Hepatitis B virus X antigen promotes transforming growth factor-β1 (TGF-β1) activity by up-regulation of TGF-β1 and down-regulation of α2-macroglobulin

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Hepatitis B virus (HBV) X antigen (HBxAg) may contribute to the development of hepatocellular carcinoma (HCC) by activation of signalling pathways such as NF-κB. To identify NF-κB target genes differentially expressed in HBxAg-positive compared to -negative cells, HepG2 cells consistently expressing HBxAg (HepG2X cells) were stably transfected with pZeoSV2 or pZeoSV2–IκBz. mRNA from each culture was isolated and compared by PCR select cDNA subtraction. The results showed lower levels of α2-macroglobulin (α2-M) in HepG2X-pZeoSV2 compared to HepG2X-pZeoSV2–IκBz cells. This was confirmed by Northern and Western blotting, and by measurement of extracellular α2-M levels. Elevated transforming growth factor-β1 (TGF-β1) levels were also seen in HepG2X compared to control cells. Serum-free conditioned medium (SFCM) from HepG2X cells suppressed DNA synthesis in a TGF-β-sensitive cell line, Mv1Lu. The latter was reversed when the SFCM was pretreated with exogenous, activated α2-M or with anti-TGF-β1. Since elevated TGF-β1 promotes the development of many tumour types, these observations suggest that the HBxAg-mediated alteration in TGF-β1 and α2-M production may contribute importantly to the pathogenesis of HCC.


INTRODUCTION

Chronic hepatitis B virus (HBV) infection may result in the development of liver diseases, including hepatitis, cirrhosis and hepatocellular carcinoma (HCC). There is increasing evidence that the HBV-encoded X antigen, HBxAg, contributes importantly to the development of HCC, although the mechanisms whereby it does so are incompletely understood (Feitelson, 1999). HBxAg is a trans-activating protein that functions by binding to and altering the activity of transcription factors in the nuclei of infected cells and by constitutively activating a number of cytoplasmic signalling pathways (Henkler & Koshy, 1996; Feitelson, 1999). Among the latter, HBxAg activates phosphorylation-dependent signal transduction pathways involving Ras/Raf/MAPK (Cross et al., 1993; Benn & Schneider, 1994; Natoli et al., 1995a), diacylglycerol (DAG)/protein kinase C (PKC) (Cross et al., 1993; Kekule et al., 1993), Jak1/STAT (Lee & Yun, 1998) and NF-κB (Su & Schneider, 1996). The findings that NF-κB blocks hepatocellular apoptosis (Beg et al., 1995; Beg & Baltimore, 1996), that HBxAg protects liver cells from apoptosis by inducing NF-κB (Pan et al., 2001), and that HCC is resistant to apoptosis (Natoli et al., 1995b), suggest that HBxAg activation of NF-κB may play an important role in hepatocarcinogenesis.
suppresses anti-tumour immune responses, promotes the development of drug resistance and enhances metastasis (Teicher, 2001), through activation of the Smad and/or other signalling pathways that override apoptosis (Akhurst, 2002). These observations suggest that the regulation of TGF-β1 activity may contribute importantly to the pathogenesis of HCC.

α2-Macroglobulin (α2-M) is a broad-spectrum proteinase inhibitor in serum (Hall & Roberts, 1978; Sottrup-Jensen, 1989) that binds to many cytokines (Borth, 1992), and in some cases, binding inactivates cytokine activity (Gonia et al., 1994). In other cases, α2-M–protease and α2-M–cytokine complexes are rapidly cleared by endocytosis after binding to the LRP/α2-M receptor on hepatocytes, fibroblasts and macrophages (Williams et al., 1994). Studies with α2-M gene knockout mice suggest that the primary function of α2-M in vivo may be to regulate the activities of cytokines in the TGF-β family (Umano et al., 1995; Webb et al., 1996). In HCC, α2-M expression is decreased (Paradisi et al., 2003), although it is not clear whether part of the mechanism whereby HBxAg enhances TGF-β1 activity is through decreased expression of α2-M. Hence, experiments were designed to test the hypothesis that HBxAg up-regulated TGF-β1 activity was due not only to increased TGF-β1 production, but also to a decrease in α2-M expression.

