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RAPID COMMUNICATION

Gene modulation associated with inhibition of liver regeneration in hepatitis B virus X transgenic mice

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Abstract

AIM: To analyze the modulation of gene expression profile associated with inhibition of liver regeneration in hepatitis B X (HBx)-expressing transgenic mice.

METHODS: Microarray technology was performed on liver tissue obtained from 4 control (LacZ) and 4 transgenic mice (HBx-LacZ), 48 h after partial hepatectomy. The significance of the normalized log-ratios was assessed for each gene, using robust *t*-tests under an empirical Bayes approach. Microarray hybridization data was verified on selected genes by quantitative PCR.

RESULTS: The comparison of gene expression patterns showed a consistent modulation of the expression of 26 genes, most of which are implicated in liver regeneration. Up-regulated genes included DNA repair proteins (Rad-52, MSH6) and transmembrane proteins (syndecan 4, tetraspanin), while down-regulated genes were connected to the regulation of transcription (histone deacetylase, Zfp90, MyoD1) and were involved in the cholesterol metabolic pathway and isoprenoid biosynthesis (farnesyl diphosphate synthase, Cyp7b1, geranylgeranyl diphosphate synthase, SAA3).

CONCLUSION: Our results provide a novel insight into the biological activities of HBx, implicated in the inhibition of liver regeneration.

Key words: Hepatitis B virus; Hepatitis B X (HBx) protein; Liver regeneration; Microarray analysis; Cholesterol; Isoprenoid

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INTRODUCTION

The liver has a unique capacity to regenerate after partial hepatectomy (PH) or injury[1]. Experimental evidence has confirmed that hepatocyte proliferation is responsible for liver regeneration following PH, despite the low replication rate of hepatocytes in a normal liver[1]. After a two-third partial hepatectomy, hepatocytes switch from a quiescent state to a proliferative state and re-enter cell cycle division to compensate for the loss of liver mass. Liver regeneration is a multi-step process comprising at least two critical points: the immediate early gene phase (priming) that mediates the transition of the quiescent hepatocytes to the cell cycle (G1), and progression from G1 to S phase. In mice, DNA synthesis peaks at 40-44 h after PH[1].

Numerous growth factors and cytokines regulate the regeneration process by providing stimulatory and inhibitory signals for proliferation. Tumour necrosis factor alpha (TNF-α) and interleukin-6 (Il-6), together with their downstream transcription factors Stat-3 and nuclear factor kappa B (NF-κB), are the most important initiating factors in the regenerative response[1]. TNF-

α-induced hepatocyte proliferation has been shown to involve the small GTPases (Ras and RhoA) necessary for cell cycle progression by stimulating the degradation of Cdk inhibitors ($p21^{Cip}$ and $p27^{Kip1}$)[2]. The initiation factors governing liver regeneration partially overlap with regulators of the hepatic acute phase response (APR), which results in dramatic changes in the expression of acute phase response proteins following PH[3]. Hepatocyte growth factor (HGF) and transforming growth factor alpha (TGFα) further stimulate cell cycle progression, DNA synthesis and cell proliferation. Finally, TGF-β and activin suppress cell growth and terminate liver regeneration at a fixed point[1]. Recently, a large scale gene expression analysis, based on cDNA microarray technology, has provided a unique opportunity to elucidate the expression pattern throughout the course of liver regeneration[4-6], and the priming phase of liver regeneration in mice[7].

In a previous report, we showed that hepatitis B virus (HBV) X protein (HBx) inhibits liver regeneration after PH in HBx-expressing transgenic mice, and also demonstrated a paracrine inhibitory effect of HBx on liver cell proliferation[8]. HBx exhibits pleiotropic effects that modulate cell responses to genotoxic stress, protein degradation, cell viability and signalling pathways[9]. HBx has been shown to enhance transcription in the cytoplasm by activating various signal transduction cascades such as Ras/Raf mitogen-activated protein kinase and Src kinases, as well as in the nucleus through transcription factors like AP-1, AP-2 and NF-κB. Several studies have identified possible cellular targets of HBx, including members of the cyclic-AMP response element binding protein (CREB)/activating transcription factor (ATF) family, the TATA-binding protein, RNA polymerase subunit RPB5, the UV-damaged DNA-binding protein and the replicative senescence $p55^{sen}$[9,10]. HBx has been shown to interact with p53 and inhibit its function, but it may also induce apoptosis *via* p53-dependent and independent mechanisms[9]. Furthermore, it has been demonstrated that mutations identified in HBx-encoding sequences in the tumour cells of HCC can markedly modify the biological activity of HBx, and possibly favour cell transformation[11]. It has been shown that the HBx protein participates in the development of hepatocellular carcinoma (HCC) in HBV infected patients[12]. Indeed, HBx protein can induce HCC in certain transgenic mice and may, under certain conditions, cooperate *in vitro* with Myc or Ras oncogene[13]. However, the mechanisms involved in HBx-mediated HCC remain obscure.

