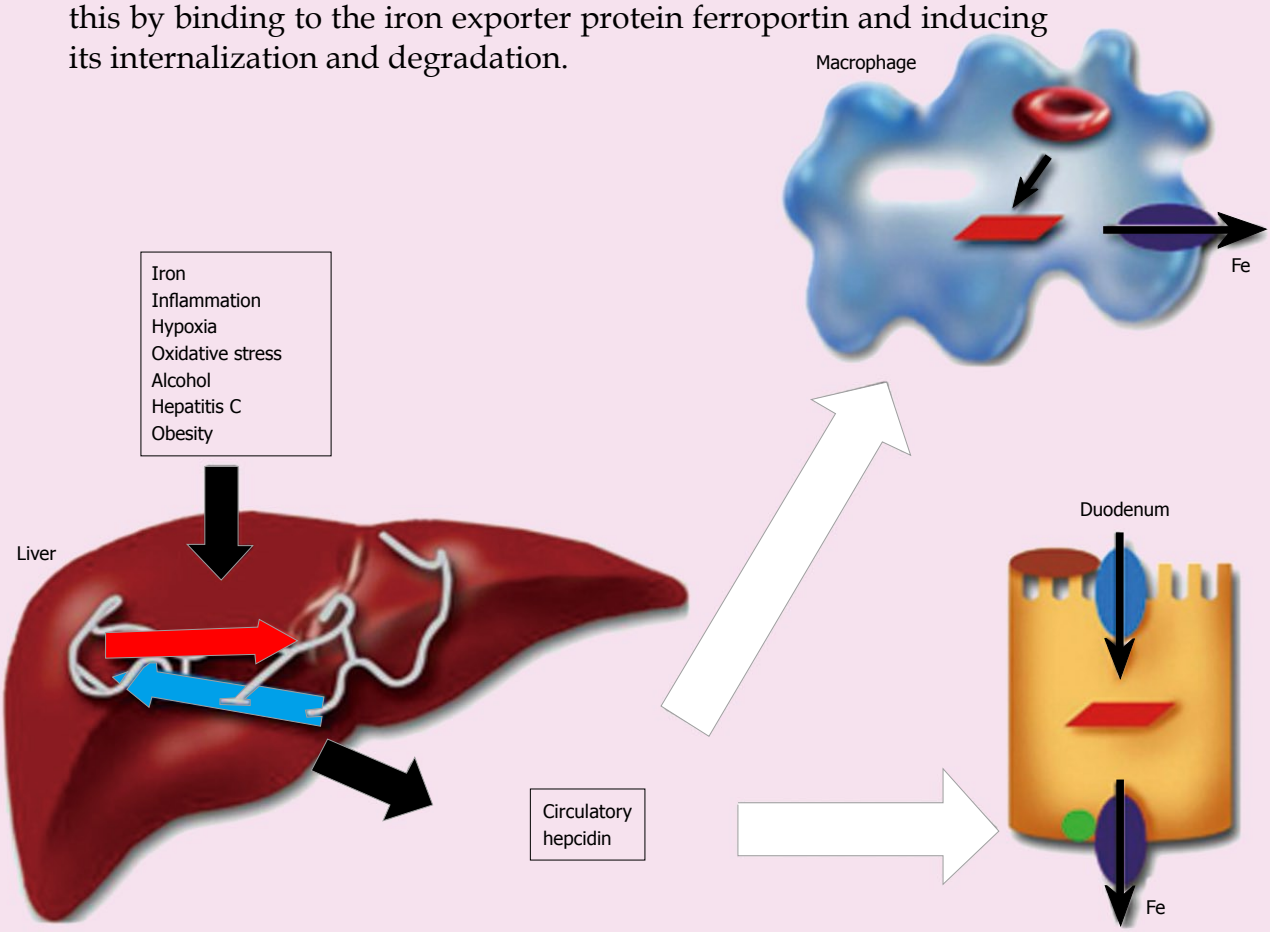
ISSN 1948-5182 (online)

World Journal of Hepatology

World J Hepatol 2010 August 27; 2(8): 295-324

A peer-reviewed, online, open-access journal of hepatology

Regulation of hepcidin and iron metabolism. The key iron-regulatory hormone hepcidin is primarily synthesized in the parenchymal cells of the liver and is subsequently released into the circulation. The transcriptional regulation of hepcidin in the liver is regulated by various factors. Hepcidin released into the circulation in turn regulates iron metabolism by controlling the iron transport in the duodenum and iron export in the macrophages. Hepcidin achieves this by binding to the iron exporter protein ferroportin and inducing its internalization and degradation.



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WJH World Journal of Hepatology

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Monthly Volume 2 Number 8 August 27, 2010

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World Journal of Hepatology
Volume 2 Number 8 August 27, 2010

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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a monthly, open-access, peer-reviewed journal supported by an editorial board of 572 experts in hepatology from 45 countries.

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NAME OF JOURNAL
World Journal of Hepatology

LAUNCH DATE
October 31, 2009

SPONSOR
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PUBLICATION DATE
August 27, 2010

CSSN
ISSN 1948-5182 (online)

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World J Hepatol 2010 August 27; 2(8): 295-301
ISSN 1948-5182 (online)

EDITORIAL

Mallory-Denk body pathogenesis revisited

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Received: May 4, 2010 Revised: July 7, 2010
Accepted: July 14, 2010
Published online: August 27, 2010

Abstract

This editorial reviews the recent evidence showing that Mallory-Denk bodies (MDBs) form in hepatocytes as the result of a drug-induced shift from the 26s proteasome formation to the immunoproteasome formation. The shift is the result of changes in gene expression induced in promoter activation, which is induced by the IFNγ and TNFα signaling pathway. This activates TLR 2 and 4 receptors. The TLR signaling pathway stimulates both the induction of a cytokine proinflammatory response and an up regulation of growth factors. The MDB- forming hepatocytes proliferate as a result of the increase in growth factor expression by the MDB-forming cells, which selectively proliferate in response to drug toxicity. All of these mechanisms are induced by drug toxicity, and are prevented by feeding the methyl donors SAMe and betaine, supporting the epigenetic response of MDB formation.

Key words: Toll-like receptor; Proinflammatory; Methyl donors; Epigenetic processes; Drug toxicity; 26s Proteasome; Immunoproteasome

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French SW, Bardag-Gorce F, Li J, French BA, Oliva J. Mallory-Denk body pathogenesis revisited. *World J Hepatol* 2010; 2(8): 295-301 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i8/295.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i8.295

INTRODUCTION

A great deal of new information on the subject has been published since the last review on Mallory-Denk body (MDB) pathogenesis published in 2007[1]. In that review it was emphasized that MDBs contain keratins K18 and 8, ubiquitin and p62. The relevant proteins and cellular processes that contribute to MDB formation include chronic stress-induced protein misfolding and consequential proteasome overload, a K8-greater-than-K18 ratio, and transamidation of K8 and other proteins. However, the mechanisms involved in the formation of the MDB aggresome have remained elusive. Three new mechanisms of MDB formation have recently been explored. The first is epigenetic mechanisms. The second is the shift from the 26s proteasome to the immunoproteasome. The third is the chronic activation of the Toll-like signaling pathways which stimulate proinflammatory and cell growth pathways. The 3 mechanisms combine to form MDBs.

EPIGENETIC MECHANISMS

The first indication that MDB formation is the result of epigenetic changes in gene expression came about when it was demonstrated that feeding S-adenosyl-methionine (SAMe) prevented MDB formation when diethyl-1,4-

dihydro-2,4,6-trimethyl-3,5-pyridine decarboxylate (DDC) was re-fed to drug-primed mice[2]. Microarray analysis of the livers from these mice showed that the drug treatment phenotype was remembered by the liver cells at 9 wk, 11 wk and 4 mo after withdrawal of the drug, suggesting that the epigenetic changes were heritable. More significant was that this memory was completely prevented by feeding SAMe with DDC because it meant that SAMe, a methyl donor, had silenced the changes in gene expression that had induced MDB formation. Methylation of H3K9 of histones and DNA leads to gene silencing. Consequently, no induction of MDBs resulted when SAMe was fed. Data mining of the microarray changes in gene expression by the MDB forming liver cells showed that SAMe feeding prevented the changes induced in gene expression caused by drug re-feeding associated with MDB formation. Most notably, SAMe prevented the up regulation of HSP70, caspase 3, Map3K14, glutathione synthase, sequestosome 1 (p62), HDAC 9, alpha fetal proteins (Afp), Kruppel-like factor 6 (KLF-6), Egr2, glutathione S transferase mu2 (Gstm2), ubiquitin D (FAT10), gamma-glutamyl transferase 1 and glutathione peroxidase 2. These changes in gene expression stimulate changes in growth factors, apoptosis, chaperones, antioxidants and preneoplasia.

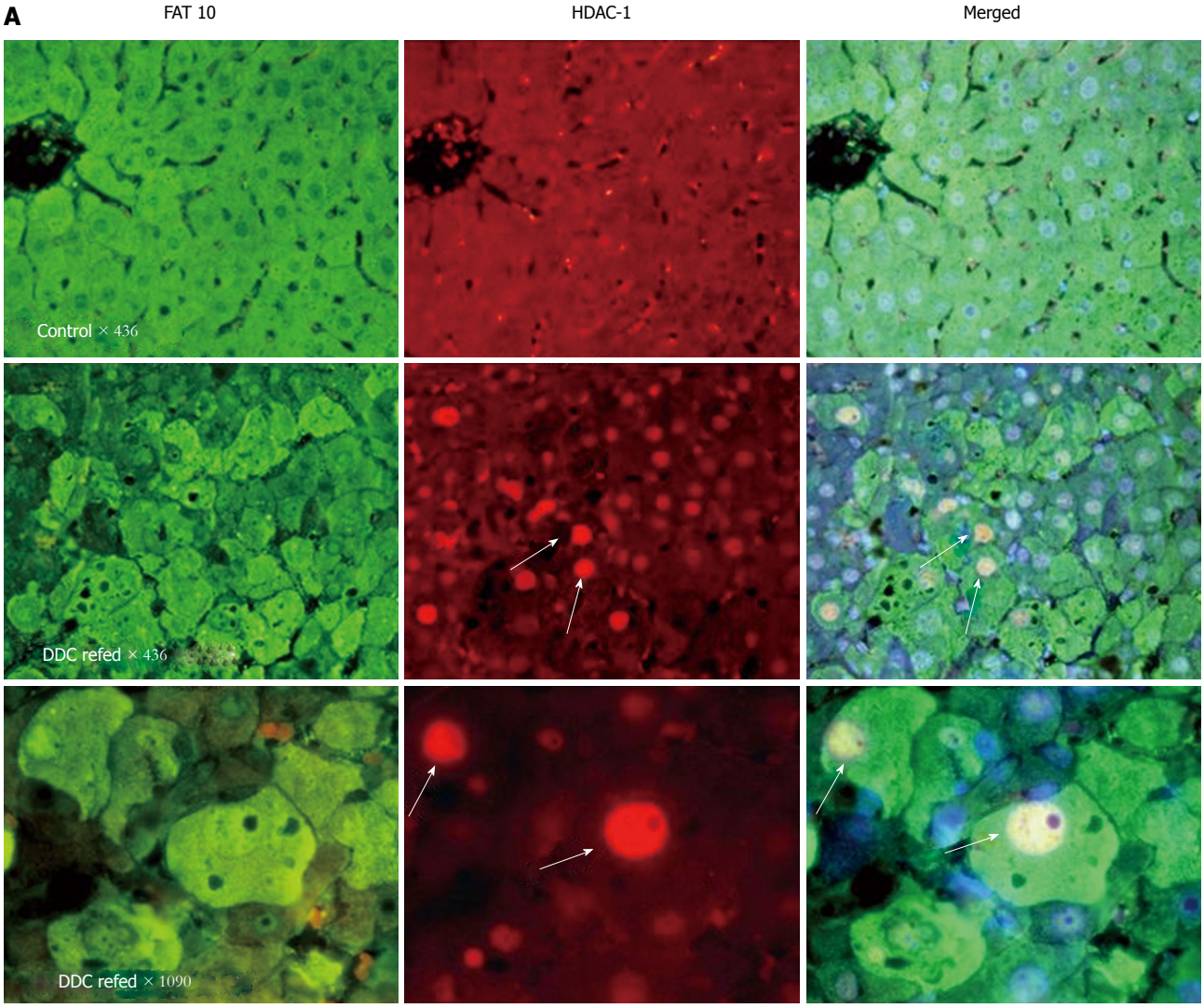
To further substantiate the changes in gene expression, qPCR was done. FAT10 and KLF6 were markedly up regulated by DDC, and this was prevented by feeding SAMe. The up regulation of FAT10, KLF6, Afp and Gstm2 was observed at all 3 time intervals (9 wk, 11 wk and 4 mo) and SAMe prevented these changes in gene expression at every time interval[2]. Feeding SAMe also affected the expression of acetylation and methylation enzymes (Dnmt3A, HDC9) induced by DDC re-feeding. Parameters involving oxidative stress (GSH levels, 4HNE, and carbonyl protein levels) were not changed during MDB formation, and were unaffected by SAMe feeding. MDB formation by drug-primed liver cells in primary cultures was totally prevented by SAMe *in vitro*. These results clearly established the role of epigenetic changes in gene expression during MDB formation and prevention by the methyl donor SAMe[2].