METHODS

Cell lines and culture. The HepG2X (encoding HBx) and HepG2CAT (encoding bacterial chloramphenicol acetyltransferase) cells were made and characterized previously (Lian et al., 1999). G418-resistant cultures were passaged by standard trypsinization. The sequences obtained were compared with sequence data of α2-M obtained at the sequencing facility on campus. The sequences obtained were compared with the sequence database.

Stable transfection of HepG2X cells with pZeoSV2-1xBα-HA. Separate cultures of HepG2X and HepG2CAT cells were stably transfected with pZeoSV2-1xBα-HA or pZeoSV2 vector, as described (Pan et al., 2001). Cells were selected in zeocin for 4 weeks. Exogenous 1xBα expression was detected by Western blotting using anti-HA as the primary antibody (Pan et al., 2001). NF-xB activity was measured in a luciferase assay by transient transfection of cells with the reporter plasmid pGL2-HIV-LTR-1. In this plasmid, luciferase expression was under control of the HIV LTR-1 promoter, which contained an NF-xB binding site that is responsive to HBxAg (Twu et al., 1989; Pan et al., 2001).

PCR select cDNA subtraction, cloning and sequencing. mRNA was isolated from HepG2X-pZeoSV2 and HepG2X-pZeoSV2-1xBz cells (see below), reverse transcribed to cDNA fragments with random primers, and then subjected to PCR select cDNA subtraction (BD Biosciences/Clontech), as described (Lian et al., 1999). The cDNA fragments obtained from subtraction were then individually cloned into pT7BlueT vector (Novagen). DNA sequencing was performed at the sequencing facility on campus. The sequences obtained were compared to those in GenBank by GCG software for homology to known genes.

Northern blot analysis. Total RNA from HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 and HepG2X-pZeoSV2-1xBz cells was isolated using the RNasy Mini Kit (Qiagen). Ten μg of total RNA from each culture was analysed by formaldehyde denaturing agarose gel electrophoresis. The α2-M cDNA fragment identified from subtraction was labelled with [α-32P]dCTP using the Prime-a-Gene Labelling system (Promega) for use as a probe. A G3PDH probe was used to normalize for RNA loading in each sample.

Western blot analysis. HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 and HepG2X-pZeoSV2-1xBz cells were treated with lysis buffer containing 50 mM Tris/HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, phosphatase inhibitors (50 mM NaF, 0.1 mM Na3VO4), protease inhibitors (1 mM PMSF, 10 μg leupeptin ml−1, 10 μg pepstatin ml−1) and 1 % Triton X-100 for 15 min on ice. Forty μg of total protein in each sample was reduced in 10 % 2-mercaptoethanol, and analysed by SDS-PAGE on 4–20 % gradient gels. Proteins were then blotted onto PVDF membranes (Millipore) and then blocked overnight in PBS containing 5 % nonfat milk. For primary antibody, rabbit anti-human α2-M polyonal antibody (A749/RSH, Accurate Chemical Co., Westbury, NY, USA) was used at a 1:2000 dilution. Mouse anti-human β-actin monoclonal antibody (Clone AC-15, Sigma) was used at 1:5000 as an internal control. For secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (1:10 000 dilution) or HRP-conjugated goat anti-mouse Ig (1:3000 dilution) (Accurate) was used. The results were visualized using the ECL detection system (Amersham).

Immunoprecipitation of α2-M in culture medium from metabolically labelled cells. Five-million HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 or HepG2X-pZeoSV2-1xBz cells in 100 mm dishes were incubated for 16 h in complete medium. After each culture had been washed twice with PBS, the cells were incubated with methionine-free medium containing 5 % dialysed FCS for 1 h, which was then replaced with 4 ml of the same medium containing 70 μCi [35S]methionine (REN) ml−1 for 16 h. Cell supernatants were collected through a 0·45 μm filter to remove cell debris, and 1·2 ml of each sample was used for immunoprecipitation. All immunoprecipitation steps were done at 4 °C. Samples preclaried with protein A–agarose beads were incubated with 15 μl of rabbit anti-human α2-M for 3 h, and then with 20 μl of protein A–agarose beads overnight. Beads were collected and washed four times with NP-40 buffer (150 mM NaCl, 50 mM Tris/HCl, pH 6·8, 1 % NP40) by centrifuging at 4000 r.p.m. for 5 min each time. Finally, samples were analysed by SDS-PAGE on 7·5 % gels, and results visualized by autoradiography.

