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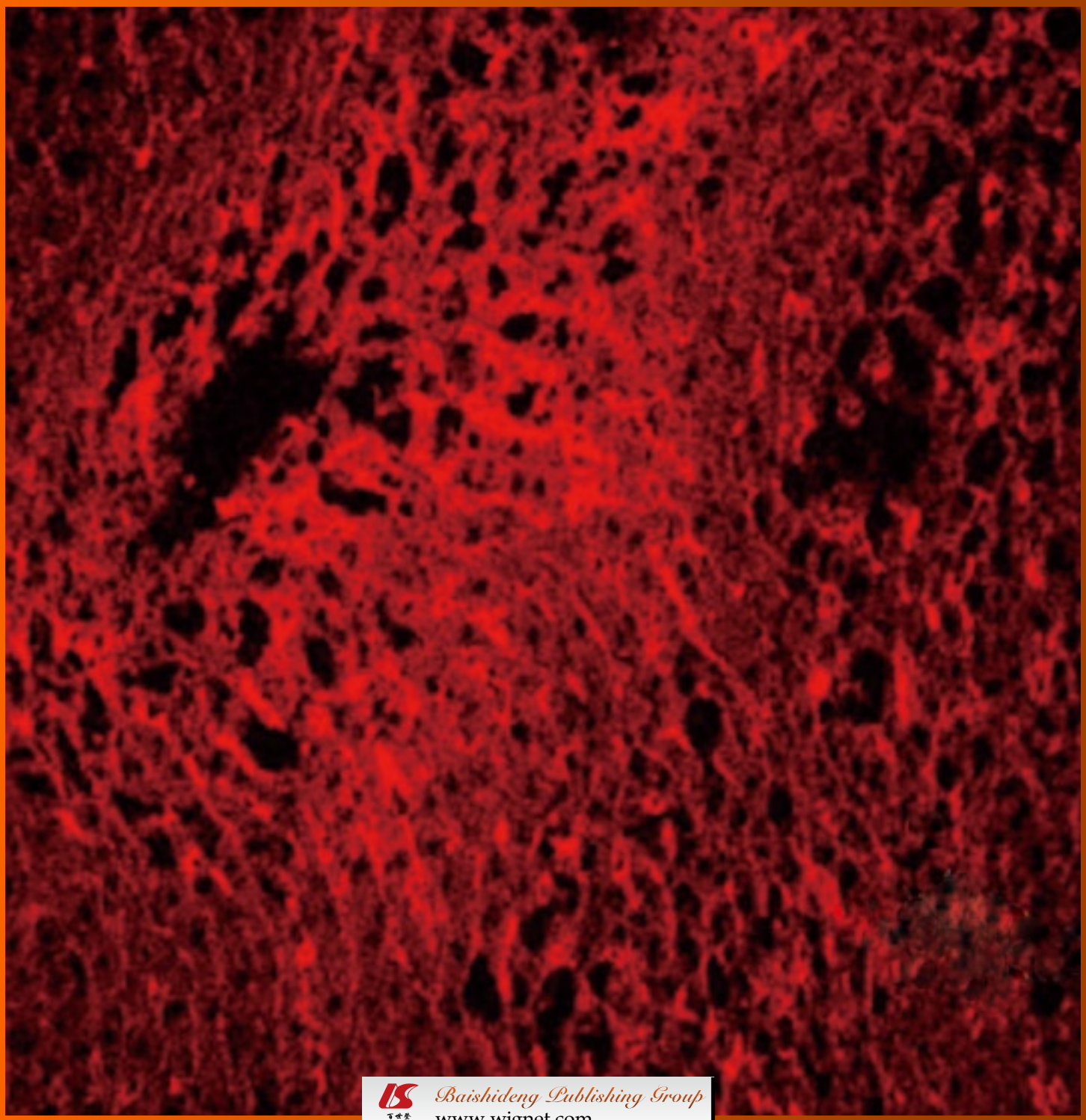

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EDITORIAL

Hepatitis B prevention and control: Lessons from the East and the West

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Abstract

Despite being ten times more common than HIV infection, viral hepatitis has so far not commanded the same public health response worldwide, so a global viral hepatitis treatment program is still a long way from becoming a reality. However, much progress has occurred over the last few decades, with the screening of blood products, sound infection control practices and the introduction of disposable needles and syringes leading to significant reductions in nosocomial hepatitis B transmission in the developed world and increasingly in other countries. The introduction of hepatitis B vaccination in the 1980s and its integration into the Expanded Immunization Program have led to substantial reductions in chronic hepatitis B infection rates in children and to millions of lives saved. The availability of effective antiviral treatment has revolutionized treatment prospects, although access to treatment remains a significant challenge for most developed countries and remains out of reach for developing nations. Some of these breakthroughs have occurred in Asian countries, others in the West, but their unifying features are innovative research, timely clinical translation and a commitment to apply their findings to improve the health of populations, not just individuals. This paper reviews some of the challenges and opportunities for hepatitis B control at the end of the first decade of the third millennium and argues for closer East - West collaborations, to bring in fresh perspectives, avoid duplications of effort and in order to help answer many of the remaining challenges in making hepatitis B history.

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Key words: Hepatitis B; Hepatocellular cancer; Hepatitis B surveillance; Vaccination; Screening

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INTRODUCTION

In May 2010, the 63rd World Health Assembly of the World Health Organization adopted a resolution calling for a comprehensive approach to the prevention and control of viral hepatitis, which kills over 1 million people every year. Viral hepatitis is 10 times more common than HIV infection[1], but despite the availability of effective treatments, a global viral hepatitis program providing access to treatment for the vast numbers of people with chronic hepatitis B (CHB) infection living in the developing world is far from becoming a reality[2].

Hepatitis B (HBV) infection is endemic in many South-East Asian countries, in some Pacific Islands, and in some African countries[1,3], with sero-prevalence rates in excess

of 8% in the general population leading to 25%-40% of those infected developing cirrhosis or hepatocellular cancer (HCC)[4]. Even in countries of intermediate HBV prevalence, such as in the Mediterranean basin and the Middle East, HBV poses significant challenges, due to the large numbers of affected individuals[5].

A comprehensive health response to hepatitis B has been slow to develop, probably because the complexity and variable natural history of the disease and its long asymptomatic phase do not command the same emergency response accorded to other infectious diseases. The link between CHB and liver cancer was described some 3 decades ago in Taiwan[6], and we owe much of our current understanding of hepatitis B epidemiology to scientists working in the East. They contributed key research findings on the impact of HBV vaccination on HCC incidence[7], the effect of antiviral therapy (lamivudine) in preventing progression to CHB-related cirrhosis[8] and in demonstrating the link between elevated viral loads and HCC development[9].

Until quite recently, two patterns of disease appeared to co-exist in different parts of the world: a chronic disease associated with significant death and disability in the East and a self-limiting, acute viral infection in the West. The introduction of the hepatitis B vaccine has led to substantial reductions of chronic hepatitis B infection in countries where infection is transmitted in early childhood, but overall, prevention strategies have failed to take into account the multifaceted epidemiology of the HBV infection[10]. An increased migration from high HBV prevalence countries to the industrialized countries in the West in the second half of the last century is linked to increased rates of HCC diagnoses among some migrant populations in the West. For example, Vietnamese American men are 11 times more likely to develop HCC than non-Hispanic Whites[1] and hepatitis-B related HCC has become the most important cancer health disparity affecting Asian Americans[1,11]. These statistics are increasingly being replicated in Australia[12], as well as in other Western countries[13-14].

In the West, HBV infection is usually acquired in adulthood, a long-term carrier state is uncommon and severe complications of chronic hepatitis-B infection remain rare in the general population. Consequently, the public health response to hepatitis B has focused mostly on the prevention and management of acute hepatitis B outbreaks. Hepatitis B surveillance systems exist in most Western countries[15-18], but approximately 50% of acute cases are missed by the infectious disease reporting systems[19], downgrading the importance of hepatitis B as a priority disease in the eyes of policy makers and program planners[2]. In part, this is due to the absence or non-specific nature of symptoms associated with acute hepatitis and compounded by the fact that HBV infection is more common in marginalized populations, with limited contact with health care providers [such as prisoners, men who have sex with men (MSM), intravenous drug users (IVDU) and some migrant populations]. As early as 1991, Margolis emphasized that eliminating HBV transmission was only possible by preventing infections acquired during early childhood, as well as those acquired by teenagers and adults[10]. Change is on the horizon though, with the Institute of Medicine recently publishing recommendations for improving the prevention and control of hepatitis B and C infections, in response to substantial increases in the number of cases of hepatitis B and C in the United States[2]. This comprehensive report is focused on the US situation and systematically examines the progress made in disease surveillance, immunization, knowledge and awareness of viral hepatitis among health care providers and communities, as well as the degree of access to hepatitis services. The Chair of this working group was Dr Palmer Beasley, whose work in Taiwan improved our understanding of disease transmission patterns, elucidated links to HCC and established the effectiveness of HBV vaccination[6,20-23]. One can only speculate how effectively a closer East-West collaboration led by someone who worked closely both in the East and the West may accelerate progress in hepatitis B prevention and control, from the current position where we are running a "a race against time"[24]. In this race, some success stories have already changed practice both in the East and the West, while in many areas, a collective approach uniting East and West may help solve some of the as yet unanswered questions on hepatitis B and its control.

PRIMARY PREVENTION

Significant successes have been recorded both in the East and the West in delivering vaccination programs, although immunization rates in rural, remote and marginalized populations lag behind those of urban populations. Within 10 years of launching the nationwide HBV vaccination program, the HBsAg carrier rate of Taiwanese children decreased 10-fold, from approximately 10% to 1%. This was accompanied by a four-fold reduction in HCC incidence rate in 6-9 year olds, demonstrating for the first time that a mass vaccination program can reduce cancer incidence in humans[7]. Similar trends have been observed in China, where the chronic HBV carrier rate in children fell from 10% in the year universal infant vaccination commenced (1992) to 1%-2% in 2006, thus preventing approximately 30 million new CHB infections[25].

In the US, acute hepatitis B incidence fell by 80% from 1987 to 2004[26], yet approximately 1000 infants (mostly children not born in medical settings- many of them Asian American) still become infected annually as a result of vertical transmission[1-2]. Reaching some of these populations remains a challenge both in the East and the West, as demonstrated by the fact that two consecutive US national surveys did not identify any reductions in hepatitis B prevalence[6], assumed to be related to an increased migration from countries where the disease is endemic[27].

The WHO-led Expanded Immunization Program (EPI) has made great strides in increasing access to the hepatitis B vaccine in the developing world, with 177 countries having nationwide hepatitis B vaccination programs in 2008. While in 1990 only 1% of infants worldwide received all 3 doses of the hepatitis B vaccine, by 2008 this figure had increased to 69%. Regional variations persist, although the proportion of fully immunized infants in South East Asia has been increasing rapidly, from 29% in 2007 to 41% in 2008[28]. Gaps in coverage persist in some countries, particularly in rural areas , with fewer than 21% of newborns in Laos are receiving their first dose of vaccine on time[29].

Substantial challenges remain, as highlighted by a global survey carried out by the World Hepatitis Alliance in conjunction with WHO: 80% of the 135 countries participating in the survey lacked resources to carry out viral hepatitis control, one third lacked any viral hepatitis prevalence data and free hepatitis testing was not available to half their populations[29]. While developed countries have been extremely successful in reducing disease transmission through their blood supply and reducing nosocomial transmission, significant resource limitations and persisting stigma constrain these efforts in many resource-limited settings[29-30].

CHALLENGES IN HEPATITIS B SCREENING AND SURVEILLANCE

Obtaining accurate estimates of the prevalence of HBV infection remains a significant challenge to epidemiological research not only in the US[31], but also in other countries. National population-based surveys can provide reliable estimates of HBV sero-prevalence, but their complexity and cost means that only a few countries such as the US and South Korea have been able to implement such programs[6,32] although, even there, survey coverage remains low in at risk populations. The advent of rapid testing technologies (recently utilised by WHO in Cambodia to evaluate the impact of HBV vaccination)[33] can improve HBV detection rates in hard-to-reach populations both in the East and West, and may provide critical data for program planning[2].

While most European countries (as well as North America and Australia) have in place surveillance systems for hepatitis B and C infection, data are not readily comparable across countries, due to differences in surveillance systems, reporting practices and data collection, as well as to different case definitions[17]. Difficulties arise in the classification of "acute" cases, due to the complex testing required for establishing a hepatitis B diagnosis and the limitations of the current testing systems, where test results are not reliably communicated to surveillance staff. Adopting pragmatic laboratory case definitions for hepatitis B and harmonizing case definitions from Canada, the UK and the US (as done in a pilot program in Manitoba, Canada) could allow more meaningful comparisons between countries and prevention strategies[34].

Although the predictive value of the "traditional" case definitions is low, it too can be improved, using additional criteria, such as specific criteria for ALT and total bilirubin levels. Applying these to the CDC acute hepatitis B cases increased the positive predictive value from 50% to over 95%[19].

In western countries, more or less systematic screening for hepatitis B has been carried out in their indigenous populations, such as in Alaskan natives in the US[35], the Canadian First nation people[36], Australian Aboriginal populations[37] and among Maori populations in New Zealand[18]. Since 1999, New Zealand has had a national screening and follow-up program for hepatitis B targeted at Maori, Pacific and Asian residents, developed in response to the high morbidity and mortality and the significant economic impact of untreated HBV infection in these subpopulations. The program has screened over 170 000 people and is providing ongoing CHB surveillance to more than 12 000 people[18,38].

Since 1996, Taiwan has been offering free hepatitis screening through outreach community-based screening programs, screening over 160 000 people over a 10-year period. Results suggest that a heavy burden of disease related to hepatitis B is to be expected in years to come, as 17% of people born before the vaccination program was instituted were CHB-positive[39].

