bp)/shRNA(19 bp)[1]	U6	Plasmid	293T, Huh 7	Transfection	> 10	[78]
NS4B	siRNA (23 bp)	-	-	Huh-7.5	Transfection	-80	[80]
NS3, NS4B, NS5A, NS5B	siRNA (21 bp)	-	-	S1179I (Huh-7)	Transfection	-23	[81]
IRES, NS3, NS5B	siRNA (23bp)/shRNA (21 bp)[2]	Dual H1	Plasmid	Huh-7	Transfection	> 9	[82]
5′-UTR, C, NS4B, NS5A, NS5B	esiRNA (15-40 bp)[3]/shRNA (19bp)	H1	Mo-MuLV	Huh-7	Transfection / Transduction	-100	[88]
5′-UTR, C, NS3, NS5B	siRNA (21 bp)/shRNA (19 bp)[4]	U6	Plasmid/Lentivirus	Huh-7	Transfection / Transduction	-7	[83]
Cellular gene							
Lα, PTB, eIF2Bγ, hVAP33	shRNA (19 bp)	U6	Plasmid/Adenovirus	Huh-7	Transfection / Transduction	-13	[91]
Cyp-A,B,C	shRNA (NR)	U6	Plasmid/Retrovirus	Huh-7	Transfection / Transduction	-10	[92]
Combination of viral and cellular genes							
5′-UTR, 3′-UTR, PSMA7, HuR	shRNA (19-21 bp)	U6	Plasmid/Retrovirus	Huh-7	Transfection / Transduction	> 2	[89]
CD81, IRES, NS5B	shRNA (19-21 bp)	H1	Lentivirus	Huh-7	Transduction	> 32	[90]
In vivo studies							
NS5B	siRNA (23 bp)	-	-	Mice	Hydrodynamic transfection	3	[84]
IRES	shRNA (19-25 bp)[5]	-	-	Mice	Hydrodynamic transfection	-50	[85]

The fold inhibition of virus production represents the most potent effect caused by a specific siRNA or combinatorial siRNAs. All siRNAs were prepared by chemical synthesis unless indicated otherwise. UTR: Untranslated region; NS: Non-structural; IRES: Internal ribosomal entry site; C: Core protein; esiRNA: Endoribonuclease-prepared siRNA; Mo-MuLV: Moloney murine leukemia virus; PTB: Polypyrimidine tract-binding protein; eIF2Bγ: Subunit gamma of human eukaryotic initiation factors 2B; hVAP-33: Human VAMP-associated protein of 33 kDa; Cyp: Cyclophilin; PSMA7: Proteasome a-subunit 7; HuR: Hu antigen R; N.R: not reported. [1]stem-loop- and tandem-type siRNA expressed from DNA-based vectors driven by one and two U6 promoters respectively. [2]shRNA expressed from a transfected plasmid under the control of two H1 tandem promoters that drive the synthesis of each of the siRNA strand. [3]esiRNA generated by *in vitro* T3/T7 promoter-mediated transcription. [4]shRNA expressed from a transfected plasmid or a lentivirus vector respectively under the control of a U6 promoter. 5 shRNA generated by *in vitro* T7 promoter-mediated transcription.

C/C, PreS/S, X gene, have been employed as targets to examine the *in vitro* efficacy of RNAi[95-99] (Table 3). Some sites have also been tested in hydrodynamic HBV model and transgenic HBV model[100-104] (Table 3). Our group has successfully designed multiple shRNAs that target DR elements and regions that code for core, polymerase, PreS, S, and X proteins. These shRNA were found to potently inhibit HBV replication and showed synergistic antiviral effects with the commonly used antiviral drug, lamivudine[105]. In a recent study, we showed that simultaneous delivery of two shRNAs that target different regions, exhibited strong synergistic antiviral effects in a hydrodynamic transgenic mice model. In this study, both S and e antigens were reduced to undetectable levels, and the viral load was reduced by greater than one hundred-fold (He *et al* unpublished observations). These results clearly demonstrate the potential of RNAi application in anti-HBV therapy.

SARS-coronavirus

Severe acute respiratory syndrome (SARS) outbreak affected nearly 30 countries during the years 2002-2003. This epidemic was caused by a novel SARS-associated coronavirus (SARS-CoV)[106-108]. SARS-CoV is a large (-30 kb), enveloped, positive-stranded RNA virus and its genome is composed of replicase (*rep*), spike (*S*), envelope (*E*), membrane (*M*), and nucleocapsid (*N*) genes. The prophylactic and therapeutic efficacies of siRNAs were tested because of the absence of any effective drugs or vaccines against SARS-CoV infection. Both *in vitro* and *in vivo* applications proved satisfactory, using synthetic siRNAs as well as vector-based shRNAs against leader sequence[109,110], 3'-UTR[110], non-structural[111] and structural genes[110,112-115] of SARS-CoV (Table 4). Another recent report revealed that the siRNA-mediated depletion of the host cellular clathrin heavy chain gene, reduced the SARS-CoV infectivity[116]. Locked nucleic acid (LNA)-modified siRNAs, an RNA-like high affinity nucleotide analogue, has been found to improve the performance of gene silencing via enhancement of siRNA biostability and specialty. The improvement was clearly apparent when siRNA was transfected into Vero cells prior to a lethal SARS-CoV attack[117].

It is worth mentioning that our group was the first to demonstrate in 2003 the remarkable inhibition and replication of SARS-CoV infection by siRNAs against *rep* gene[118]. Subsequently, we designed siRNAs that could target both *rep* and structural genes. We also evaluated the antiviral effect, dose response, duration and viral kinetics of siRNAs in foetal rhesus kidney (FRhK-4) cells[119,120]. Two of the siRNAs were further evaluated for safety and antiviral efficacy in a rhesus macaque SARS model[119].

Table 3 Strategies designed to inhibit HBV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
In vitro studies							
C	siRNA (21 bp)	-	-	Huh-7, HepG2	Transfection	-4-5	[95]
	siRNA (19 bp)	-	-	HepAD38, HepAD79	Transfection	-50	[96]
C, X	shRNA (19 bp)	hH1	Plasmid	Huh-7, HepG2.2.15	Transfection	2-20	[97]
C, S, P, X, DR	shRNA (21-24 bp)	mU6	Plasmid	HepG2.2.15	Transfection	-2	[98]
	shRNA (21 bp)	hU6	Plasmid	HepG2	Transfection	> 30	[105]
S	shRNA (19 bp)	hH1	PFV, AAV	293T.HBs, HepG2.2.15	Transduction	4-9	[99]
In vivo studies							
C, S, P, X	shRNA (25 bp)	hU6	Plasmid	Immunocompetent C57BL/6J mice, Immunocompromised NOD/SCID mice	Hydrodynamic transfection[1]	3-12	[100]
C, S	siRNA (21 bp)	-	-	Male NMRI mice	High-volume injection *via* tail vein[1]	-4	[101]
S	shRNA (19 bp)	hH1, hU6	Plasmid	BALB/c mice, HBsAg-transgenic FVB/N mice	Hydrodynamic transfection[2]	-9	[102]
P, S, X	shRNA (20 bp)	hH1	Plasmid	C57BL/6 HBV-transgenic mice	Hydrodynamic transfection[1]	19-99	[103]
P, S, X	shRNA (NR)	mU6	Adenovirus	HBV-transgenic mice	Hydrodynamic transfection	> 9	[104]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA. All siRNAs were prepared by chemical synthesis unless indicated otherwise. C: Core antigen; S: Surface antigen; P: Polymerase; X: X protein; DR: Direct repeat element; PFV: Prototype foamy virus; AAV: Adeno-associated virus; mU6: Mouse U6; hU6: Human U6; hH1: Human H1. 1shRNA expression plasmid/naked siRNA coinjected with the pHBV construct. 2shRNA expression plasmid simultaneously or subsequently injected with the pHBV/pSAg construct in BALB/c mice.

Table 4 Strategies designed to inhibit SARS-CoV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
In vitro studies							
Viral gene							
Leader, TRS, 3'-UTR, S	siRNA (21 bp)	-	-	Vero E6	Transfection	9	[110]
N	shRNA (20 bp)	U6	Plasmid	293	Transfection	NR	[112]
E, M, N	siRNA (21 bp)	-	-	Vero E6	Transfection	> 4	[113]
P	shRNA (19 bp)	H1	Plasmid	Vero	Transfection	> 100	[114]
S	shRNA (22 bp)	U6	Plasmid	Vero E6, 293T	Transfection	-6	[115]
Rep	siRNA (21 bp)	-	-	FRhk-4	Transfection	> 12	[118]
Cellular gene							
CHC	siRNA (25 bp)	-	-	HepG2, COS7	Transfection	-1	[116]
In vivo studies							
S, NSP12	siRNA (21 bp)	-	-	BALB/C mouse, Rhesus macaque (*Macaca mulatta*)	*i.t.*[1] and *i.n.*[2] *administration*	3	[119]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA. All siRNAs were prepared by chemical synthesis unless indicated otherwise. TRS: Transcription-regulating sequence; UTR: Untranslated region; S: Spike protein; N: Nucleocapsid protein; NR: Not reported; E: Envelope protein; M: Membrane protein; P: RNA polymerase; Rep: Replicase; FRhk-4: Fatal Rhesus monkey kidney cells; CHC: Clathrin heavy chain; NSP: Non-structural protein; i.t.: Intratracheal; i.n.: Intranasal. [1]siRNA and target-sequence containing reporter plasmid co-administered intratracheally into mouse lungs in D5W or Infasurf solution; [2]siRNA instilled intranasally to monkey in D5W solution with different dosing regimens.

These siRNAs relieved SARS-like symptoms, and were safe for prophylaxis and therapeutic treatment. These findings greatly encouraged the clinical testing of siRNAs as an anti-SARS therapy.

Influenza virus

Influenza virus is one of the public health scourges worldwide. Three influenza epidemics have occurred in the last century and have caused tens of millions of deaths globally. Recent outbreaks of highly pathogenic avian influenza in Asia and Europe have greatly increased public awareness, and accelerated the development of measures for the prophylaxis and therapy of this infection.

Influenza viruses are enveloped, single-stranded, segmented (7-8) RNA viruses which belong to the Orthomyxoviridae family[121]. They are classified into

influenza virus types A, B, and C, based on their nucleoproteins and matrix proteins. Influenza A virus (IAV) is the most prevalent respiratory pathogen worldwide.

Since it is an RNA virus, IAV has the ability for rapid genetic changes through antigen drift[122] or antigen shift[123]. This involves the accumulation of minor mutations within the viral genome, or reassortment of RNA segments between different viruses, which results in the emergence of new viral strains. Ge *et al*[124,125] and Tompkins *et al*[126] verified the efficacy of siRNAs which specifically target the conserved regions of the influenza virus genome (nucleocapsid and acid polymerase). They confirmed that siRNAs were potent inhibitors of the influenza virus both *in vitro* and *in vivo*, and could be administered both prior to and subsequent to a lethal IAV challenge. Moreover, Ge developed an unconventional delivery system, administering small volumes of siRNAs or DNA vectors encoding shRNA in complex with polyethyleneimine (PEI) by slow intravenous infusion[127]. This system was effective in reducing virus production in infected mice and provided helpful suggestions for future clinical application of siRNAs.

Progress of RNAi for clinical application

Since RNAi was found to have antiviral activity in transgenic plants, much evidence has emerged with regard to its pivotal role in antiviral therapeutic applications. Numerous investigations have reported successful inhibition of viral replication in cultured cells and in murine/nonhuman primate models using both transient transfection of synthetic siRNA and stable expression of shRNA. To harness the full potential of RNAi for therapeutic applications, pharmaceutical companies are actively engaged in clinical trials. In 2004, Acuity Pharmaceuticals initiated a clinical trial using RNAi in the treatment of macular degeneration; encouraging results have been obtained in the Phase Ⅰ/Ⅱ studies[128]. In 2006, Alnylam Pharmaceuticals launched a Phase I clinical trial in the U.S. of an inhaled formulation of ALN-RSVO1 (an RNAi-based drug) to combat respiratory syncytial virus (RSV) infection[129]. Other potential indications for RNAi use include asthma, Huntington's disease, spinocerebellar ataxia, and HIV, HAV, HBV and influenza virus infections, and clinical trials are under consideration in many of these conditions[130].

Challenges and perspectives

Despite the rapid progress in RNAi use, its clinical application still poses several challenges. These include target specificity, biostability, biosafety, and delivery efficacy of the RNAi system in various diseases. Recent studies have indicated off-target effects associated with the use of siRNA[131-133]. In order to improve the power of gene silencing and to avoid undesirable adverse effects induced by siRNAs, such as nonspecific gene silencing and immunoactivation[134,135], great effort has been made to improve siRNA design, including its sequence[136], size[137] and structure[138]. However, the poor pharmacokinetic properties of siRNAs have added another hurdle in the development of RNAi-based therapies. Multiple chemical modifications at different positions of the siRNA duplexes, including sugars[117,139-141], backbones[142,143], and bases of oligonucleotides[144,145] have been found to prolong siRNA half-life in serum. Conjugation of one or both strands of siRNAs with lipids[146,147] and peptides[148,149], has been shown to enhance nuclease stability and improve cellular uptake.

The systematic and site-specific deliveries of siRNA also need to be addressed. Non-viral vectors, such as cationic lipids[150-152] and polymers[153-156], have been widely used for *in vitro* and *in vivo* siRNA delivery. It has been reported that siRNAs encapsulated into stable nucleic acid lipid particles (SNALPs) improve the potency, lengthen the half-life, lower the effective dose and reduce the dosing frequency. This was observed in a study comparing unformulated siRNAs in rodents challenged with replicating virus[157,158] and non-human primates[159]. Besides, Song *et al* designed a protamine-antibody fusion protein to deliver siRNA to HIV-infected or envelope-transfected cells. This study established a systemic, cell-type specific, antibody-mediated *in vivo* delivery system of siRNAs *via* cell surface receptors[160]. The current advances have brought siRNA close to the era of clinical trials and real-life therapeutic applications in infected human subjects.

However, before RNAi-based clinical trials can be carried out, the toxicity and side-effects of RNAi, and the harmful potential of viral vectors need careful attention. It has been shown that over-expression of shRNA by double-stranded AAV8 viral vectors resulted in severe hepatic toxicity and even death. Moreover, it has been observed that over-expressed shRNA can saturate the miRNA pathway[161]. Our studies have shown that simultaneous delivery of two shRNAs using a weaker expressing viral vector (AAV2) did not produce any obvious liver toxicity or side-effects (He *et al* unpublished). Therefore, it is essential to use safer vectors and in this respect we believe that inducible viral vectors may be good candidates for future clinical studies.

Scientists in different fields, including geneticists, biochemists, pharmacologists, chemists and materials scientists, have supported the use of RNAi in clinical applications. As a part of the research force, our team while being cautious, is optimistic regarding the use of RNAi in human diseases.

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LIVER CANCER

Hepatic steatosis as a possible risk factor for the development of hepatocellular carcinoma after eradication of hepatitis C virus with antiviral therapy in patients with chronic hepatitis C

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Abstract

AIM: To elucidate risk factors contributing to the development of hepatocellular carcinoma (HCC) among patients with sustained viral response (SVR) after interferon (IFN) treatment and to examine whether HCV-RNA still remained in the liver of SVR patients who developed HCC.

METHODS: Two-hundred and sixty-six patients, who achieved SVR, were enrolled in this study. We retrospectively reviewed clinical, viral and histological features of the patients, and examined whether the development of HCC depends on several clinical variables using Kaplan-Meier Method. RT-PCR was used to seek HCV-RNA in 3 out of 7 patients in whom liver tissue was available for molecular analysis.

RESULTS: Among the enrolled 266 patients with SVR, HCC developed in 7 patients (7/266; 2.6%). We failed to detect HCV-RNA both in cancer and non-cancerous liver tissue in all three patients. The cumulative incidence for HCC was significantly different depending on hepatic fibrosis (F3-4) (P = 0.0028), hepatic steatosis (Grade 2-3) (P = 0.0002) and age ($\geqslant$ 55) (P = 0.021) at the pre-interferon treatment.

CONCLUSION: The current study demonstrated that age, hepatic fibrosis, and hepatic steatosis at pre-interferon treatment might be risk factors for developing HCC after SVR.

INTRODUCTION

Continuous eradication of hepatitis C virus (HCV) with interferon (IFN) therapy significantly inhibits development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C[1-3]. However, HCC sporadically developed even after achievement of sustained viral response (SVR) with IFN therapy, especially reported from Japan[4-14], and therefore it is of clinical importance to identify patients who are at high risk for HCC after achievement of SVR.

Several investigators have made efforts to elucidate risk factors for occurrence of HCC in patients with SVR[6,15-18], and so far several factors including age[6,15-18], sex[6,15], alcohol consumption[16,17], staging of fibrosis[15-17], platelet counts[18], and AST levels[18] at baseline were regarded as candidate risks. However, these results seem to be controversial, and there may be other crucial contributing factors. In addition, reappearance of HCV in serum after achievement of SVR has also been reported[19-22]. In some cases, HCV-RNA is still present in liver of patients without detectable HCV-RNA in serum[19-22]. Therefore, the remaining HCV-RNA in the liver may contribute to the occurrence of HCC in patients with SVR[23]. Nevertheless, the studies[6,15-18] describing risk factors for the occurrence of HCC lack any evaluation for the presence of HCV-RNA in the liver.

Furthermore, chronic HCV infection frequently results in hepatic steatosis[24]. It has been reported that HCV genotype 3 infection is likely to cause steatosis possibly due to direct effect of HCV, whereas steatosis in patients infected with HCV genotype 1 seems to be associated

with co-existing risk factors including an increased body mass index (BMI) or presence of insulin resistance, thus resembling to those non-alcoholic steatohepatitis (NASH)[25-29]. Apparently hepatic steatosis is not altered in patients infected with HCV genotype 1, even after continuous eradication of HCV with antiviral therapy[30]. In addition, obesity-related cryptogenic cirrhosis has been recently paid attention as an alternative etiology for HCC[31,32]. Taken together, it may be possible that hepatic steatosis, still present after achievement of eradication of HCV genotype 1, results in progression of fibrosis as well as occurrence of HCC. The investigators describing risk factors for HCC in patients with SVR[6,15-18] have not mentioned whether hepatic steatosis is observed in pre-treatment liver histology.

Therefore, in the current study, we aimed to elucidate contributing factors for occurrence of HCC in patients in whom serum HCV-RNA has been eradicated with IFN therapy. First, we examined whether HCV-RNA still remained in the liver at the development of HCC using sensitive reverse-transcriptase polymerase chain reaction (RT-PCR). In addition, we evaluated clinical features and histopathological fibrosis and steatosis of the patients at the pre-IFN treatment, and examined whether the occurrence of HCC depends on these factors using Kaplan-Meier model.

MATERIALS AND METHODS

Patients

This single-center study was conducted at the Department of Medicine, Teikyo University School of Medicine. Since the introduction of IFN therapy for patients with chronic hepatitis C in 1986, 1101 patients were treated with IFN alone or IFN and ribavirin combination therapy. Among them, 266 patients, who achieved SVR, defined as absence of serum HCV-RNA at 6 mo after termination of IFN therapy, and did not fulfill the exclusion criteria described below, were enrolled. We verified that anti-HCV antibody as well as HCV-RNA was detected in sera before IFN treatment in all enrolled patients. Serum anti-HCV antibody was examined using third-generation antibody to HCV (Abbott Japan, Tokyo, Japan). Serum HCV-RNA was sought with Amplicor HCV v2.0 (Nippon Roche, Tokyo, Japan), employing RT-PCR. Also, liver biopsy was performed in all cases as long as 3 mo before beginning of IFN therapy for assuring the presence of chronic hepatitis. Exclusion criteria for this study were hepatitis B virus (HBV) infection determined by seropositivity for HBsAg and/or HBcAb, autoimmune hepatitis, alcoholic abuse (daily alcohol consumption > 60 g), and presence of HCC detected by abdominal ultrasound (US) and/or computed tomography (CT).

Follow-up and diagnosis of hepatocellular carcinoma

After termination of IFN therapy, patients were regularly checked up at the out-patient clinic at every 2-3 mo. The average period of follow-up for the enrolled patients in this study was 9.9 ± 4.1 years. Blood chemistries as well as tumor markers were examined. Additionally, imaging studies, abdominal US and/or CT, were performed 1-2 times per year. The diagnosis of HCC was made if both abdominal US and CT demonstrated the presence of HCC, and was further confirmed by histopathological studies after surgical resection. In cases without resection, abdominal angiography was used to verify the diagnosis of HCC.

Variables

We selected several pre-IFN treatment valuables of patients to assess risk factors for development of HCC, including the age at pre-IFN treatment and sex of the patient, the stage of liver fibrosis and the grade of hepatic steatosis, HCV genotype and serum ALT level. The stage of liver fibrosis was determined by two independent pathologists according to the classification of the Metavir group[33] as follows: F0 (no fibrosis); F1 (portal fibrosis); F2 (few bridges); F3 (many bridges); and F4 (cirrhosis). Hepatic steatosis was also graded by two independent pathologists according to Brunt *et al*[34]. In short, steatosis observed in up to 33%, 33%-66% and more than 66% of the liver histology was determined as grade 1, 2 and 3, respectively. Hepatic steatosis, if not observed, was graded as grade 0. When two pathologists estimated the classification of fibrosis and the grading of steatosis differently, the mean value of the two was applied for statistical analysis. Genotyping of HCV-RNA was performed with HCV coregenotyping (Nippon Roche).

Detection of HCV-RNA in liver tissue

HCV-RNA in liver tissue was detected by RT-PCR. Formalin-embedded liver biopsy specimens obtained prior to IFN treatment and snap-frozen liver tissue taken at the development of HCC were used as starting materials. Total RNA was extracted using Isogen (Nippon gene, Tokyo, Japan) and was converted into cDNA using Superscript™ Ⅱ RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA). The primer specific to HCV-RNA 5' UTR region (HC1R: ACTCGCAAGCACCCTATCA, nt 293-312) was used for cDNA synthesis. Thereafter HCV-RNA was sought using "semi-nested" PCR consisting of 1st, 2nd and 3rd amplification, using one 5' forward primer (HC1F: GAGCCATAGTGGTCTGCGGA, nt 135-154) and three sets of reverse primers (HC1R, HC2R: ACT CGGCTAGCAGTCTTGCG, nt 240-259; and 2H-AS: GTTTATCCAAGAAAGGACCC, nt 188-207). The 1st PCR consisted of initial denaturing at 95℃ for 5 min, followed by 50 amplification cycles at 95℃ for 15 s for denaturing and at 62℃ for 1 min for primer annealing and extension, and a final extension at 72℃ for 7 min. The 2nd PCR was done for 35 cycles by the identical program, but annealing and extension at 63℃. The 3rd PCR consisted of initial denaturing at 95℃ for 5 min, followed by 30 amplification cycles at 95℃ for 15 s, at 58℃ for 30 s for primer annealing and at 72℃ for 1 min for extension, and a final extension at 72℃ for 7 min. The PCR products of 178, 125 and 73 bp after 1st, 2nd and 3rd PCR, respectively, were electrophoresed on 15 g/L agarose gel, and visualized by ethidium bromide staining. In addition, to ensure the quality of extracted RNA, we also amplified β-actin as positive controls using the identical liver-derived total

Table 1 Viral, clinical and histopathological profiles of patients with SVR who developed HCC

	Age/sex	HCV genotype	Pre-IFN treatment				Occurrence of HCC			Duration until HCC (yr)
			ALT (IU/L)	Fibrosis	Steatosis	BMI	Fibrosis	Steatosis	BMI	
Case 1	67/M	1b	138	F3	Grade 2	28.4	F2	Grade 1	27.4	5.4
Case 2	58/M	2a	39	F1	Grade 1	23.5	F2	Grade 0	22.5	9.5
Case 3	68/M	n.t.	111	F4	Grade 2	24.2	F2	Grade 2	25.0	9.6
Case 4	53/M	1b	82	F2	Grade 1	19.6	NT	NT	20.3	8.4
Case 5	58/M	1b	21	F3	Grade 1	23.5	NT	NT	24.8	3.3
Case 6	65/F	2a	145	F3	Grade 1	25.6	F2	Grade 1	26.7	2.9
Case 7	54/M	1b	85	F2	Grade 2	26.1	F2	Grade 1	22.4	4.9

NT: Not tested; BMI: Body mass index. In cases 4 and 5, the liver tissues with the occurrence of HCC were not obtained because HCC was not treated with surgical operation.

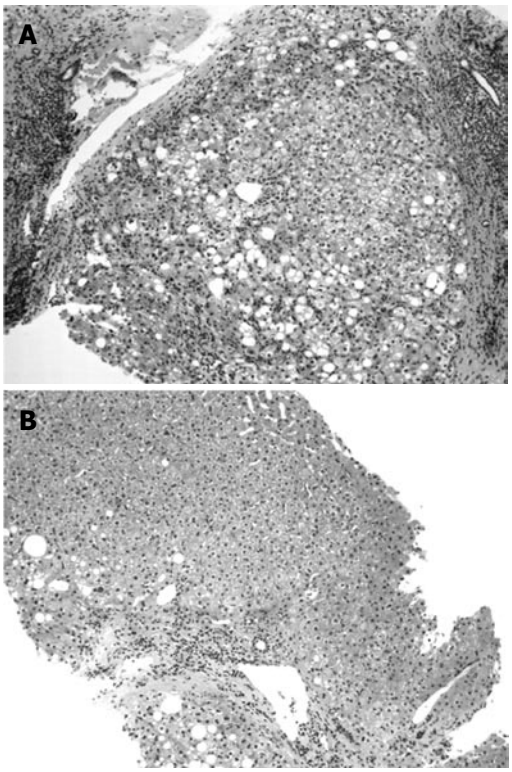

Figure 1 Liver histology of case 1 (HE staining). **A**: Pre-IFN treatment liver histology showing F3 fibrosis grade 2 steatosis; **B**: Liver histology at the occurrence of HCC showing F2 fibrosis and grade 1 steatosis.

RNA. Random primer was used for synthesis of cDNA as template for amplification of β-actin.

Statistical analysis

Statistical analysis was performed using SAS software version 9.1 (SAS Institute, Cary, NC). The kappa value was calculated to evaluate the degree of agreement in two pathologists for histological evaluation. For determining risk factors contributing the occurrence of HCC, continuous variables such as age and pre-treatment ALT value were classified into two categories: age $\geqslant$ 55 and age $<$ 55; ALT $>$ 80 IU/L and ALT $\leqslant$ 80 IU/L. Cumulative incidence for development of HCC was calculated using Kaplan-Meier method, and the differences between groups were analyzed using the log-rank test. *P* value less than 0.05. was considered statistically significant.

RESULTS

Patients with SVR who developed HCC

Of 266 patients, HCC was detected in 7 patients (6 males and 1 female) during follow-up for the patients with SVR after termination of IFN therapy. Clinical and histopathological profiles of these patients are mentioned in Table 1. The age and mean ALT value at the beginning of IFN therapy was 60.4 ± 6.2 (range, 53-68) years, and 89 ± 47 (range, 21-145) IU, respectively. The duration between IFN therapy and development of HCC was 6.3 ± 2.9 (range, 2.9-9.6) years. Undetectable serum HCV-RNA and normal ALT had been continuously maintained since IFN therapy in all patients. All patients denied excess consumption of alcoholic beverage (> 20 g/d), and were seronegative for HBsAg as well as HBcAb. The staging of liver fibrosis on pre-IFN liver biopsy was F1 in one patient, F2 in two, F3 in three and F4 in one. Hepatic steatosis was observed in all patients, grade 1 in four patients and grade 2 in three.

The comparison of liver histological findings at baseline and at the development of HCC is shown in Table 1. Overall, the grading of fibrosis was improved in cases 1, 3 and 6, unchanged in case 7, and worsened in case 2. Steatosis was decreased in cases 1, 2 and 7, and unchanged in cases 3 and 6. In cases 4 and 5, we failed to obtain liver tissue at the development of HCC since these patients were treated by transcatheter arterial embolization, instead of surgical resection of the tumor. Liver histology of case 1 before IFN therapy and at the occurrence of HCC as a representative example is shown in Figure 1.

Failure to detect HCV-RNA in HCC

In 3 patients (cases 1, 2 and 4), we tried to detect HCV-RNA in liver tissue before IFN treatment as well as at the development of HCC to elucidate whether HCV-RNA, which could remain in the liver tissue even long after successful IFN treatment[22], might be involved in hepatocellular carcinogenesis. The tumors were surgically resected in cases 1 and 2, and the liver specimens were obtained from both the cancerous as well as non-cancerous lesion and snap-frozen at -80℃ until use. In case 4, as described above, the HCC was treated by transcatheter arterial embolization, and thus the liver specimens at occurrence of HCC were not available. Instead, biopsied specimen from the liver obtained after

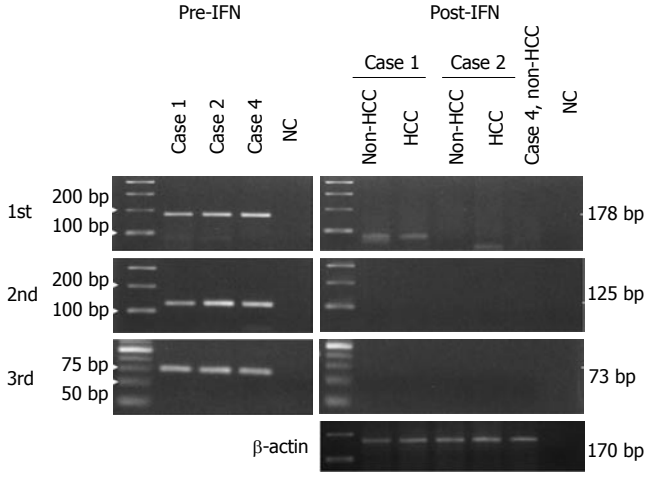

Figure 2 Amplification of HCV-RNA in liver tissues in cases 1, 2 and 4. HCV was detected in all liver tissues at the pre-IFN therapy. NC: Without template cDNA, gave no distinct band. By contrast, HCV was demonstrated in neither HCC nor non-HCC tissue in cases 1 and 2 even after 3 cycles of RT-PCR. In case 4, in whom liver tissue was obtained after IFN therapy with percutaneous biopsy, HCV was not found either. β-actin was well amplified in these post-IFN specimens, demonstrating that total RNA had not been degraded. NC: Negative control.

IFN therapy was used. HCV-RNA was successfully amplified in all specimens before IFN treatment. However, we failed to detect HCV-RNA in any sample, irrespective of cancerous and non-cancerous tissue (Figure 2). β-actin was successfully amplified in all liver tissue after IFN therapy and at HCC development (Figure 2). Therefore, we concluded that failure to detect HCV-RNA in liver tissue at development of HCC was not due to degradation of RNA, but resulted from disappearance of HCV-RNA in the liver with successful IFN treatment.

Cumulative incidence of HCC during follow-up

We demonstrated viral, clinical and histopathological profiles in all enrolled patients, the patients with and without HCC (Table 2). The cumulative incidence of HCC was analyzed for 4 variables which had been reported as risk factors before (Figure 3A-D), and pre-treatment hepatic steatosis (Figure 3E). As for the histopathological evaluation, there was an excellent agreement in the histopathological evaluation by two independent pathologists, since the kappa value was 0.70 in fibrosis and 0.78 in steatosis. The age of patients with/without HCC at baseline was 60.4 ± 6.2 and 44.8 ± 13.3 (Table 2), respectively and the patients with age ⩾ 55 were at significantly higher risk for the development of HCC (P = 0.021, Figure 3A). The staging of pre-treatment hepatic fibrosis was available in 238 of 266 patients. The number of patients with F1, F2, F3 and F4 was 1, 2, 3, 1 in patients with HCC and 102, 97, 25, 7 in patients without HCC, respectively (Table 2). Log-rank test analysis revealed that advanced hepatic fibrosis (F3-4) was a risk factor for HCC as well (P = 0.0028, Figure 3C). Pre-IFN hepatic steatosis was evaluated in 231 of 266 patients, and the number of patients in grade 0, 1, 2 and 3 was 116, 95, 19 and 1, respectively. It is notable that no patient in grade 0 (no steatosis) at the IFN pre-treatment developed HCC, whereas 3 of 19 patients with grade 2 steatosis developed HCC in the follow-up (4.9, 5.4 and 9.6 years after IFN therapy, respectively) (Table 2). Indeed, log-rank test analysis demonstrated that there was a significant difference in the grade of steatosis for cumulative incidence of HCC (P = 0.0002, Figure 3E). By contrast, we failed to demonstrate that sex and pre-treatment ALT value were risk factors for HCC (P = 0.389 and 0.251, respectively, Figure 3C and D).

DISCUSSION

Among 266 cases, who achieved SVR with IFN therapy, 7 patients developed HCC in the follow-up period. Statistical analysis using log-rank test demonstrated that the cumulative incidence of development of HCC after SVR was significantly different depending on age and hepatic fibrosis, as previously suggested, and also on hepatic steatosis in these patients.

In the previous investigations, age[6,15-18], sex[6,15], alcohol consumption[16,17], staging of fibrosis[15-17], platelet counts[18], and AST levels[18] at baseline were regarded as risk factors for occurrence of HCC in patients with SVR. In the current study, we selected age, sex, ALT and staging of fibrosis as candidate contributing variables. Excess consumption of alcohol beverage was not noted in any patient with HCC and therefore was very unlikely to be a risk factor in the enrolled population. Since it is assumed that platelet counts are equivalent to staging of fibrosis and that serum ALT levels are more specific to hepatocellular injury, we adapted fibrosis and ALT values, instead of platelet counts and AST levels.

The exact determination of stage of hepatic fibrosis as well as the grade of hepatic steatosis at pre-IFN treatment is a vital premise in the current study. In this regard, grading of fibrosis and grading of steatosis were performed by two independent pathologists. The kappa values were 0.70 in fibrosis and 0.78 in steatosis, and there was a good agreement in the decision of the two. The results appeared comparable to previous reports[18]. For instance, the proportion of the patients graded in F1, F2, F3 and F4 was 43%, 42%, 12% and 3% in the current study, and 41%, 30%, 17% and 10% in the report by Ikeda *et al*[18] who included 1056 patients with SVR. In addition, hepatic steatosis in our study was observed in 115 of 231 (50%) patients evaluated, and it is generally accepted that steatosis is present in 40%-60% of the patients with chronic hepatitis C[24]. Therefore, it is conceivable to conclude that hepatic fibrosis as well as steatosis were adequately assessed in this study.

In the current study, the incidence of the patients who developed HCC after SVR was only 2.6% (7/266). Therefore, in the current study, we performed statistical analysis with only Kaplan-Meier method, and thus the effect of confounding factors among each valuable could not be excluded. In this regard, we should be reluctant to conclude that age, hepatic fibrosis and hepatic steatosis, demonstrated in this study as statistically significant, would be the only determinants of risk for developing HCC. Nevertheless, hepatic steatosis, which has never been investigated as a risk factor for developing HCC after SVR, is identified as a statistically significant factor, and

Table 2 Pre-IFN viral, clinical and histopathological profiles of all patients with SVR

	Age (mean ± SD)	Sex (M/F)	ALT (IU) (mean ± SD)	Genotype (1/2/NT)	Hepatic fibrosis (F1/F2/F3/F4/NT)	Hepatic steatosis (Grade 0/1/2/3/NT)
All (n = 266)	46.3 ± 14.3	184/82	112 ± 99	102/142/20	103/99/28/8/28	116/95/19/1/35
HCC (n = 7)	60.4 ± 6.2	6/1	89 ± 47	4/2/1	1/2/3/1/0	0/4/3/0/0
No HCC (n = 259)	44.8 ± 13.3	178/81	113 ± 100	98/140/19	102/97/25/7/28	116/91/16/1/35

NT: Not tested.

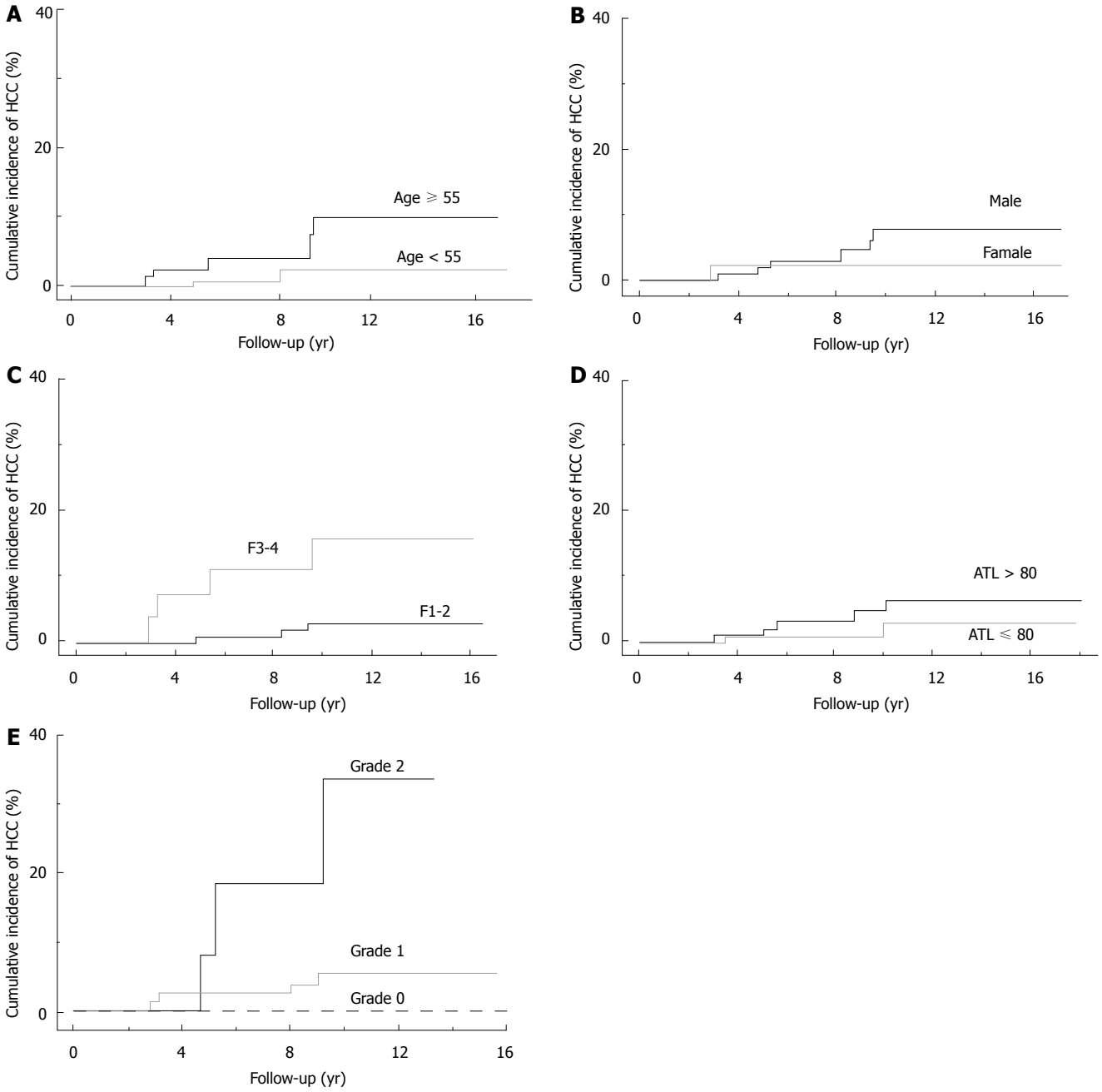

Figure 3 Cumulative incidence of HCC in SVR patients using the log-rank test. **A**: Patients with age ⩾ 55 (black line) and < 55 (gray line) years (P = 0.021); **B**: Male (black line) and female (gray line) patients (P = 0.389); **C**: patients with grading of fibrosis, F3-4 (black line) and F1-2 (gray line) (P = 0.0028); **D**: Patients with pre-treatment ALT levels, ALT > 80 IU (black line) and ⩽ 80 IU (gray line) (P = 0.251); **E**: Patients with the grading of hepatic steatosis, grade 2 (black line), grade 1 (gray line), and grade 0 (gray dotted line) (P = 0.0002 between grade 2 and grade 1 or grade 0).

we believe that it is worth evaluating its potential role for developing HCC after eradication of HCV.

Hepatic steatosis is known to be decreased after eradication of HCV in HCV genotype 3 infection. However, HCV genotype 3 is very rare in Japan, and indeed we found no patient infected with genotype 3 among the 266 subjects enrolled in the current study. By contrast, it has been reported that hepatic steatosis with HCV genotype 1

infection is not altered after successful antiviral treatment[30]. In the current study, among 6 patients who developed HCC after SVR and in whom HCV genotype was examined, 4 and 2 were infected with genotype 1 and 2, respectively (Table 1). Disappearance of hepatic steatosis (grade 0) after successful IFN therapy was noted only in 1 patient (case 2), infected with HCV genotype 2a; steatosis still remained in 4 of 5 patients whose histologies on the occurrence of HCC were investigated (Table 1). Thus, hepatic steatosis at baseline continuously remained after eradication of HCV, and might play a role in development of HCC, along with other factors, such as age and fibrosis in the liver.

It has been repeatedly reported that NASH caused by hepatic steatosis eventually could result in development of HCC[31,32,35-39], and indeed NASH is regarded as an alternative etiology of HCC worldwide[36,40,41]. It is believed that cryptogenic cirrhosis, occasionally producing HCC, may be a late complication of NASH, even though steatosis is not observed in the end-stage cirrhotic liver[31,32]. In the current study, however, liver histology on the onset of HCC was not coincident with cryptogenic cirrhosis. Rather, the staging of fibrosis was F2 in 5 of 7 patients who developed HCC, relatively at early stage. Moreover, although hepatic steatosis was noted at the pre-IFN treatment, histological hallmarks of NASH, such as perisinusoidal/pericellular fibrosis or infiltration of polymorphonuclear cells[42,43], were not observed in our patients. Therefore, hepatic steatosis in these patients should be regarded as non-alcoholic fatty liver diseases (NAFLD), instead of NASH. Taken together, the role of hepatic steatosis in the hepatocarcinogenesis after SVR seems to be different from those in NASH liver. Rather, the results in the current study suggest the possibility that steatosis and HCV infection, even if eradicated, would cooperatively operate for development of HCC.

We should be cautious, however, in concluding that hepatic steatosis would be a risk factor for developing HCC after SVR. First of all, multivariate analysis was not performed in this study and therefore the effect of confounding factors could not be excluded, as described above. Second, it is still controversial whether hepatic steatosis facilitates hepatocarcinogenesis in untreated patients with infection of HCV[44,45], and prospective well-controlled studies are required to conclude the association between hepatic steatosis and development of HCC. Third, it is also notable that HCC developed within 10 years after successful eradication of HCV in the presented cases, even though almost half of the enrolled 266 patients with SVR had been followed up more than 10 years. Since hepatic steatosis would remain in a similar manner after eradication of HCV, HCC might develop in patients after more than 10 years as well. Finally, very low amount of HCV, even undetectable by RT-PCR, had still remained in the liver after SVR and might play a crucial role in hepatocarcinogenesis. Radkowski *et al*[22] recently demonstrated that HCV-RNA was detected in 3 of 11 patients with SVR after treatment, even though previous reports describing the occurrence of HCC in patients with SVR have repeatedly shown the absence of HCV-RNA in the tumor and non-tumor liver tissue[8,9,12].

In conclusion, the current study demonstrates that development of HCC in patients after SVR depends on age, hepatic fibrosis, and hepatic steatosis at pre-IFN treatment. As discussed, the remote effect of HCV infection could be more important for hepatocarcinogenesis, and a large-scale, multi-center cooperative study is required to conclude whether hepatic steatosis at baseline is a contributing factor for development of HCC. Nevertheless, it should be kept in mind that patients with hepatic steatosis at pre-IFN treatment may be at a high risk for developing HCC, and therefore should be closely monitored after SVR.

COMMENTS

Background

Continuous eradication of hepatitis C virus (HCV), i.e., sustained viral response (SVR) with interferon (IFN) therapy greatly reduces the risk of developing hepatocellular carcinoma (HCC). However, HCC was sporadically found even in patients with SVR, and it is of clinical importance to elucidate which patients with SVR are at higher risk for HCC.

Research frontiers

In the current study, we retrospectively examined 266 patients with SVR and detected 7 patients among them (2.6%) who developed HCC after SVR. RT-PCR failed to detect HCV-RNA in the liver. The cumulative incidence for HCC was significantly different depending on hepatic fibrosis (P = 0.0028), hepatic steatosis (P = 0.0002) and higher age (P = 0.021) at the pre-interferon treatment.

Innovations and breakthroughs

Although age and fibrosis were previously reported as risk factors, hepatic steatosis was firstly demonstrated as a possible risk in the current study.

Applications

Further large-scale study is warranted to confirm the contribution of hepatic steatosis for developing HCC after SVR. For the moment, patients with high hepatic steatosis should be closely monitored for HCC, even after SVR with successful antiviral treatment.

Peer review

This is a very interesting study. There are few papers with such histological and follow up studies in SVR and the article is well written.

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VIRAL HEPATITIS

Low-dose intermittent interferon-alpha therapy for HCV-related liver cirrhosis after curative treatment of hepatocellular carcinoma

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Abstract

AIM: To assess the efficacy of low-dose intermittent interferon (IFN) therapy in patients with hepatitis C virus (HCV)-related compensated cirrhosis who had received curative treatment for primary hepatocellular carcinoma (HCC).

METHODS: We performed a prospective case controlled study. Sixteen patients received 3 MIU of natural IFN-alpha intramuscularly 3 times weekly for at least 48 wk (IFN group). They were compared with 16 matched historical controls (non-IFN group).

RESULTS: The cumulative rate of first recurrence of HCC was not significantly different between the IFN group and the non-IFN group (0% *vs* 6.7% and 68.6% *vs* 80% at 1- and 3-year, $P = 0.157$, respectively). The cumulative rate of second recurrence was not also significantly different between the IFN group and the non-IFN group (0% *vs* 6.7% and 35.9% *vs* 67% at 1- and 3-year, $P = 0.056$, respectively). Although the difference in the Child-Pugh classification score between the groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group (7.19 ± 1.42 *vs* 5.81 ± 0.75, $P = 0.0008$). The cumulative rate of deviation from objects of any treatment for recurrent HCC was also higher in the non-IFN group than IFN group (6.7% and 27% *vs* 0 and 0% at 1- and 3-year, $P = 0.048$, respectively).

CONCLUSION: Low-dose intermittent IFN-alpha therapy for patients with HCV-related compensated cirrhosis after curative HCC treatment was effective by making patients tolerant to medical or surgical treatment for recurrent HCC in the later period of observation.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Interferon therapy; Liver cirrhosis; Liver function; Recurrence; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide. Approximately 80% of Japanese patients with HCC have a history of hepatitis C virus (HCV) infection, and most such patients have liver cirrhosis[1-3]. Although recent advances in imaging techniques and treatment of HCC have improved prognosis of patients with HCV-related HCC, the outcome is still unsatisfactory; the 5-year survival rate is only 50% to 70% even after curative treatment such as hepatic resection and local ablation[4]. The reasons for this unfavorable prognosis is considered to include high intrahepatic tumor recurrence rates and biochemical deterioration by sustained hepatic damage, both resulting from persistent HCV infection[5]. Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumor recurrence within 1 year is 20% to 40%, rising to about

80% by 5 years[4,6-8]. Intrahepatic recurrences of HCC may result from intrahepatic metastasis originating from the primary HCC or from ongoing multicentric carcinogenesis related to chronic HCV infection. In addition, sustained underlying HCV-related hepatic damage may compromise hepatic functional reserve, worsening clinical outcome. Thus, prevention of HCC recurrence and preservation of liver function are both highly important priorities in improving prognosis of patients with HCV-related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective as evident by reduction of serum alanine transaminase (ALT) activity and eradication of HCV. Accordingly, IFN is valuable in minimizing hepatic necrosis, inflammation, and fibrosis, as well as reducing the likelihood of hepatocarcinogenesis[9-16]. The primary goal of treatment of patients with HCV infection is elimination of the virus. Several studies have reported recently that IFN therapy provided after curative treatment for HCV-related HCC prevents HCC recurrences and improves survival[17-23]. Such improvement of prognosis is more predominant when IFN therapy results in elimination of HCV RNA[24]. However, most patients with HCV-related HCC also have liver cirrhosis. Many centers do not advocate IFN therapy of patients with compensated cirrhosis, mainly because of the disappointing sustained virological response (SVR) rates in such patients[25]. Several studies indicated that the response of cirrhotic patients to antiviral therapy is low[26-28]. The reasons for the low SVR rate in such patients include inability to administer IFN at recommended doses due to adverse effects and dose-limiting cytopenia. On the other hand, several investigators suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients[29,30]. Furthermore, they indicated that the same therapy could improve the underlying liver histology. There is evidence to suggest that low-dose IFN therapy might be beneficial in HCV-related cirrhosis, not only because it prevents the progression of liver disease, but also because it reduces the risk of hepatocarcinogenesis[31,32]. In this regard, low-dose IFN therapy seems to be tolerable without significant life-threatening adverse effects than the standard dose of IFN.

However, it is not known whether low-dose IFN after curative treatment of primary HCC could slow disease progression or reduce the rate of clinical decompensation in cirrhotic patients, in addition to prevention of HCC recurrence. Several studies used the standard dose of IFN after HCC treatment[17,23,33], and studies using low-dose IFN therapy for HCV-related cirrhosis after HCC treatment also reported that such regimen may reduce late recurrence of HCC[34].

In this prospective case controlled trial, we assessed the efficacy of low-dose intermittent IFN therapy on HCV-related liver cirrhosis after curative treatment of primary HCC in terms of overall survival, HCC recurrence, and liver function.

MATERIALS AND METHODS

Patients

A total of 176 consecutive patients received their initial treatment for HCV-related primary HCC at Hiroshima University Hospital between August 2001 and December 2006. Of these, 67 patients with HCC underwent first medical or surgical therapeutic intervention with curative intent (defined as complete tumor eradication with no visible residual tumor in computed tomographic images, or resection of all evident tumor tissue). Medical treatments included percutaneous radiofrequency (RF) ablation and ethanol injection, while surgical procedures included hepatic resection and RF ablation under laparotomy. Among these 67 patients, 52 patients with liver cirrhosis (LC), which was diagnosed clinically and pathologically, were considered for this prospective study. Figure 1 shows our study flow. Among these 52 patients with HCV-related LC, we assessed 42 patients who met the following inclusion criteria: (1) the presence of up to three tumors with none exceeding 30 mm in diameter or a solitary tumor less than 50 mm in diameter; (2) tumor-node-metastasis (TNM) stage of Ⅰ, Ⅱ or Ⅲ; (3) detectable serum HCV RNA; (4) all seronegativity for hepatitis B marker including hepatitis B surface antigen, hepatitis B anti-core antibody and hepatitis B surface antibody ; (5) compensated cirrhosis with a Child-Pugh class A or B; (6) platelet count $\geqslant$ 40 000/μL; and (7) absence of local recurrence during the follow-up period and of any ectopic intrahepatic recurrence within 12 wk after treatment for primary HCC. We used the TNM classification system

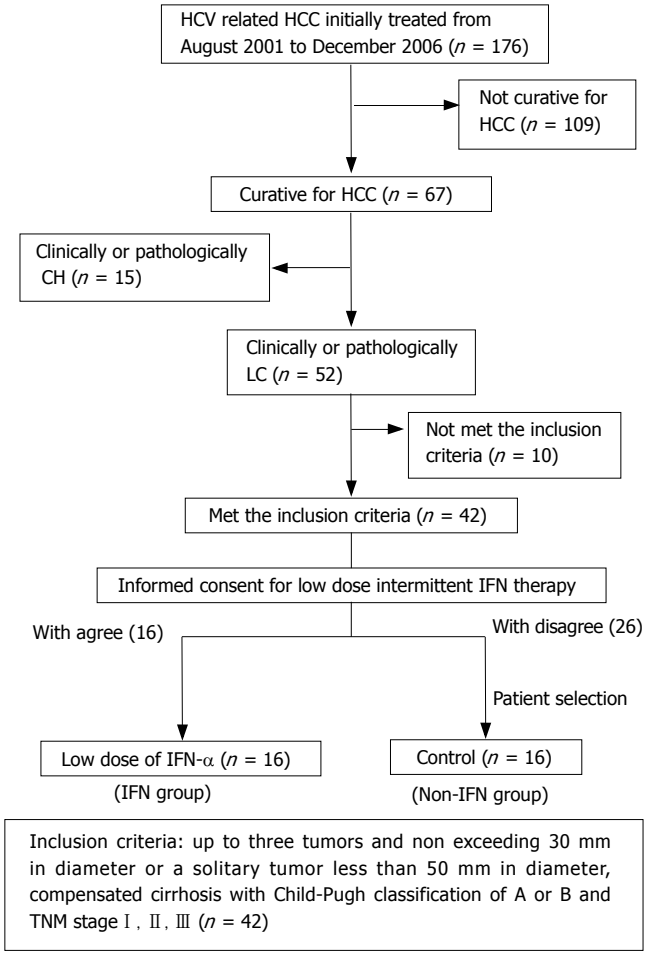

Figure 1 Schematic flow chart of enrolled patients.

Table 1 Characteristics of participating patients

	Interferon group	Non-interferon group	*P* value
No. of patients	16	16	
Age in years (range)	68.5[1] (53-73)	67.5[1] (58-75)	NS
Gender (Male/Female)	10/6	11/5	NS
Albumin (g/dL)	3.7[1] (3.0-4.8)	3.7[1] (3.0-4.5)	NS
Platelet count ($\times 10^4$/L)	8.0[1] (4.5-14.2)	8.4[1] (4.6-14.3)	NS
ICG R-15 (%)	17.3[1] (6.1-40.8)	18.2[1] (5-45)	NS
Alanine aminotransferase (IU/L)	59[1] (35-99)	58[1] (21-143)	NS
Alpha fetoprotein (ng/mL)	54[1] (5.3-293.6)	38[1] (5.0-1217)	NS
Child-Pugh score (A/B)	13/3	13/3	NS
Main tumor size (mm)	15[1] (10-50)	18[1] (10-40)	NS
No. of HCC tumors (single/multiple)	9/7	10/6	NS
Stage (Ⅰ/Ⅱ/Ⅲ)	8/3/5	7/5/4	NS
Treatment (medical/surgical)	8/8	9/7	NS
HCV genotype (1/2)	12/4	14/2	NS
Viral loads (low/high)	6/10	5/11	NS

ICG-R15: Indocyanine green retention at 15 min; Low viral loads: HCV RNA < 100 KIU/mL, high viral loads: HCV RNA ≥ 100 KIU/mL. [1]median.

of the Liver Cancer Study Group of Japan as a staging system for HCC[35]. The underlying liver condition leading to LC was identified by histopathological examination of resected tissue samples. When this was not available, laboratory tests were performed including serum albumin, platelet, prothrombin time and indocyanine green retention at 15 min (ICG-R15), and radiological examination such as ultrasonography and computed tomography.

Of the 42 patients with LC who met the above eligibility criteria, 16 patients received low-dose IFN therapy after signing a written informed consent (IFN group). Of the remaining 26 patients who rejected IFN therapy, we selected 16 patients as the control (non-IFN group). These 16 patients, who met the eligibility criteria mentioned above, were matched by age, gender, tumor size, number of tumors, TNM stage of HCC, serum albumin level, platelet counts, ICG-R15 and Child-Pugh class with patients of the IFN group. Thus, a total of 32 patients (16 in the IFN group and 16 in the non-IFN group) were enrolled in this study. All agreed to participate in the research protocol, which was approved by the hospital research ethics board. Table 1 shows the baseline characteristics of patients of the two groups. The data indicates no significant differences between the groups for age, gender, liver function, tumor characteristics, and therapeutic methods used against primary HCC.

IFN therapy

In the IFN group, patients received 3 MIU of natural IFN-alpha (human lymphoblastoid IFN; Sumiferon, Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly three times weekly for at least 48 wk as long as possible. IFN therapy commenced within 12 wk after initial treatment for HCC. Patients received post-treatment IFN therapy up to the detection of HCC recurrence, and then patients who could have curative treatment for recurrent HCC restarted IFN therapy when possible. However, patients who had advanced liver dysfunction or untreatable progressive HCC did not receive IFN therapy. In the control group, none of the patients received IFN therapy after curative treatment of HCC; instead, they were on ursodeoxycholic acid (UDCA) and stronger neo minophagen C (SNMC).

Follow-up

After curative treatment for primary HCC, all patients underwent liver function tests, serum tumor marker assays such as alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist (PIVKA)-Ⅱ, every month, abdominal ultrasonography every 3 mo, and dynamic computed tomography (CT) every 6 mo. If recurrences of HCC were suspected, additional examinations including CT during arteriography or tumor biopsy were performed. Recurrence of HCC was defined as any new nodules appearing as hyperattenuation by CT during hepatic arteriography or as hypoattenuation in CT performed during arterioportography. Hypovascular HCC was confirmed histopathologically by fine-needle aspiration biopsy. Patients with recurrent HCC were treated medically or surgically, with curative intent if possible. Patients without curative treatment of recurrent HCC then received transcatheter chemoembolization. After repeated transcatheter chemoembolization, patients were finally unable to receive any treatment for recurrent HCC.

End points

We analyzed the outcome of this prospective study in December 2006. We compared the rate of HCC recurrence and the survival rate between IFN group and control group. We assessed whether low-dose of IFN therapy was effective in inhibiting recurrence of HCC, preserving liver function and prolonging survival. In addition, we also assessed the cumulative rate of deviation from objective of any treatment against recurrent HCC due to progression of HCC and/or underlying liver dysfunction.

Statistical analysis

The Chi-square and Fisher exact tests were used for categorical variables, while Student's *t*-test and the Mann-Whitney *U* test were used for continuous and ordinal variables, as appropriate. The Kaplan-Meier method used to assess cumulative survival and recurrence rates calculated from the date of diagnosis to the date of

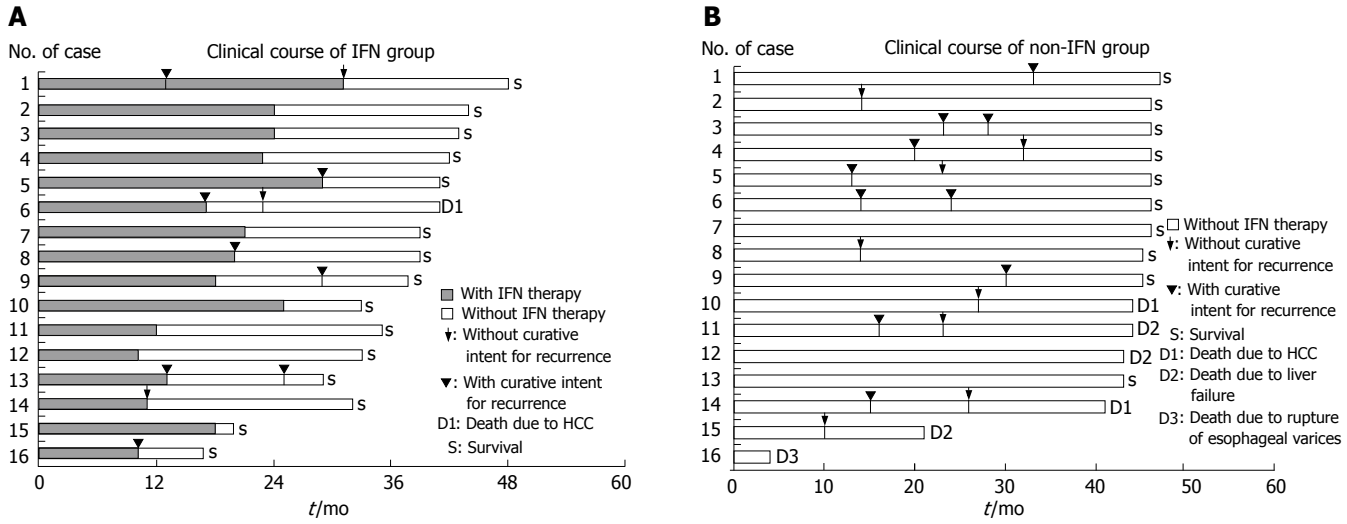

Figure 2 **A**: Clinical course of the interferon group. Patients who had a curative treatment for primary HCC received 3 MIU of natural interferon-alpha three times weekly for at least 48 wk as long as possible except Cases 12, 14 and 16. Recurrent HCCs were treated with or without curative treatment; **B**: Clinical course of the non-interferon group. Patients who had a curative treatment of primary HCC did not receive IFN therapy. Recurrent HCCs were also treated with or without curative treatment.

disease recurrence or death. Surviving patients and patients who died of causes unrelated to the liver were defined as censored cases, while patients who died of causes related to the liver were defined as noncensored cases. The log-rank test was used to compare survival and recurrence curves. *P* values below 0.05 were considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC) was used for analysis of data.