To further document the role of epigenetics in the formation of MDBs, FAT10 (UbD) was used as an immunofluorescent marker of the MDB- forming liver cell phenotype[3]. The FAT10 positive cells that formed MDBs proliferated over 7 d of DDC re-feeding to drug-primed mice. The FAT10 liver cell phenotype showed a growth advantage over the intervening normal FAT10 negative hepatocytes in the livers of the mice re-fed DDC[3]. The proliferative response was completely prevented by feeding SAMe. FAT10 protein was markedly increased by Western blot analysis, as well as by immunohistochemistry. Morphologically, the FAT10-positive MDB-forming hepatocytes are often in mitosis, and the nuclei stain positive for cyclin D1 and proliferation cell nuclear antigen, indicating that they were proliferating. The individual FAT10-positive liver cells persisted in the liver among normal hepatocytes for 9 mo after drug withdrawal, at which time FAT10-positive tumors formed. This correlated with a decrease in 8-oxyguanine DNA-glycosylase (OGG1), which would favor the failure of DNA damage repair. Nuclear extracts from drug re-fed mice showed a decrease in Dnmt-3B protein, as was predicted by the microarray results. Tissue cultures of liver cells from drug-primed mice formed MDBs spontaneously, and TSA (a deacetylase inhibitor) prevented MDB formation, whereas 5-azacytidine, a transmethylase inhibitor, did not[3].

When microarray analysis was carried out on the livers of mice fed DDC for 10 wk and re-fed DDC for 7 d after 1 mo of drug withdrawal, the number of changes in gene expression in the liver was remarkable (3343 genes). The change in expression was completely reversible after drug withdrawal for 1 mo[4]. Almost all of the KEGG functional pathways were up-regulated, especially cell adhesion molecules, actin cytoskeleton, the Toll-like receptor signaling pathway, cytokine-cytokine interaction, and the NFκB signaling pathway. The largest increases in expression were in FAT10 (119 fold), alpha fetal proteins (68 fold) and two growth factors, Ctgf and Gadd 45 g. Western blots confirmed the increased expression of Ctgf and Gadd 45 g. H1F-1 alpha, measured by means of Western blot of nuclear extracts, showed a decrease in HIF-1 alpha both in the DDC- fed for 10 wk and in the DDC-re-fed. Gene mining showed that Sirt 3, (a deacetylase) expression, was down regulated by DDC re-feeding. By Western blot, the expression of HDAC was reduced by DDC feeding. HDAC-1 was increased in the nuclei of FAT10-positive/MDB-forming hepatocytes induced by DDC re-feeding, as indicated by immunofluorescent antibody staining (Figure 1).

H3K9ac was increased by DDC feeding and refeeding. SAMe did not affect the H3K9ac response to D DC refeeding[5]. The same was true for the decrease in H3K18ac. SAMe also did not prevent the down regulation of Sirt-3 caused by DDC feeding. The histone acetyltransferase (GCN5) levels were increased after DDC refeeding but SAMe did not prevent this. On the other hand, feeding SAMe prevented the decrease in H3K9me^3 and H3K4m^3 caused by DDC refeeding. H3K9me3 causes gene silencing and H3K4me3 causes gene up regulation globally. SAMe prevented the down regulation of the histone methyltransferase SUV39H1 and the up regulation of the histone methyltransferase SET 7/9. The former methyl transferase methylates H3K9 and the latter methylates H3K4. DDC refeeding increased the expression of the histone demethylase LSD1 but SAMe did not prevent this. SAMe prevented the up regulation of H2A ubiquitination[5]. DDC refeeding altered the expression of enzymes involved in the metabolism of methionine and these changes were prevented by SAMe[5]. Refeeding DDC, up regulated MAT2a, AMD, and Mthfr and down regulated Ahcy and Gnmt. Gnmt demethylates SAMe. Mthfr is a potent inhibitor of Gnmt.

Betaine, another methyl donor, was also protective, like SAMe, when fed to drug-primed mice refed DDC[6]. This was true as assessed by microarray analysis and KEGG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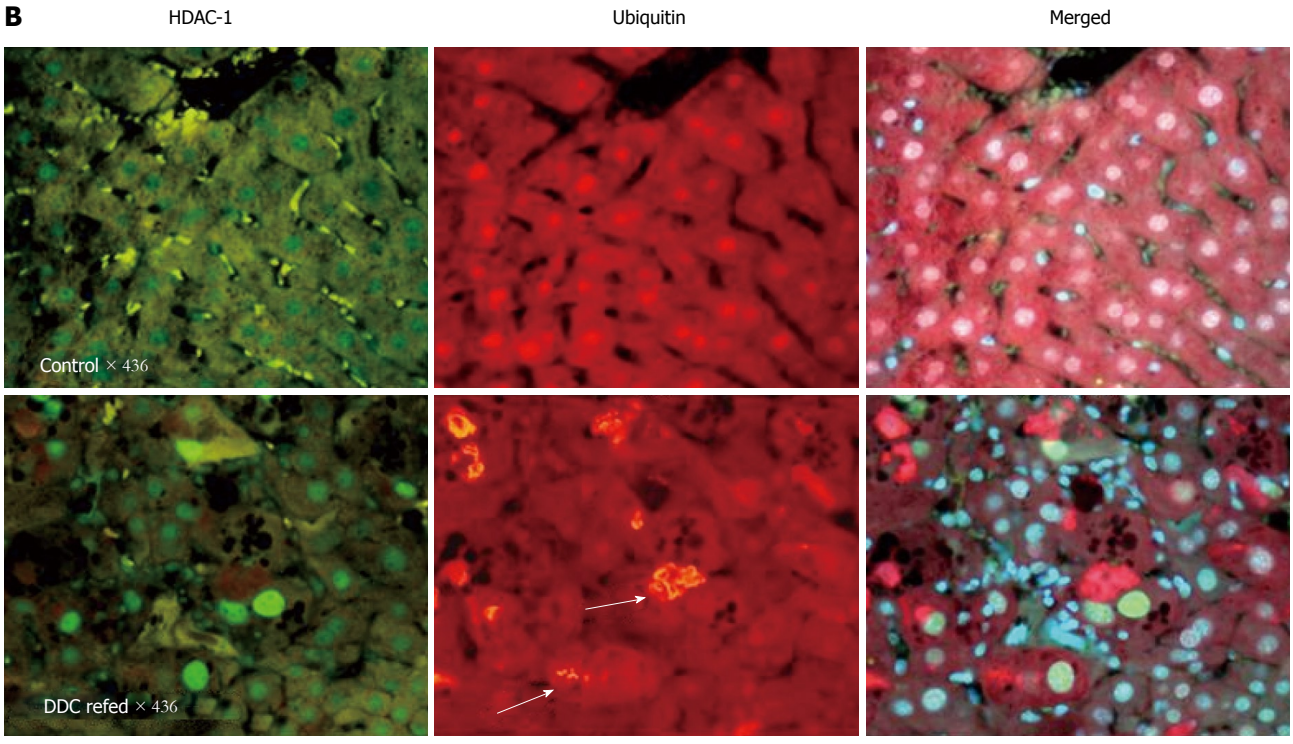
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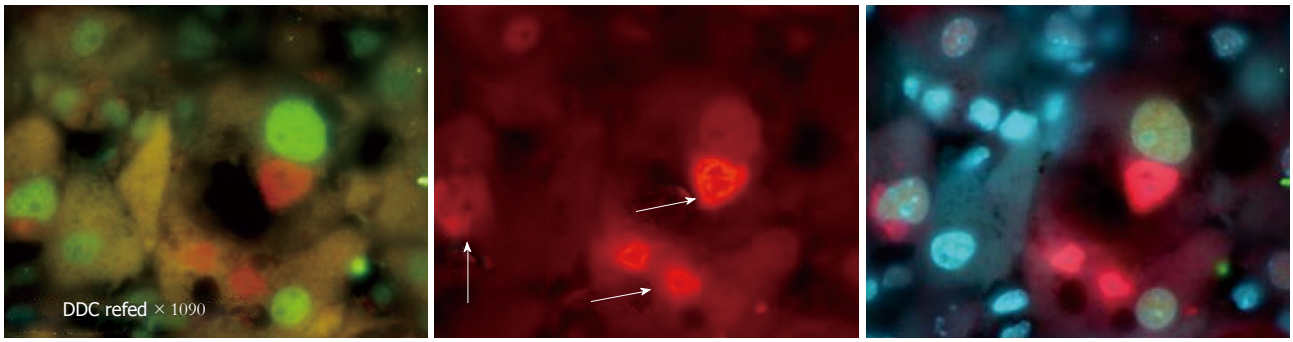

Figure 1 Mallory-Denk bodie/FAT10 positive hepatocytes were double stained with fluorescent antibodies to FAT10/histone deacetylase 1 and ubiquitin/histone deacetylase 1, showing increased nuclear staining for histone deacetylase 1. A: FAT10 positive hepatocytes (× 436); B: Hepatocytes forming ubiquitin positive Mallory-Denk bodies (× 1090). HDAC-1: histone deacetylase 1; DDC: diethyl-1,4-dihydro-2,4,6-trimethyl-3,5-pyridine decarboxylate.

functional pathways, although the effect of betaine feeding on these parameters was less dramatic. The same was true where betaine feeding prevented MDB formation induced by DDC refeeding. Betaine feeding also reduced the proliferation of FAT10 positive cells induced by DDC refeeding. This was correlated with the prevention of the up regulation of FAT10 mRNA expression induced by DDC. Betaine feeding also markedly reduced the replication of FAT10 positive MDB forming hepatocytes when DDC was refed[6].

Betaine feeding also prevented the effect of DDC refeeding on the metabolism of methionine[6]. For instance, betaine partially prevented the increase in the expression of Mhtfr induced by DDC refeeding. Mhtfr elevation inhibits Gnmt activity[7]. Betaine plus DDC refeeding increased the expression of MAT1a[6]. It prevented the decrease in expression of Gnmt, Ahcy and Bhmt caused by DDC refeeding. Bhmt converts homocysteine to methionine and reduces the levels of homocysteine. It partially prevented the decrease of AMD-1 caused by DDC refeeding[6]. Betaine feeding also prevented the decrease in SAH levels caused by DDC refeeding[6]. SAH inhibits the methylating activity of Gnmt. It is likely that betaine prevents MDBs by preventing the changes in methionine metabolism, and consequently the reduced methylation caused by DDC refeeding.

SHIFT FROM THE 26S PROTEASOME TO THE IMMUNOPROTEASOME

When DDC is refed, the activity of the 26s proteasome is decreased by the shift of the expression of the proteasome catalytic subunits from the 26s proteasome proteins to that of the immunoproteasome[8]. The consequence of the loss of 26S proteasome activity is aggresome formation (MDBs) where the lack of protein turnover leads to accumulation of altered and ubiquitinated proteins[9,10]. Microarray analysis of the livers from DDC fed mice showed the up regulation of FAT10 and the catalytic subunits of the immunoproteasome (MECL-1, LMP2 and 7) as well as the immunoproteasome regulatory subunit PA28 alpha. The up regulation was limited to FAT10 over expressing liver cells, which formed MDBs as seen by immunofluorescence microscopy[2,3]. The FAT10 stained positive hepatocytes also over expressed LMP2 and 7 and MECL-1 when viewed by confocal microscopy[8]. Thus only the MDB forming cells and not the intervening hepatocytes had shifted from the 26s proteasome to the immunoproteasome. When liver homogenates from control mice, DDC fed, DDC withdrawn 1 mo and DDC refed 7 d were assayed by Western blot there was an increase in the expression of LMP2 and 7 and MECL-1 only in the group fed DDC for 10 wk and in the group fed DDC for 10 wk, withdrawn from DDC 1 mo and then refed DDC for 7 d. This contrasted with the Western blot results for the 26s proteasome catalytic subunit B5. B5 protein (chymotrypsin-like subunit) was decreased when DDC was fed or refed. The effect of DDC feeding and refeeding on the 26s proteasome chymotrypsin-like catalytic activity was tested[8]. DDC feeding for 10 wk caused a loss of activity which returned to control levels after 1 mo withdrawal. The activity was again reduced when DDC was refed 7 d. SAMe fed with DDC refeeding prevented the loss of the 26s proteasome chymotrypsin-like activity[8]. The accumulation of polyubiquitinated proteins occurred 10 wk after DDC feeding and also after 7 d of DDC refeeding[8]. Again, SAMe fed with DDC refeeding prevented the accumulation of polyubiquitinated proteins caused by DDC. The results clearly indicated that DDC feeding or refeeding caused a switch from the 26s proteasome, which was down regulated, to the immunoproteasome which was up regulated. The immunoproteasome increased at the expense of the 26s proteasome in the MDB forming hepatocytes. The switch was completely prevented when SAMe was fed with DDC. This result was true for the catalytic subunits LMP2 and 7 and MECL-1. When qRT-PCR was done to assay the gene expression of LMP2 and 7 and MECL-1, the increases in the expression of these genes was also prevented by SAMe feeding[8].