Recently, demonstration projects have successfully targeted special migrant groups in the West, by linking screening with the provision of free or subsidized vaccination for those identified as susceptible. The programs generally target closely knit populations, defined by religious or other affiliations (e.g. users of ethnic media) and provide targeted information and education to the intended audience[38,40-42]. To be successful, the programs need to be accompanied by a shift in current perceptions of screening from that of a once-off test to to that of a way of entering into a program of regular follow up and timely institution of treatment.

San Francisco is aiming to become the first HBV-free city, with the Hep B Free Campaign providing screening, vaccination and treatment to all Asian and Pacific Islander residents (representing 30% of its population)[1]. To improve disease surveillance, the city has also established a population-based chronic hepatitis B registry, which carries out CHB enhanced surveillance and interviews cases, improving the understanding of transmission patterns and participants' ability to access hepatitis care[43]. However, such programs are very resource- intensive[15], making them unaffordable in resource-limited settings and problematic even for well-resourced countries with a huge infected population, such as China[44].

Devising cost-effectiveness screening and treatment strategies is critical to program success and recent studies have confirmed that routine screening can be cost-effective in Asians and Pacific Islanders in the US[45] and in at-risk migrant populations in the Netherlands[46]. Providing antiviral treatment to people with CHB can be more

Table 1 Challenges and solutions in the public health response to hepatitis B

Challenges	Possible solutions
Lower HBV immunization rates in remote/ marginalized populations	Develop effective outreach vaccination programs Community education
Unresponsive HBV surveillance systems	Develop uniform, pragmatic case definitions for acute HBV infection Redesign surveillance systems
Low rates of HBV screening	Reduce stigma and discrimination Increased awareness and education about HBV Replace once-off screening with systematic follow up
Cost of screening and treatment programs	Develop more cost-effective programs
Access to treatment	Increase treatment access and reduce cost of drugs Seek innovative ways of providing treatment access and know-how for less developed countries

HBV: hepatitis B virus.

cost- effective than liver cancer screening in migrant populations in Australia[47], but more work is needed to develop low-cost screening and treatment programs, which can address the needs of the developing world.

The relative merits of alternative surveillance options, such as carrying out enhanced surveillance intermittently[16] or focusing efforts on specific populations, are worthy of exploring. As early as 1994, Ruth Berkelman recommended a reevaluation of US infectious disease surveillance practices, to ensure they remain responsive to the challenges imposed by a changing disease landscape[48]. This remains as relevant today as it was 16 years ago.

Program design needs to avoid stigma and discrimination associated with testing in specific populations, particularly in certain ethnic groups. Because mandatory hepatitis B testing in mainland China has in the past led to the exclusion of HBsAg positive people from employment and study, Chinese immigrants may have serious misgivings about being tested, out of concern that this could lead to stigmatization and discrimination in their adopted country. A substantial body of research has documented migrant communities' knowledge of hepatitis B and attitudes to screening, particularly in North America[49-57], and this can inform the development of culturally sensitive screening and treatment programs, which take into account the ethnic, racial and socioeconomic disparities associated with chronic hepatitis B infection.

Unfortunately, the low levels of awareness and knowledge about hepatitis B among at-risk populations are also mirrored in low levels of knowledge among health care providers[58-61]. This is a contributing factor to low rates of testing and treatment among at-risk populations and leads to limited clinical support for the allocation of resources for hepatitis B prevention, control and surveillance efforts, even in well-resourced settings (Table 1)[62].

ACCESS TO SERVICES

The differential rates of hepatitis B–related liver cancer between Asian and Pacific Americans and the general US population represents the single most important cancer-related inequality in the US[1,62]. Canadian estimates suggest that 1%-2% of their Southeast Asian migrants have CHB and would benefit from antiviral therapy[63], but that few in this population are aware of their CHB status and of the changing treatment paradigms in hepatitis B. In Australia antiviral treatment can be accessed free of charge, yet only approximately 2% of people with CHB are receiving treatment[64]. These problems are greatly magnified in resource-limited settings by the high cost and long duration of antiviral treatments. Furthermore, identifying the people who stand to benefit the most from antiviral treatments remains problematic both in the West and the East.

WAY FORWARD

In well-resourced settings, a hepatitis B Registry model may improve the quality of CHB surveillance and provide direct links to health services, education, contact tracing and better outcomes[43]. The next step in well-resourced countries may be to integrate CHB into a system of chronic disease surveillance, as piloted in Taiwan. The program uses a specially designed health information system, collecting data on five cancers (cervical, breast, colorectal, cancer of the oral cavity and liver cancer) and three chronic conditions (hypertension, hyperlipidemia and type 2 diabetes)[65]. By the end of 2003, 60 000 participants (representing almost 30% of the target population) were enrolled in the program, demonstrating that an integrated management model of cancer and chronic disease is feasible.

To follow on from Ruth Berkelman's recommendations, the "new look surveillance" in the 21st century may combine core surveillance with targeted surveillance of specific population groups. It may involve sentinel networks[48], linking health care providers and laboratories to a central data collection and processing center by using primary care networks, as is currently done for influenza surveillance. The information provided by this system could be augmented by enhanced surveillance in at-risk populations, such as migrants, indigenous people, MSM, prisoners, or institutionalizedgroups. The creation of international networks to monitor and investigate HBV infection could increase the effectiveness of public health interventions and promote collaborative research. Survei-

Table 2 Ways to improve outcomes in hepatitis B

Ways forward
Develop Registry model of hepatitis B surveillance, linked to health service delivery and education
Integrate CHB surveillance with surveillance for other chronic diseases
Develop responsive HBV surveillance systems
Create international networks for hepatitis B monitoring
Integrate surveillance and clinical data
Develop collaborations to enhance sharing of information

CHB: chronic hepatitis B; HBV: hepatitis B virus.

llance data coupled with clinical information obtained from publicly available databases recording hospital discharge as well as morbidity and mortality, could provide a comprehensive picture of the burden of disease related to HBV infection in different populations. Sharing economic models across different countries could assist in devising cost-effective health care delivery models, commensurate with the level of available resources.

International collaborations, such as that currently trialed by the National Institute of Diabetes and Digestive and Kidney Diseases which is implementing an action plan for liver disease research across the National Institute of Health, could lead to a more efficient utilization of resources and information sharing and to the speeding up of progress (Table 2)[31].

Increasing East - West collaboration can provide new perspectives, avoid the duplication of effort and help answer the many remaining challenges posed by this protean disease. Over the last decade, WHO has supported the development of comprehensive cancer control programs in many countries, based upon four pillars: cancer prevention, early detection, appropriate treatment and relevant research. These four principles apply equally well to hepatitis B prevention and control and to this list we can add a fifth pillar: international collaboration. They are all essential in the process of "making hepatitis B history".

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DREAM 2020

Liver cancer: Targeted future options

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Abstract

Hepatocellular carcinoma (HCC) has a poor prognosis and systemic chemotherapies have disappointing results. The increasing knowledge of the molecular biology of HCC has resulted in novel targets, with the vascular endothelial growth factor and epidermal growth factor receptor (EGFR)-related pathways being of special interest. New blood vessel formation (angiogenesis) is essential for the growth of solid tumors. Anti-angiogenic strategies have become an important therapeutic modality for solid tumors. Several agents targeting angiogenesis-related pathways have entered clinical trials or have been already approved for the treatment of solid tumors. These include monoclonal antibodies, receptor tyrosine kinase inhibitors and immunomodulatory drugs. HCC is a highly vascular tumor, and angiogenesis is believed to play an important role in its development and progression. This review summarizes recent advances in the basic understanding of the role of angiogenesis in HCC as well as clinical trials with novel therapeutic approaches targeting angiogenesis and EGFR-related pathways.

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Key words: Angiogenesis; Epidermal growth factor receptor; Hepatocellular carcinoma; Targeted therapy; Vascular endothelial growth factor

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and is the seventh most frequent cause of cancer related death in Europe[1]. It is the fifth most common cancer in men and eighth most common cancer in women worldwide, resulting in at least 500 000 deaths per year[2]. HCC accounts for 90% of all liver cancers. Its crude incidence in the European Union is 8.29/100 000. Areas such as Asia and sub-Saharan Africa with high rates of infectious hepatitis have incidences as high as 120 cases per 100 000. HCC is four to eight times more common in men and usually associated with chronic liver injury such as hepatitis B HBV, hepatitis C HCV and alcoholic cirrhosis. Most HCCs arise from chronic liver disease and cirrhosis, caused mainly by viral infections, fatty liver disease or alcohol induced cirrhosis[3].

The management of HCC patients is multidisciplinary and treatment is influenced by the stage of the disease, by the liver function (underlying liver cirrhosis) and by the patient's performance status. Potential curative therapy options such as liver transplantation, liver resection and

local liver ablation are only considered for patients with early stage HCC and with preserved liver function[4]. Most HCC patients are at an intermediate or late disease stage and the therapeutic options are limited to transarterial chemoembolization (TACE) or systemic chemotherapy. However, many patients are not suitable for TACE and the efficacy of conventional systemic cytotoxic chemotherapy is modest with limited benefit. Although a few randomized trials have been conducted, no single cytotoxic regimen has emerged as superior to any other, and no drug or regimen has been shown to improve survival. Therefore new therapeutic options targeting specific pathways and new drugs are of urgently needed. New insights into the biology of hepatocarcinogenesis have been identified new therapeutic approaches like including antiangiogenesis or and inhibition of specific growth factors like such as the Epidermal growth factor receptor (EGFR) or the Insulin like growth factor receptor 1 (IGF1R)[5].

New blood vessel formation (angiogenesis) is fundamental to tumor growth and spread. In adults, physiological angiogenesis is limited to a small number of specific processes, such as wound healing, tissue repair and the female reproductive cycle[6]. Following the pioneering work of Judah Folkman it was recognized that angiogenesis plays an important role in tumor development, progression, and metastasis[7]. Tumors require nutrients and oxygen in order to grow, and new blood vessels, formed by the process of angiogenesis, provide these substrates. Tumor blood vessels are generated by various mechanisms, such as co-option of the existing vascular network, expansion of the host vascular network by budding of endothelial sprouts (sprouting angiogenesis), remodeling and expansion of vessels by the insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusceptive angiogenesis) and homing of endothelial cell precursors (EPC; CEP) from the bone marrow or peripheral blood to the endothelial lining of neovessels (vasculogenesis)[8]. Bone marrow derived progenitor cells contribute significantly to neovascularization in a variety of tumors[9-12].

The key mediator of angiogenesis is the vascular endothelial growth factor (VEGF). Therefore, VEGF and its receptors are interesting targets for anticancer therapies. VEGF signaling inhibition has been shown to result in significant tumor growth delay in a wide range of animal models[13]. Even a single VEGF allele knock-out has been shown to lead to embryonic lethality in mice[14]. The clinical benefit of this approach has also been confirmed and concentrated efforts in recent years have resulted in a number of novel anti-angiogenic agents. The humanized monoclonal anti-VEGF antibody bevacizumab is the first VEGF-targeting drug, which is officially approved as first-line therapy in patients with metastatic colorectal cancer[15].

Tight control of angiogenesis is maintained by a balance of endogenous anti-angiogenic and proangiogenic factors. VEGF has a key, rate-limiting role in promoting tumor angiogenesis and exerts its effects by binding to one of three tyrosine kinase receptors: VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 [ligands include VEGF-A, VEGF-B and placental growth factor (PIGF)] and VEGFR-2 (ligands include VEGF-A, VEGF-C and VEGF-D) are predominantly expressed on vascular endothelial cells, and activation of VEGFR-2 appears to be both, necessary and sufficient, to mediate VEGF-dependent angiogenesis and induction of vascular permeability[16]. VEGF-A binds to VEGFR-1 and VEGFR-2, whereas VEGF-B and PlGF only bind to VEGFR-1. Both receptor tyrosine kinases are expressed in all adult endothelial cells except for endothelial cells in the brain. VEGFR-1 is also expressed on hematopoietic stem cells (HSC), vascular smooth muscle cells, monocytes, and leukemic cells[17]. VEGFR-2 is also expressed on endothelial progenitor cells and megakaryocytes[18,19]. Although the exact contribution of VEGFR-1 signaling to angiogenesis is unclear, it has been shown to co-operate directly with VEGFR-2 *via* heterodimerization, as well as to bind two additional VEGF homologues, VEGF-B and PlGF[20]. VEGFR-3, which is largely restricted to lymphatic endothelial cells, binds the VEGF homologues VEGF-C and VEGF-D and may play an important role in the regulation of lymphangiogenesis.

HCC is one of the most vascular solid tumors and is characterized by an abnormal vascular structure. Therefore, proangiogenic factors such as VEGF and plateletderived growth factor (PDGF) are of major importance and are upregulated in hepatocarcinogenesis. Sorafenib, a multikinase inhibitor blocking VEGFR signaling was the first targeted agent showing an overall survival benefit in HCC patients[21] and provided a breakthrough in modern HCC therapy.

New insides into the pathological and molecular mechanisms of HCC have led to the development of numerous targeting agents. This review summarizes a selection of these new drugs.

ANTI-ANGIOGENIC THERAPIES

As already mentioned above, HCC is a highly vascular tumor and so antiangiogenic therapies are of major interest[22-23]. In a variety of solid tumors, anti-angiogenic therapies like bevacizumab or sunitinib have already proven clinical efficacy. Most of these compounds can be broadly classified into two main categories: small-molecule kinase inhibitors and monoclonal antibodies.