RESULTS

Clinical course of IFN group

Figure 2A shows the clinical course of 16 patients of the IFN group from the initial treatment of primary HCC to the date of data analysis. The duration of low-dose IFN therapy ranged from a minimum of 10 mo to a maximum of 25 mo (median 16 mo). Although 8 patients did not have HCC recurrence, HCC recurred in 8 patients after initial treatment of HCC during a median follow-up period of 37 mo. Of the recurred patients, 7 developed HCC recurrence during IFN therapy (Cases 1, 5, 6, 8, 13, 14 and 16) except 1 patient (Case 9) who had HCC recurrence after discontinuation of IFN therapy. Of the 8 patients with HCC recurrence, 4 were treated with surgical resection therapy (Cases 5, 9, 13 and 16), 3 patients with percutaneous RF ablation therapy (Cases 1, 6 and 8) and 1 patient transcatheter chemoembolization (Case 14). Of these patients, a patient with transcatheter chemoembolization (Case 14) could not have curative treatment and repeated transcatheter chemoembolization. He was excluded from the study concerning the next recurrence. Of the 7 patients with curative treatment for HCC recurrence, 2 restarted IFN therapy, one continued IFN therapy until next recurrence (Case 1), which was not curative, and the other continued until intolerant generalized fatigue (Case 8). The remaining 5 patients (Cases 5, 6, 13, 14 and 16) were followed without IFN therapy because of rejection of IFN therapy. Although one of these 5 patients was not curative for first recurrence (Case 14), he was tolerant to repeated transcatheter chemoembolization and was still alive at the date of data analysis. Two patients without curative treatment at the second recurrence (Cases 1 and 6) were also relatively tolerant to the repeated medical treatment such as transcatheter chemoembolization. Of these patients, one died of progression of HCC in spite of repeated transcatheter chemoembolization and hepatic arterial infusion (Case 6), another was alive at the date of data analysis (Case 1). Of 3 patients without curative treatment of HCC, two survivors' status of HCC were not progressive (stage Ⅱ and stage Ⅲ) and underlying liver function could be tolerant to the treatment such as transcatheter chemoembolization because of relatively preserved function (Cases 1 and 14).

The 16 patients who received IFN therapy included 2 patients with virological response (Cases 2 and 3) and 14 patients who did not get SVR [3 transient responders (Cases 8, 9 and 11), and 11 non-responders (Cases 1, 4, 5, 6, 7, 10, 12, 13, 14, 15 and 16)]. Among the 14 patients who did not show SVR, 8 were biochemical responders with normalized ALT (Cases 1, 4, 5, 7, 9, 10, 13 and 16), including 4 transient responders and 4 non-responders. Two sustained virological responders who received IFN therapy for 96 wk have viral characteristics of genotype 1 and low viral load. Among the patients who did not show SVR, 7 discontinued IFN treatment because of recurrence of HCC, while 2 patients restarted IFN therapy after the curative treatment of recurrent HCC. None of the patients who received IFN therapy developed life-threatening side effects.

Clinical course of non-IFN group

Among the non-IFN group, the first recurrence of HCC occurred in 13 patients during a median follow-up period of 45 mo (Figure 2B). HCC recurred in 6 of the 7 non-IFN patients who had a sustained normalized ALT. Of the 13 patients with recurrent HCC among the non-IFN group, 4 were treated with hepatic resection (Cases 1, 4, 9 and 11),

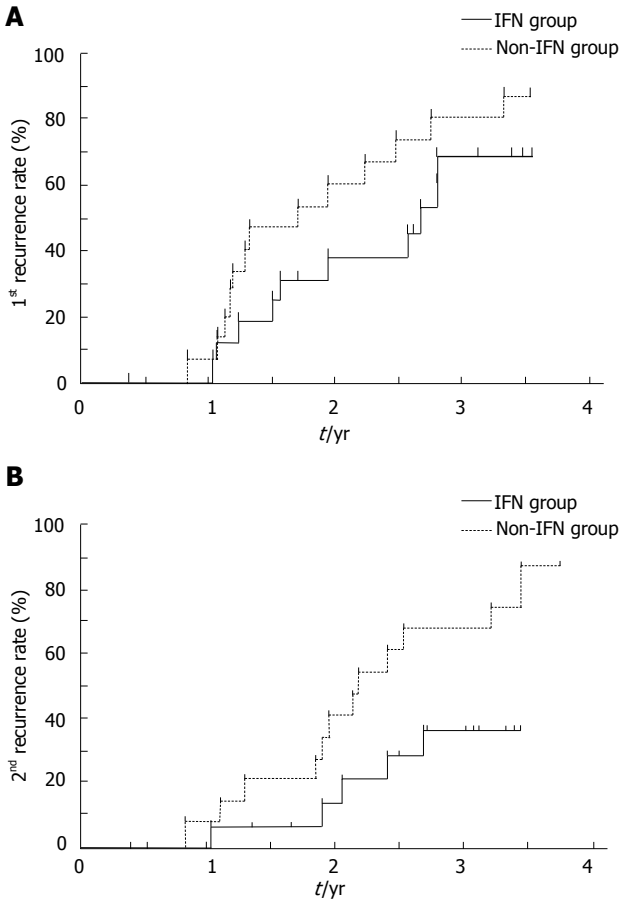

Figure 3 **A**: Cumulative rate of first recurrence. Rates of first recurrence for the IFN and non-IFN groups. The rate of first recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group (P = 0.157); **B**: Cumulative rate of second recurrence. Rates of second recurrence for the IFN and non-IFN group. The rate of second recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group (P = 0.056).

6 with local ablation including percutaneous RF ablation or ethanol injection (Cases 3, 5, 6, 7, 10 and 14) and 3 with transcatheter chemoembolization (Cases 2, 8 and 15). Of the 13 recurrent patients, 5 patients (2 received ethanol injection and 3 transcatheter chemoembolization) could not be treated curatively and was excluded from the study concerning the next recurrence. These 5 patients were treated repeatedly with transarterial chemoembolization after first recurrence. Among the remaining 8 patients who were treated curatively for first recurrence, 7 developed a second recurrence (Cases 3, 4, 5, 6, 9, 11 and 14). Among these 7 patients with second recurrence, 2 were treated curatively for HCC [1 with RF ablation (Case 3) and 1 with hepatic resection (Case 6)], while the remaining 5 patients were not (4 patients due to uncontrolled multiple HCC and one patient due to underlying liver dysfunction). The latter group of 5 patients received transarterial chemoembolization repeatedly after second recurrence.

Comparison of the first and second recurrence rates of HCC

We compared the overall cumulative rates for first and second recurrence between IFN and non-IFN groups (Figure 3). The 1-, 2- and 3- year rates of first recurrence of HCC in the IFN and non-IFN group were not different (0% *vs* 6.7%, 38.1% *vs* 60% and 68.6% *vs* 80%, respectively, Figure 3A, P = 0.156). The 1-, 2- and 3-year rates of second recurrence in the IFN and non-IFN groups were 0% *vs* 6.7%, 13.5% *vs* 33.3% and 35.9% *vs* 67%, respectively (Figure 3B, P = 0.056).

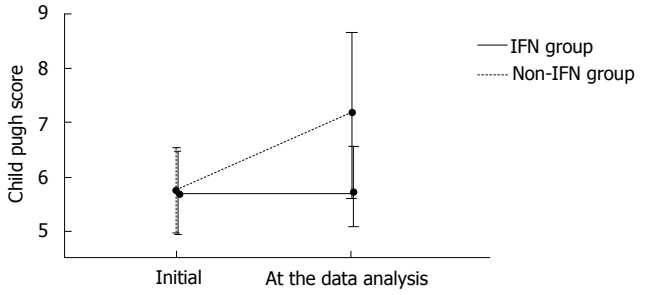

Figure 4 Effect of IFN therapy after curative treatment of HCC on Child-Pugh scores. IFN-treated patients were less likely to show deterioration of hepatic function. The average scores of Child-Pugh of the IFN group were significantly better preserved than the non-IFN group (P = 0.0008).

Liver function

Patients of the IFN group were less likely to develop worsening of hepatic dysfunction compared with the non-IFN group. We compared the average score determined for Child-Pugh classification at initial treatment of HCC with that at the time of data analysis (Figure 4). Although the difference in the Child-Pugh classification score between the two groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group (P = 0.0008).

Deviation from objects of any treatments for recurrent HCC

At the date of data analysis, patients who developed recurrent HCC were treated repeatedly, as possible, for the purpose of curative treatment including surgical resection and ablative therapy such as RF ablation and ethanol injection. Patients who were difficult to treat with curative intent received transcatheter chemoembolization or hepatic arterial infusion. Although patients with recurrent HCC received repeated treatments, some patients finally could not be treated because of excessive progression of HCC or liver dysfunction. Figure 5 shows that the cumulative rate of deviation from objects of any treatment for recurrent HCC between the IFN group and non-IFN group. In the IFN group, one patient could not receive treatment due to progressively advanced HCC in later period. On the other hand, 8 patients in the non-IFN group could not receive treatment because of underlying liver dysfunction (n = 2) and progressively advanced HCC (n = 6). The 1-, 2- and 3- year rates of deviation from objects of any treatment for recurrent HCC in the IFN and non-IFN group were 0% *vs* 6.7%, 0% *vs* 20% and 0% *vs* 27%, respectively (P = 0.048). Thus, the IFN group tended to be treatable for recurrent HCC compared with the non-IFN group.

Survival of patients

At the date of data analysis, 1 patient among the IFN

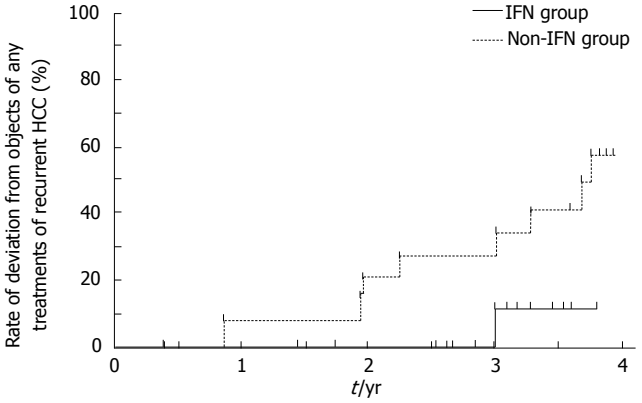

Figure 5 Cumulative rate of deviation from objects of any treatment of recurrent HCC. Recurrent HCC tended to be treatable later in the IFN group than non-IFN group ($P = 0.048$).

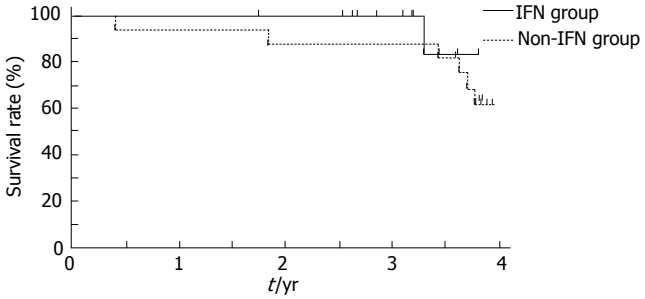

Figure 6 Cumulative survival rate. Comparison of the cumulative survival rates of the IFN and non-IFN groups. The cumulative survival rate was not significantly different between the two groups ($P = 0.45$).

group and 6 patients among the non-IFN group had died of liver disease. Of the 8 recurrence patients among the IFN group, 1 died of advanced multiple HCC and none died of liver failure. On the other hand, of the 13 recurrence patients among the non-IFN group, 2 died of advanced HCC and 2 died of liver failure in spite of the relatively early stage of HCC. Among the 3 patients without recurrent HCC of the non-IFN group, 1 died of liver dysfunction and 1 died of ruptured esophageal varices.

With regard to the cumulative survival rates of the IFN and non-IFN groups (Figure 6), the respective rates of survival were 100% *vs* 93.7% at 1 year, 100% *vs* 87.5% at 2 years, 100% *vs* 87.5% at 3 years and 83.3% *vs* 61.4% at 4 years. Thus, the cumulative survival rate was not significantly different between the two groups for first 4 years after curative treatment of HCC ($P = 0.45$). The median survival time following the first treatment of HCC was 37 mo (range, 17 to 45) for the IFN group and 45 mo (range, 4 to 47) for the non-IFN group.

DISCUSSION

HCC recurrence is still a risk even if HCV-related HCC is treated with curative intent. Most of such patients with HCC have underlying liver cirrhosis, and deterioration of underlying hepatic function may be a hindrance to treatment of recurrent HCC and be associated with prognosis. The present prospective case controlled study of cirrhotic patients shows that low-dose intermittent IFN therapy after curative treatment of HCC could preserve liver function and increase the chance of treatment for recurrent tumor.

Previous studies indicated that IFN therapy after curative treatment of HCC was effective in inhibiting or delaying the development of recurrent HCC[17-23,34,36]. Although several recent studies have reported the efficacy of chemoprevention with IFN therapy after treatment of HCV-related HCC, the basis of the benefit was not clear. Shiratori *et al*[23,33] and Ikeda *et al*[17] reported that IFN therapy in cirrhotic patients reduced recurrence of HCC and improved prognosis. Although they used standard IFN dosage per time, there are no other reports on the effect of low-dose IFN therapy after curative treatment of primary HCC in cirrhotic patients. Sakaguchi *et al*[21] reported that low-dose, long-term, intermittent IFN therapy in patients who had curative HCV-related HCC suppressed recurrence of HCC and improved survival, though it was not clear whether their patients had underlying liver cirrhosis or not. On the other hand, Mazzaferro *et al*[34] indicated that low-dose intermittent IFN therapy seemed to reduce late recurrence in patients with HCV-related cirrhosis after resection of HCC. Considered together, these results suggest that low-dose IFN therapy is potentially useful for cirrhotic patients when used as long as possible. However, our results of low-dose intermittent IFN therapy showed no significant difference in recurrence between those who received IFN therapy and those who did not. Unfortunately, since the difference in treatment outcome between the above three studies might be due to the use of different IFN regimens (e.g., dosage and frequency), and background characteristics of cirrhotic patients (e.g., performance status), the results varied and no standard IFN regimen to pursue after curative treatment of HCV-related HCC could be advocated.

The design of the present study was not randomized controlled type, and differed in details of the IFN protocol and characters of patients from the other studies. Although there was no significant difference in the recurrence rate between the IFN and non-IFN groups, the recurrence rate in the later period of observation including second recurrence appeared to be lower in patients with IFN therapy. Furthermore, the recurrent HCC in patients on IFN therapy did not seem to be aggressive compared with that in patients without IFN therapy, probably because they could be treated with curative intent during the observation period. Thus, low-dose intermittent IFN therapy seemed to have delayed or reduced the chance of development of recurrent HCC in the later period of observation, although IFN did not completely inhibit HCC recurrence in our cirrhotic patients.

Most cirrhotic patients cannot receive a standard full-dose IFN regimen due to underlying liver dysfunction and unfavorable complication such as cytopenia. Hence, it could be difficult to achieve SVR in most cirrhotic patients on low-dose intermittent IFN therapy. Valla *et al*[37] performed a randomized, controlled trial of IFN-alpha 2b but the results showed a lack of any benefits in terms of sustained biochemical response, liver function test

results, histology, occurrence of decompensation or HCC, or prolongation of survival. On the other hand, Everson and coworkers[29,30] suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients. Several recent studies have reported that IFN therapy following HCC treatment improved liver function of patients with HCV-related HCC, although it is not clear which specific IFN action is important for these benefits. We also demonstrated that preservation of liver function was significantly better in the IFN group than in the non-IFN group even when HCV was not completely eradicated. Thus, hepatic functional preservation increases the chance of treatment for recurrent. Therefore, the cumulative rate of deviation from objects of any treatment for recurrent HCC might be lower in patients with IFN therapy than in patients without IFN therapy as we showed that low-dose IFN resulted in less advanced recurrence and hepatic functional preservation. Although the survival rates were not significantly different between the two groups in our observation period, we need a longer observation to determine differences in survival rates. Although we also assessed the correlation between the observed beneficial effects of the low-dose intermittent IFN therapy and HCV genotype, we could not reach the clear conclusion due to small sample size. In the future, the study with large sample size may be needed to conclude.

In our study, only about 12.5% (2/16) of patients who received IFN therapy had sustained viral elimination. And there were no significant difference in population of patients with normalized ALT between the IFN and non-IFN group (n = 10, n = 7, respectively). In spite of these results, patients treated with low-dose intermittent IFN therapy have a hepatic functional preservation greater than IFN untreated patients who received continuous medication with UDCA or SNMC after curative treatment of HCC. Although the mechanism of this reason is not well known, we suggested that the anti-inflammatory activity by low-dose intermittent IFN therapy may be stronger than medication with UDCA or SNMC and induce regression or retardation of underlying hepatic fibrosis, and finally, inhibits the progression of hepatic dysfunction.

Adverse effects such as reduction in blood counts by low-dose of IFN-alpha were not observed in our study, although neutropenia and/or thrombocytopenia were identified before IFN therapy. Furthermore, none of the patients required dose reduction in our study. Although 4 patients discontinued IFN therapy because of generalized fatigue, 2 of these patients restarted IFN therapy after that. Therefore, low-dose intermittent IFN-alpha therapy can be used relatively safely for cirrhotic patients with thrombocytopenia. However, patients who can not receive even low-doses of IFN also exist due to severe cytopenia or advanced liver cirrhosis. Medication with UDCA or SNMC or phlebotomy may be useful in decreasing ALT level for those patients.

Most cirrhotic patients who had received curative treatment for primary HCC have a limited hepatic reserve or thrombocytopenia. Therefore, low-dose intermittent IFN therapy might be effective for better prognosis. However, further studies of larger samples followed-up for longer periods should be conducted to establish a definite conclusion about the effect of low-dose IFN therapy for the prevention of progressive liver disease and effect of treatment for recurrent HCC.

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H pylori

A novel phenol-bound pectic polysaccharide from *Decalepis hamiltonii* with multi-step ulcer preventive activity

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Abstract

AIM: To investigate H^+, K^+-ATPase inhibition, anti-*H pylori*, antioxidant, and the *in vivo* antiulcer potential of a pectic polysaccharide from Swallow root (*Decalepis hamiltonii*; SRPP).

METHODS: SRPP, with known sugar composition [rhamnose: arabinose: xylose: galactose in the ratio of 16:50:2:32 (w/w), with 141 mg/g of uronic acid] was examined for anti-ulcer potency *in vivo* against swim/ethanol stress-induction in animal models. Ulcer index, antioxidant/antioxidant enzymes, H^+, K^+-ATPase and gastric mucin levels were determined to assess the anti-ulcer potency. Anti-*H pylori* activity was also determined by viable colony count and electron microscopic studies.

RESULTS: SRPP, containing phenolics at 0.12 g GAE/g, prevented stress-induced gastric ulcers in animal models by 80%-85%. Down regulation of gastric mucin 2-3 fold, antioxidant/antioxidant enzymes and upregulation of 3 fold of H^+, K^+-ATPase in ulcerous animals were normalized upon treatment with SRPP. Histopathological analysis revealed protection to the disrupted gastric mucosal layer and epithelial glands. SRPP also inhibited H^+, K^+-ATPase *in vitro*, at an IC_{50} of 77 μg/mL as opposed to that of 19.3 μg/mL of Lansoprazole and *H pylori* growth at Minimum Inhibitory Concentration (MIC) of 150 μg/mL. In addition, free radical scavenging (IC_{50}-40 μg/mL) and reducing power (3200 U/g) activities were also observed.

CONCLUSION: SRPP, with defined sugar composition and phenolics, exhibited multi-potent free radical scavenging, antioxidant, anti-*H pylori*, inhibition of H^+, K^+-ATPase and gastric mucosal protective activities. In addition, SRPP is non-toxic as opposed to other known anti-ulcer drugs, and therefore may be employed as a potential alternative for ulcer management.

INTRODUCTION

Ulcer is a common global problem with increasing incidence and prevalence. Worldwide 14.5 million people have ulcers with a mortality of 4.08 million (http://digestive.nidk.nih.gov/statistics/statistics.htm/peptic ulcer prevalence). The increasing incidence and prevalence of ulcers have been attributed to several factors encountered during day-to-day life, such as stress[1], exposure to bacterial infection[2], and use of non-steroidal anti-inflammatory drugs (NSAIDS)[3]. Indeed, NSAIDs are used daily by approximately 30 million people world wide, constituting a world market in excess of $2 billion. Associated serious side effects are ulceration and gastric bleeding, which are due to inhibiting cyclooxygenase-1 activity that is required for mucosal protection[4]. Gastric lesions develop due to loss of the delicate balance between gastro-protective and aggressive factors. Reduction in gastroprotective factors, such as mucus, bicarbonate secretion and gastric mucosa-blood flow, and enhancement of aggressive factors, such as increase of acid/pepsin secretion and *H pylori* infection, results in gastric ulceration[1,2]. Mucosal damage, an initial step in ulcer development, has been known to be due to oxidative stress (OS) by Reactive Oxygen Species (ROS), hypersecretion of HCl through H^+, K^+-ATPase action[5], harboring of *H pylori* on the damaged mucin layer[6], and the blockade of the cyclooxygenase enzyme system by NSAIDS[4], as depicted in Figure 1.

A modest approach to control ulceration, therefore, is *via* stimulation of gastric mucin synthesis, enhancement of antioxidant levels in the stomach, scavenging of ROS, inhibition of H^+, K^+-ATPase and *H pylori* growth[7]. Although the antisecretory drugs, such as H^+, K^+-ATPase pump inhibitors-omeprazole, lansoprazole; histamine H_2-receptor blockers-ranitidine, famotidine, are being used to control acid secretion and acid related disorders; however, they are not the drugs of choice since they produce

potential adverse effects on human health[8].

In light of the above, it is pertinent to study natural products from food/plants as potential anti-ulcer compounds. Due to the lack of side effects compared to synthetic drugs, approximately 60% of the world's population relies entirely on such natural medications. In Indian traditional medicine, several plants have been employed to treat gastrointestinal disorders, including gastric ulcers[9]. Antiulcer properties have been attributed generally to phenolics[10,11] and occasionally to polysaccharides[12-14] of plant extracts.

In this paper we report a pectic polysaccharide from *Decalepis hamiltonii* (Swallow root) containing a sulfonamide group and phenolics as an effective antiulcer compound *in vivo*. We envisage a multi-potent role for this phenolic-polysaccharide in the upregulation of mucin, antioxidant levels, modulation of oxidative status, inhibition of H^+, K^+-ATPase activity against swim and ethanol stress-induced ulcers in experimental animal models, in addition to its ability to inhibit *H pylori*. This paper reveals the potency and multi-step action of phenolic polysaccharide in preventing gastric ulceration.

MATERIALS AND METHODS

Chemicals

Monoclonal anti-gastric mucin from Sigma Chemicals (St. Louis, MO, USA), Ham's F-12 media from HiMedia (Mumbai, India), Alkaline phosphatase conjugated-rabbit anti mouse IgG secondary antibody from GENEI (Bangalore, India). All other reagents were of analytical grade purchased from Qualigens fine chemicals (Mumbai, India).

Plant

Decalepis hamiltonii Wight & Arn. (*Asclepiadaceae*) roots (Batch No. 6, 2005) were procured from a local vendor at Devaraja market, Mysore, India, originally collected from the Gumballi forest range located between 11-13 N and 77-78 E, South-East corner of Mysore district in July 2005 and identified by a taxonomist in the herbarium of Vivekananda Girijana Kalyana Kendra, B.R. Hills, Chamaraja Nagar, Karnataka, India, where a voucher specimen is deposited.

Isolation of pectic polysaccharide from swallow root

Pectic polysaccharide was isolated from defatted powder of swallow root as described previously[15]. Briefly, 10 g of defatted powder were depleted with proteins, amylose and amylopectins by specific enzymatic (protease, termamylase and glucoamylase) digestions at their optimum reaction conditions and centrifuged. Further, the residue was extracted with 200 mL of 0.25% (w/v) ammonium oxalate solution and filtered; the filtrate was precipitated by ethanol at 4℃. The precipitate was resuspended in 10 mL of water and lyophilized to obtain pectic polysaccharide. Total sugar content was estimated by a Phenol-sulphuric acid method. A total yield of 6% was obtained as carbohydrate and this pectic polysaccharide of swallow root is designated as SRPP. Sugar composition analysis revealed the presence of rhamnose: arabinose: xylose: galactose in the ratio 16:50:02:32, in addition to 141 mg uronic acid/g of SRPP.

Determination of the phenolic content and antioxidant activity of SRPP

Since phenolics are generally found to be associated with polysaccharides, we evaluated the phenolic content in SRPP using a Folin-Ciocalteu reagent as described previously[16]. Gallic acid was used as a standard for the generation of a calibration curve. Total phenolic content is expressed as Gallic Acid Equivalents (GAE) in mg/g of SRPP.

The reducing power and free radical scavenging activity of SRPP was determined according to the method described previously[16]. SRPP at 10-100 μg was employed for determining the reducing power and free radical scavenging activity. Capability to scavenge the DPPH radical was calculated using the following equation.

Scavenging effect (%) =(Absorbance of control at 517 nm - Absorbance of sample at 517 nm)/ Absorbance of control at 517 nm × 100

Characterization of SRPP by Fourier transform infra-red spectroscopy (FTIR)

Pectic polysaccharides, particularly with sulfur, also have been shown to exhibit antioxidant activity and SRPP was subjected to FTIR study. The samples were prepared in the form of pellets by mixing with dry KBr. Potassium bromide discs containing 1% (w/w) of film material were scanned at 4 mm/s with a resolution of 4/cm over 400-4000/cm, averaging over 128 scans for each type of film and determined the presence of sulphur group.

Inhibition of H^+, K^+-ATPase in vitro

Fresh sheep stomach was obtained from a local slaughterhouse at Mysore and an enzyme extract was prepared[17]. The enzyme extract was incubated with different fractions of swallow root polysaccharide, in a reaction mixture containing 16 mmol/L Tris buffer (pH 6.5). The reaction was initiated by adding substrate (2 mmol/L ATP, 2 mmol/L $MgCl_2$ and 10 mmol/L KCl) and after 30 min of incubation at 37℃, the reaction was stopped by the addition of an assay mixture containing 4.5% ammonium molybdate and 60% perchloric acid. Inorganic phosphate formed was measured spectrophotometrically at 400 nm. Enzyme activity was calculated as micromoles of Pi released per hour at various doses of SRPP.

Toxicity studies

Toxicity studies were carried out in Albino Wistar rats that were orally fed with SRPP at 1 mg/kg b.w. for 15 d. Analysis showed biochemical changes as described previously[17].

Assessment of antiulcer potential of SRPP against swim/ethanol stress induced ulcers

Wistar albino rats, weighing about 180-220 g and maintained under standard conditions of temperature, humidity and light, were provided with standard rodent pellet diet (Amruth feeds, Bangalore, India) and water *ad libitum*. The study was approved by the institutional ethical committee, which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India.

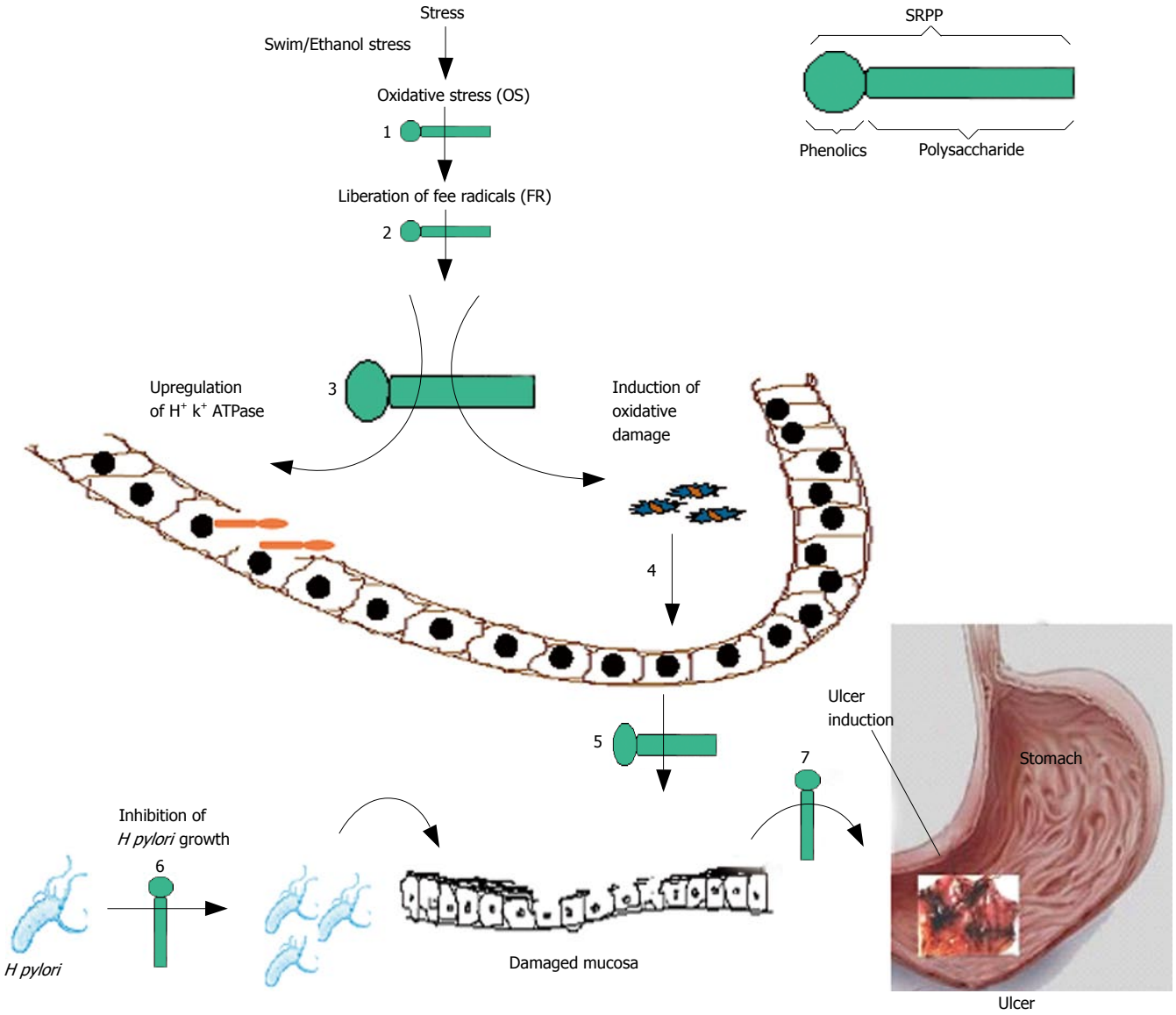

Figure 1 Scheme representing various steps of ulcer pathogenicity and multi-step anti-ulcer action by SRPP (•-); (•) and (-) represents phenolic and polysaccharide portions of SRPP respectively. Swim/Ethanol stress leading to OS (1) and liberation of FR (2). FR upregulated H^+, K^+-ATPase (3) and induced oxidative damage to mucosa (4) leading to mucosal damage (5). *H pylori* may invade on to damaged mucosa and together may cause ulcers (7). SRPP has ability to inhibit steps 1-7 including the growth of *H pylori in vitro* (6).

All the animals were categorized into two sets of five groups with 6 animals in each group (n = 6). SRPP and ranitidine at indicated doses were administered orally twice daily for 14 d. At the end of 14 d, animals were fasted for 18 h and then subjected to the ulcer inducing treatment. In the first set, ulcers were induced by forced swim stress per a published protocol[18], in which rats were briefly subjected to forced swim stress by making them swim in a jar of 30 cm height and 10 cm diameter containing water up to 15 cm height for 3 h. In the second set, ulcers were induced in rats by administering 100% ethanol (5 mL/kg b.w.) for 1 h[19]. Animals were sacrificed under deep ether anesthesia and stomachs were examined for mucosal integrity and occurrence of ulcers. Lower to higher gradings were assigned to milder to severe symptoms, respectively. The following are descriptions of ulcer scores: 0.5-red coloration, 1.0-spot ulcers, 1.5-hemorrhagic streaks, 2.0-ulcers more than 3 mm and less than 5 mm, 3.0-ulcers more than 5 mm. Mean ulcer scores for each experimental group were calculated and expressed as the ulcer index (UI)[20]. Stomach/liver tissues were used for enzyme assays. Serum was collected from the blood of all animals and analyzed for various biochemical parameters.

Preparation of extracts from tissues for biochemical analysis

The stomach and liver tissues were collected, weighed and homogenized in chilled Tris-buffer (10 mmol/L, pH 7.4) at a concentration of 5% (w/v). The homogenates were centrifuged at 1000 × g at 4℃ for 20 min using a high speed cooling centrifuge (REMI C 24, Mumbai, India). The clear supernatant was used to analyse biochemical parameters[21].

Assessment of gastric mucin and H^+, K^+-ATPase

Gastric mucin was isolated from the glandular segments of stomach and quantitated employing a monoclonal anti-human gastric mucin antibody (MAb-GM) by ELISA[5], as well as by Alcian blue dye binding methods[22]. Histological

Table 1 Toxicity studies with Swallow root pectic polysaccharide (n = 6) mean ± SD

Parameters	Control	SRPP treated
Total protein	348[a] ± 32.21	361.81[a] ± 28.10
SGOT (U/mg protein)	18.34[a] ± 1.55	16.22[a] ± 1.34
SGPT (U/mg protein)	21.31[a] ± 2.70	23.21[a] ± 2.29
ALP (U/mg protein)	35.52[a] ± 3.879	33.62[a] ± 2.95
TBARS (n moles/mg protein)	0.166[a] ± 0.08	0.186[a] ± 0.11

SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase; ALP: Alkaline phosphatase; TBARS: Thiobarbituric acid reactive substances. [a]$P < 0.05$ between control and SRPP treated groups.

and immunohistological evaluation was done as described previously[17]. Equal weight of gastric tissue from animals of each group was homogenized using Tris-HCl buffer pH 7.4. The gastric membrane vesicles enriched in H^+, K^+-ATPase were prepared and the activity was assessed as described above.

Assessment of oxidant/antioxidant and antioxidant enzymes

Lipid peroxidation products of serum, stomach and liver homogenates were determined as TBARS and the malondialdehyde (MDA) that formed was quantitated using the molar extinction coefficient of the MDA molecule[21].

Glutathione (GSH) content was determined as described by Das and Banerjee[21]. The activity of superoxide dismutase (SOD) was assayed by measuring the reduction in the NBT in the presence of SOD[23] and catalase (CAT) was assayed by decomposition of H_2O_2 in the presence of catalase at 240 nm[24]. Glutathione peroxidase (GPx) was estimated based on the degradation of H_2O_2 in the presence of GSH and the decrease in absorbance was read at 340 nm[25]. SOD and CAT activity was expressed as units per milligram protein per min. The activity of GPx was expressed as nanomoles of NADPH oxidized per min per milligram of protein. The protein content of the homogenate was determined by Lowry's method[26].

Determination of anti-Helicobacter pylori activity of SRPP

H pylori is a major ulcerogen, and anti-*H Pylori* activity was therefore determined. *H pylori* was obtained by endoscopic samples of gastric ulcer patients from KCDC (Karnataka Cardio Diagnostic Centre, Mysore, India) and cultured on Ham's F-12 agar medium supplemented with 5% FBS at 37℃ for 2-3 d in a microaerophilic condition[27]. *H pylori* culture was characterized by specific tests as described by Siddaraju and Shylaja[16].

Anti-*H pylori* activity of an aqueous solution of SRPP (200 μg/mL) was determined by a viable colony count method[28]. 100 μL of SRPP treated *H pylori* were also processed for scanning electron microscopy (SEM)[29] and examined by SEM (Model No. LEO 425 VP, Electron microscopy LTD, Cambridge, UK) with an acceleration voltage of 20 KV. Multiple fields of visions were viewed. The MIC value was determined by a conventional broth dilution method[16]. MIC was defined as the lowest concentration to restrict the growth to less than 0.05 absorbance units at 625 nm.

Statistical analysis

All values are expressed as mean ± SE. Significance was calculated with student's t test (parametric). When several groups were compared, significance was calculated using an ANOVA. Enzyme estimations were carried out as described and results were tabulated. After calculating means and standard deviations, Dennett's test was performed to obtain the significance between the treated groups and the control groups. A value of $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Toxicity studies

Toxicity studies with aqueous solution of SRPP were carried out in rats for safety evaluation. These studies indicated no lethal effect up to 1 g/kg b.w. when orally fed for 14 d. There were no significant differences in total protein, TBARS levels, SGPT, SGOT and ALP between normal and SRPP treated rats (Table 1), indicating no adverse effect on the major organs. After the above treatment schedules, animals remained as healthy as control animals with normal food and water intake, body weight gain and behavior.

SRPP prevented swim stress/ethanol induced gastric ulcer

Healthy rats showed no lesions in their stomachs (Figure 2A), while rats treated with forced swim stress for 3 h or ethanol stress showed damage in the gastric wall with a hemorrhagic form of lesions and intraluminal bleeding (Figure 2B and C). Rats treated with only SRPP (Figure 2D) also showed no lesions, which is similar to the controls. Oral treatment of SRPP at 100 and 200 mg/kg b.w., as well as Ranitidine at 30 mg/kg b.w., showed protection in a dose dependent manner with no intraluminal bleeding and an insignificant number of gastric lesions (Figure 2E-I). Ranitidine protected both ethanol/swim stress-induced ulcers up to 66%-87% at 30 mg/kg b.w., while SRPP protected up to 80%-85%, respectively, indicating an ulcer preventive effect. Quantitative reduction in the ulcer index (%) in treated rats, compared to either ulcer induced or healthy, is depicted in Figure 3.

SRPP prevents gastric mucosal damage; Alcian blue binding/ELISA/histo-and Immunohistological studies

Gastric wall mucus is damaged during ulcer development and becomes the first target of stress-induced reactive oxygen species. Mucin oxidation or degradation takes place and subsequently looses the protective effect. In the current study, we evaluated the effect of *in vivo* ingestion of SRPP on protection of gastric wall mucus during ulceration induced by swim/ethanol stress. Since Alcian blue binds to carboxylated mucopolysaccharides as well as sulfated and carboxylated glycoproteins, any disruption results in reduction in the dye binding, which can be quantitated. The gastric mucin of stomach tissue was decreased to 17 and 16 mg/g in swim stress/ethanol stress-induced ulcerous rats, respectively, when compared to that of controls (45

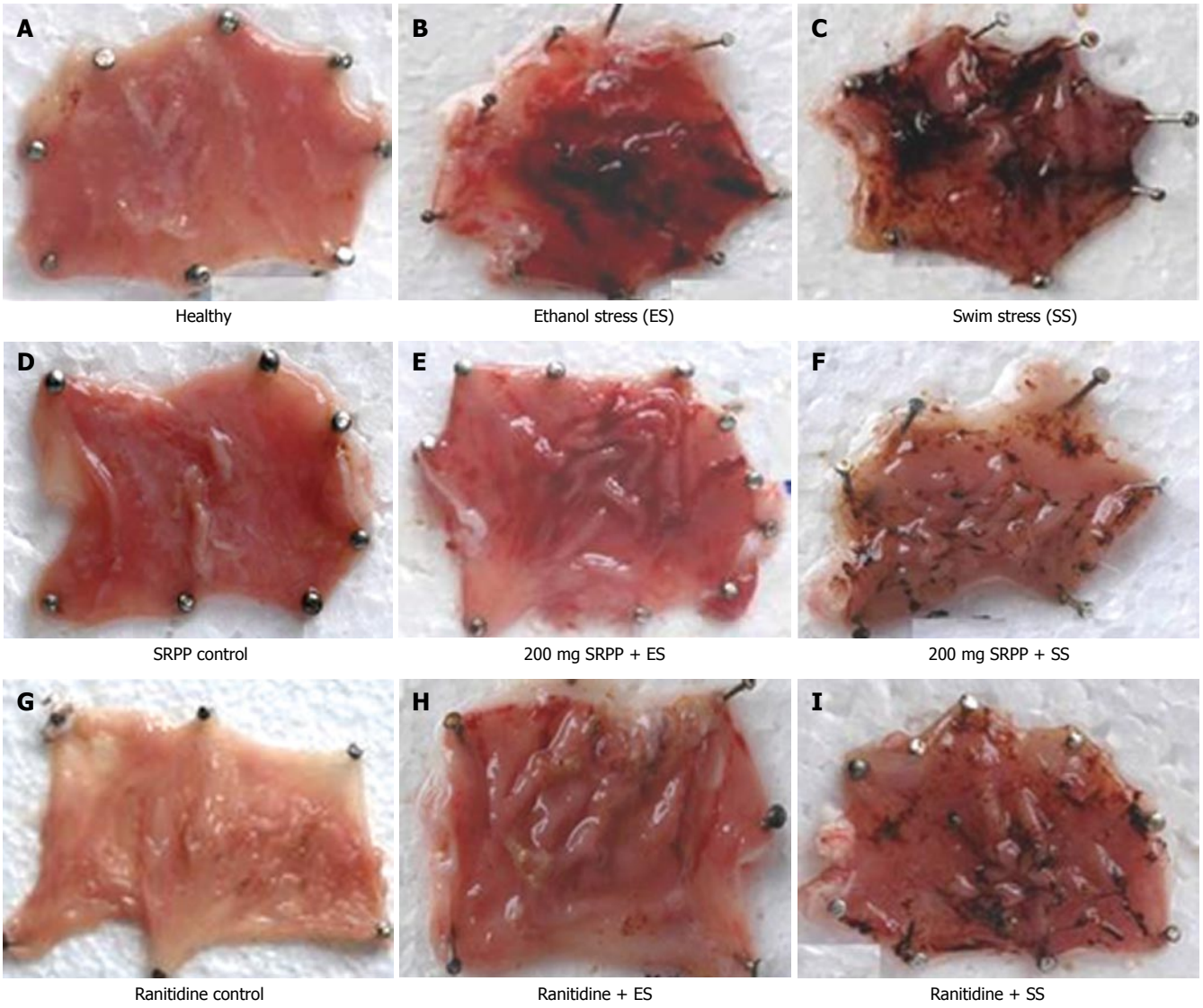

Figure 2 Macroscopic observation of Ulcers in ulcer induced/protected stomachs in swim stress/ethanol stress induced ulcer models; Ulcer was induced in animals by either swim stress (SS) or ethanol stress (ES) in group of pretreated/untreated animals at indicated concentrations. In healthy (**A**), SRPP control (**D**), Ranitidine control (**G**)-no ulcer lesions or damage in the stomach tissue were observed. In ethanol stress (**B**) and swim stress (**C**) induced animals ulcers score were very high. SRPP (**E** and **F**) and ranitidine (**H** and **I**) treated animals showed reduced stomach lesions.

mg/g) (Table 2). Results were substantiated by observing increased gastric mucin content by monoclonal antibody-based immuno histological studies (Figure 4). Hematoxylin and eosin staining of stomach tissue sections in control animals indicated intact structures (Figure 4A). Ulcer induction showed damage in the mucosal epithelium, destruction of regular glandular organization, very high inflammatory exudates, proliferated fibroblasts, infiltration of leucocytes and cellular debris (Figure 4B). SRPP and ranitidine treated rats showed recovery in the mucosal epithelium, regained glandular structure and mucosal regeneration (Figure 4C and D). Immunohistological analysis clearly revealed the intact mucosal epithelium in the control group (Figure 4E) and complete loss or eradication in ulcer-induced tissue sections (Figure 4F). Complete recovery was observed upon treatment with SRPP (Figure 4G) and partial recovery is depicted during ranitidine treatment (Figure 4H).

Evaluation of SRPP potential on oxidant and antioxidant status in ulcerous and treated animals

Tables 3 and 4 indicate antioxidant, antioxidant enzymes and TBARS levels in stomach/liver homogenate and the serum of swim/ethanol stress models. SOD and GPx levels increased in stomach (2 fold) and CAT and GSH decreased (1.8 fold) during stress-induced ulcerous conditions and were normalized upon treatment with SRPP in a dose dependent manner. An approximately 4 fold increase in TBARS levels depicts lipid peroxidation or damage of stomach tissue in ulcerous animals and was recovered up to 80% upon treatment with SRPP. Ranitidine, although showing protection against ulcer, showed no significant improvement in GSH or antioxidant enzyme levels. Similar changes in antioxidant enzymes except catalase was also observed in serum and liver homogenates. A 2 fold increase in TBARS levels was shown in the ulcer condition and, SRPP treatment at 200 mg/kg b.w. showed up to 90% recovery.

Inhibition of H pylori

Initially, anti-*H pylori* activity was assayed by a viable colony

Groups of animals (n = 6)	Control	Ulcer induced	SRPP 100 mg/kg b.w.	SRPP 200 mg/kg b.w.	Ranitidine 50 mg/kg b.w.
Swim stress mean ulcer index ± SE	000.0 ± 0.00	86.0 ± 6.8	52.2^{a} ± 5.1	13.3^{b} ± 1.4	11.2^{b} ± 1.5
Protection (%)	-	0	40	85	87
Ethanol stress mean ulcer index ± SE	000.0 ± 0.00	67.4 ± 3.2	28.2^{a} ± 2.5	13.4^{b} ± 1.8	22.4^{b} ± 1.2
Protection (%)	-	0	58	80	66

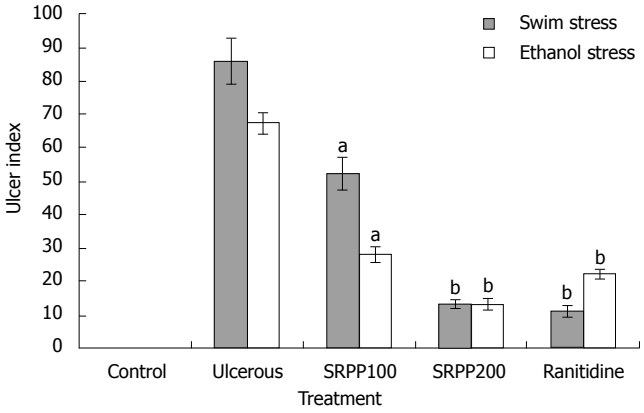

Figure 3 Effect of SRPP on gastric lesions in swim/ethanol stress induced ulcer models; Ulcers were scored as described under the methods and expressed as ulcer index. Maximum ulcer index observed during stress induction was controlled in a concentration dependent manner. Reduction in ulcer index and percent protection is depicted. $^{a}P < 0.05$ and $^{b}P < 0.01$ between ulcerated and treated groups.

Table 2 Gastric mucin and H^+, K^+-ATPase levels in healthy, ulcerated and protected rats (n = 6) mean ± SD

Group, n = 6	Mucin content (mg/g)	H^+, K^+-ATPase (μmoles Pi released/mg/h)
Healthy	45.04^{d} ± 4.128	0.807^{d} ± 0.072
Swim stress induced ulcer model		
Swim stress induced	17.78^{a} ± 2.557	2.209^{a} ± 0.152
SRPP 100 mg/kg b.w.	27.13^{b} ± 4.082	1.771^{b} ± 0.081
SRPP 200 mg/kg b.w.	35.35^{c} ± 3.221	1.601^{b} ± 0.091
Ranitidine 50 mg/kg b.w.	$31.42^{b,c}$ ± 2.327	1.621^{b} ± 0.092
Ethanol stress induced ulcer model		
Ethanol stress induced	16.32^{a} ± 3.821	2.621^{a} ± 0.211
SRPP 100 mg/kg b.w.	32.13^{b} ± 3.457	2.123^{b} ± 0.241
SRPP 200 mg/kg b.w.	$39.53^{b,c}$ ± 3.082	1.512^{c} ± 0.121
Ranitidine 50 mg/kg b.w.	37.13^{b} ± 1.507	1.485^{c} ± 0.124

Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups. Range was provided by Duncan multiple test at $P < 0.05$. a: Less significant; b: Moderately significant; c: Very significant and d: Most significant.

count method. SRPP showed up to 95% inhibition at a 200 μg/mL concentration, which is equivalent to that of a susceptible antibiotic amoxicillin at 10 μg/mL. MIC, determined by a broth dilution method, indicated significant anti-*H pylori* activity at 55 μg/mL ($P = 0.003$) (Figure 5A).

SEM observations

Normal *H pylori* shows uniform rod shaped cells (Figure 5B), whereas the cells treated with SRPP (200 μg/mL) changed from a helical form to coccoid and became necrotic (showed in arrows in Figure 5C). A similar coccoid form was observed with *H pylori* treated with amoxicillin (Figure 5D) and this form has been known to result in a loss of infectivity[30]. A coccoid form with blebs in the bacterial surface, appearance of vacuoles, granules and an area of low electron density in the cytoplasm (shown in arrow marks) were observed in SRPP treated samples indicating the lysis of *H pylori*. Substantiating this viable colony test indicates the loss of more than 95% viability upon treatment with SRPP, supporting an antimicrobial nature of SRPP.

Effect of SRPP on H^+, K^+-ATPase activity

An approximately 3 fold increase in H^+, K^+-ATPase activity in ulcer-induced stomach homogenate was brought to normal levels in a concentration dependent manner by SRPP at 100 and 200 mg/kg b.w. Approximately 58% and 62% (1.5 fold) were reduced at 200 mg/kg b.w. in both ethanol and swim stress-induced ulcer models (Table 2).

To further validate the inhibition of H^+, K^+-ATPase enzyme by SRPP, sheep stomach parietal cells were used. Inhibition of H^+, K^+-ATPase *in vitro* was examined with different polysaccharide fractions of swallow root including SRPP. Only SRPP inhibited H^+, K^+-ATPase activity, with an IC_{50} of 77 μg as opposed to that of Lansoprazole (19.3 μg), whereas other polysaccharide fractions did not show inhibitory activity (Figure 6A).

Characterization of SRPP, an antiulcer compound from swallow root and its relation to antiulcer activity

FTIR spectra obtained using a FTIR spectrometer (Perkin-Elmer 2000 spectrophotometer) equipped with TGS detector with solid samples at a concentration of 1-10 mg provides a signal at 1329 and 1145 cm^{-1} indicating the presence of sulfonamides where sulfate may be found attached to aminosuagars of pectic polysaccharide (Figure 6B).

We evaluated its phenolic content and subsequently its antioxidant property. 0.12 g GAE/g of SRPP yielding 12% of phenolics in SRPP is intriguing since this is the first report of pectic polysaccharides containing such a high level of phenolics. This could be due to the presence

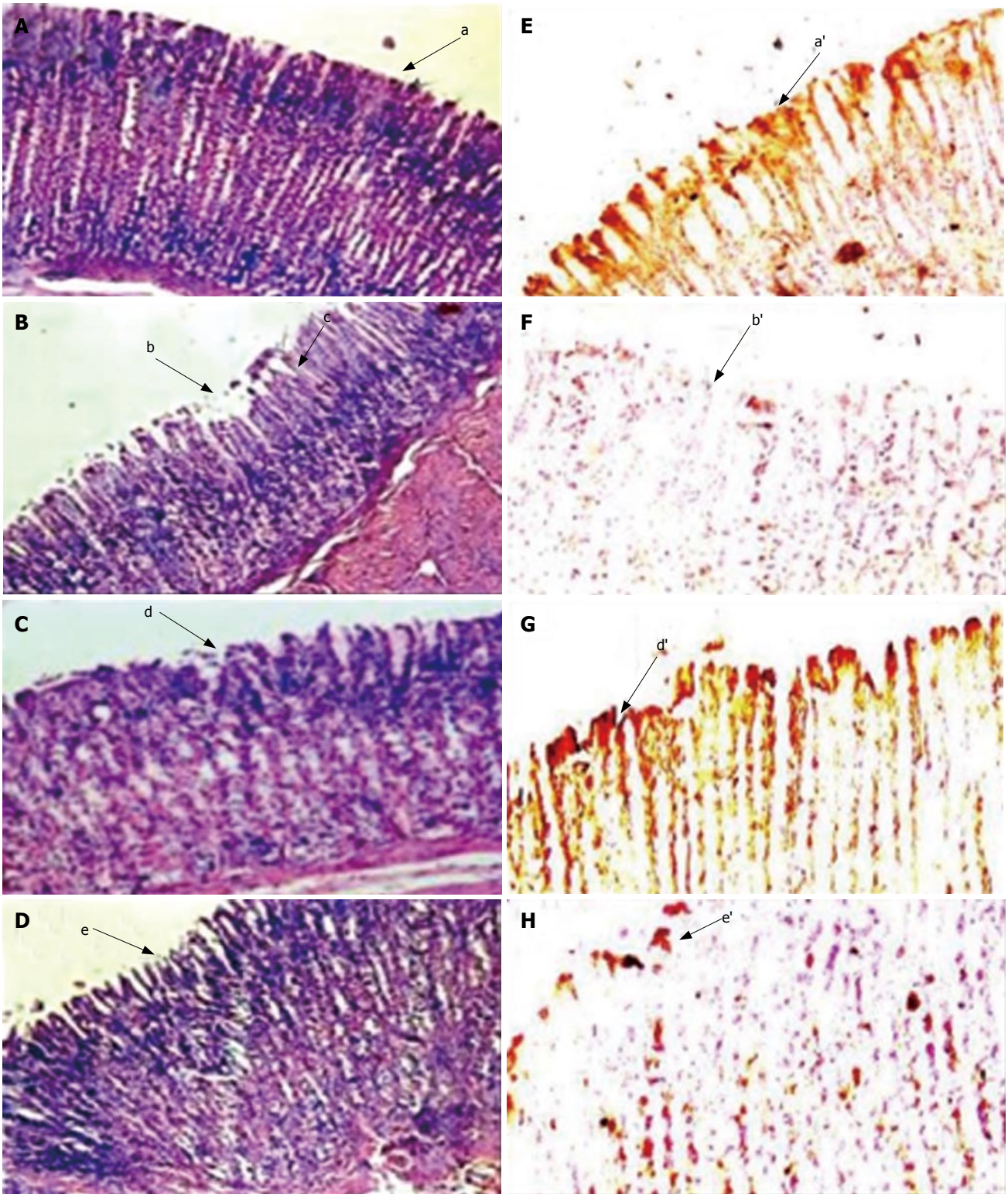

Figure 4 Histopathologic/Immunohistopathologic observation of stomach from ulcer induced/SRPP and Ranitidine treated animals; **A**-**D** indicates HE staining sections (× 40), while **E**-**H** reveal anti-gastric mucin stained sections (× 40, and magnified the selected portion in computer photoshop). Control (**A**, **E**) shows intact mucosal epithelium with organized glandular structure (a) and intense brown staining for gastric mucin by antibody (a'). Ulcer induction (**B**, **F**) showed damaged mucosal epithelium (b) and disrupted glandular structure (c), loss of brown staining (b') in figure **F** indicate the loss of gastric mucin. Complete recovery of mucosal damage (d and d' of **C**, **G**) by SRPP and partial recovery by ranitidine (e and e' of **D**, **H**) treatments were observed.

of higher levels of phenolics (34 mg/g) in swallow root per se. Presence of higher levels of phenolics was substantiated by expression of potent reducing power ability with 3200 absorbance Units/g of SRPP (Figure 6C). In addition, dose dependent free radical scavenging activity was also found (Figure 6D) with an IC_{50} of 40 μg/mL.

DISCUSSION

Recently, phytomedicines from medicinal plants and nutraceuticals from food sources have become attractive sources of new and natural drugs. However, the active

Table 3 Antioxidant/antioxidant enzymes and TBARS levels in swim stress induced ulcer model (n = 6) mean ± SD

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	Glutathione Peroxidase (ηmoles/g)	GSH (nmoles/mg)	TBARS ηmoles
Stomach						
Healthy	$2.23^{c} \pm 0.21$	$9.86^{a} \pm 1.1$	$829.2^{c} \pm 41.6$	$0.21^{a} \pm 0.009$	$224^{c} \pm 10.0$	$0.31^{a} \pm 0.01$
Ulcerated	$1.95^{a} \pm 0.13$	$19.10^{c} \pm 1.8$	$462.4^{a} \pm 30.2$	$0.49^{d} \pm 0.01$	$121^{a} \pm 18.9$	$1.12^{c} \pm 0.20$
SRPP 100 mg/kg	$1.90^{a} \pm 0.09$	$16.32^{b,c} \pm 2.1$	$488.1^{a,b} \pm 32.8$	$0.34^{b} \pm 0.02$	$174^{b} \pm 22.1$	$0.94^{c} \pm 0.10$
SRPP 200 mg/kg	$2.10^{b} \pm 0.19$	$13.06^{b} \pm 2.6$	$679.6^{b} \pm 9.9$	$0.22^{a} \pm 0.01$	$208^{c} \pm 16.5$	$0.55^{a,b} \pm 0.00$
Ranitidine	$2.16^{b} \pm 0.22$	$15.22^{b} \pm 1.2$	$505.5^{a,b} \pm 35.5$	$0.39^{c} \pm 0.01$	$136^{a} \pm 12.1$	$0.92^{b} \pm 0.10$
Serum						
Healthy	$6.62^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9$	$0.221^{a} \pm 0.004$	$23.6^{c} \pm 3.0$	$0.165^{a} \pm 0.01$
Ulcerated	$6.84^{a} \pm 0.53$	$264.6^{d} \pm 32$	$22.90^{a} \pm 3.1$	$0.286^{c} \pm 0.02$	$11.1^{a} \pm 1.8$	$0.326^{d} \pm 0.02$
SRPP 100 mg/kg	$6.35^{a} \pm 0.59$	$201.1^{c} \pm 36$	$28.63^{b} \pm 2.3$	$0.298^{d} \pm 0.03$	$16.5^{b} \pm 2.1$	$0.261^{c} \pm 0.03$
SRPP 200 mg/kg	$6.95^{a} \pm 0.48$	$168.2^{b} \pm 21$	$40.12^{c} \pm 3.8$	$0.268^{b} \pm 0.03$	$19.8^{b,c} \pm 12.9$	$0.162^{a} \pm 0.01$
Ranitidine	$6.35^{a} \pm 0.63$	$196.3^{b,c} \pm 23$	$30.82^{b} \pm 2.9$	$0.226^{a} \pm 0.02$	$12.8^{a} \pm 2.6$	$0.186^{b} \pm 0.01$
Liver						
Healthy	$24.2^{c} \pm 0.31$	$261.5^{b} \pm 41$	$28.42^{d} \pm 3.1$	$0.32^{a} \pm 0.02$	$414^{c} \pm 51$	$0.98^{a} \pm 0.13$
Ulcerated	$21.9^{a} \pm 0.23$	$142.4^{a} \pm 18$	$22.18^{b,c} \pm 2.6$	$0.58^{c} \pm 0.05$	$221^{a} \pm 26$	$2.41^{d} \pm 0.23$
SRPP 100 mg/kg	$23.1^{b} \pm 0.28$	$164.2^{a} \pm 13$	$19.63^{b,c} \pm 2.4$	$0.36^{a,b} \pm 0.03$	$315^{b} \pm 36$	$1.84^{c} \pm 0.16$
SRPP 200 mg/kg	$23.9^{b} \pm 0.28$	$361.5^{d} \pm 39$	$15.54^{a} \pm 2.1$	$0.28^{a} \pm 0.02$	$214^{a} \pm 24$	$1.26^{b} \pm 0.11$
Ranitidine	$23.6^{b} \pm 0.26$	$314.4^{c,d} \pm 36$	$17.34^{a} \pm 1.9$	$0.32^{a} \pm 0.02$	$254^{a} \pm 28$	$1.41^{b} \pm 0.12$

SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups.