The question was then addressed, what was the mechanism that drives the switch in the proteasome expression? The fact that SAMe prevented the switch indicated that an epigenetic mechanism was responsible. However, it remained to be determined as to what changes in gene expression induced the switch. An increase in tumor ne-

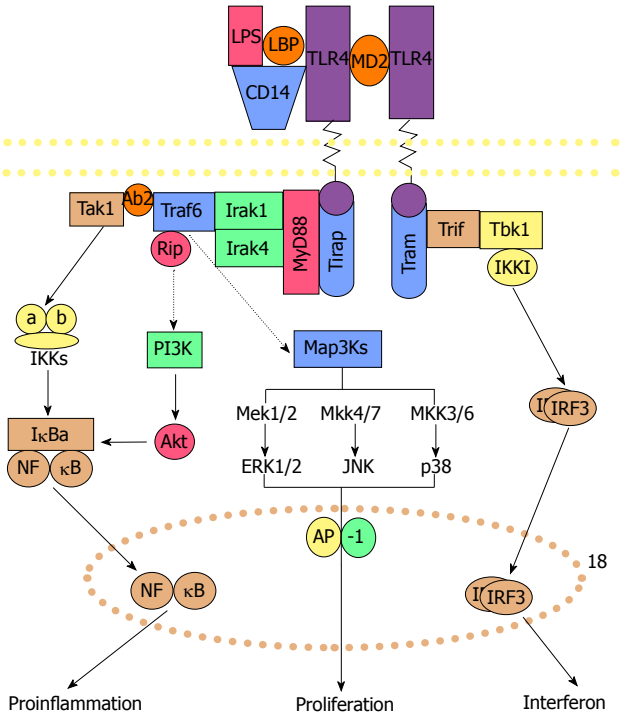

Figure 2 Diagram of the Toll-like recepter 4 signaling pathway modified from Wikipedia Commons.

crosis factor α (TNFα) and interferon γ (IFNγ) expression stimulates the expression of LMP2 and FAT10[11]. Because of this, the gene expression of the TNFα and IFNγ receptors (IFNγR) was measured by quantitive polymerase chain reaction[8]. The expression of IFNγR1 and IFNγR2, and TNFR2 and 21a were markedly increased by DDC refeeding. SAMe fed with DDC refeeding completely prevented these changes. The expression of TNFα was markedly increased by DDC refeeding and SAMe prevented the increase when fed with DDC refeeding[8]. IFNγ added to primary hepatocyte cultures from DDC-withdrawn mice induced a 4-fold increase in the number of hepatocytes that formed MDBs *in vitro*[8].

Further *in vitro* studies on the mouse Hepa1-6 cell line were done to test whether TNFα and IFNγ would induce the expression of the immunoproteasome catalytic subunits and FAT10[12]. It was found that IFNγ but not TNFα alone induced the expression of FAT10, LMP2 and 7 and MECL-1. However, there was a further synergistic increase in the expressions of FAT10, LMP2 and 7 and MECL-1 when both IFNγ and TNFα were added to the culture. The same response was found when downstream phosphorylation of STAT3 and pSTAT1 was measured by Western blot. TNFα, IFNγ and the combination of TNFα and IFNγ activated D1 on the promoter of FAT10. The ISRE sequence (interferon sequence responsive element) was involved in the increased expression of FAT10. In the absence of the D1 ISRE sequence, the treatment with the TNFα-IFNγ co-treatment could not induce the activity of the promoters D2 and D3. The Hepa1-6 cells were subjected to long term treatment with TNFα and IFNγ to see if MDBs would form *in vitro* in response to these cytokines. MDB-like aggresomes formed, beginning at 21 d of culture. The aggresomes stained positive for FAT10, ubiquitin and CK8, as is characteristic for MDBs[12]. In summary: the data supports the hypothesis that proinflammatory cytokines are responsible for up regulation of the immunoproteasome and consequently, down regulation of the 26s proteasome, which causes the accumulation of undigested proteins and MDB formation in FAT10 positive hepatocytes.

CHRONIC ACTIVATION OF TOLL-LIKE SIGNALING PATHWAYS LEADS TO PROINFLAMMATORY AND GROWTH PATHWAYS WHICH INDUCE MDB FORMATION

An explanation for the increase in TNFα and IFNγ stimulation of the proinflammatory and cell growth response that leads to MDB formation is needed. TNFα and IFNγ activate the Toll-like receptor (TLR) signaling pathway[13-15]. We therefore investigated this possibility in our model. Mice refed DDC developed an up regulation of the expression of both TLR 2 and 4 as indicated by qPCR[16]. SAMe feeding prevented the up regulation of both TLRs by DDC refeeding. Western blot confirmed the increase in TLR 4 and 2 induced by DDC refeeding and the prevention by SAMe. Downstream components of the TLR signaling pathway were also up regulated, including MyD88 and TRAF-6. SAMe feeding prevented both of these up regulations[16]. To test if the proinflammatory response was activated by DDC refeeding, interleukin (IL)-1 beta was measured by Western blot. DDC markedly increased the expression of IL-1 beta and, conversely, feeding SAMe prevented this[16]. CD-14, which binds lipopolysaccharide (LPS) at the TLR 4 receptor, was also up regulated by DDC refeeding and this was prevented by SAMe feeding. These increased gene expressions would increase the response of the TLR signaling pathway to LPS in the DDC-fed mice.

How would the increase in the proinflammatory signaling pathway and growth of the FAT 10 positive hepatocytes cause the formation of MDBs? The TLR pathway activity stimulates both activation of NFκB mediated proinflammatory response and the AP-1 mediated growth response, as illustrated in Figure 2.

NFκB activation is increased in response to DDC feeding both *in vivo* and in primary hepatocyte cultures when MDBs form[17-20]. Activation of p38, pERK and JNK pathways have been shown to be up regulated *in vitro* when MDBs were formed by DDC-withdrawn hepatocytes in primary culture[20-21]. JNK, p38 and ERK activate AP-1 (Figure 2) which leads to cell proliferation. It has been shown that DDC feeding also causes AP-1 activation[19].

Thus both the proinflammatory pathways and growth pathways are activated as a consequence of TLR signaling,

which is enhanced by TNFα and IFNγ generated by NFκB activation. Growth of FAT10 positive cells results when AP-1 is activated. MDBs are formed in FAT10 positive hepatocytes as a result of the IFNγ stimulated switch in the expression of the catalytic subunits from the 26s proteasome to the immunoproteasome.

CONCLUSION

There are two major components involved in the mechanism of MDB formation. The first component is the switch of the metabolism of methionine away from the S-adenosylmethionine-methyltransferase activity pathway to the decarboxylated S-adenosylmethionine pathway[10] and by down regulating Gnmt expression which catalyzes SAMe utilization in the methyltransferase methylation of histones H3K4 and H3K9[5] and DNA[3]. Gnmt activity was also inhibited by the upregulation of Mthfr which is a potent inhibitor of Gnmt[6]. SAMe[5] and betaine[6] both prevented this shift in methionine metabolism, as well as the epigenetic changes in histone and DNA methylating enzymes induced by DDC refeeding. This argues strongly in favor of the epigenetic changes playing a major role in the mechanism of MDB formation.

The second component involves the methyl donors S-adenosylmethionine and betaine. Both prevented the shift from the 26s proteasome to the formation of the immunoproteasome[8], and prevent the up regulation of the TLR 2/4 signaling pathways[16]. Consequently MDB formation was prevented[2,6]. Blocking the up regulation of the TNFα and IFNγ receptor up regulation[16] and TLR2/4 signaling prevented the activation of the NFκB and API up regulation of growth and proinflammation genes.

In conclusion, since both the methyl donors SAMe and betaine were proven to be effective in blocking the two mechanisms involved in MDB formation and the associated growth and proinflammatory gene expression, both donors should be effective in preventing MDB formation in liver in tumor formation in patients if used in clinical trials in the prevention and treatment of alcoholic liver disease.

ACKNOWLEDGEMENTS

The authors thank Adriana Flores for typing the manuscript.

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S- Editor Zhang HN **L- Editor** Herholdt A **E- Editor** Liu N

World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
doi:10.4254/wjh.v2.i8.302

World J Hepatol 2010 August 27; 2(8): 302-310
ISSN 1948-5182 (online)

REVIEW

Gender-related variations in iron metabolism and liver diseases

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Received: May 17, 2010 Revised: June 30, 2010
Accepted: July 7, 2010
Published online: August 27, 2010

Abstract

The regulation of iron metabolism involves multiple organs including the duodenum, liver and bone marrow. The recent discoveries of novel iron-regulatory proteins have brought the liver to the forefront of iron homeostasis. The iron overload disorder, genetic hemochromatosis, is one of the most prevalent genetic diseases in individuals of Caucasian origin. Furthermore, patients with non-hemochromatotic liver diseases, such as alcoholic liver disease, chronic hepatitis C or nonalcoholic steatohepatitis, often exhibit elevated serum iron indices (ferritin, transferrin saturation) and mild to moderate hepatic iron overload. Clinical data indicate significant differences between men and women regarding liver injury in patients with alcoholic liver disease, chronic hepatitis C or nonalcoholic steatohepatitis. The penetrance of genetic hemochromatosis also varies between men and women. Hepcidin has been suggested to act as a modifier gene in genetic hemochromatosis. Hepcidin is a circulatory antimicrobial peptide synthesized by the liver. It plays a pivotal role in the regulation of iron homeostasis. Hepcidin has been shown to be regulated by iron, inflammation, oxidative stress, hypoxia, alcohol, hepatitis C and obesity. Sex and genetic background have also been shown to modulate hepcidin expression in mice. The role of gender in the regulation of human hepcidin gene expression in the liver is unknown. However, hepcidin may play a role in gender-based differences in iron metabolism and liver diseases. Better understanding of the mechanisms associated with gender-related differences in iron metabolism and chronic liver diseases may enable the development of new treatment strategies.

Key words: Alcohol; Hepcidin; Hepatitis C; Hemochromatosis; Non-alcoholic steatohepatitis

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Harrison-Findik DD. Gender-related variations in iron metabolism and liver diseases. *World J Hepatol* 2010; 2(8): 302-310 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i8/302.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i8.302

INTRODUCTION

Clinical data suggest that men and women exhibit differences regarding the progression of certain liver diseases such as alcoholic liver disease, chronic hepatitis C and non-alcoholic steatohepatitis. Sex hormones and their effect on metabolic processes and oxidative stress have been suggested to play a role in this process. Interestingly, patients with alcoholic liver disease, chronic hepatitis C or non-alcoholic steatohepatitis often display elevated serum iron and mild to moderate hepatic iron overload. Recently, alcohol, hepatitis C viral proteins and obesity have all been shown to affect the expression of the key

iron regulatory protein, hepcidin. Oxidative stress and sex-specific differences have also been postulated to be involved in the regulation of hepcidin expression by alcohol in the liver. However, it is unclear whether the pathophysiological differences observed between men and women with chronic liver disease are associated with gender-based variances in iron metabolism. This review will highlight gender-related differences in liver diseases and iron metabolism including the role of the key iron-regulatory hormone, hepcidin.

IRON

Iron is essential for an array of key biological processes including erythrocyte production, DNA synthesis and cellular respiration[1-3]. The normal iron content of the body in an adult male is 35 to 45 mg of iron per kilogram of body weight. The majority of the iron is bound to hemoglobin in erythrocytes. Macrophages of the reti culoendothelial system supply the iron to the plasma transferrin pool to be delivered to bone marrow (~24 mg/d) for hemoglobin synthesis in red blood cell precursors[4-6]. About 20% of women, 50% of pregnant women and 3% of men do not have adequate iron stores. Based on the differences between the amount of iron available for absorption and the increased requirement for iron, most females of reproductive age, especially in the developing world, exhibit iron deficiency anemia[7]. Pregnant women require more iron due to the increasing iron demands of the growing fetus, the placenta and the elevated red cell mass of the mother[8]. However, it must also be noted that there is no regulated pathway for the excretion of iron in the body except by blood loss or desquamated intestinal cells. Parenchymal cells of the liver and reticuloendothelial macrophages serve as depots for excess iron storage. Liver not only carries the main burden of iron overload but also acts as the central organ in the regulation of body iron stores[9].

PRIMARY AND SECONDARY IRON OVERLOAD, GENDER-DIFFERENCES AND LIVER DISEASES

Hepatic iron overload is common in many liver diseases where iron is a risk factor in disease progression[10-16]. Genetic hemochromatosis (GH) is a prevalent iron overload disorder among the Caucasian population. Mutations in the Hfe gene are the main cause of primary iron overload observed in GH[14]. Patients with genetic hemochromatosis absorb more than the normal amount of iron through the intestine. Iron accumulation subsequently results in organ damage including liver injury[17,18]. GH is not a gender-specific disease. However, more males than females present with symptoms of hemochromatosis. Men accumulate more iron and have a higher incidence of liver injury. Iron overload also affects the hypothalamic-pituitary axis eventually leading to hypogonadism, exposure of sperm to oxidative injury and infertility[19]. The clinical symptoms of GH usually start later with women, possibly due to blood loss experienced with menstruation and childbirth. The majority of patients exhibiting the clinical symptoms of GH are homozygous for a Cys282-Tyr (C282Y) mutation in GH gene, Hfe[20]. Of note, a male-specific association of C282Y mutation with childhood acute lymphoblastic leukemia has also been reported[21]. The C282Y mutation inhibits the heterodimer formation of Hfe with the beta2-microglobulin (β_2M) light chain and its delivery to the plasma membrane[22]. Interestingly, female mice deficient in β_2M expression have been shown to exhibit more hepatic iron loading than male β_2M-deficient mice which is in contrast to that observed with genetic hemochromatosis patients[23]. However, it should be noted that unlike humans, female laboratory mice do not experience menstrual bleeding and live in a controlled environment. The observed sex differences in β_2M-deficient mice may be due to a possible protective effect of the Y chromosome or to hormonal differences[23].