Sorafenib

Sorafenib is an oral tyrosine kinase inhibitor (RTKI) blocking several receptors including VEGFR1-3, PDGFR β, c-KIT and FLT-3. Having shown anti HCC activity in several preclinical models Sorafenib[24-26], progressed to clinical studies. The results of four phase I studies were summarized in a review describing the tolerability and the pharmacokinetics of sorafenib in pre-treated patients[27]. Sorafenib was well tolerated and the maximal tolerated dose (MTD) was 400 mg twice daily. The most common adverse events were fatigue, diarrhea, rash and hand-foot

skin reaction. Based on these results, phase Ⅱ and Ⅲ studies with 400 mg twice a day were started. The phase Ⅱ trials confirmed the antitumor efficacy and tolerability of the drug[28].

In two large randomized, multicenter, controlled clinical phase Ⅲ trials sorafenib given as first systemic agent showed an overall survival benefit for patients with unresectable HCC[21]. In the sorafenib HCC assessment randomized protocol (SHARP) patients not eligible for locoregional therapy were randomly assigned to sorafenib 400 mg twice daily or placebo. The study included 602 primarily European patients (sorafenib n = 299, placebo n = 303) and inclusion criteria were ECOG performance status ≤ 2, Child Pugh liver function class A as well as no prior systemic therapy. The results of the study showed a significant prolongation of the time to progression (TTP) from 2.8 to 5.5 mo [HR = 0.58, 95% confidence interval (CI) = 0.45 - 0.74, P < 0.0001] and an improvement of survival from 7.9 to 10.7 mo (HR = 0.69, 95% CI = 0.55 - 0.87, P < 0.0001) in the sorafenib treatment arm.

A similar study was performed in mainly Asian patients where 271 patients were allocated to sorafenib or placebo[29], randomized in a 2:1 ratio. The outcome of this study showed a median overall survival of 6.5 mo with sorafenib treatment compared to 4.2 mo in the control group. The discordance in the OS benefit between the SHARP and Asian could be related to a divergence in selection of patients. In the Asian population unfavorable prognostic factors including the rate of Hepatitis B virus infections, the stage of disease (Asian population showed more level C Barcelona clinic liver criteria), age (Asians were younger) and performance status (Asian included more ECOG 2) were more often observed. The adverse event profile of the two large phase Ⅲ trails was similar, with hand-foot syndrome (8% Europe, 11% Asian), fatigue (8%-10%), and diarrhea (9%) the most common.

Sunitinib

Sunitinib is an oral RTKI, targeting the VEGFR1-3, the platelet derived growth factor receptor PDGFR α and β and the stem cell factor receptor (KIT). In several preclinical studies sunitinib showed anti HCC activity[30]. To date, Sunitinib is approved for the treatment of advanced renal cell carcinoma (RCC) and gastrointestinal stroma tumors (GIST) after disease progression or intolerance to imatinib mesylate[31]. In RCC and GIST sunitinib is administered at a dose of 50 mg/d for 4 wk followed by 2 wk of no treatment. The optimal treatment dose assessed in preclinical studies was used in phase Ⅰ studies and was well tolerated. Described adverse events of fatigue, hypertension and skin toxicity with sunitinib are typical for VEGFR tyrosine kinase inhibitors[32].

Suntinib was evaluated in HCC in two phase Ⅱ trials using different doses of the drug. Zhu *et al* analysed 34 patients and reported a 2.9% response rate, a median progression free survival (PFS) of 3.9 mo, and a median overall survival (OS) of 9.8 mo. The administered dose of sunitinib was 37.5 mg daily for 4 wk (d1-d28) at time intervals of 6 wk[33]. In another study by Faivre *et al*[34] where 37 patients were included, 50 mg sunitinib was given daily for 4 wk followed by two weeks off-treatment in 6 wk cycles. The authors reported similar results with a response rate of 2.7%, PFS of 5.2 mo and an OS of 11.2 mo. A higher dose of suntinib used in the study by Zhu revealed a high toxicity rate and 10% of deaths were treatment related. Therefore the authors concluded that 50 mg/d sunitinib is not appropriate and that the dose should be reduced to 37.5 mg without the 2 wk wash-out phase[32].

A direct comparison of sunitinib and sorafenib in a randomized phase Ⅲ study in advanced HCC was discontinued in April 2010 after the first review by an independent data monitoring committee. The study was terminated based on higher incidence rates of serious adverse events in the sunitinib treatment arm compared to the sorafenib arm and because the preliminary data did not meet the primary study endpoints (sunitinib did not improve survival compared to sorafenib) (Pfizer press release April 22, 2010).

Cediranib

Cediranib (AZD2171, Recentin®) is a potent inhibitor of both VEGFR-1 and VEGFR-2. It also has activity against c-kit, PDGFR-β, and FLT4 at nanomolar concentrations[35]. Cediranib has been shown to inhibit VEGF signaling. In our study, cediranib was well tolerated up to 45 mg/d in patients with a broad range of solid tumors[36]. The most common toxicities include diarrhea, dysphonia, and hypertension. In a phase Ⅱ study with cediranib in 28 patients with advanced HCC, 19 patients were evaluable for toxicity[37]. The main adverse events were fatigue, hypertension and anorexia.

Vatalanib

Vatalanib (formerly PTK787/ZK 222584) is an oral angiogenesis inhibitor that is active against VEGFR and PDGFR tyrosine kinases, thereby offering a novel approach to inhibiting tumor growth[38]. This drug interferes with the ATP binding sites of VEGF receptors. In a phase Ⅰ study by us, vatalanib was well tolerated and showed clinical activity in a variety of solid tumors[39]. Preclinical studies suggested anti-angiogenic and angiogenesis-independent effects on HCC growth arrest[40]. In a phase Ⅰ study of vatalanib in 18 patients with unresectable HCC, nine patients had a best response of stable disease (SD), and nine patients had progressive disease (PD)[41].

Bevacizumab

Bevacizumab (Avastin®) is a humanized monoclonal antibody IgG1. It was created from a murine anti-human VEGF monoclonal antibody that blocks the binding of human VEGF to its receptors, thereby disrupting autocrine and paracrine survival mechanisms mediated by VEGFR-1 and VEGFR-2[42]. Bevacizumab is the first VEGF targeting drug, which is officially approved for cancer therapy. Initially, Bevacizumab demonstrated survival benefits in patients with metastatic colon cancer when combined with conventional chemotherapy[15].

Since then, it has been tested in several other cancer types. In patients with HCC, bevacizumab was examined as monotherapy or in combination therapies. In a phase Ⅱ study, monotherapy with bevacizumab was examined in 46 patients with advanced HCC[43]. Six patients had objective responses (13%) and 65% were progressive after 6 mo. Median progression-free survival was 6.9 mo and overall survival rate was 53% at 1 year. The main adverse events were hypertension, thrombosis. Bevacizumab was associated with significant reductions in tumor enhancement by dynamic contrast-enhanced magnetic resonance imaging and reductions in circulating VEGF-A and stromal-derived factor-1 levels. In a phase Ⅱ study, bevacizumab was studied in combination with gemcitabine and oxaliplatin in patients with advanced HCC[44]. The overall response rate was 20% in evaluable patients. An additional 27% of patients had SD with a median duration of 9 mo. The median overall survival was 9.6 mo and the median progression-free survival was 5.3 mo. Main bevacizumab-related side effects were hypertension, bleeding, and proteinuria.

TARGETING THE EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY

The epidermal growth factor receptor is upregulated in HCC and plays an important role in tumor progression[45,46]. The ligands of the EGFR EGF and TGF-α have been identified as key stimuli for HCC cell proliferation. The inhibition of EGFR signalling can either be by extracellular neutralizing antibodies such as cetuximab and panitumumab or by receptor tyrosine kinase inhibitors such as gefitinib, erlotinib and lapatinib. To date these targeted agents are being assessed in clinical trials.

Gefitinib

Gefitinib is an oral EGFR tyrosine kinase inhibitor approved for the treatment of non small cell lung cancer patients having an activating mutation in the EGFR gene[47]. The beneficial effect of Gefitinib is well analysed in many solid tumors including lung cancer, colorectal cancer, breast cancer. However, only a few studies have evaluated the effect of Gefitinib in HCC. The only reported study is a phase Ⅱ study with 31 advanced HCC patients presented at the 2006 ASCO conference in which Gefitinib induced 3% of objective responses and 22.6% of stable disease. Median PFS and OS were 2.8 and 6.8 mo, respectively[48]. The final outcome of this study is not yet published in MedLine. At the 2010 ASCO conference, a pilot study, analyzing the feasibility of gefitinib in adjuvant treatment of HCC patients was presented[49]. The study protocol includes a large biomarker program to identify prognostic as well as predictive markers and first results are awaited.

Erlotinib

Erlotinib is another oral EGFR RTKI that has showed clinical efficacy in the therapy of HCC. In two phase Ⅱ studies reported by Philip *et al*[50] and by Thomas *et al*[51] erlotinib showed antitumor activity and a PFS of 3.2/3.1 mo respectively and an OS 13/10.75 mo respectively. These studies included 38/40 patients respectively, with advanced nonresectable HCC. The side effects reported in these studies of erlotinib were rash, diarrhea or other skin events (acne, dry skin, pruritus).

In a recently published phase Ⅱ study in 40 HCC patients, a combination therapy of erlotinib with bevacizumab was assessed[52]: The rationale of this combination is based on preclinical models where a dual inhibition of VEGFR and EGFR showed additive effects. The combination therapy showed antitumor activity and the 16 wk PFS was 62.5% (primary end point), with 10 patients achieving a partial response, giving a confirmed overall response rate of 25%. The median PFS was 39 wk (95% CI, 26 to 45 wk; 9.0 mo), and the median overall survival was 68 wk (95% CI, 48 to 78 wk; 15.65 mo). Compared these results with the sorafenib studies (phase Ⅱ and SHARP trials) the authors identified more favourable results from combination therapy compared to monotherapy.

These retrospective comparisons are of minor scientific relevance and should be tested in prospective studies. Several new studies comparing combination therapies of EGFR and VEGFR TKIs with the standard VEGFR TKI sorafenib (bevacizumab + erlotinib versus sorafenib; erlotinib and sorafenib versus sorafenib) will identify the most effective treatment with the best tolerability.

Further anti-EGFR-based approaches include cetuximab, a chimeric monoclonal antibody against EGFR, and lapatinib, a selective dual inhibitor of both EGFR and ErbB2 tyrosine kinases. Both agents are currently being evaluated in clinical trials for patients with HCC.

EVALUATION OF BIOMARKERS

A major focus of research on targeted therapies should be the definition of predictive biomarkers which will allow identification of potential responders. To date there is no direct evidence on which HCC patients respond to targeted therapies. Various possible biomarkers have been postulated, but adequate valuation in prospective studies is still lacking. Possible candidates that could be considered are clinical parameters like blood pressure increase, various proteins assessed by biochemical methods [e.g. phosphorylated extracellular signal regulated kinase (pERK)] or levels of circulating endothelial cells and progenitor cells (CEC, CEPs). In addition, angiogenic factors and new imaging strategies as DCE-MRI[53] are currently being evaluated as possible biomarkers.

About Alfa *et al*[28] showed that standard RECIST criteria for the evaluation of response to sorafenib therapy in HCC patients are not ideal, because sorafenib-treated tumors do not decrease in size, although the necrotic index increases. In the same study, better responses to sorafenib were observed in patients with high mitogen

activated protein kinase (MAPK) activity and resulted in a prolonged time to progression. An increased activity of MAPK was defined by elevated tumor cell pERK immunohistochemical staining intensity (2 - 4 +) compared to normal (0 - 1 +) at baseline assessment before treatment. Greater activation of the Ras signalling pathway could be due to loss of sprouty and spreads[54,55]. Sprouty and spreads downregulation may reduce the threshold for cells to acquire malignant features. Lung cancer patients with upregulated Ras activity (battle trial) also show good response rates to sirafenib.

Hypertension is a common side effect of antiangiogenic therapies. In renal cell cancer, studies identified a correlation between blood pressure elevation and better overall survival benefit on axitinib therapy[56]. Kim *et al*[57] assessing the predictive effect of hypertension in patients taking sorafenib for advanced HCC, identified elevated blood pressure levels as a positive predictive marker for sorafenib therapy.

A recently published study proposed early alpha- fetoprotein (AFP) as a predictive marker for therapeutic effects in HCC patients treated with antiangiogenic therapies[58].

One of the largest biomarker programs integrated in a phase Ⅱ trial by Zhu *et al*[33] included the assessment of angiogenic serum markers, CEPs and CECs, DCE- MRI and immunhistochemical analyses of tumor samples. In HCC patients, the authors found that rapid changes in vascular permeability and in circulating inflammatory biomarkers reflected response or resistance to sunitinib therapy. Thus, these candidate biomarkers should continue to be actively explored in trials of antiangiogenic agents in patients, with the goal of improving and individualizing cancer therapy[53].

CONCLUSION

Molecular targets are of relevance in the treatment of HCC. Angiogenesis is upregulated in HCC and provides a target for novel agents. Therefore, VEGF and its receptors comprise the most important pathway in regulating neoangiogenesis, vasculogenesis and recruitment of endothelial progenitor cells. Further, VEGF stimulates proliferation, migration and survival of HCC cells directly and is of prognostic relevance. Further targets such as EGFR provide the possibility of combination therapies in order to enhance treatment efficacy. A number of clinical trials are underway to examine these novel agents in the hope of improving treatment modalities in advanced HCC.

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ORIGINAL ARTICLE

Cellular fibronectin stimulates hepatocytes to produce factors that promote alcohol-induced liver injury

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Abstract

AIM: To examine the consequences of cellular fibronectin (cFn) accumulation during alcohol-induced injury, and investigate whether increased cFn could have an effect on hepatocytes (HCs) by producing factors that could contribute to alcohol-induced liver injury.

METHODS: HCs were isolated from rats fed a control or ethanol liquid diet for four to six weeks. Exogenous cFn (up to 7.5 μg/mL) was added to cells cultured for 20 h, and viability (lactate dehydrogenase), apoptosis (caspase activity) and secretion of proinflammatory cytokines (tumor necrosis factor alpha, TNF-α and interleukin 6, IL-6), matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) was determined. Degradation of iodinated cFn was determined over a 3 h time period in the preparations.