In the present study, we analyzed further the impact of HBx on gene expression profile in the liver of transgenic mice, following PH. For this purpose, we employed HBx-expressing transgenic mice mentioned above, and adopted a transcriptome approach to analyse gene expression modulation, 48 h after PH. We observed that impaired DNA synthesis and reduction in liver mass in HBx-transgenic mice was associated with a weaker expression of genes involved in the cholesterol and nonsterol isoprenoid pathways as well as in impaired expression of serum amyloid A.

MATERIALS AND METHODS

Source of RNA

Two groups of mice (the same as described previously[8]) were used as liver RNA source. HNF1-LacZ transgenic mice carrying the nls-LacZ gene driven by the hepatocyte nuclear factor (HNF-1) promoter acted as controls. Test animals AX16/HNF1-LacZ double transgenic mice, were generated by crossing homozygous AX16 transgenic mice carrying ORF HBx linked to the promoter/enhancer of human anti-thrombin Ⅲ gene, with HNF1-LacZ transgenic mice. PH was performed on 21-35 d old LacZ and HBx-LacZ (with confirmed expression of HBx) transgenic mice. Frozen liver tissue obtained from 4 control (LacZ) and 4 treated (HBx-LacZ) mice was pulverized in liquid nitrogen, 48 h after partial hepatectomy. Total RNA extraction was performed using RNAble (Eurobio), according to the manufacturer's instructions and purified by RNeasy kit (Qiagen) followed by on-column DNase digestion (RNase-free DNase set). Only RNAs with an absorbance ratio of 260/280 over 1.9 and intact ribosomal 28S and 18S rRNA bands, evaluated by 1% agarose gel electrophoresis, were utilized for the microarray study.

Microarray assay

The aminoallyl indirect labelling method was used to obtain Cyanin-3 (Cy-3) or Cy-5 labelled cDNA. Briefly, 20 μg of total RNA and 5 μg of oligo-dT primers were incubated at 70℃ for 10 min and snap-cooled in ice, in order to generate aminoallyl-labelled cDNA. Reverse transcription was then performed by adding 0.5 mmol/L dATP, dCTP, dGTP, 0.3 mmol/L dTTP, 0.2 mmol/L aminoallyl-dUTP (aa-dUTP), 400 U SuperScript Ⅱ, 10 mmol/L DTT, and 1 × first strand buffer. This mixture was incubated at 42℃. After RNA hydrolysis (1 U of RNaseH at 37℃ for 15 min), unincorporated aa-dUTP was removed by the QIAquick PCR purification kit (Qiagen). The resulting aminoallyl -cDNAs were diluted in 0.1 mol/L sodium carbonate (pH 9.0) and coupled with 100 μg of Cy-3 or Cy-5 monoreactive dye (Amersham), prepared in dimethyl sulfoxide (DMSO) for 1 h. Cy3 and Cy5 labelled cDNA targets were concentrated in a Microcon filter device (Millipore) and suspended in 15 μL of hybridization buffer (0.7% SDS, 70% formamide in 3.6 × Denhardt solution). The cDNA microarray consisting of 5376 mice and rat cDNA spotted onto glass slides (Functional Genomic Service, CEA) was prehybridized in buffer containing: 3.5 × sodium chloride-sodium citrate buffer (SSC), 1% Bovine Serum Albumin (BSA) and 0.1% sodium dodecyl sulfate (SDS) for 1 h at 50℃. For each experiment, test cDNA was mixed with control cDNA, labelled with Cy3. Mixed, labelled cDNA, after preheating to 42℃ in hybridization buffer, was overlaid on dried glass cDNA array and incubated overnight at 42℃. After hybridization, the slides were washed once in 2 × SSC/0.2% SDS buffer and twice in 0.2 × SSC buffer, and dried by centrifugation.