β-thalassemia is a genetic hematological disorder whereby repeated blood transfusions and dysregulated iron homeostasis lead to secondary iron overload[24,25]. Distinct from GH, patients with β-thalassemia also exhibit iron deposition in the pituitary gland and hypothalamus[26,27]. Thalassemic males develop hypogonadotropic hypogonadism whereas females have amenorrhea due to pituitary and gonadal damage caused by iron overload[26,28]. However, paternity has been shown to be less common in males including those with normal sperm counts[27-29].

Patients with non-hemochromatotic liver diseases such as chronic hepatitis C, alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) frequently display an increase in serum iron values and mild to moderate elevation of hepatic iron concentrat ion[11-13,15,30-37]. Studies with HCV-infected chimpanzees also demonstrate that the viral infection leads to an increase in body iron levels[38]. Furthermore, as shown by *in vitro* studies, iron alters HCV replication[39,40]. In male patients with chronic hepatitis C over 50 years of age, iron has been implicated to be a fibrinogenetic factor in comparison to female patients of the same age[41]. Menstruating or iron deficient women with chronic hepatitis C have been reported to have a slower rate of disease compared to men of comparable age and women with normal iron status[42]. Population-based studies indicate differences in HCV clearance rates and the severity of disease between men and women[43-45]. IL-10 promoter polymorphisms have also been postulated to be associated with gender susceptibility to HCV infection[46].

In vivo whole-body retention studies have demonstrated a two-fold increase in intestinal iron absorption in chronic alcoholics[13]. Recently, even mild to moderate alcohol consumption has been shown to elevate the indices of iron stores[12]. Experimental animal models of ALD have also been reported to exhibit increased iron content in Kupffer cells which leads to the activation of the

transcription factor, nuclear factor-kappa (NF-κB), and increased expression of the proinflammatory cytokine, tumor necrosis factor-alpha (TNF-α)[47,48]. These effects are abolished by iron chelation, thereby indicating a role for iron-mediated cell signaling in the pathogenesis of experimental alcoholic liver disease[49]. There are sex-specific differences in the metabolism and elimination of ethanol both in humans and rodents[50,51]. The rates of ethanol elimination are higher in women[50]. The activity of the alcohol metabolizing enzyme, alcohol dehydrogenase (ADH) in rodent livers is elevated in females and castration of males increases ADH activity[52]. Moreover, men with prostatic metastatic carcinoma who have undergone therapeutic orchiectomy have been shown to exhibit an increase in ethanol elimination[53]. Clinical studies demonstrate that females exhibit a greater susceptibility to alcohol-induced liver injury than men[54]. Estrogens, endotoxin and inflammatory processes have been suspected to play a role. However, it is unknown whether there is any association between iron and the gender-related differences observed in alcohol-induced liver injury. Alcohol suppresses the expression of the key iron regulatory molecule, hepcidin in the liver, which leads to an increase in duodenal iron transport[55-58]. Interestingly, male mice have been reported to display significantly lower hepcidin expression compared to female mice following acute alcohol exposure[55].

NAFLD is the hepatic manifestation of metabolic syndrome[59-61]. NAFLD ranges from benign steatosis to nonalcoholic steatohepatitis (NASH) which is differentiated by histopathologic evaluation[62]. NASH is the severe manifestation of disease which can lead to liver fibrosis and hepatocellular carcinoma[63,64]. Increased iron stores have been reported in NAFLD/NASH[15,36,37,65,66]. However, the relevance of iron accumulation in disease progression is unclear[15,36,37,65,66]. Excess hepatic iron is postulated to cause insulin resistance[16,67]. Interestingly, iron depletion *via* phlebotomy in patients with NAFLD has been shown to have a positive effect on insulin resistance and to reduce serum TNF-α levels[68,69]. Serum ferritin levels are also positively associated with BMI and serum glucose levels[70-73]. However, it should be noted that ferritin is an acute phase protein and may not accurately reflect the extent of iron overload in NAFLD. There is a relationship between gender and NAFLD. However, the data from several studies are conflicting regarding the prevalence of NAFLD among men and women[74-79]. Population-based studies suggest a protective role for endogenous estrogens in non-alcoholic hepatic steatosis[80]. The prevalence of NAFLD increases in women over 50 years of age[81]. Interestingly, the deletion of histone variant macroH2A1 which is enriched on the inactive X-chromosome in females has been postulated to cause female-specific steatosis in mice[82].

IRON REGULATORY PROTEINS

Since there is no physiological pathway of excretion for excess iron in the body, the uptake, transport and storage of iron must be tightly regulated. Divalent metal transporter 1 (DMT1), a multi-transmembrane protein, is responsible for importing dietary non-heme iron through the apical site of absorptive enterocytes in the duodenum[83,84]. Conversely, the iron transporter ferroportin is responsible for exporting iron into the circulation[85]. The ferroportin Q248H polymorphism is associated with increased serum ferritin levels in Sub-Saharan Africans and African Americans[86]. The frequency of ferroportin Q248H polymorphism has been reported to be higher in African American males with elevated serum ferritin levels compared to those with normal serum ferritin. However, these differences were not observed among African American women. Furthermore, men with elevated serum ferritin were three times more likely to have Q248H polymorphism than women with elevated serum ferritin[86].

In the duodenum, the basolateral transport of iron from the enterocytes into the bloodstream also requires hephaestin, a transmembrane-bound multicopper ferroxidase[87,88]. Like its homolog ceruloplasmin in the liver, hephaestin also links copper and iron metabolism[89]. Sex-linked anemia is an X-linked inherited iron deficiency anemia, first observed in the male descendants of an irradiated mouse[90]. Sex linked anemia (*sla*) mice are impaired in intestinal iron transport and contain a deletion in *Heph* gene yielding a truncated hephaestin protein[87].

In the plasma, iron circulates by binding to the glycoprotein, transferrin[91]. There are different glycosylated forms of transferrin which are different in the number of N-linked oligosaccharide chains[92,93]. Heavy alcohol drinkers display abnormal serum transferrin profile[94,95]. Males with high alcohol intake have been shown to display higher amounts of disialotransferrin in the serum when compared to females. There are no gender-related differences in serum disialotransferrin levels between nondrinker males and females[96]. Iron-bound transferrin is taken up into the cell by transferrin receptors 1 and 2 (TrfR1, TrfR2)[97,98]. TrfR1 is ubiquitously expressed whereas TrfR2 is mainly expressed in the liver[98]. The regulation of iron metabolism involves multiple organs including the duodenum, liver and bone marrow. Hepcidin is the iron-regulatory hormone which mediates iron homeostasis between these distant organs[2,99].

Hepcidin is a circulatory antimicrobial peptide, synthesized in the hepatocytes of the liver as an 84 amino acid precursor protein[100,101]. It is subsequently cleaved into the 25 amino acid cysteine-rich mature (biologically active) peptide form[102,103]. Hepcidin achieves the regulation of iron homeostasis by binding to the iron exporter ferroportin and thereby inhibiting the iron trans port in the duodenum and the release of iron from reticuloendothelial macrophages (Figure 1)[104]. During pregnancy, iron is transferred from the mother to the fetus and hepcidin regulates maternofetal iron transport across the placenta[105]. Transgenic mice studies have confirmed the role of hepcidin in the regulation of iron metabolism[106,107]. Hepcidin synthesis in the liver is

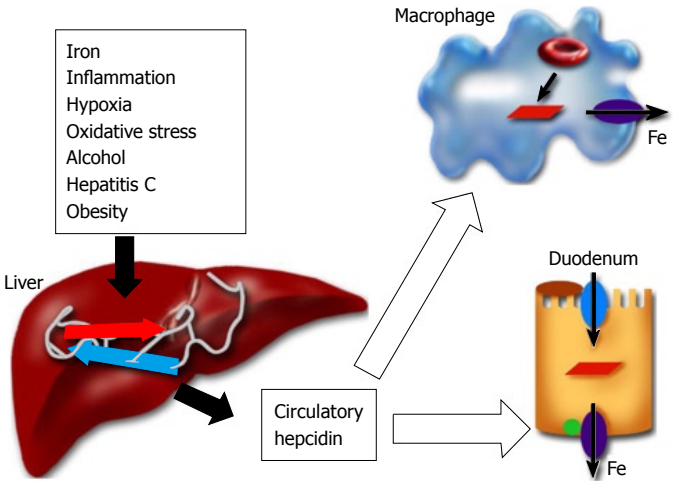

Figure 1 Regulation of hepcidin and iron metabolism. The key iron-regulatory hormone hepcidin is primarily synthesized in the parenchymal cells of the liver and is subsequently released into the circulation. The transcriptional regulation of hepcidin in the liver is regulated by various factors. Hepcidin released into the circulation in turn regulates iron metabolism by controlling the iron transport in the duodenum and iron export in the macrophages. Hepcidin achieves this by binding to the iron exporter protein ferroportin and inducing its internalization and degradation.

sensitive to body iron levels; increasing with iron overload and decreasing in the case of iron deficiency[2]. Hepcidin levels in humans have been reported to correlate with the liver iron concentration and the parameters of hepatic function (e.g. serum albumin)[108]. Furthermore, inflammatory signals and the inflammatory cytokines IL-1 and IL-6 elevate hepcidin expression in the liver[109,110]. Conversely, hypoxia and anemia down-regulate hepcidin expression[111]. The decrease in hepcidin expression in the liver leads to increased iron absorption through the duodenum and the mobilization of iron from reticuloendothelial stores to meet the demands of erythrocyte production[2]. The synthesis of hepcidin in the liver is modulated by upstream regulators. Transferrin receptor2, Hfe, the juvenile hemochromatosis gene product, Hjv, and bone morphogenetic protein 6 are positive regulators of hepcidin expression[112-118]. On the other hand, TMPRSS6 (matriptase 2), a transmembrane serine protease, is the negative regulator of liver hepcidin expression[119,120]. Patients expressing TMPRSS6 mutations exhibit iron-refractory iron deficiency anemia due to elevated hepcidin production[119].

HEPCIDIN, SEX DIFFERENCES AND LIVER DISEASES

Human hepcidin gene (*HAMP, HEPC*, OMIM 606464) is located on the long arm of chromosome 19 at position 13.1[2,100]. Unlike humans or rats, mice have 2 hepcidin genes, *hepc1* and *hepc2*, and both genes are located on mouse chromosome 7[106,121]. Hepcidin expression in the liver has been reported to differ by gender[122]. Female mice express significantly higher hepcidin levels in the liver than males[122,123]. Both hepcidin1 and hepcidin2 respond to iron. The higher level of hepcidin expression in female mice is also associated with elevated liver and spleen iron concentrations[122,123]. However, it is unclear whether the elevated expression of hepcidin in female mice is due to the increase in iron stores. It is also not known whether women and men differ in the level of hepcidin expression in the liver. Women usually have lower iron stores than men mainly due to the physiological loss of blood. A study utilizing enzyme-linked immunoabsorbent assay reported lower serum hepcidin levels in healthy female volunteers compared to those measured in males[124]. The level of serum hepcidin has been postulated to correlate with that of serum ferritin levels[124]. However, it should be noted that besides iron, hepcidin is also regulated by other stimuli which may also play a role in sex-specific expression of hepcidin in the liver.

Accumulating evidence suggests hepcidin as the modifier gene in genetic hemochromatosis. Hepcidin mRNA expression is reduced in patients with GH and in Hfe knockout mice[125,126]. Some patients with Hfe C282Y homozygosity have been reported to carry additional mutations in hepcidin gene (*HAMP*)[127-129]. GH patients subjected to acute oral iron challenge have been shown to display a blunted hepcidin response compared to healthy control subjects[130]. Constitutive expression of hepcidin has been shown to prevent iron overload in Hfe knockout mice[131]. Hepcidin is also altered in other non-Hfe-related forms of hemochromatosis. Hemochromatosis patients harboring mutations in transferrin receptor 2 gene have lower urinary hepcidin levels[112]. Mutations in the hepcidin gene and the juvenile hemochromatosis gene, hemojuvelin (*Hjv*), have been identified in juvenile hemochromatosis patients[114,132]. In contrast to hepcidin, *Hjv* does not respond to iron levels but its inactivation results in hepcidin deficiency[114,132-134]. *Hjv* acts as a bone morphogenic protein (BMP) co-receptor[118]. Furthermore, BMP6 regulates hepcidin expression[115,116].