Table 4 Antioxidant/antioxidant enzymes and TBARS levels in ethanol induced ulcer model (n = 6) mean ± SD

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	Glutathione Peroxidase (ηmoles/g)	GSH (U/mg)	TBARS ηmoles
Stomach						
Healthy	$2.23^{a} \pm 0.21$	$09.86^{a} \pm 1.1$	$829.2^{c} \pm 41.6$	$0.21^{a} \pm 0.009$	$224^{d} \pm 23.2$	$0.31^{a} \pm 0.1$
Ulcerated	$2.32^{a} \pm 0.09$	$17.86^{c} \pm 2.4$	$201.5^{a} \pm 18.9$	$0.30^{c} \pm 0.01$	$102^{a} \pm 12.6$	$1.26^{d} \pm 0.3$
SRPP 100 mg/kg	$2.16^{a} \pm 0.16$	$16.21^{c} \pm 1.0$	$193.3^{a} \pm 62.5$	$0.26^{b} \pm 0.01$	$162^{b} \pm 15.5$	$0.92^{c} \pm 0.1$
SRPP 200 mg/kg	$2.41^{a} \pm 0.20$	$11.09^{b} \pm 1.0$	$540.5^{b} \pm 40.2$	$0.33^{c} \pm 0.02$	$196^{c} \pm 16.4$	$0.54^{b} \pm 0.1$
Ranitidine	$2.42^{a} \pm 0.19$	$12.42^{b} \pm 1.4$	$468.6^{c} \pm 31.6$	$0.22^{a} \pm 0.03$	$152^{b} \pm 16.3$	$0.96^{c} \pm 0.2$
Serum						
Healthy	$6.62^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9$	$0.221^{a} \pm 0.04$	$23.6^{d} \pm 3.0$	$0.165^{a} \pm 0.01$
Ulcerated	$6.52^{a} \pm 0.69$	$282.3^{d} \pm 26$	$28.36^{a} \pm 3.2$	$0.315^{c} \pm 0.03$	$09.6^{a} \pm 1.2$	$0.465^{d} \pm 0.03$
SRPP 100 mg/kg	$6.35^{a} \pm 0.70$	$228.4^{c} \pm 32$	$34.25^{a,b} \pm 3.3$	$0.286^{b} \pm 0.03$	$18.6^{c} \pm 2.2$	$0.321^{c} \pm 0.04$
SRPP 200 mg/kg	$6.24^{a} \pm 0.56$	$172.3^{b} \pm 2$	$39.60^{b} \pm 4.51$	$0.243^{b} \pm 0.02$	$18.2^{c} \pm 1.9$	$0.181^{a} \pm 0.02$
Ranitidine	$6.32^{a} \pm 0.69$	$210.7^{c} \pm 28$	$34.12^{ab} \pm 4.6$	$0.252^{b} \pm 0.03$	$14.6^{b} \pm 1.6$	$0.214^{a,b} \pm 0.02$
Liver						
Healthy	$24.2^{a} \pm 0.31$	$261.5^{b} \pm 1.1$	$28.42^{c} \pm 3.1$	$0.32^{b} \pm 0.02$	$414^{c} \pm 51$	$0.98^{a} \pm 0.13$
Ulcerated	$24.3^{a} \pm 0.31$	$118.1^{a} \pm 16$	$19.64^{b} \pm 2.2$	$0.48^{b,c} \pm 0.03$	$392^{b,c} \pm 41$	$2.98^{d} \pm 0.31$
SRPP 100 mg/kg	$23.5^{a} \pm 0.21$	$121.8^{a} \pm 15$	$18.32^{b} \pm 1.6$	$0.39^{b} \pm 0.03$	$268^{b} \pm 25$	$2.15^{c} \pm 0.22$
SRPP 200 mg/kg	$26.4^{a} \pm 0.41$	$325.4^{c} \pm 34$	$13.17^{a} \pm 1.6$	$0.29^{a} \pm 0.02$	$241^{a,b} \pm 28$	$1.65^{b} \pm 0.14$
Ranitidine	$26.8^{a} \pm 0.29$	$254.5^{b} \pm 26$	$14.24^{a} \pm 1.8$	$0.31^{a} \pm 0.03$	$211^{a} \pm 28$	$1.61^{b} \pm 0.16$

Range was provided by Duncan multiple test at $P < 0.05$. a: Less significant; b: Moderately significant; c: Very significant and d: most significant. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups.

ingredients and mode of action have been rarely established, which is very crucial for understanding the long-term potency of these antiulcer sources. Among the majority of identified sources, flavonoids[11,31], and occasionally polysaccharides, have frequently been implicated as antiulcer agents[12-14]. We previously reported on a non-toxic, edible antioxidant source[17] - *Decalepis hamiltonii*, a significant antiulcerogenic properties *in vitro* and *in vivo*. High levels of antioxidant properties, probably just little less than that found in green tea, with multiple compounds[32] may play a critical role in inhibiting oxidative induced mucosal damage in ulcers[33]. In the current paper, we report the antiulcerogenic potential of a combinational molecule, which is a pectic polysaccharide with bound phenolics from swallow root. In human nutrition, pectic polysaccharides play a key role as low energy foods and break down products have been known to have health beneficial properties.

Gastric ulcers have multiple etiopathogeneses. Stress ulcers are due to both physiological and psychological factors, which affect gastrointestinal defense and increased accumulation of acid due to influx of H^+ into the lumen of the stomach by parietal cell plasma membrane bound H^+, K^+-ATPase leading to autodigestion of the gastric mucosa[34], and generation of free radicals. Ethanol stress, on the other hand, is known to act on the gastric mucin

A Samples	MIC in μg/mL
Control	No inhibition
Amoxicillin	26 ± 3.2
SRPP	55 ± 4.8

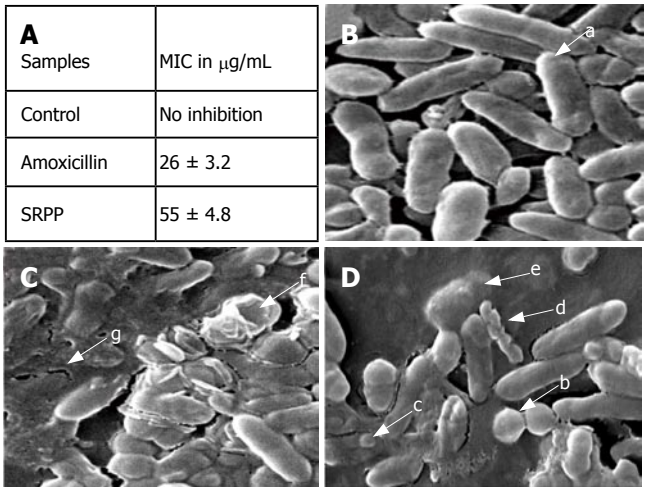

Figure 5 Effect of SRPP on *H pylori*; Minimum Inhibitory Concentration (MIC) (**A**) was established by serial dilution technique; **B-D** indicate the scanning electron microscopic pictures at 15 k magnification of control (**B**), SRPP (**C**) and amoxicillin (**D**) treated *H pylori*. Untreated control cultures indicate uniform rod shaped (a) *H pylori* cells. Amoxicillin treatment showed coccoid form (b), blebbing (c), fragmented (d) and lysed (e) cells. SRPP treatment in addition indicates cavity formation (f) with disrupted structures (g).

directly, affecting mucosal defense. Nevertheless, in both cases the causes of severe ulcerations are depicted in the current study in addition to observations from other investigators[35,1]. Our earlier studies indicated that phenolic antioxidants were efficient in inhibiting upregulated H^+, K^+-ATPase and recovering the depleted levels of antioxidant and antioxidant enzymes[17].

The effect of SRPP on gastric ulcers induced by swim and ethanol stress was investigated in *in vivo* rat models. Oral administration of 100 and 200 mg/kg b.w. reduced gastric lesions. It is evident from our data (Figures 2-4 and Tables 2-4) that swim stress and ethanol stress induced gastrointestinal effects, such as gastric erosions, gastric or duodenal ulcerations, gastrointestinal hemorrhages and perforations. These effects were modulated by the inhibition of upregulated H^+, K^+-ATPase, and enhancement of down regulated gastric mucin, antioxidant and antioxidant enzyme levels. Histological studies indicated that characteristic ulcerogenic pathogenicity, with a distinct ulcer margin formed by the adjacent non-necrotic mucosa, the epithelial component, and granulation tissue at the ulcer base, was normalized upon treatment with SRPP. Current data, together with the results of our previous paper[17], indicate clearly that phenolic antioxidants of SRPP may contribute to H^+, K^+-ATPase inhibition, rather than the polysaccharide per se since swallow root antioxidants inhibited H^+, K^+-ATPase at 36 μg/mL as apposed to that of SRPP (77 μg/mL).

Ethanol induced gastric lesions are thought to arise as a result of direct damage to gastric mucosal cells, resulting in the development of free radicals and hyperoxidation of lipids. Recently, it was discovered that *Solanum nigrum* extract provides significant antioxidant activity as one of the possible gastroprotective mechanisms against ethanol-induced gastric ulceration[19]. SRPP may also act similarly in reducing ulcerations in stomach since it showed potent antioxidant properties.

In addition, SRPP is a safer source since toxicity studies indicated no lethal effect up to an oral dose of 1 g/kg b.w. for 14 d. To understand the potential role of SRPP in gastric mucosal protection, it is important to know that mucin is an insoluble adherent mucus gel, which is quite stable and has significant buffering capacity for neutralization of luminal acid in the presence of bicarbonate. SRPP showed 2 fold upregulation of gastric mucin as revealed by immunohistological/biochemical and ELISA methods, indicating the stabilization of the mucosal layer.

Further, SRPP possessed H^+, K^+-ATPase inhibitory activity, although not as potent as that of phenolic fractions. Phenolics present in SRPP together with those reported in the literature revealed that phenolic antioxidants are potent H^+, K^+-ATPase blockers[11]. The significant levels of phenolics present in SRPP may also contribute towards inhibition of H^+, K^+-ATPase activity, which plays a tremendous role in reducing an acidic condition in the gastric lumen.

Results are intriguing that SRPP also showed potential anti-*H pylori* activity. The results are in accordance with the observation made by Lee *et al*[36], where inhibition of *H pylori* growth by pectic polysaccharide was reported. However the mechanism still needs to be established. Several mechanisms may be proposed for potential inhibition of *H pylori* by SRPP. SRPP phenolics may inhibit microbial activity as phenolics were thought to exert their antimicrobial effect by causing (1) hyper acidification at the plasma membrane interface of the micro organism, or (2) intracellular acidification, resulting in the disruption of H^+, K^+-ATPase required for ATP synthesis of microbes, or (3) may be related to inactivation of cellular enzymes causing membrane permeability changes[10,37]. The rate of inactivation of microbial cellular enzymes is dependent on the rate of penetration of phenolic antioxidants into the cell. In the case of *H pylori*, phenolics may be inactivating the urease enzyme, which is specifically expressed at its surface to neutralize hyperacidification to survive in the gastric environment of the stomach[38]. It is thus clear that SRPP is creating a cavity in the organism (Figure 5C) with the loss of cellular contact resulting in loss of viability of *H pylori*.

There are several schools of thought that indicate the ulcer healing component must be proliferative, amplify cell migration, and enhance angiogenesis in order to enhance re-epithelialization in the ulcer healing process. However, antiulcer compounds with proliferative ability, and the ability to enhance angiogenesis, may be carcinogenic also. This statement is also substantiated by observation of induction of cancer upon the usage of antiulcer drugs on a long-term basis[8]. In this context, SRPP, although found to be antiulcerogenic, has been shown to be anticancerous (Unpublished observation, 2006). Hence the treatment of ulcer by SRPP even for longer periods of time may not pose side effects.

Generally antioxidants have been known to be antimicrobial by binding to the microbial membrane leading to disruption[39]. SRPP, by virtue of phenolics, may be antimicrobial. In addition, SRPP may also participate in enhancement of gastric mucin. The enhancement of gastric mucin contents, as measured by ELISA and Alcian blue binding, may suggest that enhancement is most probably

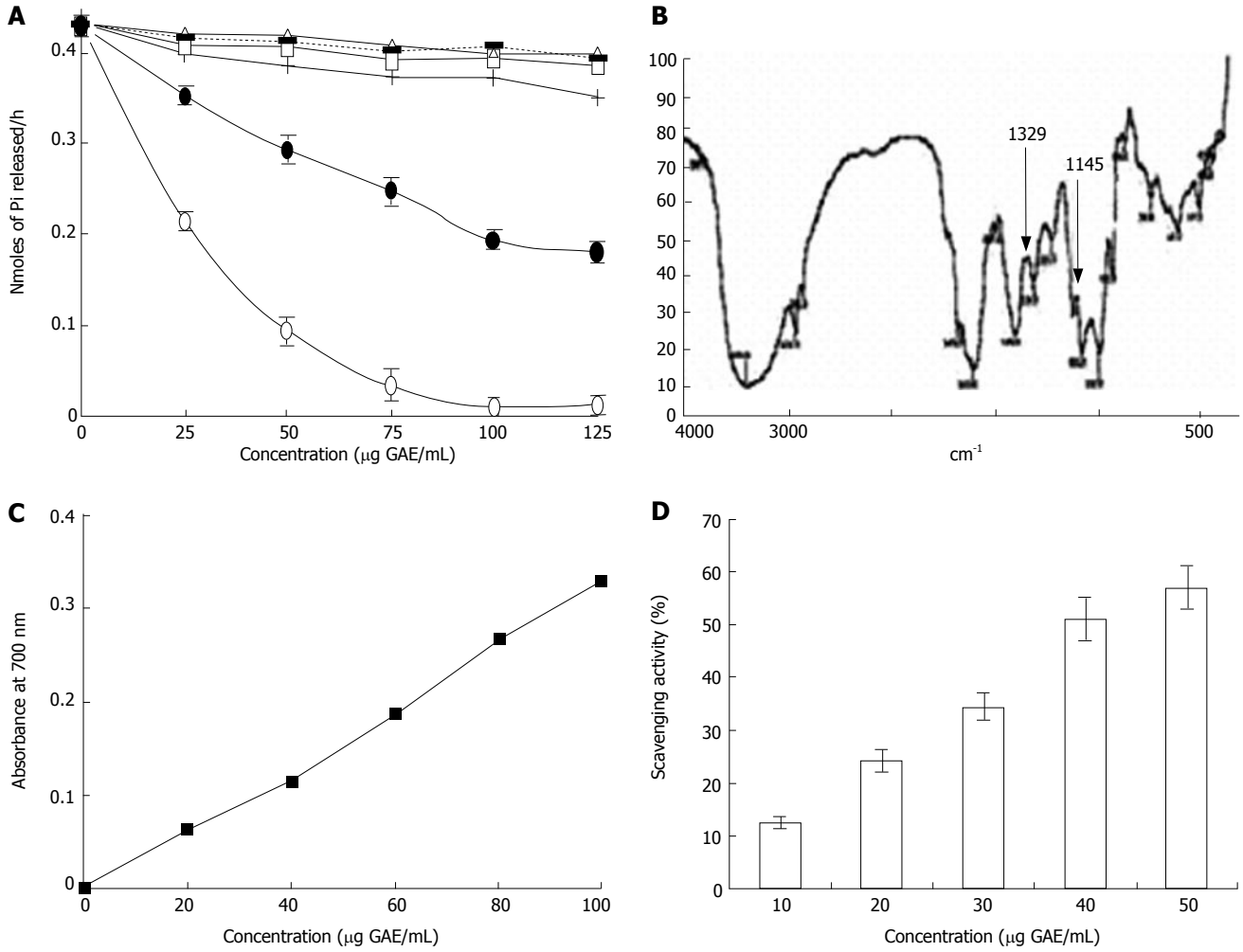

Figure 6 H^+, K^+-ATPase (**A**), Fourier Transform Infra-Red Spectroscopy (**B**), Reducing power (**C**) and Free radical scavenging activity (**D**) of SRPP. A: inhibition of H^+, K^+-ATPase only by SRPP (•) and not by other polysaccharides of Swallow root; SR water soluble polysaccharide (▪), SR Hemicellulose A (+), Hemicellulose B (□), SR alkali insoluble residue (◊) and inhibition by lansoprazole (o) a known blocker is also depicted in the figure; B: arrow at 1329 and 1145 cm^{-1} indicate the presence of sulfonamide group in FTIR spectrum. Dose dependent antioxidant activity evaluated as reducing power ability (**C**) and free radical scavenging ability (**D**) indicates potential antioxidant activity by phenolics of SRPP.

due to prevention/protection of mucosal injury during ulceration rather than direct increase in synthesis. This is supported by no upregulation of gastric mucin in SRPP controls where animals were fed with SRPP without inducing ulcers. However, regulated synthesis might occur, which may be evaluated by tracer techniques. Antioxidant potency may also be contributed by both phenolics and sulfonamide groups containing polysaccharides[40,41]. Further, it is also possible that SRPP, by virtue of its anionic nature, may bind effectively to positively charged amino acid residues of gastric mucin as well as sucralfate and other polysaccharides[42]. This binding may avoid gastric mucin damage and subsequent ulceration. SRPP thus can be a safe and promising multi-step ulcer blocker (Figure 1).

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causative factors being unavoidable such as stress, use of non-steroidal anti-inflammatory drugs; and the side effects of available antiulcer drugs; alternatives that are safer, but effective in ulcer prevention must be envisaged.

Research frontiers

Frontiers of research on development of antiulcer drugs must emphasize on (a) detection and diagnosis of *H pylori*-a major ulcerogen; (b) identification of ulcerogens and antiulcerogens from diet since some components of food are ulcerogens; (c) antiulcer drugs with less or no side effects, so that it can be used by subjects who are using NSAIDS and also alcohol.

Innovations and breakthroughs

Herbal/dietary sources are the challenging alternatives for potential ulcer management. Since herbal medicines include extracts from either edible or non-edible source, side effect has been a threat. Dietary sources are therefore

optional. We report a novel potent multi-step ulcer blocker which inhibits acid secretion/growth and invasion of *H pylori* and enhances mucosal defense.

Applications

The antiulcer component identified is inexpensive, effective and nontoxic; hence can be directly applied to human health. Single compound with multi-potency implies a potential reduction in the drug load during ulcer treatment.

Terminology

Ulcer, H^+,K^+-ATPase, pectic polysaccharide, *H pylori*, antioxidant, gastric mucin, mucosal injury, non-steroidal anti-inflammatory drugs.

Peer review

This is a nicely done description of the effects of SRPP. Through *in vivo* studies, the authors concluded that SRPP with defined sugar composition and phenolics exhibited multi-potent free radical scavenging, antioxidant, anti-*H pylori*, inhibition of H^+, K^+-ATPase and gastric mucosal protective activities.

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BASIC RESEARCH

CTGF, intestinal stellate cells and carcinoid fibrogenesis

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Abstract

AIM: To investigate the role of small intestinal carcinoid tumor-derived fibrotic mediators, TGFβ1 and CTGF, in the mediation of fibrosis *via* activation of an "intestinal" stellate cell.

METHODS: GI carcinoid tumors were collected for Q RT-PCR analysis of CTGF and TGFβ1. Markers of stellate cell desmoplasia were identified in peritoneal fibrosis by immunohistochemistry and stellate cells cultured from fresh resected fibrotic tissue. CTGF and TGFβ1 were evaluated using quantitative tissue array profiling (AQUA analysis) in a GI carcinoid tissue microarray (TMA) with immunostaining and correlated with clinical and histologically documented fibrosis. Serum CTGF was analyzed using a sandwich ELISA assay.

RESULTS: Message levels of both CTGF and TGFβ1 in SI carcinoid tumors were significantly increased (> 2-fold, $P < 0.05$) versus normal mucosa and gastric (non-fibrotic) carcinoids. Activated stellate cells and markers of stellate cell-mediated fibrosis (vimentin, desmin) were identified in histological fibrosis. An intestinal stellate cell was immunocytochemically and biochemically characterized and its TGFβ1 (10-7M) initiated CTGF transcription response (> 3-fold, $P < 0.05$) demonstrated. In SI carcinoid tumor patients with documented fibrosis, TMA analysis demonstrated higher CTGF immunostaining (AQUA Score: 92 ± 8; $P < 0.05$), as well as elevated TGFβ1 (90.6 ± 4.4, $P < 0.05$). Plasma CTGF (normal 12.5 ± 2.6 ng/mL) was increased in SI carcinoid tumor patients (31 ± 10 ng/mL, $P < 0.05$) compared to non-fibrotic GI carcinoids (< 15 ng/mL).

CONCLUSION: SI carcinoid tumor fibrosis is a CTGF/TGFβ1-mediated stellate cell-driven fibrotic response. The delineation of the biology of fibrosis will facilitate diagnosis and enable development of agents to obviate its local and systemic complications.

Key words: Carcinoid; Connective tissue growth factor; fibrosis; Small intestine; Stellate cell; TGFβ

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INTRODUCTION

Carcinoid (neuroendocrine) tumors are enigmatic, generally slow growing malignancies that occur most frequently (67%) in the GI tract[1]. They are not rare lesions, arising in 1.68 of every 100 000 people[1]. The commonest gut tumor is the SI carcinoid tumor[1,2], which is derived from neuroendocrine enterochromaffin (EC) cells. SI carcinoid tumors are usually identified based on their characteristic paroxysmal symptomatology of flushing, sweating and diarrhea. They are often, however, detected at surgery for unexplained bowel obstruction[3], as a consequence of the fibrosis that they engender[4]. The etiology of this desmoplastic response is unknown but is a consequence of conversion of the normally filmy and flexible mesentery into a contracted fibrous adhesive mass with bands and even retroperitoneal desmoplasia[5,6]. These events are due both to tumor invasion and the ability of secretory products of the EC cell to initiate fibrosis by activating local cells to produce a desmoplastic response[7]. SI carcinoid tumor patients also develop distant (cardiac) fibrosis suggesting that the bioactive agents involved in the process have both a paracrine and a systemic effect[6]. In contrast, neither gastric carcinoids (derived from the neuroendocrine EC-like (ECL) cell) nor pulmonary carcinoids are associated with extensive local or systemic desmoplastic responses[8].

The mechanism whereby such fibrosis occurs is unknown although serotonin has previously been suggested as a mediator[6]. TGFβ1 and CTGF are well-characterized fibrotic factors[9-12]. TGFβ1 is a profibrotic mediator that

induces CTGF expression[10]. Together, these factors stimulate over-production of collagen synthesis[13,14]. The target cells of TGFβ1 and CTGF are activated myofibroblasts, also known as stellate cells[15,16]. In the pancreas, TGFβ1 activates pancreatic stellate cells (PSCs) in both experimental and human pancreatic fibrosis; these cells are the main cellular source of collagen in chronic pancreatitis[17-19]. SI neuroendocrine tumors express TGFβ1 and its receptors, while stromal cellular elements around tumor nests express the TGFβ receptor[20]. This suggests a mechanism by which tumor cells can interact with and alter the character of the surrounding stroma.

We hypothesized that tumor TGFβ1 and CTGF produced by EC cells is involved in the mechanism of SI carcinoid tumor fibrosis via activation of an "intestinal" stellate cell. The aims of this study were to: (1) quantify CTGF and TGFβ1 message in carcinoid tumor tissue; (2) examine protein expression levels of CTGF and TGFβ1 and matrix proteins using immunohistochemistry in SI carcinoid tumors and intestinal fibrosis; (3) isolate and characterize the "intestinal" stellate cell; (4) examine the effects of TGFβ1 on this cell type; (5) quantitatively analyze CTGF and TGFβ1 protein levels on a GI carcinoid tissue microarray by AQUA analysis; and 6) determine whether serum CTGF discriminated SI carcinoid tumor patients with fibrosis from other non-fibrotic GI carcinoids.

MATERIALS AND METHODS

These studies were approved by the Human Investigations Committee at the Yale University School of Medicine.

Tissue specimens

Tissue for molecular analysis: Tumor tissue from ten GI carcinoid patients (M:F = 6:4; median age [range] = 60 years [40-78]) diagnosed with either SI EC cell carcinoid tumors (n = 5) or gastric ECL cell carcinoids (n = 5) were collected for this study (Table 1). None of the patients had received therapy (surgery or somatostatin analogues) prior to tissue procurement. Paired normal tissue samples were also obtained from adjacent, macroscopically normal, non-tumor mucosa in nine cases from these patients.

Tissue for cell culture analysis: Tumor tissue and mesenteric fibrotic tissue was obtained from a patient with a fibrotic SI carcinoid tumor (male, 43 years; sample #6) operated on at Yale University (by IMM). This patient had not received medical therapy (somatostatin analogues) prior to surgery and was a de novo case of SI fibrosis.

GI Carcinoid TMA: Formalin-fixed paraffin-embedded tissue blocks containing GI carcinoids (stomach: n = 7; and SI: n = 36) diagnosed between 1965 and 2001 at the Yale University School of Medicine Department of Pathology were retrieved. Follow-up information was available (median follow-up: 110 mo, range: 24-456 mo) for all patients. The TMA consisted of primary GI carcinoids, matched normal mucosa and peritoneal fibrotic material and was represented by 2 cores/case. Complete clinical details including fibrosis were known for all patients. Clinically significant fibrosis was determined at surgery, and all samples were examined by a pathologist (RLC) to histologically confirm fibrosis.

Table 1 Clinical details of carcinoid tumors used for mRNA analysis

No	Sex	Age[2]	Race	Tumor site	Lymph node involvement	Liver involvement	Fibrosis[3]
1[1]	M	71	H	G	N	N	N
2[1]	M	45	W	G	N	N	N
3[1]	F	74	W	G	N	N	N
4[1]	M	78	W	G	N	N	N
5	F	40	W	G	N	N	N
6[4]	M	43	W	SI	N	Y	Y
7[1]	F	60	W	SI	16/22	N	Y
8[1]	M	59	W	SI	N	Y	N
9[1]	M	73	W	SI	1/9	Y	N
10[1]	F	53	W	SI	1/12	N	N

[1]Normal tissue available, [2]Age at time of procedure, [3]Identified at surgery; [4]Used to isolate and culture intestinal stellate cell. H: Hispanic; W: White; G: Gastric ECL carcinoid; SI: SI EC cell carcinoid tumor.

Serum: Twenty-nine subjects (median age [range] = 42 years [20-83]; M:F = 17:12) attending the Neuroendocrine Referral, Oncology and Surgery outpatient clinics at Yale University School of Medicine were recruited for serum analysis. These included 29 patients with GI carcinoids: SI EC cell carcinoid tumors (n = 16), gastric ECL cell carcinoids (n = 7), and six other GI carcinoids [rectal: n = 2, parotid: n = 1, appendiceal: n = 2, duodenal: n = 1]. Serum samples from ten age-, sex-matched control subjects were also collected.

Tissue techniques

Quantitative RT-PCR: Total RNA was isolated from frozen carcinoid tumor tissue (n = 10) and normal mucosa (n = 9) with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. RNA was dissolved in DEPC water, measured spectrophotometrically and an aliquot analyzed on a denaturing gel using electrophoresis to check the quality of RNA isolated.

CTGF and TGFβ1 message were quantitatively measured in the ten tumor and nine control samples as described[21,22]. Briefly, Q RT-PCR was performed using the ABI 7900 Sequence Detection System. Total RNA from each sample was subjected to reverse transcription using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). 2 μg of total RNA in 50 μL of water was mixed with 50 μL of 2X RT mix containing Reverse Transcription Buffer, dNTPs, random primers and Multiscribe Reverse Transcriptase. RT reaction was carried out in a thermal cycler for 10 min at 25℃ followed by 120 min at 37℃. Real time PCR analysis was performed in triplicate[21,22]. cDNA in 7.2 μL of water was mixed with 0.8 μL of 20 × Assays-on-Demand primer (*CTGF* = Hs00170014, *TGFβ1* = Hs00171257, *GAPDH* = Hs99999905) and probe mix, 8 μL of 2 × TaqMan Universal Master mix in a 384 well optical reaction plate. The following PCR conditions were used: 50℃ for 2 min, then 95℃ for 10 min, followed by 40 cycles at 95℃/0.15

min and 60℃/1 min. A standard curve was generated for each gene using cDNA obtained by pooling equal amounts from each sample (n = 19). The expression level of target genes was normalized to internal GAPDH. Data was analyzed using Microsoft Excel and calculated using the relative standard curve method (ABI, User Bulletin #2).

Immunohistochemistry: Serial sections (5 μm) encompassing SI carcinoid tumors or fibrotic tissues were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval purposes, sections were immersed in citrate buffer (10 mM sodium citrate, pH 6.0), and subjected to 1 × 10 min high temperature-high pressure treatment followed by treatment with 0.3% H_2O_2 in methanol for 30 min at 37℃ to inactivate endogenous peroxidase. In some studies, sections were incubated with goat antiserum to CTGF (1:250) or TGFβ1 (1:1000) (both from Santa Cruz Biotechnology, Santa Cruz, CA) diluted in Tris-buffered saline containing BSA and a monoclonal antibody against CgA (0.5 μg/mL) or serotonin (2 μg/mL) (both from DAKO, Carpinteria CA) for 24 hr at 4℃ and then with Alexa 488-labeled anti-mouse IgG (1:100 dilution) for 1 hr at RT. Donkey anti-goat antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp, Carpinteria, CA) was used as a secondary reagent. HRP-amplification was performed. CTGF or TGFβ1 was visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA). Dual-positive cells (CTGF + serotonin or CTGF + CgA) were counted in a minimum of 5-well orientated sections and expressed as a percentage. In other studies, fibrotic areas from the peritoneum of patients with SI carcinoid tumors were stained with mouse anti-α-smooth muscle actin (1:1000) or desmin (1:1000, both DAKO), goat anti-vimentin (1:1000), collagen Ⅲ (1:1000) or CTGF (1:250). Stromal (myofibroblast) cells were separable from tumor cells that were identified by the use of a fluorescently tagged anticytokeratin antibody cocktail (AE1/AE3; DAKO Corp). Nuclei were visualized by 4', 6-diamidino-2-phenylindole (DAPI 10 mg/mL). Localization of expression of products was used to determine whether stromal (non-cytokeratin staining) or tumor cells expressed these products.

Intestinal stellate cell culture and analysis: Stellate cells were isolated using a modification of the method by Bachem *et al*[15]. Briefly, cells were isolated from the fibrotic tumor specimen (hand dissected, digested in collagenase (0.25 mg/mL)/DNAse (100 U/mL) solution for 60 min at 37℃ under constant aeration) and were cultured on 10 cm^2 uncoated culture wells in 10% fetal calf serum in a 1:1 (vol/vol) mixture of DMEM and Ham's F12 medium supplemented with 2% L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% amphotericin. Twenty-four hours after seeding, the culture medium was changed and the myofibroblasts remained attached to the plastic. After reaching confluence, cells were subcultured by trypsinization using a 0.025% trypsin solution containing 0.01% EDTA in PBS. For immunofluorescence microscopy, cells were seeded on 1 cm^2 glass coverslips in six-well (10 cm^2/well; 2 mL medium) plates (2-3 glass coverslips per well). Phase-contrast microscopy was used to identify the translucent fat droplets in the cytoplasm and stellate-like morphology that typifies stellate cells[15]. These studies were undertaken within the first 3-d as culturing cells results in a transdifferentiation from a vitamin A-storing phenotype to a myofibroblastic phenotype[15]. For immunocytological characterization, cells cultured on uncoated glass coverslips were fixed for 30 min in -20℃ acetone and air-dried. Coverslips were preincubated for 15 min in TBS (pH 7.4) with 3% bovine serum albumin and 0.3% hydrogen peroxide. Incubations with the primary antibody (mouse monoclonal: α-smooth muscle actin 1:1000) was performed at room temperature in a humidified chamber for 1 h. Non-specific staining was controlled by omitting the primary antibody and including mouse, non-immune serum at the same dilution as used for the specific primary antibody. After rinsing (three times for 5 min with TBS/Tween-0.5%), the second antibody (HRP goat anti-mouse, diluted 1:100) was added and incubated for 1 h at room temperature. Cy5-labelled tyramide (TSA; NEN Life Science Products, Boston, MA) was used with DAPI (10 mg/mL) to stain nuclei and cells observed with a fluorescence microscope. For RNA studies, cultured cells were stimulated with TGFβ1 (10^{-7} M) for 24 h. Thereafter, RNA was isolated and Q RT-PCR performed as described above to quantitatively measure TGFβ1-stimulated CTGF message.

AQUA Analysis of CTGF and TGFβ1 in the carcinoid TMA: Tissue microarray slides were stained as described[21,23]. Antigen retrieval and immunostaining for CTGF, TGFβ1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), cytokeratin and nuclei were as above. Protein expression (CTGF or TGFβ1) was determined using an automated tissue microarray reader. Automated image acquisition and analysis using AQUA has been described previously[21,23]. In brief, monochromatic, high-resolution (1024 × 1024 pixel; 0.5-μm) images were obtained of each histospot. Areas of tumor separate from stromal elements were distinguished by creating a mask from the cytokeratin signal. Coalescence of cytokeratin at the cell surface localized the cell membranes, and DAPI was used to identify nuclei. The Cy-5 signal from the membrane area of tumor cells was scored on a scale of 0-255 and expressed as signal intensity divided by the membrane area. Histospots containing < 10% tumor, as assessed by mask area (automated), were excluded from further analysis. Previous studies have demonstrated that the staining from a single histospot provides a sufficiently representative sample for analysis[24].

Serum techniques

CTGF serum ELISA: Serum CTGF-W (whole molecule) and CTGF-N (N-terminal fragment) were assayed by two separate sandwich enzyme-linked immunosorbent assays (ELISA). The CTGF-W ELISA uses a capture mAB reactive to the amino terminus of CTGF, and detects the bound CTGF-W with an alkaline phosphatase labeled mAb reactive to the carboxyl- terminal region of CTGF. A second ELISA uses two non-cross blocking monoclonal antibodies reacting to distinct NH_2-terminal epitopes of CTGF. This assay detected both CTGF-W and CTGF N fragment, so-called CTGF N + W, as described previ-

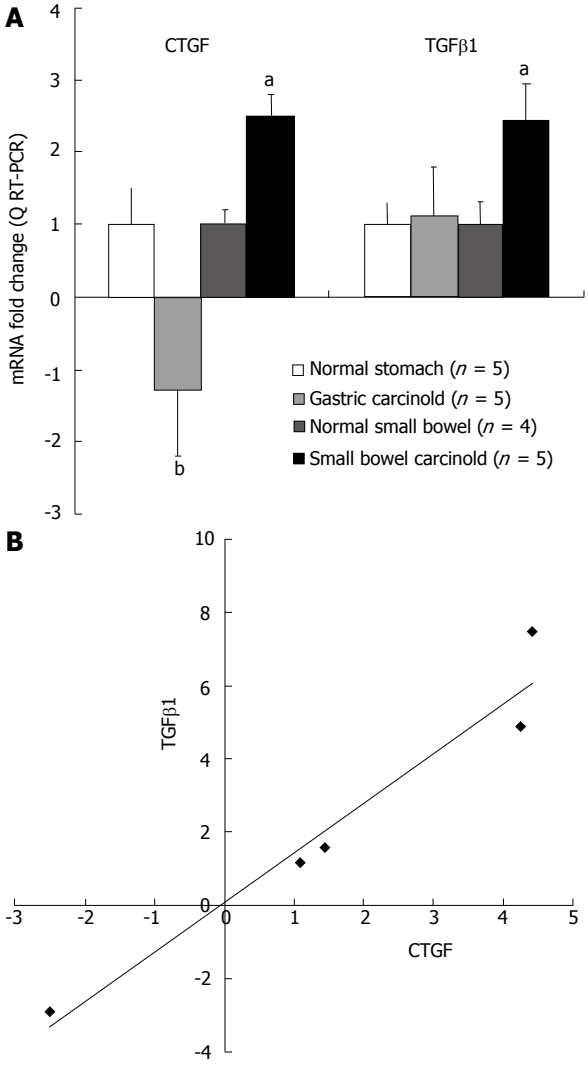

Figure 1 **A**: Message levels of both *CTGF* and *TGFβ1* determined by Q RT-PCR. Levels were corrected against expression of the housekeeping gene, *GAPDH*, compared to similarly corrected gene levels in normal mucosa, and represented as fold increase over normal (1.0). *TGFβ1* was significantly over-expressed (about 2.5-fold) in SI carcinoid tumor samples compared to normal mucosa ($^aP < 0.05$) but not the gastric carcinoids. CTGF was significantly over-expressed (about 2.5-fold) in SI carcinoid tumor samples compared to normal mucosa ($^aP < 0.05$) while gastric carcinoids had significantly decreased CTGF compared to SI carcinoid tumors ($^bP < 0.01$). Mean ± SE; **B:** Correlation analysis of QRT-PCR results in SI EC cell carcinoid tumors. There was a good correlation between CTGF and *TGFβ1* transcript levels in tumor samples ($R^2 = 0.9445$, $P < 0.01$, $n = 5$).

ously[25]. CTGF-N is a value calculated by subtracting CTGF-W from the CTGF N + W level measured by the second assay. Standards for both assays were made from purified full-length CTGF and expressed in nanograms per milliliter. The intra- and interassay coefficient of variation was 5 and 15%, respectively, for both ELISA tests. Data on CTGF-W is presented in this study.

Statistical analysis

Results are expressed as mean ± SE; *n* indicates the number of patients in each study group. Statistical significance was calculated by the Student's test for unpaired values or non-parametric statistics as appropriate. On the TMA, the unpaired 2-tailed Student's *t*-test was used to identify statistically significant differences in fibrotic protein expression between different patient groups (patients with clinical evidence of fibrosis versus non-fibrosis, fibrosis versus gastric carcinoid).

RESULTS

Quantitative RT-PCR

Q RT-PCR analysis was undertaken using Assays on Demand (Applied Biosystems) on the RNA isolated from SI EC cell carcinoid tumors (fibrosis associated) ($n = 5$); gastric ECL cell tumors (little fibrosis) ($n = 5$); normal SI mucosal samples ($n = 4$) and normal gastric mucosa ($n = 5$) to quantitatively measure the levels of CTGF and TGFβ1 mRNA expression in these two different tumor types. Transcript levels of both CTGF and TGFβ1 were significantly elevated in the five SI carcinoid tumor samples ($P < 0.05$ *vs* normal mucosa) (Figure 1A). In contrast, TGFβ1 message was not different (+ 1.13-fold) in gastric carcinoid tumor samples compared to normal, and message levels of CTGF were significantly decreased (-1.3-fold; $P < 0.01$) compared to SI carcinoid tumors (Figure 1A). There was a good correlation ($R^2 = 0.95$) between CTGF and TGFβ1 message levels in the SI carcinoid tumor samples demonstrating that transcription of these growth factors was tightly associated in this tumor tissue (Figure 1B). No relationship was noted between TGFβ1 mRNA levels and CTGF mRNA levels in gastric carcinoids ($R^2 = 0.01$). These results demonstrate while both gastric and SI carcinoid tumors express mRNA for TGFβ1, CTGF mRNA is over-expressed only in SI carcinoid tumors. CTGF and TGFβ1 transcript levels are associated in SI carcinoid tumors.

Immunohistochemistry

CTGF and TGFβ1 in tumor samples: CTGF was localized in the cytoplasm of SI carcinoid tumor cells (Figure 2). Co-staining with anti-CgA (Figure 2A) or anti-serotonin (Figure 2B) antibodies demonstrated a significant co-localization with CTGF and either antibody (80 ± 12% and 93 ± 6% respectively) in tumor mucosa. Like CTGF, TGFβ1 was cytoplasmic and was present in > 75% of tumor cells (Figure 2C). These results demonstrate that TGFβ1 and CTGF expression are characteristic features of SI EC cell carcinoid tumors.

Matrix production in fibrosis: α-smooth muscle actin-positive cells were identified interspersed with carcinoid tumor cells in areas of fibrosis (Figure 3A). α-smooth muscle actin is a marker for activated myofibroblasts (or stellate cells) and indicates that, as for the pancreas, stellate cells are present in peritoneal fibrotic material associated with SI carcinoid tumor mesenteric invasion[15]. Vimentin, desmin and collagen-Ⅲ positivity was identified with stellate cells (Figure 3B-D). These are markers of a TGFβ1-mediated stellate-cell driven fibrosis[15,19,26], and indicate that this response occurs in SI carcinoid tumors. CTGF was present in both tumor cells and stellate cells (Figure 3E and F), consistent with the expression of this fibrotic mediator in both cell types.

Intestinal stellate cell isolation and culture

Myofibroblasts from SI carcinoid tumor fibrotic surgical

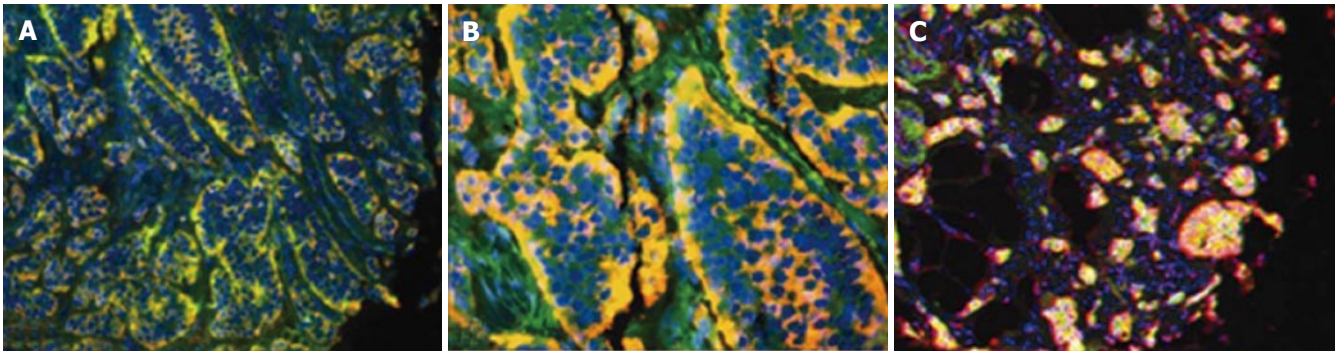

Figure 2 **A:** Triple color staining of nuclei (blue-DAPI), CgA (green-Alexa 488) and CTGF (red-Cy5) in a SI carcinoid tumor from the carcinoid TMA. Staining for both CgA and CTGF was cytoplasmic. Dual-stained (CgA + CTGF) cells are yellow. A majority of CgA cells (about 80%) were also CTGF positive (x 400); **B:** Triple color staining of nuclei, serotonin (green-Alexa 488) and CTGF in a carcinoid tumor from the TMA. Staining for both Serotonin and CTGF was cytoplasmic. Dual-stained (Serotonin + CTGF) cells are yellow. A majority of the serotonin cells (about 95%) were also CTGF positive. (x 600); **C:** Triple color staining of nuclei (blue-DAPI), cytokeratin (green - Alexa 488) and TGFβ1 (red-Cy5) in a carcinoid tumor from the TMA. Staining for TGFβ1 was cytoplasmic. A majority of the carcinoid tumor cells (cytokeratin-positive) (about 85%) were also TGFβ1 positive (x 200).

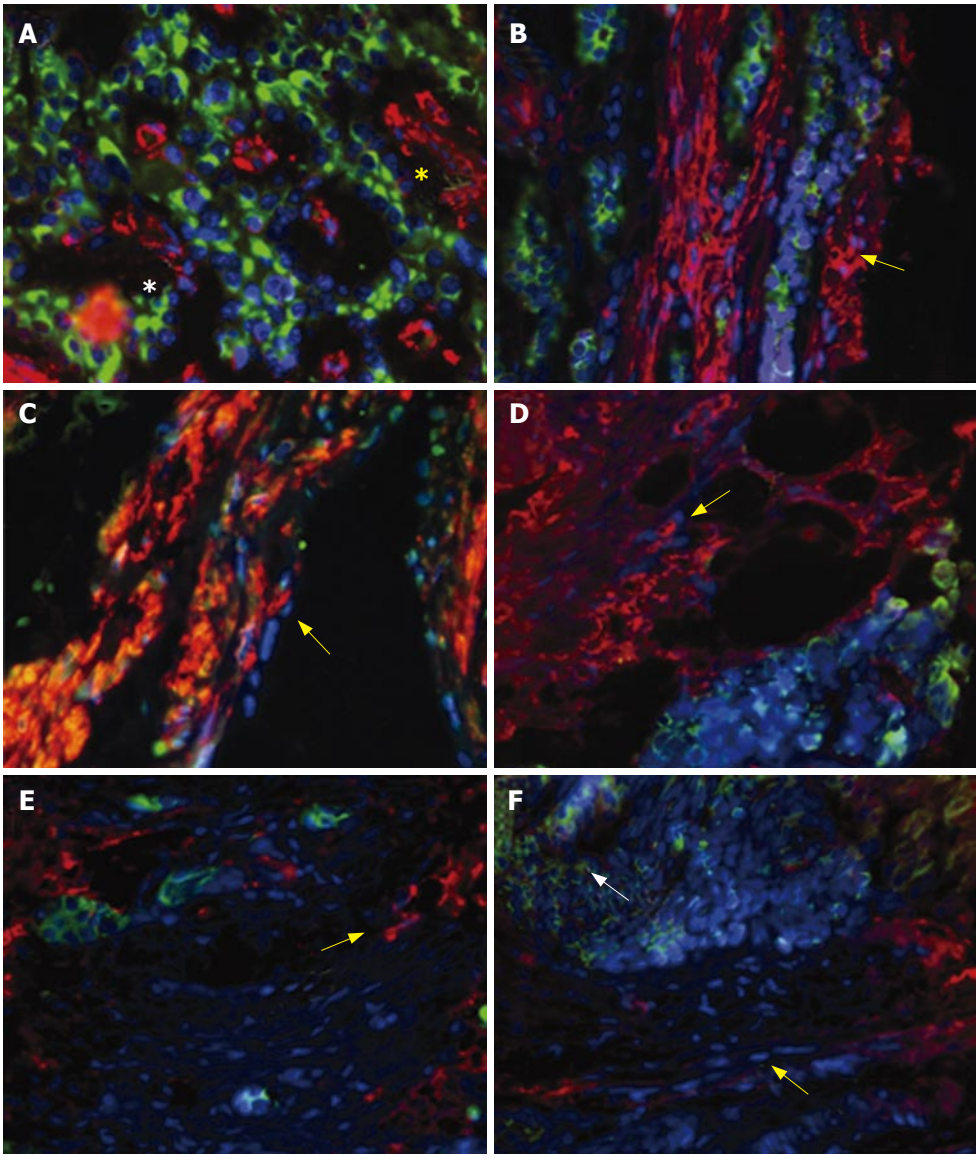

Figure 3: Immunostaining of areas of SI carcinoid tumor fibrosis with α-smooth muscle actin (**A**), vimentin (**B**), desmin (**C**), collagen III (**D**) and CTGF (**E/F**). Triple color staining of nuclei (blue-DAPI), cytokeratin-carcinoid tumor cells (green-Alexa 488) and the protein of interest (red-Cy5). (**A**) Discrete α-smooth muscle actin-positive cells (yellow star) were noted interspersed with tumor cells (white star) in areas of fibrosis. Cells consistent with myofibroblasts were associated with vimentin (**B**), desmin (**C**), collagen-III (**D**) and CTGF (**E/F**) production (yellow arrows). Within the fibrosis, carcinoid tumor cells were also CTGF-positive (**F**) (white arrow) (400 × magnification).

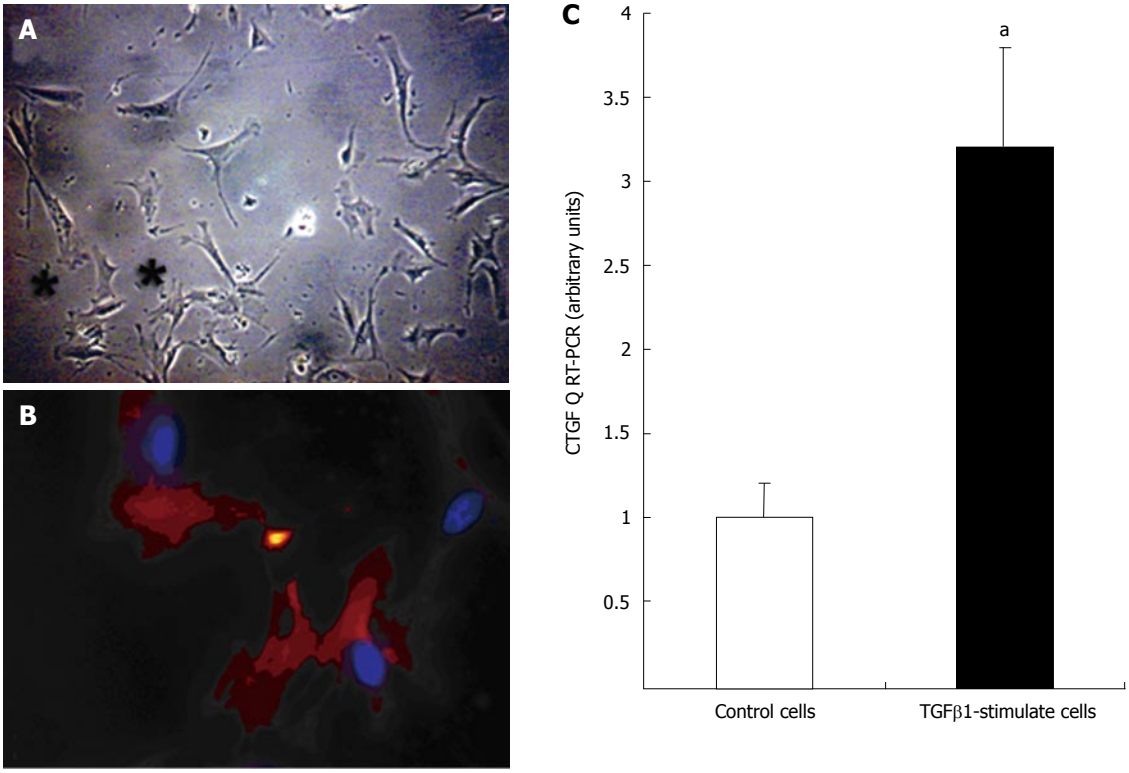

Figure 4 Micrographs of primary cultured human myofibroblasts isolated from human fibrotic material (SI carcinoid tumor). **A:** Light microscopy identified the typical stellate shape (black stars) in 5-day cultured cells (200 × magnification); **B:** Immunostaining with α-smooth muscle actin (Cy-5-red stain; nuclei are blue-DAPI) in same cells after 7-d culture (x 600); **C:** Message levels of *CTGF* determined by Q RT-PCR in primary cultured human myofibroblasts. *CTGF* was significantly over-expressed (about 3-fold) in TGFβ1 (10^{-7} mol/L, 24 h) stimulated cells compared to control (un-stimulated) cells ($^{a}P < 0.05$), mean ± SE, $n = 3$.

tissue were cultured on plastic as described. Cells in primary cultures flattened and developed long, cytoplasmic extensions. During the 5-7 d in culture, cells developed the typical stellate shape (Figure 4A) and became positive (100%) for α-smooth muscle actin-α marker of myofibroblasts (Figure 4B). This is the classical stellate cell (myofibroblast) activation pathway[15,19]. Stimulating the cells with TGFβ1 (10-7 mol/L) for 24 h significantly increased CTGF mRNA expression (3.2 ± 0.7, $P < 0.05$ *vs* un-stimulated cells) (Figure 4C).

AQUA Analysis of CTGF and TGFβ1

An examination of the CTGF-stained histospots from the 36 patients with SI carcinoid tumors demonstrated that CTGF expression levels ranged from: AQUA score: 49.7-186.3. Higher levels of CTGF staining (AQUA score: 92.5 ± 8.2; $P = 0.017$) were identified in the fifteen SI carcinoid tumor patients with clinical (surgical) and histologically documented evidence of peritoneal fibrosis compared to the twenty-one patients (AQUA score: 72.7 ± 3.2) with no evidence of fibrotic disease (Figure 5). CTGF levels in non-tumor, non-fibrotic normal SI mucosal tissue were significantly lower (59 ± 4, $P < 0.005$) than in patients with clinically and histologically documented fibrotic disease.

An examination of the CTGF-stained histospots from the seven patients with gastric carcinoids assessed by AQUA demonstrated that expression levels were not elevated in these patients compared to normal matched gastric mucosa (64 ± 3 *vs* 72 ± 3) but were significantly lower than in SI carcinoid tumors associated with fibrosis ($P < 0.03$).

An examination of the TGFβ1-stained histospots from patients with SI carcinoid tumors demonstrated that although TGFβ1 expression levels were elevated in patients with documented fibrosis (AQUA score: 90.6 ± 4.4) compared to the patients with no evidence of fibrotic disease (AQUA score: 82.7 ± 4.0) this did not reach statistical significance ($P = 0.08$). TGFβ1 levels were, however, lower in the matched normal SI mucosal samples (65 ± 4, $P < 0.05$ versus fibrotic tumor samples). In the gastric mucosa, expression levels were not elevated in patients with gastric carcinoids compared to normal matched mucosa (61 ± 5 *vs* 64 ± 3) but, as for CTGF, values in these non-fibrotic samples were significantly lower than in SI carcinoid tumors associated with fibrosis ($P < 0.03$).

CTGF serum ELISA

Serum levels of CTGF ranged from 7.2-171 ng/mL. Significantly higher serum CTGF levels were found in patients with SI carcinoid tumors (31.0 ± 10) than in patients with ECL cell carcinoids (12.5 ± 4.9, $P < 0.03$), other GI carcinoids (12.9 ± 0.6, $P < 0.04$) and control patients (12.4 ± 4, $P < 0.02$) (Figure 6). A comparison of serum CTGF levels with tissue levels of CTGF (AQUA scores) (where available) identified a strong correlation between these two measurements ($R^2 = 0.91$, $P < 0.005$, $n = 9$).

DISCUSSION

In the current study, we present data in support of our hypothesis that fibrosis is associated with invasion of

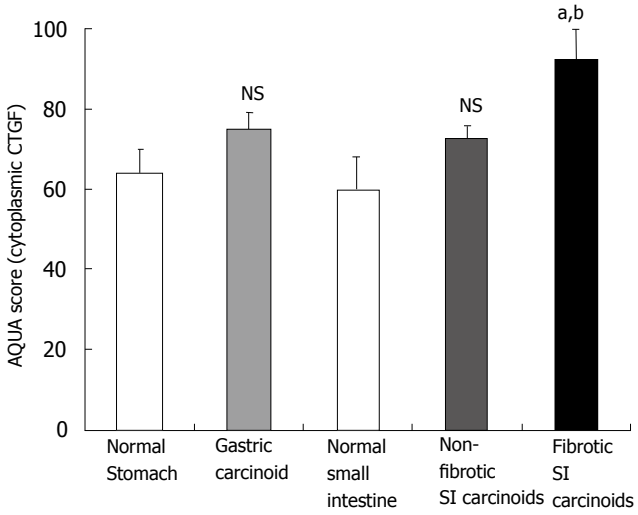

Figure 5 AQUA scores for CTGF protein expression in the TMA. Levels in tumors from carcinoid patients with clinically or histologically documented fibrosis (fibrotic SI carcinoid tumors) were significantly higher than tumors from patients with no evidence of fibrosis (non-fibrotic SI carcinoid tumors and gastric carcinoids) and normal mucosa. No differences in expression were noted between either non-fibrotic SI carcinoid tumors or gastric carcinoids and normal mucosa respectively. ($^aP < 0.05$ *vs* non-fibrotic SI carcinoid tumors, $^bP < 0.01$ *vs* normal SI mucosa). NS = not significant. mean ± SE.

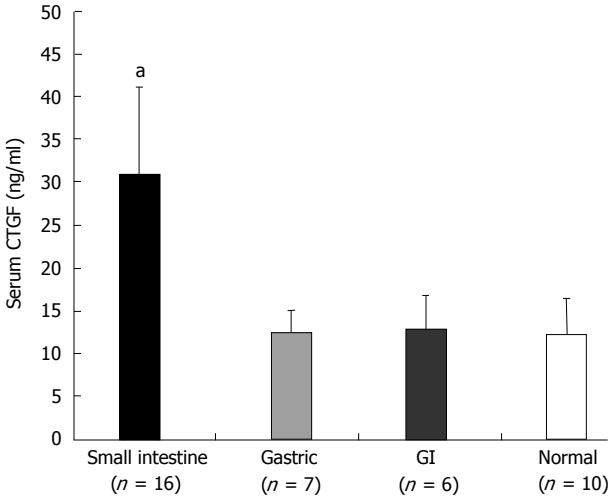

Figure 6 Serum levels of CTGF in patients with SI EC cell carcinoid tumors, gastric ECL cell carcinoids, other GI carcinoids [hepatic, rectal or appendiceal] and normal controls. Levels (ng/mL) were significantly elevated (> 2-fold versus all other patient groups) in patients with SI EC cell carcinoid tumors compared to the other GI carcinoid tumors. $^aP < 0.05$ *vs* all other samples. mean ± SE.

the mesentery by SI carcinoid tumor cells and is a consequence of the secretory activity of these cells. In addition we have demonstrated that the mechanism may be due to CTGF production, and TGFβ related events that activate an intestinal stellate (myofibroblastic) cell resulting in a local desmoplastic response. The latter is responsible for the clinical consequences of mesenteric fibrosis and adhesive obstruction noted in SI carcinoid tumors.

In our studies, Q RT-PCR demonstrated that all samples from patients with SI carcinoid tumors had elevated CTGF message levels (+ 1.1 to + 4.4-fold). In contrast, non-fibrotic gastric ECL cell carcinoids had significantly decreased CTGF levels. This analysis demonstrates that CTGF was quantitatively over-expressed in SI tumors. Message levels for TGFβ1 were elevated in SI carcinoid tumor samples but not in gastric samples. These results indicate that CTGF and TGFβ1 are potentially functionally related in the tumor EC cell but not in the ECL cell. We have previously reported that type I gastric (ECL cell) carcinoids (with no evidence of fibrosis) failed to express detectable levels of CTGF message by standard RT-PCR[27]. These results suggest that CTGF message produced by a transformed neuroendocrine cell (the SI EC cell) is associated with fibrosis.

Immunohistochemistry demonstrated that the majority (> 75%) of SI carcinoid tumor cells expressed CTGF. In normal mucosa, CTGF immunostaining was restricted to the basal third of the SI crypts with either CgA or serotonin-positive cells. Approximately one-third of serotonin-expressing (EC) cells were CTGF-positive (data not shown). It is likely that the remainder of the CTGF-staining cells are myofibroblasts in the crypts. CTGF-positive myofibroblasts have previously been demonstrated in the rectum[28].

Carcinoid tumor cells also express TGFβ1, and presumably this growth factor is secreted by these cells during mesenteric invasion. This was previously noted by Chaudhry *et al*[20] who reported that stromal cells expressed the TGFβ receptor. This suggests a mechanism by which tumor cells can interact with stromal cells and influence their function. Our immunohistochemical analysis demonstrated that stromal cells in areas of mesenteric fibrosis were α-smooth muscle actin positive. α-smooth muscle actin is a marker for activated myofibroblasts (or stellate cells[15,19]) and indicates that fibrosis-induction in the small intestine is associated with a stellate cell phenotype. This is a typical phenotype of both pancreatic- and hepatic-associated fibrosis[17,19], and suggests this may be an archetypical GI fibrotic phenomenon. This postulate is supported by evidence that vimentin, desmin and collagen-Ⅲ, all markers of a stellate-cell driven fibrosis, were present in SI fibrosis.