Preparation of serum-free conditioned medium (SFCM). Cells were seeded in 100 mm dishes (4 × 106 cells per dish) in complete medium. The next day, the cultures were washed twice with PBS, and then incubated overnight in serum-free Eagle’s MEM containing 0·02 % BSA. Cells were then incubated in fresh serum-free medium for an additional 24 h. Cell culture supernatants were collected, filtered, aliquoted and stored at −80 °C until use.

DNA synthesis. To activate TGF-β in SFCM, the pH was adjusted to 1·5 with HCl. Samples were incubated at room temperature for 15 min, and then neutralized with NaOH/HEPES to pH 7·4–7·5. Activated SFCM was diluted to 75 % with fresh serum-free medium (to allow for addition of other reagents) for DNA synthesis assays. In some experiments, 75 % SFCM was incubated with 10 μg TGF-β antibody ml−1 (clone 1D11, which binds all TGF-β isoforms; R & D Systems) for 1 h at 37 °C prior to evaluation in the DNA synthesis assay. Mouse IgG (Sigma) was used as a control in place of TGF-β antibody. In other experiments, 75 % SFCM was incubated with methylene-treated α2-M (α2-M-MA) (BioMac, Leipzig, Germany)
The Mv1Lu cells were seeded in 96-well plates (1 × 10⁴ cells per well) in complete medium for 24 h. Medium was changed to 75% of activated SFCM with or without TGF-β1 neutralizing antibody, and with or without z2-M-MA. After incubation for 18 h at 37°C, [3H]thymidine (1 μCi per well) was added for an additional 7 h. Cells in each well were harvested onto glass fibre filters using a cell harvester and the radioactivity incorporated in DNA was determined by scintillation counting. Each experiment was done in quadruplicate.

The findings that HBxAg stimulates NF-κB (Su & Schneider, 1996), that NF-κB is hepatoprotective (Beg et al., 1995), in part, by inhibiting apoptosis (Beg & Baltimore, 1996), that HBxAg confers resistance to apoptosis in a number of cell lines tested (Shih et al., 2000; Pan et al., 2001), and that an important characteristic of HCC is its resistance to apoptosis (Natoli et al., 1995b), suggest that the constitutive activation of NF-κB in HBxAg-positive cells may contribute importantly to hepatocarcinogenesis. To examine this in more detail, HepG2X cells (Lian et al., 1999) were stably transfected with pZeoSV2 or pZeoSV2-IxBz (Pan et al., 2001). Following selection in zeocin, exogenous IxBz expression was demonstrated by Western blotting, and suppressed NF-κB activity in a transient luciferase reporter gene assay with HepG2X cells (Pan et al., 2001).

Identification and verification of z2-M as a gene down-regulated in HepG2X-pZeoSV compared to HepG2X-pZeoSV-IxBz cells

To discern differences in gene expression triggered by NF-κB in HepG2X cells, PCR select cDNA subtraction was performed with whole cell mRNA isolated from HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IxBz cells. The results yielded three cDNA fragments that may represent differentially expressed genes. Among the two up-regulated genes, one consisted of a 792 bp fragment that had 99-4% homology over 621 bp with the human fibronectin gene (GenBank accession no. A14133), which encodes an important extracellular matrix component of the fibrogenic response. The other up-regulated gene consisted of a 540 bp fragment that was 98-9% homologous over a 444 bp overlap with a human fetal liver cDNA clone (GenBank accession no. H49417). Cloning and expression of the full-length cDNA from the latter, referred to as up-regulated gene 7 (URG7), is stimulated nearly 5-fold in HepG2X cells. HBxAg blocks anti-Fas-mediated apoptosis in HepG2 cells, and it was found that most of this was due to the NF-κB-dependent expression of URG7 (Lian et al., 2001). Sequence analysis of the cDNA fragment that was strongly down-regulated in HepG2X-pZeoSV2 cells compared to HepG2X-pZeoSV2-IxBz cells showed 100% homology over a 500 bp region with human z2-M cDNA when compared to entries in GenBank (Benson et al., 1997). This fragment, spanning nucleotides 3026–3525, based upon GenBank accession no. A21185, was used as a probe for further studies. To verify the results of subtractive hybridization, Northern blotting was performed with RNA isolated from the cells used for PCR select cDNA subtraction. The results show that the levels of z2-M RNA in HepG2X-pZeoSV2-IxBz cells were 4-7-fold higher than in HepG2X-pZeoSV2 cells following normalization with G3PDH. In comparison, the levels of z2-M mRNA in HepG2CAT-pZeoSV2 control cells were 6-9-fold higher than in HepG2X-pZeoSV2 cells (Fig. 1a). Western blotting for z2-M yielded similar results following normalization to β-actin (Fig. 1b). Hence, HepG2X-pZeoSV2