Hepcidin expression is also altered in other liver diseases. Patients with alcoholic liver disease or chronic hepatitis C and animal models of alcohol and HCV display reduced hepcidin expression[35,56-58,135,136]. Hepcidin has been reported to be expressed in adipose tissue and the expression was increased in obese patients; correlating with the body mass index (BMI)[137]. The pathogenesis of nonalcoholic steatohepatitis is associated with insulin resistance and metabolic syndrome[59,60,79]. However, hepcidin expression in the livers of these patients was unchanged[137]. High levels of leptin accompany insulin resistance which is suggested to play a role in the progression

of NAFLD to NASH[138-140]. Interestingly, an *in vitro* study performed with Huh7 human hepatoma cells showed that the adipokine, leptin, increased the expression of hepcidin through the Jak2/Stat3 signaling pathway[141].

The liver is sensitive to the action of sex hormones including estrogens[142-144]. There is some evidence that estrogens can increase the production of reactive oxygen species in the liver[145]. Recently, oxidative stress has been reported to regulate hepcidin transcription in the liver[55]. It is therefore possible that estrogens may play a role in sex-specific regulation of hepcidin expression in the liver. A study of patients with chronic hepatitis reported higher c-myc expression in the livers of patients in which the liver expressed a variant form of the estrogen receptor that exhibits constitutive transcriptional activity compared to patients in whom the liver expressed wild type estro gen receptor[145,146]. Estrogen has also been reported to cause c-myc overexpression in hamster kidneys[147]. c-myc belongs to the basic helix-loop-helix/leucine zipper (bHLH/zip) family of transcription factors which also includes upstream stimulatory factor (USF) and transcription factor E (TFE)[148]. These transcription factors bind to E-Box motifs in the regulatory elements of promoter sequences of target genes[148]. Hepcidin genes are located directly downstream of the *Usf2* gene. An involvement of USF1, USF2 and c-myc in the transcriptional regulation of human and mouse hepcidin genes has been postulated[106,107,149]. Moreover, the mutation of E-box motifs in the human hepcidin gene promoter has been shown to abolish the transcriptional regulation by USF1, USF2 or c-myc[149]. However, it remains to be seen whether estrogen plays a role in the transcriptional regulation of hepcidin and in sex-based differences observed regarding the expression of hepcidin in the liver.

CONCLUSION

Iron is essential for many biological processes. However, excess iron is harmful and can lead to tissue injury. The liver acts as a storage depot for iron and plays a central role in the regulation of iron metabolism. The key iron re gulatory hormone, hepcidin, is synthesized in the liver. Genetic hemochromatosis (GH) is a prevalent iron overload disorder in the Caucasian population. Patients with non-hemochromatotic liver diseases such as alcoholic liver disease, chronic hepatitis C and non-alcoholic steatohepatitis also frequently exhibit evidence of iron overload. Hepcidin is suggested to play a role in GH and has been shown to be modulated by alcohol, hepatitis C viral proteins and obesity. Genotypic and sex differences have been shown to be involved in the regulation of liver hepcidin expression in mice. Men and women exhibit clinical differences in the severity of various liver diseases. Women of childbearing age usually have lower iron stores compared to men mainly due to the physiological loss of blood. However, an association between body iron levels and the gender-specific differences observed in the progression of chronic liver diseases has yet to be established. Gender-specific regulation of hepcidin synthesis in the liver may play a role in this process. Further understanding of the mechanisms underlying the gender-based differences in the pathophysiology of chronic liver diseases may lead to the development of novel diagnostic markers and treatment strategies.

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S- Editor Zhang HN **L- Editor** Roemmele A **E- Editor** Liu N

World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
doi:10.4254/wjh.v2.i8.311

World J Hepatol 2010 August 27; 2(8): 311-317
ISSN 1948-5182 (online)

ORIGINAL ARTICLE

Effect of mycophenolate mofetil plus adriamycin on HepG-2 cells

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Received: February 23, 2010 Revised: August 12, 2010
Accepted: August 17, 2010
Published online: August 27, 2010

Abstract

AIM: To investigate the influence of mycophenolate mofetil (MMF) plus adriamycin (ADM) on hepatocellular carcinoma (HCC) cells.

METHODS: HCC cells were treated with 100 μg/ml of MMF alone (MMF group), 1 μg/mL of adriamycin (ADM group) alone, or a combination of the drugs (MMF + ADM group). We performed an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay to measure the growth inhibition rate of HCC cells. Flow cytometry was used to determine the percentage of cells in different phases of the cell cycle and the number of apoptotic cells. Hoechst 33258 staining revealed the morphological changes associated with apoptosis in HCC cells.

RESULTS: The results of MTT assays revealed that monotherapy with ADM or MMF showed inhibition of cell growth, while MMF + ADM therapy afforded an inhibition rate of more than 90% with cell distribution in G1 and G2/M phase greater than that in S phase. MMF + ADM treatment also downregulated Bcl-2 expression markedly. The growth of HCC cells was markedly inhibited and apoptosis was enhanced in all the 3 groups. Compared with other 2 groups, the MMF + ADM group showed more obvious apoptosis of cells.

CONCLUSION: The MMF plus ADM combination exerts remarkable inhibitory effects on the growth of HCC cells.

Key words: Mycophenolate mofetil; Adriamycin; Hepatocellular carcinoma; Cell cycle; Apoptosis

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Chu YK, Liu Y, Yin JK, Wang N, Cai L, Lu JG. Effect of mycophenolate mofetil plus adriamycin on HepG-2 cells. *World J Hepatol* 2010; 2(8): 311-317 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i8/311.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i8.311

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most wide spread malignant diseases. It is currently the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death. Therefore, finding new strategies for treatment of HCC is an important objective. Many methods have been established to treat HCC including surgery, chemotherapy with new antitumor drugs, interventive therapy, liver transplantation.

Among these treatments, liver transplantation has been considered as one of the most curative options for HCC. It is reported that the current 1- and 5-year survival rates for HCC patients undergoing orthotopic liver transplantation are 77.0% and 61.1%, respectively. The 5-year survival rate has steadily improved from 25.3% in 1987 to 61.1% during the most recent period studied. Liver transplantation is now considered to be an effective method for the treatment of HCC, although there is still a significant rate of recurrence after transplantation.

Patients who have received immunosuppression following organ allotransplantation display an increased incidence of de novo neoplasms. Studies have identified some definite risk factors for malignancy, namely: aging, the quantity/quality of the administered immunosuppressive drugs. Mycophenolate mofetil (MMF) is a potent, reversible, noncompetitive inhibitor of leukaryotic inosine monophosphate dehydrogenase (IMPDH). This drug has the ability to inhibit T- and B-cell proliferation through the de novo pathway for synthesis of guanosine nucleotides. Mycophenolic acid has antiproliferative activity *in vitro* against a variety of tumor-cell lines and *in vivo* against murine leukemias, lymphomas, and solid tumors. A serious concern is whether the increased power of immunosuppressive combinations administered to patients who have had organs transplanted has now led to an increased risk for tumor development.

Adriamycin is a hydroxy derivative of Daunorubicin, a commonly and widely used antitumor drug. In order to explore whether the mycophenolate mofetil and adriamycin have antiproliferative activity on hepatocellular carcinoma cells and possible clinical application in cancer therapy, we examined the anti-proliferation effects of mycophenolate mofetil with adriamycin on hepatocellular carcinoma cells *in vitro*.

MATERIALS AND METHODS

Materials

HepG-2 cell line was provided by Experimental Facility Core of Tangdu Hospital, The Fourth Military Medical University, China.

We purchased RPMI-1640 from GIBCO (USA); calf serum (CS) (Sijiqing biological product company, Hangzhou, China); mycophenolate mofetil (MMF) from Shanghai Company; and Hoechst 33258 staining solution was purchased from Xi'an Company.

Cell culture and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay

HepG-2 cells were cultured in RPMI-1640 medium (pH 7.2-7.3) supplemented with 10% inactivated fetal calf serum (FCS), 100 μg/mL penicillin and l00 μg/mL streptomycin, and incubated in humid conditions under 5% CO_2 at 37℃. Cells in the logarithmic growth phase were used for further experiments. We established 4 experimental groups as follows: MMF group (0.1-100 μg/mL), adriamycin (ADM) group (1 μg/mL), MMF (0.1-100 μg/mL) + ADM group (1 μg/mL), and control group (untreated).

HepG-2 cells in the logarithmic growth phase were collected, and 200 μL of cell suspension was dispensed at a density of 4×10^6 cells per well in 96-well plates. We maintained 6 parallel wells for each group, and cultured the cells for 0, 24, 48, and 72 h. Finally, we added 20 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) to each well and incubated the plates for 4 h. Water-insoluble formazan crystals which formed were solubilized by adding 150 μL of dimethylsulfoxide (DMSO) to each well. We determined the absorbance (A-value) of the culture solutions at 570 nm (test wavelength) and 490 nm (reference wavelength) within 20 min (i.e. before the disappearance of the colored particles). The cell growth inhibition rate was calculated according to the formula: Cell growth inhibition rate = (A-value of control group - value of experimental group)/A-value of control group × 100%.

Determination of the number of cells by flow cytometry

Cells were detached from the wells by digestion with 0.25% trypsin and then centrifuged. The cell pellets were washed twice with phosphate-buffered saline (PBS) and fixed with 70% cold alcohol for 24 h at 4℃. The solution was centrifuged again to remove alcohol. After washing with PBS, the cells were mixed with 200 μg/mL of RNase. The number of cells in each phase of the cell cycle was determined by flow cytometry (FCM). Data were processed by the MAC analytical system program.

[^{3}H]-Thymidine uptake assay

We performed [^{3}H]-thymidine uptake assays to evaluate the effect of MMF on cell growth. Briefly, cells treated with different drug concentrations were collected at various times. Then, 200 μL of cell suspension (1×10^6 cells) was dispensed in each well of the 96-well culture plates. Next, [^{3}H]-thymidine (1 μCi/well) was added and the cells were incubated for additional 6 h at 37℃. Cellular DNA was collected on a glass fiber filter by using an automatic cell collector. The glass fiber filter was dried at room temperature and placed in a thermal bag containing 10 mL of scintillation fluid. The Cpm was finally determined using β-scintillation counter.

Hoechst 33258 staining

After digestion with 0.25% trypsin, the cells treated with different drug concentrations were collected at various times, centrifuged (3000 rpm, 5 min), and dried. The cells were fixed with 40 g/L formaldehyde for 10 min, washed twice with distilled water, and then dried at room temperature. The cells were stained with Hoechst 33258 (5 mg/L), and the morphological changes were observed under a fluorescence microscope (Ex360 nm/Em450 nm).

Cell adhesion experiment

The 96-well-plate was first coated with 20 mg/L of

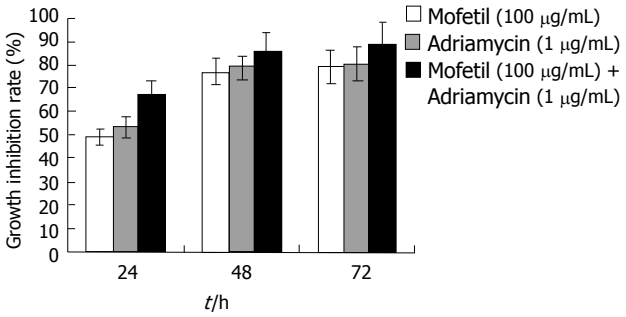

Figure 1 The inhibitory effects of mofetil + adriamycin treatment on the growth of HepG-2 cells.

fibronectin (FN) and incubated at 37℃ for 1 h; subsequently, the plate was coated with 10 mg/L of FN (Sigma, USA) and incubated at 4℃ overnight. A few wells were left uncoated and were used as negative controls. Further, the wells were washed twice with a washing buffer, blocked with a blocking buffer, and finally the plate was incubated at 37℃ for 1 h. The plate was washed again with washing buffer and frozen on ice. Then, 50 μL of cells (4×10^5/mL) were added to each well and the plates were incubated at 37℃ for 30 min. Subsequently, the plate was shaken, washed, and fixed with 4% paraformaldehyde at room temperature (RT) for 10-15 min. Finally, the plate was stained with crystal violet for 10 min, washed, dried completely, and incubated at RT for 30 min after the addition of 2% sodium dodecyl sulfate (SDS). The plate was read using a microplate reader at 550 μm. The ratio of inhibition of cell adhesion was calculated as follows: inhibition ratio = [(*A*-value of experimental group/*A*-value of BSA) - 1] × 100.