In order to confirm whether stellate cells were present in this tissue and played a role in fibrosis, we isolated and characterized a cell type from a patient with SI carcinoid tumor fibrosis that exhibited the hallmarks of a stellate cell[15]. During primary culture, this cell flattened, initially developed long, cytoplasmic extensions, and subsequently, the typical stellate shape of activated myofibroblasts. The presence of α-smooth muscle actin staining confirmed the stellate cell phenotype. Addition of TGFβ1 resulted in activation of CTGF message and demonstrated the cell type to be functionally responsive to this growth factor. These functional data, together with the immunohistochemical evidence of activated intestinal stellate cells in situ, strongly suggest that carcinoid-induced fibrosis is a stellate-cell induced phenomenon. It is possible that the "intestinal stellate" cell could be derived from precursor cells in blood stream and there is some evidence that bone marrow-derived cells can migrate into the SI[29]. A study of hepatic stellate cells, however, conclusively identified that these cells were not derived from bone marrow derived fibrocytes[30]. The latter did not stain for a-smooth muscle

actin or desmin and were considered a separate population within the liver. This, as well as our immunohistochemical results strongly suggests the presence of an endogenous intestinal stellate cell population.

Having established that mesenteric fibrosis was associated with elevated CTGF and TGFβ1 in SI carcinoid tumors and identified a mesenteric target cell (intestinal stellate cell), we next used TMA analysis to both quantitate the protein expression as well as the cellular source of CTGF and TGFβ1 and statistically determine whether these proteins were related to clinically and histologically documented evidence of fibrosis. Our results demonstrated that TGFβ1 levels were elevated in patients with fibrosis, and were significantly increased compared to normal SI mucosa and to gastric carcinoids. The difference in protein expression between fibrotic SI carcinoid tumors and non-fibrotic gastric carcinoid samples identified on the TMA further supports a role for TGFβ1 in the etiology of this fibrosis. The role of CTGF was confirmed by the unambiguous relationship between increased expression of CTGF protein in primary SI carcinoid tumors and fibrosis. It is of interest to note that five patients who initially had exhibited elevated CTGF AQUA scores (87 ± 5) on the TMA subsequently developed fibrosis.

In order to identify a clinically useful tool to recognize patients at risk for fibrosis, we sought to measure CTGF in serum. Secreted CTGF protein could be identified in patient serum and was elevated in patients with SI carcinoid tumors compared to patients with gastric ECL cell carcinoids. Serum levels of CTGF from the latter patient group were similar to values in control subjects as might be predicted given that the gastric carcinoids are not associated with carcinoid fibrosis. The highest levels of serum CTGF in this study were identified in two patients with SI carcinoid tumors who also had the typical carcinoid "flushing" symptoms consistent with disseminated disease. This suggests this protein is identifiable in serum and can discriminate SI from gastric carcinoids. Prospective longitudinal studies in patients with and without fibrosis are needed to determine whether plasma levels have clinical significance in the detection, or prediction of peritoneal or cardiac fibrosis.

In conclusion, SI carcinoid tumors over-express CTGF and TGFβ1 mRNA and synthesize CTGF and TGFβ1 protein which are significantly elevated in patients with clinically documented fibrosis. In addition, SI carcinoid tumors secrete CTGF, which is readily detectable in the serum. We have also immunohistochemically identified and biochemically characterized intestinal stellate cells from mesenteric fibrosis. These cells respond to TGFβ1 with CTGF mRNA transcription. In addition, matrix production in SI carcinoid tumor fibrosis was similar to that identified in other stellate cell-driven reactions (e.g., liver or pancreas)[15,17,19]. We postulate that intestinal stellate cells are the target cells that are activated by profibrotic mediators (TGFβ1 and CTGF) synthesized and secreted by invasive SI carcinoid tumor cells. Furthermore, once activated, these stellate cells may auto-regulate the fibrotic phenotype (by production of CTGF). The detection of blood levels of CTGF may ultimately provide a diagnostic opportunity to predict the development of fibrosis and pre-empt its local and systemic complications.

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BASIC RESEARCH

Exogenous sphingomyelinase causes impaired intestinal epithelial barrier function

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Abstract

AIM: To test the hypothesis that hydrolysis of sphingomyelin to ceramide changes the composition of tight junctions (TJs) with increasing permeability of the intestinal epithelium.

METHODS: Monolayers of Caco-2 cells were used as an *in vitro* model for the intestinal barrier. Permeability was determined by quantification of transepithelial flux and transepithelial resistance. Sphingolipid-rich membrane microdomains were isolated by a discontinuous sucrose gradient and characterized by Western-blot. Lipid content of microdomains was analysed by tandem mass spectrometry. Ceramide was subcellularly localized by immunofluorescent staining.

RESULTS: Exogenous sphingomyelinase increased transepithelial permeability and decreased transepithelial resistance at concentrations as low as 0.01 U/mL. Lipid analysis showed rapid accumulation of ceramide in the membrane fractions containing occludin and claudin-4, representing TJs. In these fractions we observed a concomitant decrease of sphingomyelin and cholesterol with increasing concentrations of ceramide. Immunofluorescent staining confirmed clustering of ceramide at the sites of cell-cell contacts. Neutralization of surface ceramide prevented the permeability-increase induced by platelet activating factor.

CONCLUSION: Our findings indicate that changes in lipid composition of TJs impair epithelial barrier functions. Generation of ceramide by sphingomyelinases might contribute to disturbed barrier function seen in diseases such as inflammatory, infectious, toxic or radiogenic bowel disease.

INTRODUCTION

Abnormal mucosal permeability is a hallmark of inflammatory bowel disease (IBD)[1,2]. The epithelial barrier function and its relevance in IBD pathophysiology has gained increasing attention in recent years[2] as it may contribute to increased bacterial translocation and subsequent inflammatory responses in the mucosa.

Many signaling molecules involved in the pathogenesis of IBD such as TNF-α or IFN-γ may cause an alteration of the lipid composition in the cell membrane by activation of sphingomyelinases (SMases)[3,4]. SMases are characterized by a specific optimal pH and accordingly are divided into acid, neutral and basic sphingomyelinase species. The acid sphingomyelinase (ASM) contributes to lysosomal sphingomyelin turnover and is also secreted upon cellular treatment with inflammatory stimuli[5]. In contrast, neutral SMase is membrane-bound, alkaline SMase is found in the bile. Activation of SMases is followed by rapid hydrolysis of plasma membrane sphingomyelin to the second messenger ceramide[6]. The sphingolipid ceramide is an important messenger involved in many signaling pathways with influences on cell differentiation, growth suppression and apoptosis. It is generated upon ligation of receptors like cluster of differentiation (CD) 40, CD95, IL-1 or TNF receptor[7-10], as response to ionizing radiation[11-13], ischemia-reperfusion injury[14] and infections with bacteria like *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*[15-17] or viruses like Sindbis- or Rhinovirus[18,19]. Ceramide alters the composition of cholesterol- and sphingolipid-enriched membrane microdomains[20] and thereby promotes transmembrane signaling[7-9]. It also has the capacity to restructure the membrane to allow the release of vesicles[21].

Grassme *et al*[7,8] have demonstrated that cellular stimulation triggers a translocation of the acid sphingomyelinase from intracellular stores onto the extracellular leaflet of the cell membrane. Surface ASM initiates a release of ceramide which mediates clustering of sphingolipid-rich membrane domains, termed "lipid rafts". Lipid rafts are also described as detergent insoluble cholesterol- and glycosphingolipid-enriched membrane microdomains (DIGs) because the tight packing of the lipids renders rafts resistant to solubilization by non-ionic detergents at low temperatures[22,23].

Intestinal permeability is influenced by the lipid content of epithelial cells. Dietary fatty acids are known to affect barrier function of the mucosa[24,25]. Clinical studies showed that omega 3-fatty acids may be of beneficial effect on the course of Crohns disease[26,27]. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight-junction (TJ). TJs have been identified as microdomains in the plasma membrane with similar characteristics as DIGs[28,29]. Depletion of cholesterol from Caco-2 cell layers increases permeability[30], suggesting that cholesterol is critical in maintaining the barrier function.

The proinflammatory cytokines TNF-α and IFN-γ, known to induce ceramide-generation, have recently been shown to disrupt the barrier function of epithelial cells independent from their apoptosis-inducing property[31]. With regard to the finding that ceramide displaces cholesterol from sphingolipid-enriched microdomains[20], we hypothesized that formation of ceramide may be an initial event leading to structural lipid-rearrangements of TJs with impaired barrier integrity.

To test this hypothesis we used the intestinal epithelial cell line Caco-2 to assess the effect of exogenous SMase on barrier function and the accompanying lipid composition of TJs.

MATERIALS AND METHODS

Cells and reagents

The human intestinal epithelial cell line Caco-2 was obtained from American Type Culture collection (HTB 37). Cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids 1% sodium pyruvate in an atmosphere containing 10% CO_2 at 37℃. Sphingomyelinase from staphylococcus aureus, platelet activating factor (PAF) and deoxycholic acid were purchased from Sigma-Aldrich, Germany.

Antibodies

The following antibodies were used for Western-blot analysis: Goat polyclonal anti-occludin (C-19) and goat polyclonal anti-claudin-4 (C-18) from Santa Cruz, CA, USA; mouse anti-E-cadherin (clone HECD-1) from Calbiochem, CA, USA; rabbit polyclonal anti p38 mitogen activated protein kinase (MAPK) from New England Biolabs, Beverly, MA, USA. The rabbit polyconal anti-toll like receptor 4 (TLR4) Ab was a kind gift from Dr. Werner Falk, Regensburg, Germany. For immunohistochemical staining of tight junctions we used the rabbit polyclonal anti-ZO-1 Ab from Zymed lab. Inc., CA, USA. For visualization and neutralization of ceramide, the monoclonal mouse IgM anti-Ceramide Ab (MID 15B4) from Alexis, Germany was used.

Measurement of transepithelial permeability and transepithelial resistance

For permeability assays, Caco-2 cells were seeded in 12 well plates with a growth area of 1.0 cm^2 and a pore size of 3 μm (Transwell® permeable supports by Corning Incorporated, MA, USA) at a density of 2×10^5 cells/cm^2. Media was replaced every 3 or 4 d. Experiments were performed 13-15 d after cells reached confluency with a transepithelial electrical resistance (TEER) between 500-750 $\Omega \cdot cm^2$. Permeability was quantified by measuring the transepithelial flux of fluorescein-sulfonic acid (Molecular Probes Inc., Germany). After treatment of Caco-2 monolayers with the indicated substances, fluorescein-sulfonic acid was added to the apical side of the monolayers at a final concentration of 100 μg/mL. After incubation for 4 h 100 μL aliquots of medium were removed from the basolateral chambers, and fluorescence signal by fluorescein was measured using a fluorescence microplate reader. TEER of the Caco-2 monolayers was measured using a Millicell®-ERS epithelial voltohmmeter by Millipore with a pair of chopstick electrodes. Untreated monolayers were used as negative controls. All measurements were performed in duplicate.

Caspase-3/7 activity assay

Caco-2 cells were incubated with the indicated substances 6 h before measurement of caspase-activity. As a positive control for induction of apoptosis, cells were treated with deoxycholic acid (DCA) for 1 h. DCA was sonicated at 40℃ for 20 min prior to the experiments. The colorimetric caspase-3/7-activity assay Apo-ONE™ (Promega, WI, USA) was used according to the manufacturer's recommendations. Activity was quantified using a fluorescence microplate reader with appropriate wavelengths for excitation (485 nm) and emission (530 nm).

Fluorescence microscopy

For fluorescence studies Caco-2 cells were grown on chamber slides (Nunc, Germany) and stimulated with 0.25 U/mL SMase for 10 min. After stimulation the cells were washed with phosphate buffered saline (PBS) at 4℃ and fixed for 15 min in 1 % (w/v) paraformaldehyde in PBS at room temperature. Then cells were washed three times with PBS and blocked with Tween 20 0.05% in PBS. Cells were washed again and then incubated with anti-ceramide mAb 15B4 (1/50 dilution) and anti ZO-1 Ab for 30 min in washing buffer (PBS with 2% fetal calf serum, 0.01% NaN_3) to block sites of non-specific binding. The anti-ceramide Ab was visualized with a PE-labeled anti-mouse IgM, the anti-ZO-1 Ab with a fluorescein-isothiocyanate (FITC)-anti rabbit Ab. Fluorescence staining was viewed with an Axiovert 100 fluorescence microscope (Zeiss, Germany).

Isolation of detergent resistant microdomains

Confluent layers of Caco-2 cells were stimulated in 75 cm^2 flasks in 5 mL of culture media. Stimulation was terminated by washing the cells with 5 mL ice-cold TNE (25 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, and 5 mmol/L EDTA) on ice. Cells were washed twice with TNE at 4℃ and lysed for 20 min in 1.5 mL of ice-cold TNE containing 1% of Triton X-100 and protease inhibitors (10 μg of aprotinin and leupeptin and 200 μmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were then further homogenized with 20 strokes in a Wheaton loose fitting Dounce-homogenizer. Nuclei and cellular debris were pelleted by centrifugation at 600 g for 5 min, 4℃. To isolate low-density, Triton X-100-insoluble complexes, the supernatant was adjusted to 40% sucrose, transferred to an ultracentrifugation tube, and overlaid with a 35% and 5% discontinuous sucrose gradient. Ultracentrifugation was performed at 29 000 r/min (110 000 × *g*) at 4℃ for 16 h in a Beckman SW41 rotor. Gradient fractions (1 mL) were either collected from the top of the tube or from the 35%/5%-interface to compare the effect of cell stimulation on raft fractions. For mass spectrometry, fractions were prepared as described below. For Western blotting, proteins were precipitated using 400 μL of 10% trichloroacetic acid, neutralized and separated by 10% SDS-PAGE.

Immunoblotting

Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Hybond). Blots were blocked with 5% nonfat dry milk powder and incubated overnight at 4℃ with the indicated primary antibodies. All antibodies were diluted 1:750 in Tris-buffered saline supplemented with 0.1% Tween 20. The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized using a commercial chemiluminescence detection kit (ECL plus, Amersham Biosciences).

Tandem mass spectrometry

Lipids were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode as described previously[32-35]. Samples were quantified by direct flow injection analysis using the analytical setup and the data analysis algorithms described by Liebisch *et al*[34]. A parent ion scan of m/z 184 specific for phosphocholine-containing lipids was used for phosphatidylcholine, sphingomyelin[33] and lysophosphatidylcholine[33]. Neutral loss scans of m/z 141 and m/z 185 were used for phosphatidylethanolamine and phosphatidylserine, respectively. Ceramide was analyzed similar to a previously described method[35] using N-heptanoyl-sphingosine as internal standard. Free cholesterol and cholesteryl esters were quantified using a fragment ion of m/z 369 after selective derivatization of free cholesterol[36]. Quantification was achieved by calibration lines generated by addition of naturally occurring lipid species to cell homogenates[32-36].

Statistical analysis

Data are shown using vertical scatter plots with Box-Whisker plots (25% and 75% values), generated in the basic module of the program SigmaPlot. Statistical analysis was performed by Mann-Whitney *U*-test, with $P < 0.05$ considered statistically significant. Data are given as means ± SE (SD in case of lipid analysis).

RESULTS

Exogenous sphingomyelinase enhances permeability in Caco-2 epithelial cell layers

To study a potential regulation of intestinal permeability by sphingomyelinases, Caco-2 cell layers were exposed to different concentrations of exogenous SMase. Transepithelial permeability was determined by measurement of transepithelial flux of fluorescein-sulfonic acid across a monolayer grown on permeable supports. Incubation with SMase to the apical chamber induced a concentration-dependent increase of permeability which could be detected at concentrations as low as 0.01 U/mL SMase (181.6% ± 16.7%, $P < 0.01$) (Figure 1A). Using 0.05 U/mL SMase, permeability was increased by 201.1% ± 15.8% ($P < 0.01$) and by 224.0% ± 18.0% ($P < 0.01$) when 0.125 U/mL SMase were used. Increase of SMase-concentration to 0.25 U/mL did not further increase transepithelial flux (192.0% ± 15.3%, $P < 0.01$) (Figure 1A). In a different set of experiments with the same experimental conditions, PAF was used as a positive control. At a concentration of 5 μmol/L, PAF increased permeability by 162.8% ± 13.0% (Figure 2).

To gain insight into the mechanisms of ceramide-mediated permeability we measured the transepithelial electrical resistance (TEER). Exogenous SMase produced a significant decline in TEER at concentrations as low as 0.01 U/mL (17.5% ± 6.2%, $P < 0.05$) (Figure 1B). The fall in TEER with 0.05 U/mL was much higher (38.1% ± 6.0%, $P < 0.01$). Using 0.125 U/mL SMase or 0.25 U/mL SMase did not further decrease TEER (32.2% ± 7.3%, $P < 0.01$ and 33.2% ± 6.4%, $P < 0.01$, respectively).

To exclude apoptotic or necrotic cell death caused by SMase within the time frame of our experiments, caspase-3/7-activity and LDH release assays were performed. As shown in Figure 1C, 0.25 U/mL SMase induced no activation of caspase-3/7 within 6 h. Deoxycholic acid (500 μmol/L for 1 h) was used as a positive control. Release of LDH from Caco-2 monolayers by SMase was also not detectable (data not shown).

Neutralization of surface ceramide prevents permeability-increase induced by PAF

Next, we investigated whether the increased permeability induced by PAF might be linked to rearrangement of tight-junctional lipids. Incubation of the monolayers with 5 μmol/L PAF increased permeability by 162.8% ± 13.0% (Figure 2). To examine the role of ceramide in PAF-mediated permeability we co-incubated Caco-2 cell layers with ceramide-antiserum (1/100 dilution). Co-incubation of the Caco-2-monolayer with ceramide-antiserum prevented the increase of permeability induced by 5 μmol/L PAF (111.6% ± 9.86%, $P < 0.05$) (Figure 2), indicating a stabilization of tight-junctional complexes by the IgM-anti-ceramide Abs.

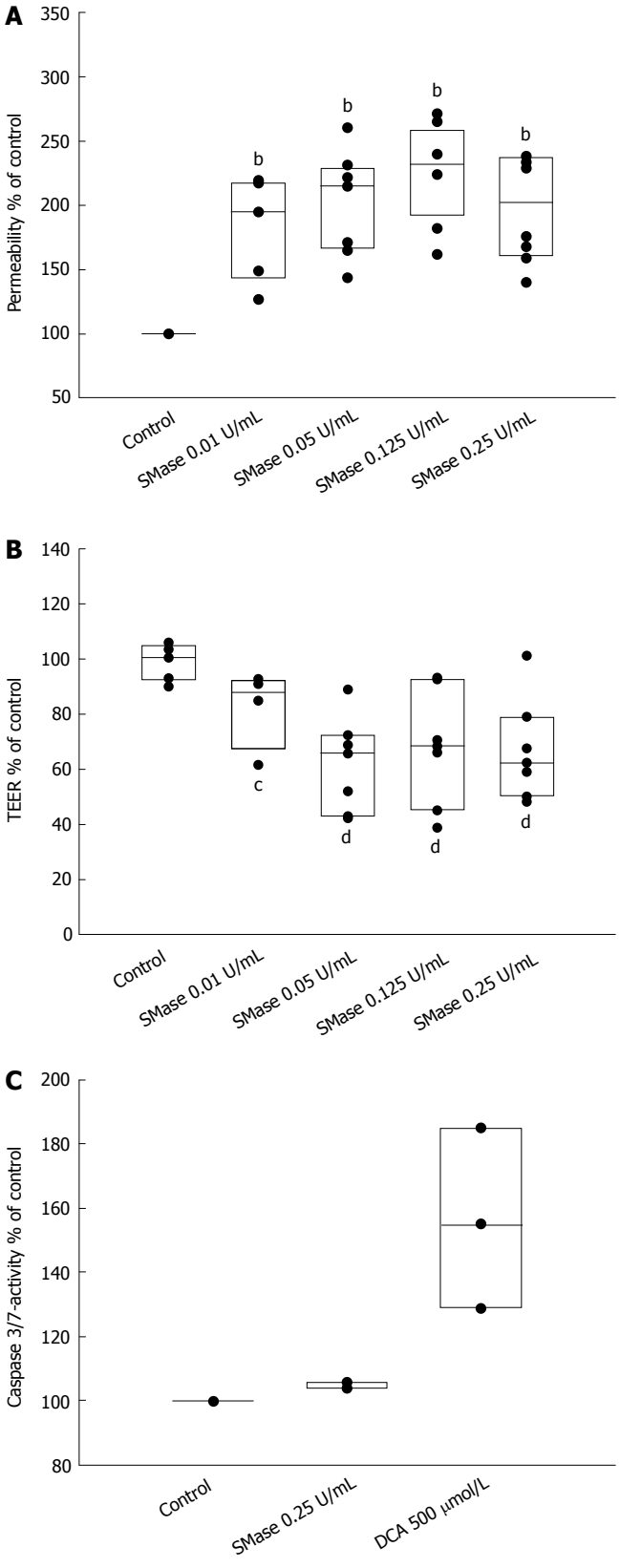

Figure 1 Exogenous sphingomyelinase increases permeability of Caco-2 epithelial monolayers. **A**: Caco-2 monolayers were incubated with different concentrations of exogenous SMase. $^{b}P < 0.01$ between control and treated samples; **B**: Transepithelial electrical resistance (TEER) was measured across the cell monolayers 4 h after treatment of the monolayers with exogenous SMase. The data were compared with TEER before treatment and are expressed as % of untreated, $^{c}P < 0.05$, $^{d}P < 0.01$; **C**: Epithelial apoptosis was monitored 6 h after incubation with 0.25 U/mL SMase by measurement of caspase-3/7-activity with colorimetric assays. As a positive control, deoxycholic acid (DCA) was used for 1 h.

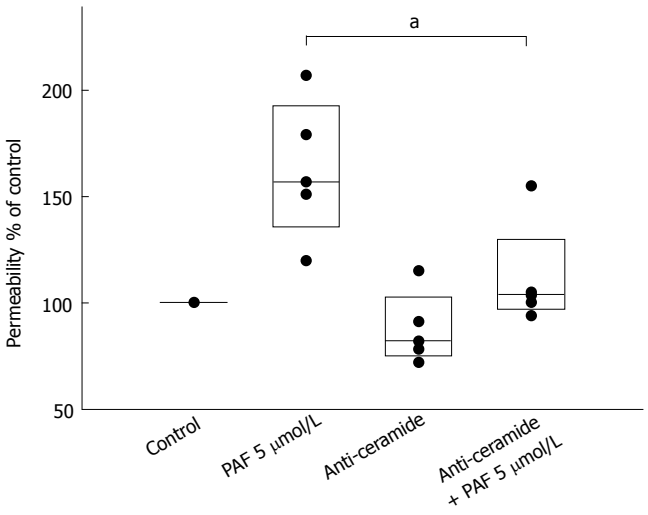

Figure 2 Neutralization of surface-ceramide prevents PAF-mediated increase of permeability. Caco-2 cell layers were incubated with the IgM ceramide-antiserum (15B4) 30 min prior to stimulation with PAF. Permeability was determined by measurement of transepithelial flux of fluorescein sulfonic acid from the upper chamber of the Transwell insert to the lower chamber across the Caco-2-monolayer after 4 h. The data are expressed as % of control. $^{a}P < 0.05$ between PAF-treated samples.

Detergent insensitive glycosphingolipid-enriched domains (DIGs) contain major pools of tight junction proteins like occludin and claudin 4

To further test our hypothesis, DIGs were isolated using sucrose gradient techniques and analysed for their lipid- and protein-composition. Analysis of the lipid composition by tandem mass spectrometry revealed the presence of high amounts of sphingomyelin (12.5% ± 1.4%), cholesterol (44.9% ± 4.9%) and ceramide (0.74% ± 0.18%) in DIGs with only 3.8% ± 0.6%, 19.2% ± 3.9% and 0.19% ± 0.03% in the total cell lysate (including DIGs), respectively (Figure 3A, values are given as % of analysed lipids ± SD). This indicated a good separation of DIGs from other membrane components. To ascertain isolation of tight junctions within the Triton X-100 insoluble preparations, Western-blot experiments were performed to prove the presence of tight-junctional proteins. As shown in Figure 3B, major pools of the tight-junctional proteins occludin and claudin-4 were present in Triton X-100 insoluble preparations and to a lesser extent in cell lysate. The basolateral membrane protein E-cadherin was also present in DIGs but was not as prominent. To exclude contamination with proteins not associated with DIGs, Western-blots of TLR4 and p38MAPK were performed which could not be detected in preparations of DIGs (Figure 3B).

Ceramide accumulates in DIGs, whereas sphingomyelin and cholesterol decrease

Tandem mass spectrometry was used to analyze the effect of SMase on the lipid content of DIGs which overlap with tight junctions. Incubation of Caco-2 monolayers with 0.25 U/mL SMase resulted in rapid increase of ceramide in DIGs by 5.0% ± 2.4% after 10 min ($P < 0.01$) and 7.3% ± 2.6% after 30 min ($P < 0.01$), whereas the increase was

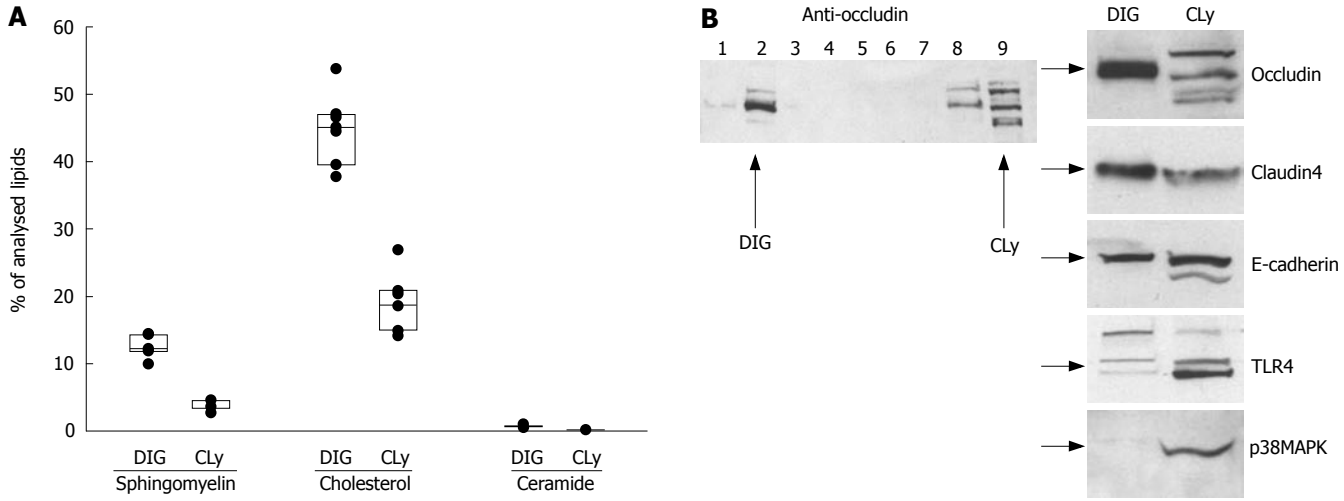

Figure 3 Preparations of detergent insensitive glycosphingolipid-enriched domains (DIGs) contain TJ-proteins. **A**: Lipid analysis of DIGs from non-stimulated Caco-2 cells by tandem mass spectrometry revealed the presence of high amounts of sphingomyelin, cholesterol and ceramide when compared to the total cell lysate; **B**: Gradient fractions were separated by 10% SDS-PAGE and analyzed by Western blot. Fraction No. 2 represents the raft-containing fraction at the 35%/5% interface (DIG); No. 9 represents the soluble fraction (cell lysate = CLy). The presence of high amounts of TJ-proteins occludin and claudin-4 and, to a lesser extent, E-cadherin show a clear association of TJ-proteins with DIGs. The simultaneous absence of other proteins like TLR4 and p38MAPK excludes significant contamination with non-raft fractions.

only 0.84% ± 0.14% and 1.25% ± 0.23% in the whole cell lysate (including DIGs), respectively (Figure 4A, data shown as % of analysed lipids). In the same preparations of DIGs the concentration of sphingomyelin decreased by 4.73% ± 1.4% after 10 min ($P < 0.01$) and 7.6% ± 2.2% after 30 min ($P < 0.01$) (Figure 4B). The concentration of cholesterol declined by 5.0% ± 3.2% after 10 min and 4.8% ± 2.3% after 30 min (Figure 4C). This reflects a decrease of the percentage of cholesterol by 10.9% ± 7.3% after 10 min ($P < 0.05$) and 10.6% ± 4.8% after 30 min ($P < 0.01$).

Clusters of ceramide colocalize with tight junctions

Fluorescence microscopy was performed to demonstrate the localization of ceramide accumulation. We induced generation of ceramide by incubation of Caco-2 cells with 0.25 U/mL SMase for 10 min. After stimulation, staining of the cells with anti-ceramide 15B4 antibodies revealed the formation of ceramide-clusters (Figure 5A and B) that were frequently located at the sites of cell-cell-contact. Costaining for the tight-junction protein ZO-1 confirmed colocalization of ceramide-clusters with the junctional complexes (Figure 5B).

These data indicate that the generation of ceramide by SMase leads to localized accumulation of ceramide at the sites of cell/cell-contact with increased intestinal permeability and suggest a central role of ceramide in the regulation of barrier integrity.

DISCUSSION

Our data indicate that hydrolysis of sphingomyelin to ceramide increases intestinal epithelial cell permeability in a well-established model of Caco-2 cell monolayers[37]. To obtain naturally occurring long chain ceramides, formation of ceramide was induced by addition of bacterial sphingomyelinase (SMase). This approach was used because neutral as well as acid SMase are capable of generating ceramide in the outer leaflet of the cell membrane by rapid hydrolysis of membrane sphingomyelin. Previous studies suggested that, upon cellular stimulation, the acid sphingomyelinase (ASM) translocates onto the outer surface of sphingolipid-rich membrane microdomains[7,8]. The fact that ASM activity is also increased in the serum of mice treated with endotoxin[38] or PAF[39], indicates a possible role of extracellularly located ASM. Another advantage of using exogenous SMase is the enzymatic cleavage of sphingomyelin at the sites of sphingomyelin-enriched domains in the cell membrane, whereas the addition of long chain ceramides might lead to lateral assembly of ceramide to pre-existing DIGs (and/or TJs) without affecting the structure inside these microdomains. Apart from that, exogenous ceramides are likely to be rapidly metabolised, thus having a rather transient effect on lipid composition. Exogenous SMase was effective in terms of increased permeability and decrease of TEER, indicating a reduction of the paracellular barrier by hydrolysis of sphingomyelin. Other possible effects of SMase include an increase of transcellular transport of vesicles. Therefore, we measured intracellular fluorescence after exposure of the cells with SMase and fluorescein-sulfonic acid with colorimetric assays of the cell lysates and performed transepithelial flux experiments with simultaneous colchicine-treatment (data not shown). In both cases we did not observe any differences, making transcellular transport unlikely. To exclude damage of monolayers as a possible cause for increased permeability, activity of caspase-3/7 in the cell lysate and lactate dehydrogenase (LDH) in the supernatant were measured without significant results.

Lipid analysis of Triton X-100 insoluble preparations clearly demonstrated the separation of membrane portions which were enriched in cholesterol, sphingomyelin and ceramide. Western-blot experiments of DIGs revealed that these preparations contained major pools of the TJ-proteins occludin and claudin-4. Upon stimulation with

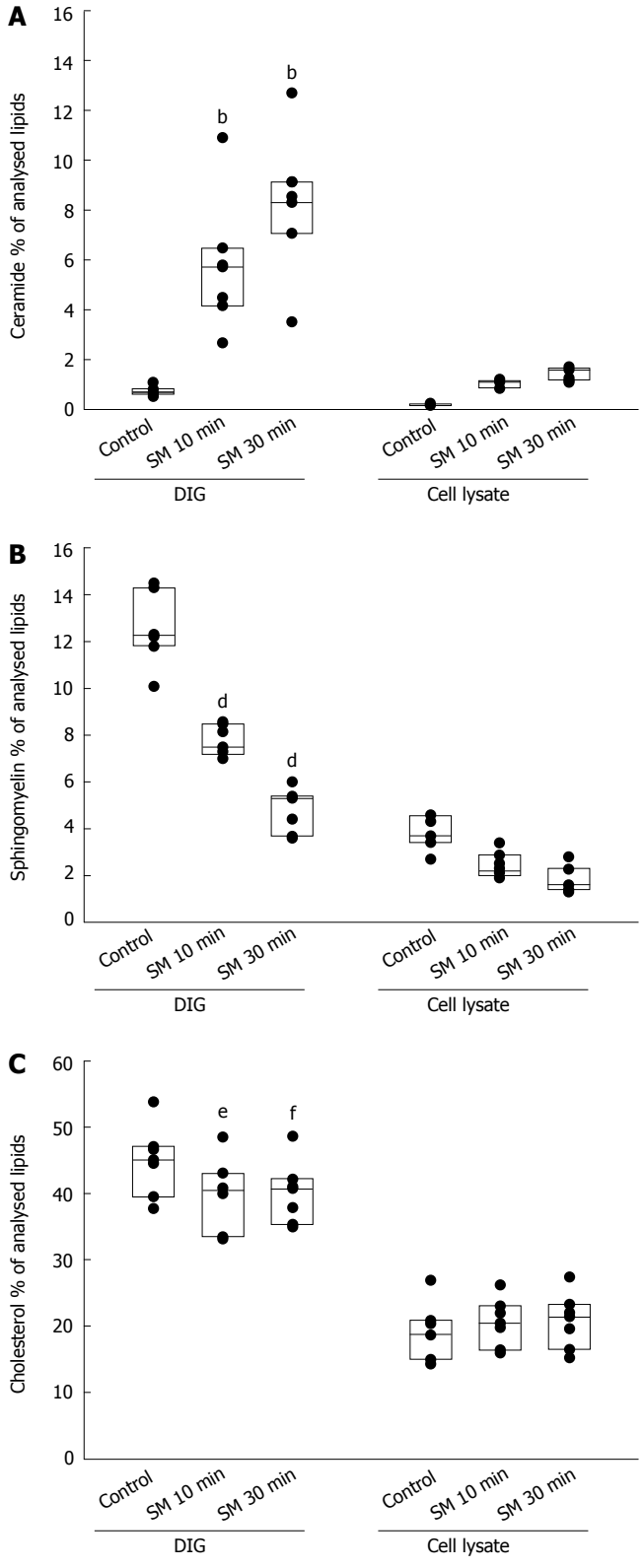

Figure 4 **A**: Lipid analysis by tandem mass spectrometry revealed a rapid accumulation of ceramide in preparations of DIGs after incubation with 0.25 U/mL SMase for 10 or 30 min whereas the increase of ceramide in the cell lysate was only small in comparison ($^{b}P < 0.01$); **B**: The content of sphingomyelin in DIGs decreased at the same time with a relative reduction comparable to the generation of ceramide ($^{d}P < 0.01$); **C**: Cholesterol also decreased in DIGs whereas the concentration in the cell lysate remained constant ($^{e}P < 0.05$ and $^{f}P < 0.01$).

SMase we detected a rapid accumulation of ceramide in the same membrane fractions, indicating selective accumulation of ceramide in sphingolipid-enriched membrane microdomains, including junctional complexes.

Previous studies suggested displacement of cholesterol from DIGs by ceramide[20,40,41]. To our knowledge, there is no data in the available literature about the modulation of ceramide and cholesterol in TJs by sphingomyelinases. Therefore we compared the content of cholesterol in DIGs which overlap with TJs. In accordance with these studies we also observed a decrease of cholesterol in these microdomains with increasing concentrations of ceramide, suggesting a rearrangement of tight junctional lipids by ceramide with impaired barrier function.

Lambert *et al*[30] showed a decrease of TEER by 80%-90% after extraction of 40%-45% of cholesterol from Caco-2 monolayers. Compared to these results the decrease of tight-junctional cholesterol by ceramide is moderate. Nevertheless, these changes could explain early disturbances of tight-junctional integrity upon cellular stress, deriving from toxic, infectious or immunologic challenges of the intestinal epithelium. Therefore, we propose the following pathophysiologic model: Cellular stimulation leads to localized accumulation of ceramide with concomitant decrease of sphingomyelin and cholesterol leading to destabilization of tight junctional strands and loss of barrier integrity.

Long chain ceramides also have the unique property of fusing membrane domains and tend to form cluster, which was also observed in our studies. Mechanisms of ceramide to modify the structure of bilayers and their effect in promoting efflux and release have been studied in several models[21,42,43]. Montes *et al*[21] demonstrated release of molecules with a molecular mass up to 20 kDa upon treatment of unilamellar vesicles with sphingomyelinase. A possible mechanism for this release was suggested by Siskind and Colombini who detected formation of large stable channels by ceramide in planar bilayers through electrophysiological methods[43]. The influence of ceramide on the architecture of TJs also seems to be important in the PAF-mediated increase of permeability. Pretreatment of Caco-2 cells with a monoclonal IgM ceramide-antiserum prevented the loss of barrier function induced by 5 μmol/L PAF.

Together, these findings provide evidence for a new aspect of cellular ceramide generation. Lipid rearrangements triggered by enzymatically catalysed formation of ceramide upon cellular stimulation may be a frequent process for localized exposure of intraluminal antigens to the immune system. Repeated stimulation or extension of the stimulated area may lead to an imbalance of the lipid geometry producing a stronger leakiness of the affected junctional complexes resulting in initiation of an inflammatory process which may further perpetuate itself. Better knowledge of the relevant lipids which control this "lipid-barrier" and their modifying enzymes would be helpful to develop treatment strategies to strengthen this barrier. Lipid-enriched diets or inhibition of relevant lipid-modifying enzymes could be possible treatment modalities to control chronic inflammation of the intestine as seen in IBD[44].

In summary, our data demonstrate that hydrolysis

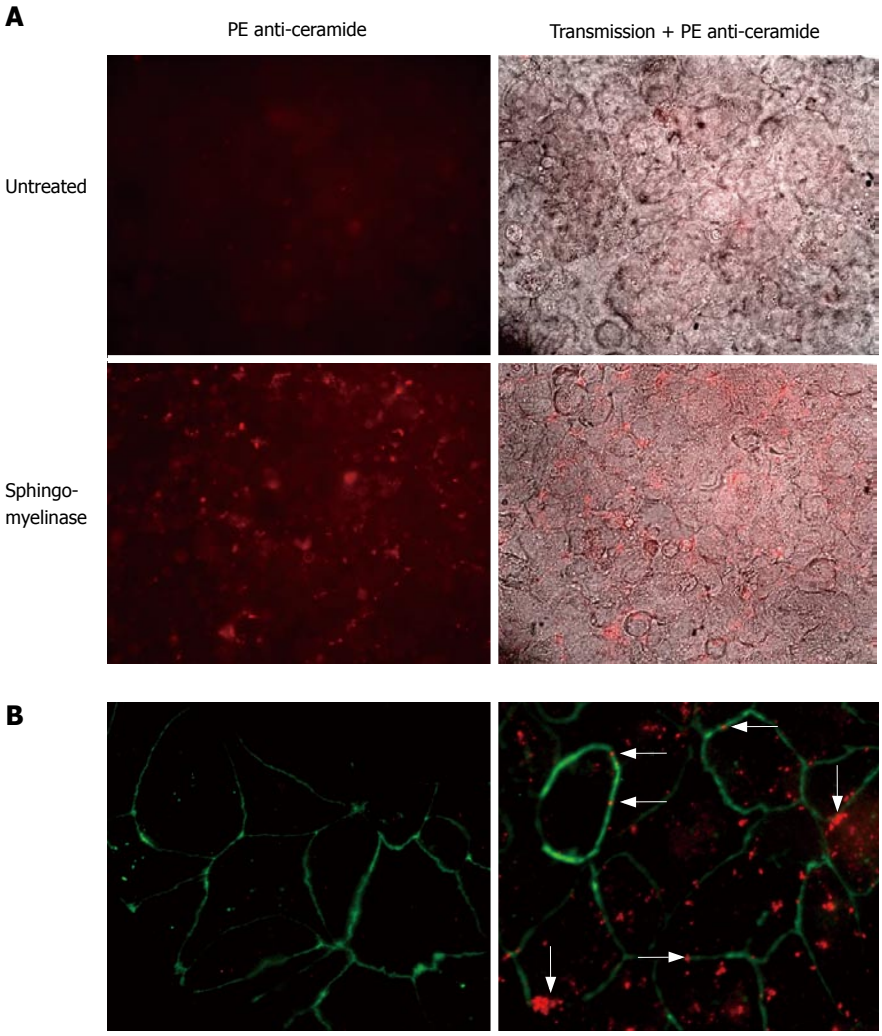

Figure 5 Ceramide clusters at the sites of cell-cell contacts. Caco-2 cell layers were incubated with 0.25 U/mL SMase for 10 min or left untreated. After fixation of the cells with paraformaldehyde, ceramide was visualized by staining of the cells with the monoclonal anti-ceramide 15B4 antibody and PE-coupled anti-mouse antibodies. **A**: Top, left: Non-stimulated cells displayed a certain level of background staining that was used to determine the time of exposure. Top, right: Overlay of fluorescence and transmission. Bottom, left: Incubation with SMase resulted in formation of multiple ceramide-clusters. Bottom, right: Overlay of fluorescence and transmission. Close inspection of the clusters revealed a frequent localization of the clusters at the sites of cell/cell-contact; **B**: Costaining of tight junctions with FITC-labeled anti ZO-1 Abs. Left: non-stimulated. Right: Predominant colocalization of ceramide-clusters with tight junctions upon stimulation with SMase (arrows indicate ceramide-clusters at the sites of cell-cell contact).

of sphingomyelin to ceramide affects transepithelial permeability and resistance. Long chain ceramides, generated by SMase, accumulate in cholesterol- and sphingolipid-enriched membrane microdomains which include TJs. These microdomains fuse to large, ceramide-enriched clusters, located on the surface of the cells and at the sites of cell-cell contact, explaining the effect of long-chain ceramides on barrier integrity. Finally, the effect of PAF on paracellular permeability is inhibited by neutralization of surface ceramide, suggesting a regulation of the paracellular permeability by the arrangement of membrane ceramide.

COMMENTS

Background

The sphingolipid ceramide, generated by signal-activated sphingomyelinases, has emerged as a second messenger of stimuli as diverse as ligation of various receptors, ionizing radiation, chemotherapy or infection with some bacteria and viruses. Upon stimulation of sphingomyelinases, ceramide is generated in distinct sphingolipid-enriched membrane microdomains of the cell membrane, termed "lipid rafts". Tight junctions (TJs) are structurally related to lipid rafts, and thus, we hypothesized that hydrolysis of sphingomyelin to ceramide changes the composition of TJs with increasing permeability of the intestinal epithelium.

Research frontiers

Intestinal permeability is influenced by the lipid content of epithelial cells and some lipids may be beneficial for the course of inflammatory bowel disease. Modification of the lipid content of TJs may be a possible strategy to improve intestinal barrier functions.

Innovations and breakthroughs

Our data indicate that hydrolysis of sphingomyelin to ceramide by sphingomyelinase (SMase) increases intestinal epithelial cell permeability. Lipid analysis after stimulation with SMase demonstrated rapid accumulation of ceramide in the membrane fractions which contain the TJ-proteins occludin and claudin-4, while sphingomyelin and cholesterol decrease. Pretreatment of cells with a monoclonal IgM ceramide-antiserum prevented the loss of barrier function induced by platelet activating factor.

Applications

Better knowledge of the relevant lipids and their modifying enzymes that control this "lipid-barrier" of the intestine would be helpful to develop treatment strategies to strengthen this barrier. Lipid-enriched diets or inhibition of relevant lipid-modifying enzymes could be possible treatment modalities to control chronic inflammation of the intestine as seen in inflammatory bowel disease.

Terminology

Sphingomyelinase is an enzyme which hydrolyses sphingomyelin to ceramide upon various cellular stimuli. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight-junction (TJ). Platelet activating factor is a lipid messenger which increases paracellular permeability in intestinal epithelial cells.

Peer review

The authors set out to test the hypothesis that hydrolysis of sphingomyelin to ceramide changes the composition of tight junctions (TJs) with increasing permeability of the intestinal epithelium. Their findings suggested that changes in lipid composition of TJs impair epithelial barrier functions. Generation of ceramide

by sphingomyelinases might contribute to disturbed barrier function seen in diseases such as inflammatory, infectious, toxic or radiogenic bowel disease.

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BASIC RESEARCH

Propolis reduces bacterial translocation and intestinal villus atrophy in experimental obstructive jaundice

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Abstract

AIM: To investigate the effects of propolis on bacterial translocation and ultrastructure of intestinal morphology in experimental obstructive jaundice.

METHODS: Thirty Wistar-Albino male rats were randomly divided into three groups, each including 10 animals: group Ⅰ, sham-operated; group Ⅱ, ligation and division of the common bile duct (BDL); group Ⅲ, BDL followed by oral supplementation of propolis 100 mg/kg per day. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and transmission electron microscopic examination on postoperative 7th d after sacrification.

RESULTS: The mean number of villi per centimeter and mean mucosal height of the propolis group were significantly different in the BDL group (P = 0.001 and 0.012, respectively). The electron microscopic changes were also different between these groups. Sham and BDL + propolis groups had similar incidence of bacterial translocation (BT). The BDL group had significantly higher rates of BT as compared with sham and BDL + propolis groups. BT was predominantly detected in MLNs and the most commonly isolated bacteria was *Escherichia coli*.

CONCLUSION: Propolis showed a significant protective effect on ileal mucosa and reduced bacterial translocation in the experimental obstructive jaundice model. Further studies should be carried out to explain the mechanisms of these effects.

Key words: Obstructive jaundice; Bacterial translocation; Ileal morphology

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INTRODUCTION

The cytotoxicity of bile salts and the toxicity of high levels of intracellular bilirubin are suspected as mediators of some of the systemic consequences of obstructive jaundice. Recent data suggest that more complex mechanisms involving changes in gut flora, mucosal integrity, and macrophage-immune system interactions may be responsible for the complications of obstructive jaundice[1].

The gastrointestinal tract is not only a passive organ of nutrient absorption, but it additionally displays important endocrine, immunologic, metabolic, and barrier functions[2]. Bacterial translocation is the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes or other extraintestinal organs and sites[3]. Obstructive jaundice impairs intestinal barrier function leading to bacterial and endotoxin translocation in experimental and clinical studies. It affects the three levels of gut barrier globally; the immune barrier, the biological barrier, and and the mechanical barrier[2]. Moreover, biliary obstruction in the rats results in a significant depression of the reticuloendothelial system (RES) phagocytic function, which may cause impaired systemic bacterial clearance and is associated with decreased survival following *E. coli* endotoxemia[4].

Propolis is a natural product collected by honey bees from various plant sources. It has antibacterial,

antifungal, scolicidal, antiinflammatory, antioxidant, immunomodulatory, antiviral and anticarcinogenic properties[5-13].

According to these properties, we planned to use propolis for determining the effects on bacterial translocation and the ultrastructure of intestinal morphology in experimental obstructive jaundice.

MATERIALS AND METHODS

Animals

Thirty Wistar-Albino male rats, weighing 250 ± 25 g, were housed under constant temperature (21℃ ± 2℃) individually in wire cages with a 12 h light-dark cycle. Twelve hours before anesthesia, animals were deprived of food, but had free access to water two hours before anesthesia. No enteral or parenteral antibiotics were administered at any time. The rats that died during the experiment were excluded from the experiment and no new rat was included. The procedures in this experimental study were performed in accordance with the National Guidelines for The Use and Care of Laboratory Animals and approved by Animal Ethics Committee of Ankara Research and Training Hospital.

Study groups

Rats were randomly divided into three groups, each including 10 animals: group Ⅰ, sham-operated; group Ⅱ, ligation and division of the common bile duct (BDL); group Ⅲ, BDL followed by oral supplementation of propolis 100 mg/kg per day (Balparmak LTD, Istanbul, Turkey) with a nasogastric tube that was inserted daily and taken off after propolis supplementation. The first dose of propolis was given on postoperative d 1 and continued until the rats were sacrificed. Animals were sacrificed on postoperative d 7 by high-dose diethyl ether inhalation. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and transmission electron microscopic examination.

There is no standard dose for propolis based on previous experimental studies. In previous studies the dose ranged between 30-200 mg/kg per day[14,15]. We gave propolis at a dose of 100 mg/kg per day to each rat.

Operative procedure

Animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylasine (Rompun®, Bayer, Istanbul, Turkey). Midline laparotomy was performed under sterile conditions. In the sham-operated group (group Ⅰ) the common bile duct (CBD) was freed from the surrounding soft tissue and was manipulated without ligation and transection. In group Ⅱ and Ⅲ, CBDs of the rats were identified, double ligated with 5-0 silk, and divided between the ligatures. The same surgeon performed all procedures. The abdominal incisions were closed in two layers with continuous 3-0 silk sutures. Animals were allowed to feed after the operation.

Microbiological examination

The mesenteric lymph nodes, spleen and liver were chopped with sterile instruments under aseptic conditions. Then the tissue samples were weighed and placed in tubes containing 1.5 mL broth (thioglycollate, Oxoid, UK) and homogenized. Subsequently, 0.01 ml tissue samples were inoculated on blood agar (Oxoid, UK) and Levine Eosine Methylene Blue (EMB) agar (Oxoid, UK). Plates were incubated at 37℃ for examination of bacterial growth. The growth of bacteria in quantitative culture was observed at 24 and 48 h.

One mL blood samples taken from inferior vena cava of rats were inoculated on the media of aerobic and anaerobic blood cultures. The aerobic and anaerobic blood cultures were observed by incubation in BACTEC 9240 blood culture system (Becton Dickinson, USA) at 37℃ for seven days. Samples taken from the blood culture bottle, which gives a positive signal, was cultured by inoculating on blood agar and EMB agar. The subcultures were inoculated at 37℃ under aerobic and anaerobic conditions and examined at 24 and 48 h.

Histopathological examination

For light microscope analyses, tissue samples from the terminal ileum were obtained from all animals. In order to avoid mucosal suffering, the intestinal lumen was carefully cannulated and gently washed with normal saline solution before the sampling. The ileal samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm by Leica RM 2125 RT, and stained with hematoxylin and eosin (HE) for routine light microscopic examination. Histopathological examinations were performed by a pathologist who was blinded to the study design and photographs were taken with Nikon Eclipse E 600. The number of villi per centimeter (V/cm) and the total mucosal thickness (measured from the tip of the villus to the muscularis mucosa) were assessed in all groups. The mucosal thickness was measured in a minimum of 20 well-preserved villi in each randomly selected sample from each tissue block.

For transmission electron microscopic (TEM) analyses, samples were fixed with phosphate buffered (pH: 7.3) 2.5% glutaraldehyde and 2% PFA mixture solution for 2 h at room temperature. They were washed with phosphate-buffered saline solution (PBS) (pH: 7.3) and were fixed with 1% osmium tetraoxide for 2 h as secondary fixation. After washing, they were embedded in Araldite 6005 and were cut with Leica EM FCS (Vien-Austria) ultramicrotome. 1 μm semi-thin sections were stained with Toluidin blue-Azur Ⅱ to select the region of interest for the following procedures. 60-70 nm thin sections were stained with uranil acetate and lead citrate. They were examined and photographed using a LEO 906 E TEM (80 kV-Oberkochen-Germany).

Statistical analysis

Differences between the numbers of positive cultures from the groups were evaluated by Chi-square test and *P* values of less than 0.05 were considered to be significant. Scores of total mucosal thickness and number of villi per centimeter were presented as mean ± SD and compared by One-Way ANOVA or Kruskal-Wallis variance analysis. If the *P* values of the variance analyses were statistically

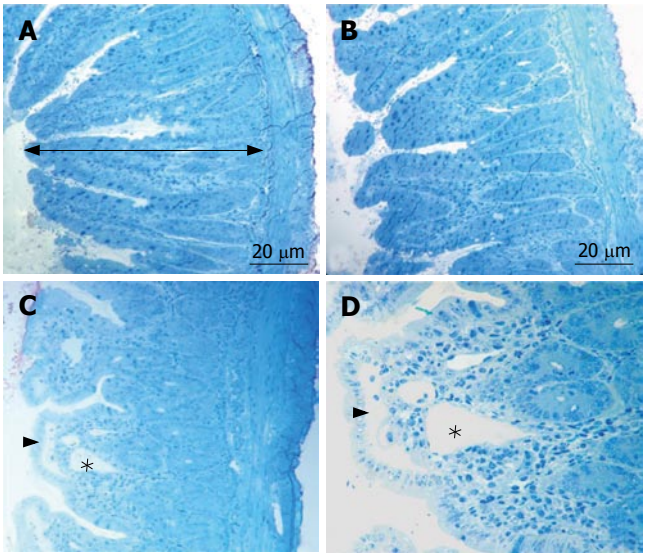

Figure 1 The micrographs of light microscope stained with toluidin blue. **A**: Typical structure of villi and the total mucosal thickness (arrow) in the group Ⅰ; **B**: The normal villous architecture in group Ⅲ; **C**, **D**: Blunting of the villi, the subepithelial edema (arrow head) and the dilated the lacteal (*) in group Ⅱ.

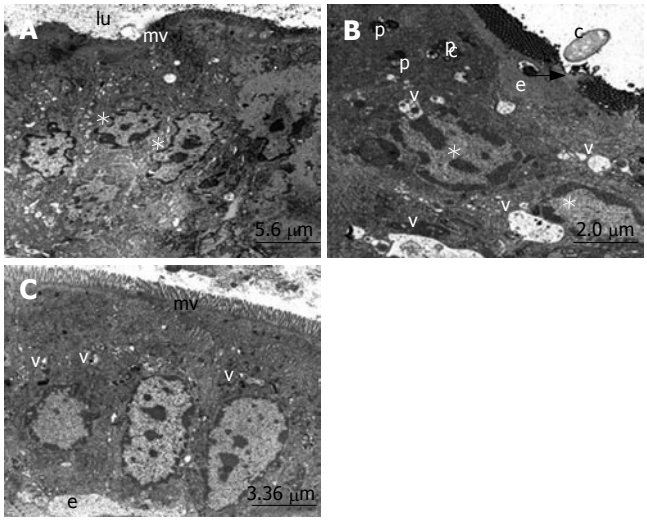

Figure 2 These transmission electron microscope (TEM) micrographs illustrate the main ultrastructural features of enterocytes, and the absorptive cells of the ileum. **A**: The regular structure of microvilli (mv), lumen (lu) and the nuclei of the enterocytes (*). **B**: The subepithelial edema (e), phagosomes (p), vacuoles (v), desquamation of epithelial tissue (arrow), nuclei of enterocytes (*) and candida (c). **C**: The regular structure of microvilli (mv), small vacuoles (v) and subepithelial edema (e).

Table 1 Mean number of villi per cm

Groups	*n*	Mean number of villi per cm	*P* values
Sham (Group Ⅰ)	10	84.40 ± 3.75	< 0.001[b]
BDL (Group Ⅱ)	8	73.01 ± 2.83	0.001[d]
BDL + Propolis (Group Ⅲ)	9	80.89 ± 3.87	

[b]P < 0.001 group Ⅰ *vs* Ⅱ; [d]P = 0.001 group Ⅱ *vs* Ⅲ. BDL: Bile duct ligation.

Table 2 Mean height of mucosa (μm)

Groups	*n*	Mean height of mucosa	*P* values
Sham (Group Ⅰ)	10	640.02 ± 43.72	0.002[b]
BDL (Group Ⅱ)	8	567.50 ± 34.54	0.012[a]
BDL + Propolis (Group Ⅲ)	9	612.78 ± 29.69	

[b]P = 0.002 < 0.01 group Ⅰ *vs* Ⅱ; [a]P = 0.012 < 0.05 group Ⅱ *vs* Ⅲ. BDL: Bile duct ligation.

significant, differences between groups were analysed with the Mann-Whitney U test. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 13.0 for Windows (SPSS Inc., Chicago, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

All rats were sacrificed on postoperative d 7. Two rats from group Ⅱ (BDL group) and one from group Ⅲ (BDL + propolis group) died in the early postoperative period probably due to anesthesia.

Intestinal morphology

In all specimens of the sham group, the histologic features showed regular appearence of ileal tissue. When we evaluated the specimens systematically, including assessment of villous architecture, surface and crypt epithelia, lamina propria constituents and submucosal structures, no alteration was found in the sham group (Figure 1A). Non specific morphological abnormalities were evident in the intestinal mucosa of the BDL group. The specimens of the BDL group presented villous blunting associated with reduced mucosal thickness. We indicated subepithelial edema mostly located at the tip of the villi, but also extended throughout the villus, with the epithelial layer moderately lifted from the lamina propria. We observed that the crypts were generally preserved. The number of villi per centimeter (V/cm) (villus density) was decreased in the BDL group (Figure 1B). In group Ⅲ, the subepithelial edema still existed, but villous blunting was not evident. Further, the crypts generally appeared to be preserved (Figure 1C). Although the number of villi per centimeter and the height of the mucosa were higher in the sham group, there was no statistically significant difference between sham and propolis groups ($P > 0.05$). On the other hand, there was a statistically significant difference between the BDL group and other groups ($P < 0.05$). The mean number of villi per centimeter and mean mucosal height of the groups are given in Table 1 and Table 2.

The ultrastructure of the ileum was observed by electron microscopy. The intestinal surface epithelium showed regular architecture with a large number of microvilli exposed to the lumen and the enterocytes, which are tightly bound by junctional complexes, showed the regular architecture of the small intestine surface epithelium in the sham group (Figure 2A). When we evaluated the micrographs of group Ⅱ, we observed the desquamation of the epithelial tissue, cytoplasmic vacuoles with the inclusion of lipid droplets and phagosomes.

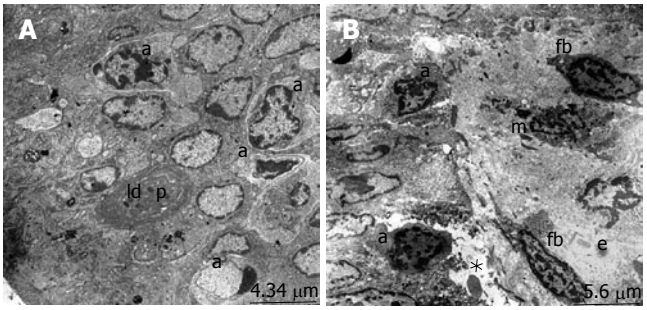

Figure 3 **A**: The apoptotic cell nuclei (a) and phagosomes (p) with lipid droplets (ld). **B**: The the apoptotic cell nuclei (a), fibrocytes (fb), macrophage (m) and subepithelial edema (e) with the seperation of the basal membrane (*).

The nucleus of the enterocytes were flattened possibly due to both apical surface and subepithelial edema. Ultrastructural findings indicating the invasion of candida, especially located in the desquamated epithelial, were detected in many areas (Figure 2B). The number of apoptotic cells, characterized by cytoplasmic condensation and nuclear fragmentation, was qualitatively increased. The phagosomes, including lipid droplets, were observed in the cytoplasm of the epithelial cells. The infiltration of macrophages and fibrocytes were evident. Basal membranes were separated due to subepithelial edema (Figure 3). Structure of the enterocytes and microvilli were regular in the propolis group. The junctional complexes were in common apppearance and adjoining outer membranes of surface absorptive cells were close to each other. The cytoplasmic vacuoles were present but they were smaller than the vacuoles in the BDL group. The nuclei of surface absorptive epithelial cells were regular, in accordance with the regression of the apical surface and subepithelial edema. Findings supporting the invasion of candida were not evident (Figure 2C).

Bacterial translocation

The rates of bacterial translocation (BT) to liver, spleen, MLNs, and blood for all groups are summarized in Table 3. Sham and BDL + propolis groups had similar incidence of BT. The BDL group had significantly higher rates of BT as compared with sham and BDL + propolis groups. Only BT rates to spleen were not statistically different between the BDL and BDL + propolis groups. BT was predominantly detected in MLNs and the most commonly isolated bacteria was *E. coli*.