RESULTS: cFn degradation is impaired in HCs isolated from ethanol-fed animals, leading to its accumulation in the matrix. Addition of exogenous cFn did not affect viability of HCs from control or ethanol-fed animals, and apoptosis was affected only at the higher concentration. Secretion of MMPs, TIMPs, TNF-α and IL-6, however, was increased by exogenously added cFn, with HCs from ethanol-fed animals showing increased susceptibility compared to the controls.

CONCLUSION: These results suggest that the elevated amounts of cFn observed in alcoholic liver injury can stimulate hepatocytes to produce factors which promote further tissue damage.

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Key words: Alcoholic liver diseases; Hepatocytes; Fibronectin; Asialoglycoprotein receptor; Inflammation; Fibrosis

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INTRODUCTION

Chronic alcohol consumption is a major health problem in the United States, and a leading cause of end-stage liver disease. Though much evidence exists which suggests that ethanol itself and/or its metabolites directly induce the cascade of pathophysiological responses from the cells of the liver that contribute to the development of steatosis, inflammation and fibrosis, the precise mechanism(s) behind these events has not yet been completely described[1-3]. To this end, our laboratory has largely focused on determining the specific processes underlying aberrations in hepatocellular protein trafficking resulting from ethanol administration. In recent studies, we have demonstrated that ethanol-induced impairments occur in the receptor-mediated endocytosis (RME) of the hepatocyte-specific asialoglycoprotein receptor (ASGP-R)[4-7].

Of interest, cellular fibronectin (cFn), a purported natural ligand for the hepatic ASGP-R, is a matrix component known to activate hepatic stellate cells (HSCs), and has been found, by our laboratory, as well as by others, to be increased in the livers of ethanol-fed animals[8,9]. Fibronectins are high molecular weight glycoproteins that exist as soluble dimers circulating in the blood and other tissue fluids, or as insoluble multimeric fibrils that are incorporated into the extracellular matrix (ECM). They are involved in numerous cellular processes that include cytoskeletal organization, cell adhesion, migration, growth and differentiation, that are mediated by interactions with integrin and non-integrin cell surface receptors[10]. Two major types of fibronectin are found *in vivo*, having distinct structures that define their respective functions. Plasma fibronectin (pFn), which predominantly exists as a soluble dimer produced by hepatocytes, has terminal carbohydrate residues capped by sialic acid. In contrast, 80%-85% of cFn, which is synthesized in soluble form by mesenchymal, epithelial and inflammatory cells prior to deposition in the ECM to form fibrils, contains terminal galactose residues that are not capped by sialic acid[11]. Studies by Rotundo *et al*[9] show the direct participation of the ASGP-R in the rapid *in vivo* removal of this desialylated fibronectin from the blood. In their study, Gillis and Nagy[8] suggest that accumulating levels of cFn after long-term ethanol administration in a rat model may be an early response to liver injury, and could lead to the activation of a fibrogenic response in hepatic stellate cells (HSCs). Overall, it is believed that changes in cFn levels prompt the remodeling of the hepatic ECM, which in turn may lead to the stimulation of factors involved in the initiation and/or progression of liver fibrosis[12,13]. The presence and accumulation of cellular fibronectin after ethanol administration may be involved in a variety of autocrine and paracrine responses within liver cells. Specifically, accumulating fibronectin generated in part by altered ASGP-R clearance may further stimulate the activity of cells to release chemokines, cytokines or matrix factors that are associated with ethanol-induced pathological changes in the liver[8,14-16]. This accumulation of cFn during liver injury may be attributed not only to impaired ASGP-receptor mediated clearance and increased cellular output, but also to alterations in the balanced interaction between matrix metalloproteinases (MMPs) that are largely responsible for the proteolytic degradation of the ECM, and their associated inhibitors, the tissue inhibitors of metalloproteinases (TIMPs)[17,18].

Matrix metalloproteinases (MMPs) are the principal enzymes involved in the remodeling of ECM components. Expressed in a cell or tissue-specific pattern, these highly specialized proteases are important in many biological and pathological processes. Of specific interest to our laboratory is the knowledge that, under certain conditions, the balanced interaction between TIMPs and the associated MMPs is altered with consequent changes to the ECM. More specifically, liver fibrosis, which is characterized by changes in the composition and extent of the ECM, is regulated by the activity of MMPs and TIMPs both of which play a role in healing after acute injury[17,18]. As previously mentioned, fibronectin deposition has been suggested to be an early indicator of liver fibrosis, and, as such, it may be a trigger for certain mechanisms that lead to changes in MMP/TIMP expression[8,13]. Cytokines have also been implicated in the regulation of MMP/TIMP activity, and the consequent remodeling of the ECM distinctive in liver injury[19-21]. Notably, production of these soluble ligands is influenced by cellular interactions with ECM molecules[22].

To date, no studies have examined the effect of cellular fibronectin accumulation following chronic ethanol administration on the functionality of hepatocytes in the liver. Any associations that have been drawn focus on non-parenchymal cellular activity, with little parenchymal cell involvement. In the present study, our goal is to examine the effect of exogenous cFn on hepatocyte performance including cytokine release and the MMP/TIMP relationship. We demonstrate, for the first time in a model of ethanol induced liver injury, a response by hepatocytes to elevated concentrations of exogenous cFn, such as is found in tissue exposed to alcohol, that reveals the ability of these cells to play an active role in the promotion of damage.

MATERIALS AND METHODS

Materials

William's Eagle culture medium, Percoll, lipopolysaccharide (LPS), type IV collagen, HEPES, BSA (fraction V), collagenase, phosphotungstic acid (PTA), trichloroacetic acid (TCA), human plasma fibronectin (pFn), and NADH were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal Bovine Serum (FBS) was obtained from Gemini (West Sacramento, CA). Human cFn was received from Millipore (Temecula, CA). Penicillin/streptomycin was obtained from Cellgro (Manassas, VA) and L-glutamine and gentamicin were purchased from Gibco BRL (Grand Island, NY). $Na^{125}I$ was obtained from Amersham-Pharmacia. The antibodies for MMP-2 and TIMP-2 came

from Calbiochem (San Diego, CA), and ICN (Costa Mesa, CA) supplied the cFn antibody. All other materials not specifically identified were of reagent grade. Nutritionally adequate liquid diets were formulated according to the method of Lieber and DeCarli[23] and purchased from Dyets Inc. (Allentown, PA). The caloric distribution of the ethanol-containing diet was 18% as protein, 35% as fat, 11% as carbohydrate and 36% as ethanol. In the isocaloric control diet, additional carbohydrates replaced ethanol.

Animals and diet administration

Male Wistar rats weighing 175-200 g, purchased from Charles River Labs (Portage, Michigan) were paired according to weight, and housed in individual cages in the Animal Research Facility at the Omaha Veterans Affairs Medical Center. After three days acclimatization on a control Lieber-DeCarli liquid diet, one animal from each pair was gradually introduced to a liquid diet containing 6.4% ethanol by volume as 36% of total calories. Each counterpart was pair-fed the isocaloric control diet. This feeding regimen was carried out for 12 wk for one group of rats, and 4-6 wk for all others, after which time the animals were sacrificed. As the purpose of this study was to ascertain whether cFn contributes to the development of advanced liver injury, animals were fed for a shorter duration of 4-6 wk, sufficient for the development of the early stages of alcoholic liver injury, but not prolonged enough for substantial cFn accumulation to have already taken place[8]. All animals received humane care in accordance with the guidelines established by the American Association for the Accreditation of Laboratory Animal Care. All protocols were approved by the Animal Studies Subcommittee of the Omaha Department of Veterans Affairs Medical Center. At necropsy, the livers of these animals were either removed completely and frozen for immunohistochemical analysis (12 wk fed group) or perfused for hepatocyte isolation in subsequent studies (4-6 wk fed group).

Immunohistochemistry

Cellular fibronectin was detected in control and ethanol rat livers using monoclonal antibody (Clone DH1) specific to the extra domain of cellular fibronectin (EDA-sequence). Liver tissue was removed from the rats that were fed for 12 wk, and flash frozen in liquid N_2. A piece of frozen tissue was embedded in freezing media and frozen tissue sections were sliced into 6-micron sections and affixed to slides (Fisher, Superfrost Plus). The sections were fixed in acetone for 10 min at -20℃ and subsequently washed with TBS (15 mmol/L Tris, 150 mmol/L NaCl, pH 7.6) at room temperature. Monoclonal antibody at 1:50 dilution in TBS was added to each section and incubated overnight at room temperature in a humidified chamber. Mouse IgG1 (Sigma, St. Louis, MO) was used as a negative control. After a series of washes with TBS the sections were further incubated with rhodamine (TRITC) conjugated anti-mouse IgG (H + L) (Jackson Immunoresearch, West Grove, PA) at 1:50 dilution at room temperature for 3 h in a humidified chamber. The slides were further washed, then mounted using Vectashield (Vector Laboratories, Burlingame, CA), viewed, and quantified, using a confocal-laser scanning microscope (Carl Zeiss LSM 410 inverted microscope with an argon-krypton laser with DIC capabilities) at the appropriate wavelengths.

Isolation of hepatocytes

Hepatocytes (HC) were obtained from control and ethanol-fed rats by the collagenase perfusion method[24] as described in our previous work[25]. The isolated cells were washed with Seglen suspension buffer and purified over a 35% (controls) or 33% (ethanol) Percoll gradient. Hepatocyte viability, determined by trypan blue dye exclusion was routinely > 85% for all the experimental groups. These cells were used for cFn degradation studies, or were cultured overnight to determine the biological effects of exogenously added cFn.

Degradation of cellular fibronectin

Preparation of iodinated cellular fibronectin: Iodinated cellular fibronectin was prepared according to the procedure of Fraker and Speck[26]. Briefly, 10 μL of a 1 mg/mL solution of 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril was evaporated under nitrogen gas in a glass tube (12 mm × 75 mm). To the tube (on ice) was then added 200 μL of a solution of cFn (1 mg/mL) in phosphate-buffered saline (PBS) followed by 7.5 μL of $Na^{125}I$ (750 μCi). This mixture was swirled on ice for 10 min; after mixing, the sample was loaded onto a column (1 cm × 12 cm) of Sephadex G-25 (medium) and eluted at room temperature with PBS.

Measurements of ^{125}I-cFn degradation: Isolated hepatocytes were pre-incubated in suspension buffer with 2% BSA at 37℃ for 30 min in a metabolic shaker before use in order to increase and equilibrate the number of cell surface receptors. The hepatocytes were then suspended in Williams Eagle's medium, pH 7.4, with 10 mol/L HEPES and 0.5% BSA at a concentration of 2×10^6 cells/mL. ^{125}I-cFn, at a final concentration of 2.5 μg/mL, was added to the cell suspension, which was subsequently incubated at 37℃ with gentle swirling. At 0, 15, 25, 60, 85, 120, 150, and 180 min respectively, an aliquot was removed to an ice-cold suspension buffer, pH 7.4, with 25 mmol/L EDTA, and incubated on ice to remove surface-bound ligand. After a minimum of 10 min on ice, the cells were pelleted (900 *g*, 4℃, 3 min), and an aliquot of the supernatant was placed in an equal volume of ice-cold 2% PTA in 20% TCA. The PTA/TCA mixture was incubated for a further 10 min on ice, then centrifuged (900 *g*, 4℃, 10 min), after which the radioactivity of an aliquot of the resulting supernatant was determined (^{125}I-cFn degradation).

Assays of cultured hepatocytes

Plating and incubation: Hepatocytes were plated a density of 7.5×10^5 viable cells/well on type Ⅳ collagen coated

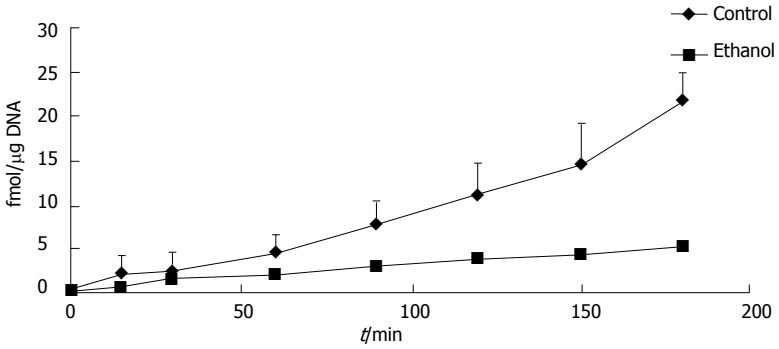

Figure 1 Ethanol administration delays degradation of iodinated cellular fibronectin in rats. Isolated hepatocytes were obtained from rats fed a control or ethanol-containing liquid diet for 4-6 wk. The degradation of iodinated cellular fibronectin (^{125}I-cFn) that has been internalized by the hepatocytes was monitored over a 3-h time period as described in Material and Methods. Data are presented as femtomoles bound per μg DNA and are means ± SEM (n = 3 experiments).