![Fig. 1. Levels of z2-M mRNA were determined by Northern blot analysis (a) while levels of z2-M protein were determined by Western blot analysis (b) in lysates from HepG2CAT-pZeoSV2 (lane 1), HepG2X-pZeoSV2 (lane 2) or HepG2X-pZeoSV2-IxBz cells. The results shown in each panel are from one of three experiments.](http://vir.sgmjournals.org)
HBxAg up-regulates TGF-β1 in HepG2 cells

Given that TGF-β1 is transcriptionally activated by HBxAg (Yoo et al., 1996; Lee et al., 2001), the levels of TGF-β1 were assayed by quantitative ELISA in SFCM from HepG2X-pZeoSV2, HepG2X-pZeoSV2-IxBz and HepG2CAT-pZeoSV2 cells. In SFCM from HepG2CAT-pZeoSV2 cells, the levels of TGF/β1 were some 7- to 8-fold lower than the SFCM from HepG2X-pZeoSV2 (P<0.001) and HepG2X-pZeoSV2-IxBz cells (Fig. 3). As expected, when extracellular HBxAg was assayed by ELISA, the levels were highest in SFCM of HepG2CAT-pZeoSV2 cells, about 7-fold lower in the SFCM from HepG2X-pZeoSV2 cells (P<0.001), but less than 2-fold reduced in the SFCM from HepG2X-pZeoSV2-IxBz cells (Fig. 3). TGF-β2, also assayed by ELISA, was not detectable in any of the SFCM, suggesting HBxAg alters only TGF-β1 but not TGF-β2 expression. Hence, TGF-β1 expression is up-regulated in the supernatants of HBxAg-positive cells, but its increased production, unlike that of HBxAg, is not NF-κB dependent.

When the HBxAg: TGF-β1 ratio was determined on a molar basis in the SFCM of HepG2CAT-pZeoSV2 cells (Fig. 3), it was about 1440, while for HepG2X-pZeoSV2 cells it was 29. The ratio of these numbers showed that the introduction of HBxAg into HepG2 cells resulted in an almost 50-fold change in the relative amounts of HBxAg and TGF-β1 in the SFCM. The question now became, what were the functional consequences of this altered HBxAg: TGF-β1 ratio?

Decreased HBxAg: TGF-β1 ratio was associated with increased TGF-β activity

To determine whether there is a net difference in TGF-β activity in HBxAg-positive compared to -negative HepG2 cells, SFCM from HepG2CAT-pZeoSV2 or HepG2X-pZeoSV2 supernatant from HepG2CAT-pZeoSV2 cells with normal IgG resulted in no discernable band (Fig. 2, lane 4), demonstrating that the immunoprecipitations were specific. These results suggest that the decreased levels of HBxAg within HepG2X cells were also decreased in the supernatant from the same cultures, and that a significant fraction of the HBxAg could be recovered when HepG2X cells were stably transfected with pZeoSV2-IxBz.