Table 3 Bacterial translocation rates of the groups

Groups	Liver	Spleen	MLNs	Blood
Sham (Group Ⅰ)	0/10 (0%)	0/10 (0%)	1/10 (10.0%)	0/10 (0%)
BDL (Group Ⅱ)	6/8 (75.0%)	4/8 (50.0%)	7/8 (87.5%)	4/8 (50%)
BDL + Propolis (Group Ⅲ)	2/9 (22.2%)	2/9 (22.2%)	3/9 (33.3%)	0/9 (%)
P values				
Ⅰ *vs* Ⅱ	0.002	0.023	0.002	0.023
Ⅱ *vs* Ⅲ	0.044	> 0.05	0.036	0.029
Ⅰ *vs* Ⅲ	> 0.05	> 0.05	> 0.05	> 0.05

BDL: Bile duct ligation; MLNs: Mesenteric lymph nodes.

DISCUSSION

Propolis is a resinous material collected by bees from various plants. Once collected, this material is enriched with salivary and enzymatic secretions of bees. Propolis is used by bees to cover hive walls, fill cracks or gaps and embalm killed invader insects. Hundreds of chemical compounds have been identified from propolis. Propolis contains a variety of flavonoids, phenols, alcohols, terpenes, sterols, vitamins and amino acids. Antimicrobial properties of propolis seem attributable mainly to the flavonoids, pinocembrin, galangin, and pinobanksin. Pinocembrin also exhibits antifungal properties. Flavonoids are well-known plant compounds that have antioxidant, antibacterial, antifungal, antiviral, and antiinflammatory properties[16-18].

Bacterial translocation is defined as the phenomenon by which bacteria, their products, or both bacteria and products, cross the intestinal barrier. The first site encountered by the microorganisms or products undergoing translocation is the mesenteric lymph node. Subsequently, extension to the liver, spleen, and systemic circulation may occur. The mechanisms which are proposed to promote bacterial translocation are small bowel bacterial overgrowth, immune deficiency states, and physical damage to the intestinal mucosa and vasculature that causes increased permeability. Certain enteric organisms such as *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, seem to undergo translocation more easily. When any of the three mechanisms that promote translocation becomes severe, prolonged, or combined with other mechanisms, bacterial translocation may lead to sepsis and death in experimental animals[19].

Obstructive jaundice is a common clinical entity complicated by intestinal failure and endotoxemia, leading to high morbidity and mortality rates[2]. Kuzu *et al*[20] collected peritoneal swab, mesenteric lymph node, portal venous blood, liver wedge biopsy, and bile samples for culture in patients with obstructive jaundice, and they demonstrated translocation of enteric bacteria despite common use of preoperative antibiotics. Welsh *et al*[21] demonstrated a reversible impairment in gut barrier function in patients with cholestatic jaundice by using the lactulose/mannitol permeability test and concluded that these data might directly identify an important underlying mechanism contributing to the high risk of sepsis in jaundiced patients.

Obstructive jaundice globally affects three levels of gut barrier: the immune barrier, the biological barrier, and the mechanical barrier. Obstructive jaundice depresses Kupffer cell clearance capacity and natural killer cell activity, reduces T cells in intestinal epithelium, alters intestinal mucosal immunity and deprives the gut from biliary secretory IgA and from other specific and nonspecific antibodies contained in bile that inhibit adhesion of enteric bacteria on the intestinal wall. Bile salts exert bacteriostatic properties, and therefore their absence from the intestinal lumen results in quantitative and qualitative disruption of the indigenous microflora. Absence of intraluminal

bile also deprives the gut from their trophic effect resulting in intestinal atrophy[2]. The cellular alterations of the mechanical barrier are associated with significant disturbances of intestinal oxidative status, with increased lipid peroxidation, protein oxidation and oxidation of non-protein and protein thiols. These biochemical changes are indicative of high oxidative stress in the intestine after biliary obstruction and represent another significant parameter of intestinal injury leading to barrier failure[22].

Propolis has antibacterial, antioxidant and anti-inflammatory properties. There are a number of studies documenting the antimicrobial functions of propolis, its extracts, and constituents. This is a broad spectrum activity against Gram-positive (*S. aureus, S. pyogenes, S. viridens, D. pneumoniae,* and *C. diphtheria*) and Gram-negative (*E. coli, K. pneumoniae, P. vulgaris, P. aeruginosa, S. typhi, S. paratyphi*-A, *S. paratyphi*-B, and *S. flexneri*) rods and cocci, *Helicobacter pylori*, *Tryponosoma cruzi*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Candida*, *Saccharomyces*, *Cryptococcus*, *Mycobacteria*, as well as viruses (HIV, Herpes viruses, influenza viruses, adenovirus, poliovirus type 2)[5]. Propolis also possesses potent antimicrobial activity, providing an alternative therapy against infections caused by resistant strains such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*[23]. Although the chemical composition of propolis varies depending on the site of its collection, antimicrobial properties seem attributable mainly to the flavonoids pinocembrin, galangin, and pinobanksin. Pinocembrin also exhibits antifungal properties. Other active compounds are ester of coumaric acid and caffeic acids. Prenylated *p*-coumaric and diterpenic acids possess antibacterial and cytotoxic activities. Caffeoylquinic acid derivatives show immunomodulatory and hepatoprotective actions and furofuran lignans inhibit the growth of some bacteria[24].

Propolis has been used in the treatment of cutaneous lesions such as burns, wounds, and ulcers. Morales *et al*[25] used a hypoallergic formula of propolis and obtained a very satisfactory evolution and cicatrization in cases of wounds with and without infection. A fast cure, shorter treatment period, and less septic complications were also obtained. The cicatrization was evident between the 4th and 5th d by the early formation of granulation tissue. The antimicrobial capacity was evident with a fast regression of the septic component of the supurated wounds. Propolis exerts a wound healing effect by minimizing acute inflammatory exudate, stimulating macrophage activity, promoting collagen production, and stimulating epithelialization. In our present study, the mean number of villi per centimeter and mean mucosal height of the propolis group were significantly different from the BDL group (P = 0.001 and 0.012, respectively). The electron microscopic changes were also different between these groups (Figures 1-3). We conclude that enhancement of wound healing capacity by the use of propolis might be the reason for decreased atrophy of intestinal mucosal villi.

Propolis also has immunomodulatory activity. In an experimental study, a water-soluble derivative of propolis (WSDP) and its components stimulated macrophages thus influencing specific and nonspecific immune defence mechanisms[13,26]. In another study, it was shown that WSDP treatment induced extensive proliferation of nucleated cells in the spleen and bone marrow, which are mainly macrophages and hematopoietic cells[17].

Ara *et al*[27] found that intraperitoneal administration of CAPE reduced tissue levels of malondialdehyde and myeloperoxidase, but increased levels of glutathione in the ileum after bile duct ligation. Additionally, CAPE decreased interleukin-1α, interleukin-6, tumor necrosis factor-α, and intestinal mucosal injury, but the effect of CAPE on bacterial translocation was not revealed. In this study, although bacterial translocation in CAPE-treated rats was lower than in the control group, the difference was not significant.

In our study, bacterial translocation was reduced significantly. The BDL group had significantly higher rates of BT as compared with sham and BDL + propolis groups ($P < 0.05$). Propolis may reduce bacterial translocation by enhancing mucosal barrier function, supporting generalized immune function, and reducing bacterial overgrowth. As we mentioned before, these three mechanisms are the main mechanisms responsible for bacterial translocation.

In conclusion, propolis showed a significant protective effect on ileal mucosa and reduced bacterial translocation in an experimental obstructive jaundice model. Further studies should be carried out to explain the mechanisms of these effects.

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BASIC RESEARCH

In vitro pancreas duodenal homeobox-1 enhances the differentiation of pancreatic ductal epithelial cells into insulin-producing cells

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Abstract

AIM: To observe whether pancreatic and duodenal homeobox factor-1 enhances the differentiation of pancreatic ductal epithelial cells into insulin-producing cells *in vitro*.

METHODS: Rat pancreatic tissue was submitted to digestion by collagenase, ductal epithelial cells were separated by density gradient centrifugation and then cultured in RPMI1640 medium with 10% fetal bovine serum. After 3-5 passages, the cells were incubated in a six-well plate for 24 h before transfection of recombination plasmid XlHbox8VP16. Lightcycler quantitative real-time RT-PCR was used to detect the expression of PDX-1 and insulin mRNA in pancreatic epithelial cells. The expression of PDX-1 and insulin protein was analyzed by Western blotting. Insulin secretion was detected by radioimmunoassay. Insulin-producing cells were detected by dithizone-staining.

RESULTS: XlHbox8 mRNA was expressed in pancreatic ductal epithelial cells. PDX-1 and insulin mRNA as well as PDX-1 and insulin protein were significantly increased in the transfected group. The production and insulin secretion of insulin-producing cells differentiated from pancreatic ductal epithelial cells were higher than those of the untransfected cells *in vitro* with a significant difference (1.32 ± 0.43 *vs* 3.48 ± 0.81, $P < 0.01$ at 5.6 mmol/L; 4.86 ± 1.15 *vs* 10.25 ± 1.32, $P < 0.01$ at 16.7 mmol/L).

CONCLUSION: PDX-1 can differentiate rat pancreatic ductal epithelial cells into insulin-producing cells *in vitro*. *In vitro* PDX-1 transfection is a valuable strategy for increasing the source of insulin-producing cells.

Key words: Pancreatic ductal epithelial cells; Islet; Differentiation; Pancreas duodenal homeobox-1

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INTRODUCTION

Diabetes mellitus is a common endocrine and metabolic disease in the world. At present, insulin injection therapy is used in the treatment of type 1 and some type 2 diabetes. However, the results are not satisfactory. Transplantation of islets is one of the promising therapies for type 1 diabetes and can effectively prevent diabetic nephropathy, retinopathy and other complications[1]. Since it is limited by the shortage of islets, expanding the sources of islet cells (especially beta cells) has become a hot field of research in pancreas transplantation.

In recent years, great efforts have been made to differentiate embryonic stem cells, pancreatic ductal epithelial multipotent progenitor cells and bone marrow stem cells into islet cells[2-4]. However, it was reported that the most promising way is to differentiate pancreatic stem cells into islet cells, because the process of cell differentiation and growth is shorter than that of bone marrow stem cells and embryonic stem cells, and the induction method *in vitro* is relatively simple[5]. The amount of islets differentiated from pancreatic stem cells *in vitro* is minimal and insulin released by islets is insufficient to meet the clinical needs[6]. How to enhance the efficiency of differentiation *in vitro* and increase the output of insulin-producing cells and insulin-release needs to be studied.

The pancreatic and duodenal homeobox factor-1 (PDX-1), also known as islet/duodenum homeobox-1/somatostatin-DE transactivating factor 1/insulin promoter

factor DE 1, a homeodomain containing transcription factor, is homologous to a Xenopus endoderm-specific homeodomain protein, XlHbox 8. It plays a central role in regulating pancreatic development and insulin gene transcription[7]. Transfection of PDX-1 gene into rat intestinal epithelial cell line IEC-6 can produce insulin[8]. Ferberr *et al*[9] showed that PDX-1 can endow some cells in the liver with pancreatic beta-cell characteristics *in vivo* using recombinant adenovirus-mediated gene delivery. Bonner-Weir *et al*[10] reported that PDX-1 protein can permeate pancreatic duct and islet cells due to an Antennapedia-like protein transduction domain sequence in its structure and transduced PDX-1 functions similarly to endogenous PDX-1. PDX-1 protein transduction is a safe and valuable strategy for enhancing insulin gene transcription and facilitating differentiation of ductal progenitor cells into insulin-producing cells without gene transfer technology. Recently, Yamada *et al*[11] showed that mature liver cells can also be induced into insulin-producing cells by *in vitro* PDX-1 gene transfection. These studies have highlighted the potential usefulness of PDX-1 as a reprogramming factor of non-beta-cells toward beta-cell-like cells that can be used in diabetes cell/gene therapy.

Due to the high homology of PDX-1 sequence in different species[12], we investigated the role of exogenous PDX-1 in pancreatic ductal epithelial cells in adult rats by transfecting exogenous PDX-1 (XlHbox8) into pancreatic ductal epithelial cells *in vitro*. The results demonstrate that we can enhance the differentiation of pancreatic ductal epithelial cells into insulin-producing cells and insulin-release via PDX-1 (XlHbox8-VP16) transfection.

MATERIALS AND METHODS

Isolation of cells and culture conditions

Rat (adult male S-D rats, weighing 250-300 g) pancreatic tissue was digested in 1 g/L type V collagenase (Sigma) and incubated at 37℃ for 40 min with intermittent shaking and then terminated by Hank's solution, followed by centrifugation at 1000 r/min for 5 min. After purification on a Ficoll gradient, 50%-95% islets were found on the top interface (1.062/1.096 densities) with varying amounts of duct and degranulated acinar tissues, and 1%-15% islets were found on the middle interface (1.096/1.11 densities). Duct, degranulated acini and pellet were composed of well-granulated acinar tissue with less than 1% islets. In the top and middle layers, there were sheets of ductal epithelium from larger ducts whereas the clumps of exocrine cells found in all layers consisted of small intercalated ducts continuing into the acini. The epithelial cells were washed 3 times with Hank's solution containing 5% fetal bovine serum (FBS, Gibco), then put into RMPI 1640 culture medium (Hyclone) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell suspensions were put into non-treated T-75 flasks and incubated at 37℃ in an atmosphere containing 5% CO_2. After 48 h, the nonadherent tissue (both viable and dead) was removed. The medium was changed. The adherent or residual cells were expanded for up to 1 wk with media changed every 2-3 d. The adherent or residual cells were continuously cultured in an atmosphere containing 5% CO_2 and 95% humidity. After 3-5 passages, the ductal epithelial cells were used in experiments.

Transfection of plasmids

Plasmid pCS2-TTR-XlHbox8VP16 was kindly provided by Professor J M.W.Slack (Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath)[13]. Plasmids were transfected into pancreatic ductal epithelial cells with Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. The medium was replaced with a fresh medium after 24 h of transfection. The cells were cultured for a further 7 d, and analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) or Western blotting at variable time points.

In vitro induction of differentiation

To induce differentiation of pancreatic ductal epithelial cells to insulin-producing cells, forty-eight hours after transfection, pancreatic ductal epithelial cells were transferred to a medium supplemented with 200 μg/mL G418, 10 mmol/L nicotinamide, and insulin/transferrin/selenium(ITS, Sigma). G418-resistant colonies were found about 4 wk after transfection. The resulting clusters were cultured for 1-5 d in RPMI 1640 supplemented with 10% FBS, 10 mmol/L nicotinamide, 200 μg/mL G418, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 5.6 mmol/L glucose[2].

Lightcycler quantitative real-time PCR

Fifty mg pancreatic tissues and 1 mL TRIzol were mixed with 0.3 mL chloroform added, and then centrifuged. Isopropanol (0.5 mL) was added to supernatant and centrifuged with supernatant discarded. Deposit was washed with 70% ethanol, and dissolved in DEPC-treated water to obtain total mRNA. In RT-PCR, 4 μL of mRNA and 0.5 μL of Oligo (dt) were added to 6.5 μL of distilled water. After annealing at 70℃ for 5 min and immediate cooling on ice, 4.0 μmol/L of 5 × first strand buffer, 2.0 μL of 10 mmol/L dNTP, 0.5 μL of RNasin and 0.5 μL RTase were added to get a total reaction volume of 20 μL. The reaction was allowed to proceed at 37℃ for 60 min, followed by at 95℃ for 5 min to inactivate the enzyme. RT-PCR assay was performed three times for each cDNA sample. The total PCR volume consisted of 1 μL of cDNA, 1 μL of SYBRGreen PCR I, 5 μL 10 × buffer, 1.6 μL of primers (rat PDX-1: sense 5'-CTTGGGTATGGATCTGTGG-3' and antisense 5'-CGGACTCATCGTACTCCTGCTT-3'; Xenopus PDX-1 homologue XlHbox8: sense 5'-TGCCAACTTCATCCCAGCCC-3' and antisense 5'-GGCAGATGAAGAGGGCTC-3'; insulin: sense 5'-GCTACAATCATAGACCATC-3' and antisense 5'-GGCGGGGAGTGGTGGACTC-3'; beta-actin: sense 5'-CTTGGGTATGGAATCCTGTGG-3' and antisense 5'-CGGACTCATCGTACTCCTGCTT-3'), 7 μL of $MgCl_2$, 0.5 μL of Taq DNA polymerase, 1 μL of dNTP and 33 μL of distilled water. After denaturation of the enzyme at 94℃ for 2 min, 45 cycles of PCR assay were carried out, with denaturation at 94℃ for 30 s, annealing at 57℃ for 30 s and extension at 72℃ for 30 s. Fluorometric PCR was

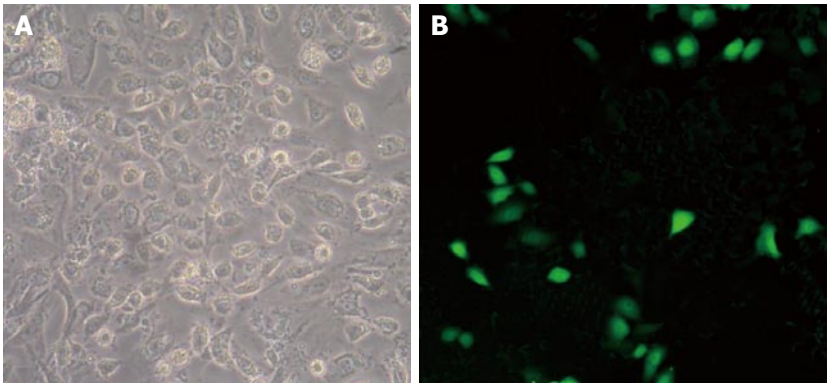

Figure 1 Transfection of recombination plasmids into cultured (**A**) and non-cultured (**B**) pancreatic ductal epithelial cells.

performed with the FTC-2000 system. The expression of each transporter protein gene determined was relative to the β-actin RNA gene.

Western blotting

Total protein was obtained as previously described[14] at various time points (d 0, 1, 3, 5, 7, 14, 21, 28 after transfection). Proteins in samples were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and blocked overnight in a blocking solution (5% dry milk in PBS). The membrane was incubated at 37℃ for 1 h with goat anti PDX-1 antibody (1:500, Santa Cruz) and goat anti insulin antibody (1:500, Santa Cruz), washed with PBS and incubated at 37℃ for 1 h with horseradish peroxidase-labeled secondary antibody (1:2500). Finally, proteins were visualized on a film with the ECL method.

Dithizone staining

A dithizone (DTZ; Sigma) stock solution was prepared with 50 mg of DTZ in 5 mL of dimethylsulfoxide (DMSO) and stored at -15℃[15]. *In vitro* DTZ staining was performed by adding 10 μL of the stock solution to 1 mL of culture medium. The staining solution was filtered through a 0.2 μm filter and used as the DTZ working solution which was added to culture dishes and incubated at 37℃ for 15 min. Clusters were examined under a phase contrast microscope.

Secreted insulin measurement

Differentiation of cells transfected with or without TTR-XlHbox8VP16 was induced with either normal dose glucose (5.5 mmol/L) or high dose glucose (16.7 mmol/L). Insulin concentration was measured using an insulin RIA kit (Linco).

Statistical analysis

The data were expressed as mean ± SD. Individual treatment was compared using Student's *t*-test and ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Transfection of XlHbox8 plasmids into cells

Green fluorescence could be seen in pancreatic ductal epithelial cells transfected with XlHbox8. The distribution of green fluorescence in nuclei and cytoplasm indicated the characteristics of location of cytokines in these cells. Twenty-four hours after transfection, the expression of GFP fluorescence was observed under fluorescence microscope (Figure 1). Green fluorescent cells were calculated. The transfection efficiency of pancreatic ductal epithelial cells was 3%-5%. These results suggest that exogenous XlHbox8 could be transfected into nuclei and cytoplasm of pancreatic ductal epithelial cells.

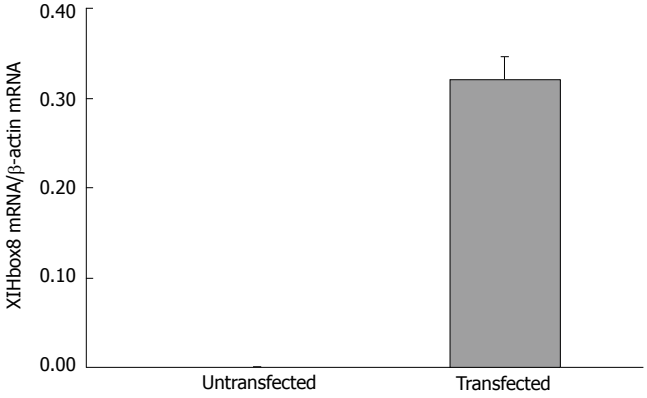

Figure 2 XlHbox8 mRNA expression 24 h after transfection of recombination plasmids.

Expression of PDX-1 and insulin in transfected cells

To investigate the change in gene expression patterns caused by exogenous PDX-1 (XlHbox8) expression in pancreatic ductal epithelial cells, we performed RT-PCR analysis. As shown in Figure 2, the cells transfected with TTR-XlHbox8VP16 showed the expression of XlHbox8 mRNA, which was not detectable in the untransfected cells. In addition, the results also showed that the expression of PDX-1 and insulin mRNA increased between the 1st and 7th d after transfection (Figure 3), which is consistent with the reported results[13]. In contrast, the expression of PDX-1 and insulin mRNA was not detectable in the untransfected cells.

Western blotting showed that PDX-1 and insulin protein were expressed in the transfected cells but not in the untransfected cells. After induction, the untransfected cells also began to express PDX-1 and insulin protein. Moreover, the expression increased between the 14th and 28th d in the transfected cells. The expression of PDX-1

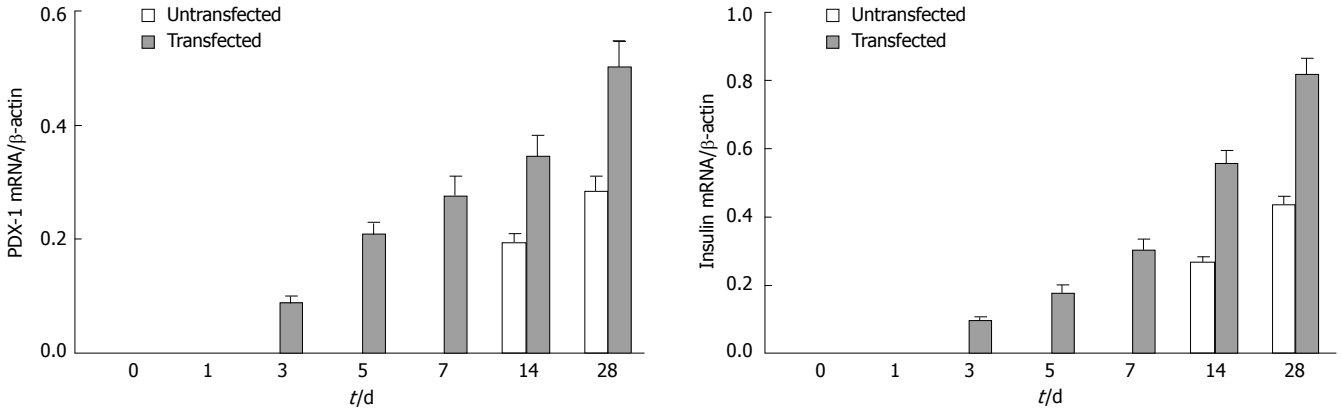

Figure 3 PDX-1 and insulin mRNA expression after transfection of recombination plasmids.

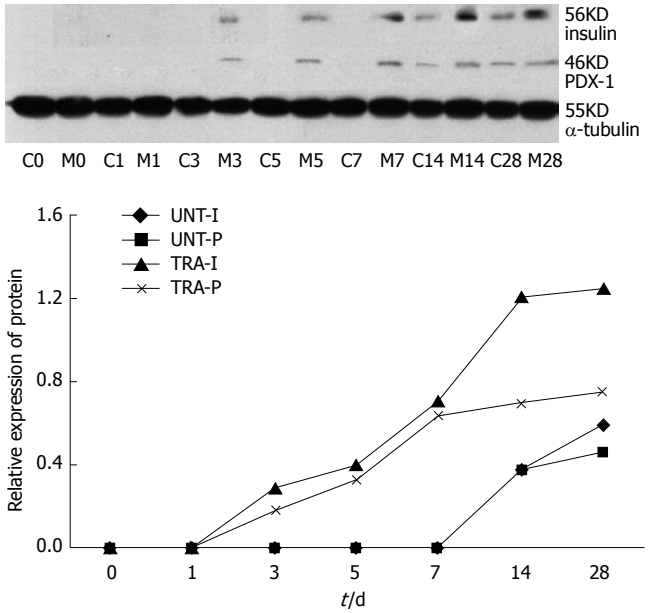

Figure 4 Western blot analysis of PDX-1 and insulin protein showing the increased expression of PDX-1 ($P < 0.01$) and insulin ($P < 0.01$) protein in the transfected cells. C0-C28: Untransfected group on d 0-28; M0-M28: Transfected group on d 0-28; UNT-I: Insulin expression in untransfected group; UNT-P: PDX-1 expression in untransfected group; TRA-I: Insulin expression in transfected group; TRA-P: PDX-1 expression in transfected group.

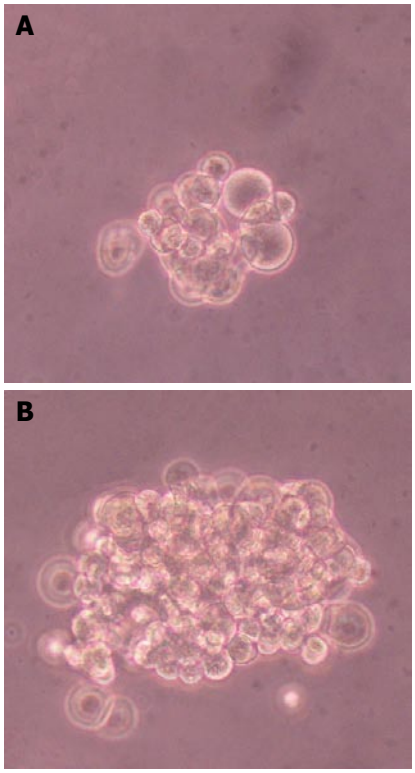

Figure 5 Dithizone staining of insulin-producing cells in untransfected group (**A**) and transfected group (**B**).

and insulin protein in the transfected cells increased more obviously (Figure 4).

These results suggest that once XlHbox8 was transfected into pancreatic ductal epithelial cells, endogenous PDX-1 gene transcription was amplified by this XlHbox8 and might enhance differentiation of pancreatic ductal epithelial cells into insulin-producing cells.

Induction of XlHbox8-expressing pancreatic ductal epithelial cells to insulin-producing cells

Pancreatic ductal epithelial cells were transfected with XlHbox8 expression plasmid, and stable transfectants were selected according to G418 resistance. Further differentiation of pancreatic ductal epithelial cells was induced in serum-free medium supplemented with ITS, keratinocyte growth factor (KGF, Sigma) and basic fibroblast growth factor (bFGF, Sigma). During induction, most insulin-producing cells could be seen under microscope. Dithizone staining showed more insulin-producing cells in the transfected group than in the untransfected group (Figure 5).

Response of differentiated insulin-producing cells to physiological stimuli

The most important and characteristic property of insulin-producing cells is their ability to secrete insulin in response to an elevated external glucose concentration. We examined the insulin concentration in the medium of differentiated cells after challenged with 5.6 and 16.7 mmol/L glucose, using a RIA kit (Figure 6). The results showed that insulin secretion was significantly increased in the transfected group compared with the untransfected group.

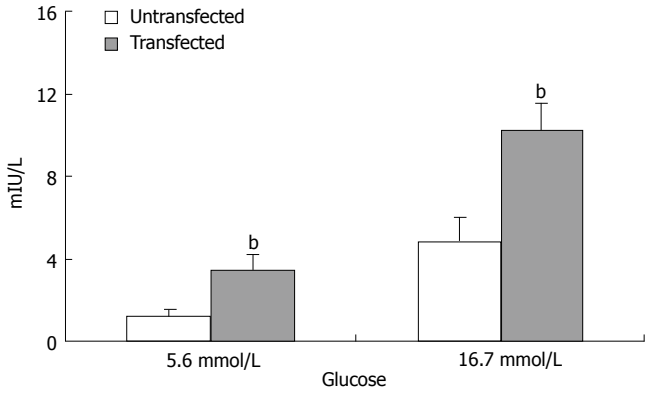

Figure 6 Response of insulin secretion to physiological stimuli. Insulin secretion was significantly increased in the transfected group compared with the untransfected group ($^bP < 0.01$).

DISCUSSION

Pancreas duodenum homeobox-1 (PDX-1) is also known as islet/duodenal homeobox-1(IDX-1), insulin promoter factor-1 (IPF-1), insulin upstream factor-1 (IUF-1), somatostatin transactivating factor-1 (STF-1) and glucose-sensitive factor (GSF)[7]. PDX-1 is a transcription factor encoded by Hox-like homeodomain gene and also one of the specific signals of pancreatic stem cells. Human PDX-1 gene consists of two exons and spans a region of about 6 kb in humans and 282 amino acids[16]. In mice lacking PDX-1, pancreas development is blocked at its very early stage, and there are no or diminished endocrine cells in the rostral duodenum and stomach[17,18], suggesting that PDX-1 plays a very important role in the development and maintenance of normal pancreatic beta cell function, which also has a potential value in gene therapy for diabetes. Amplifying and inducing differentiation of non-islet cells into insulin-producing cells by PDX-1 has become a hot field of study. Miyazaki *et al*[19] reported that islet-like cellular clusters are significantly increased and insulin-producing cells can release more insulin by transfecting exogenous PDX-1 into embryonic stem cells. Further investigation found that PDX-1 can enhance the expression of insulin 2, somatostatin and HNF6 genes in the differentiated cells[19]. XlHbox8 is the homologous of PDX-1. Horb *et al*[20] transfected Xlhbox8-VP16 into liver cells and found that some liver cells could be trans-differentiated into pancreatic cells.

Bonner-Weir *et al*[3] reported human pancreatic ductal epithelial cells can expand and differentiate into islet cells *in vitro*. In this study, most of early adherent cells and a few fibroblasts were found to be pancreatic ductal epithelial cells. PDX-1 may express in the whole pancreas of an early embryo. However, in pancreas of adult rats, PDX-1 expresses primarily in beta and delta cells, but not in pancreatic ductal epithelium. PDX-1 can also express in activated pancreatic ductal epithelial cells[12,14]. When pancreatic ductal epithelial cells are transfected with exogenous homologous of PDX-1 and XlHbox8, endogenous PDX-1 gene can be activated, increasing insulin mRNA and protein expression[9], suggesting that once XlHbox8 is transfected into pancreatic ductal epithelial cells, endogenous PDX-1 gene transcription is activated by XlHbox8 and insulin gene, thus enhancing its expression. In our study, the expression of insulin mRNA and protein was enhanced after treatment with exogenous XlHbox8, indicating that XlHbox8 may promote differentiation of pancreatic ductal epithelial cells into insulin-producing cells. When pancreatic ductal epithelial cells were transfected with exogenous PDX-1, endogenous PDX-1 gene transcription was amplified, promoting differentiation of pancreatic ductal epithelial cells into insulin-producing cells, which is in agreement with the reported results[10]. In the present study, after XlHbox-VP16 was transfected into pancreatic ductal epithelial cells, differentiation of pancreatic ductal epithelial cells to insulin-producing cells was significantly increased, suggesting that this method can produce more insulin-producing cells. It was reported that unmodified PDX-1 is insufficient to activate transcription in the absence of other cofactors, such as Pbx and Meis[20]. Beta cells are able to secrete insulin in response to an elevated external glucose concentration. In this study, the results of RIA show that the treated cells can produce more insulin than the untreated cells. However, whether differentiated insulin-producing cells have their physiological function *in vivo* still needs to be further tested *in vivo*. Moreover, the low transfection efficiency also limits the production of insulin-producing cells.

In conclusion, PDX-1 (XIHbox8) can significantly promote *in vitro* differentiation of pancreatic ductal epithelial cells into insulin-producing cells. This new method can be used in the treatment of diabetes.

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COMMENTS

Background

Diabetes mellitus is a common endocrine and metabolic disease in the world. Although transplantation of islets is one of the promising therapies for type 1 diabetes, its application is limited due to the shortage of islets.

Research frontiers

Expanding the sources of islet cells (especially beta cell) is a hot field of research in pancreas transplantation. Great efforts have made to differentiate embryonic stem cells, pancreatic ductal epithelial progenitor cells and bone marrow stem cells into islet cells. The most promising method is to differentiate pancreatic stem cells into islet cells, but the amount of islets obtained through differentiation of pancreatic ductal cells *in vitro* is minimal and insulin released by islets is insufficient.

Innovations and breakthroughs

The pancreatic and duodenal homeobox factor-1 (PDX-1) plays a central role in regulating pancreatic development and insulin gene transcription. In this study, we transfected PDX-1 (XlHbox8VP16) plasmid into human pancreatic ductal cells.

Applications

PDX-1 (XlHbox8) can significantly promote *in vitro* differentiation of pancreatic ductal epithelial cells into insulin-producing cells. This new method may be used in the treatment of diabetes.

Peer review

This manuscript provides direct evidence for the enhanced differentiation of pancreatic ductal cells into insulin-producing cells. The study is well designed and the results are convincing.

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CLINICAL RESEARCH

Maintenance of remission with infliximab in inflammatory bowel disease: Efficacy and safety long-term follow-up

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Abstract

AIM: To evaluate the safety and efficacy of a long-term therapy with infliximab in Crohn's disease (CD) and ulcerative colitis (UC) patients retrospectively.

METHODS: The medical charts of 50 patients (40 CD and 10 UC), who received after a loading dose of 3 infliximab infusions scheduled re-treatments every 8 wk as a maintenance protocol, were reviewed.

RESULTS: Median (range) duration of treatment was 27 (4-64) mo in CD patients and 24.5 (6-46) mo in UC patients. Overall, 32 (80%) CD and 9 (90%) UC patients showed a sustained clinical response or remission throughout the maintenance period. Three CD patients shortened the interval between infusions. Eight (20%) CD patients and 1 UC patient underwent surgery for flare up of disease. Nine out of 29 CD and 4 out of 9 UC patients, who discontinued infliximab scheduled treatment, are still relapse-free after a median of 16 (5-30) and 6.5 (4-16) mo following the last infusion, respectively. Ten CD patients (25%) and 1 UC patient required concomitant steroid therapy during maintenance period, compared to 30 (75%) and 9 (90%) patients at enrolment. Of the 50 patients, 16 (32%) experienced at least 1 adverse event and 3 patients (6%) were diagnosed with cancer during maintenance treatment.

CONCLUSION: Scheduled infliximab strategy is effective in maintaining long-term clinical remission both in CD and UC and determines a marked steroid sparing effect. Long-lasting remission was observed following infliximab withdrawal.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Infliximab therapy; Steroid sparing

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic-relapsing diseases the clinical course of which is characterized by periods of remission and periods of acute flare up. The clinical symptoms have a strong impact on the quality of life of patients with inflammatory bowel disease (IBD)[1-3]. Although many drugs have been used in the treatment of IBD, none has, so far, been shown to modify the natural history of the diseases or to maintain a stable remission over time[4,5]. For many years, corticosteroids have represented the cornerstone of therapy for induction of remission in IBD, having demonstrated efficacy in inducing a rapid clinical response, in CD as well as in UC; however, long-lasting remission was not achieved and the side-effects emerging with long-term use exceeded the clinical benefits[6]. Immunomodulators have been demonstrated to be efficacious as adjunctive therapy and as steroid-sparing agents; but their slow onset of action precludes their use in the active clinical setting as a sole therapy[7].

The introduction of biological agents in the therapeutic armamentarium for CD and UC has significantly changed the treatment strategies and clinical outcomes. Infliximab (IFX) is a chimeric monoclonal IgG1 antibody directed against tumor necrosis factor-α (TNF-α) able to almost completely neutralize its biological activity[8]. Several placebo-controlled trials have demonstrated the efficacy of IFX treatment in active luminal and fistulizing CD[9-13]. Recently, IFX has become an alternative choice also in the treatment of UC. Indeed, following the conflicting conclusions of 2 placebo-controlled studies performed in patients with moderately severe steroid-resistant UC[14,15], showing opposite clinical effects, another two randomized controlled trials have since then been published, demonstrating the clinical efficacy of IFX therapy in patients with moderate-to-severe UC[16,17].

Some safety issues are associated with IFX use, mostly related to the development of adverse events (e.g., opportunistic infections, autoimmune disorders, and infusion reactions)[18-20]. Major concerns are related to the reactivation

of latent tuberculosis and development of malignancy, even if there is no clear evidence that the use of IFX increases the incidence of solid cancers[21,22]. Although the efficacy of a therapeutic strategy consisting of a loading dose of 3 IFX infusions (5 mg/kg) at 0, 2, and 6 wk and, thereafter, every 8 wk is supported by several placebo-controlled studies, very few data are available on the use of IFX for > 12 mo or > 8 doses, either in active CD or in active UC[23,24].

The aim of the present single-centre study was to retrospectively analyze the prospectively collected data on the safety and efficacy of long-term therapy with IFX in CD and UC patients treated with a scheduled regimen.

MATERIALS AND METHODS

Subjects

The medical charts of 79 patients affected by IBD (59 CD and 20 UC patients, mean age 47.7 years) and treated with IFX (Remicade; Centocor Inc., Malvern, PA, USA) between January 1999 and September 2005 at the Department of Digestive Diseases of the Campus Bio-Medico University Hospital were retrospectively reviewed (Table 1). Diagnosis of IBD was based on the standard combination of clinical, endoscopic, histological, and radiological criteria. Patients were classified into one of 3 groups of treatment, according to IFX infusion administration: (1) episodic therapy only in 10 patients (5 CD, 5 UC), (2) episodic followed by on-demand maintenance in 19 patients (14 CD, 5 UC), and (3) induction therapy followed by scheduled maintenance therapy every 8 wk in 50 patients (40 CD, 10 UC).

Patients presented with mild to moderate IBD, as defined by a score of 150-350 (or less in steroid-dependent patients) on the Crohn's Disease Activity Index (CDAI) for CD patients[25], and a score of > 10 (or less in steroid-dependent patients) on the Clinical Activity Index (CAI) for UC patients[26]. Indications for IFX treatment were disease severity, steroid-dependent disease (unable to reduce prednisolone < 10 mg/d), steroid-refractory disease (active disease with prednisolone up to 0.7 mg/kg per day), or contraindication to steroids (e.g., diabetes, osteoporosis, acne, mood disturbance and severe hypertension). Exclusion criteria were cancer or history of cancer, pregnancy, chronic heart failure, previous tuberculosis, or presence of symptomatic intestinal strictures or abscesses.

All patients were allowed to continue concomitant therapies such as 5-aminosalicylates, immunosuppressive agents [azathioprine (AZA), 6-mercaptopurine (6-MP), methotrexate], and antibiotics; patients on steroids discontinued treatment using a tapering regimen of 10 mg weekly starting at the first IFX infusion.The study protocol was approved by the University Ethic Committee.

Pretreatment protocol

Clinical and instrumental assessment was performed within the 3 wk before IFX induction treatment. Blood samples were collected, after overnight fasting, prior to IFX infusion for routine laboratory tests, including inflammatory [C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)] and biochemical parameters. A chest X ray evaluation was performed before the first IFX infusion in order to exclude previous or latent tuberculosis infection. Patients' symptoms were routinely recorded before each infusion, in order to calculate the CDAI and CAI.

Table 1 Demographic and clinical characteristics of the study population

Clinical characteristics	IBD (CD + UC)	Crohn's disease	Ulcerative colitis
n	50	40	10
Gender (M/F)	23/27	18/22	4/6
Age (yr)[1]	47.7 ± 15.6	45.4 ± 17.9	52.7 ± 8.9
Median disease duration (yr) (range)	10.8 (0.3-22.2)	9.2 (0.3-22.2)	9.3 (2.1-15.1)
Disease activity (T0)[1]	N/A	245.0 ± 99.3 (CDAI)[2]	12.9 ± 2.8 (CAI)
Site of disease *n* (%):			
Small bowel/pouch		13 (32.5)	
Large bowel		4 (10)	10
Ileum-colon		23 (57.5)	
Steroid therapy at enrolment (%) (≥ 0.7 mg/kg per day)	39 (78)	30 (75)	9 (90)
Concomitant medications *n* (%):			
Salicylates	29	21 (52.5)	8 (80)
6-MP/azathioprine	31	28 (70)	3 (30)
Antibiotics	25	24 (60)	1 (10)

[1]Mean ± SD; [2]5 steroid-dependent patients had baseline Crohn's disease activity index (CDAI) < 150; CAI: Clinical activity index; T0: Baseline time; N/A: Not applicable.

Infliximab administration

All 50 IBD patients were treated with an induction regimen consisting of 3 intravenous (iv) infusions of IFX at a dose of 5 mg/kg for induction of remission (wk 0, 2, 6 for CD; wk 0, 4, 8 for UC). Eight weeks after the third infusion, clinical assessment was repeated in all patients to evaluate treatment efficacy: in CD patients, the clinical response was defined as a ≥ 70 point reduction in the CDAI score, and clinical remission was defined as a CDAI score ≤ 150; in UC patients, clinical response was defined as a CAI score ≤ 10 points, and inactive disease as a CAI score ≤ 4. Thereafter, all responders (both CD and UC patients) received IFX maintenance infusions at 8-wk intervals. Written patient's consent was obtained before every IFX infusion.

All adverse events were recorded by means of direct questioning of patients. Infusion reactions to IFX were classified as either acute or delayed. Any adverse reaction, during or within 24 h of an initial or subsequent IFX infusion, was considered as an acute infusion reaction. A delayed infusion reaction refers to any adverse reaction occurring from 24 h to 14 d after re-treatment with IFX. Acute and delayed reactions were further defined as mild, moderate, or severe, according to the severity of signs and symptoms reported by the patient. Serum sickness-like reactions included any IFX-related event that occurred 1 to 14 d after infusion and consisted of a cluster of symptoms such as myalgias and/or arthralgias with fever and/or rash. Severe infection, cancer, and death were considered as serious adverse events if potentially related to IFX treatment.

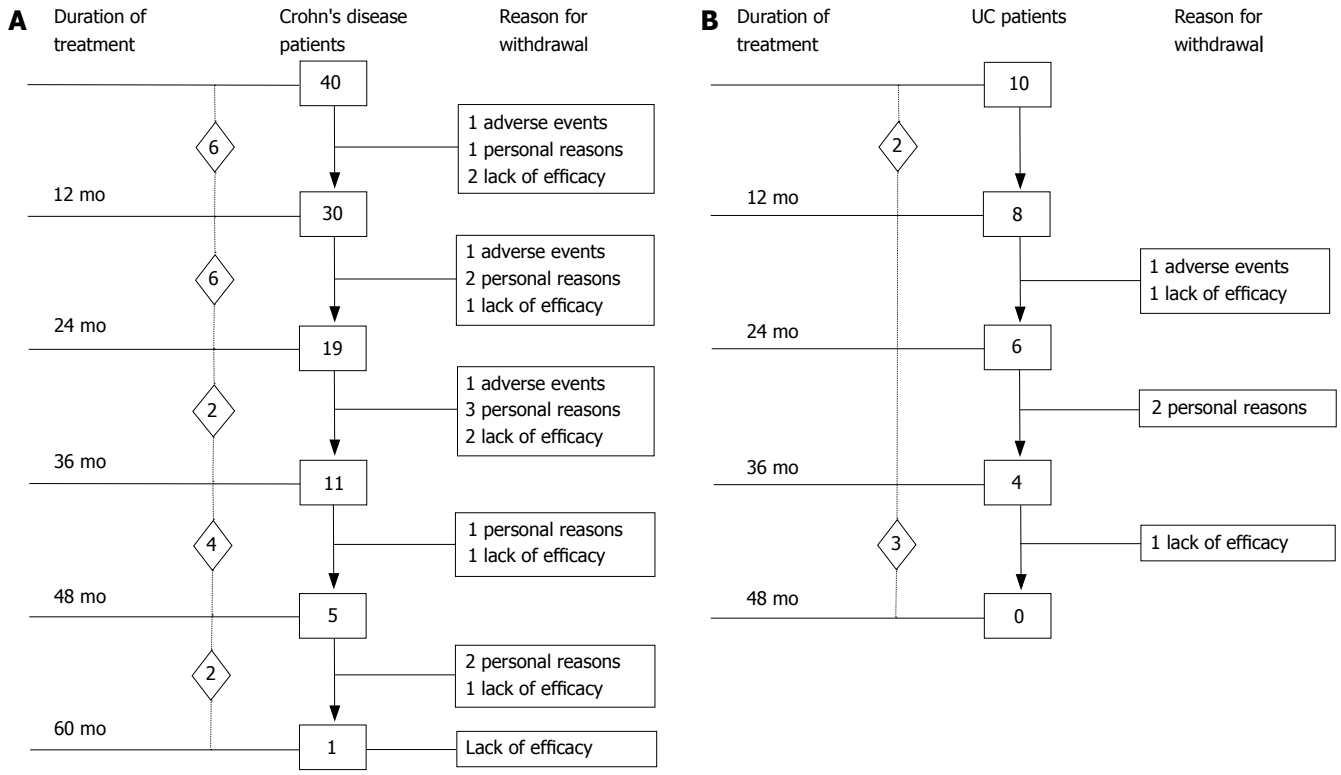

Figure 1 Patients observed at 12-mo intervals (endpoints) during IFX maintenance therapy. **A:** CD; **B:** UC. Numbers in boxes refer to patients reaching each endpoint. Numbers in rhombics refer to patients not reaching 12-mo endpoints.

Table 2 Efficacy profile of maintenance protocol

	IBD (CD + UC)	Crohn's disease	Ulcerative colitis
IFX infusions total number	637	493	144
Median number (range)	15 (4-25)	15 (4-25)	14.5 (6-22)
Median duration (mo) of IFX therapy (range)	27 (4-64)	27 (4-64)	24.5 (6-46)
No. patients maintaining remission with IFX (%)	41/50 82	32/40 80	9/10 90
Median duration (mo) of remission during IFX treatment (range)	25 (4-59)	25.5 (4-59)	25 (6-46)
No. patients discontinuing therapy during clinical remission (%)	13/38 34	9/29 31	4/9 44
Median duration (mo) of remission after treatment discontinuation (range)	15.5 (4-30)	16 (5-30)	6.5 (4-16)
No. patients on corticosteroids[1] during maintenance with IFX (%)	1/50 2	10/40 25	1/10 10

[1]Median corticosteroid dose ≤ 0.25 mg/kg per day.

Statistical analysis

Results related to continuous data are expressed as median (range). The cumulative probability of relapse was calculated using the Kaplan-Meier method.

RESULTS

Baseline demographic and clinical data of the 50 patients (40 CD, 10 UC) who underwent induction therapy followed by scheduled maintenance therapy every 8 wk are outlined in Table 1. The summary of IBD patients' outcome observed at 12-mo intervals (endpoints) during IFX maintenance therapy is shown in Figure 1. A total of 637 IFX infusions were administered (493 in CD, 144 in UC). The median duration of scheduled IFX treatment in the whole group of IBD patients was 27 (range, 4-64) mo.

Crohn's disease patients

The 40 CD patients undergoing the maintenance treatment strategy had a median disease duration of 9.2 years (range, 0.3-22.2). At baseline, all patients presented luminal disease; 6 patients had also draining fistulas (4 perianal and 2 recto-vaginal fistulas). Median number of IFX infusions was 15 (range, 4-25). The median duration of IFX treatment was 27 (range, 4-64) mo. Of the 40 CD patients, 35 (87.5%) underwent scheduled infusion strategy every 8 wk. In 3 patients (7.5%), showing a progressive loss of response (i.e., increase in CDAI and serum CRP level), the dose intervals were reduced to 6 wk; in 2 patients (5%), on account of stable clinical remission, the infusion intervals were prolonged to 12 wk. In 29 out of 37 CD patients (78%) the initial clinical improvement remained unchanged during IFX scheduled treatment, with CDAI below the remission level (< 150) throughout the maintenance period (Table 2). The median duration of remission during IFX treatment was 25.5 (range, 4-59) mo. Of the 29 CD patients, the 9 (31%) who chose to discontinue IFX scheduled treatment, were still relapse-free at a median of 16 (range, 5-30) mo after the last infusion; all these patients were on concomitant immunomodulatory treatment; 2 CD patients were lost at follow-up. Twenty CD patients are currently on IFX treatment with a median duration of therapy of 20 (range, 5-57) mo. Of the 40 CD patients, 8 patients (20%) who showed worsening of clinical symp-

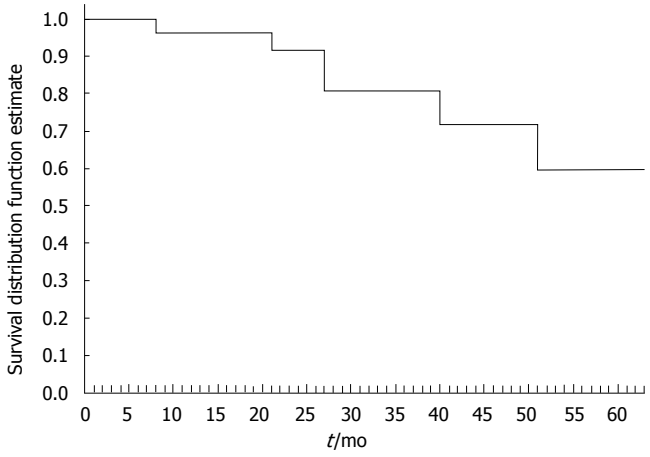

Figure 2 Kaplan-Meier survival curve for CD patients (n = 40). The cumulative probability of being free of relapse in CD patients with complete response was: 97.2% (CI: 91.8%-100%) at 12 mo, 90.3% (CI: 79.7%-100%) at 24 mo, 81.7% (CI: 66.9%-96.5%) at 36 mo, 73.5% (CI: 53.3%-93.7%) at 48 mo, and 61.3% (CI: 33.6%-88.9%) at 51 mo.

toms underwent surgery for intestinal strictures and/or abdominal abscesses. These patients presented with higher baseline values of acute phase reactants - in terms of serum ESR and CRP levels - compared to the patients not undergoing surgery. The difference was not significant. The cumulative probability curve of maintaining the initial response in those patients with clinical benefit at the third infusion is shown in Figure 2. The cumulative probability of being free of relapse in CD patients with complete response was: 97.2% (CI: 91.8-100%) at 12 mo, 90.3% (CI: 79.7-100) at 24 mo, 81.7% (CI: 66.9-96.5%) at 36 mo, 73.5% (CI: 53.3-93.7%) at 48 mo, and 61.3% (CI: 33.6-88.9%) at 51 mo. In this study, fistula closure was not considered as a goal of the clinical outcome.

Ulcerative colitis patients

Ten UC patients who underwent scheduled re-treatment every 8 wk as maintenance protocol had a median disease duration of 9.3 (range, 2-15) years (Table 2). The median number of IFX infusions was 14.5 (range, 6-22). Median duration of IFX treatment was 24.5 (range, 6-46) mo. In 9/10 (90%) UC patients, the CAI score remained below the remission level (< 4) throughout the maintenance period. The median duration of remission during IFX treatment was 25 (range, 6-46) mo. Of the 9 UC patients, 4 withdrew from scheduled treatment due to non compliance (n = 2) and to adverse events (n = 2) after a median time of 24.5 (range, 21-46) mo and were still relapse-free after a median time of 6.5 (range, 4-16) mo after the last IFX infusion; 3 of these patients were on concomitant immunomodulatory treatment. One UC patient, who showed a gradual loss of benefit, underwent surgery for flare up of the disease 24 mo after starting scheduled treatment. Five UC patients are currently on IFX therapy with a median duration of therapy of 36 (range, 6-39) mo. In patients who discontinued IFX treatment, endoscopic evaluation performed before withdrawal revealed complete mucosal healing in all cases.

Steroid-sparing effect

At baseline, 39 (78%) IBD patients were on steroid therapy; namely, 30/40 (75%) CD patients and 9 out of 10 (90%) UC patients required a median corticosteroid dose of 0.7 mg/kg per day. All patients were able to discontinue corticosteroids following IFX induction therapy, but 10/40 (25%) CD patients and 1/10 (10%) UC patients required reintroduction of steroid therapy during IFX scheduled treatment. Moreover, in patients who required concomitant steroid therapy, the median daily corticosteroid dose decreased to 0.25 mg/kg per day. The 10 CD and 4 UC patients, who discontinued scheduled treatment and remained relapse-free, did not require steroid therapy after the last IFX infusion.

Safety and infusion reactions

Of the 50 IBD patients, 16 (32%) experienced at least 1 adverse event: number and type of adverse events are listed in Table 3. Of the 50 IBD patients, 3 (6%) experienced moderate acute infusion reactions characterized by headache, dizziness, nausea, flushing, chest pain, dyspnoea, and pruritus, which developed after the second infusion. All 3 patients were successfully reinfused following intravenous hydrocortisone (200 mg) premedication before any subsequent IFX infusion. One patient presented serum sickness-like symptoms within 10 d of the third IFX infusion, developing arthralgias, myalgias, and fever; laboratory tests showed non-viral acute liver failure. The patient was treated with a high dose of iv corticosteroids and fully recovered after prolonged hospitalization. IFX treatment was discontinued in this patient.

Infections

Of the 50 IBD patients, 9 (18%) presented opportunistic infections during maintenance therapy. Six cases of viral infections occurred (5 herpes simplex virus and 1 varicella-zoster virus) in 5 CD patients and 1 UC patient had herpes simplex virus. Atypical pneumonia developed in 2 CD patients; full recovery was achieved in both patients following antibiotic therapy. All these patients were on concomitant immunomodulatory treatment which was withdrawn upon diagnosis. All infections developed within the first year of treatment and, in 3 patients, viral infections recurred during IFX maintenance treatment.

Malignant disorders

Three patients (6%) developed neoplasia during maintenance treatment (Table 4). Median time interval between the first infusion and diagnosis of neoplasia was 21 (range, 14-24) mo. Median number of IFX infusions was 10 (range, 6-13). Two patients with neoplasia were on concomitant immunomodulatory treatment (azathioprine). The histological types of neoplasia were gastric adenocarcinoma (T2aN0M0), which subsequently developed into peritoneal carcinomatosis and Krukemberg tumor, and endometrioid carcinoma (T1cN0M0), both in CD patients; breast cancer (T2mN3M0) developed in one UC patient.

Deaths

One CD patient, aged 68 years, who withdrew from the maintenance treatment protocol (9 infusions within 13 mo) because of complete disease remission, died of acute myocardial infarction 10 mo after the last IFX infusion. The patient did not present with any cardiovascular risk factors,

Table 3 Summary of IFX related adverse events

Adverse event	IBD (CD + UC)	Crohn's disease	Ulcerative colitis
Acute infusion reaction (%) (Mild, moderate, severe)	3/50 (6)	3/40 (7.5)	0
Delayed infusion reaction (%) (Serum sickness-like)	1/50 (2)	1/40 (2.5)	0
Opportunistic infection (%)	9/50 (18)	8/40 (20) 5 HSV 1 VZV 2 atypical pneumoniae	1/10 (10) 1 HSV
Lymphoproliferative diseases	0	0	0
Malignant disorder (%)	3/50 (6)	2/40 (5)	1/10 (10)

HSV: Herpes simplex virus; VZV: Varicella-zoster virus.

Table 4 Clinical characteristics of patients developing malignant disorders during IFX treatment

Patients	1	2	3
Gender	F	F	F
Disease	Ulcerative colitis	Crohn's disease	Crohn's disease
Disease duration (yr)	22	3	3
Site of disease	Left colon	Ileum-colon	Ileum-colon
Type of neoplasia (TNM)	Breast cancer (T2mN3M0)	Gastric cancer (T2aN0M0)	Endometrial cancer (T1cN0M0)
Concomitant medication	Azathioprine	Azathioprine/ cyprofloxacine/ metronidazole	Azathioprine/ cyprofloxacine/ metronidazole
N. IFX infusions	13	6	10
Months since first IFX infusion	21	24	14

TNM: Tumour, nodes, metastasis.

except for mild chronic renal failure, not requiring medical therapy and/or dialysis. The event was considered unrelated to IFX as it occurred 10 mo after the last infusion.

DISCUSSION

Despite the large amount of literature demonstrating the efficacy of IFX for the induction of remission in moderate to severe IBD, few data are available regarding the use of IFX for more than 12 mo or for more than 8 doses in IBD patients. Results of this retrospective study confirm current data on the efficacy of IFX in inducing a rapid clinical response in CD and support the finding, emerging from uncontrolled study data, of prolonged clinical efficacy in maintaining long-lasting remission beyond 1 year of treatment. While the efficacy of IFX therapy in the treatment of CD it is well established, only few studies have been performed on the use of IFX in UC. Järnerot *et al*[16] found IFX to be an effective rescue therapy in patients experiencing an acute flare up of UC, leading to a significant reduction in the emergency colectomy rate. Moreover, Rutgeerts *et al*[17] were the first to demonstrate that IFX can induce and maintain a clinical response and remission up to 54 wk of scheduled treatment in patients with moderately to severely active UC showing an inadequate response to conventional therapy. No data are yet available concerning the efficacy and safety of IFX treatment lasting for more than 1 year in UC. In our study population the median duration of IFX treatment was 27 mo in CD patients and 24.5 mo in UC patients, during which most of the patients (80 % of CD patients and 90% of UC patients) remained relapse free, following the clinical response or remission obtained with the induction treatment phase. It is worthwhile stressing that at enrolment, all these patients had active disease despite conventional therapy (or had a steroid-dependent disease). At baseline, considering all IBD patients, 58.5% were receiving 5-aminosalicylates, 78% corticosteroids, and 75.6% immunosuppressants. Moreover, compared to the 78% of patients on corticosteroids at baseline, only 25% needed concomitant corticosteroid therapy during IFX maintenance treatment, with a decreased median daily corticosteroid dose (0.7 mg/kg per day *vs* 0.25 mg/kg per day). The steroid-sparing effect of IFX was another important finding emerging from our study, which confirmed the efficacy of a scheduled treatment regimen in avoiding the well-known morbidity associated with long-term corticosteroid therapy[4,7].

Interestingly, long-term IFX therapy in IBD has been demonstrated to potentially modify the course of the disease. Indeed, 9 out of the 29 CD and 4 out of the 9 UC patients, who discontinued IFX scheduled treatment were still relapse-free after a median of 16 (range, 5-30) and 6.5 (range, 4-16) mo since the last IFX infusion, respectively. All these patients were off steroids and 12 were on immunosuppressants. Before stopping therapy, endoscopic evaluation was performed only in the subgroup of UC patients, showing complete mucosal healing in all. This result could account for the sustained clinical benefit maintained after withdrawal of treatment.

Long-term safety is an emerging and important issue as IFX use and indications are rapidly increasing worldwide. Recently, large retrospective studies have evaluated the incidence of serious adverse events and onset of neoplasia in CD patients treated with IFX[18-24,27]. Colombel *et al*[27], who studied 500 CD patients for a median of 17 (range, 0-48) mo with a median of 3 IFX infusions, reported the presence of 8.6% serious adverse events, 6.0% of which were considered possibly related to IFX therapy. In our study, 32% of IBD patients experienced at least 1 adverse event; 8% of the patients had an infusion reaction (3 patients experienced an acute infusion reaction and 1 a serum sickness-like disease). Moreover, in our study population, the incidence of opportunistic infections was 18%, which is higher than that observed in the study by Colombel *et al*[27] (8.2%). This finding could be due both to the longer scheduled treatment protocol and the concomitant use of immunomodulatory treatment (AZA or 6-MP). Albeit, the long duration of scheduled treatment associated with the concomitant use of immunomodulators may give rise to the risk of opportunistic infections; regular maintenance therapy has been proven to reduce the number of infusion reactions with respect to episodic dosing, which is more immunogenic, as demonstrated by the results of the ACCENT I trial[11]. In fact, in the study by Hanauer *et al*[11], the antibodies to IFX (ATI), which may be associated with decreased clinical response and increased risk of infusion reactions, were detected at higher rate in the episodic-

treated group (30%) compared to the maintenance strategy protocol (8%). Comparable results emerged from the studies by Baert *et al*[19] and Farrell *et al*[28], in which IFX was used episodically, thus demonstrating that the formation and concentrations of ATI were correlated with lower post-infusion serum IFX concentrations and with the need to shorten infusion intervals. Moreover, in patients who underwent bowel surgery after IFX withdrawal, no increase in the number of severe infections or surgical complications was observed in the perioperative period (data not shown).