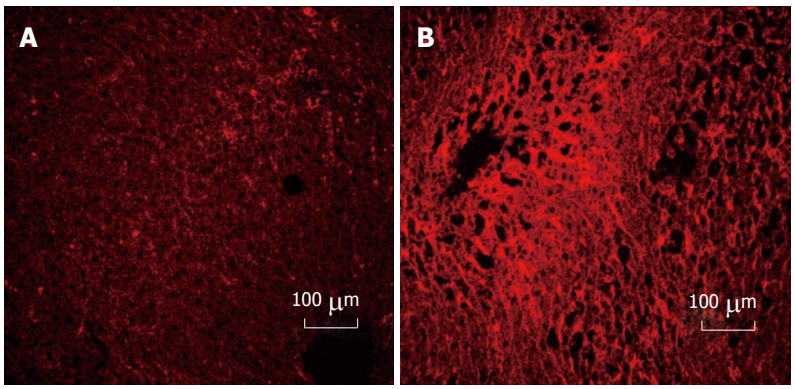

Figure 2 Immunohistochemical detection of cellular fibronectin in rat liver. Cellular fibronectin was detected by rhodamine conjugated antibody staining in liver sections from rats which had been fed control diet (A) or in ethanol-fed (B) animals for 12 wk. No staining was seen for isotype controls. Increased cellular fibronectin staining was observed (60% increase in intensity) in liver sections after ethanol administration. Values were determined using Carl Zeiss LSM 410 inverted confocal-laser scanning microscope.

6-well plates as detailed in Tuma *et al*[27] in Williams Eagle media supplemented with 10% FBS, penicillin/streptomycin (100 IU), 2 mmol/L *L*-glutamine and 40 mg/L gentamicin, and allowed to equilibrate at 37℃, 5% CO_2 for 2 h. Non-adherent cells were aspirated, and fresh serum-free Williams E media was added to the wells. The hepatocytes were subsequently treated with different concentrations of cFn (0 μg/mL, 0.2 μg/mL, 0.75 μg/mL, 4.0 μg/mL and 7.5 μg/mL), and 40 ng/mL LPS, a known activator of liver cells, as a positive control, and incubated at 37℃, 5% CO_2 for 20 h.

Viability assessment: Hepatocyte viability was assessed following the 20-h incubation for necrotic cell death as indicated by lactate dehydrogenase (LDH) leakage, as well as for caspase-3 activity, which is an upstream indicator of an activated programmed cell death (apoptosis) cascade. The leakage of LDH from the cells into the cell culture supernatant was measured by assaying the rate of change in the absorbance of NADH as it is oxidized (through the enzymatic activity of LDH) to NAD^+ in the culture media and comparing this value to that activity originally in the cells. The cell lysate was further assayed for caspase-3 activity, by measuring the ability to cleave a fluorogenic substrate Ac (N-acetyl)-DEVD-AMC (7-amino-4-methylcoumarin) (BD Biosciences, San Jose, CA), and quantified by spectrofluorometric analysis as previously demonstrated[28].

Cytokine assay: BD OptEIA rat TNF and BD OptEIA rat IL-6 ELISA sets (BD Biosciences, San Jose, CA) were used to measure TNF-alpha and IL-6 levels in the media obtained from HC cultures. The assays were performed according to the manufacturer's directions.

Western blot analysis: Cell culture supernatant was prepared in Laemmli[29] denaturing sample buffer and electrophoresed on 15% polyacrylamide gels using Mini-Protean Ⅱ Cell (Bio-Rad, Hercules, CA). Proteins were transferred (30 V, 4℃, 16 h) onto 0.2-μm nitrocellulose membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) and probed with specific antibodies against MMP-2 and TIMP-2. Briefly, the nitrocellulose blots were incubated at room temperature for 1 h in Odyssey blocking buffer (Licor Biosciences, Lincoln, NE), followed by exposure to either MMP-2 or TIMP-2 antibody (1:500 or 1:250 respectively diluted in blocking buffer) overnight at 4℃. After washing in PBS with 1% Tween-20, the blots were incubated in 1:5000 diluted IRDye 800 CW labeled-goat anti mouse IgG for 1 h at room temperature. Subsequent to a final wash, the immunoreactive proteins were visualized and quantified using LICOR Odyssey Infrared Imaging System.

Gelatin zymography: The total activity of MMP-2 released in the cell culture supernatant was measured by gelatin zymography. Culture media was prepared in non-reducing sample buffer without boiling, then electrophoresed on 7.5% polyacrylamide gels containing 0.1% gelatin using Mini-Protean Ⅱ Cell (Bio-Rad, Hercules, CA). Proteins were renatured (30 min to 1 h) in 2.5% Triton-X100 (Sigma, St. Louis, MO), after which the gels were incubated in an activation buffer (50 mmol/L Tris, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.02% Brij-350, pH 8.0) for 24 h at 37℃.

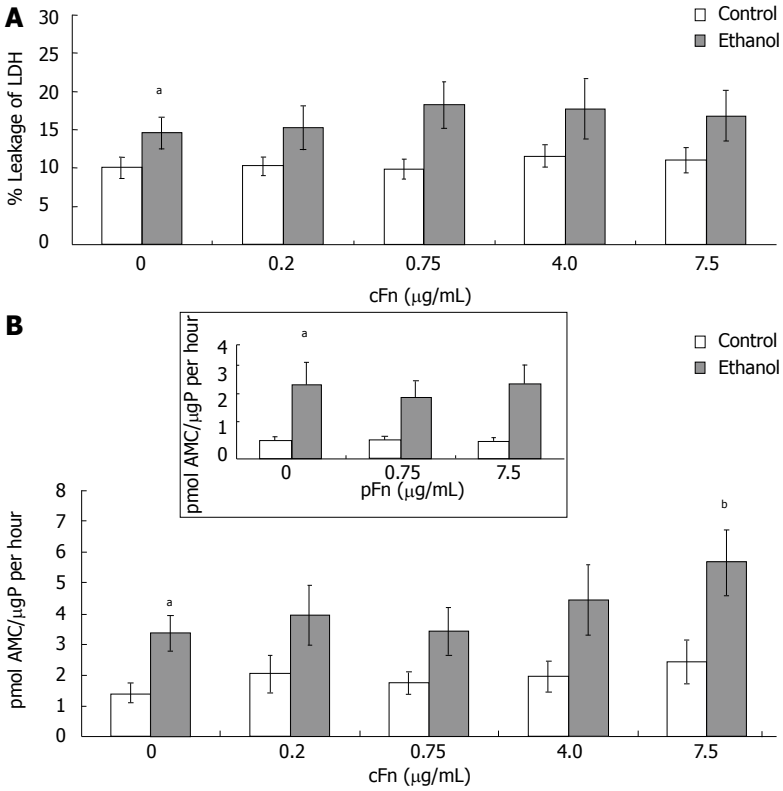

Figure 3 Effect of cellular fibronectin on hepatocyte viability. A: Percentage leakage of the cytosolic lactate dehydrogenase (LDH) enzyme into the cell culture supernatant was used to test for severe irreversible cell damage (necrosis) as described in Material and Methods. Incidence of necrosis was consistently higher in cultured cells from ethanol-fed animals (n = 8 - 12); B: Caspase-3 activity in the cells was determined as described in Material and Methods. Basal level caspase-3 activity was significantly higher in hepatocytes from ethanol-fed animals when compared with controls. Enzyme activity is presented as picomoles of fluorogenic AMC product released upon cleavage of the caspase-3 substrate Ac-DEVD-AMC, per μg protein in the cell lysate in an hour. At the higher dose of exogenous cellular fibronectin (cFn) (7.5 μg/mL), caspase-3 activity was significantly induced in cells from the ethanol-fed animals. No changes were seen in the control cells (n = 5 - 14). Plasma fibronectin exhibited no effect on either cell type (inset). Data are represented as means ± SEM. Ethanol values significantly different from those of the control are expressed as $^{a}P < 0.05$, and treatment significantly different from untreated cultures is expressed as $^{b}P < 0.05$. μgP: μg protein.

The gels were washed in deionized water and stained with Coomassie Blue (40% Methanol, 10% Acetic Acid, 0.5% Brilliant Blue R-250), and subsequently dried. MMP activity, represented by unstained bands was quantified by scanning densitometry using Quantity-One analysis software (Bio-Rad, Hercules, CA).

Statistical analysis

Results refer to the average from 3-15 experiments reported as mean ± SEM. Groups were compared using Student t-test, with values $P < 0.05$ considered significant.

RESULTS

Degradation of cellular fibronectin is delayed and accumulates in the liver after ethanol feeding

Ethanol administration alters the amount of cFn degraded by hepatocytes. When compared with the cells from control animals, the cells isolated from ethanol-fed rats degraded significantly less iodinated cFn (50%-75%) (Figure 1). Soluble pFn, however, was not degraded by any of the cells (data not shown). Immunohistochemical data revealed a dramatic up-regulation in cFn accumulation after ethanol feeding in our rat model of liver injury (Figure 2). Little or no staining was evident in the liver sections of animals fed control diets, while a dramatic increase in cFn staining (60%) was observed in the liver sections from the ethanol-fed rats.

Consequences of elevated levels of cellular fibronectin on hepatocyte function

Exogenous cellular fibronectin has minimal effect on hepatocyte necrosis but increased apoptotic caspase-3 activity: The viability of isolated hepatocytes from control and ethanol-fed animals incubated with different concentrations of cFn as described in Material and Methods was characterized to determine whether cFn treatment produced a cytotoxic effect. The present data (Figure 3A) reveals a significantly higher percentage of LDH in the media of cultured cells from ethanol-fed animals when compared with the control group. However, the presence of exogenously added cFn did not induce an additional effect. The activity of caspase 3, a death protease, in the cell lysates of hepatocytes from control and ethanol-fed animals was determined by quantifying the release of the fluorogenic AMC, which was produced by cleavage of the highly specific Ac-DEVD-AMC synthetic tetrapeptide caspase-3 substrate. From our data (Figure 3B), it is apparent that a significantly elevated level of basal caspase-3 activity exists in cultured hepatocytes from ethanol-fed rats, compared to that of the control rats. Similar results were obtained from assays of freshly isolated hepatocytes (data not shown). Furthermore, the hepatocytes from ethanol-fed animals were more susceptible to the effect of cFn at the high concentration of 7.5 μg/mL. However, cFn appeared to have no effect on the viability of cells derived from the control animals. We also included data of another isoform of fibronectin, plasma fibronectin (pFn), that is increased after alcohol administration but is neither a ligand of the ASGP-R, nor does it activate non-parenchymal cells. This isoform did not have an effect on either cell type.

Cellular fibronectin stimulates the secretion of MMP-2 and its corresponding inhibitor TIMP-2 by hepatocytes isolated from control and ethanol-fed rats: As outl-

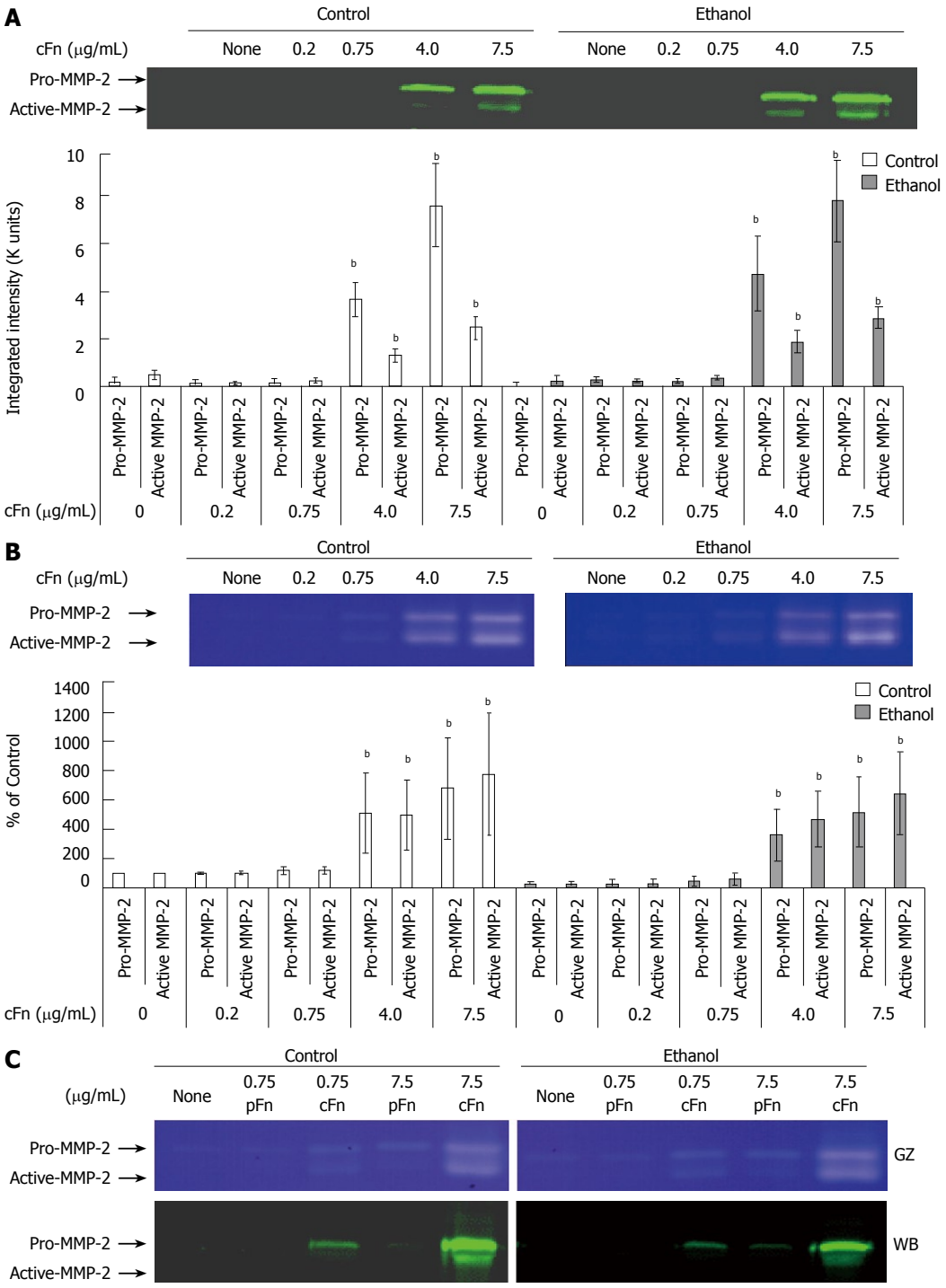

Figure 4 Effect of cellular fibronectin on the secretion of pro- and active-matrix metalloproteinase-2 by cultured hepatocytes isolated from control and ethanol-fed rats. Protein and activity levels of pro- and active-matrix metalloproteinase-2 (MMP-2) in the cell culture media of hepatocytes after 20 h of treatment with cellular fibronectin (cFn) were determined as described in Material and Methods. In the presence of higher concentrations of exogenous cFn (4 μg/mL and 7.5 μg/mL), cells from both control and ethanol-fed animals released elevated levels of pro- and active-MMP-2 (A) with corresponding higher MMP-2 activity (B). Plasma fibronectin treatment produced no effect on either cell type (C). GZ: gel zymography; WB: western blot. Data are represented as means ± SEM. Ethanol values significantly different from those of the control are expressed as $^{a}P < 0.05$, and treatment significantly different from untreated cultures is expressed as $^{b}P < 0.05$. (n = 3 - 11 experiments).