HBxAg suppresses accumulation of extracellular α2-M

Since extracellular HBxAg binds to and inactivates TGF-β, it was important to see whether the suppression of HBxAg expression in HBxAg-positive HepG2 cells also resulted in decreased accumulation of HBxAg in the tissue culture supernatant. Accordingly, HepG2CAT-pZeoSV2, HepG2X-pZeoSV2 and HepG2X-pZeoSV2-IxBz cells were radio-labelled with [35S]methionine. Equal volumes of tissue culture supernatants were immunoprecipitated with anti-human α2-M, and then analysed by SDS-PAGE and autoradiography. The results showed that there was 7-7-fold more HBxAg in the supernatant of HepG2CAT-pZeoSV2 cells (Fig. 2, lane 1) compared to that of HepG2X-pZeoSV2 cells (P<0.001) (Fig. 2, lane 2). In comparison, the amount of HBxAg recovered from the supernatants of HepG2X-pZeoSV2-IxBz cells (Fig. 2, lane 3) was approximately 4-fold higher than the levels observed in HepG2X-pZeoSV2 supernatants (P<0.003). Immunoprecipitation of the supernatant from HepG2CAT-pZeoSV2 cells with normal IgG resulted in no discernable band (Fig. 2, lane 4), demonstrating that the immunoprecipitations were specific. These results suggest that the decreased levels of HBxAg within HepG2X cells were also decreased in the supernatant from the same cultures, and that a significant fraction of the HBxAg could be recovered when HepG2X cells were stably transfected with pZeoSV2-IxBz.

Fig. 3. α2-M, TGF-β1 and TGF-β2 were each detected by specific ELISAs in serum-free medium (SFM) alone (black bar), or in the supernatants of HepG2CAT-pZeoSV2 cells (white bar), HepG2X-pZeoSV2 cells (grey bar) or HepG2X-pZeoSV2-IxBz cells (striped bar). The data shown are from one of three experiments, each done in duplicate.

Fig. 2. Immunoprecipitation of α2-M labelled with [35S]methionine from cell-culture supernatants of HepG2CAT-pZeoSV2 (lane 1), HepG2X-pZeoSV2 (lane 2) and HepG2X-pZeoSV2-IxBz (lane 3). In lane 4, the immunoprecipitation in lane 1 was repeated with an equivalent amount of normal IgG. The results shown here are from one of three independent experiments.
cells was added to cultures of Mv1Lu cells. DNA synthesis (\[^{3}H\]thymidine uptake) in this cell line is very sensitive to inhibition by TGF-\(\beta\). In this assay, acidification followed by neutralization of the SFCM was performed in order to activate TGF-\(\beta\). Accordingly, when this experiment was done with serum-free medium, which contained no TGF-\(\beta\), high levels of DNA synthesis were observed (Fig. 4, group 1). When unacidified SFCM from HepG2CAT-pZeoSV2 or HepG2X-pZeoSV2 was tested, no inhibition of DNA synthesis was observed (Fig. 4, group 1), since all of the TGF-\(\beta\) in unacidified SFCM was inactive. In group 2, in which TGF-\(\beta\) was activated, serum-free medium again showed no inhibition of DNA synthesis. In contrast, there was about a 3-5-fold inhibition of \[^{3}H\]thymidine incorporation when SFCM from HepG2CAT-pZeoSV2 cells was tested (white bar), while a 15-18-fold decrease in proliferation was observed when the assay was repeated with SFCM from HepG2X-pZeoSV2 cells. Each of these differences was highly significant compared to the values for serum-free medium alone \((P<0.001)\). Comparison of the inhibitory activities of SFCM from HepG2CAT-pZeoSV2 and HepG2X-pZeoSV2 showed that the latter inhibited 5-6-fold more than the former \((P<0.001)\) (Fig. 4, group 2). To determine whether inhibition of DNA synthesis was related to TGF-\(\beta\) activity, the SFCM samples from group 2 were preincubated with normal IgG, which had no effect upon the inhibition of DNA synthesis (Fig. 4, group 3), or with anti-human TGF-\(\beta\) (Fig. 4, group 4), which completely reversed the inhibitory effect of the SFCM samples. These results suggest that the HBxAg-mediated increase in expression of TGF\(\beta\) (and suppression of \(\alpha_2\)-M expression) resulted in a stronger inhibition of DNA synthesis on Mv1Lu reporter cells compared to the same experiment done with SFCM isolated from HBxAg-negative HepG2 cells, and that all of the inhibition observed in this assay was due to TGF-\(\beta\).

**Fig. 4.** Effect of SFCM on DNA synthesis in Mv1Lu cells. Mv1LU cells were incubated with serum-free medium (grey bars), SFCM from HepG2CAT-pZeoSV2 cells (white bars) or SFCM from HepG2X-pSV2neo cells (black bars). Test medium was added to the reporter cell line unacidified (group 1), acidified and neutralized (group 2), acidified, neutralized and preincubated with normal IgG (10 \(\mu\)g ml\(^{-1}\)) (group 3), or acidified, neutralized and preincubated with anti-TGF-\(\beta\) (10 \(\mu\)g ml\(^{-1}\)) (group 4). The data presented are means of three experiments, each done in triplicate.