An interesting finding emerging from our study, for which further investigation is necessary, concerns the relatively large number of malignancies (6%) observed in our IBD patients treated with IFX. In fact, the high incidence of solid tumors is somewhat inconsistent with the results reported so far. Biancone *et al*[21], in their multi-centre case-controlled study, evaluated the risk of developing neoplasia in IFX-treated CD patients: the incidence of newly diagnosed neoplasia was comparable in the 2 groups of CD patients, treated (2.2%) or not treated (1.73%) with IFX. Colombel *et al*[27], in a retrospective study found that 3 out of 500 of the CD patients treated with IFX had a malignant disorder, possibly related to biologic therapy. The fact that our study did not include a control group and consisted of a rather heterogeneous and relatively small population makes it difficult to establish a direct cause-effect relationship between anti-TNF-α therapy and the increased risk of developing malignancies. However, it should be pointed out that a slight difference was observed in terms of mean number of IFX infusions between patients who developed malignancies and those who did not (9.66 *vs* 14.6, respectively). This result would appear to suggest that there is no linear dose-dependent increase in risk.

Another important issue concerns the risk of hematologic malignancies related to the use of biologic therapies in IBD. It is well known that patients with long-standing IBD treated with immunomodulatory drugs may be more susceptible to developing lymphoproliferative disease[29-32]. Even though the use of TNF-α blocking agents has been associated with an increased risk of developing lymphoma[32], a finding, however, not confirmed by the data of a large US-based CD registry (TREAT)[22], we did not observe any cases of hematologic malignancy in our study population.

The results from this study need to be interpreted with an understanding of both the strengths and limitations of retrospective analysis of prospectively collected data. Although it allowed us to have a long term follow-up, the patients could have not been monitored as rigorously as prospective, randomized controlled studies. In any event, these limitations are not relevant to the analysis of efficacy of IFX treatment in IBD and of serious infections reported in this setting.

In conclusion, IFX scheduled treatment has proven to be an effective strategy in our IBD patient population for long-term maintenance of clinical remission. The scrupulous selection of patients to be started on IFX therapy is a fundamental issue, not only to obtain maximum efficacy, but also to avoid serious adverse events. A note of caution is mandatory when considering the possible risk of malignancy associated with the use of anti-TNF-α therapy. Further studies on larger series are needed to further clarify these important aspects.

COMMENTS

Background

For many years, corticosteroids have represented the cornerstone of therapy for induction of remission in inflammatory bowel disease (IBD); however, long-lasting remission was not achieved and the side-effects emerging with long-term use exceeded the clinical benefits. Immunomodulators have been demonstrated to be efficacious as adjunctive therapy and as steroid-sparing agents. The introduction of biological agents in the therapeutic armamentarium for Crohn's disease (CD) and ulcerative colitis (UC) has significantly changed the treatment strategies and outcomes of these patients.

Research frontiers

Despite the large amount of literature demonstrating the efficacy of infliximab (IFX) for the induction of remission in moderate to severe IBD, few data are available regarding the use of IFX for more than 12 mo or for more than 8 doses in IBD patients. Some safety issues are associated with IFX use, mostly related to the development of adverse events (e.g., opportunistic infections, autoimmune disorders, and infusion reactions). Major concerns are related to the reactivation of latent tuberculosis and development of malignancy, even if there is no clear evidence that the use of IFX increases the incidence of solid cancers.

Related publications

Colombel *et al*[27] studied 500 CD patients for a median of 17 mo (range, 0-48) with a median of 3 IFX infusions and reported the presence of 8.6% serious adverse events, 6.0% of which were considered possibly related to IFX therapy. Hanauer *et al*[11] found that the antibodies to IFX, which may be associated with decreased clinical response and increased risk of infusion reactions, were detected at higher rate in the episodic-treated group (30%) compared to the maintenance strategy protocol (8%). Comparable results emerged from the studies by Baert *et al*[19] and Farrell. Biancone *et al*[21], in their multi-centre case-controlled study, evaluated the risk of developing neoplasia in IFX-treated CD patients: the incidence of newly diagnosed neoplasia was comparable in the 2 groups of CD patients, treated (2.2%) or not treated (1.73%) with IFX.

Innovations and breakthroughs

IFX scheduled treatment has proven to be an effective strategy in our IBD patient population for long-term maintenance of clinical remission. The scrupulous selection of patients to be started on IFX therapy is a fundamental issue, not only to obtain maximum efficacy, but also to avoid serious adverse events.

Terminology

Infliximab is a chimeric monoclonal IgG1 antibody directed against TNF-α able to almost completely neutralize its biological activity. Crohn's Disease Activity Index was used to monitor Crohn's disease activity and the Clinical Activity Index was used for assessing UC activity.

Peer review

This article contains valuable information that would be useful for practicing clinicians.

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CLINICAL RESEARCH

Transcutaneous cervical esophagus ultrasound in adults: Relation with ambulatory 24-h pH-monitoring and esophageal manometry

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Abstract

AIM: To determine the gastroesophageal refluxate in the cervical esophagus (CE) and measure transcutaneous cervical esophageal ultrasound (TCEUS) findings [anterior wall thickness (WT) of CE, esophageal luminal diameter (ELD), esophageal diameter (ED)]; to compare TCEUS findings in the patient subgroups divided according to 24-h esophageal pH monitoring and manometry; and to investigate possible cut-off values according to the TCEUS findings as a predictor of gastroesophageal reflux (GER).

METHODS: In 45/500 patients, refluxate was visualized in TCEUS. 38/45 patients underwent esophagogastroduodenoscopy (EGD), 24-h pH monitoring and manometry.

RESULTS: The 38 patients were grouped according to 24-h pH monitoring as follows: Group A: GER-positive (n = 20) [Includes Group B: isolated proximal reflux (PR) (n = 6), Group C: isolated distal reflux (DR) (n = 6), and Group D: both PR/DR (n = 8)]; Group E: no reflux (n = 13); and Group F: hypersensitive esophagus (HSE) (n = 5). Groups B + D indicated total PR patients (n = 14), Groups E + F reflux-negatives with HSE (n = 18), and Groups A + F reflux-positives with HSE (n = 25). When the 38 patients were grouped according to manometry findings, 24 had normal esophageal manometry; 7 had hypotensive and 2 had hypertensive lower esophageal sphincter (LES); and 5 had ineffective esophageal motility disorder (IEM). The ELD measurement was greater in group A + F than group E (P = 0.023, 5.0 ± 1.3 *vs* 3.9 ± 1.4 mm). In 27/38 patients, there was at least one pathologic acid reflux and/or pathologic manometry finding. The cut-off value for ELD of 4.83 mm had 79% sensitivity and 61% specificity in predicting the PR between Groups B + D and E (AUC = 0.775, P = 0.015).

CONCLUSION: Visualizing refluxate in TCEUS was useful as a pre-diagnostic tool for estimating GER or manometric pathology in 71.1% of adults in our study, but it was not diagnostic for CE WT.

Key words: Ambulatory 24-h pH monitoring; Cervical esophageal ultrasound; Gastroesophageal reflux; Esophageal manometry; Esophageal refluxate

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INTRODUCTION

Esophageal ultrasonography (US) is a non-invasive, readily available, repeatable, cheap, fast and highly sensitive technique[1-4] in the diagnosis of gastroesophageal reflux (GER) in infants and children[5-8]. The esophageal US studies in GER have mainly focused on the evaluation of the gastroesophageal junction (GEJ)[9-11] and esophageal motility[12-14]. These studies were performed by transabdominal or endoluminal routes. Although cervical US is a part of neck US, it has not been routinely used in infants and adults for diagnosis of GER[9]. There are only a few studies about the transcutaneous cervical esophagus ultrasonography (TCEUS), but these were in normal[15,16] and pathologic conditions[17,18].

Intraluminal refluxate can be recognized by US images. Sonographic GER diagnosis was made by backward movement of gastric content into the esophagus and the visualization of the clearance of refluxate material[15,19,20]. The visualization of GER episodes or gastroesophageal reflux disease (GERD) estimation in the GEJ region in US provided the background for our study. The aims of this study were to evaluate the possible pathologies in 24-h (h) pH monitoring and esophageal manometry in patients with refluxate in the lumen of the cervical esophagus

(CE) during TCEUS; to compare TCEUS findings in the patient subgroups divided according to 24-h esophageal monitoring and manometry; and to investigate possible cut-off values according to the TCEUS findings as a predictor of GER.

MATERIALS AND METHODS

Study design

Patient features: Five hundred patients (45.82 ± 14.15 years, 163 M/337 F) who were admitted to the outpatient clinic between the years from January 2006 to January 2007 with complaints other than of the gastrointestinal system underwent TCEUS. Refluxate material was found in the esophageal lumen in 45 (9%) of the 500 patients during TCEUS. Forty-five patients were questioned regarding GERD symptoms, and all had reflux symptoms.

Thirty-eight of the 45 patients underwent esopha gogastroduodenoscopy (EGD), 24-h pH monitoring and esophageal manometry [7 patients were excluded as follows: pH monitoring not accepted (n = 5), nasal cannulation could not be performed due to nasal operation history (n = 1), inability to continue the 24-h pH monitoring/pH catheter extracted (n = 1)]. The period between the TCEUS and the pH monitoring was 1-3 d.

Patients had no history of weight loss, gastrointestinal bleeding, gastrointestinal motility disorder, pneumonic dilatation, collagen vascular disease, any operation around the cervical region, or gastrointestinal operation. None of the patients was taking medications known to affect esophageal motor function, including promotility agents, antacids, H2 receptor antagonist, or proton-pump inhibitors (PPI); 3 patients had been taking PPI but they had been discontinued for two weeks before manometric investigation and 24-h pH monitoring.

Test techniques

Questionnaire for GERD: All patients were evaluated for typical (acid regurgitation and heartburn) and extraesophageal (hoarseness, asthma-like clinical presentation, nocturnal cough, and nocturnal wake-up) GERD symptoms.

Esophagogastroduodenoscopy (EGD): The procedure was performed by Pentax EG 2940 with 2% xylocaine topical anesthesia after a 12-h overnight fast. Reflux esophagitis was evaluated according to Los Angeles classification[21]. The presence of hiatal hernia and the distance between GEJ and diaphragmatic impression were recorded.

TCEUS: Each patient was given an 800 kcal standard meal (15% protein, 50% carbohydrate, and 35% fat) and TCEUS was performed at postprandial 1-2 h with patient in supine position (Hitachi EUB, 6-13 MHz linear probe). TCEUS was performed as defined by Zhu and Mateen[15,16]. The esophagus was demonstrated at thyroid cartilage level with the guidance of thyroid gland acoustic window up to the supraclavicular level to thoracal inlet (manubrium sterni) by linear probe in transverse and longitudinal sections without a pillow under the neck. CE was evaluated by using a slightly flexed neck position with head turned 45° to the opposite side by left and right lateral approaches over 15 min to determine the presence of refluxate (the luminal anechoic fluid and/or linear bright stratifying small lines indicating gas in refluxate) and its to-and-fro movement, with the patients not swallowing[15,20,22]. Then all patients were required to swallow and the clearance of refluxate was observed. The presence of comet-tail artifact (during swallowing, the presence of saliva mixed with air and downward movement of refluxate generated a strong echogenic appearance[15,20,22]) was observed in patients. After the clearance of refluxate was observed, a few patients had backward flow of refluxate into the esophagus, which can perhaps be considered by the terminology "re-reflux"[23].

Anterior wall thickness (WT) of the esophagus (distance between adventitia and the mucosa, with 5-7 esophageal wall layers), esophageal diameter (ED) [distance between the adventitia (outer to outer)], and esophageal luminal diameter (ELD) with or without refluxed material [distance between the mucosa (inner to inner)] were measured in longitudinal section at left lateral cervical approach. The GEJ was not evaluated during US in this study. The TCEUS appearance with or without refluxate is given in Figure 1.

Ambulatory 24-h pH monitoring: pH monitoring was performed using Synetics Digitrapper MHⅢ, and double-channel, 15 cm antimony catheter. The esophageal pH catheter was placed 5 cm above the upper border of the lower esophageal sphincter (LES). Findings were evaluated by Microsoft esophagram version 2.04. The pathologic measurements were evaluated as follows: Proximal reflux (PR): The upper esophageal sphincter (UES) localization was determined by manometry and PR was determined by the proximal probe localization and UES. If proximal probe was localized in the UES or above it, a single acid reflux synchronously occurring with distal probe was accepted as pathologic acid reflux; if the probe was localized under the UES, acid contact time above 1% of total time was accepted as pathologic in PR. De Meester score > 14.72 and acid contact greater than 4.0% of total time below pH 4 were accepted as pathologic in distal reflux (DR). Hypersensitive esophagus (HSE) was defined if symptom index (SI) for distal measurements (SI = number of symptoms in pH < 4/total number of symptoms) was ⩾ 50% while there was no measurable DR or PR[23-25].

Esophageal manometry: Esophageal manometry was performed using MMS (ver. 8.4i Beta) and eight-channel Dent-sleeve catheter. After calibration, catheter was sent through the nose to the stomach and advanced 65 cm by swallowing. When all channels were in stomach, with patient in supine position, UES and LES were determined as the catheter was slowly withdrawn back into the esophagus. LES pressure (LESP), relaxation, esophageal body pressure, body contractions, contraction amplitudes and duration, peristalsis and upper esophageal contractions were recorded. Manometric findings were grouped as: normal, spastic (hypertensive LES, if LESP > 45 mmHg),

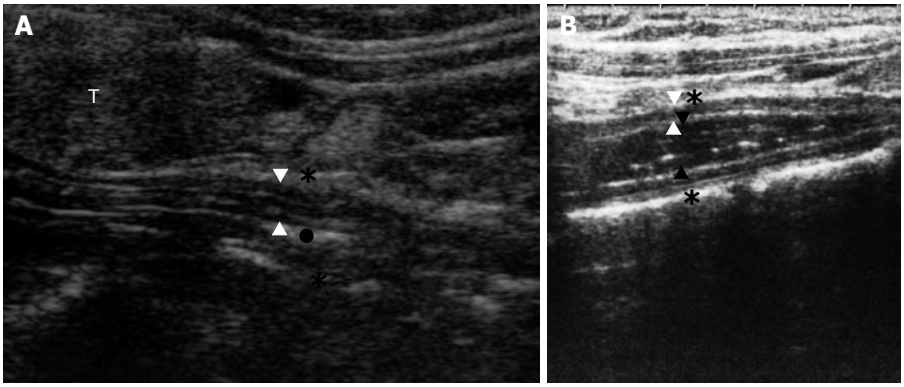

Figure 1 The TCEUS appearance with and without refluxate. **A**: A pattern showing no anechoic fluid in the lumen. Note anterior esophageal wall (distance between the open arrowheads), esophageal lumen without fluid inside and hyperechogenic bands representing collapsed lumen and mucosa (dark dot). T: thyroid; **B**: A pattern showing anechoic fluid in the lumen. Note esophageal wall (distance between the open arrowheads), refluxate in the esophageal lumen (distance between the dark arrowheads) and esophageal diameter (distance between the asterisks).

non-spastic [hypotensive LES, if LESP < 10 mmHg) or ineffective esophageal motor contractions (IEM), if contraction amplitude was < 25 mmHg in > 30% of wet swallows][26].

Statistical analysis

Descriptive and comparative statistical analyses were performed using statistical software system (SPSS v11.0). Where appropriate, average data were presented as mean ± SD. Comparison between groups was performed by Kruskal Wallis analysis. All possible pair-wise comparisons were done by Mann-Whitney *U* test with Bonferroni correction. Fisher-Freeman-Halton test generalized at Fisher's exact test to mxn tables was used for categorical variables. The cut-off values were determined using receiver operating characteristic (ROC) curve for TCEUS parameters between all possible patient group pairs according to pH-metry and manometry to determine reflux or any pathologic manometry finding. The sensitivity and the specificity were determined according to the measured cut-off values. The significance of the area under curve (AUC) was tested ($P < 0.05$).

Investigators interpreting sonography, 24-h pH monitoring and esophageal manometry were blinded to the patients' features. None of the patients was sedated during EGD. All patients provided written informed consent and the study conformed to the guidelines of the Helsinki Declaration.

RESULTS

Forty-five (9%) of 500 patients who underwent TCEUS were found to have anechoic fluid and/or air echogenicities of refluxate in the cervical esophageal lumen. CE was not visualized clearly in 1 (0.2%) of the 500 patients due to neck anatomy.

Esophagitis (all grade A), hiatal hernia, antral gastritis, and grade 1 bulbitis were diagnosed in 10.7%, 10%, 14%, and 3%, respectively, in EGD. None of the patients had malignancy, or gastric or duodenal ulcer disease.

Thirty-eight patients were grouped according to 24-h pH monitoring as follows: Group A: Acid reflux-positive ($n = 20$, 52.7%) [includes Group B + Group C + Group D] [Group B: Isolated PR but no DR ($n = 6$, 15.8%); Group C: Isolated DR but no PR ($n = 6$, 15.8%); Group D: Both PR and DR ($n = 8$, 21.1%)]; Group E: No acid reflux ($n = 13$, 34.2%); and Group F: patients with hypersensitive esophagus (HSE) ($n = 5$, 13.1%). Group B + D indicated total patients with PR ($n = 14$, 36.9%) and Group E + F: acid reflux-negatives with HSE ($n = 18$, 47.3%) and Group A + F: Acid reflux-positives with HSE ($n = 25$, 65.8%). The demographic and TCEUS findings of subjects grouped according to 24-h pH monitoring are given in Table 1.

When the 38 patients were grouped according to manometry findings, 24 (63.2%) patients had normal esophageal manometry; 7 (18.4%) had hypotensive and 2 (5.3%) had hypertensive LES; 5 (13.1%) had ineffective esophageal motility disorder (IEM). Demographic and TCEUS findings of subjects grouped according to manometric results are given in Table 2.

Patient symptoms are given in Table 3. None of the patients had complaints of dysphagia or asthma-like dyspnea.

Statistical analysis was performed between (1) 24-h pH monitoring subgroups, (2) esophageal manometry subgroups, and (3) categorized groups according to combined 24-h pH monitoring and esophageal manometry findings as acid reflux/abnormal, acid reflux/normal, no acid reflux/abnormal, and no acid reflux/normal with the following parameters: Age, sex, body mass index (BMI), LES localization defined during manometry, and TCEUS findings (WT, ED, ELD).

There were no significant differences in BMI, LES localization, and typical and extraesophageal symptoms between subjects grouped according to 24-h pH monitoring and according to esophageal manometric findings. There was no correlation between the TCEUS findings and sex or presence of hiatal hernia or esophagitis.

When 24-h pH monitoring subgroups were compared according to TCEUS findings, the ELD measurement was greater in group A + F than group E ($P = 0.023$). No significant differences were determined between the other subgroups when compared regarding TCEUS findings.

There was a positive significant correlation between ED and ELD ($r = 0.889$, $P = 0.000$) and ED and WT ($r = 0.499$, $P = 0.001$) (Pearson correlation analysis).

There were 2 patients with hypertensive LES. Excluding this group, when the nonspastic esophageal motor disorder group (hypotensive LES, IEM) was compared with the normal manometric group, there was no significant difference with regard to TCEUS findings and the LES localization between subgroups.

There was no significant difference according

Table 1 Demographic and TCEUS findings in groups divided according to pH monitoring (mean ± SD)

	Group A (n = 20)	Group B (n = 6)	Group C (n = 6)	Group D (n = 8)	Group E (n = 13)	Group F (n = 5)	Group B + D (n = 14)	Group E + F (n = 18)	Group A + F (n = 25)	P
Age (yr)	43.4 ± 12.0 (24-72)	40.0 ± 11.4 (24-58)	47.8 ± 12.7 (38-72)	42.6 ± 12.5 (25-62)	43.17 ± 9.6 (25-65)	37.2 ± 18.8 (15-64)	41.5 ± 11.7 (24-629	40.5 ± 10.7 (16-65)	42.3 ± 13.7 (15-72)	NS
Sex (F/M) n (%)	8/12 (40/60)	3/3 (50/50)	1/5 (16.7/83.3)	4/4 (50/50)	11/2 (84.6/5.4)	5/0 (100/0)	7/7 (50/50)	2/16 (88.9/11.1)	12/13 (52/48)	NS
BMI (kg/m^2)	27.4 ± 4.5 (19.6-37.4)	27.4 ± 4.4 (19.6-32)	27.4 ± 4.4 (19.6-32)	27.9 ± 5.6 (20.6-37.5)	27.7 ± 4.6 (17.6 ± 35.0)	24.9 ± 2.1 (22.7-27.4)	27.7 ± 4.9 (19.6-37.4)	26.9 ± 4.24 (17.6-35.0)	26.9 ± 4.3 (19.6-37.5)	NS
ED (mm)	9.4 ± 1.4 (6.1-12.1)	9.7 ± 0.9 (8.2-10.8)	9.1 ± 1.6 (8.0-12.0)	9.6 ± 1.71 (6.1-11.4)	8.54 ± 1.82 (5.5-11.1)	10.6 ± 1.3 (8.8-12.4)	9.6 ± 1.4 (6.2-11.4)	9.1 ± 1.9 (5.5-12.4)	9.7 ± 1.5 (6.1-12.4)	NS
ELD (mm)	5.0 ± 1.2 (3.0-7.7)	5.1 ± 1.1 (3.00-6.00)	4.6 ± 1.6 (3.4-7.7)	5.2 ± 1.18 (3.0-6.4)	3.9 ± 1.4 (1.5-6.2)	5.3 ± 1.7 (3.4-7.9)	5.16 ± 1.10 (3.0-6.4)	4.3 ± 1.6 (1.5-7.9)	5.0 ± 1.3 (3.0-7.9)	0.023[a]
Esophageal WT (mm)	2.2 ± 0.2 (1.5-2.6)	2.3 ± 1.2 (2.2-2.6)	2.2 ± 0.1 (2.0-2.3)	2.1 ± 0.3 (1.5-2.5)	2.3 ± 0.3 (2.0-3.3)	2.6 ± 0.7 (2.2-4.0)	2.2 ± 0.3 (1.6-2.6)	2.4 ± 0.50 (2.0-4.0)	2.3 ± 0.4 (1.6-4.0)	NS
DeMeester score	19.5 ± 12.1 (1.7-46.2)	7.9 ± 3.1 (4.6-13.2)	24.3 ± 14.4 (1.7-46.2)	24.5 ± 9.1 (14.4-38.9)	6.4 ± 4.7 (0.8-14.7)	7.5 ± 4.9 (1.4-17.3)	17.4 ± 10.9 (4.6-38.9)	6.7 ± 4.7 (0.8-14.7)	17.7 ± 11.8 (1.4-46.2)	NS

TCEUS: Transcutaneous cervical esophagus ultrasonography; ED: Esophageal diameter; ELD: Esophageal luminal diameter; Esophageal WT: Esophageal wall thickness; BMI: Body mass index. [a]$P < 0.05$ between group A + F and group E. NS: Non-significant.

Table 2 Demographic and TCEUS findings of the groups divided according to esophageal manometry (mean ± SD)

	Normal (n = 24)	IEM (n = 5)	Hypo LES (n = 7)	Hyper LES (n = 2)	Total patients (n = 38)	P
Age (yr)	40.1 ± 11.4 (15-64)	50.6 ± 11.9 (39-65)	48.9 ± 11.3 (38-72)	29.0 ± 4.3 (26-32)	42.5 ± 12.2 (15.0-72.0)	NS
Sex (F/M), n (%)	8/16 (66.7%/33.3%)	4/1 (80%/20%)	2/5 (28.6%/71.4%)	2/0 (100%/0%)	24/14 (63.2%/36.8%)	NS
BMI (kg/m^2)	26.7 ± 4.6 (17.6-375)	26.7 ± 4.1 (20.6-31.1)	29.58 ± 3.93 (23.1-35.0)	25.4 ± 2.7 (23.4-27.3)	27.2 ± 4.3 (17.6-37.4)	NS
ED (mm)	9.1 ± 1.59 (6.0-12.4)	9.5 ± 2.3 (5.5-11.4)	9.8 ± 1.7 (7.5-12.1)	9.6 ± 1.1 (8.9-10.4)	9.3 ± 1.7 (5.5-12.4)	NS
ELD (mm)	4.5 ± 1.3 (2.0-7.9)	4.7 ± 1.9 (1.5-6.4)	5.1 ± 1.7 (3.1-7.7)	4.7 ± 0.9 (4.1-5.4)	4.68 ± 1.43 (1.5-7.9)	NS
Esophageal WT (mm)	2.2 ± 0.4 (1.6-4.0)	2.4 ± 2.2 (2.0-2.6)	2.3 ± 0.4 (2.0-3.3)	2.35 ± 0.2 (2.2-2.5)	2.3 ± 0.4 (1.6-4.0)	NS

IEM: Ineffective esophageal motility; Hypo LES: Hypotensive lower esophageal sphincter; Hyper LES: Hypertensive lower esophageal sphincter; ED: Esophageal diameter; ELD: Esophageal luminal diameter; Esophageal WT: Esophageal wall thickness; BMI: body mass index. NS: Non-significant.

Table 3 Symptoms of patients according to esophageal monitoring n (%)

	Group A (n = 20)	Group E (n = 13)	Group B + D (n = 14)	Group E + F (n = 18)
Extra-esophageal symptom	12 (60.0)	7 (53.8)	9 (64.3)	12 (66.6)
Cough	6 (30.0)	6 (46.2)	4 (28.5)	8 (44.4)
Hoarseness	5 (25)	3 (23.1)	4 (28.6)	4 (22.2)
Nocturnal wake-up with reflux	10 (50)	5 (38.5)	8 (57.1)	9 (50.0)
Typical symptom	20 (100)	12 (92.3)	14 (100.0)	17 (94.4)
Heartburn	18 (90)	12 (92.3)	12 (85.7)	17 (94.4)
Regurgitation	15 (75)	10 (76.9)	12 (85.7)	13 (72.2)

Table 4 Distribution of patients according to 24-h pH monitoring and manometric findings n (%)

	Manometric findings (abnormal)	Manometric findings (normal)
24-h pH monitoring (Acid reflux)	7 (18.4)	13 (34.3)
24-h pH monitoring (No acid reflux)	7 (18.4)	11 (28.9)

to TCEUS findings when 24-h pH monitoring and esophageal manometry subgroups were evaluated together (Table 4). Table 5 shows the detailed 24-h pH monitoring and manometric findings. In 27/38 (71.1%) patients, there was at least an acidic reflux and/or pathologic manometry finding. 11/38 (28.9%) with refluxate in CE had no acid reflux and normal esophageal manometric findings.

We tried to find cut-off values in order to differentiate total GER, PR or the other reflux subgroups from the reflux-negatives and to differentiate each manometry subgroup according to TCEUS parameters. The groups which had significant cut-off values (AUC, $P < 0.05$) are given in Table 6 with their sensitivity and specificity rates for ELD in determining reflux. The ROC curve is given in Figure 2 according to TCEUS findings in patients with total PR (group B + D) (n = 14) and in patients without reflux (group E) (n = 13).

DISCUSSION

The esophagus is a 23-24 cm muscular channel. The longitudinal scan of the esophagus shows a tubular structure with hypoechogenic muscular layer on the wall and one or two echogenic inner layer(s) representing the mucosa and the collapsed lumen of the esophagus[3,10,15,16,20,22].

US evaluation is performed at four sites of the esophagus: GEJ[4,9,27,28], thoracal esophagus[22], CE[15,16], and upper esophageal sphincter[29].

Table 5 24-h pH monitoring and esophageal manometry results

24-h pH monitoring	IEM (*n* = 5)	Hypo LES (*n* = 7)	Hyper LES (*n* = 2)	Normal manometry (*n* = 24)
PR + DR	2	2		4
PR				6
DR		3		3
Reflux negatives	3	2	1	7
HSE			1	4

IEM: Ineffective esophageal motility; Hypo LES: Hypotensive lower esophageal sphincter; Hyper LES: Hypertensive lower esophageal sphincter ; PR: Proximal reflux; DR: Distal reflux; HSE: Hypersensitive esophagus.

Table 6 Cut-off values for esophageal luminal diameter (ELD) to determine acid reflux

Between groups	Cut off (mm)	AUC	*P*	Sensitivity (%)	Specificity (%)
Group B + D (total PR) (*n* = 14)/Group E (reflux negative) (*n* = 13)	4.83	0.775	0.015[a]	79	61
Group B + D (total PR) (*n* = 14)/Group E + F (reflux negative with HSE) (*n* = 18)	4.95	0.708	0.046	71	77
Group A (*n* = 20)/Group E (reflux negative) (*n* = 13)	4.95	0.721	0.034	60	77

HSE: Hypersensitive esophagus; PR: Proximal reflux; AUC: Area under the curve. *P* values show significance of AUC ([a]*P* < 0.05).

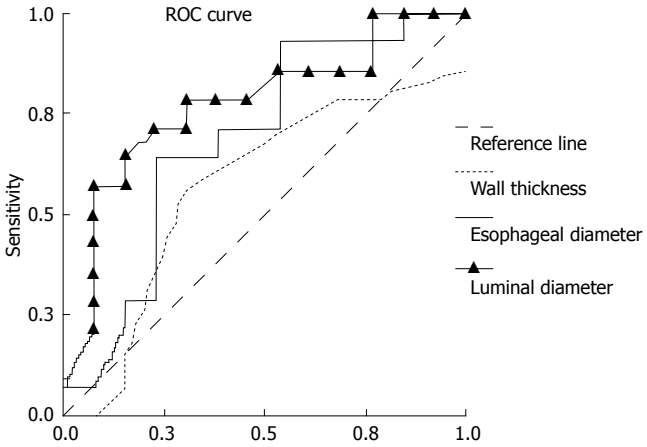

Figure 2 The ROC curve showing the relation between TCEUS findings according to total PR group (B + D) (*n* = 14) and reflux-negative group (E) (*n* = 13) (AUC = 0.775, *P* = 0.015 for ELD). PR: Isolated proximal reflux; ELD: Esophageal luminal diameter.

GERD arises from increased exposure and/or sensitivity of the esophageal mucosa to gastric contents[30,31], and affects 5%-40% of the population[32,33]. The content of refluxate can be isolated liquid (acid or non-acid nature), isolated gas, or gas/liquid mixture. 24-h pH monitoring and multichannel intraluminal impedance (MII) are the gold standard techniques to evaluate GER[19,34,35].

US has been used in GERD since 1984[36]. The GEJ was first described by Westra[6] and Gomes[1] during US by transabdominal route. The first-line use of esophageal GEJ US in GERD for infants and children was established by multiple studies[2,4,6,37]. Sonographic sensitivity was 81%-94%[3,4]. US provides a morphologic and functional approach. In infants and children, 24-h pH monitoring and esophageal US are the complementary techniques of choice[2,4].

Zhu points out the importance of conventional US to evaluate the GEJ, but the use of the CE was defined to be restricted. Zhu defined the normal sonographic parameters of the CE (7.5-12 MHz transducer) transcutaneously[15]. Mateen *et al* used a modified technique which differed from the normal neck US to evaluate the CE. Visualization failure of the right lateral two-thirds CE was decreased from 36% to 2% using this modified technique[16]. In our study, use of this modified technique resulted in failed visualization in only 1 (0.2%) of 500 patients due to the deformed anatomy of the patient.

GERD was diagnosed in 26% of a healthy population of infants and children according to US[3]. In our study, postprandial refluxate was seen in 45 of 500 (9%) adults. Furthermore, 20/38 (52.63%) of the patients who admitted to the hospital for other than gastrointestinal symptoms had refluxate in TCEUS and acidic reflux according to the 24-h pH monitoring. Since non-acid reflux was not evaluated, the 18 other patients were not evaluated in this respect.

Cool *et al*[38] showed that respiratory and ear, nose and throat symptoms were especially related with gas reflux with weak acidity and not abnormal proximal acid reflux. We did not find any correlation in our study between proximal acid reflux, GER and any symptoms.

During the first hour after a meal, 20% more reflux episodes reach a higher proximal extent than during the fasting period and the late postprandial period (after 1 h). Acid reflux can reach 15 cm above the LES in approximately 6.8-21 s[39,40]. We performed the sonography at the postprandial 1st-2nd h.

The content of liquid refluxate, whether acidic or not, did not affect the sonographic appearance[41]. The new studies have pointed out that gas reflux with weak acidity is quite often determined in PR studies performed by pharyngeal impedance-pH recordings. Mixed reflux of gas and fluid is more frequent than pure fluid reflux[39].

In the study of Mittal performed simultaneously with high frequency endoscopic US (HFEUS) and pH-metry, five US refluxate patterns were identified, as fluid, gas, first gas later fluid transition, first liquid later gas transition, or no luminal opening[19]. In our study, we observed these patterns as fluid with or without gas and its to-and-fro movement during a period patients did not swallow. During swallowing, the comet-tail artifact was detectable in 42.8% of the cases[15,19]. We observed it in approximately half of the patients. A few patients had reverse movement of refluxate to the esophagus after swallowing. This could be US documentation of a new terminology, "re-reflux"[23].

Jang *et al* found no correlation between reflux number in 15 min counted during US and the reflux index in the 24-h pH monitoring. Sonographic reflux number was not considered as a specific indicator of disease severity[2]. We did not count the reflux episodes in our study. Only one reflux episode in CE during US was included. Dickmann *et*

al[42] had shown that the acid reflux period below pH 4 was significantly lower when the distal pH probe was located 16 cm above the LES than 1, 6 or 11 cm above the LES in non-erosive reflux disease (NERD).

Transcutaneous CE WT has been reported as 2.3 ± 0.3 (1.3-4.1) mm in healthy adults[15]. Mateen reported right-side thickness as 2.8 ± 0.4 mm (upper limit of normal, 3.6 mm) and left-side thickness as 2.9 ± 0.2 mm (upper limit of normal, 3.3 mm). We determined the left anterior WT as 2.29 ± 0.38 mm (1.57-4.0) in TCEUS (Table 1).

Dogan *et al*[43] reported an increase in distal esophageal WT in conjunction with increasing age (1.56 ± 0.32 mm *vs* 1.29 ± 0.24 mm). In our study, we found no correlation between age, sex and TCEUS findings in subjects with refluxate.

Endosonographically, GEJ and 10 cm above thicknesses were given as 2.43 ± 0.16 and 2.28 ± 0.21 mm in healthy adults, respectively. The distal esophagus wall was thicker than the proximal[28]. In reflux esophagitis, total esophageal WT and the smooth muscle layer were observed to be thicker than in normal subjects[19,28,32]. Changchien measured normal GEJ WT as 3.8 ± 1.2 (2-5) mm using real time US. During acute severe inflammation in reflux esophagitis, the GEJ wall was observed as 7.6 ± 2.1 (5-10) mm, which was significantly thicker than normal[9]. The submucosal healing due to lansoprazole in GERD was evaluated by US and the WT had decreased significantly in the GEJ region[32,44]. We did not determine any significant difference in WT between patients with or without reflux according to 24-h pH monitoring. The distal esophageal WT increased with reflux according to the literature as described above, but we could not confirm this observation for the proximal esophagus in GER. Although HSE is a new terminology in the GERD spectrum, there was no significant difference between the HSE subgroup and the other subgroups with regard to CE WT.

Zhu reported normal transverse ED as 11.1 ± 1.6 (7.1-13.9) mm and anteroposterior diameter as 7.5 ± 1.2 mm (4.9-10.1)[15]. Mateen measured the transverse diameter as 6.8 ± 2.7 mm (max 12.2)/10.7 ± 4.0 mm (18.7) and anteroposterior diameter as 6.5 ± 1.1 (max 8.7) mm/7.4 ± 1.5 (10.4) mm with right and left approaches, respectively, using the modified technique[16]. We measured the cervical anteroposterior ED as 5.5-12.4 mm, and the ELD with refluxate was 1.5-7.9 mm in patients longitudinally (Tables 1 and 2). No significant differences were determined between proximal and total reflux patient groups and the other subgroups with regard to ED. ELD measurements with refluxate were statistically greater in group A + F than group E ($P = 0.023$). There was no difference between the other groups regarding ELD. Peak ED was given as 22 mm during physiologic swallows with 15 mL water[19]. PR volume has not been accurately diagnosed to date, though esophageal continuous aspiration and scintigraphic studies have been used in an effort to obtain results about the reflux volume[39]. In our study, the ED and ELD measurements may be an indirect indicator of reflux amount.

The distal esophageal distension and the cross-sectional area (CSA) are known to be wider than the proximal esophagus[19]. Mittal reported that healthy asymptomatic individuals had comparable esophageal diameter and CSA measurements according to spontaneous fluid GER and 5 mL swallow. It is difficult to differentiate between the ingested fluid and the refluxate of esophageal content. Mittal made the differentiation by looking at transient LES relaxations synchronously. In our study, our patients did not drink water during the TCEUS measurements. We measured the esophageal refluxate during a non-swallowing period. Our ED measurements (Table 1) were compatible with the 5 mL water intake in Mittal's studies[19].

Mittal *et al* observed many reflux episodes determined by pH probe but not concomitant sonographic reflux by HFEUS. Similar observations were also made using impedance techniques. They concluded the gas-dominant or mixed reflux episodes could be the contributory factor[19]. In our study, we did not perform TCEUS and the 24-h pH monitoring concurrently. This finding points out that US in GERD has some shortcomings. In contrast to this finding is the short reflux period which was determined by color Doppler (CD) US but not by pH monitoring[45].

There was a positive significant correlation between ED and ELD ($r = 0.889$, $P = 0.000$) and ED and WT ($r = 0.499$, $P = 0.001$) (Pearson correlation analysis). The positive relation could be explained by presence of refluxate in the esophageal lumen. Although there was positive correlation between ED and WT, no significant difference was found between groups. This could be explained by the small patient groups.

Esophageal motor disorders can cause abnormal fluid or viscous bolus transit[37,46]. Esophageal dysmotility can cause reflux esophagitis and reflux can cause esophageal dysmotility[12,37]. Patients with normal esophageal motility, diffuse esophageal spasm (DES) and achalasia had 35%, 67%, and 100% abnormal fluid or viscous bolus transit, respectively[37]. The possible manometric disorders that could be responsible for the PR were also evaluated in our study. We did not observe any patients with achalasia, DES or nutcracker esophagus. The manometric abnormality prevalence in patients with cervical refluxate during TCEUS was 36.84% (14/38 patients) [5 (13.16%) IEM, 7 (18.4%) hypotensive LES, 2 (5.4%) hypertensive LES] (Table 2).

There is a gradual increase in muscle thickness, thickening of the muscularis propria and increase in CSA from the proximal to distal esophagus in primary spastic esophageal motor disorders like achalasia, DES, nutcracker esophagus, hypertensive LES, and atypical LES relaxation, and in non-spastic esophageal motor disorders like hypotensive LES, IEM, and incomplete LES relaxation[12,15,37,41]. WT according to disorder was achalasia > DES > nutcracker esophagus[19,41,43]. The normal basal esophageal WTs at 2 cm and 10 cm above the GEJ were measured as 1.45 ± 0.31 mm and 1.24 ± 0.23 mm, respectively, by Dogan *et al*. The corresponding abnormal values were 2.08 mm and 1.71 mm[43].

We found no difference in anterior CE WT in patient subgroups divided according to esophageal manometry. In our study, the esophageal measurements were taken at the thyroid gland level, while corresponding values in the literature were measured at the GEJ or 2 or 10 cm above

the GEJ. IEM is characterized by low amplitude esophageal contractions, which could cause ineffective acid clearance and aid the reflux pathogenesis[37]. In our study, 7 of 14 (50%) patients with abnormal esophageal manometry had acid reflux (3 had DR and 4 had both PR and DR) in 24-h pH monitoring. Two of 5 patients who had IEM disorder, 5 of 7 patients with hypotensive LES and 0 of 2 patients with hypertensive LES had reflux (Table 5). The PR rate was higher than DR rate (70%, 30%) in group A. None of the patients with isolated PR had manometric impairment. Twenty-seven patients (71.1%) had at least one pathology in pH monitoring (acid reflux) and/or manometry. We did not observe any pathology which could cause impairment in esophageal transit in 11 of 38 patients (28.9%). Since we did not investigate non-acid reflux, the probable reflux patterns in these 11 patients are unknown.

We aimed to determine the possible cut-off values for TCEUS findings in patients with refluxate as a predictor of GER or pathologic manometry finding. ELD but not WT and ED showed cut-off values (AUC, $P < 0.05$). ELD (with refluxate) of 4.95 mm had 71% sensitivity and 77% specificity in the estimation of total PR patients (Table 6).

The fact that 24-h pH monitoring and manometry were not performed in subjects without refluxate during TCEUS is a limitation of this study.

Esophageal refluxed material can be recognized in ultrasonographic images. TCEUS can not substitute for 24-h pH monitoring or esophageal manometry, but it can serve as a complementary technique by aiding in the estimation of proximal reflux, GER and motility disorders which could cause impairment in bolus transit.

To our knowledge, there is no study in the available literature showing refluxate presence in the cervical esophageal lumen and measuring the TCEUS parameters at the thyroid gland level transcutaneously while correlating pH monitoring and esophageal manometry findings in adults.

ACKNOWLEDGMENTS

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COMMENTS

Background

Esophageal refluxed material can be recognized in ultrasonographic images. The content of refluxate can be isolated liquid (acid or non-acid nature), isolated gas, or gas/liquid mixture. The content of liquid refluxate, whether acidic or not, did not affect the sonographic appearance. Liquid can be present in the esophageal lumen in gastroesophageal reflux (GER) and esophageal motility disorders. Esophageal ultrasonography is currently being used to evaluate the gastroesophageal junction by transabdominal route, especially in newborns and children, and endosonographic studies have been used especially for motility disorders. The relation between the presence of refluxate in the cervical esophageal lumen and the esophageal pH-metry/manometry findings using transcervical esophageal ultrasonography (TCEUS) has not been studied previously in children and adults.

Research frontiers

We evaluated the possible pathologies in 24-h pH monitoring and esophageal manometry in patients with refluxate in the lumen of the cervical esophagus during TCEUS. In 27/38 (71.1%) patients with refluxate in TCEUS, there was at least one pathologic acid reflux and/or pathologic manometry finding. 24 h pH-metry and esophageal manometry subgroups were compared statistically according to TCEUS findings [anterior wall thickness (WT) of the esophagus, esophageal diameter (ED), esophageal luminal diameter (ELD)].

Innovations and breakthroughs

Our study is distinct from other studies evaluating the GER and esophageal manometry pathologies with ultrasonographic methods due to our usage of TCEUS. We performed esophageal manometry and 24-h pH monitoring in patients with refluxate in the esophageal lumen. The shortcomings of the study were that 1) we did not perform manometry or pH monitoring in patients without refluxate and 2) we did not evaluate the non-acid reflux.

Applications

Different patient groups and volunteers without refluxate can be evaluated for different study designs.

Terminology

The presence of refluxate in esophageal lumen in TCEUS: The luminal anechoic fluid and/or linear bright stratifying small lines indicating gas in refluxate with the patients not swallowing; The presence of comet-tail artifact: During swallowing, the presence of saliva mixed with air and downward movement of refluxate generated a strong echogenic appearance; TCEUS parameters: Wall thickness (WT) of the esophagus: Distance between adventitia and the mucosa, with 5-7 esophageal wall layers; Esophageal diameter (ED): Distance between the adventitia (outer to outer); Esophageal luminal diameter (ELD) with or without refluxate: Distance between the mucosa (inner to inner).

Peer review

The authors studied transcutaneous cervical esophageal ultrasound (TCEUS) as a possible diagnostic procedure in gastroesophageal reflux. TCEUS has been forgotten in the diagnosis of esophageal diseases, however, it is a non-invasive, available and high sensitive technique. This manuscript is in principle an interesting topic for the readers of World Journal of Gastroenterology.

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CLINICAL RESEARCH

Early nasogastric enteral nutrition for severe acute pancreatitis: A systematic review

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Abstract

AIM: To evaluate the effectiveness and safety of early nasogastric enteral nutrition (NGEN) for patients with severe acute pancreatitis (SAP).

METHODS: We searched Cochrane Central Register of Controlled Trials (Issue 2, 2006), Pub-Medline (1966-2006), and references from relevant articles. We included randomized controlled trials (RCTs) only, which reported the mortality of SAP patients at least. Two reviewers assessed the quality of each trial and collected data independently. The Cochrane Collaboration's RevMan 4.2.9 software was used for statistical analysis.

RESULTS: Three RCTs were included, involving 131 patients. The baselines of each trial were comparable. Meta-analysis showed no significant differences in mortality rate of SAP patients between nasogastric and conventional routes (RR = 0.76, 95% CI = 0.37 and 1.55, P = 0.45), and in other outcomes, including time of hospital stay (weighted mean difference = -5.87, 95% CI = -20.58 and 8.84, P = 0.43), complication rate of infection (RR = 1.41, 95% CI = 0.62 and 3.23, P = 0.41) or multiple organ deficiency syndrome (RR = 0.97, 95% CI = 0.27 and 3.47, P = 0.97), rate of admission to ICU (RR = 1.00, 95% CI = 0.48 and 2.09, P = 0.99) or conversion to surgery (RR = 0.66, 95% CI = 0.12 and 3.69, P = 0.64), as well as recurrence of re-feeding pain and adverse events associated with nutrition.

CONCLUSION: Early NGEN is a breakthrough in the management of SAP. Based on current studies, early NGEN appears effective and safe. Since the available evidence is poor in quantity, it is hard to make an accurate evaluation of the role of early NGEN in SAP. Before recommendation to clinical practice, further high qualified, large scale, randomized controlled trials are needed.

Key words: Enteral nutrition; Nasogastric tube; Severe acute pancreatitis; Systematic review; Meta-analysis

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INTRODUCTION

Acute pancreatitis (AP) is one of the most common pancreatic diseases, with an incidence rate of 4.9-80/100000 per year, and there was a trend to increase during the past two decades[1]. Around 80% of patients with mild acute pancreatitis (MAP) are treatable with a short period of bowel rest, simple intravenous hydration, and analgesia[1,2]. However, severe acute pancreatitis (SAP) is complicated by systemic inflammatory response syndrome (SIRS), leading to hypermetabolism and high protein catabolism[3]. Consequently, nutritional stores are rapidly consumed and about 30% of patients with SAP undergo malnutrition[3,4]. Acute malnutrition is expected to increase morbidity and mortality due to impaired immune function, increased risk of sepsis, poor wound healing, and multiple organ failure[3]. Thus, current therapy for AP has shifted to intensive medical care, nutrition support, infection control and medicine administration, while early invasive intervention as surgery has been reserved for defined clinical indication[5,6]. Nutritional management for AP is an important issue and regarded as an indispensable approach.

Oral or enteral feeding may be harmful in AP and is thought to stimulate exocrine pancreatic secretion and consequently autodigestive process[4]. Up to the mid 1990s, total parenteral nutrition (TPN) and gastrointestinal tract rest have been comprehensively recommended in the acute phase of pancreatitis, which make pancreas at rest to reduce pancreatic exocrine secretion and also meet nutritional need[6,7]. Intestinal mucosa atrophies during fasting as TPN phase, which would induce bacteria translocation in gastrointestinal tract and cause pancreatic necrotic tissue infection[8]. Animal experiments and several human studies

have shown that enteral nutrition (EN) is safe and can preserve the integrity of intestinal mucosa to decrease the incidence of infectious complications and other severe complications, such as multiple organ deficiency syndrome (MODS)[8-10]. Furthermore, EN does not stimulate pancreatic exocrine secretion, if the feeding tube is positioned in the jejunum by nasojejunal or jejunostomy routes[8,11]. Therefore, TPN or jejunal EN is considered the mainstream of nutritional support for AP.

Recently, some researchers have considered the feasibility of EN through nasogastric (NG) tube to improve the nutrition status of patients with AP in the early phase[7,12]. However, this breakthrough is potentially opposing to the requirement of pancreatic rest in the acute inflammation phase. The present study was to confirm whether nasogastric EN is safe and effective for patients with AP.

MATERIALS AND METHODS

Search strategy and update

We searched electronic databases of Cochrane Central Register of Controlled Trials (Issue 2, 2006), Pub-Medline (1966-2006), and references from relevant articles. The search strategy used was "Enteral Nutrition" (MeSH) AND ["Pancreatitis" (MeSH) OR "Pancreatitis, Acute Necrotizing" (MeSH)], with limitations to Randomized Controlled Trial, Humans. There was no limitation of publication language. This systematic review will be updated if more randomized controlled trials (RCTs) can be found through the monthly automatic search procedure from National Center for Biotechnology Information (NCBI) at the National Library of Medicine.

Inclusion and exclusion criteria

Only randomized controlled trials were eligible. Eligible patients include those who diagnosed as acute pancreatitis by Atlanta classification, and those with severe diseases assessed by APACHE Ⅱ criteria, and/or Ranson criteria, and/or Balthazar computer tomography criteria. Any etiology was eligible, and there was no limitation of age, race, and sex distribution. Comparator intervention was considered an early enteral nutritional route through nasogastric tube (NGEN), while control intervention was considered one of the conventional pancreatic-rest nutritional support routes, such as total parenteral nutrition (TPN) or enteral nutrition by nasojejunal tube (NJEN) or jejunostomy tube (JSEN). Additionally, other combined treatments included gastrointestinal decompression, prophylactic antibiotics, fluid management, artificial ventilation or renal replacement therapy for MODS, endoscopic retrograde cholangio-pancreatography with endoscopic sphincterotomy for selected biliary patients, and surgery for indicated patients. The primary outcome measure of effectiveness was overall mortality, the secondary outcome measures of effectiveness was hospital stay, complications and their management, while the outcome measures of safety included re-feeding pain recurrence and adverse events related to nasogastric enteral nutrition.

Quality assessment and data collection

To evaluate the methodological quality of included studies, two reviewers (Jiang K and Chen XZ) assessed the quality of methods used in studies independently. According to the Cochrane Handbook for Systematic Review 4.2.6[13], we assessed the quality of RCTs using random allocation concealment as adequate (A), unclear (B), inadequate (C), or not used (D); blinding process as double blind (A), single blind (B), unclear (C) or not used (D); intention-to-treat (ITT) as yes (A), unclear (B), or not used (C), or loss, withdrawal, dropout, cross-intervention not reported (D). The criteria proposed by Jadad *et al*[14] were also used to evaluate the quality of trials.

Data were collected by the two reviewers independently, including study sample (number of each arm), interventions (nutrition management, approach and regimens) and outcomes (overall mortality, time of hospital stay, complications of systematic or local infection, or MODS defined as failure in no less than 2 organs, re-feeding pain defined as pain requiring discontinuation of feeding, elevated serum amylase levels at least two-fold higher than normal[7], and adverse events related to nutrition), as well as the publication year and country of studies, and the number of withdrawals and dropouts and the reasons.

Any disagreement in quality assessment and data collection was discussed and solved by a third reviewer as the referee.

Statistical analysis

Meta-analysis was performed with the Cochrane Collaboration's RevMan 4.2.9 software. All *P*-values were two-sided and $P < 0.05$ was considered statistically significant. For dichotomous variables, the risk ratio (RR) was calculated with 95% confidence intervals (CI); for continuous variables, weighted mean difference (WMD) was calculated with 95% confidence intervals. Heterogeneity was determined by chi-square test. If any heterogeneity existed, the following techniques were employed to explain it: (1) random effect model, (2) sub-group analysis including different control arms as TPN or EN through nasojejunal or jejunostomy tube, (3) sensitivity analysis performed by excluding the trials which potentially biased the results. Any patients randomly assigned in each trial, but not analyzed in the present meta-analysis, were calculated.

RESULTS

Three RCTs[15-17] were eligible for the inclusion criteria, and 131 participants were included. Of them, 67 were randomly assigned to NGEN group and 64 to conventional nutritional route group. Conventional routes included TPN and NJEN, but not JSEN. The mean number of samples for each trial was 43.7 (31-50). The baselines of each trial were comparable. The details of included trials are listed in Table 1, and the results of quality assessment in Table 2.

Effectiveness

Overall mortality: Three included RCTs reported the mortality. The overall mortality rate of early NGEN group and conventional route group was 14.9% (10/67) and 18.8% (12/64) respectively, which is consistent with the reported rate[18]. No heterogeneity was detected (P =

Table 1 Details of included RCTs

Reference	Yr	Country	Number of intervention/control	Inclusion/exclusion criteria of participants	Drop-out/withdrawal
[15]	2005	Scotland	27 (NGEN)/23 (NJEN)	Inclusion: both a clinical and biochemical presentation of AP (abdominal pain and serum amylase at least 3 times the upper limit of the reference range), and objective evidence of disease of disease severity (Glasgow prognostic score ≥ 3, or APACHE Ⅱ ≥ 6, or CRP > 150 mg/L). Exclusion: patients under 18 yr of age and pregnant females. Inclusion: severity was defined according to the Atlanta criteria (ie, presence of organ failure and APACHE score of ≥ 8 or CT severity score ≥ 7).	One excluded in NJEN group for misdiagnosed and 2 in NJEN group received NGEN for failure of NJ tube placement.
[16]	2006	India	16 (NGEN)/15 (NJEN)	Exclusion: if there was a delay of more than 4 wk between the onset of symptoms and presentation to the hospital, if they were already taking oral feeding at presentation, if there was acute exacerbation of chronic pancreatitis, or if they were in shock (ie, systolic blood pressure < 90 mmHg at the time of randomization).	One excluded in NJEN group for failure of NJ tube placement.
[17]	2006	Sweden	24 (NGEN)/26 (TPN)	Inclusion: abdominal pain, amylase ≥ 3 times upper limit of normal, onset of abdominal pain within 48 h, APACHE Ⅱ score ≥ 8 and/or CRP ≥ 150 mg/L and/or peripancreatic liquid shown on CT. Exclusion: AP due to surgery, inflammatory bowel disease, stoma, short bowel, chronic pancreatitis with exacerbation, and patients under 18 yr of age.	One patient from each group was considered as protocol violators due to surgery performed after study inclusion on d 2 in 1 case, and a dislocated tube not accepted to be replaced in the other one

Abbreviations: RCT: Randomized controlled trial; NGEN, nasogastric enteral nutrition; NJEN, nasojejunal enteral nutrition; TPN, total parenteral nutrition; AP, acute pancreatitis; SAP, severe acute pancreatitis; APACHE, acute physiology and chronic health evaluation; CTSI, computer tomography severity index; CRP, C-reactive protein.

Table 2 Quality of included RCTs[1]

Study	Randomization method	Allocation concealment	Blind method	ITT method	Jadad score
Eatock *et al*[15], 2005	Computer-generated random numbers	Adequate	Not used	Yes	3
Kumar *et al*[16], 2006	Computer-generated random numbers	Unclear	Not used	Unclear	3
Eckerwall *et al*[17], 2006	Not specified	Adequate	Not used	Yes	2

[1]According to the Cochrane Handbook for Systematic Review 4.2.6[13].

0.61). Meta-analysis showed no significant difference in overall mortality between early NGEN and conventional route groups (RR = 0.76, 95% CI = 0.37 and 1.55, P = 0.45) (Figure 1). Sub-group analysis showed no significant difference in overall mortality between NGEN and NJEN groups[15,16] (RR = 0.67, 95% CI = 0.32 and 1.40, *P* = 0.28), as well as between NGEN and TPN groups[17] (RR = 3.24, 95% CI = 0.14 and 75.91, *P* = 0.47).

Hospital stay: All the included RCTs reported the mean time of total hospital stay, but 2 trials did not mentioned the standard difference (SD)[15,17], and were excluded from the meta-analysis. There was no significant difference in the mean time of total hospital stay between NGEN and NJEN groups (WMD = -5.87, 95% CI = -20.58 and 8.84, *P* = 0.43) (Figure 2). The weighted mean time of hospital stay of patients in early NGEN and conventional route groups was 15.4 d and 15.3 d, respectively.

Complications and management: Two RCTs reported the detailed data on infective complications or MODS[16,17]. The results of meta-analysis showed no significant difference in infective complications, such as sepsis and infected pancreatic necrosis (RR = 1.41, 95% CI = 0.62 and 3.23, *P* = 0.41) (Figure 3), as well as in heterogeneity (*P* = 0.15). There was no significant difference in MODS (RR = 0.97, 95% CI = 0.27 and 3.47, *P* = 0.97) (Figure 3), as well as in heterogeneity (*P* = 0.93). The rate for admission to intensive care unit was reported in 2 RCTs[15,17], which was 21.6% (11/51) and 20.4% (10/49) in early NGEN and conventional groups respectively (RR = 1.00, 95% CI = 0.48 and 2.09, *P* = 0.99) (Figure 4). Two trials[16,17] reported that the rate for surgery was 5.0% (2/40) and 7.3% (3/41) in early NGEN and conventional route groups, respectively with no significant difference between the two groups (RR = 0.66, 95% CI = 0.12 and 3.69, *P* = 0.64) (Figure 4).

Safety

Re-feeding pain recurrence: Two RCTs[16,17] reported the cases needing to withdraw oral feeding due to recurrent re-feeding pain. Meta-analysis showed no significant difference in the recurrent re-feeding pain (RR = 0.94, 95% CI = 0.06 and 13.68, *P* = 0.96) (Figure 5). The pain recurrence rate was 2.5% (1/40) and 2.4% (1/41) in NGEN and conventional route groups, respectively.

Adverse events associated with nutrition: Meta-analysis showed that the main adverse events associated with nutrition support were diarrhea (RR = 1.59, 95% CI = 0.51 and 4.91, *P* = 0.42), tube displacement (RR = 0.45, 95% CI = 0.09 and 2.30, *P* = 0.33), and withdrawal of feeding due to severe complications (RR = 0.30, 95% CI = 0.03 and 2.76, *P* = 0.29) (Figure 6). Other nutrition-associated complications were found in patients of the NJEN group

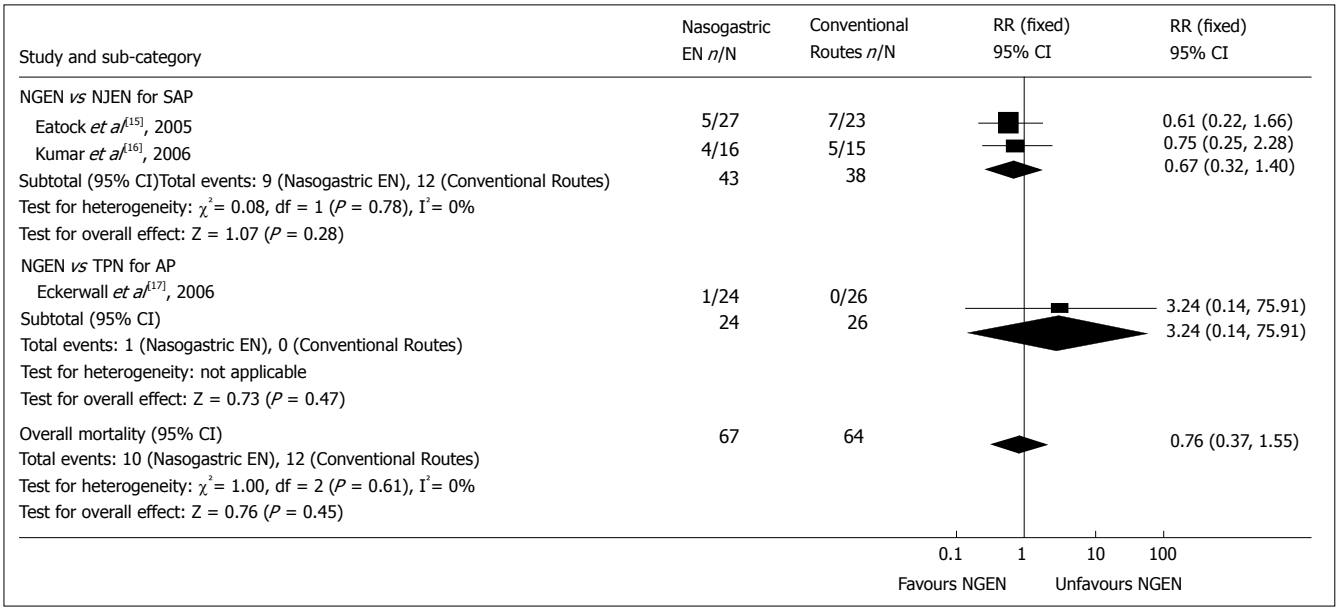

Figure 1 Comparison of overall mortality between nasogastric enteral nutrition and conventional nutritional routes. Abbreviations: AP, acute pancreatitis; SAP, severe acute pancreatitis; EN, enteral nutrition; TPN, total parenteral nutrition; NGEN, nasogastric enteral nutrition; NJEN, nasojejunal enteral nutrition; RR, risk ratio; CI, confidence interval.