Microarray analysis

For each slide, the fluorescent images of hybridized microarray were scanned with a Gene Pix 4000B scanner.

Table 1 Primer's sequence and PCR conditions used for the microarray results validation

Name of gene	Sequences of forward (F) and reverse (R) primers	Source of primers	PCR product (bp)	T anneling (℃)	Mg^{2+} (mmol/L)
Serum amyloid A3	F: TCAGCACATTGGGATGTTTAGG R: CAGAGGACTCAAGAGCTGACCA	UniSTS: 219434	206	53	3
Creatine kinase muscle	F: CCTCCTGGAAGTCCAATCAT R: GGCCATCACGGACTTTTATT	UniSTS: 159603	150	53	3
Peroxiredoxin 1	F: GAGCAGCCAGAAGAAACTCTTG R: AGAAGATTGGTCTGCCCAAAA	UniSTS: 144118	153	53	3
Retinoblastoma-like 2	F: TGGCTGAGTCCTGTAACAAC R: CCAACACCTTTCTGAGGC	UniSTS: 162222	374	53	2
Interleukin-6-receptor, 80-kD	F: AAGCAGCAGGCAATGTTACC R: CATAAATAGTTCCCAGTGTCG	[18]	120	55	3
DNA mismatch repair protein MSH6	F: ATATGTCCTAGGCGCACACAAA R: CTAGCATACTCAGGCATGCGAC	UniSTS: 211063	208	56	4
Peroxisome proliferator-activated receptor-α	F: CATCGAGTGTCGAATATGTGG R: GCAGTACTGGCATTTGTTCC	[19]	172	55	4
β-actin	F: CGTGACATTAAGGAGAAGCTGTGC R: CTCAGGAGGAGCAATGATCTTGAT	[20]	374	53	2

MSH6: DNA mismatch repair protein 6.

Image analysis was performed using a Gene Pix Pro 40054 to quantify the arrays. Spots flagged automatically by Genepix or by visual inspection of the array scans were excluded from the analysis. Global normalization was applied to correct the artefacts caused by different incorporation rates or scanner settings for two dyes. Scatter plots in a log scale were performed to visualize fold changes between two channels by plotting Cy5 intensity against Cy3 intensity. Normalization steps were performed using Bioconductor array packages[14]. After removal of the background, we normalized the log2 of the cy5/cy3 ratio using a print-tip loess approach[15]. For each gene, the average of the normalized log-ratios was computed and the significance was assessed using robust *t*-tests under an empirical Bayes approach[16] as implemented in the limma package. *P*-values were then corrected by the FDR approach[17] to take account of test multiplicity. Differentially expressed genes were considered to be those with a false discovery rate (FDR) of less than 5% and an expression ratio > 1.4.

Real-time PCR

In order to verify microarray hybridization data, real-Time PCR was performed using a Light Cycler rapid thermal system (Roche Diagnostics). Random cDNA was transcribed from 10 μg total RNA with 400 U of Superscript Ⅱ in the presence of 1 μg random primers, according to the manufacturer's instructions. All PCR experiments were carried out on the same cDNA preparation. Reactions were performed according to the manufacturer's instructions in a 20 μL volume containing 2 μL of 10-fold diluted cDNA, 0.5 μmol/L of primers, and an $MgCl_2$ concentration optimized between 2 and 4 mmol/L. A typical protocol included 10 min of initial denaturation followed by 40 cycles of 95℃ denaturation for 20 s, annealing at 53℃-56℃ for 15 s and a 72℃ extension for 5-12 s (depending on the PCR product). For each set of primers, a template without reverse transcription was amplified as a negative control. The details of primer sequences and amplification conditions are summarized in Table 1. The quantification of gene expression was based on a standard curve prepared from gene-specific, purified PCR product (PCR clean-up kit, Macherey-Nagel). The specificity of each PCR product was controlled by a melting curve analysis and subsequent agarose gel electrophoresis of the PCR product. For each experiment, relative concentrations were obtained after normalization with β-actin values. Calculation of the ratio of each mRNA expression was based on the relative concentration of specific cDNA found by RT-PCR in samples originating from control and HBx-expressing livers.