Exogenous \(\alpha_2\)-M blocks the TGF-\(\beta\) inhibition of DNA synthesis in SFCM

If in natural HBV infections the levels of \(\alpha_2\)-M in serum regulate the activity of TGF-\(\beta\), then addition of exogenous activated (methylamine-treated) \(\alpha_2\)-M or \(\alpha_2\)-M-MA to SFCM from HepG2X-pZeoSV2 cells should inactivate the TGF-\(\beta\) and relieve the inhibition of DNA synthesis in Mv1Lu. When this experiment was done, the SFCM from HepG2X-pZeoSV2 cells significantly inhibited proliferation relative to the SFCM from HepG2CAT-pZeoSV2 cells \((P<0.001)\) (Fig. 5), as expected. However, when the amount of exogenous \(\alpha_2\)-M-MA added to the SFCM from HepG2X-pZeoSV2 cells was increased to levels similar to that found in normal human serum (which is in the mg % range) (LaMarre et al., 1991), TGF-\(\beta\) activity was blocked, and control levels of DNA synthesis were observed (Fig. 5). These observations show that the inhibitory effects of TGF-\(\beta\) on DNA synthesis are reversible in the presence of activated \(\alpha_2\)-M.

**DISCUSSION**

The observations that HBxAg activates NF-\(\kappa\)B (Henkler & Koshy, 1996; Su & Schneider, 1996), that NF-\(\kappa\)B is hepatoprotective (Beg et al., 1995), in part, by blocking apoptosis (Beg & Baltimore, 1996), and that HBxAg inhibits both Fas and TGF-\(\beta\) triggered apoptosis (Shih et al., 2000; Pan et al., 2001), suggest that the constitutive activation of NF-\(\kappa\)B by HBxAg may contribute importantly to the development of preneoplastic foci and HCC. Using PCR select cDNA subtraction, this study revealed that HBxAg suppressed the expression of \(\alpha_2\)-M, which is known to bind to and inactivate TGF-\(\beta\). In fact, \(\alpha_2\)-M is a major negative regulator of TGF-\(\beta\).
regulatory factor for the TGF-β family of proteins (LaMarre et al., 1991; Gonias et al., 1994; Webb et al., 1996, 1998). \(x_2\)-M expression was suppressed 5- to 6-fold in HepG2X-pZeoSV2 compared to HepG2CAT-pZeoSV2 cells (Figs 1 and 2). Similar differences were observed in SFCM from these cells (Fig. 3), while TGF-β1 expression increased 7- to 8-fold (Fig. 3). This resulted in a significant increase in the TGF-β1 DNA synthesis inhibitory activity in a reporter cell line (Fig. 4). Therefore, HBxAg increases TGF-β1 signalling through trans-activating the TGF-β1 gene (Yoo et al., 1996), by stimulating Smad4 (which mediates TGF-β1) signalling (Lee et al., 2001) and by reducing expression of the major TGF-β1 inhibitor, \(x_2\)-M (this study). The latter is likely to be another mechanism whereby HBxAg promotes TGF-β1 activity during CLD.

TGF-β1 is elevated in the blood of HBV carriers with HCC (Castilla et al., 1991; Ito et al., 1991; Song et al., 2002), while a recent microarray-based study demonstrated a consistent suppression of \(x_2\)-M gene expression in HCC (Paradis et al., 2003). \(x_2\)-M gene expression is also reportedly reduced or absent in several human hepatoma cell lines (Kondo et al., 1998). Hence, the observations made here are consistent with those of increased TGF-β1 and decreased \(x_2\)-M made in clinical studies. Collectively, these observations suggest that the increase in TGF-β1 and decrease in \(x_2\)-M by HBxAg parallels the shift in expression of these proteins in vivo. Moreover, \(x_2\)-M gene expression has been reported to be depressed in adenocarcinomas and squamous cell carcinomas of the lung (McDoniels-Sivers et al., 2002), suggesting that the phenomenon observed here may occur in other tumour types, although by other mechanisms.