ined in the Material and Methods section, the media from cultured hepatocytes was collected after a 20-h incubation, clarified and assayed on SDS-PAGE gels, followed by Western blot analysis for MMP-2 and its corresponding inhibitor, TIMP-2 expression, and via gelatin zymography for MMP-2 activity. Data from Figure 4A and B show that MMP-2 expression and activity levels from both control and ethanol-fed animals, in a basal condition of incubation, are very low in hepatocytes. At low concentrations of exogenous cFn, there is very little response. However, at the higher concentrations of 4 and 7.5 μg/mL cFn, secreted levels of MMP-2 are significantly increased in

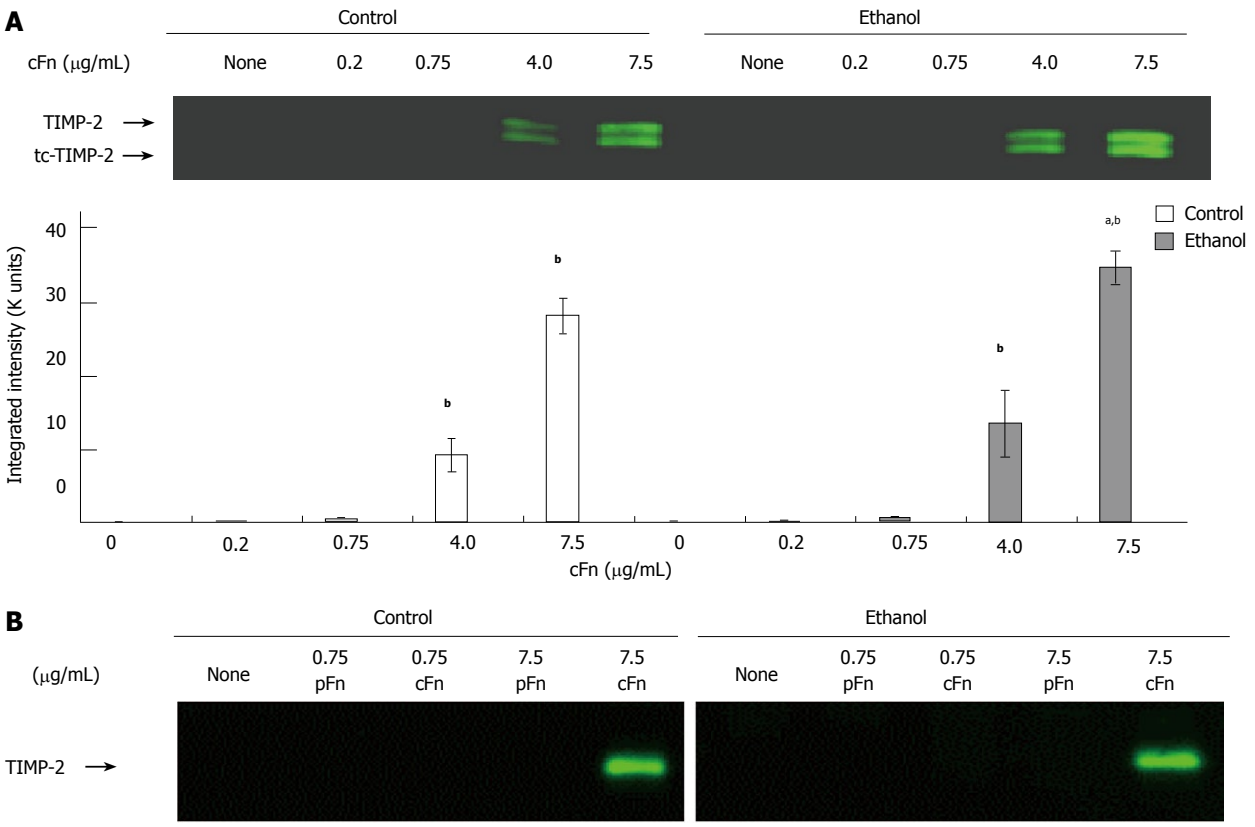

Figure 5 Effect of cellular fibronectin on the secretion of tissue inhibitor of metalloproteinase-2 by cultured hepatocytes isolated from control and ethanol-fed rats. The level of tissue inhibitor of metalloproteinase (TIMP)-2 released into the cell culture media of hepatocytes after 20 h of treatment with cellular fibronectin (cFn) was determined as described in the Material and Methods. At the higher concentrations of exogenous cFn (4 μg/mL and 7.5 μg/mL), hepatocytes from both control and ethanol-fed animals secreted significantly higher levels of TIMP-2 forms than corresponding untreated cells, and those incubated in the presence of low concentrations of cFn. The cells from ethanol-fed animals cultured in the presence of 7.5 μg/mL cFn were significantly more responsive than those of the controls (A). Plasma fibronectin treatment produced no effect on either cell type (B). Data are represented as means ± SEM. Ethanol values significantly different from those of the control are expressed as $^{a}P < 0.05$, and treatment significantly different from untreated cultures is expressed as $^{b}P < 0.05$. ($n = 11$ experiments).

the media of both cultured cell-types. This increase in expression corresponds with the gelatin zymography data, which shows a higher level of MMP-2 activity in the media of hepatocytes from both control and ethanol-fed rats. However, there appears to be no distinction in the response between the two cell types. Similarly, in the absence of cFn and at low concentrations, the secretion of TIMP-2 by hepatocytes from both control and ethanol-fed animals is very low (Figure 5). In the presence of higher concentrations of exogenous cFn (4 μg/mL and 7.5 μg/mL), there is a substantial response from both cell types, with hepatocytes from ethanol-fed animals secreting significantly more TIMP-2 in the presence of 7.5 μg/mL cFn than in the corresponding control animals. As shown in Figures 4C and 5B, overall, pFn did not produce an effect.

Production of pro-inflammatory cytokines by hepatocytes isolated from control and ethanol-fed rats increased in response to cellular fibronectin: Hepatocytes were cultured in the presence of low (0.2 and 0.75 μg/mL) and high (4 and 7.5 μg /mL) concentrations of cellular fibronectin, and the secretion of TNF-α and IL-6 into the cell culture media was determined by ELISA, as described in Material and Methods. The results show that hepatocytes cultured in the presence of exogenous cFn are stimulated to release TNF-α and IL-6 (Figure 6A and B). In the absence of cFn, cells from ethanol-fed rats produced significantly higher amounts of both cytokinesin comparison with hepatocytes from control-fed animals. The level of IL-6 in the media of cultured hepatocytes from the ethanol-fed rats was consistently higher, regardless of the presence of cFn. However, only at the higher concentrations of cFn (4 and 7.5 μg/mL), did chronic ethanol administration result in a significant enhancement of the secretion of TNF-α. There was a marked difference in the levels of cytokines secreted by hepatocytes from control animals and ethanol-fed animals in the presence of 7.5 μg/mL cFn, in comparison with corresponding untreated cells (4-fold increase in TNF-α and 2-fold increase in IL-6). Treatment of both cell types with pFn did not produce an effect (inserts Figure 6A and B).

DISCUSSION

The results of this study demonstrate that the accumulation of cellular fibronectin in the liver tissue of rats subject to chronic ethanol administration elicits a response in the parenchymal cells that leads to the increased secretion

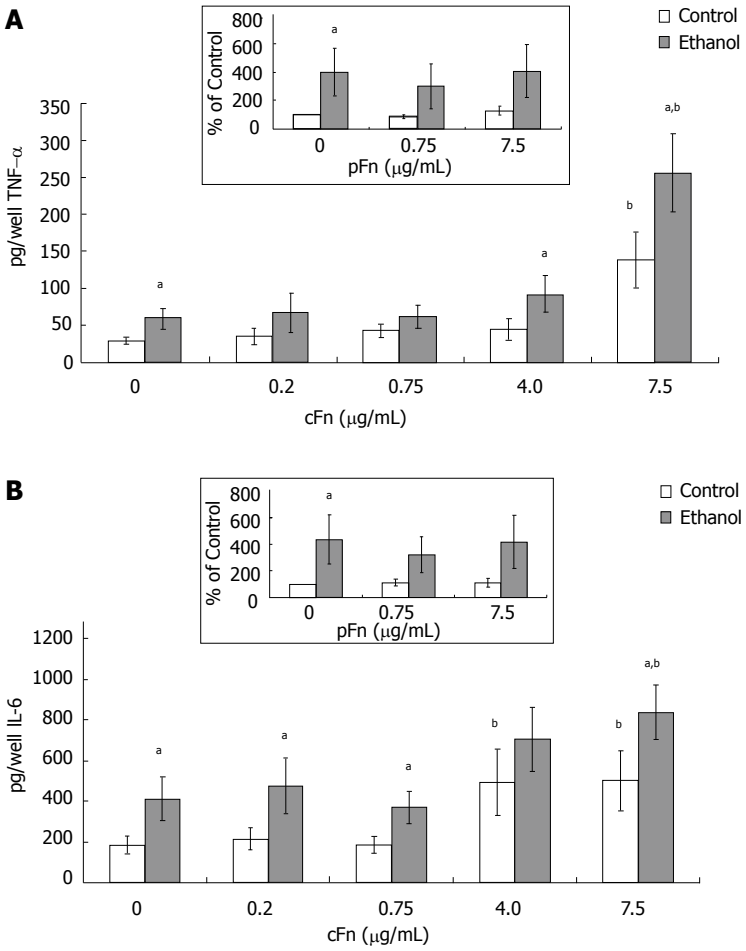

Figure 6 Secretion of cytokines tumor necrosis factor-alpha and interleukin-6 by cultured hepatocytes isolated from the livers of rats that were pair-fed control and ethanol diets and stimulated with cellular fibronectin. Hepatocytes were cultured in the presence of different concentrations of exogenous cultured hepatocytes (cFn) (0 μg/mL, 0.2 μg/mL, 0.75 μg/mL, 4.0 μg/mL and 7.5 μg/mL) for 20 h. After this time, the level of each cytokine (pg/well) released by the cells into the culture supernatant was determined by ELISA, as described in Material and Methods. In the presence of 7.5 μg/mL cFn, cells from both control and ethanol-fed animals released elevated levels of tumor necrosis factor-alpha (TNF-α) (A) and Interleukin-(IL)-6 (B) compared with corresponding untreated cells, with hepatocytes from ethanol-fed rats secreting significantly higher levels of both cytokines compared with controls. Plasma fibronectin treatment produced no effect on either cell type (inserts, A and B). Data are represented as means ± SEM. Ethanol values significantly different from those of the control are expressed as $^{a}P < 0.05$, and treatment significantly different from untreated cultures is expressed as $^{b}P < 0.05$. (n = 8 - 15 experiments).

of factors that could contribute to the progression of alcohol-induced liver injury. Prior studies from our laboratory have shown that chronic ethanol administration can alter the function and expression of the hepatocyte-specific asialoglycoprotein receptor[4-7]. A consequence of the impaired function of this receptor is the inability to adequately bind and internalize respective ligands for vesicular transport to the lysosome where they are degraded. Of particular interest is cellular fibronectin, cFn, a ligand for the ASGP-R and a protein that is known to participate in alcohol-induced fibrogenesis. We first examined the degradation of iodinated cellular fibronectin in isolated hepatocytes from control and ethanol-fed rats and found a significant disparity in the ability of each cell type to degrade cFn, in that hepatocytes from ethanol-fed animals were markedly impaired. The impaired degradation of cFn would contribute to the previously identified accumulation of cFn in the livers of ethanol-fed animals, and may be a factor in alcohol-induced liver pathology[30-34]. In addition, impaired degradation could lead to an increase in the concentration of circulating cFn, which has been identified in patients, and reflects a corresponding increase in tissue matrix changes and endothelial cell activation in the liver, events which are known to occur during ethanol-induced liver injury[27,34].

In our investigation, we investigated the addition of exogenous cFn to isolated HC preparations that had been obtained from rats fed ethanol or a control diet for 4-6 wk. We used a range of cFn from 0.2-7.5 μg/mL, for which the low concentration (0.2 μg/mL) represented circulating cFn levels in healthy subjects, while the higher concentrations (4 and 7.5 μg/mL) represented characteristic pathological levels of cFn as determined by previous studies[35,36].

In our study, HCs from both control and ethanol-fed livers were fairly resistant to any additional death by either necrosis or apoptosis in the presence of added cFn, so the cFn was not considered to be toxic on its own. The increased level of caspase-3 activity observed in hepatocytes from ethanol-fed rats at the highest concentration of cFn tested, suggest that this ECM protein may contribute in some way to the induction of apoptosis. It is thus plausible that, at elevated levels, cFn could facilitate apoptosis in hepatocytes whose viability has already been otherwise compromised by the effects of ethanol.