Detection of Bcl-2, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 gene expression by reverse transcriptase-polymerase chain reaction

The total RNA was extracted from cells using the RNase Mini kit (Qiagen, Valencia, CA, USA). The extracted RNA samples were analyzed by measuring the absorbance at 260 nm using a Genequant spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). Next, 1 μg of total RNA was added to a solution containing 0.5 μg/μL oligo dT, 10 mmol/L dNTP (Promega), and diethylpyrocarbonate (DEPC)-treated water (total volume: 13 μL), and this mixture was then incubated for 5 min at 65℃. Next, we prepared a mixture for reverse transcription, which included 4 μL of 5 × first-strand buffer (Promega), 1 μL of 0.1 mol/L dithiothreitol (DTT) (Promega), 1 μL of 40 U/μL RNasin (Promega), and 1 μL of 200 U/μL Superscript Ⅲ (Invitrogen). After mixing, the samples were incubated at 37℃ for 45 min, 95℃ for 5 min, and 4℃ for 5 min. Then, 2 μL of cDNA (reverse transcription product) was added to 48 μL of polymerase chain reaction (PCR) mixture containing 10 μmol/L primers for *Bcl-2*, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Invitrogen); 10 mmol/L dNTP (Promega); 10 × PCR buffer (Promega); 25 mmol/L $MgCl_2$ (Promega); 5 U/μL Go Taq (Promega); and DEPC-treated water. The primer sequences, length of the amplified product, and annealing temperature were as follows. For *Bcl-2*: forward primer, 5'-GACTGAATCGGAGATGGAGACC-3'; backward primer, 5'-CGATCCGACTCACCAATACC-3'; length of the amplified product, 380 bp; and annealing temperature, 57℃. For *ICAM-1*: forward primer, 5'-AGCCAATTTCTCGTGCCG-3'; backward primer, 5'-AGGAGTCGTTGCCATAGGTG-3'; length of the amplified product, 267 bp; and annealing temperature, 58℃. For *VCAM-1*: forward primer, 5'-TGGGAATCTACAGCACCT-3'; backward primer, 5'-AATGGTAGGGATGAAGGTC-3'; length of the amplified product, 355 bp; and annealing temperature, 50℃. For *GAPDH* (internal control): forward primer, 5'-CACCCACTCCTCTACCTTCGA-3'; backward primer, 5'-TCGTCCTCCTCTGGTGCTCT-3'; and length of the amplified product, 76 bp. The PCR products were subjected to 2% agarose gel electrophoresis and visualized using ethidium bromide (EtBr) staining. The densitometric data of PCR products was processed using the Gel-Pro Analyzer Software. The relative transcript abundance was expressed as the ratio of the target transcript level to the level of GAPDH transcripts in terms of percentage.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test with the SAS 6.12 software. The data was statistically significant if $P < 0.05$.

RESULTS

Inhibitory effects of MMF + ADM treatment on the growth of HepG-2 cells

As shown in Figure 1, treatment with MMF, ADM, and MMF + ADM inhibited the growth of HepG-2 cells in a dose- and time-dependent manner (all $P < 0.05$).

Changes in the percentages of cells in different stages of the cell cycle

In MMF 100 μg/mL group, as treatment time increased, the cell percentage at G0-G1 phase was increased and that at S phase was decreased while no obvious effect was found at G2-M phase. In addition, apoptosis rate of HCC cells was significantly higher in treatment group than control group. In, similar results was also obtained in ADM 1 μg/mL group, except that the cell percentage at G2-M phase was increased, but not unchanged. In MMF + ADM group, cell percentage at G0-G1 phase or at G2-M phase significantly increased and that at S phase significantly decreased. The apoptosis rate was dramatically increased in a time-dependent manner ($P < 0.05$) (Table 1).

Effects on cell proliferation

As shown in Figure 2, the proliferation of HCC cells in

Table 1 Effect of mycophenolate mofetil combined with adriamycin on the cell cycle distribution of HepG-2 cells

Group	*n*	G0/G1 (%)	S (%)	G2/M (%)	Apoptosis (%)
Control group	3	32.07 ± 1.32	66.20 ± 1.26	1.73 ± 0.62	2.86 ± 0.03
MMF	3	68.22 ± 1.37	27.98 ± 0.63	3.80 ± 1.03	13.34 ± 0.31
ADM	3	70.52 ± 2.52	17.53 ± 0.78	11.90 ± 0.51	21.56 ± 0.56
MMF + ADM	3	73.36 ± 2.73	12.15 ± 0.59	14.13 ± 0.38	28.33 ± 0.76

MMF: mofetil; ADM: adriamycin.

the MMF + ADM group was significantly inhibited within 72 h after treatment with a cpm value of 1892.23 ± 142 (mean ± SD), suggesting that the combination of MMF and ADM exerted a profound inhibitory effect on the proliferation of HepG-2 cells ($P < 0.01$).

Morphological changes of apoptotic cells

Treatment of apoptotic HepG-2 cells with different drugs for 24, 48, and 72 h revealed obvious morphological changes such as condensation of chromatin and nuclear fragmentations on Hoechst 33258 staining (Figure 3). In addition, after 48 h of treatment, the number of apoptotic cells in MMF + ADM group gradually increased relative to those in the other 2 groups.

Effect on cell adhesion

HepG-2 cells in logarithmic growth phase were treated with different drugs at different concentrations for 48 h. We found that the capacity for cell adhesion decreased significantly in a dose-dependent manner as shown in Figure 4 ($P < 0.05$ Figure 4).

Effect on Bcl-2 gene expression

After treating HepG-2 cells with MMF or ADM for 72 h, the ratios of *Bcl-2* gene expression of the experimental group and the control group were 0.61 ± 0.06 and 0.56 ± 0.05, respectively. Further, the ratio in MMF + ADM group was 0.38 ± 0.10, which was reduced by 32.14% as compared to the ratio in the ADM group ($P < 0.01$; Figure 5).

Effect on ICAM-1 and VCAM-1 gene expression

When the drug concentration was 100μg/mL, the ICAM-1/VCAM-1 expression ratio in the experimental group and control group was 0.42 ± 0.05 and 0.51 ± 0.03, respectively. The *ICAM-1* and *VCAM-1* gene expression was lowered in a dose-dependent manner ($P < 0.01$; Figure 6).

DISCUSSION

The outcomes of liver transplantation have shown improvement in recent years. Cherqui[1] and other researchers have reported that the survival rates of non-cancer patients and those of patients with small tumors (> 5 cm in diameter) after transplantation are almost identical(1-3 years). Annually, liver transplantation is performed on

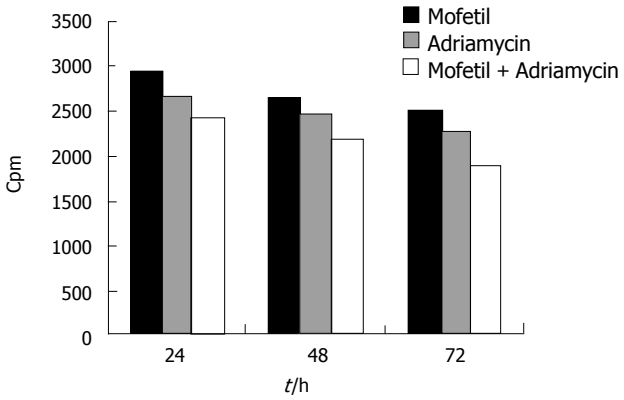

Figure 2 Effects of mofetil + adriamycin treatment on the proliferation of HepG-2 cells.

approximately 4 00 patients across China, among which 30%-40% are referred for liver transplantation of HCC. Tumor recurrence is one of the chief concerns after liver transplantation. Even though the pathological liver and the primary site of HCC are removed during liver transplantation, tumor recurrence and metastasis may occur. In 1999, Bismuth reported that the liver cancer recurrence rate was as high as 22% even after selecting optimal liver transplantation. The average life span of patients after tumor recurrence was only 15.3 mo which was significantly shorter than the life span of patients who underwent liver resection (31.6 mo). The selection of immunosuppressive drugs is one of the main factors influencing liver cancer recurrence. Theoretically, long-term use of immunosuppressive drugs potentially inhibits the immune response and natural defense, thereby contributing to tumor recurrence and growth[2,3]. However, recent reports suggest that not all immunosuppressants promote tumor growth, and in fact, a few might also exhibit anti-tumor properties.

Many immunosuppressive drugs used in clinical therapy, such as rapamycin[4,5] have been proved to possess anti-tumor effects[4]. As early as 1994, Tressler RJ[6] found that MMF possessed anti-rejection effect as well as anti-tumor effect, and MMF could inhibit the growth of Ka posi's sarcoma[7]. In addition, Tobias[8] found that MMF exerted different anti-tumor effects on HT-29 colon can cer cells and DU-145 prostate cancer cells. Liu C[9] found that MMF exerted an inhibitory effect on the proliferation of epithelial cells of the intrahepatic bile duct. However, the effect was not statistically significant, thereby suggesting that the drug exerted a stronger effect on tumor cells than on normal cells. Moreover, MMF exerted a mild toxic effect on the liver, kidneys, and bone marrow; further, MMF treatment lowered the probability of acquiring an infection. Meanwhile, fewer incidences of tumor induction have been reported in patients treated with MMF as compared to those in patients treated with cyclosporine and FK506.

ADM, which is a glucoside antibiotic, is commonly used for antitumor treatment and plays an important role in chemotherapy. It can cause necrosis by direct

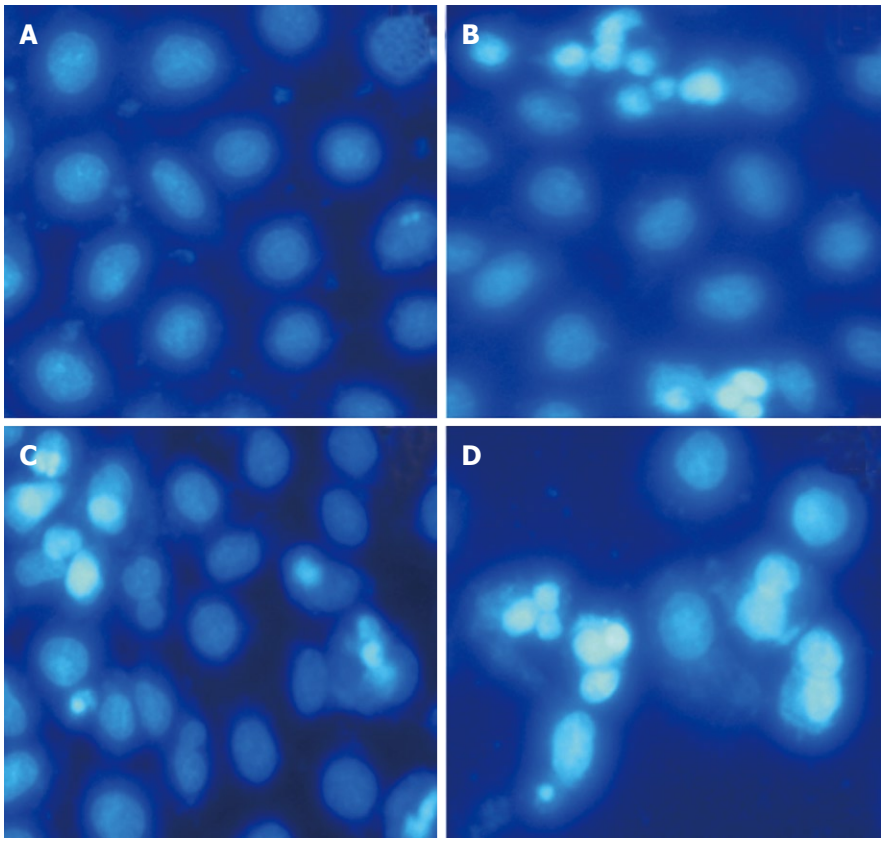

Figure 3 Morphological changes in the apoptotic cells after Hoechst 33258 staining (× 200). A: Untreated HepG-2 cells; B: HepG-2 cells treated with 100 μg/mL mofetil (MMF) for 48 h; C: HepG-2 cells treated with 1 μg/mL adriamycin (ADM) for 48 h; D: HepG-2 cells treated with MMF + ADM for 48 h.

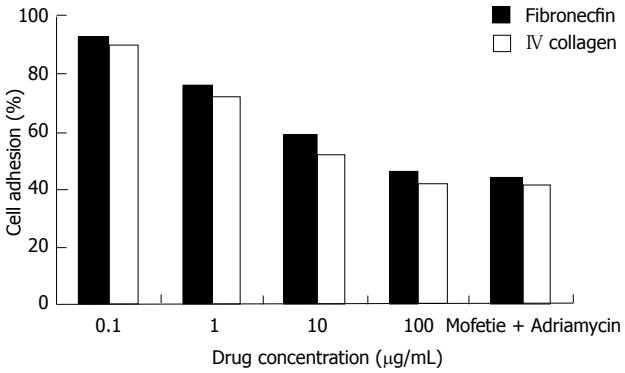

Figure 4 Effect of mofetil + adriamycin treatment on the adhesion properties of HepG-2 cells.

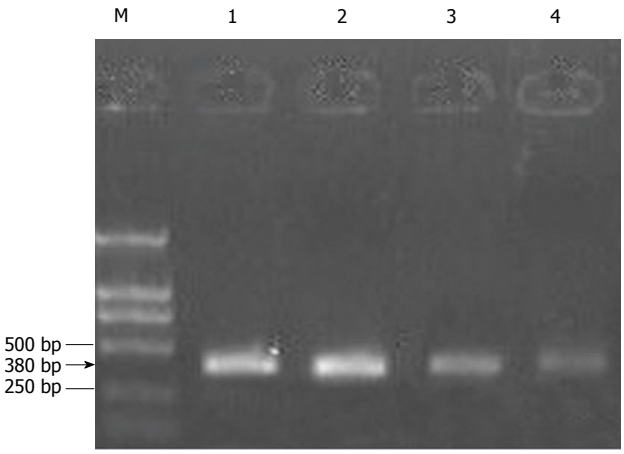

Figure 5 Effect of mofetil + adriamycin treatment on Bcl-2 gene expression in HepG-2 cells. M: Standard reference; 1: Untreated HepG-2 cells; 2: HepG-2 cells treated with 100 μg/mL mofetil (MMF) for 72 h; 3: HepG-2 cells treated with 1 μg/mL adriamycin (ADM) for 72 h; 4:HepG-2 cells treated with MMF (100 μg/mL) + ADM (1 μg/mL) for 72 h.

killing of cells or induce apoptosis mainly by affecting topoisomerase II activity. Further, ADM induces nucleopore changes in the peripheral sensory ganglion cells. However, ADM treatment exerts serious clinical adverse effects such as inhibition of bone marrow, depletion of white blood cells and platelets, toxic effects on cardiac muscles, and damage to liver and kidney. Although ADM is recommended as a chemotherapeutic drug for liver cancer, its clinical efficacy is not satisfactory due to a partial remission rate of only 16%[10,11].