RESULTS

Microarray analysis of gene expression in the liver of HBx-transgenic mice after partial hepatectomy

As previously reported, HBx expression inhibits liver regeneration after PH in AX16/HNF1-LacZ transgenic mice, by a combination of intracellular and paracrine effects[8]. In the present study, we investigated, using microarray analysis consisting of 5376 murine genes, the impact of HBx expression on gene expression profile, 48 h after partial hepatectomy to analyze any changes in gene expression which may be involved in the observed inhibition of liver regeneration in HBx transgenic mice. This time point of gene expression analysis was chosen in order to determine alterations in gene expression associated with the DNA synthesis phase[1]. In addition, BrdU incorporation analysis performed on SCID mice transplanted with HBx-expressing hepatocytes, 48 h after partial hepatectomy, had indicated a marked (5.5-fold) reduction in the cellular DNA synthesis[8].

RNAs isolated from the livers of four HBx-LacZ mice were labelled separately with Cy5, and RNAs from the livers of four LacZ mice were mixed together and labelled with Cy3 as the reference. Thus, the expression profile of each of the four tested RNA was compared with the expression of control RNA by the microarray assay (performed in duplicate). 82% of the clones spotted

Table 2 Upregulated genes in HBx-LacZ transgenic mice 48 h after partial hepatectomy

Gene name (Abbreviation)	Unigene ID	GB Acc.	Fold difference HBx-LacZ/LacZ	*P* value	Function
Aven: caspase activation inhibitor	Mm.292041	BF662037	1.50	0.030	Caspase inhibitor
RAD52 homolog	Mm.149	U12135	2.14	0.035	DNA repair/double-strand break
Mismatch repair protein MSH6	Rn.16755	XM345633	1.51	0.009	DNA repair/mismatch
Epidermal growth factor	Rn.6075	NM012842	1.40	0.014	Growth factor
DC-SING (CD209)	Mm.32510	NM133238	1.40	0.009	Transmembrane protein/Cell adhesion
Leucyl-tRNA synthetase	Hs.432674	NM020117	1.41	0.009	Metabolism/protein synthesis
Syndecan 4	Mm.3815	BC005679	1.42	0.014	Transmembrane protein/Focal adhesion
Transmembrane 4 superfamily member	Mm.18590	BC050153	1.45	0.009	Tetraspanin interacting with beta-1 integrin
Golgi SNAP receptor complex member 2	Rn.13518	BC061994	1.42	0.011	Transmembrane protein/Vesicular transport of Golgi

Abbreviations: RAD52: DNA double-strand break repair and recombination protein; DC-SING: Dendritic Cells-specific intercellular adhesion molecule 3-grabbing nonintegrin.

Table 3 Downregulated genes in HBx-LacZ transgenic mice 48 h after partial hepatectomy

Gene name (Abbreviation)	Unigene ID	GB Acc.	Fold difference HBx-LacZ/LacZ	*P* value	Function
Serum amyloid A3 (Saa3)	Mm.14277	X03479	-1.95	0.016	Acute phase response protein cholesterol transport
Cadherin 16 (Cdh 16)	Mm.19423	AF016271	-2.64	0.010	cell recognition protein
Creatine kinase (Ckm)	Mm.2375	AI325205	-1.65	0.040	ATP synthesis
Glutathione S-transferase, pi2 (Gstp2)	Mm.299292	AI325120	-1.85	0.043	Detoxification and drug metabolism
Progastricsin (pepsinogen C) (Pgc)	Mm.22957	AK008959	-1.73	0.009	Digestion enzyme
Phospholipase A2 group 1B (Pla2g1b)	Mm.20190	AI327450	-1.58	0.016	Digestion enzyme
Tripsin Ⅱ precursor (Try2)	Mm.301947	AI386046	-1.54	0.014	Digestion enzyme
Farnesyl diphosphate synthase (Fdps)	Mm.39472	W76783	-1.98	0.020	Enzyme of cholesterol pathway
Cytochrom P450, 7b1 (Cyp7b1)	Mm.278588	U36993	-1.61	0.009	Enzyme of cholesterol pathway
Geranylgeranyl diphosphate synthase 1 (Ggps 1)	Mm.148039	AB016044	-1.41	0.034	Enzyme of cholesterol pathway
Aldolase1, A isoform (Aldo 1)	Mm.275831	AI327494	-3.35	0.015	Enzyme of glycolysis pathway
Peptidylglycine alpha-amidating monooxygenase	Mm.5121	AI323455	-1.73	0.029	Posttranslational modification
Mouse integrase gene (IN)	NF	X52622	-1.43	0.021	Replication
Myogenic differentiation1 (MyoD1)	Mm.1526	M84918	-1.52	0.032	Transcription
Zinc finger protein 90 (Zfp 90)	Mm.295582	X79828	-2.06	0.040	Transcription
Histone deacetylase 10 (Hdac 10)	Mm.346413	AI323 456	-3.73	0.014	Transcription
Prion protein (Prnp)	Mm.648	M13685	-1.40	0.021	CNS protein