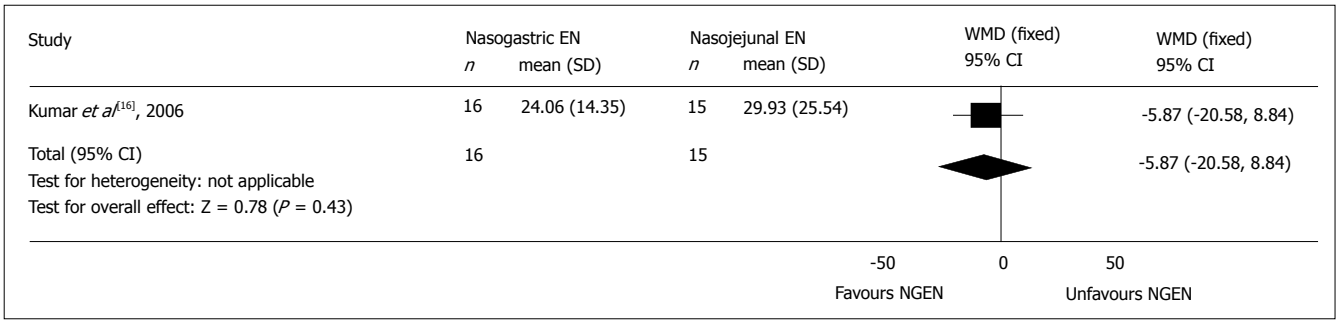

Figure 2 Comparison of total hospital stay (days) between nasogastric and nasojejunal enteral nutrition. WMD, weighted mean difference; SD, standard deviation.

who suffered from cardiorespiratory arrest at the moment of endoscopic tube placement which were successfully resuscitated[14] and in patients of the NJEN group with sweating and palpitation[15].

DISCUSSION

The present systematic review was intended to find the feasibility and safety of early nasogastric enteral nutrition in the management of severe acute pancreatitis. We selected 3 randomized controlled trials involving comparison between early NGEN and NJEN or TPN. The total number of samples was limited. All the trials did not perform a blinding process due to the nature of interventions. Two trials reported detailed randomization assignment methods[15,16], and two were put into practice based on allocation concealment and intention-to-treat method[15,17]. Sensitivity analysis showed negative results. Potential biases of the present systematic review included selection bias due to the severity criteria of one trial (Eatock 2005)[15] set at APACHE Ⅱ > 6, on an international symposium[19]. Moreover, another concern is the high mortality (24.5%) in patients with the severity of the illness[20].

Treatment of SAP has been evolving from routine early aggressive surgical management towards conservative care for patients without evidence of pancreatic infection[21,22]. However, SAP remains a disease with a poor prognosis[23]. Artificial nutrition can prevent and provide a long term nutritional support for SAP[24]. Enteral nutrition is preferred to parenteral nutrition for improving patient outcomes[25,26], and has largely replaced the parenteral route[27]. However, early nasogastric enteral nutrition is regarded as a potentially pancreatic-unrest nutritional support route, which is harmful to the early acute phase of AP[15]. Eatock *et al*[12] first introduced the early nasogastric feeding into nutritional management of SAP, and then Pandey *et al*[7] applied oral re-feeding in patients with SAP, suggesting that the nasogastric feeding is feasible in up to 80% cases[15].

The present meta-analysis showed that early NGEN would be as effective and safe as early NJEN or TPN in SAP patients, without increase in mortality. Pancreatic infection, sepsis, and MODS are the complications

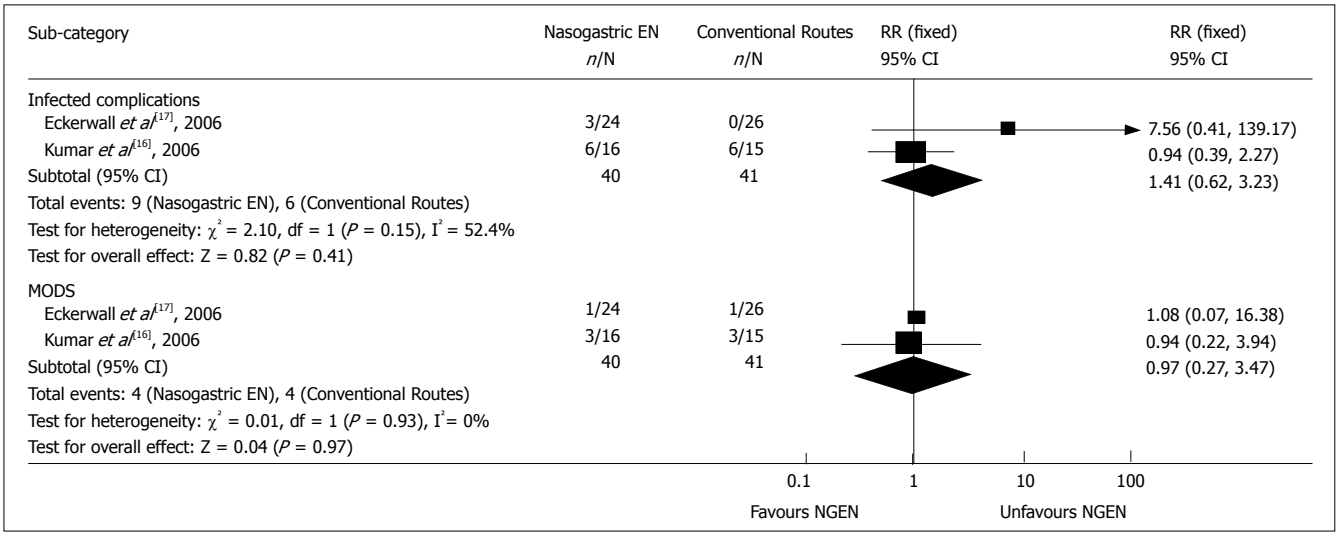

Figure 3 Comparison of complications of acute pancreatitis between nasogastric enteral nutrition and conventional nutritional routes. MODS, multiple organ deficiency syndrome.

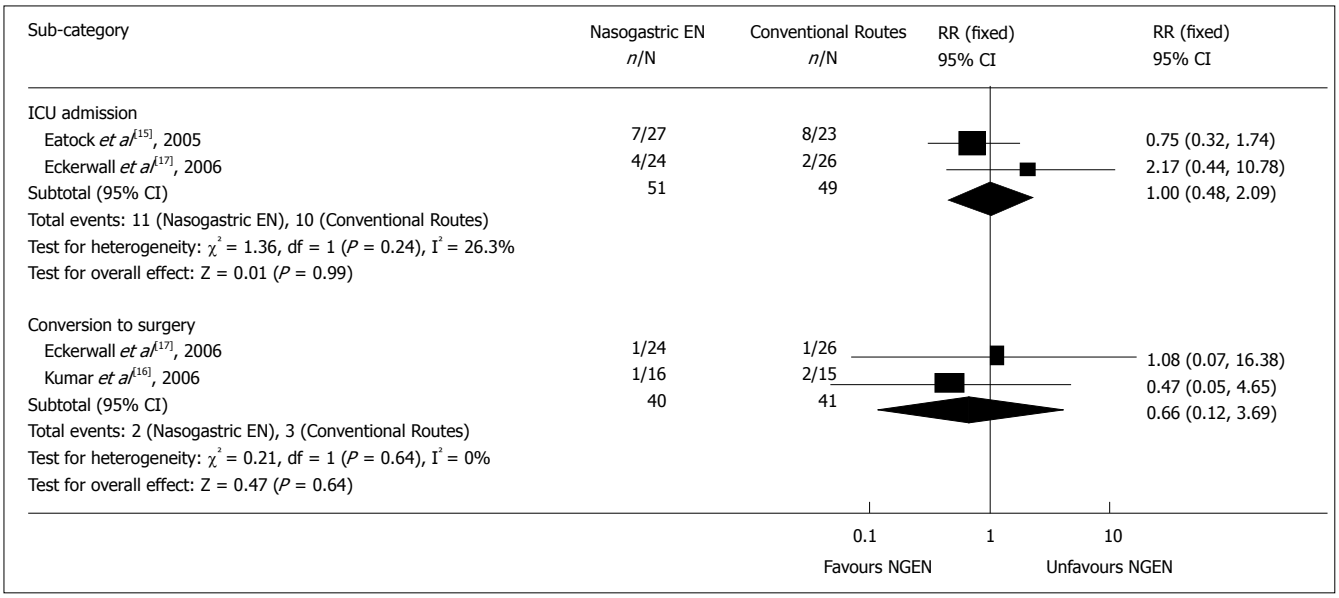

Figure 4 Comparison of management of complications between nasogastric enteral nutrition and conventional nutritional routes. ICU, intensive care unit.

of SAP[28]. Duration of organ failure during the first week of predicted severe acute pancreatitis is strongly associated with the risk of death or local complications[29]. Besides, NGEN did not increase severe complications and prolong hospital stay. Bacterial infection is the common complication of acute pancreatitis, and bacterial translocation from the gut is probably the first step in the pathogenesis of these infections[30]. NGEN could preserve the intestinal permeability including duodenum, proved by the assessment of excretion of polyethylene glycol and antiendotoxin core antibody IgM levels[17], which would be the best barrier for prevention of certain complications. Interleukin-6 serum levels are elevated very early in patients with necrosis infection[31], and C-reactive protein (CRP) is considered a valuable independent predictor of mortality[32]. IL-6 and CRP levels play a similar role in the control of systematic inflammatory response of early NGEN and TPN groups at each time point[17]. Moreover, biochemical nutritional parameters, such as serum albumin and prealbumin concentration in early NGEN and NJEN groups, are both well preserved without any significant difference[16].

After three-month follow-up, about 92% patients in the early NGEN group have no symptoms related to SAP, compared with 82% patients in the TPN group, but all pseudocysts occur in the early NGEN group[17], suggesting that early nasogastric enteral nutrition is potentially feasible in SAP patients. The clinical outcomes are quite similar to conventional routes, such as early nasojejunal or total parenteral nutrition. However, the incidence of late complications such as pseudocysts is likely higher in early nasogastric enteral nutrition group.

Enteral nutrition has no benefit to the mild acute pancreatitis subset[24]. However, oral re-feeding is feasible,

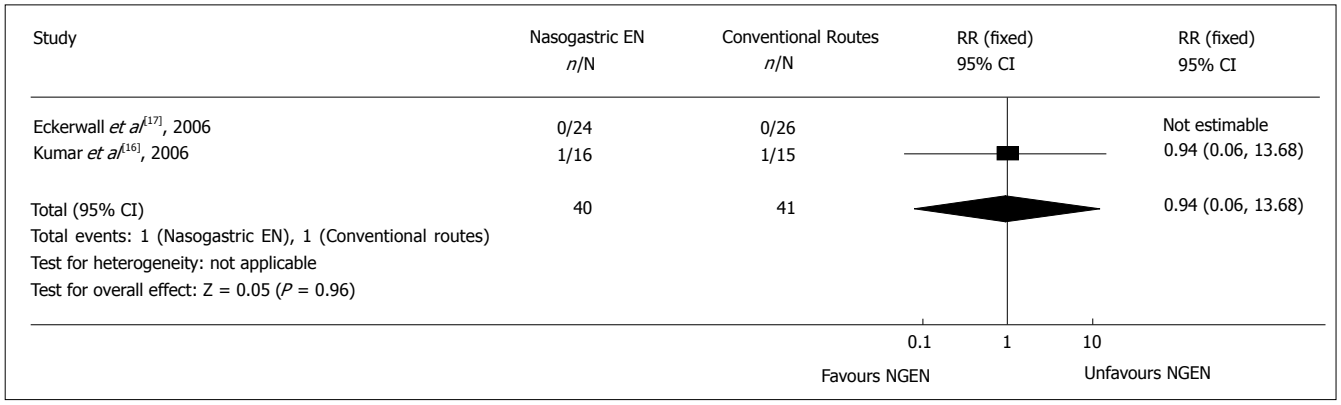

Figure 5 Comparison of refeeding pain recurrence between nasogastric enteral nutrition and conventional nutritional routes.

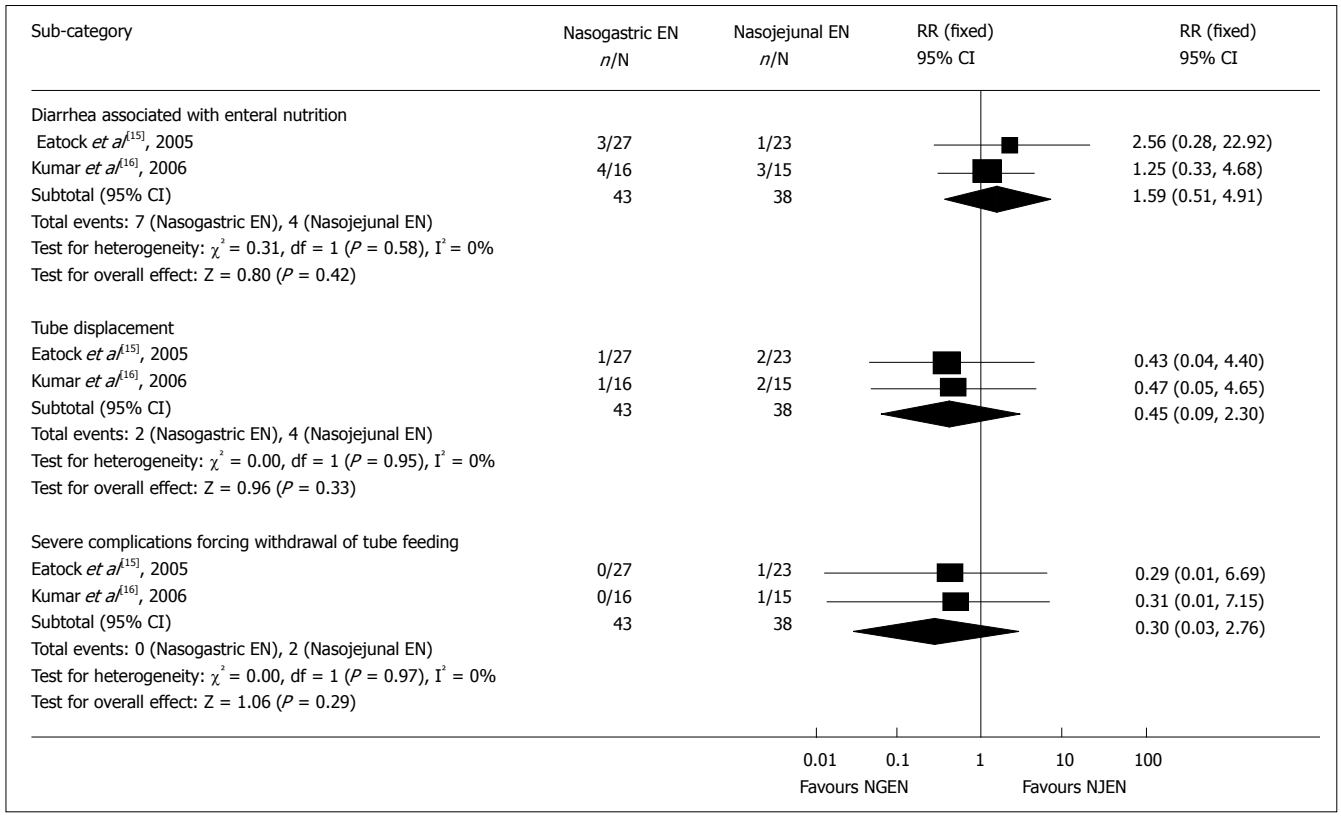

Figure 6 Comparison of nutrition associated adverse events between nasogastric and nasojejunal enteral nutrition (NJEN).

but the proper time of commence needs to be further investigated. Oral re-feeding given 1 wk after onset of the disease is safe in selected MAP patients[4], while there is no evidence that early oral re-feeding within 1 wk is a feasible management for SAP.

In conclusion, early nasogastric enteral nutritional support route is potentially feasible and safe, which does not increase the rate of mortality, complication and re-feeding pain recurrence in patients with severe acute pancreatitis, and prolong the hospital stay. No major innovations in the treatment of SAP have been introduced in recent years[23]. It is a breakthrough in enteral nutrition management of severe acute pancreatitis, with a bright future because it is more convenient and cheaper. However, before it is applied in clinical practice, further investigation is necessary to validate its effectiveness, safety and cost-effectiveness.

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COMMENTS

Background

Nutritional management of severe acute pancreatitis is an important issue

and regarded as an indispensable approach. Total parenteral nutrition and gastrointestinal tract rest have been recommended in the management of severe acute pancreatitis (SAP) sine the mid 1990s. Enteral nutrition through jejunal route has been accepted as a safe and effective approach to the management of SAP by preserving the integrity of intestinal mucosa and preventing bacterial translocation. Moreover, some studies have attempted to find the feasibility and safety of early enteral nutrition through nasogastric route.

Research frontiers

Gastrointestinal and pancreatic rest has been regarded as an important factor for management of severe acute pancreatitis. Nevertheless, nasogastric enteral nutrition disobeys this discipline. Whether nasogastric route is able to gain the similar results needs to be further investigated. If possible, serological, radiological or histological appraisal should be considered for the effectiveness and safety of early nasogastric enteral nutrition in the treatment of severe acute pancreatitis.

Innovations and breakthroughs

Early nasogastric enteral nutrition is a breakthrough in the management of severe acute pancreatitis. Previously, it was forbidden for the sake of potentially opposing to the requirement of pancreatic rest in the acute inflammation phase. However, the present systematic review of three randomized controlled trials showed that nasogastric route does not worsen the clinical outcomes compared with the conventional total parenteral or jejunal enteral routes.

Applications

Nasogastric route is much more convenient in clinical practice. Moreover, it is obviously cheaper than nasojejunal tube placement. Based on the present results, nasogastric enteral nutrition can be applied in the early management of severe acute pancratitis. However, before its application in clinical practice, further investigation is necessary to validate its effectiveness and safety.

Terminology

Severe acute panceatitis (SAP) is usually accompanied with pancreatic necrosis, systematic inflammatory response syndrome, or organ failure. Total partenteral nutrition (TPN) is the way to give nutrient substances intravenously, bypassing the digestive system. Nasogastric enteral nutrition (NGEN) is the way to provide nutrient substances for patients through a tube placed in the nose up to the stomach. Nasojejunal enteral nutrition (NJEN) is the way similar to NGEN, but the tube is placed up to the jejunum.

Peer review

The authors evaluated the effectiveness and safety of early nasogastric enteral nutrition (NGEN) for severe acute pancreatitis (SAP) compared with conventional nutritional routes. Based on the current studies, early NGEN appears effective and safe, but the available evidence is limited.

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RAPID COMMUNICATION

Expression of periostin and its clinicopathological relevance in gastric cancer

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Abstract

AIM: To investigate the expression and localization of periostin in gastric cancer and its clinical relevance.

METHODS: Reverse transcriptase polymerase chain reaction was used to measure periostin mRNA expression. Western blotting was carried out to detect periostin protein expression. Immunohistochemistry was performed to localize and quantify the expression of periostin in benign gastric diseases and gastric cancer, and immunostaining results were correlated with gastric cancer pathological stages.

RESULTS: Periostin expression was low at both mRNA and protein levels in normal gastric tissues, but was overexpressed in gastric cancer tissues. Immunohistochemical staining revealed that periostin was overexpressed in primary gastric cancer, as well as in metastatic lymph nodes, but only faint staining was found in benign gastric ulcers. By quantitative analysis of the immunostaining results, periostin expression was increased 2.5-4-fold in gastric cancer, compared to that in benign gastric disease, and there was a trend toward increasing periostin expression with tumor stage.

CONCLUSION: This observation demonstrated that periostin was overexpressed in gastric cancer and lymph node metastasis, which suggests that periostin plays an important role in the progression and metastasis of gastric cancer.

Key words: Cell growth; Gastric cancer; Metastasis; Oncogene; Periostin

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INTRODUCTION

Gastric cancer remains the fourth most common cancer worldwide. Due to its high incidence and malignant behavior, and the lack of major advances in treatment strategy, it is still the second most frequent cause of death from malignant diseases[1]. A variety of clinicopathological characteristics may affect the prognosis of gastric cancer, and accumulating evidence has revealed that numerous genetic markers influence the biological behavior of gastric cancer. The abnormal expression of certain genes in cancer cells is closely related to various aspects of tumor progression, including tumor growth, invasion and metastasis, and inactivation of tumor suppressor genes, particularly the p53 or adenomatous polyposis coli gene[2].

Recently, great efforts have been made to determine the gene expression pattern differences between various types of human cancer and their corresponding normal tissues, although the alterations of a certain number of tumor suppressor genes and oncogenes have been shown to be closely associated with the progression of human cancer. Less is known about the functions of a large number of other genes whose expression patterns are also significantly changed during the tumorigenic process. Particularly interesting is the mesenchyme-specific gene family, normally associated with osteoblasts, which are highly expressed in various types of human cancer[3,4]. One such candidate gene is that for periostin, which is overexpresed in several types of human cancer, such as breast, colon and ovarian cancer[5-7]. Furthermore, it is supposed that, at the molecular level, periostin functions as a ligand for alpha (V), beta (3), and alpha (V) beta (5) integrins to support adhesion and migration of ovarian epithelial cells[5], and periostin activates the Akt/PKB signaling pathway through the alpha (V) beta (3) integrins, to increase cellular survival[7]. While integrin expression level is supposed to be related to the metastasis and relapse of gastric cancer[8], the expression pattern of periostin in gastric cancer is still unknown. In this paper we investigated periostin expression profile in gastric cancer and its clinical relevance.

MATERIALS AND METHODS

Patients and tissue collection

Samples from 35 gastric cancers, including five metastatic lymph node samples, were obtained from 25 male and 10 female patients (median age, 56 years; range, 45-79 years) who underwent gastric resection at the University Hospital of the Affiliated Zhong-Da Hospital, Southeast University. Five benign gastric ulcer tissue samples were obtained from three male and two female patients, in whom gastrectomy was performed (median age, 55 years; range, 25-67 years). According to the TNM classification of the Union International Contre le Cancer (UICC), the 35 gastric cancers were classified as follows: five tumors were stage Ⅰ, eight were stage Ⅱ, 16 were stage Ⅲ, and six were stage Ⅳ. The vast majority of the tumors were located in the distal part of the stomach (21 cases), and one-third were located in the proximal part of the stomach. Immediately upon surgical removal, tissue samples were either snap-frozen in liquid nitrogen and then maintained at 80℃ until use (for RNA extraction), or fixed in 5% formalin and embedded in paraffin after 24 h. All studies were approved by the ethics committees of the Affiliated Zhong-Da Hospital, Southeast University.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from human gastric tissues was isolated either by the Trizol Reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions. Potentially contaminating DNA was removed by RNAse-free DNAse I treatment. Primers for the genes of interest were: periostin193 bp (5'-GCCATCACATCGGACA TA-3' and 5'-CTCCCATAATAGACTCAGAACA-3'), and β-actin 621 bp (5'-ACACTGTGCCCATCTACGAG G-3' and 5'-AGGGGCCGGACTCGTCATACT-3'). The RT-PCR conditions were: for the first strand synthesis of cDNA, 48℃ for 45 min and 94℃ for 2 min to denature the template; and for second strand synthesis and DNA amplification, 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 1 min, for a total of 32 cycles, followed by a single step at 68℃ for 7 min.

The products were visualized on 1.5% agarose gels stained with ethidium bromide, and signals were quantified by densitometry using the MetaView analyzing system (version 4.5 Universal Imaging, West Chester, PA, USA). Periostin expression was standardized to β-actin expression assessed from the same cDNA in separate PCR reactions, and run on the same gels. The standardized mean of each triplicate PCR was then expressed relative to the levels in β-actin cDNA. The density value was analyzed by Labimage software (Halle, Germany). $P < 0.05$ was considered as statistically significant.

Protein extraction

Tissues were lysed in 0.5 mL lysis buffer containing 50 mmol/L Tris/HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0) and 1% SDS with proteinase inhibitors (one tablet/10 mL, Roche Molecular Biochemicals, USA). Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA).

Western blot analysis

Fifty micrograms of protein was separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% non-fat milk in 20 mmol/L Tris/HCl, 150 mmol/L NaCl, 0.1% Tween-20; TBS-T), followed by incubation with rabbit anti-periostin antibodies (Abcam, Cambridge, MA, USA; 1:500) at 4℃ overnight. The membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Life Sciences, Amersham, UK) for 1 h at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction (Amersham Life Sciences).

Immunohistochemistry

Immunohistochemistry was performed using the strepavidin-peroxidase technique and the DAKO EnVision System (Dako Cytomation, Hamburg, Germany). Consecutive paraffin-embedded tissue sections (4-μm thick) were deparaffinized and rehydrated. Antigen retrieval was performed by pretreatment of the slides in citrate buffer (pH 6.0) in a microwave oven for 12 min. Thereafter, slides were cooled to room temperature in deionized water for 5 min. Endogenous peroxidase activity was quenched by incubating the slides in methanol containing 0.6% hydrogen peroxide, followed by washing in deionized water for 3 min, after which the sections were incubated for 1 h at room temperature with normal goat serum, and subsequently at room temperature for 1 h with the primary anti-periostin antibody (Abcam; 1:100). Next, the sections were rinsed with washing buffer (Tris-buffered saline with 0.1% BSA) and incubated with biotinylated goat anti-rabbit IgG and streptavidin-peroxidase complex, followed by reaction with diaminobenzidine and counterstaining with Mayer's hematoxylin. In addition, to confirm the specificity of the primary antibody and the technique used, tissue sections were incubated in the absence of the primary antibody and with negative control rabbit IgG. Under these conditions, no specific immunostaining was detected. The quantitative analysis of the immunohistochemistry staining of periostin expression in gastric tissues was performed as previously described[9]. The staining of each tissue was analyzed to determine the mean optical density (MOD), which represents the concentration of the stain per positive pixels.

RESULTS

Periostin mRNA expression is upregulated in gastric cancer tissues

To clarify the expression of periostin in human gastric tissues, we initially examined the expression of periostin mRNA in five gastric cancer tissues (T) versus normal gastric tissues (N) obtained from the same patients. The expression of β-actin was examined as an internal control. As shown in Figure 1, periostin mRNA was expressed in all five gastric cancer tissues at different levels. In contrast, its staining was detected at a very low level in normal gastric tissues, and quantitative analysis showed that there

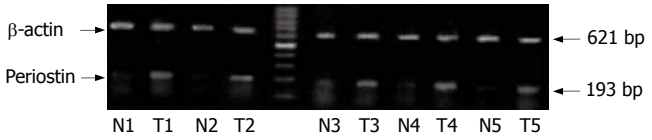

Figure 1 RT-PCR analysis of periostin mRNA expression in human gastric cancer (T) and normal gastric tissues (N). Periostin was differentially expressed in gastric cancer tissues (T) compared with normal gastric tissues (N) from the same patient. The expression of β-actin was used as an internal control.

was a significant difference in periostin expression between gastric cancer and normal tissues ($P < 0.05$, the density ratio between N and T was 0.1912 *vs* 0.8804).

Periostin protein expression in gastric cancer and normal tissues

To determine if the higher level of periostin mRNA expression revealed by RT-PCR analysis was directly linked to increased levels of periostin protein expression, we performed Western blot analysis with protein extracts from matched tissue sections (from which the mRNA samples were extracted). As shown in Figure 2A, periostin was found to be highly expressed in gastric cancer tissues from five different patients (T), whereas only faint periostin expression was found in the normal gastric tissues, with a fivefold overexpression of periostin in cancer tissues compared with normal tissues ($P < 0.05$, the density ratio was 0.8354 *vs* 4.5773) (Figure 2B). Taken together, these data demonstrate that periostin is highly expressed at both mRNA and protein levels in gastric cancer tissues.

Cellular localization of periostin in gastric tissues and its clinical relevance

To investigate the significance of periostin overexpression in gastric cancer further, immunohistochemistry was carried out on five benign gastric ulcers and 35 cases of gastric cancer, which included: five stage I tumors, eight stage Ⅱ, 16 stage Ⅲ, and six stage Ⅳ tumors, and five metastatic lymph nodes. As shown in Figure 3, the immunostaining indicated that high levels of periostin were present in the areas surrounding cancer cells, as well as in some cancer cells themselves. Periostin was also prominently stained in metastatic lymph nodes. In contrast, periostin expression was low in benign gastric ulcer tissues. Quantitative analysis of the periostin immunohistochemistry results indicated that the average MOD of periostin staining in the stage I -Ⅳ tumors was much higher than that in benign gastric ulcers in each group ($P < 0.05$). Furthermore, there was a trend for increasing periostin expression in stage I -Ⅳ tumors, although the difference was not statistically significant. In addition, there was no correlation between periostin expression and patient age and gender (data not shown). Taken together, these observations indicated that higher levels of periostin expression were associated with cancer progression and metastasis.

DISCUSSION

Many studies have already demonstrated that interactions between integrins of the tumor cell surface and adhesion molecules in the extracellular matrix (ECM) microenvironment may play a critical role in tumor cell migration, survival and growth[10-12]. The ECM-integrin interactions may also trigger intracellular signaling and activation of certain genes that leads to tumor cell proliferation during metastatic growth[13], and the formation of blood vessels within the tumor mass[14]. It is believed that integrins are also important in the attachment and metastasis of gastric cancer[8]. As a mesenchymal gene, periostin was formerly called osteoblast-specific factor-2, and was originally identified as an 811-amino acid protein secreted by osteoblasts[15]. It has structural homology with insect fasciclin I and can bind heparin and support adhesion of osteoblasts[16], which leads to the hypothesis that it functions to recruit and attach osteoblasts to the periosteum.

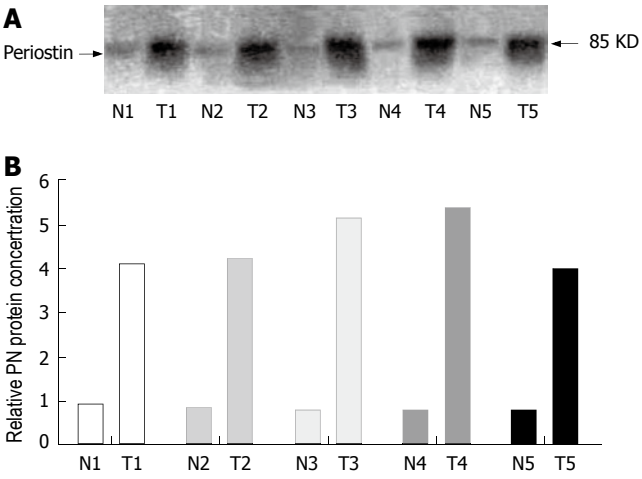

Figure 2 Periostin protein expression in normal and cancer tissue samples. Tissue extracts from normal or gastric cancer tissue samples were subjected to immunoblot analysis with a polyclonal antiperiostin antibody. The results shown in the T and N lanes are for cancer and normal tissues, respectively, from which RT-PCR was performed (**A**). Over fivefold more periostin was detected in gastric cancer tissues than in normal tissues from the same patient (**B**).

Periostin is upregulated in colorectal and breast cancer, and their liver metastases, which suggests that it plays a role in promoting growth in these tumors; furthermore, anti-periostin antibodies activate apoptosis and potentiate the effects of 5-fluorouracil chemotherapy in colorectal cancer[17,18]. Sasaki has reported[6] that periostin serum level is elevated in breast cancer with bone metastases, and has suggested it as a tumor marker in breast cancer. Since periostin functions as a ligand for integrins, and promotes ovarian cancer cell migration and adhesion[7], it is reasonable to investigate its role in gastric cancer.

In the present study, we found that periostin was expressed in normal gastric and gastric cancer tissues, while its expression was markedly elevated in cancer tissues. The basal mRNA and protein level of periostin in normal and benign gastric tissues suggest that it plays a role in the normal physiology of the gastrointestinal epithelium, as it was also found that periostin was expressed in normal colon tissue (data not shown). The dramatic increase in periostin in gastric cancer suggests its role in cancer progression. We found that periostin level

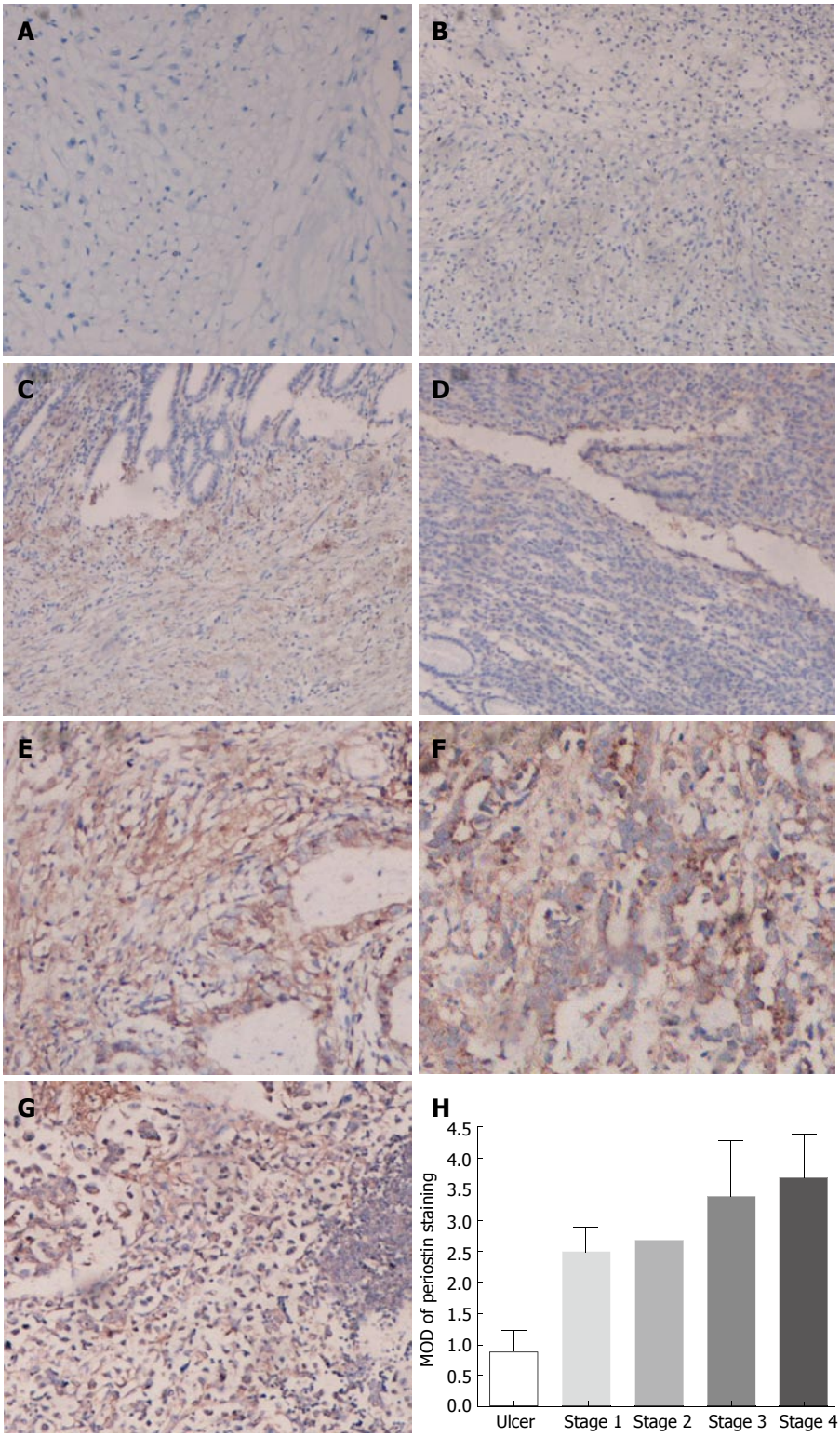

Figure 3 Immunohistochemical analysis of periostin expression in normal gastric tissues, benign gastric ulcers, gastric cancer tissues, as well as lymphoid metastasis from gastric cancer. The tissue sections were immunostained with a polyclonal antibody. The positive staining for periostin protein is shown with a brown color. All sections were counterstained with hematoxylin showing a blue color. (**A**) Negative control; (**B**) benign gastric ulcer; (**C**) stage Ⅰ gastric cancer; (**D**) stage Ⅱ gastric cancer; (**E**) stage Ⅲ gastric cancer; (**F**) stage Ⅳ gastric cancer; (**G**) lymph node metastasis. The average MOD of periostin staining from stage Ⅰ-Ⅳ gastric cancer was significantly higher than that from normal gastric tissues in each group (**H**) ($P < 0.05$).

was significantly increased in cancer tissues compared with gastric ulcers, and periostin level also increased with tumor-stage progression. As observed in other cancers, increased expression of periostin was associated with advanced stage and cell proliferation, adhesion and migration[5,19-27], and these results demonstrate that periostin may play a role in the progression of gastric cancer.

However, it has been reported that decreased expression of periostin is associated with progression of bladder cancer in humans, and expression of periostin mRNA is markedly reduced in a variety of human cancer cell lines[28,29]. These results differed from our findings that showed that periostin was upregulated in gastric cancer tissues at both the mRNA and protein level. We speculate that there are several explanations for the present findings. First, the expression profile of cancer cell lines may not reflect the *in vivo* expression pattern in certain tumors; second, periostin may have different functions according to different histopathological types of cancer; and third, it is possible that periostin may function differently by

expressing alternative splicing events at the C-terminal region, as five different spliced transcripts of periostin are produced[15].

In summary, periostin expression was greater in gastric cancer tissues and metastatic lymph nodes compared to that in normal gastric tissues and benign gastric diseases (ulcers), and this increased expression was closely correlated with the TNM stage of gastric cancer. Our results strongly suggest that periostin plays a role in the progression of gastric cancer.

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COMMENTS

Background

The pathogenesis of gastric cancer has been extensively investigated in recent years, and the expression of many genes changes in the progression of carcinogenesis, cell invasion and metastasis. Periostin was originally identified in a mouse osteoblastic library. Its role in tumorigenesis is still unclear.

Research frontiers

Periostin has been suggested to be involved in cell adhesion and tumor formation. The human periostin gene has been shown to be overexpressed in lung cancer. Serum periostin levels are elevated in many kinds of human malignancy, and are correlated with poor prognosis. All of these observations suggest that periostin plays a role in tumorigenesis.

Innovations and breakthroughs

The expression of periostin is variable throughout the gastrointestinal tract. The basic level of this protein expression suggests that it plays a role in the normal physiology of the gastrointestinal epithelium. However, the expression of periostin mRNA differs between primary tumors and their respective cell lines. For example, periostin mRNA expression is low in colorectal cancer and head and neck squamous cell carcinoma cell lines, but higher in the primary tumors. The possibility is that stromal components play a role in stimulating periostin expression. Furthermore, opposite findings have been reported, periostin is down-regulated in bladder cancer, and invasiveness and metastasis are suppressed by periostin.

Applications

This study has implications for the future investigation of mesenchyme-specific genes in the formation of gastrointestinal cancer. Regarding the higher expression of periostin in gastric cancer, at both the mRNA and protein level, it is possible that periostin plays a role in the development of gastric cancer, and further study of this molecule in gastric cancer and its role as a biological marker is warranted.

Peer review

This is a well-written study which investigated the expression of the periostin gene. There has not been much research on this molecule in gastric cancer, and this study revealed that periostin was more highly expressed at the mRNA and protein level in gastric cancer, and the expression level was positively correlated with clinical tumor stage. Further investigation regarding the function and prognostic significance of periostin will be interesting, and this paper is a valuable addition to the literature on gastric cancer.

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RAPID COMMUNICATION

Serum transforming growth factor-β1 level reflects disease status in patients with esophageal carcinoma after radiotherapy

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Abstract

AIM: To evaluate the relationship between changes in serum transforming growth factor β1 (TGFβ1) level and curative effect of radiotherapy (RT) in patients with esophageal carcinoma.

METHODS: Ninety patients with histologically confirmed esophageal carcinoma were enrolled. Serum samples for TGFβ1 analysis were obtained before and at the end of RT. An enzyme-linked immunosorbent assay was used to measure serum TGFβ1 level. Multivariate analysis was performed to investigate the relationship between disease status and changes in serum TGFβ1 level.

RESULTS: Serum TGFβ1 level in patients with esophageal carcinoma before RT was significantly higher than that in healthy controls ($P < 0.001$). At the end of RT, serum TGFβ1 level was decreased in 67.82% (59/87) of the patients. The overall survival rate at 1, 3 and 5 years was 48.28% (42/87), 19.54% (17/87) and 12.64% (11/87), respectively. Main causes of death were local failure and regional lymph node metastasis. In patients whose serum TGFβ1 level decreased after RT, the survival rate at 1, 3 and 5 years was 61.02% (36/59), 28.81% (17/59) and 18.64% (11/59), respectively. The survival rate at 1 year was 17.86% (5/28) in patients whose serum TGFβ1 level increased after RT, and all died within 18 mo ($P < 0.01$).

CONCLUSION: Serum TGFβ1 level may be a useful marker for monitoring disease status after RT in patients with esophageal carcinoma.

Key words: Esophageal carcinoma; Prognosis; Radiotherapy; TGFβ1

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INTRODUCTION

Esophageal carcinoma is one of the most common malignant diseases in China. However, for a large number of patients, treatment is only palliative. The 5-year survival rate has remained about 10% for patients treated with conventionally fractionated radiotherapy (CR) alone. The poor prognosis is the result of both loco-regional treatment failure, seen in up to 80% of cases, and early disease dissemination[1,2]. Failure of local control remains a significant clinical problem. Therefore, local control to improve survival of patients with esophageal carcinoma patients has been focused on by most investigators. Much emphasis has been placed on the role of physical factors (e.g., total dose and dose per fraction) in improving local control and survival rate. In China, investigators have published their results on esophageal carcinoma, using a schedule named late-course hyperfractionation accelerated radiation therapy (LCHART)[3-7]. In this way, the survival rate of patients with esophageal carcinoma has been increased from 10% to 30%. However, acute toxicity reactions, mainly esophagitis, have increased. Although physical factors are important, none of these models considers the molecular biological events that may be responsible for the observed heterogeneity in tumor tissue response.

Cytokines play a key role in regulation of cells of the immune system and have also been implicated in the pathogenesis of malignant diseases. Transforming growth factor beta-1 (TGFβ1) is a cytokine with multiple biological functions. It influences the proliferation rate of many cell types, and acts as a growth inhibitor in most but not all cases. In addition, TGFβ1 controls

the process of epithelial cell differentiation. In normal cells, TGFβ1 generally enhances adhesion through increased cell matrix production and decreased proteolysis. Resistance to the negative growth-regulating properties of TGFβ1 has been observed in epithelial and mesenchymal tumors. In addition to acting as a stimulator of angiogenesis, TGFβ1 also influences the growth of tumor cells directly or indirectly. Tumor cells can escape the inhibitory effects of TGFβ1 on normal cells at the post-transcription, receptor or post-receptor level. When tumor cells are insensitive, TGFβ1 can also promote tumor metastasis by enhancing angiogenesis, and adjusting the character of the matrix, or adjusting the body's immune response to tumor growth. Animal experiments and clinical observations have demons-trated the functions of TGFβ1 in radiation-induced injury of normal tissues[8,9]. It has been implicated in the injury of several organs after irradiation, including the lungs and breasts, especially in radiation-induced pneumonitis (RP)[10-13]. It also has been proposed that serial measurements of plasma TGFβ1 can be valuable for estimating the risk of RP and deciding whether additional dose-escalation can be safely applied. In recent studies, a relationship between prognosis of many tumors and this cytokine has also been found[14-17]. These suggest that TGFβ1 may be a promising prognostic marker for some cancer patients. However, Fukai's data have suggested that an elevated systemic TGFβ1 level is not related to tumor progression in esophageal cancer[18]. For this reason, they think that systemic inflammation or chronic disease, in addition to the tumor itself, may influence plasma TGFβ1 level. However, another study has shown a significant correlation between TGFβ1 level measured in the azygos vein and distant lymph node metastasis in esophageal cancer[19]. Based on these findings, we hypothesized that serial blood TGFβ1 measurements can be used to identify disease status in patients with esophageal carcinoma treated with conventional doses of radiotherapy (RT), and as a potential predictive marker that may allow us to stratify patients into different treatment groups. Here, we detected serum TGFβ1 level in 90 patients with esophageal carcinoma before and at the end of RT, to investigate the relationship between changes in serum TGFβ1 level and disease status in patients with esophageal carcinoma after RT.

MATERIALS AND METHODS

Patients and pretreatment characteristics

From August 1997 to June 1998, 90 unresectable or medically inoperable patients were enrolled into our clinical trial. Only patients with histologically confirmed esophageal carcinoma were eligible. The additional criteria for eligibility were age ⩽ 75 years, Karnofsky performance status ⩾ 70, white blood cell and hemoglobin levels within the normal range, and no prior treatment. The patients' clinical characteristics are listed in Table 1. The pretreatment evaluation generally included medical history and physical examination, complete blood cell count, chest radiography and/or chest computed tomography (CT), esophageal barium examination, and ultrasound examination of the abdomen, including the liver, kidneys, spleen and retroperitoneal lymph nodes. Based on the examinations mentioned above, patients were staged according to the TNM staging system of the 1997 American Joint Committee on Cancer staging system.

Table 1 Patient characteristics

Characteristic	Number of patients
Gender	
Male	64
Female	26
Age (yr)	
Range	42-75
Median	57.80
Pathology	
Squamous cell carcinoma	88
Undifferentiated carcinoma	2
Location	
Upper-thoracic	37
Middle-thoracic	41
Lower-thoracic	12
Length (cm)	
Median	5.91
Range	2-12
Stage (UICC 1997)	
Ⅰ	8
Ⅱa	31
Ⅱb	45
Ⅲ	6

The study, including the criteria for patient eligibility, diagnostic procedures, fractionation schemes for treatment techniques, collection of blood samples, and tests, was approved by the Ethical Committee of Changhai Hospital. All patients received full information concerning the aim of the study, diagnostic and treatment procedures, medical care, and risks of acute and late sequelae before they entered the trial, and all patients voluntarily gave informed consent.

RT

All patients were given RT alone. A 10 MV X-ray linear accelerator was used for treatment. The design of the radiation fields was based on the diagnosis by CT and barium examination. For all patients, a three-field approach was administered: one anterior and two posterior oblique portals. The width of the fields was adjusted to cover gross tumors with 2-3 cm extended margins, so as to include subclinical lesions. The length of the field covered clinical tumors with a 3-5 cm extended margin at both ends of the lesion. All patients received conventional fractions, 2.0 Gy per fraction, five fractions per week. The total dose given to the tumor was 60-70 Gy/6-7 W. Lung corrections were not performed in this study.

Clinical evaluation of radiation response

At the end of RT, all patients received esophageal barium examination and the clinical radiation response was evaluated according to standard X-ray diagnosis of esophageal carcinoma after RT[5]. A complete response (CR) was the disappearance of the mass shadow, no narrowing observed in the esophageal lumen, and none or slight

Table 2 Changes in serum TGFβ1 levels before and after RT

Groups	*n*	Mean value (ng/mL)	*t* value	*P* value
Control	15	9.53 ± 6.45	5.287	0.001
All patients				
Before RT	90	41.13 ± 15.41		
After RT	87	36.52 ± 19.26	1.365	0.072
Decreased group				
Before RT	59	42.93 ± 14.37		
After RT	59	25.98 ± 8.39	2.481	0.006
Increased group				
Before RT	28	40.25 ± 16.29		
After RT	28	51.61 ± 19.75	1.827	0.039

rigidity of the esophageal wall without residual ulceration. Partial response (PR) was > 50% reduction in tumor bulk, but < 100% resolution of the disease and a residual shallow ulcer with a diameter < 1.5 cm, despite the disappearance of the mass shadow. Minor response (MR) was definite improvement in the barium esophagogram, but with < 50% regression, with a large residual ulcer crater and/or narrowing of the esophageal lumen, regardless of the residual state of the mass shadow. No change (NC) was no improvement in the X-ray findings, with a deep and large residual ulcer or complete obstruction of the esophageal lumen, regardless of the residual state of the mass shadow.

The main endpoint in this analysis was the relationship between survival rate and change in serum TGFβ1 level. Death from any cause was calculated from the starting date of RT until death or the last follow-up evaluation. After treatment, follow-up included medical oncology visits at 3-mo intervals for 1 year, and then every 6 mo thereafter up to 5 years. The relationship between 1, 3 and 5-year survival rates and change in serum TGFβ1 level was observed.

Preparation of blood samples and tests for TGFβ1

Blood samples (2 mL each) were collected in EDTA tubes and stored for 1-3 h at 4℃, until the samples were centrifuged for plasma removal. Blood samples were centrifuged at 2000 *g* for 20 min, and only the top 0.5-1.0 mL plasma supernatant (serum) was removed to avoid platelet contamination. The serum samples were kept frozen at -70℃ until assayed for TGFβ1. An enzyme-linked immunosorbent assay (ELISA) was used to determine TGFβ1 level. The TGFβ1 ELISA kit was purchased from R&D Systems (Shanghai, China). Serum samples were not subjected to acid/ethanol extraction, and active TGFβ1 was measured using the kit according to the manufacturer's recommended procedures. The control population consisted of 15 samples from normal blood donors.

Statistical analysis

The Statistical Package for Social Sciences, version 10.0. was used for statistical analysis. Serum TGFβ1 levels were expressed as means ± SD. Two sample means were statistically compared using Student's *t* test, assuming an unequal variance. Multiple comparisons between the mean TGFβ1 concentrations were performed using analysis of variance. The Kaplan-Meier model was used to estimate survival, and the differences between them were compared by the log-rank test.

Table 3 Immediate response to RT and serum TGFβ1 levels (%)

TGFβ1	*n*	CR	PR	MR	NC
Decrease	59	32.20 (19/59)	50.85 (30/59)	13.56 (8/59)	3.39 (2/59)
Increase	28	21.43 (6/28)	46.43 (13/28)	25.00 (7/28)	7.14 (2/28)

RESULTS

All patients were followed until death or the time of analysis. Three patients were interrupted during RT and were removed from the statistical analysis, and five patients were lost to follow-up, who were counted as being dead at the time they disappeared. Eighty-two patients were followed for 5 years and the follow-up rate was 94.25%.

Changes in serum TGFβ1 level before and after RT

As shown in Table 2, before RT, mean serum TGFβ1 level in patients with esophageal cancer was 41.13 ± 15.41 ng/mL. This concentration was significantly higher than that in controls (9.53 ± 6.45 ng/mL) ($P < 0.001$). At the end of RT, a decreased serum TGFβ1 level was found in 67.82% (59/87) of all patients that completed the schedule. This suggested that TGFβ1 in the blood was produced by the esophageal tumor, and that its decrease may be due to the tumors being controlled by RT.

Clinical response to RT and serum TGFβ1 level

The relationship between clinical response to RT and serum TGFβ1 level is listed in Table 3. In patients with a reduced TGFβ1 level, tumor response rate to RT (CR plus PR) was 83.05% (49/59), and it was 67.86% (19/28) in those showing an increase ($P < 0.05$).

Changes in serum TGFβ1 level and disease status

It was found in stage Ⅲ patients that serum TGFβ1 level was significantly decreased at the end of RT compared to that before RT. Mean serum TGFβ1 levels before and after therapy in these patients were 42.38 ± 13.65 and 34.76 ± 15.62, respectively ($P < 0.05$). Three months after RT, 11.5% (10/87) of patients had local treatment failure and regional lymph node metastasis. Among these patients, nine out of 28 (32%) patients' serum TGFβ1 levels increased, while only one out of 59 (1.7%) patients had a decreased TGFβ1 level. Comparatively, the difference between change in serum TGFβ1 level and local treatment failure and metastasis was significant ($P < 0.001$). The patterns of failure and metastasis are listed in Table 4.

Changes in serum TGFβ1 level and survival rate

Five patients were lost to follow-up between 1 and 5 years after RT, and were counted as deaths from the day that they died. The overall survival rate at 1, 3 and 5 years was 48.28% (42/87), 19.54% (17/87) and 12.64% (11/87), respectively. Main causes of death were local treatment failure and regional lymph node metastasis. In patients with

Table 4 Characteristics of ten patients

Case	Location	Stage	Response to RT	Failure or metastasis	TGFβ1 (ng/mL)	
					Before	After
1	Upper	Ⅲ	NC	Lymph nodes M	30.10	42.80
2	Middle	Ⅱb	NC	Lymph nodes M	51.95	67.65
3	Upper	Ⅱa	PR	Lymph nodes M	18.60	38.20
4	Upper	Ⅲ	PR	Lymph nodes and bone M	25.93	28.73
5	Middle	Ⅲ	PR	Trachea M	29.45	29.55
6	Lower	Ⅱa	PR	Regional failure	23.05	27.18
7	Middle	Ⅱa	PR	Regional failure	40.68	19.88
8	Middle	Ⅱb	PR	Regional failure	30.08	62.65
9	Upper	Ⅰ	CR	Regional failure	40.73	49.03
10	Upper	Ⅰ	PR	Lymph nodes M	13.00	32.65

decreased serum TGFβ1 level after RT, survival rate at 1, 3 and 5 years was 61.02% (36/59), 28.81% (17/59) and 18.64% (11/59), respectively. The survival rate at 1 year was 17.86% (5/28) in patients with increased serum TGFβ1 levels after RT, and all died within 18 months ($P < 0.01$). The survival curves for patients in the three groups are shown in Figure 1.

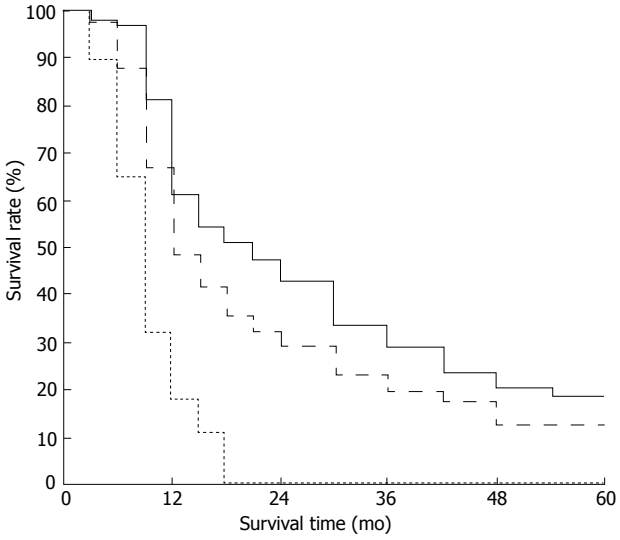

Figure 1 Survival status of all patients, and those with decreased and increased serum TGFβ1 levels. These data show statistically significant differences in the increased group compared with the decreased group and all patients ($P < 0.01$). Solid line indicates the decreased group; broken line indicates all patients; and dashed line indicates the increased group.

DISCUSSION

In the present study, we investigated the relationship between changes in serum TGFβ1 level and curative effect of RT in patients with esophageal carcinoma, and the function of the cytokine TGFβ1 in treatment prognosis or disease status after RT. Data showed that average serum TGFβ1 level in patients with esophageal carcinoma before RT was significantly higher than that of healthy controls, which suggests that TGFβ1 in the blood is produced by esophageal tumors. After RT, serum TGFβ1 level was reduced in 67.82% (59/87) of patients compared to that before RT. This may be because the tumors were controlled by RT. This is believed to be the first report that there is a relationship between serum TGFβ1 level and esophageal carcinoma.

As mentioned above, much emphasis has been placed on the role of physical factors (e.g., total dose and dose per fraction) in improving local control and survival rate of esophageal carcinoma. Although physical factors are important, molecular biological events may be responsible for the observed heterogeneity in tumor tissue response between patients.

Recently, investigators have shown that changes in blood levels of certain cytokines, such as TGFβ1, may predict the risk of radiation-induced lung injury and association with disease progression[20-22]. In a study of 73 patients receiving high-dose thoracic RT for lung cancer, Anscher *et al*[23] found that those patients whose plasma TGFβ1 level was normal at the completion of RT were at low risk for subsequent radiation-induced lung injury, whereas the risk of symptomatic lung damage was increased in patients whose TGFβ1 level remained elevated. Subsequent analysis showed that these changes in plasma TGFβ1 correlated with the risk of pulmonary injury, independent of the volume of lung irradiated. TGFβ1 seems to affect tumor angiogenesis and play an important role in tumor progression in non-small cell lung carcinoma. Kong *et al*[24] measured plasma TGFβ1 concentrations before, during and after RT in 54 patients with lung cancer non-small cell lung cancer (NSCLC), to determine the kinetics of TGFβ1 expression during and after RT, and to correlate plasma TGFβ1 level with disease status after treatment. The results show that in those patients with an elevated plasma TGFβ1 level at diagnosis, monitoring this level may be useful in detecting both disease persistence and recurrence after therapy. Ivanovic *et al*[25] examined the association between elevated plasma TGFβ1 level and disease progression in advanced breast cancer. Follow-up of six patients indicated a relationship between plasma TGFβ1 and treatment response.

In patients with cervical cancer treated with RT alone, pretreatment plasma TGFβ1 level is a significant prognostic factor for survival and local control, but not for radiation toxicity[26]. Using concurrent chemoradiotherapy, Yang *et al*[27] treated 42 patients with biopsy-proven squamous cell carcinoma or adenocarcinoma of the cervix, and assessed serum TGFβ1 level weekly. They have found that sudden elevation of serum TGFβ1 level after the first fraction of brachytherapy is accompanied by greater RT-related morbidity. Lower pretreatment TGFβ1 levels are associated with tumor response to chemoradiation. The conclusion is that serial changes in serum cytokines during chemoradiation may correlate with tumor regression and treatment morbidity.

In breast, gastric, colorectal, prostate, renal and liver cancers, a similar relationship to ours has been found between plasma TGFβ1 level and treatment response[28-31]. However, in advanced head and neck cancer and NSCLC, no similar relationship between plasma TGFβ1 level and tumor burden was found, and neither to treatment response[26]. Thus, for these cancers, it can be hypothesized that healthy tissues and the immune system are responsible for the major part of TGFβ1 production and that cancer

cells make only minor contribution to the total plasma TGFβ1 level.

Among patients with local treatment failure and regional lymph node metastasis after RT, there were nine with increased serum TGFβ1 (Table 4). There was a positive relationship between changes in serum TGFβ1 level and local treatment failure and regional lymph node metastasis. All patients with increased serum TGFβ1 died within 18 months after RT, which strongly suggests that serum TGFβ1 level can predict progression in patients with esophageal carcinoma. Our results have been confirmed by other studies on different kinds of carcinoma[16, 24].