To improve the efficiency of controlling tumor recurrence after liver transplantation, we evaluated the effects of MMF + ADM therapy in several *in vitro* experiments. The results of MTT assay revealed that monotherapy with ADM or MMF showed inhibition of cell growth, while MMF + ADM therapy afforded an inhibition rate of more than 90% with cell distribution in G1 and G2/M phase greater than that in S phase.

The *Bcl-2* gene (B-cell lymphoma/leukemia-2 gene) is a proto-oncogene that inhibits cellular apoptosis. *Bcl-2* exerts marked effects on the mitochondrial and pore complex signal molecules, thereby contributing to the control of signal transduction and prolonged cell life[12]. We found that MMF + ADM treatment markedly downregulated *Bcl-2* expression.

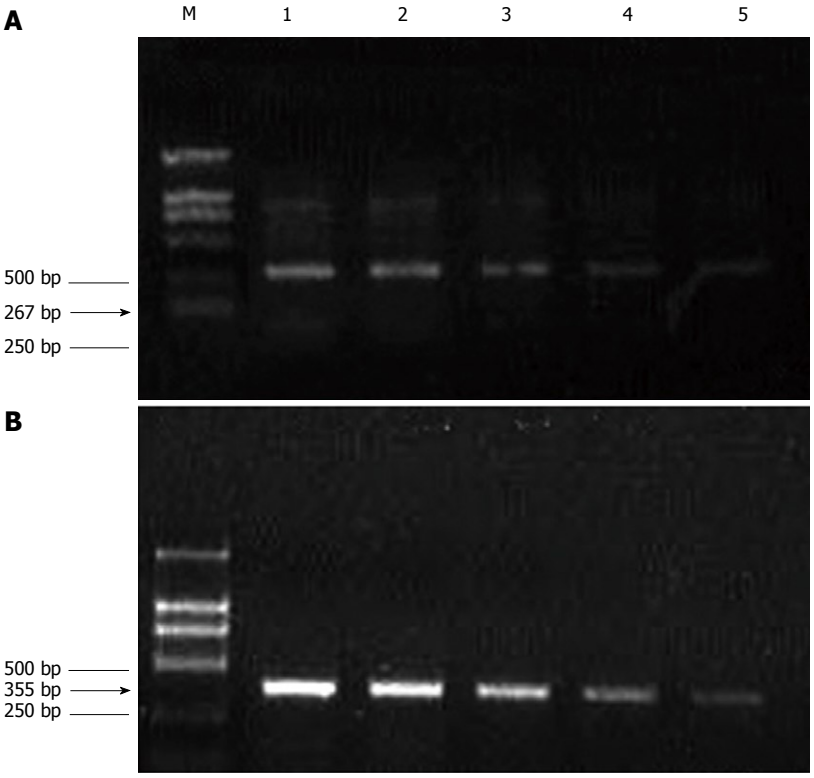

Figure 6 Effect of mofetil + adriamycin treatment on gene expression in HepG-2 cells. A: Intercellular adhesion molecule-1; B: Vascular cell adhesion molecule-1; M: Standard reference; 1: Untreated HepG-2 cells; 2: HepG-2 cells treated with 0.1 μg/mL mofetil (MMF) for 72 h; 3: HepG-2 cells treated with 1 μg/mL MMF for 72 h; 4: HepG-2 cells treated with 10 μg/mL MMF for 72 h; 5: HepG-2 cells treated with 100 μg/mL MMF for 72 h.

The findings of our study suggest that MMF and ADM inhibit the growth of HCC cells and promote cellular apoptosis. These findings can help in the development of new modalities of chemotherapy and help prevent immunological rejection after liver transplantation. However, we need to conduct experiments with a greater number of animals and determine the optimal strategy for treatment of liver cancer patients after liver transplantation.

COMMENTS

Background

Hepatocellular carcinoma (HCC) has become increasingly important all over the world. Liver transplantation is an effective method nowadays in the treatment of HCC. Patients who receive chronic immunosuppression following organ allotransplantation display an increased incidence of de novo neoplasms. So, it becomes a challenge to find a proper drug. Recently, some researchers reported that mycophenolic acid had antiproliferative activity against a variety of tumor cell lines in vitro and against murine leukemias, lymphomas, and solid tumors in vivo. Adriamycin (ADM) is a chemotherapeutic agent used widely in treatment of malignant tumors. Combined use of mycophenolate mofetil (MMF) and ADM may exert remarkable effect.

Research frontiers

Liver transplantation and the application of the immunosuppression are hotspots in the research field related to this article.

Innovations and breakthroughs

In this study, we have educed that MMF can inhibit HepG-2 cell growth, adhesion, and induce its apoptosis in a concentration-dependent manner. We also found combination of MMF and ADM exerted remarkable inhibitory effects on the growth of HCC cells.

Applications

MMF is an immunosuppressive agent, which has been used in organ allotransplantation area. ADM is a chemotherapeutic agent used widely in the treatment of malignant tumors. If MMF plus ADM is used after liver transplantion, a double action of anti-immunological rejection and anti-tumor may be achieved.

Peer review

The authors present interesting results on the combined effects of MMF and ADM on HepG2 cell lines. The findings indicate that MMF does indeed has some effects on some specific genes on HepG2 cells. Overall, this is a great study clearly presented. Maybe in the future, the authors should repeat the study on at least three different human HCC cell lines to document the broader applicability of their findings. Anyway, it should be accepted for publication.

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World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
doi:10.4254/wjh.v2.i8.318

World J Hepatol 2010 August 27; 2(8): 318-321
ISSN 1948-5182 (online)

CASE REPORT

Surgical treatment of HCC in a patient with lamivudine-resistant hepatitis B cirrhosis with adefovir dipivoxil

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Received: April 16, 2010 Revised: July 1, 2010
Accepted: July 8, 2010
Published online: August 27, 2010

Abstract

We describe a 77-year-old woman with chronic hepatitis B who became resistant to lamivudine. She was started on adefovir (10 mg daily) while still continuing lamivudine therapy. Four mo later her liver function improved and serum Hepatitis B virus (HBV)-DNA level became undetectable. Three years after the start of additional adefovir treatment, hepatocellular carcinoma (HCC) was detected and the patient underwent a successful hepatectomy. Our findings suggest that the addition of adefovir to ongoing lamivudine therapy cannot completely suppress hepatocarcinogenesis, but is useful for improving liver function in patients with lamivudine-resistant HBV-related cirrhosis, allowing HCC surgery.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Hepatocarcinogenesis; Lamivudine; Adefovir dipivoxil

Peer reviewers: Sandro Vento, MD, Professor of Internal Medicine, Department of Internal Medicine, School of Medicine, Faculty of Health Sciences, University of Botswana, Private Bag 0022, Gaborone, Botswana; Jordi Muntane, PhD, Unidad de Investigacion, Hospital Universitario Reina Sofia, Av. Menéndez Pidal s/n, Cordoba 14004, Spain

Akima T, Tamano M, Yamagishi H, Kubota K, Fujimori T, Hiraishi H. Surgical treatment of HCC in a patient with lamivudine-resistant hepatitis B cirrhosis with adefovir dipivoxil. *World J Hepatol* 2010; 2(8): 318-321 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i8/318.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i8.318

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide and the third leading cause of cancer death, next to lung and stomach cancer. Hepatitis B virus (HBV) infection is one of the major causes of HCC, with interferon as one of several therapeutic options for chronic HBV infection. Recently, lamivudine, a nucleoside analogue, has been used to treat chronic HBV infection[1]. However, long-term lamivudine therapy may result in the emergence of genotypic resistance in the form of tyrosine-methionine- aspartate-aspartate (YMDD) mutations, which is occasionally associated with severe, or even fatal, breakthrough hepatitis[2,3]. Thus, there is a clear need for alternative or additional therapies. Adefovir dipivoxil (adefovir) is a nucleotide analogue that converts to an active metabolite, adefovir diphosphate[4,5]. In order to prevent breakthrough hepatitis induced by lamivudine-resistant HBV mutants, additional adefovir dipivoxil is recommended[6].

CASE REPORT

A 77-year-old Japanese female patient had been followed

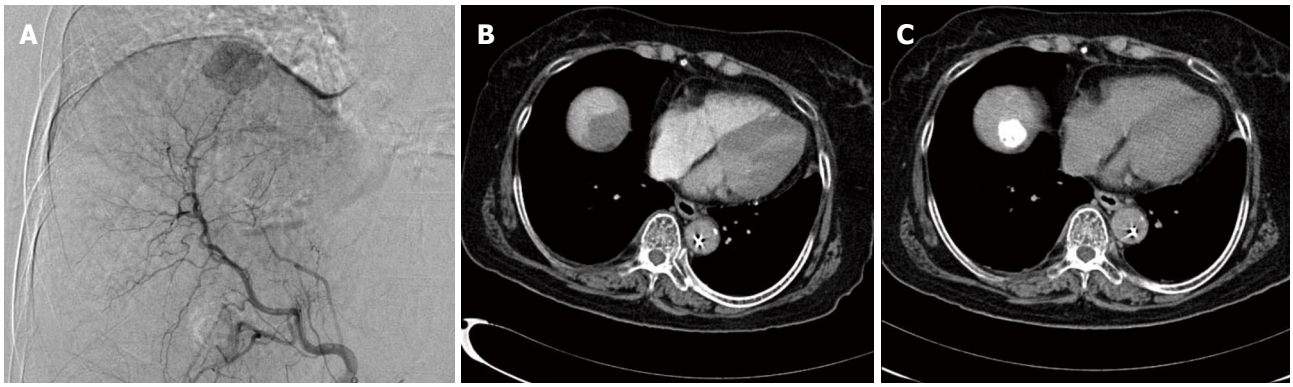

Figure 1 Digital subtraction angiography. A: Digital subtraction angiography showed tumor staining close against the diaphragm 20 mm in diameter; B: Computed tomography (CT) during arterial portography showed a perfusion defect in segment 8; C: CT during hepatic arteriography showed a hypervascularity lesion in the corresponding region.

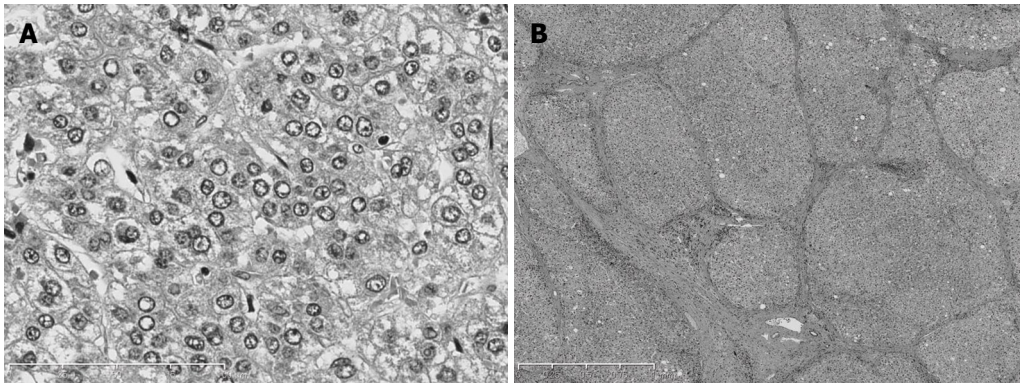

Figure 2 Histological findings of specimens obtained by subsegmentectomy. A: The tumor is a moderately differentiated hepatocellular carcinoma (H&E stain, × 100). B: The non-tumorous liver tissue around the tumor shows cirrhosis (H&E stain, × 40).

since 1977 for HBV-related chronic hepatitis. In 2002, her serum ALT level fluctuated between 100-400 IU/L (normal 4-40 IU/L), the HBV-DNA level was shown to be 6.2 log genome equivalent (LGE)/ml by transcription-mediated amplification, and alpha-fetoprotein (AFP) level was elevated to 537 ng/mL (normal upper limit 5 ng/mL). However, the desgamma carboxy prothrombin (DCP) level was within normal limits. Abdominal ultrasonography and dynamic computed tomography (CT) revealed no evidence of HCC. From December 2002, the patient started to receive lamivudine at a dose of 100 mg per day. The serum ALT and AFP levels decreased to the normal range, HBV-DNA decreased to undetectable levels. After 30 mo of lamivudine treatment, the serum HBV-DNA level increased rapidly to 5.1 LGE/mL with serum ALT elevation (127 IU/L). The lamivudine-resistant YVDD mutant strain of HBV was detected. The patient was then treated with adefovir at a dose of 10 mg daily in addition to lamivudine from July 2005.