on the microarray revealed hybridization signals, and the data shown in Tables 2 and 3 was obtained after the elimination of genes that did not comply with the criteria for expression, normalization and statistical analysis (see Material and Methods). In order to validate the microarray analysis, real-time PCR was performed on selected genes for which the expression was found to be up- or down-regulated or unmodified in LacZ-HBx mice when compared with LacZ mice. As shown in Table 4, the RT-PCR results were similar to the data obtained from cDNA microarrays; we therefore did not perform RT-PCR on all the genes listed in Tables 2 and 3.

As shown in Table 2, the majority of the up-regulated genes in HBx-expressed transgenic mice corresponded to the expression of DNA repair and transmembrane proteins. RAD52 is responsible for DNA double-strand break repair and mitotic recombination and MSH6 for DNA mismatch repair[21]. The transmembrane protein genes implicated in focal adhesion (syndecan 4), viral internalization (CD209), transport of protein between the medial-and trans-Golgi compartment (Golgi SNAP receptor) and in interactions with beta-1 integrin (transmembrane 4 superfamily member 2) were found to be up-regulated. In addition, changes in the expression of the epidermal growth factor and Aven, a caspase inhibitor, were also observed.

Genes whose expression was found to be down-regulated in the liver of HBx-expressed transgenic mice (Table 3) consisted of two main classes: those connected to the transcription control, such as Myogenic differentiation1, Zinc finger protein 90 and Histone deacetylase 10 (HDAC 10), and genes involved in cell metabolism, such as serum amyloid A3 (SAA3), creatine kinase, pepsinogen C, aldolase A, glutathione S-transferase and phospholipase A2. A subclass of down-regulated genes, connected with cholesterol metabolism and isoprenoid synthesis, such as farnesyl diphosphate synthase (FPPS), geranylgeranyl diphosphate synthase (GGPPS) and Cyp7 were also observed. In the group of genes that was found to be down-regulated in the liver of HBx-transgenic mice, we observed several genes like SAA3, aldolase A, creatine kinase, phospholipase A2 that have been reported elsewhere as up-regulated during the course of liver regeneration[22-24].

Interestingly, down-regulation of histone deacetylase and up-regulation of DNA repair genes (Rad52 and MSH6)

Table 4 Results from RT-real time PCR 48 h after PH for selected genes

Gene	Mice[1]	RT-real time PCR		Microarrays fold difference HBx-LacZ/LacZ
		Relative value[2]	Fold difference HBx-LacZ/LacZ	
SAA3	LacZ	0.37 ± 0.41	-10.9	-2.00
	HBx-LacZ	0.03 ± 0.02		
Creatine Kinase	LacZ	1.36 ± 1.29	-1.6	-1.65
	HBx-LacZ	0.87 ± 0.91		
MSH6	LacZ	1.08 ± 0.64	1.5	1.50
	HBx-LacZ	1.62 ± 0.64		
Rb2	LacZ	0.47 ± 0.15	1.2	1.20
	HBx-LacZ	0.59 ± 0.23		
Peroxiredoxin	LacZ	0.08 ± 0.02	1.5	1.40
	HBx-LacZ	0.12 ± 0.08		

SAA3: Serum Amyloid A3; MSH6: DNA mismatch repair protein 6; Rb2: Retinoblastoma like-2. [1]Four animals in control (LacZ) group and four animals in treated (HBx-LacZ) group; [2]for each experiment relative values were obtained after normalization with beta actin expression.