The increased deposition of cFn is implicated in the induction of matrix remodeling activity, that may contribute to the progression of liver injury towards fibrosis[8,13]. Correspondingly, we observed an increase in secreted MMP-2 levels and activity when excess cFn was introduced to cultured HCs. This output was observed in both cell types, with little variance between the two populations, and was seen as a likely regulatory response to excess cFn. The secreted proteases should break down the fibronectin molecule, rendering it non-functional, and decreasing its accumulation about the cell. However, even fragments of

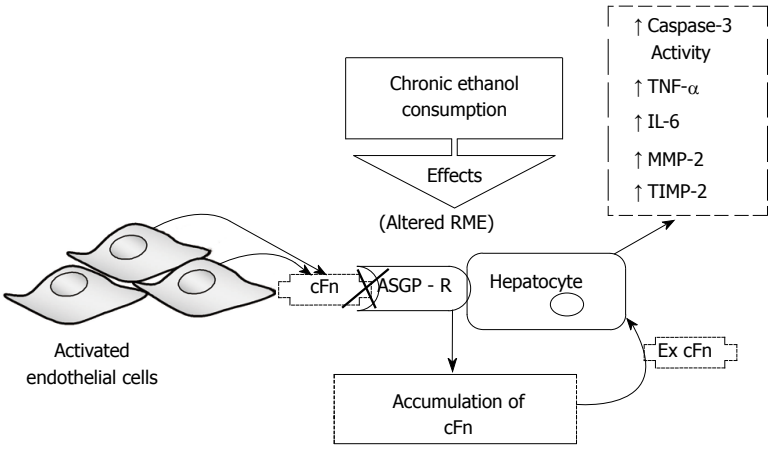

Figure 7 Schematic representation of the proposed model of ethanol-induced liver injury linking altered asialoglycoprotein receptor clearance of cellular fibronectin with hepatocyte activation by the accumulating protein. Subsequently, there is an increase in caspase-3 activity and an elevated secretion of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as well as secretion of matrix metalloproteinase-2 (MMP-2) and its corresponding inhibitor tissue inhibitors of metalloproteinase-2 (TIMP-2). RME: receptor-mediated endocytosis; ASGP-R: asialoglycoprotein receptor.

fibronectin have been reported to be involved in signal transduction events, thus the effect of excess cFn may not necessarily be neutralized[37-39].

Our data also show that the cells from ethanol-fed animals exposed to high concentrations of cFn, secreted substantially more TIMP-2 than corresponding control and untreated cells. These cells from the ethanol-fed animals appear to be more susceptible to cFn-induced up-regulation of TIMP-2 secretion, and are thus more likely to produce a disproportionate amount of TIMP-2 relative to MMP-2, that could lead to inhibited metalloproteinase activity and facilitate further build-up of cFn, as well as other ECM proteins characteristic of the early stages of fibrosis. An additional form of TIMP-2, two-chain (tc)-TIMP-2, a more potent inhibitor of MMP-2 function, was also detected at elevated levels[40]. The increased presence of tc-TIMP-2 would also contribute to a further reduction in the number of active-MMP-2 that could be involved in ECM degradation.

It should be noted that although our data demonstrates a seemingly similar release of MMP-2 and TIMP-2 by cells from both control and ethanol-fed animals, in normal control livers cFn accumulation does not occur. By introducing cFn to control cell cultures, we are reproducing conditions found in livers subject to ethanol-induced injury. This analysis allows us to identify ethanol-induced alterations that may exist in a cell's response to homeostatic challenge.

Generally, fibrosis and inflammation are coordinate events in liver injury; mediators of fibrosis may operate in concert with pro-inflammatory factors[20]. Although the output of pro-inflammatory factors TNF-α and IL-6, have been previously observed from primary cultured hepatocytes in other models[20,41], our results demonstrate for the first time the *in vitro* secretion of these cytokines by rat hepatocytes in a study of alcohol liver disease. We demonstrate a greater responsiveness by hepatocytes from ethanol-fed animals, that appears to be exacerbated in the presence of the higher concentrations of cFn.

Collectively, the data is summarized in Figure 7, which depicts a model of ethanol-induced liver injury linking altered asialoglycoprotein receptor clearance of cellular fibronectin with hepatocyte activation by the accumulating protein. Activation leads to a subsequent increase in caspase-3 activity, and an elevated secretion of pro-inflammatory cytokines, TNF-α and IL-6, as well as that of MMP-2 and its corresponding inhibitor, TIMP-2. These results, in conjunction with those from the aforementioned studies, suggest that autocrine and paracrine effects could exist that would produce a feedback relationship as a result of fibronectin-mediated changes in metalloproteinases and associated factors. For example, TNF-α has been shown to induce signaling that leads to apoptosis, thus it is plausible that the cFn-induced activation of caspase-3, observed at the higher cFn concentration, in cells from ethanol-fed animals, may in part be attributed to the associated cFn-induced increase in the secretion of this proinflammatory cytokine[42]. As a consequence of such reinforcing interactions, the promotion of tissue injury can be enhanced.

Conventionally, hepatocytes have been regarded merely as recipients and respondents of action taken by their other more reactive non-parenchymal counterparts; the results described here have been mainly ascribed to non-parenchymal cells only. Though it has been shown that *in vitro* conditions can evoke changes in cell-signaling and protein expression profiles in hepatocytes that cause them to exhibit behavior less representative of intact *in vivo* tissue, this study supports the contention that hepatocytes may be more involved in the orchestration of liver injury than previously considered.

In summary, our results demonstrate an effect resulting from accumulating cellular fibronectin, due in part to altered ASGP-R mediated clearance, on hepatocytes, that is enhanced by ethanol-induced injury. Further characterization of these responses, especially in the non-parenchymal cells of the liver, as well as further investigation of the mechanisms governing these responses, will help elucidate the specific role cFn has in promoting the progression of alcoholic liver disease.

COMMENTS

Background

Alcohol abuse is the leading risk factor for terminal liver disease worldwide. Though this association has long been established, the mechanisms of alcohol-induced liver injury remain poorly understood. To date, no effective strategies exist to counter the progression of alcoholic liver disease. It is known that alcohol-induced alterations to the character and function of the cells of the liver contribute to the build-up of cellular fibronectin in hepatic tissue. On the other hand, the

significance of this excess cellular fibronectin, and its effect on the liver during a condition of prolonged alcohol-induced damage, is not known.

Research frontiers

The increased deposition of cellular fibronectin in the liver is implicated in the development of liver fibrosis, the onset of which is characterized by remodeling of the extracellular matrix, as well as the myofibroblastic transformation of hepatic stellate cells. The composition of the extracellular matrix is regulated by the balanced interaction between matrix metalloproteinases and their inhibitors. Alterations in this balance often have pathological consequences. This fibrotic response is also preceded by an increase in pro-inflammatory cytokine levels, which in turn may contribute to the activation of stellate cells, as well asinfluencing the activity of extracellular matrix regulatory factors. The increased production of these cytokines may occur as a direct response to higher cellular fibronectin levels. The evaluation of these effects of cellular fibronectin and their potential roles in the development and progression of alcoholic liver disease is the focus of this research.

Innovations and breakthroughs

In the present study, we identifythese increased levels of cellular fibronectin not only as a symptom of progressive disease, but also as a contributing event to the development of alcohol-induced injury. Moreover, we demonstrate, for the first time in a model of alcohol-induced liver injury, a response by hepatocytes to elevated concentrations of exogenous cellular fibronectin, such as is found in tissue exposed to alcohol, that reveals the ability of these cells to play an active role in the promotion of damage.

Applications

Understanding how elevated levels of cellular fibronectin affect the cells of the liver, specifically, in this study, hepatocytes, during a condition of alcohol abuse could yield additional targets for the development of new strategies to prevent and treat alcoholic liver disease.

Terminology

Hepatocytes are the chief functioning cells of the liver, and key targets for mediators of injury. Cellular fibronectin is an extracellular matrix glycoprotein that is normally present at low levels in the body, and is only briefly up-regulated during tissue repair. A sustained elevation in cellular fibronectin levels has been observed during several pathological conditions, including that of alcoholic liver disease.

Peer review

This paper describes the effect of cellular fibronectin on hepatocytes isolated from control and ethanol-fed rats. Overall, this paper is interesting, well written and provides new information for the field, though several points need clarification and/or correction.

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CASE REPORT

A mutation of the start codon in the X region of hepatitis B virus DNA in a patient with non-B, non-C chronic hepatitis

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Abstract

There are cases of hepatitis involving occult hepatitis B virus (HBV) infection in which, even though the HB surface antigen (HBsAg) is negative, HBV-DNA is detected by a polymerase chain reaction (PCR). We conducted a sequence analysis of the entire HBV region in a case of non-B non-C chronic hepatitis in a 46-year-old female. A diagnosis of non-B non-C chronic hepatitis was made. Although HBV markers, such as HBs antibody (anti-HBs), anti-HBc, HBeAg and anti-HBe, were negative, HBV-DNA was positive. Nested PCR was performed to amplify the precore region of HBV-DNA and all remaining regions by long nested PCR. Sequence analysis of the two obtained bands was conducted by direct sequencing. Compared with the control strains, the ATG (Methionine) start codon in the X region had mutated to GTG (Valine). It is assumed that a mutation at the start codon in the X region may be the reason why HBV markers are negative in some cases of hepatitis that involve occult HBV infection.

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Key words: Hepatitis B virus; X region; Mutation; Non-B non-C chronic hepatitis; Occult infection

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INTRODUCTION

The existence of hepatitis involving occult hepatitis B virus (HBV) infection in which, even though the HB surface antigen (HBsAg) is negative, HBV-DNA is detected by a polymerase chain reaction (PCR), has been proposed

as one type of non A-E type hepatitis of unknown origin[1,2]. Among the cases of non-B non-C chronic hepatitis treated in our hospital, one case of occult HBV infection was detected as positive by supersensitive assay for HBV-DNA, the Direct Method[3]. Amplification products in the HBV-DNA precore (preC) region as well as in all remaining HBV-DNA regions were obtained by PCR and long PCR, respectively. Direct sequencing of these products was carried out, in order to compare them with the reported sequences of already registered genomes of HBV, as well as to consider the cause of HBV markers becoming negative. As a result, a mutation at the start codon in the X region was observed, which may be one cause of occult HBV infection, as reported below.

CASE REPORT

A 46-year-old female visited our hospital in April 1995, wishing to undergo a thorough examination for liver damage. Her biochemical values were high: aspartate transaminase (AST) 41 IU/L, alanine transaminase (ALT) 62 IU/L, gamma-glutamyl transferase (γGT) 39 IU/L, and zinc sulfate turbidity test 17.7 U. Platelet counts were low, at 12.8 × 10^4/μL and indocyanine green retention rate at 15 min was high at 12%. These findings which seemed to confirm chronic hepatitis. However, HBsAg, HBs antibody (anti-HBs), anti-HBc, HBeAg and anti-HBe were all negative, and so was hepatitis C virus antibody. The patient did not have a history of alcohol use and anti-nuclear antibody and anti-mitochondrial antibody tests were also negative (Table 1). The abdominal ultrasonographic findings also suggested chronic hepatitis. A diagnosis of non-B non-C chronic hepatitis was made and administration of ursodeoxycholic tacid (UDCA) was commenced in January 1996. As a result of the continuous administration of UDCA (600 mg/d), although ALT once rose to 80 IU/L, it remained less than 40 IU/L from September 1999 onwards throughout the course of treatment. Occult HBV infection was suspected and, during the course of treatment, HBsAg was reexamined, HB core related antigen was examined, and HBV-DNA was measured by AMPLICOR HBV MONITOR Assay[4]. The results of all these procedures were negative. However, a newly developed, supersensitive assay for HBV-DNA, the Direct Method[3], showed a positive result at 45 IU/mL (equivalent to 1.7 log copies/mL).

Using stored serum from the patient, and based on a report by Omata *et al*[5], a primer set that may amplify the HBV preC region was developed and nested PCR was performed to amplify the HBV-DNA segment[6]. Also, in order to amplify all remaining HBV-DNA regions other than the preC region, reverse primer sets were developed in the preC region and in its vicinity, and by altering the methods applied by Tellier *et al*[7] as well as by Günter *et al*[8], amplication of HBV-DNA was attempted by long nested PCR (Table 2). In the amplification of the preC region by PCR, a band of 0.2 kb was obtained. Also, in the amplification of all remaining regions by long PCR, a band of 3.0 kb was obtained. Sequence analysis of PCR amplification products was conducted by direct sequencing. The results of the sequences analysis of these two products were combined and, as a result, the base sequence of all HBV-DNA regions was identified (Figure 1). DNASIS^R gene analysis software (Hitachi Electronics Engineering Co., Ltd.) was applied to compare the base sequence obtained by us with the sequences of already registered genomes of four HBV strains, DQ478885, AP011098, AB367417 and AB246344[9-13], using National Center for Biotechnology Information Basic Local Alignment Search Tool. As a result, the sequence for the genotype was 2C and matched 98% of the sequences of each of these 4 control strains. The results from comparing the 4 control strains revealed mutation of the ATG (Methionine) start codon in the X region to GTG (Valine) in this case. However, the start codon in the S, P, C regions was intact, as with the other 4 strains. Further, the start codon in the preX region was also intact, as with the other 4 strains, although a mutation of TGA (stop codon) to CGA (Arginine) was identified. Of these 4 control strains, only the AP011098 strain was TGA and all the other 3 stains were CGAs.