Abdominal ultrasonography and dynamic CT were performed every 6 mo. In July 2008, dynamic CT showed a tumor in segment 8 of the liver measuring 10 mm in diameter. The patient was admitted for further examination in September 2008. Digital subtraction angiography showed a tumor staining 20 mm in diameter (Fi gure 1A), CT during arterial portography showed a perfusion defect in segment 8 (Figure 1B), and CT during hepatic arteriography showed a hypervascular lesion in the corresponding region (Figure 1C). The tumor was diagnosed typical stage I HCC. Laboratory data upon admission is shown in Table 1. The patient's Child-Pugh grade was A. Since the tumor was not detected by ultrasonography, percutaneous ethanol injection and radiofrequency ablation were not performed. After outlining the treatment plan, including surgery and transcatheter arterial chemoembolization (TACE), the patient opted to undergo surgery and subsegmentectomy was successfully performed in October 2008. The tumor was a nodular lesion measuring 16 mm × 13 mm. Microscopically, the tumor was a moderately differentiated HCC with a trabecular pattern (Figure 2A). The non-tumorous liver tissue showed cirrhosis (Figure 2B). HBs Ag and HBc Ag were not detected by immunohistological staining in either the tumorous or non-tumorous portions.

DISCUSSION

The epidemiological association of HBV with HCC is well established. In recent studies, it was revealed that HBsAg carriers have a 25-37 fold increased risk of developing HCC compared to non-infected people.

In 1998, lamivudine was approved as the first nucleotide analogue treatment of chronic hepatitis B[7]. However, drug-resistant mutants arise over the duration of lamivudine treatment, at 12.5% after 1 year, 43.8% after 3 years, and 62.5%-70.2% after 5 years[8,9]. For preventing breakthrough hepatitis induced by lamivudine-resistant HBV mutants, additional adefovir dipivoxil

Table 1 Laboratory data on admission

Blood chemistry	Value	Hematological analysis	Value	Viral markers	Value	Coagulation	Value	Tumor markers	Value
TP (g/dL)	7.8	WBC (× 103/L)	3.60	HBs Ag (IU/mL)	5.56	PT (%)	67	AFP (ng/dL)	< 5
Alb (g/dL)	4.1	RBC (× 106/L)	4.59	HBs Ab (mIU/mL)	1.20			PIVKA-Ⅱ (mAU/mL)	22
AST (IU/dL)	33.0	Hb (g/dL)	13.00	HBe Ag (S/CO)	0.30				
ALT (IU/dL)	28.0	Ht (%)	40.60	HBe Ab (%Inh)	89.60				
LDH (IU/dL)	168.0	Plt (× 103/L)	8.80	HBV-DNA	Undetectable				
ALP (IU/dL)	313.0								
GGT (IU/dL)	30.0								
T-bil (mg/dL)	0.6								
ZTT (KKU)	8.7								

TP: total protein; Alb: albumin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactic dehydrogenase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; T-bil: total bilirubin; ZTT: zinc turbidity test; WBC: while blood cells; RBC: red blood cells; Hb: hemoglobin; Ht: hematocrit; Plt: platelets; PT: prothrombin time; AFP: alpha-fetoprotein; PIVKA-Ⅱ: protein induced by vitamine K absence or antagonist-Ⅱ.

(10 mg daily) has been recommended[10] as it is more effective than switching to adefovir monotherapy and has fewer chances of developing drug-resistant mutants[11,12]. Lamivudine-resistant patients treated with lamivudine add-on adefovir can achieve both an excellent virological and biochemical response, but cannot completely suppress hepatocarcinogenesis[13,14]. It was reported that HCC development was observed in 7.3% of patients who received long-term adefovir add-on lamivudine over periods of up to 5 years. Serum ALT levels (≥ 70 IU/L), YMDD mutants, cirrhosis and age were independent factors for the development of HCC[15]. In the present case, serum ALT levels were within the normal range after receiving adefovir add-on lamivudine therapy, although the YMDD mutant was present. Furthermore, the patient was a senior citizen and her liver was cirrhotic at the start of lamivudine therapy. Therefore, this patient was at high hepatocarcinogenesis risk in the adefovir add-on lamivudine therapy group. HCC was detected about three years after starting additional adefovir treatment. The patient's clinical status and liver function were sufficiently improved when HCC was detected, allowing for successful surgical resection.

In conclusion, our experience suggests that adefovir add-on lamivudine therapy cannot completely suppress hepatocarcinogenesis, but is effective for reversing hepatic decompensation. The continuation of the combined lamivudine and adefovir treatment maintains stable liver function, permitting subsequent surgery for HCC.

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S- Editor Zhang HN **L- Editor** Hughes D **E- Editor** Liu N

World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
doi:10.4254/wjh.v2.i8.322

World J Hepatol 2010 August 27; 2(8): 322-324
ISSN 1948-5182 (online)

CASE REPORT

Spontaneous rupture of a recurrent hepatic cystadenoma

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Received: April 23, 2010 Revised: July 6, 2010
Accepted: July 13, 2010
Published online: August 27, 2010

Abstract

Biliary cystadenoma is a rare cystic tumor of the middle aged woman that usually arises in the liver or occasionally in the extrahepatic bile ducts. It has a strong potential for recurrence and for malignant tr ansformation. The lack of specific clinical and biological features hinders diagnosis before surgery. The spontaneous rupture of a hepatobiliary cystadenoma is a very rare and potentially life-threatening complication, with only two reported cases in the English literature. We report a case with spontaneous rupture of a recurrent hepatobiliary cystadenoma in a 32 year-old woman.

Key words: Cystadenoma; Liver; Spontaneous rupture

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Elfadili H, Majbar A, Zouaidia F, Elamrani N, Sabbah F, Raiss M, Mahassini N, Hrora A, Ahallat M. Spontaneous rupture of a recurrent hepatic cystadenoma. *World J Hepatol* 2010; 2(8): 322-324 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i8/322.htm DOI: http://dx.doi.org/10.4254/wjh.v2.i8.322

INTRODUCTION

Biliary cystadenomas are rare benign tumors, although prone to malignant degeneration. Clinical and imaging features are non specific. Consequently, they are preoperatively misdiagnosed in 50% to 70%of cases, commonly resulting in delayed and inaccurate treatment[1]. We report a case of spontaneous rupture of a recurrent biliary cystadenoma.

CASE REPORT

A 32-year-old woman with a symptomatic hepatic cyst was referred to our department. Five months earlier, she had been operated on in another hospital for suspicion of hydatid cyst of the liver in the fourth segment. A conservative treatment was carried out (unroofing). Histological examination of the cystic wall revealed a biliary cystadenoma with mesenchymal stroma. 4 mo later, she presented right hypochondrium pain. Abdominal US revealed a heterogeneous mixed-echoic tumor in the fourth hepatic segment. An abdominal CT scan revealed a 10 cm × 20 cm cyst with septations in liver segments 3, 4 and 5 (Figure 1). Surgical treatment was decided for recurrent hepatic cystadenoma. 2 d before surgery, the patient presented with severe abdominal pain. Abdominal ultrasound showed diffuse peritoneal fluid. The patient had an emergency laparotomy for cyst rupture. After bilateral sub-costal incision, we found 2 liters of serous fluid in the abdomen and exploration showed a huge perforated cyst in the third, fourth and fifth hepatic seg-

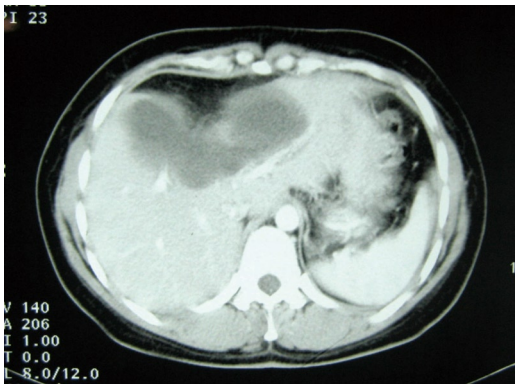

Figure 1 Computed tomography showing the hepatic cyst in segments Ⅲ, Ⅳ and Ⅴ with septations and intimate contact with portal veins.

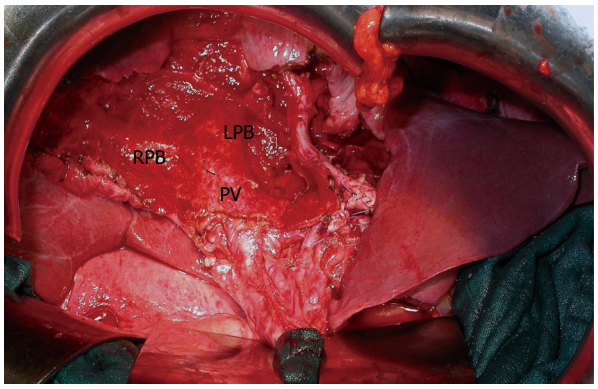

Figure 2 Operative photography after complete removal of the cyst: central hepatectomy. PV: portal vein; RPB: right portal vein; LPB: left portal vein.

ments. We performed an atypic hepatic resection achieving complete removal of the cyst (Figure 2). The immediate postoperative period was uneventful and the patient was discharged 6 d after surgery. Histological examination confirmed benign cystadenoma (Figure 3). There was no recurrence after 14 mo follow-up.

DISCUSSION

Cystadenoma is a benign tumor, supposedly originating in intrahepatic (and more rarely extrahepatic) embryonic tissue precursors of biliary epithelium[2]. With its malignant counterpart (cystadenocarcinoma), it accounts for less than 5% of all cystic lesions of the liver[3]. These tumors usually present in middle aged women with a mean age of 50 years and have a great variability in size, ranging from 1.5 cm to 30 cm[3].

The majority of patients are asymptomatic, but in the case of large tumors, they may present with a palpable mass and cause symptoms such as upper abdomen pain, dyspepsia, anorexia, nausea and fever[2-4].

The most widely used diagnosis methods are abdominal ultrasound and computed tomography[2,5]. Ultrasound will reveal an anechoic mass with sharp demarcations and often with fine internal septations[6]. Computed

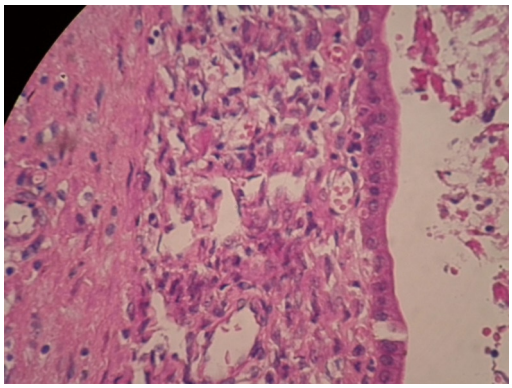

Figure 3 Photomicrograph demonstrating a cuboidal epithelial lining with dense spindle cell (mesenchymal) stroma.

tomography usually shows a well-defined mass with low-density and internal septa. Its fibrous capsule and internal septations are often visible and help distinguish the lesion from a simple cyst. The presence of mural nodules or wall thickening should trigger the suspicion of cystadenocarcinoma[5]. Unilocular lesions have been reported and are often incorrectly diagnosed, resulting in inadequate therapy[7]. Hydatid cyst is one of the disorders most likely confused with hepatic cystadenoma[5,8,9] specially in endemic hydatid disease regions. In our case, the patient had conservative surgical treatment for suspicion of hydatid cyst. Differential diagnosis may sometimes be difficult especially when serology is negative and for type Ⅱ (cystic lesion with internal septa) and Ⅳ lesions according to OMS Classification[10]. These lesions have thick walls and contain intra cystic material[11].

Establishment of the correct diagnosis is crucial be cause application of inadequate treatment, including partial excision or fenestration, results in an unacceptably high rate of recurrence[12]. Surgical resection is the treatment of choice for hepatobiliary cystadenoma because it avoids the risks of recurrence and malignant transformati on[1,3,5,13].

To our knowledge, this is only the third case of spontaneous rupture of hepatobiliary cystadenoma reported in the English literature[14]. This complication is potentially life-threatening and requires an emergency laparotomy.

In conclusion, hepatic cystadenomas are rare and sh ould be expected when radiological imaging studies sug gest a multilocular cystic hepatic lesion, especially in women. It can rarely present in an acute setting and rupture is an exceptional complication.

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S- Editor Zhang HN **L- Editor** Hughes D **E- Editor** Liu N

World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
www.wjgnet.com

World J Hepatol 2010 August 27; 2(8): I
ISSN 1948-5182 (online)

Acknowledgments to reviewers of *World Journal of Hepatology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Hepatology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Online Submissions: http://www.wjgnet.com/1948-5182office
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World J Hepatol 2010 August 27; 2(8): I
ISSN 1948-5182 (online)

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January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

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September 16-18
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Prague Hepatology Meeting 2010

September 23-26
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October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
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Online Submissions: http://www.wjgnet.com/1948-5182office
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World J Hepatol 2010 August 27; 2(8): I-V
ISSN 1948-5182 (online)

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Name of journal

World Journal of Hepatology

CSSN

ISSN 1948-5182 (online)

Indexed and Abstracted in

PubMed Central

Published by

Baishideng Publishing Group Co., Limited

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method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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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]

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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

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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

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