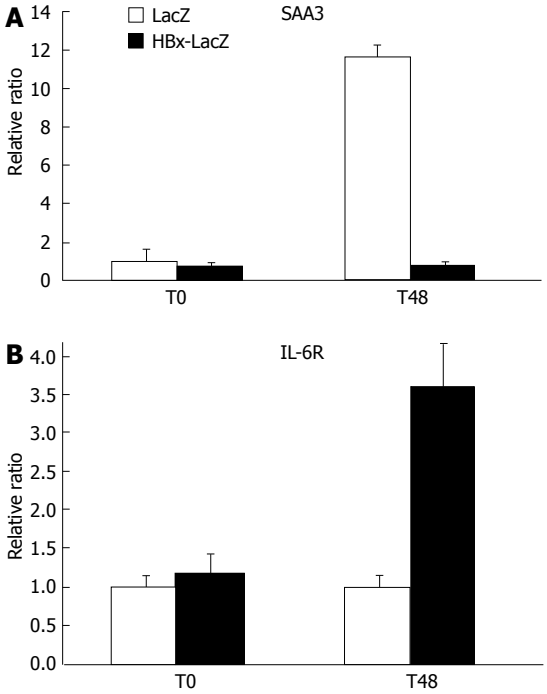

Figure 1 Expression levels of SAA3 (**A**) and IL-6R (**B**) transcripts in livers obtained from LacZ and HBx-LacZ transgenic mice. RNA was isolated before (T0) and 48 h after (T48) partial hepatectomy from the liver of two HBx-expressing (HBx-LacZ) and two control (LacZ) transgenic mice. The level of transcript expression was analyzed by quantitative RT-PCR and expressed as relative ratios after normalization with β actin values. The means of the relative ratio values and standard error were obtained from at least two independent experiments performed in duplicate.

observed in our study in HBx-transgenic mice after PH have, in agreement with other reports, similar effect on the cell cycle by inducing cell cycle arrest[25-28]. Furthermore, it has previously been reported that HBx expression may interfere with nucleotide excision repair mechanisms[29,30] and interact with the DNA repair protein[31,32].

In the next subsections we will focus on selected modulated genes implicated in cholesterol regulation and discuss their possible impact on the inhibition of liver regeneration.

Modulation of serum amyloid A expression and liver regeneration

SAA3 is a multifunctional protein involved in acute phase response, inflammation, and cholesterol transport. However, the precise function of SAA in liver regeneration is unclear. The physiological transient induction of SAA appears to be indispensable for the precise regeneration rate after PH[3]. To investigate whether HBx expression was able to completely or partially inhibit SAA3 expression during PH, real time RT-PCR was performed on liver biopsies obtained at the time of PH (T0) and 48 h later from two LacZ or HBx-LacZ transgenic mice. As expected, in the control LacZ animals, a marked increase in SAA expression (more than 10-fold) was observed 48 h after PH as compared to T0 (Figure 1A). By contrast, in HBx-LacZ mice, PH was not associated with an increase in the expression of SAA3 transcript. Thus, HBx induces complete inhibition of SAA response during PH. This may constitute an important factor involved in the impaired liver regeneration previously observed in these animals.

Regulation of SAA expression during the acute phase response depends on hepatic mitogens (Il-6, IL-1, TNF-α)[3]. As the expression of these cytokines is not significantly modified by HBx 48 h after PH, they are probably not implicated in the observed inhibition of SAA3 transcription in HBx-LacZ transgenic mice. To define whether any modulation of the expression of Il-6 receptor (IL-6R), not incorporated in our microarray, is related to the inhibition of SAA expression in HBx-LacZ mice, we used RT-PCR assay to determine the expression of IL-6R in LacZ and HBx-LacZ transgenic mice, at the time of PH and 48 h later. As shown in Figure 1B, prior to PH, no difference in IL-6R expression was observed in HBx-LacZ mice compared to control mice. By contrast, 48 h after PH there was greater than 3-fold up-regulation in HBx-LacZ mice as compared to controls. These findings suggest that HBx is able to modulate Il-6 receptor expression in association with a factor activated during PH and confirms that the lack of SAA response after PH in HBx-expressing transgenic mice is independent of cytokine activity.