Table 1 Laboratory findings at first visit

Parameter	Value	Partameter	Value	Parameter	Value
Peripheral blood		Biochemistry		Viral marker	
WBC (/μL)	3800	AST (IU/L)	41	HBsAg (COI)	1.4
RBC (× 10^4/μL)	365	ALT (IU/L)	62	Anti-HBs (COI)	0
Hb (g/dL)	12.2	ZTT (U)	17.7	Anti-HBc (S/CO)	< 1.0
Ht (%)	37.5	LDH (IU/L)	213	HBeAg (COI)	< 0.5
Plt (× 10^4/μL)	12.8	ChE (U/L)	5.8	Anti-HBe (%)	< 35.0
		γGT (IU/L)	39	Anti-HCV (COI)	0.2
Coagulation		ALP (IU/L)	265		
HPT (%)	87	TBil (mg/dL)	0.8	Autoantibody	
		DBil (mg/dL)	0.3	ANA	(-)
Urinalysis		UN (mg/dL)	15	AMA	(-)
Protein	(-)	Cr (mg/dL)	0.5		
Sugar	(-)	TP (g/dL)	8.6	Others	
Bilirubin	(-)	Alb (g/dL)	4.9	ICG-R15 (%)	12
Urobilinogen	(±)	TC (mg/dL)	235	AFP (ng/mL)	2.6
		TG (mg/dL)	40		

WBC: while blood cells; RBC: red blood cells; Hb: hemoglobin; Ht: hematocrit; Plt: platelets; HPT: hepaplastin test; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ZTT: zinc turbidity test; LDH: lactic dehydrogenase; ChE: choline esterase; γGT: gamma-glutamyl transpeptidase; ALP: alkaline phosphatase; TBil: total bilirubin; DBil: direct bilirubin; UN: urea nitrogen; Cr: creatinin; TP: total protein; Alb: albumin; TC: total cholesterol; TG: triglyceride; HBsAg: hepatitis B surface antigen; anti-HBs: hepatitis B surface antibody; anti-HCV: hepatitis C virus antibody; ANA: anti-nuclear antibody; AMA: anti-mitochondrial antibody; ICG-R15: indocyanine green retention rate at 15 min; AFP: alpha-fetoprotein.

DISCUSSION

Using the stored serum of patients diagnosed with non-A, non-B, non-C acute or fulminant hepatitis treated at our hospital, PCR assays were performed to amplify the HBV-DNA segment. As a result, with a primer set which can amplify the preC region, amplification products were obtained at high rates, thereby suggesting the involvement of occult HBV infection[6]. In consideration of this result,

Table 2 Primers for the amplification of the precore region and all remaining regions of hepatitis B virus DNA in nested polymerase chain reaction

	Primer	Sequence	Position[a]
PreC region	1st forward	5'GGGAGGAGATTAGGTTAA3'	1744
	1st reverse	5'GGCAAAAAAGAGAGTAACTC3'	1959
	2nd forward	5'TAGGAGGCTGTAGGCATAA3'	1774
	2nd reverse	5'GCTCCAAATTCTTTATA3'	1932
	Cycling protocol: 94℃ 1 min - 55℃ 1 min - 68℃ 3 min (25 cycles)		
All remaining regions (long PCR)	1st forward	5'CCTATAAAGAATTTGGAGC3'	1914
	1st reverse	5'TTTATGCCTACAGCCTCC3'	1793
	2nd forward	5'GAGTTACTCTCTTTTTTGC3'	1940
	2nd reverse	5'ACCTTTAACCTAATCTCCT3'	1765
	Cycling protocol: 95℃ 1 min - 57℃ 1 min - 68℃ 3 min (35 cycles)		

[a]HBV DNA is composed of 3215 bases and starts from the Eco RI cleavage site within pre-S/S open reading frame[9]. PCR: polymerase chain reaction.

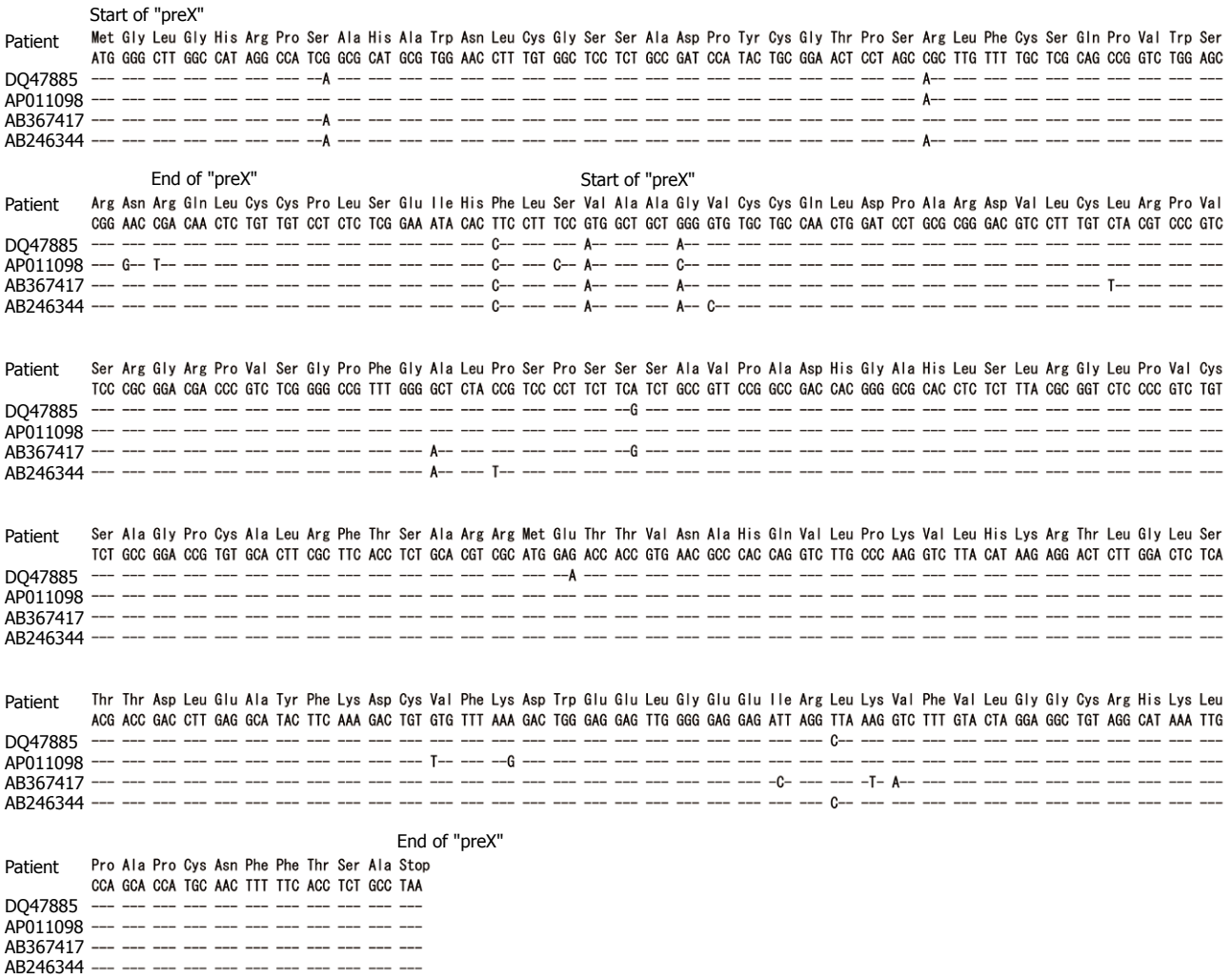

Figure 1 Nucleotide and amino acid sequences of the preX and X regions. DNASISR® gene analysis software was applied to compare the base sequence with the sequences of already registered genomes of 4 hepatitis B virus strains: DQ478885, AP011098, AB367417 and AB246344[10-13], using Basic Local Alignment Search Tool.

the involvement of occult HBV infection was examined in patients at our hospital who had been diagnosed with non-B, non-C chronic hepatitis. In the case described above, in which HBV-DNA was detected as positive by the supersensitive Direct Method, PCR assays were performed with a view to amplifying the HBV-DNA segment with a primer set that could amplify the preC region. As a result, PCR amplification products were observed.In the aforementioned patients with acute or fulminant hepatitis, using a primer set that could amplify the preC region,

PCR amplification products were obtained at high rates, although the rates were lower for other regions[6]. This result suggested that, in cases of occult HBV infection, mutations or defects might occur in regions other than the preC region, thereby obstructing PCR amplification. Therefore, in order to amplify the other remaining regions, reverse primer sets were developed in the preC region and in its vicinity, with which the amplification of genes by long nested PCR was attempted. As a result, a 3.0 kb amplification product was obtained by long PCR.

In this case, the result of analysis of the base sequence of all HBV-DNA regions demonstrated that the ATG (Methionine) start codon in the X region had been mutated to GTG (Valine). X protein (HBx) produced by X-open reading frame (ORF) is known as a multifunctional regulator that interacts with host factors and, as a result thereof, modulates transcription, signal transduction, protein degradation pathways, apoptosis and genetic stability[14]. Evaluating the role of HBx in the viral life cycle of HBV, Xu *et al*[15] utilized HBV transgenic mice that could not produce HBx and reported that HBx activates the viral genome expression, thereby enhancing viral replication. In addition, Bouchard *et al*[16] transfected an HBV genome that could not express HBx into HepG2 cells and observed that HBV replication decreased 5 to 10 fold. Furthermore, Tang *et al*[17] demonstrated that ectopically expressed HBx could stimulate HBV transcription and replication with the X-defective replicon to the same level as with the wild-type. On the other hand, Reifenberg *et al*[18] reported that in X-deficient HBV transgenic mice, HBx was not required for HBV replication or for virion secretion. In addition, Meier *et al*[19] reported that the DHBV strain with a knockout mutation in the X-ORF was not different from the wild-type strain in terms of infectivity and *in vivo* growth. From these reports, it is concluded that, although HBx may not be essential for HBV replication and for the synthesis of virus particles, it may play an important role in stimulating HBV replication. Since no significant mutation was observed in other regions in this case, it is assumed that, although HBV particles are produced, the detection of each HBV marker becomes difficult due to the X region not being translated along with HBx not being synthesized and HBV proliferation decreasing.

ATG (start codon) in the preX region proposed by Takahashi *et al*[20] was intact in our case, as it was in 4 control strains. On the other hand, in our case TGA (stop codon) was mutated to CGA (Arginine) in the preX region. In the 4 control strains from patients, only the AP011098 strain was TGA and the other 3 stains were all CGAs. In the same report, it was noted that a mutation [TGA to CGA or TGA to AGA (Arginine)] had been observed in 21% of the asymptomatic HBV carriers and in 64% of the patients with chronic hepatitis B[20]. It is suggested that a variant without a stop codon in the preX region may be translated linearly from the preX through the X regions, like the translation within the preC and core regions. In this case, it is possible that the lack of a stop codon in the preX region may supplement the fact that there was no start codon in the X region and HBx was not produced.

Occult HBV infection infers an infected state of HBV in which, even though HBsAg is negative, HBV-DNA is detected by PCR[1,2]. It was reported that, after recovery from acute hepatitis B, HBV virions form an immune complex with the anti-HBs in blood and, as a result, the state of HBsAg negative occult HBV infection persists for a long period of time[21,22]. On the other hand, it was reported that, in patients with chronic liver diseases of unknown origin, HBV-DNA is detected by PCR at a rate of 10%~30%[23-25]. In the majority of these cases of occult HBV infection, the amount of virus is small and a supersensitive assay is required to detect HBV-DNA[24,25]. It is said that, in cases of chronic hepatitis B, when the amount of virus becomes smaller, hepatitis is usually mitigated. However, in some of these cases of occult HBV infection, chronic inflammation continued and cirrhosis subsequently developed[23,24]. In the featured case, although the amount of HBV-DNA was small, persistent hepatitis, which requires medication, was observed. The mutation of HBV-DNA in such cases of occult HBV infection has been reported. Preisler-Adams *et al*[26] reported that, for cases in which HBV markers had not been detected, single base variation in the enhancer I, substitution of 9 amino acids in the P region, and substitution of 3 amino acids in the X region had been observed. Also, Fukuda *et al*[27] reported that, in cases of non-B, non-C hepatitis, deletion mutation of 8 nucleotides was observed at high rates in the distal part within the X region. In our case, a mutation at the start codon in the X region was observed. These results suggest that, although the mechanism by which HBsAg becoming negative in patients that involves occult HBV infection is not uniform, it is likely that a mutation of HBV-DNA may negatively affect replication and expression of the virus. We would suggest that, as was seen in this case, prohibition of HBx production due to a mutation at the start codon in the X region is one possible cause of occult HBV infection. In the future, we would like to conduct further research to examine whether similar mutations can be seen in cases in which occult HBV infection is involved. In conclusion, in a case of non-B non-C chronic hepatitis, following identification of the base sequence in all HBV-DNA regions, a mutation at the start codon in the X region was observed. It is concluded that this may be the cause of HBV markers being negative, as seen in some cases of hepatitis that involve occult HBV infection.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011
Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011
San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11
Regensburg 93053, Germany

January 28-29, 2011
9. Gastro Forum München
Munich, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference
Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011
Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation
Dublin, Ireland

March 3-5, 2011
42nd Annual Topics in Internal Medicine
Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings
Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011
Birmingham, England, United Kingdom

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011
Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform
Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011
Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 94143, United States

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition
Riyadh, Saudi Arabia

May 7-10, 2011
Digestive Disease Week
Chicago, IL 60446, United States

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course
Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011
Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS
Napoli, Italy

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer
Barcelona, Spain

October 19-29, 2011
Cardiology & Gastroenterology Tahiti 10 night CME Cruise
Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week
Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course
Washington, DC 20001, United States

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Instructions to authors

GENERAL INFORMATION

World Journal of Hepatology (*World J Hepatol, WJH*, online ISSN 1948-5182, DOI: 10.4254), is a monthly, openaccess, peer-reviewed journal supported by an editorial board of 573 experts in hepatology from 46 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results.

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The columns in the issues of *WJH* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in hepatology; (9) Brief Article: To briefly report the novel and innovative findings in hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJH*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice in hepatology.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Standard abbreviations should be defined in the abstract and

on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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