To the best of our knowledge, few of these studies have shown the predictive power of TGFβ1 for disease progression in patients with esophageal carcinoma after RT. The present study demonstrates that a higher serum TGβ1 level after RT is strongly associated with residual or recurrent tumor or lymph node metastasis. These data suggest that serum TGFβ1 may be useful as a marker for monitoring tumor response to therapy and disease progression.

In conclusion, our findings provide preliminary evidence that significantly elevated serum TGFβ1 level in patients with esophageal carcinoma is associated with poor prognosis. Although the complete mechanism of action and the role of TGFβ1 in esophageal carcinoma remain to be elucidated, our results suggest that this biomarker may be useful for monitoring tumor response to therapy and diseases progression in patients with esophageal carcinoma.

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COMMENTS

Background

TGFβ1 is a cytokine with multiple biological functions. In recent studies, a relationship between prognosis of many tumors and this cytokine has also been found. These suggest that TGFβ1 may be a promising prognostic marker for some cancer patients. Based on these findings, we hypothesized that serial blood TGFβ1 measurements can be used to identify disease status in patients with esophageal carcinoma treated with conventional doses of radiotherapy (RT), and as a potential predictive marker that may allow us to stratify patients into different treatment groups.

Research frontiers

A study has shown a significant correlation between TGFβ1 level measured in the azygos vein and distant lymph node metastasis in esophageal cancer. Few of studies have shown the predictive power of TGFβ1 for disease progression in patients with esophageal carcinoma after RT.

Innovations and breakthroughs

Our findings provide preliminary evidence that significantly elevated serum TGFβ1 level in patients with esophageal carcinoma is associated with poor prognosis.

Applications

TGFβ1 may be useful for monitoring tumor response to therapy and diseases progression in patients with esophageal carcinoma. It may allow us to stratify patients into different treatment groups.

Terminology

Serum TGFβ1 level in esophageal carcinoma reflects disease status. A higher serum TGFβ1 level after RT is strongly associated with residual or recurrent tumor or lymph node metastasis.

Peer review

This paper is a well done and interesting study regarding TGF-beta serum levels and response/survival to radiation therapy in patients with esophageal cancer. The science seems interesting and sound.

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RAPID COMMUNICATION

Early steroid withdrawal after liver transplantation for hepatocellular carcinoma

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Abstract

AIM: To evaluate the impact of early steroid withdrawal on the incidence of rejection, tumor recurrence and complications after liver transplantation for advanced-stage hepatocellular carcinoma.

METHODS: Fifty-four patients underwent liver transplantation for advanced-stage hepatocellular carcinoma from April 2003 to June 2005. These cases were divided into a steroid-withdrawal group (group A, n = 28) and a steroid-maintenance group (group B, n = 26). In group A, steroid was withdrawn 3 mo after transplantation. In group B, steroid was continuously used postoperatively. The incidence of rejection, 6-mo and 1-year recurrence rate of carcinoma, 1-year survival rate, mean serum tacrolimus trough level, and liver and kidney function were compared between the two groups.

RESULTS: In the two groups, no statistical difference was observed in the incidence of rejection (14.3 *vs* 11.5%, $P > 0.05$), mean serum tacrolimus trough levels (6.9 ± 1.4 *vs* 7.1 ± 1.1 μg/L, $P > 0.05$), liver and kidney function after 6 mo [alanine aminotransferase (ALT): 533 ± 183 *vs* 617 ± 217 nka/L, $P > 0.05$; creatinine: 66 ± 18 *vs* 71 ± 19 μmol/L, $P > 0.05$], 6-mo recurrence rate of carcinoma (25.0 *vs* 42.3%, $P > 0.05$), and 1-year survival rate (64.2 *vs* 46.1%, $P > 0.05$). The 1-year tumor recurrence rate (39.2 *vs* 69.2%, $P < 0.05$), serum cholesterol level (3.9 ± 1.8 *vs* 5.9 ± 2.6 mmol/L, $P < 0.01$) and fasting blood sugar (5.1 ± 2.1 *vs* 8.9 ± 3.6 mmol/L, $P < 0.01$) were significantly different. These were lower in the steroid-withdrawal group than in the steroid-maintenance group.

CONCLUSION: Early steroid withdrawal was safe after liver transplantation in patients with advanced-stage hepatocellular carcinoma. When steroids were withdrawn 3 mo post-operation, the incidence of rejection did not increase, and there was no demand to maintain tacrolimus at a high level. In contrast, the tumor recurrence rate and the potential of adverse effects decreased significantly. This may have led to an increase in long-term survival rate.

Key words: Hepatocellular carcinoma; Liver transplantation; Steroids; Tumor recurrence

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INTRODUCTION

Liver transplantation is well recognized as a treatment for prolonging survival in patients with advanced-stage hepatocellular carcinoma[1]. Obviously, tumor recurrence is the main reason for the poor long-term survival after transplantation in these patients. It has been shown that long-term immunosuppression can facilitate the growth and spread of malignant cells[2]. There is evidence that steroids play an important role in tumor recurrence after liver transplantation for hepatoma[3], but whether steroids can be safely withdrawn remains controversial. In this study, we contrasted patients with early steroid withdrawal with those using continuous steroids, in order to establish the validity of the steroid-withdrawal regimen.

MATERIALS AND METHODS

Patients

Fifty-four patients suffering from advanced-stage hepatoma (all exceeding the Milan criterion) underwent liver transplantation between April 2003 and June 2005. There were two immunosuppressive protocols: 28 patients (group A) were given an early steroid-withdrawal protocol and 26 patients (group B) were given a steroid-maintenance protocol. Factors such as age at transplantation, stage of carcinoma, Child-Pugh score, graft cold ischemic time, anhepatic phase, operation time, and mean level of liver function before operation were noted, and these parameters were well matched in both groups (Table 1).

Table 1 Preoperative and intraoperative data for patients in the 2 groups

Parameter	Group A (n = 28)	Group B (n = 26)	P value
Sex (F/M)	1/27	0/26	0.3370
Mean age at OLT	45.7 ± 3.5	47.4 ± 6.3	0.2310
TNM stage of carcinoma			0.9914
Ⅱ	1 (3.5%)	1 (3.8%)	
Ⅲ A	8 (28.6%)	7 (26.9%)	
Ⅲ B	5 (17.9%)	4 (15.4%)	
Ⅳ A	14 (50.0%)	14 (53.8%)	
Child-Pugh class			0.5259
A	20 (71.4%)	21 (80.8%)	
B	7 (25.0%)	5 (19.2%)	
C	1 (3.5%)	0 (0.0%)	
Liver and kidney function			
ALT (nka/L)	935.1 ± 383.3	1010.2 ± 536.8	0.5545
T-Bil (μmol/L)	23.1 ± 11.2	20.1 ± 10.8	0.3314
Creatinine (μmol/L)	67.8 ± 22.2	59.8 ± 24.3	0.2218
Graft cold ischemic time (min)	481.6 ± 97.0	462.1 ± 88.0	0.4464
Anhepatic phase (min)	51.5 ± 3.4	50.8 ± 3.1	0.4339
Operation time (min)	375.2 ± 98.1	391.5 ± 116.7	0.5799

OLT: Orthotopic liver transplantation.

Immunosuppressive regime

All the patients took tacrolimus, with a target serum trough level of 6-8 μg/L until 12 mo, and 4-6 μg/L thereafter. Mycophenolate mofetil was prescribed for 1 year at a dose of 0.5-1.0 g/d. Methylprednisolone was given at 500 mg/d intravenously for 3 d, during and after transplantation. Patients in group A received a rapid steroid reduction with the intention of withdrawing steroid by 3 mo. Patients in group B received a slow taper of steroid to prednisone 10 mg/d at 3 mo, and were maintained on this dose thereafter.

Postoperative treatment

ALT, creatinine, total cholesterol and fasting blood sugar were noted regularly after operation. Biopsies were used to establish the diagnosis of rejection on a histological basis when biochemical analysis suggested rejection. The following adjuvant chemotherapy regimen was adopted: E-ADM 40-60 mg/m^2 on d 1 and C-DDP 20-40 mg/m^2 on d 2-5, with 28 d as a cycle and 6 cycles in all. Tumor recurrence was confirmed by computed tomography or magnetic resonance imaging.

Statistical analysis

Statistical analysis was performed on preoperative and intraoperative data in the two groups, such as sex, age, stage of carcinoma, Child-Pugh score, liver and kidney function, Graft cold ischemic time, anhepatic phase and operation time. The rejection rate, tumor recurrence rate, patient survival rate and mean levels of biochemical parameters were compared between the two groups. Statistical analyses were conducted using the Statistical Package for the Social Sciences computer program (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). The Student's t test and χ^2 test were used to determine statistical significance between the groups. $P < 0.05$ was considered significant.

Table 2 Rejection, tumor recurrence, and survival rate

Group n	Rejection rate	Tumor recurrence rate		1-yr survival rate (%)
		6-mo	1 yr	
A 28	14.3	25.0	39.2[a]	64.2
B 26	11.5	42.3	69.2	46.1

[a]$P < 0.05$ *vs* Group B.

RESULTS

There were no significant differences between the two groups for rejection rate and 6-mo tumor recurrence rate. One-year tumor recurrence rate (39.2 *vs* 69.2%, $P < 0.05$) was significantly higher in the steroid-maintenance group. One-year survival rate was higher in group A than in group B, but the difference was not statistically significant (64.2 *vs* 46.1%, Table 2).

At 6 mo, the mean serum tacrolimus trough level was (6.9 ± 1.4) μg/L in group A and (7.1 ± 1.1) μg/L in group B, although the difference was not significant. There was no difference in liver and kidney function (ALT and creatinine) between the two groups. However, at 6 mo post operation, the mean levels of total serum cholesterol and fasting blood sugar were significantly lower in group A (Table 3).

DISCUSSION

Corticosteroids, with their multifaceted immunosuppressive properties, have long been considered as a linchpin in the prevention and treatment of transplant rejection. In addition to inhibiting the release and function of cytokines, such as interleukin-2, steroids can also regulate T- and B-lymphocyte apoptosis[4]. However, there are well-known adverse effects that result in significant morbidity, including hypertension, diabetes, hyperlipidemia, obesity, and infectious complications. The adverse effects of long-term steroid use, even at a low dose, have stimulated interest in the feasibility of steroid-free maintenance immunosuppressive regimens.

This randomized clinical study was focused on a particular group of recipients who suffered from advanced-stage hepatocellular carcinoma before liver transplantation. In this group of patients, the high tumor-recurrence rate may cause the long-term survival rate to decrease sharply. Indisputably, the use of steroids has exacerbated this problem, either *via* a direct negative impact and/or by its adverse effects. One multicenter study has shown that when steroids were withdrawn 3-6 mo after liver transplantation, tumor recurrence was reduced to its lowest level[2]. A retrospective study of three centers in Italy has found that the risk of hepatoma recurrence in patients with permanent use of steroids was almost fourfold when compared with patients made steroid-free not later than 6 mo after liver transplantation[3]. Steroids may contribute to tumor recurrence. The potential

Table 3 Biochemical indicator after operation (mean ± SD)

Group *n*	FK506 trough levels (μg/L)	ALT (nka/L)		Creatinine (μmol/L)		Total cholesterol (mmol/L)	Blood-fasting sugar (mmol/L)
		3-mo	6-mo	3-mo	6-mo	6-mo	6-mo
A (*n* = 28)	6.9 ± 1.4	567 ± 233	533 ± 183	69 ± 18	66 ± 18	3.9 ± 1.8[b]	5.1 ± 2.1[b]
B (*n* = 26)	7.1 ± 1.1	500 ± 350	617 ± 217	75 ± 15	71 ± 19	5.9 ± 2.6	8.9 ± 3.6

[b]$P < 0.01$ *vs* Group B.

mechanism of this may be that steroids can inhibit malignant-cell apoptosis and promote migration of these cells. Yazawa *et al*[5] have reported that glucocorticoids can inhibit human neutrophil-mediated tumor cell cytostasis. Ho *et al*[6] have found that, in patients with hepatoma, the survival rate is higher when hepatoma cells are negative for corticosteroid receptors, compared with those that are positive. In our study, the 1-year tumor recurrence rate in the steroid-withdrawal group was lower than that in the steroid-maintenance group. This demonstrates that early steroid withdrawal can reduce tumor recurrence. The 1-year survival rate was higher in the steroid-withdrawal group. However, because of the small sample size and short time of follow-up, the difference was not statistically significant.

Early steroid-withdrawal regimens do not increase the rejection rate[7-11]. Padbury[12] has reported that, when steroids were withdrawn safely in 140/197 patients (71%), the acute and chronic rejection rate was 4.5 and 3.9%, respectively, and this was similar to the reported rates with steroid-containing regimens. In Jane's study[13], 499 liver transplant recipients accepted early steroid-withdrawal immunosuppression, and only 9.8% of patients had steroid reintroduction. Stegall's study has shown that early steroid withdrawal after liver transplantation does not increase the fatality rate and the rate of chronic graft dysfunction[14,15]. In our study, the incidence of acute rejection during the withdrawal phase did not increase, and each episode of rejection had only a modest effect and was steroid-responsive. No graft was lost to immunological causes. The tacrolimus trough levels were similar in the two groups. There was no demand to increase serum tacrolimus levels to prevent extra rejection. Thus, this early steroid-withdrawal protocol was safe in most patients.

The toxicity of steroids includes increased susceptibility to infection (particularly opportunistic organisms), hyperlipidemia, hypertension, diabetes mellitus, osteoporosis and aseptic necrosis, acne, Cushingoid facies, and growth retardation in children. The cumulative toxicity of immunosuppressive agents remains a major source of morbidity and mortality after liver transplantation, therefore, a protocol eliminating the steroid component has been a goal. Stegall's study has shown that steroid withdrawal after adult liver transplantation reduces diabetes, hypertension and hypercholesterolemia, without causing graft loss[15]. In our study, total serum cholesterol and fasting blood sugar were significant lower in the early steroid-withdrawal group. This effect suggests that corticosteroids are a major causative agent in new-onset diabetes and hypercholesteremia in liver transplantation recipients. Considering the adverse effects of steroid treatment, steroid should be withdrawn earlier, except in patients who use prednisone preoperatively, such as primary biliary cirrhosis and sclerosing cholangitis[13].

This study indicates that steroid withdrawal at 3 mo after liver transplantation is safe and necessary. Early steroid withdrawal does not lead to a high incidence of rejection or a high level of immunosuppressive drugs. In addition, steroid withdrawal may lead to a decreased incidence of tumor recurrence, new-onset diabetes and hypercholesteremia. The decrease in tumor recurrence and adverse effects may lead to a higher survival rate for liver transplantation recipients with hepatocellular carcinoma. However, the 1-year survival rate in the steroid-withdrawal and steroid-maintenance groups was not significantly different. This result may have been due to the small sample size and short follow-up. Therefore, large long-term follow-up (several years), prospective, randomized and multicenter trials will be necessary to confirm the potential benefit of this regimen for the incidence of tumor recurrence, adverse events, and graft and patient survival.

COMMENTS

Background

Steroids have been the pillars of immunosuppression in organ transplantation for over 50 years. However, the fact that immunological graft loss is rare after liver transplantation, combined with the severe adverse effects of long-term prednisone therapy, supports steroid withdrawal in liver transplantation patients. Especially for patients suffering from advanced-stage hepatoma before liver transplantation, the long-term use of steroids may exacerbated the problem of reduced graft survival and patient survival. However, few clinical studies have focused on this group of recipients.

Research frontiers

Many clinical trails have proven the necessity of steroid withdrawal. The main findings of this study were that early withdraw of steroids was confirmed as a positive posttransplant action with a significant influence in reducing hepatoma recurrence.

Related publications

The present study was a randomized clinical trial of steroid withdrawal after liver transplantation in patients with advanced-stage hepatocellular carcinoma. We have cited several articles from other investigators that report research on steroid withdrawal after liver transplantation.

Innovations and breakthroughs

In prior studies of liver transplantation, little attention has been paid to the immunosuppression of hepatoma transplant recipients. This present clinical trail studied a steroid-withdrawal protocol for this group of recipients. We reached the conclusion that tumor recurrence can be reduced when steroids are withdrawn at 3 mo postoperatively. This protocol can be used as the guide for hepatoma transplant recipients.

Applications

In patients suffering from advanced-stage hepatocellular carcinoma, immuno-

suppression with early steroid withdrawal can reduce tumor recurrence after liver transplantation. In addition, total serum cholesterol and fasting blood sugar decrease sharply in steroid-withdrawal patients. This means that the use of steroids is one of the major causes of new-onset diabetes and hypercholesteremia after liver transplantation. Therefore, this steroid-withdrawal protocol can also be used following liver transplantation for other indications.

Terminology

Advanced-stage hepatocellular carcinoma is the end stage of a primary malignant neoplasm of the liver. Liver transplantation is the treatment for this disease. However, because of the high incidence of tumor recurrence, the outcome is poor. Steroids are a group of hormones that affect carbohydrate, fat and protein metabolism. They also possess pronounced anti-inflammatory activity. They have been used for immunosuppression for over 50 years.

Peer review

This is a brief but well-executed study which underscores what Starzl and others have been writing about for some time: the need to reduce immunosuppression in liver transplant recipients. In the authors' study, steroids were withdrawn successfully 3 mo after operation. This protocol caused a reduction in tumor recurrence and incidence of diabetes and hypercholesteremia after liver transplantation.

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CASE REPORT

Fever as the only manifestation of hypersensitivity reactions associated with oxaliplatin in a patient with colorectal cancer Oxaliplatin-induced hypersensitivity reaction

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Abstract

Hypersensitivity reactions (HSR) to oxaliplatin in patients with colorectal cancer include facial flushing, erythema, pruritis, fever, tachycardia, dyspnea, tongue swelling, rash/hives, headache, chills, weakness, vomiting, burning sensations, dizziness, and edema. We report a patient with fever as the sole manifestation of initial HSR, review the literature and discuss the management of HSR. A 57-year-old female with T3N2M0 rectal adenocarcinoma received modified FOLFOX-6. She tolerated the first 8 cycles without any toxicities except grade 1 peripheral neuropathy and nausea. During 9th and 10th infusions, she developed fever to a maximum of 38.3℃ with stable hemodynamic status despite medications. During 11th infusion, she developed grade 3 HSR consisting of symptomatic bronchospasm, hypotension, nausea, vomiting, cough, and fever. On examination, she was pale, cyanotic, with a temperature of 38.8℃, BP dropped to 95/43 mm Hg, pulse of 116/min and O_2 saturation of 88%-91%. She was hospitalized for management and recovered in 24 h. Fever alone is not a usual symptom of oxaliplatin HSR. It may be indicative that the patient may develop serious reactions subsequently, as did our patient who developed hypotension with the third challenge. Treatment and prevention consists of slowing the infusion rate, use of steroids and antagonists of Type 1 and 2 histamine receptor antagonists, whereas desensitization could help to provide the small number of patients who experience severe HSR with the ability to further receive an effective therapy for their colorectal cancer.

Key words: Oxaliplatin; Hypersensitivity reaction; Fever; Colon cancer

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INTRODUCTION

Oxaliplatin ($C_8H_{14}N_2O_4$) (Eloxatin; Sanofi-Aventis), is an organoplatinum in which the platinum atom is complexed with diaminocyclohexane and with an oxalate ligand. It is a third generation platinum which is indicated for the first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil (5-FU) and leucovorin (LV)[1] and for the adjuvant treatment of stage Ⅲ colorectal cancer[2]. Oxaliplatin is safely administered in the outpatient setting but hypersensitivity reactions (HSR) can occur. Hypersensitivity is defined as an unexpected reaction that can not be explained by the known toxicity profile of the chemotherapeutic agent[3]. HSR are seen usually with the taxanes (paclitaxel), the platinum compounds, asparaginase, the epipodophyllotoxins and procarbazine[3]. Doxorubicin and 6-mercaptupurine have also been associated with these reactions. Acute onset and delayed reactions have been described which can cause flushing, pruritis, hypotension, dyspnea, nausea, back pain and rash[3,4].

We report a patient with fever as the sole manifestation of initial hypersensitivity reaction, who subsequently developed a serious HSR including hypotension with the third challenge.

CASE REPORT

The patient is a 57-year-old female diagnosed with T3N2M0 (Stage Ⅲ/Dukes C) rectal adenocarcinoma who was treated with oxaliplatin-based regimen in the adjuvant setting. The tumor was 5.7 cm in size and six lymph nodes were positive for cancer. She was started on treatment with adjuvant chemotherapy with modified FOLFOX-6 (mFOLFOX-6; oxaliplatin 85 mg/m^2 with concurrent LV 400 mg/m^2 on d 1 followed by Ⅳ bolus 5-FU 400 mg/m^2 followed by a single continuous infusion of 5-FU 2400 mg/m^2 over 46 h every 2 wk). As per

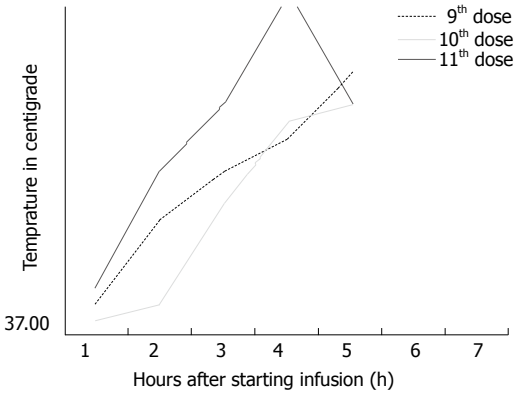

Figure 1 Fever in relation to infusion of oxaliplatin in the present case during 9th-11th cycles of modified FOLFOX-6 regimen in a patient with Stage Ⅲ rectal cancer.

Table 1 Common terminology criteria for adverse events grading of hypersensitivity reactions (version 3.0)[26]

Grade	Hypersensitivity reactions
1	Transient flushing or rash; drug fever < 38℃
2	Rash; flushing; urticaria; dyspnea; drug fever ≥ 38℃
3	Symptomatic bronchospasm, with or without urticaria; parenteral medication(s) indicated; allergy-related edema/angioedema; hypotension
4	Anaphylaxis

institutional standard premedication, she received dexamethasone 20 mg and ondansterone 16 mg intravenously. She tolerated 8 infusions without any complications. One and a half hour after starting the 9th infusion, she developed fever of 37.7℃. Other toxicities were grade 1 vomiting and grade 1 diarrhea. She was treated with promethazine (25 mg Ⅳ), hydrocortisone (100 mg Ⅳ), lorazepam (1 mg Ⅳ) and acetaminophen (650 mg PO). During this episode, her BP was 100/60 mm of Hg and pulse of 100/min. Despite the treatment, the fever continued to rise and reached a maximum of 38.8℃ (Grade 2). She was discharged with the infusional 5-FU pump after observation and once fever came down to 37.8℃.

Two weeks later when she returned for the 10th infusion, she again developed fever of 37.7℃ towards the end of the 2 h infusion. She also had emesis (grade 1) with nausea (grade 1) during oxaliplatin infusion. She was treated with promethazine and acetaminophen. Temperature rose to maximum of 38.4℃ (Grade 2). The pulse and BP were preserved. Work-up for infectious etiology including line infection was done and ruled out. Fever defervesced and patient was discharged and returned home.

The patient was pre-medicated with promethazine before cycle 11th. She was planned to receive oxaliplatin over 2-h infusion. One and half hour into the infusion, she complained of itching for which diphenhydramine (25 mg) was given. After this she developed nausea (Grade 1) and vomiting (Grade 1) for which she received promethazine. One hour later she complained of cough and gagging sensation and on examination was found to be pale and cyanosis was visualized on the nail bed. Her temperature rose to 38.8℃ (Figure 1) and soon her BP dropped to 95/43 mm Hg, pulse of 116/min and O_2 saturation of 88%-91% (Grade 3). Two liters of oxygen were administered with nasal canula and the saturations improved to 91%. She was admitted to the hospital. Fever defervesced overnight and blood and urine cultures were negative. Hypotension responded to fluids and patient was discharged the next day. Desensitization was offered but patient refused further therapy with oxaliplatin.

Table 2 Incidence of HSR in pivotal studies of oxaliplatin in metastatic and adjuvant treatment of colorectal cancer

Study	Number of patients	Patients with HSR (%)	Patients with Grade 3/4 HSR (%)
Data from first-line[10]	259	12	2
Data from second-line[10]	150	10	< 1
MOSAIC (adjuvant)[2]	1123	10.3	2.9
Siu *et al*[1] all patients	180	15	2.2
Siu *et al*[11] adjuvant/ first-line metastatic	88	10.2	
Siu *et al*[11] second-line metastatic	92	19.6	

HSR: Hypersensitivity reactions.

DISCUSSION

HSR have been reported to occur in a minority of patients (12%) who receive oxaliplatin, and as less than 0.55% of the patients develop grade 3 or 4 reactions[5-8] (Table 1). However, the incidence of HSR to oxaliplatin is rising recently as a result of increasing clinical use both in the adjuvant and metastatic treatment of colorectal cancer. The reactions usually occur after multiple infusions (mean 2-17) cycles of therapy with variable and unpredictable clinical features[9]. Patients can experience flushing, alterations in heart rate and blood pressure, bronchospasm, back pain, chest discomfort, fever, pruritis, erythema, nausea, and rash[3,5]. Usually the symptoms are mild but life-threatening anaphylactic reactions can occur. For this reason the FDA and Sanofi-Aventis has included a BOX warning for such reactions[10]. The incidence of HSR in pivotal phase Ⅲ trials of oxaliplatin both in metastatic and adjuvant setting is show in Table 2[2,10,11].

All platinum compounds are known to cause HSR[12-15]. Being a platinum derivative, it is not surprising that oxaliplatin can also develop HSR[5-9]. The differences in the reactions have been summarized in the Table 3.

The pathophysiology of HSR is not well understood. Development of this reaction after multiple infusions in most patients suggests the need for sensitization. Some investigators have described these reactions as Type Ⅰ (IgE-mediated) allergic reactions[16]. Santini *et al* reported a case of an idiosyncratic reaction; the serum analysis of the patient showed elevated TNF-alpha and IL6. The investigator postulated it to be a T-cell mediated reaction[17]. It was also postulated that oxaliplatin acted as a super-antigen on mononuclear cells and resulted in the release of these cytokines. Other mechanisms that have been suggested include binding of the platinum salts to different peptides of major histocompatibility complex (MHC). In fact, HLA phenotype is a significant determinant of occupational

Table 3 Characteristics of HSR with different Platinum compounds

	Cisplatinum[12,13]	Carboplatinum[4,15]	Oxaliplatin[5-9]
Incidence	5%-20% (Increased with radiation)	16%	12% (14), < 1% grade 3-4
Initial onset	-	After 6 cycles(range 2-12)	After 7 cycles (range 2-25)
Time of onset	Minutes	Minutes-days	Minutes-hours
Symptoms	Variable(fever, anxiety, pruritus, cough, dyspnea, diaphoresis, angioedema, vomiting, bronchospasm, rash and pruritus, and hypotension	Variable (itching ,rash, chest tightness, Blood pressure changes, facial swelling)	Variable (flushing, alterations in heart rate, dyspnea, back pain, fever, pruritis, erythema, nausea, rash)
Can it be re-introduced?	-	Yes, with slowing the infusion rate (6% discontinued)	Yes, with pre-medications, slowing the infusion rate, and/or desensitization

sensitization to inhaled hapten of complex platinum salts and the strength of this association varies according to the intensity of exposure[18]. Furthermore, the relationship between hypersensitivity reactions and HLA-haplotype has been described for other drugs[19]. Additional factors are deemed to be necessary to the immune system for developing the reaction after several infusions.

Fever alone is not a usual symptom of oxaliplatin hypersensitivity. In another study among 39 patients who received a FOLFOX regimen in first line or beyond, the most common manifestations of an allergic reaction included: respiratory (50%), cutaneous (40%), and an anaphylactic shock (7.6%)[20]. Ulrich-Pur *et al*[21] reported a case of 74-year-old man who developed fever hours after receiving the third infusion of oxaliplatin (5-FU + LV d 1 and d 5 every 28 d + mitomycin C d 1). The temperature was recorded to 39℃ and lasted for 3 d. Patient had similar episodes of fever with 4th to 6th infusion. A rise in serum levels of IL 6 levels with 5th and 6th infusion corresponded to the rise in temperature. Pre-medication with dexamethasone, clarithromycin and metamizol for 3 d prior to therapy did not prevent the febrile reaction. Because the reactions occurred 1.5 to 2 h after infusion, the authors did not consider it to be hypersensitivity reactions, and thought to be definitely some kind of acquired allergic reaction because the 1st and the 2nd infusions were well-tolerated. It is to be noted that mitomycin C can also cause fever as a reaction in some cases. Thomas *et al*[5] reported hypersensitivity reactions in 3 patients; one of them reported fever of 39.6℃ 2 h after the infusion of the 9th cycle of oxaliplatin. Fever defervesced overnight and cultures were negative. The patient again developed fever of 39.0℃ with rigors and chest tightness several hours after the next cycle. Before the next cycle, the patient was premedicated with dexamethasone 20 mg, 6 and 12 h. Thirty minutes before the infusion the patient received solumedrol 125 mg, diphenhydramine 50 mg and cimetidine 30 mg. Despite this, the patient still developed a single spike of 38.3℃ without associated symptoms[5].

When a HSR occurs, the infusion of oxaliplatin should be immediately stopped and replaced by a saline infusion, an intravenous antihistaminic drug and a low-dose corticosteroids administration. In the case of more severe reactions (dyspnoea, sweating, bronchospasm, laryngospasm), immediately administer a high dose of steroid. The steroid dose range between 100 and 1000 mg of hydrocortisone[9]. After the reaction disappears, the oxaliplatin infusion should not be restarted and the decision to administer the other scheduled drugs must be taken evaluating the clinical status of the patient after the reaction, the risk of additional toxicity and the clinical utility of the chemotherapy. Mild hypersensitivity reactions to oxaliplatin and other platinum compounds can be ameliorated in some patients through the use of steroids and antihistamines before administration of subsequent cycles. However, premedication cannot prevent all hypersensitivity reactions, and mild reactions may escalate to severe reactions even when steroids and antihistamines are administered prior to oxaliplatin infusion[22].

Over the years many protocols have been devised to desensitize patients against these life-threatening reactions so that they can benefit from Oxaliplatin. The various approaches have been summarized in the Table 4. One of the approaches is to increase the duration of the infusion. This was described in a retrospective study by Brandi *et al*[6] In the review of 124 patients the author reported fewer hypersensitivity reactions as the infusion time was increased from standard 2 to 6 h. In this review, 17 patients out of 124 (13.7%) developed hypersensitivity. Out of the 17 only 2 developed reactions at the end of infusion while in the rest the reaction appeared 10-15 min from start of the infusion. Six of the 17 patients were successfully re-exposed after pre-medication with steroids and antihistaminic drugs. Five patients developed hypersensitivity symptoms again and only one had no further reactions.

Another approach is desensitization to the drug. Lydia *et al*[23] reported a 53-year-old female with metastatic colon cancer who was treated with CAPOX (capecitabine 1000 mg/m^2 per day Monday to Friday with 2 weekly 85 mg/m^2 oxaliplatin) and bevacizumab (Avastin) 10 mg/kg. With the cycle 5th she developed diaphoresis, hypotension, hypoxia, nausea, and abdominal cramps 12 min from the start of infusion. She was immediately resuscitated with fluid bolus, oxygen, diphenylhydramine (25 mg Ⅳ) and dexamethasone (10 mg Ⅳ). The patient received cycle 5th through 7th without oxaliplatin. Because she had partial response to combination chemotherapy with oxaliplatin and she refused to take irinotecan (camptosar; Pfizer) due to toxicity profile, it was decided to desensitize her. On d 1 of desensitization, the patient was given prednisone 20 mg every 6 h for 4 doses and was hospitalized the same day. On d 2, 45 min before infusion 8 mg of ondansterone and 20 mg of dexamethasone were administered. Then 30 min before infusion, diphenyhydramine 50 mg and cimetidine 300 mg was administered. Oxaliplatin was administered over 8 h in serial dilutions from 1:10 000 to 1:1 and she

Table 4 Published cases of hypersensitivity associated with oxaliplatin

Reference	Presenting Features	Premedication	Oxaliplatin dose and serial dilution if used	Duration of therapy
Lydia *et al*[23]	Diaphoresis, hypotension, nausea, abdominal cramping rash, coryza	D1-prednisone 20 mg Po Q6 h × 4 D2 45 min pre chemo ondansterone 8 mg + dexamethasone 20 mg iv 30 min prior diphenylhydramine 50 mg iv + cemetidine 300 mg iv	140 mg on d 2 dilutions from 1:10000 to 1:1	8 h
Thomas *et al*[5]	Erythematic, flushing	Dexamethasone 20 mg 6 h and 12 h prior 30 min pre infusion: solumedrol 125 mg iv, diphenylhydramine 50 mg iv, cimetidine 50 mg iv	-	2-4 h
Bhargava *et al*[22]	Palpitation, flushing, hypotension	Dexamethasone starting 24 h prior 30 min before infusion received dexamethasone, diphenylhydramine, hydrocortisone 100 mg iv	1:10000, 1:1000, 1:100, 1:10 each bag infuse over one hour	6-8 h
Meyer *et al*[7]		dexamethasone-famotidine and diphenylhydramine	90% of total dose 1:1000, 1:100 and 1:10 dilution over 90 min	6 h
Lim *et al*[24]	Abdominal distension, heat, pruritis	D 1 diphenylhydramine 50 mg QID 30 min prior to infusion metoclopramide 9 mg, morazepam 2 mg, dexamethasone 5 mg iv	Fixed dose infusion over 24 h with dilute solution (0.15 mg/mL)	24 h
Present case	Fever	-	Refused further oxaliplatin therapy	-

received a total dose of 140 mg. Intravenous epinephrine (1:1000) and diphenyhydramine and methylprednisolone 125 mg were placed at bedside. The patient tolerated the therapy without complications. The next infusion was given as outpatient. Thus desensitization helped this patient to receive an additional three doses. In this report 24-h pre-medication schedule with serial dilution of oxaliplatin over longer period of time was used based on reports used for desensitization of carboplatinum. Lim *et al*[24] also published his case with successful desensitization.

With carboplatin, an intradermal skin test after 6th dose is a good predictor for occurrence of this reaction. However, no intradermal test is recommended before oxaliplatin administration. An intradermal skin test for hypersensitivity to oxaliplatin has been reported in small series to be 75%-80% accurate[25]. The investigators suggested that desensitization might be considered for patients with a mild to moderate skin reaction in whom oxaliplatin would be beneficial. The investigators also suggested that a rechallenge should not be attempted for patients with markedly positive skin test reactions. Garufi *et al* reported the skin test to be negative in 15 patients with no previous reactions to oxaliplatin. A positive skin-test reaction is helpful; however, negative results may be seen in some patients who experience hypersensitivity[25]. Two of 8 patients with prior hypersensitivity reactions to oxaliplatin were reported by Meyer *et al*[7] to have negative skin tests with oxaliplatin, as did 1 of 3 patients described by Thomas *et al*[5].

It is expected to see a rising incidence of HSR to oxaliplatin as has been observed as a result of increasing clinical use. Siu *et al*[11] recently reported epidemiological and clinical features of these reactions in his institution. Among 180 patients, 15% were labeled as allergic to oxaliplatin, the proportion being higher among those receiving oxaliplatin in palliative second-line or above settings (19.6%) than in adjuvant or palliative first-line settings (10.2%). Overall, 2.2% of them developed grade 3-4 reactions. Re-exposure to oxaliplatin in 14 patients resulted in 28.6% HSR with 14.3%reactions of grade 3-4 intensity.

Although the reported incidence of HSR is about 12% of the patients who receive oxaliplatin (1%-2% grade 3 or 4 in severity), the recent rising incidence of HSR to oxaliplatin observed is the result of increasing clinical use. It is also important to remember that fever can be the sole manifestation of initial HSR in few cases akin to ours; a harbinger that this patient can subsequently develop serious HSR with continued use of oxaliplatin. Therefore, proper recognition and management can prevent a serious HSR. Few patients can be managed with pre-medication with steroids and antihistamines, but the majority of patients require intensive desensitization. Due to the desensitization regimens many patients can successfully continue to receive this agent. Reintroductions have only been reported as single case studies or small cohorts. Large scale validation on desensitization strategies are still missing. Knowledge of this rare but real toxicity of oxaliplatin is paramount since the use of this drug is wide-spread both for metastatic and adjuvant settings in the treatment of colorectal cancer-the second leading cause of cancer mortality in USA.

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CASE REPORT

Retrograde jejunoduodenogastric intussusception due to a replacement percutaneous gastrostomy tube presenting as upper gastrointestinal bleeding

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Abstract

Percutaneous endoscopic gastrostomy (PEG) tube complications can be serious or life threatening. Retrograde intussusception is a very rare complication of PEG tubes with only 9 cases reported in the literature. We describe a case of retrograde intussusception, associated with the use of a Foley catheter as a replacement gastrostomy tube, presenting with upper gastrointestinal bleeding. To our knowledge, this is the first reported case of PEG-related retrograde intussusception successfully managed in a non-surgical manner. Retrograde intussusception likely occurred due to migration of the replacement tube with resultant securing and invagination of the proximal jejunum when the gastrostomy tube was anchored to the abdominal wall.

Key words: Percutaneous endoscopic gastrostomy; Intussusception; Migration and upper gastrointestinal bleeding

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INTRODUCTION

Since introduction by Gauderer in 1980, percutaneous endoscopic gastrostomy (PEG) has become the preferred procedure for establishing enteral feeding in most clinical situations[1]. The complication rate ranges from 3% to 14% depending on the definition used, and mortality approaches 1%[2]. Retrograde intussusception, defined as the telescoping or invagination of a distal segment of intestine (the intussusecptum) into the receiving proximal end (the intussusipiens), is a very rare complication of gastrostomy tube migration. We report here the first case treated successfully in a non-surgical manner.

CASE REPORT

An 89-year-old female nursing home resident was hospitalized with generalized abdominal pain, coffee ground emesis and change in mental status. Past history revealed hypertension, mild dementia, coronary artery disease, transvaginal hysterectomy and percutaneous endoscopic gastrostomy (PEG) placement six months prior due to poor oral intake. Four weeks prior to admission, the patient's PEG tube was dislodged unintentionally and replaced at the bedside using a 22f Foley catheter, inflating the balloon with 10 CC of sterile water. No testing was performed to confirm intraluminal placement of the Foley at the nursing home.

Physical examination revealed a cachetic elderly woman who was somnolent but arousable. She was afebrile and normotensive. Abdominal examination revealed a distended, diffusely tender abdomen with evidence of erythema and exudate at the ostomy site and normal bowel sounds. The remainder of the physical examination was unremarkable. Pertinent laboratory studies included white blood cell count of 12.9 thousand/μL and hemoglobin of 10.7 gm/dL. Abdominal flat and upright radiographs revealed only fecal impaction. An upper endoscopy was performed within 24 h of hospitalization with detection of a large amount of brownish foul smelling fluid within the stomach. A twisted loop of small bowel with mucosal discoloration and sloughing was seen protruding through the pylorus into the gastric body (Figures 1 and 2). A 22 fr Foley catheter was seen tethering the loop of bowel to the body of the stomach. The pyloric channel was not visualized and the intubation of the duodenum was not attempted. A water contrast X-ray series performed immediately after endoscopy showed an antral filling defect with typical coil spring appearance of intussusception. A barium study through the PEG tube showed its tip in the proximal jejunum. The clinical diagnosis of retrograde intussusception was made at this time. A general surgery

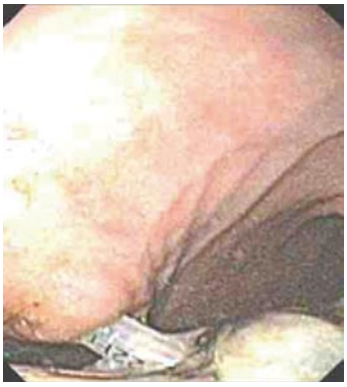

Figure 1 Intussuscepted loop of small bowel and size 22 Fr Foley catheter tethering loop to ostomy site in the gastric body.

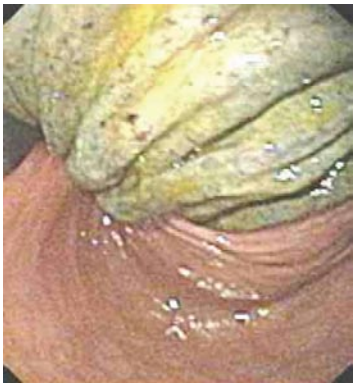

Figure 2 Close up view of intussuscepted small bowel.

opinion was obtained based on the endoscopic and radiologic findings, but a conservative approach was taken at the request of the patient's family. This included deflation and removal of the Foley catheter, placement of a button replacement PEG at the ostomy site, intravenous hydration, packed red blood cell transfusion and institution of broad-spectrum antibiotics.

There was no overt worsening of her clinical status after deflation/removal of the Foley catheter and button PEG replacement. Repeat upper endoscopy was performed three days after PEG tube replacement. Examination distal to the second portion of the duodenum revealed a segment of continuous mucosal necrosis and discoloration. Biopsies obtained revealed mucopurulent exudates and debris with dense inflammatory infiltrates only. Feeding through the button PEG began the day after the repeat endoscopy and was tolerated well during the reminder of her hospital stay. The patient continued to improve clinically and was discharged, on hospital d 14, initially to a skilled nursing unit for a 2 wk observation period followed by eventual return to her permanent assisted living facility.

DISCUSSION

PEG tube insertion is a simple endoscopic procedure performed worldwide. Multiple complications have been reported (major and minor) including those occurred while placing the PEG and postprocedure[2]. Common complications include pain at the insertion site, peristomal leak and infection, tube extrusion, transient ileus and cutaneous ulceration[2,3]. Major complications include gastric or colonic perforation, peritonitis, gastrocolic or colocutaneous fistula, peritonitis and necrotizing fascitis[4-8]. Bleeding and aspiration are complications that have the potential of turning into serious or life threatening situations[4]. Retrograde intussusception is very rare and can occur as a complication of gastrostomy tube migration. In the English literature on this subject, only 9 cases have been reported[9-17]. Nearly all of these cases were secondary to distal migration of a Foley catheter gastrostomy tube lacking an external fixation device or support.

The diagnosis in these cases was made based on radiologic imaging (contrast or barium study, abdominal ultrasound or CT scan) with endoscopy used in 2 cases. All except 1 (postmortem diagnosis) required surgical resection. The exact mechanism leading to retrograde intussusception is unknown. Oswald *et al*[11] suggested that migration of the gastrostomy tube is the initiating event resulting in telescoping of the mobile jejunum over the tip of an impacted gastrostomy tube into the proximal duodenum. Lamont and Rode[12] have proposed a different mechanism with passage of an inflated Foley gastrostomy catheter migrating past the pylorus into the small bowel, resulting in balloon fixation. Retraction attempts with the balloon inflated result in invagination of the small bowel into the duodenum. Finally, Gasparri and associates[15] suggest a 3rd potential mechanism with balloon migration resulting in increased intraluminal traction and invagination of the jejunal wall. Attempts at repositioning result in the inflated balloon acting as a lead point with retrograde telescoping of the jejunum back through the duodenum and into the gastric lumen. Following these case reports, new gastrostomy tubes are now fitted with external fixation devices or bolster to prevent distal migration.

Our case illustrates a rare but preventable complication of a gastrostomy tube with uniqueness in terms of therapeutic approach. Unlike previous cases reported in the literature, our patient did not undergo surgical exploration, making a full recovery without evidence of overt sepsis or peritonitis and was eventually discharged to her nursing home. We hypothesize that following replacement of the PEG tube with a Foley catheter, the catheter migrated into and past the C-loop of the duodenum and was pulled back by NH staff without first deflating the balloon, leading to invagination of the mobile portion of the jejunum. The tube was then likely anchored in some manner leading to mucosal ischemia and necrosis but not in a transmural fashion. After deflation of the Foley catheter balloon, blood supply was restored allowing for revascularization of the mucosa.

We recommend that intussusception should be ruled out in a patient with a PEG tube who presents with symptoms of nausea, vomiting, hemetemesis or abdominal pain by direct visualization of gastric and duodenal mucosa with EGD and upper GI series with water-based contrast. If intussusception is diagnosed, surgery remains the treatment of choice. However, if identified early or patient's surrogates decline a surgical approach, rapid deflation of the Foley catheter and button PEG replacement can result in a successful conservative approach to retrograde jejunoduodenal

intussusception with a positive outcome. Prevention of such a complication may be simply achieved by anchoring, with an external bolster, any type of replacement tube to prevent such a migration.

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CASE REPORT

Acute pancreatitis and cholangitis: A complication caused by a migrated gastrostomy tube

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Abstract

Percutaneous endoscopic gastrostomy (PEG) is generally considered safe with a low rate of serious complications. However, dislocation of the PEG-tube into the duodenum can lead to serious complications. An 86-year old Japanese woman with PEG-tube feeding sometimes vomited after her family doctor replaced the PEG-tube without radiologic confirmation. At her hospitalization, she complained of severe tenderness at the epigastric region and the PEG-tube was drawn into the stomach. Imaging studies showed that the tip of PEG-tube with the inflated balloon was migrated into the second portion of the duodenum, suggesting that it might have obstructed the bile and pancreatic ducts, inducing cholangitis and pancreatitis. After the PEG-tube was replaced at the appropriate position, vomiting and abdominal tenderness improved dramatically and laboratory studies became normal immediately. Our case suggests that it is important to secure PEG-tube at the level of skin, especially after replacement.

Key words: Percutaneous endoscopic gastrostomy; Complications; Tube migration; Pancreatitis; Cholangitis

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) has gained broad acceptance as an effective method for achieving enteral access in patients who need chronic nutritional support. The feeding through PEG-tube is convenient, safe, and agreeable. Most of complications of PEG-tube feeding are minor, except for those arising at tube-exchange, which is necessary for a long-term feeding because of degradation of tubes, or incidental tube-removal. It is better to exchange PEG-tubes under fluoroscope in hospital for preventing complications. However, many primary doctors perform at the bed side in patient's home through unavoidable circumstances. This is one of the reasons why serious complications occur when a long-term PEG-tube feeding is needed.

In this report, we describe a case of pancreatitis and cholangitis induced by dislocated PEG-tube, which are the very rare complications of PEG-tube feeding.

CASE REPORT

An 86-year-old Japanese woman with PEG-tube feeding was referred to our hospital for reiterated emesis. She had an attack of cerebral infarction and cerebral embolism at the age of 84 years. Since the disease caused her dysphagia and continuous consciousness disturbance, she received chronic nutritional support by PEG-tube feeding. One month before her hospitalization, a gastrostomy tube (Gastrostomy-tube, Bard Access Systems, Salt Lake City, UT, USA) with a diameter of 18 F, which is fixed by an intragastric balloon (20 mL of water) and an external disc bumper, was replaced by her family doctor in her home without radiologic confirmation, and she sometimes vomited gastric juice and bile without enteric nutrient. Her family noticed that the PEG-tube was sometimes drawn into the stomach. At her hospitalization, she complained of severe tenderness at the epigastric region and the PEG-tube was drawn into the stomach. The distance between the balloon and external disc bumper was 8 cm measured by a scale indicated on the PEG-tube. Laboratory studies revealed 10.4 $\times$ 10^9/L white blood cells (normal range: 5.0-8.0 $\times$ 10^9/L), 97.9 mg/L C-reactive protein (normal range: < 3 mg/L), 1.24 μmol/L total serum bilirubin (normal range: 0.49-2.16 μmol/L), 213 U/L aspartate aminotransferase (normal range: 10-34 U/L), 254 U/L alanine aminotransferase (normal range: 5-40 U/L), 553 U/L alkaline phosphatase (normal range: 100-358 U/L), 238 U/L lactate dehydrogenase (normal range: 104-224 U/L), 1191 U/L amylase (normal range: 32-112 U/L), 176 U/L gamma-glutamyltranspeptidase (normal range: 7-29 U/L). A diagnosis of acute pancreatitis and cholangitis was made based on the physical examination and laboratory findings.

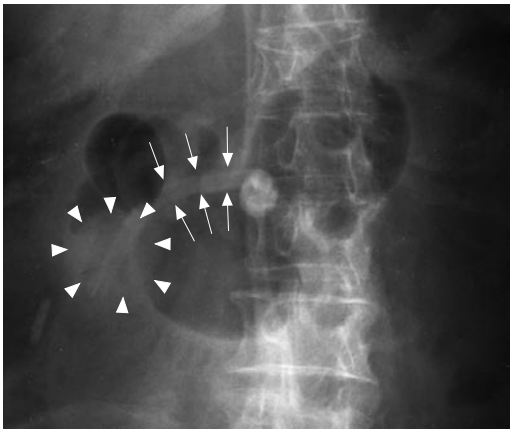

Figure 1 A plain abdominal radiograph showing collected gas in the stomach and duodenum with the tube balloon situated in the second portion of duodenum (tube: arrow, balloon: arrowhead).

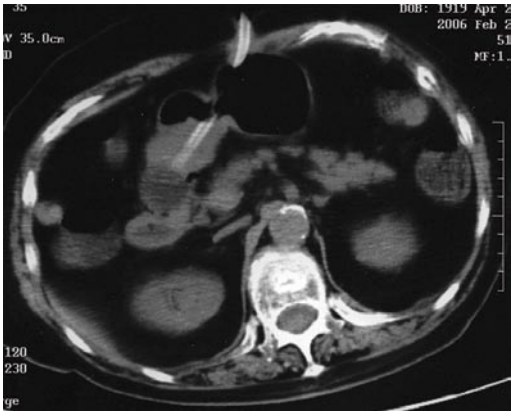

Figure 2 A plain CT scan demonstrating the PEG-tube balloon in the second portion of duodenum.

Abdominal roentgenography and computed tomography (CT) showed that the tip of PEG-tube with the inflated balloon was migrated into the second portion of the duodenum (Figures 1 and 2), suggesting that it might have disturbed the flow of bile and pancreatic juice at the papilla, and thus was thought to be the cause of cholangitis and pancreatitis. No stone or tumor was found at this region in these studies.

Once the balloon was deflated and fixed at the appropriate position by the balloon re-inflation after the tube was retracted into the stomach. After the PEG-tube was replaced at the appropriate position, vomiting and abdominal tenderness improved on the next day and laboratory studies became normal after two days.

DISCUSSION

PEG has become the preferred method in patients requiring long-term enteral nutrition because of its ease and safety of placement. Previous studies reported that complications are infrequent and a procedure-related mortality is less than 1%[1,2]. Common procedure-related complications include wound infection, aspiration, hemorrhage, pneumoperitoneum, peritonitis, and common long-term complications include leakage, granulation tissue, unintentional removal, buried bumper syndrome[3-7]. Obstructive pancreatitis and cholangitis induced by migrated PEG-tube are the very rare complications.

Although PEG-tube feeding is generally considered safe with a low rate of serious complications, dislocation of the PEG-tube into the duodenum can lead to symptoms of obstructive pancreatitis and cholangitis. Because a balloon with PEG-tube is hard to pass through the pyloric ring, a migrated balloon may obstruct the pyloric ring and cause vomiting. In this case, the family doctor might have inserted the PEG-tube too deep into the duodenum, so the tube passed through the pyloric ring and the balloon was inflated in the duodenum. As enteral peristalsis moved the balloon up to papilla of Vater, the flow of bile and pancreatic juice might have been obstructed. These speculations were supported by the frequent PEG-tube traction into the stomach noticed by her family, and her repeated vomiting due to small bowel obstruction by the balloon. Roentgenography could easily show the dislocation of PEG-tube which might have been noticed by checking carefully the length of PEG-tube over the skin.

Five cases of acute pancreatitis related to gastrostomy tube migration have been reported[8-12]. Foley catheter has been used as PEG-tubes in 4 cases[8-10,12]. This catheter is more likely to migrate because it has no external bumper which prevents dislocation of the tube. In another case using a gastrostomy tube with an external bumper, spontaneous loosening of the external bumper caused the tube migration[11]. In this case, a technical error might have caused tube dislocation when a new tube was replaced blindly, although the tube has an external bumper.

This case demonstrates that a malpositioned PEG-tube can be an iatrogenic cause of acute pancreatitis and cholangitis. It is important to secure PEG-tube at the level of skin, especially a couple of days after its replacement.

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LETTERS TO THE EDITOR

Portal vein thrombosis: Etiology and clinical outcome of cirrhosis and malignancy-related non-cirrhotic, non-tumoral extrahepatic portal venous obstruction

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Abstract

The etiology and pathogenesis of portal vein thrombosis are unclear. Portal venous thrombosis presentation differs in cirrhotic and tumor-related versus non-cirrhotic and non-tumoral extrahepatic portal venous obstruction (EHPVO). Non-cirrhotic and non-tumoral EHPVO patients are young and present with well tolerated bleeding. Cirrhosis and tumor-related portal vein thrombosis patients are older and have a grim prognosis. Among the 118 patients with portal vein thrombosis, 15.3% had cirrhosis, 42.4% had liver malignancy (primary or metastatic), 6% had pancreatitis (acute or chronic), 5% had hypercoagulable state and 31.3% had idiopathy, 12% had hypercoagulable state in the EHPVO group.

Key words: Portal vein thrombosis; Cirrhosis; Malignancy; Extrahepatic portal venous obstruction

Jain P, Nijhawan S. Portal vein thrombosis: Etiology and clinical outcome of cirrhosis and malignancy-related non-cirrhotic, non-tumoral extrahepatic portal venous obstruction. *World J Gastroenterol* 2007; 13(39): 5288-5289

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TO THE EDITOR

We read with great interest the article "Portal hypertension due to portal venous thrombosis: Etiology, clinical outcomes" by Harmanci *et al*[1] in the May 14, 2007 issue of World Journal of Gastroenterology. We agree that portal vein thrombosis (PVT) should be considered under two different categories [acute or chronic PVT (noncirrhotic and nontumoral), (b) PVT due to cirrhosis and tumors], as the presentation, clinical course and prognosis are different in the two categories.

Table 1 Clinical and laboratory findings in 118 patients with portal vein thrombosis

Parameters	Extrahepatic portal vein obstruction (*n* = 50)	Cirrhosis and tumor-related portal vein thrombosis (*n* = 68)
Male:Female	33:17	51:17
Age (yr)[2]	25 (9-40)	52 (23-80)
Hematemesis	36 (72%)	10 (14.7%)
Hypersplenism	30 (60%)	6 (8.8%)
Pain abdomen	18 (36%)	42 (61.8%)
Abdominal distension	8 (16%)	36 (52.9%)
Awareness of splenomegaly	6 (20%)	-
Jaundice	5 (10%)	18 (26.5%)
Cholangitis	1 (2%)	5 (7.4%)
Splenomegaly[2] cm[1]	4 (2-14); (*n* = 35)	2 (2-4); (*n* = 7)
Hepatomegaly[2] cm[1]	2 (1-3); (*n* = 8)	3 (2-6); (*n* = 16)
Etiology		
Hepatocellular carcinoma	-	31
Cirrhosis liver	-	18
Metastases liver	-	6
Pancreatic carcinoma	-	7
Cholangio carcinoma	-	3
Carcinoma gallbladder	-	2
Duodenal carcinoid	-	1
Esophageal Varices	35 (70%)	32 (47%)
Gastric Varices	11 (22%)	3 (4.4%)
GoVI	1	2
GoV2	8	1
IGV1	2	
Portal hypertensive gastropathy	8 (16%)	48 (70.4%)
Mild	6	40
Severe	2	8
Hemoglobin[2] (gm/dL)	7.2 (1.7-14.7)	8.0 (6.0-11.6)
Total leucocyte count[2] (10^3/mm^3)	2.9 (1.0-6.8)	6.4 (1.36-29.71)
Platelet count[2] (10^5/mm^3)	0.49 (0.14-1.54)	1.52 (0.29-2.59)
Bilirubin[2] (mg/dL)	1.0 (0.4-18.5)	1.2 (0.5-15.1)
AST[2] (U/L)	38 (18-231)	79 (39-961)
ALT[2] (U/L)	34 (18-254)	65 (18-1146)
SAP[2] (U/L)	264 (110-2849)	348 (140-3140)
Protein/albumin[2] (mg/dL)	6.8 (5-7.5)/3.8 (3-4)	6 (5.3-8)/3.3 (2-4.6)
Prothrombin time prolongation[2] (seconds)	2 (1-3)	4 (2-14)
Portal biliopathy	4 (8%)	
Choledocholithiasis	2	
CBD stricture	2	
EVL sessions[2]	3 (2-4)	-
Glue injection (*n*)	4	-
Superior mesenteric vein thrombosis	11 (22%)	6 (8.8%)
Inferior vena cava thrombosis	4 (8%)	3 (4.4%)
Splenic vein thrombosis	4 (8%)	4 (5.6%)
Deep vein thrombosis	-	1 (1.4%)
HBsAg	0	27 (39.7%)
Anti-HCV	0	3 (4.4%)

[1]Centimeter below costal margin; [2]Median (range).

Of the 118 cases of PVT admitted to our hospital over the 2-year period (from January 1, 2005 to December 31, 2006), 50 were due to extrahepatic portal vein obstruction (EHPVO) and 68 were due to cirrhosis and tumors. The clinical and laboratory characteristics of 50 patients with EHPVO are given in Table 1.The patients were young and commonly presented with features of hematemesis, hypersplenism, abdominal pain and distension. Ten patients had acute PVT and 2 had presentation as acute Budd-Chiari syndrome, 2 patients had pregnancy and delivered the fetus at term with supportive treatment. Thirty-six patients who presented with hematemesis were managed with endoscopic variceal ligation (2-4 sessions), and four patients were treated with gastric varices glue injection. These patients were maintained on beta-blockers and follow-up endoscopic surveillance. Four patients with symptomatic portal biliopathy were managed with stent placement (n = 2) and common bile duct (CBD) stone extraction (n = 2). Antithrombin-Ⅲ deficiency was present in 2 patients, antiphospholipid antibody in 2 patients, factor V Leiden (FVL) mutation in 1 patient and paroxysmal nocturnal hemoglobinuria in 1 patient. All the 6 patients were started on heparin and warfarin with warfarin continued to maintain a 2-3 INR. Four (8%), 2 (4%) and 1 (2%) patients had chronic pancreatitis, acute pancreatitis and liver abscess, respectively. During the mean follow-up period of 9 mo (3-24 mo), none of the patients had symptomatic hypersplenism.

EHPVO affects young individuals who present with well-tolerated bleeding. The etiology and pathogenesis of PVT are unclear. It was initially proposed that umbilical sepsis or catheterization of umbilical veins in the neonatal period is responsible for PVT[2]. In recent years, the presence of congenital or acquired prothrombotic conditions has been considered an interesting hypothesis for the causation of PVT. FVL mutation is known to be less common among Asians as compared to the population of European descent. Koshy *et al*[3,4] reported that FVL mutation has been found in 3% patients with portal vein thrombosis and prothrombin G20210A gene in patients with portal vein thrombosis in a south Indian study. In our study, FVL mutation was present in 2% of cases.

The clinical and laboratory features of 68 patients in cirrhosis and tumor groups are given in Table 1. The clinical presentation was abdominal pain and distension and jaundice. During a mean follow-up period of 7 mo (range 1-24 mo), 48% of the patients died.

Of 118 cases of PVT admitted to our hospital, 15.3% had cirrhosis, 42.4% had liver malignancy (primary or metastatic), 6% had pancreatitis (acute or chronic), 5% had hypercoagulable state and 31.3% had idiopathy. The higher percentage in idiopathic group might be due to a low prevalence of hypercoagulable factors, abnormality and attribution of umbilical sepsis in childhood. The role of JAK2 mutation in early diagnosis of overt or silent myeloproliferative disease cannot be undermined but requires standardization[5].

In conclusion, the presentation, etiology and prognosis in non-cirrhotic and non-tumoral EHPVO patients are different from those in cirrhosis and tumor-related portal vein thrombosis patients.

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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