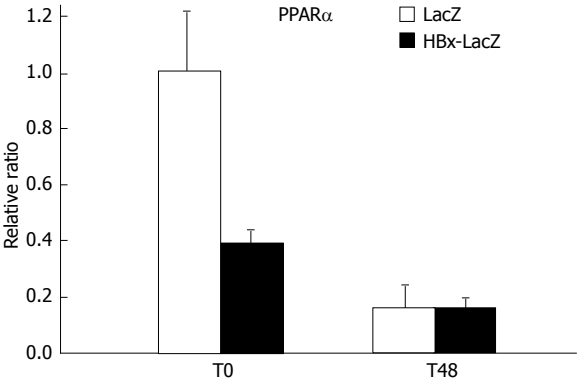

Figure 2 Expression levels of PPARα transcripts in the liver of two HBx-expressing (HBx-LacZ) and two control (LacZ) transgenic mice, before (T0) and 48 h after (T48) partial hepatectomy. The level of transcript expression was analyzed by quantitative RT-PCR and expressed as relative ratios after normalization with β actin values. The means of the relative ratio values and standard error were obtained from at least two independent experiments performed in duplicate.

Impaired expression of isoprenoid pathway and peroxisome proliferator-activated receptor-α

We observed that after PH of HBx-transgenic mice, the expression of enzymes implicated in the isoprenylation biosynthesis (FPPS and GPPS, and to a lesser extent HMG-CoA synthase) was reduced in the liver. Peroxisome proliferator-activated receptor-α (PPARα) is a transcription factor that controls lipid metabolism and glucose homeostasis. PPARα regulates the expression of enzymes implicated in isoprenoid synthesis, and its role in liver regeneration after PH was demonstrated via mechanisms involving prenylation of small GTPases Ras and RhoA independent of TNF-α and IL-6 activation[33-35]. In addition, it was observed that PPARα is involved in the expression of FPPS and HMG-CoA synthase after PH[33]. Thus, we wanted to investigate whether the decrease in the expression of enzymes implicated in isoprenylation observed in HBx-transgenic mice can be explained by a decrease in PPARα expression. We used RT-PCR to assess PPARα expression in LacZ and HBx-LacZ transgenic mice, both at the time of PH and 48 h later. At the time of PH, a 60% decrease in the expression of PPARα gene was observed in HBx-LacZ compared to LacZ transgenic mice (Figure 2). It has been reported that the absence of PPARα expression in mice contributes to impaired liver regeneration[36]. As previously reported, there is a general decrease in PPARα expression after PH[37]. In accordance with the microarray data, there was no difference in PPARα expression between LacZ and HBx-LacZ transgenic mice, 48 h post PH. Thus, the reduced expression of PPARα before PH may be involved in the inhibition of liver regeneration in HBx-LacZ transgenic mice.

DISCUSSION

In the present study, we used liver biopsies of HBx-expressing transgenic mice, 48 h after partial hepatectomy, to assess the factors implicated in the inhibition of liver regeneration in HBx-transgenic mice, by determining the alterations in mRNA profile. By that means, we extended the results obtained in *in vivo* studies, demonstrating inhibition of liver regeneration by HBx-expression in HBx-transgenic mice[8]. The present study demonstrates the influence of altered expression of genes involved in transcription control, DNA reparation, cholesterol metabolic pathway and isoprenoid biosynthesis in the reduction of liver mass observed in HBx-transgenic mice[8].

Although the data is controversial, it has been shown that HBx may up-regulate the expression of $p21^{waf1/cip1}$ and prolong G1→S transition[38,39]. A recent study on liver regeneration performed on transgenic mice showed that HBx can block G1/S transition, and so cause the lower liver mass restoration[40]. In this context, we showed that HBx down-regulated HDAC10 and up-regulated DNA repair gene (Rad52 and MSH6) expression, which may have a similar impact on the cell cycle. Indeed, HDAC inhibition causes cell cycle arrest due to an increase in the expression of p21 WAF1/Cip1 and Rb[25,26,41,42]. Moreover, it has been reported that Rad52 over-expression affects cell cycle regulation by delaying the exit from G1[27], and that MSH6 protein induces apoptosis by activation of p53[28]. Thus, one of the possible mechanisms that has emerged from our study is in accordance with previously reported activity of HBx, inhibition of cell cycle progression throughout the modulation of pathways implicated in DNA repair and transcription control.

During regeneration induced by tissue loss, a complex of growth factors and cytokines induce hepatocyte progression from G0 to cell cycle to restore the original liver mass. At the same time, Il-6, IL-1 and TNF-α, known to be hepatic mitogens, induce the acute-phase response (APR)[3,43]. During APR, the hepatic biosynthesis of SAA is up-regulated by proinflammatory cytokines, and the circulating concentration of SAA can increase up to 1000 fold. SAA is induced transiently and returns to its normal low basal level within 72-96 h of the initial stimulus[44]. It has been reported earlier that the mRNA for APR protein such as serum amyloid A increases dramatically during liver regeneration[45]. When we analyzed the changes in the mRNA profile in liver 48 h after PH we also observed a greater than 10-fold increase in the level of SAA mRNA, but only in the control mice. SAA expression level in HBx-transgenic mice 48 h after PH remained unchanged (even slightly lower) when compared to T0. Although the exact function of SAA is still unclear, the physiologic transient induction of SAA appears to be indispensable for proper regeneration after PH. Thus, the lack of SAA response in HBx-transgenic mice after PH may constitute an alternative factor in the impaired regeneration.

The activity of HMG-CoA synthase, farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate synthase was found to be indispensable for the isoprenylation process and the subsequent cell cycle progression and cell proliferation[33,34]. Furthermore, FPPS and GGPPS are implicated in cell cycle progression via their association with the isoprenylation process of GTPases Ras and RhoA proteins[33,34,46]. In addition, PPARα plays an important role in regulating lipid homeostasis. Studies of PPARα null mice have revealed the importance of PPARα in the hepatic lipid metabolism and liver regeneration[33,36]. Based on our cDNA microarray

results we determined that after PH of HBx-transgenic mice the expression level of these enzymes in the liver is down-regulated. As shown in previous studies, inhibition of enzymes involved in cholesterol and isoprenoid synthesis leads to the upregulation of cdk inhibitors, reduction in cdk, hypophosphorylation of Rb, followed by cell cycle arrest in G1[47,48]. Thus, the tempered liver regeneration in HBx-transgenic mice can be explained by the inhibition of the isoprenylation process.

Taken together, our microarray experiments performed on HBx-transgenic mice have provided new mechanisms for HBx-mediated inhibition of liver regeneration. The relevance of our results in the context of HBV-dependent cell cycle inhibition, required for an efficient HBV replication, awaits further evaluation.

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COMMENTS

Background

The liver has a unique capacity to regenerate after partial hepatectomy (PH) or injury. Experimental evidence supports the hypothesis that proliferation of hepatocytes is responsible for liver regeneration after PH, despite the low replicative rate of hepatocytes in the normal liver. In a previous report we had shown that HBx expression inhibits liver regeneration after PH in HBx expressing transgenic mice. We also demonstrated a paracrine inhibitory effect of HBx on liver cell proliferation. Indeed, transplantation of HBx-expressing liver cells or injection of serum from HBx-transgenic mice into control mice is sufficient to inhibit liver regeneration after PH. Thus, HBx protein may act by combining the intracellular and paracrine effects, and inhibiting the proliferation of both infected and uninfected liver cells in the context of liver regeneration.

Research frontiers

Hepatitis B Virus (HBV) infection is a worldwide health problem. It is estimated that 350 million people are chronic carriers of hepatitis B virus (HBV). According to the WHO, hepatitis B is responsible for 1.2 million deaths each year. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma. The HBx protein of HBV exhibits pleiotropic effects, that modulate transcription, cell responses to genotoxic stress, protein degradation, cell viability and signalling pathways. Several studies have suggested that HBx protein of HBV, participates in the development of HCC, although the precise role(s) in this process remain unclear.

Innovations and breakthroughs

In the present report, we examined the impact of HBx on the gene expression profile of liver cells in HBx-expressing transgenic mice, 48 h after partial hepatectomy. We used cDNA microarray containing over 5 thousand mouse and rat cDNA elements to determine genes that are altered in the presence of reduced liver regeneration. By demonstrating molecular mechanisms involved in HBx-mediated inhibition of liver regeneration, the data provides a better understanding of the role of the HBx protein in the physiopathology of chronic liver disease caused by HBV infection.

Applications

Although these observations were made in the specific context of liver regeneration triggered by partial hepatectomy, they may represent an important mechanism of HBV-dependent liver carcinogenesis in chronic hepatitis and the associated stimulation of liver cell proliferation. Thus, this study may contribute to the development of new approaches based on controlling the cellular effects of viral replication and ultimately provide new tools to control HBV-induced liver disease.

Peer review

This is a well written, interesting report on gene modulation associated with inhibition of liver regeneration in hepatitis B x protein transgenic mice.

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