The impact of \(x_2\)-M expression levels in HepG2 cells upon TGF-β1 activity was measured using a [\(^3\)H]thymidine incorporation assay in a mink lung epithelial cell line that is highly sensitive to the inhibitory effects of TGF-β1 upon DNA synthesis. The results in Fig. 4 show that the TGF-β1 levels in the SFCM from HepG2CAT-pZeoSV2 cells clearly inhibited DNA synthesis compared to serum-free medium, but that when SFCM from HepG2X-pZeoSV2 cells was used, the inhibition was significantly greater, suggesting that altered \(x_2\)-M:TGF-β1 ratios in the supernatants of HepG2X-pZeoSV2 compared to HepG2CAT-pZeoSV2 cells (Fig. 3) also had functional consequences. In this context, it is proposed that HBxAg-positive hepatocytes, being relatively TGF-β1 resistant, would acquire a survival advantage over HBxAg-negative hepatocytes during chronic infection. This would promote the persistence of virus-infected cells in the face of ongoing immune responses, which would be important for the maintenance of the carrier state despite multiple bouts of CLD. This would also promote the accumulation of HBxAg-positive cells in carriers with CLD, which is an important early step in tumourgenesis (Koike et al., 1994; Ueda et al., 1995). These observations would predict a strong correlation between HBxAg staining in the liver and severity of CLD, which has already been reported (Feitelson, 1999; Jin et al., 2001).

There are additional ramifications of increased TGF-β1 activity in HBxAg-positive liver cells. For example, TGF-β1 represents an important mediator of liver fibrosis (Czaja et al., 1989; Gressner & Bachem, 1995) by stimulating the synthesis of collagen, fibronectin (Ignotz & Massague, 1986), hyaluronan and proteoglycan (Bachem et al., 1989) in hepatic stellate cells. TGF-β1 also inhibits matrix degradation, by reducing collagenase gene expression and plasmin synthesis (Rieder et al., 1993), and by increasing the synthesis of metalloproteinase inhibitors (Edwards et al., 1987). TGF-β1 staining in hepatocytes also correlates with the degree of liver fibrosis in man (Castilla et al., 1991; Nagy et al., 1991) and rats (Czaja et al., 1989; Armendariz-Borunda et al., 1990). In addition, \(x_2\)-M inhibits TGF-β1-induced collagen synthesis in human liver myofibroblasts (Tiggelman et al., 1997), and reduces elevated matrix synthesis of cultured rat hepatic stellate cells (Schufla & Bachem, 1999). This suggests \(x_2\)-M may have an antifibrogenic effect in vivo, which is blocked in chronic HBV infection by HBxAg.

Elevated TGF-β1 activity may be immunosuppressive (Teicher, 2001). For example, in vitro experiments with peripheral blood mononuclear cells isolated from HBV carriers have shown that TGF-β1 treatment significantly inhibited HBV core antigen-stimulated proliferation, interferon-γ production and core antigen-specific cytotoxic T-cell activity (Kamura et al., 1993). Although TGF-β1 also suppresses anti-tumour immunity in other tumour types (Teicher, 2001), it is not clear whether this occurs with HCC.

Although TGF-β1 signalling negatively regulates cell growth, the constitutive activation of other signalling pathways, such as those involving NF-κB, Akt/Pi3K, c-Jun and Ras/Raf/MAPK, may block Smad-mediated growth inhibition and apoptosis, and instead promote cell survival, motility, migration and other features commonly associated with tumour cells (Akhurst, 2002). In this context, HBxAg is known to stimulate most of these signalling pathways (Henkler & Koshy, 1996; Feitelson, 1999), so that increased TGF-β1 expression in HBxAg-positive cells may result in autocrine and/or paracrine growth and survival signals to HBxAg-positive hepatocytes at the expense of uninfected cells. Akt/Pi3K, for example, is stimulated by HBxAg, and is known to block TGF-β1 apoptosis in HBxAg-positive cells, but it also known to block \(x_2\)-M transcription (Kortylewski et al., 2003). NF-κB also blocks \(x_2\)-M gene expression by blocking STAT3 binding (which stimulates \(x_2\)-M expression) to the \(x_2\)-M promoter (Bode et al., 2001). Under these conditions, increased TGF-β1 production by HBxAg-positive compared to -negative HepG2 cells will only serve to further promote the growth and survival of HepG2X (but not HepG2CAT) cells, which is observed in this study. Hence, the shift in \(x_2\)-M and TGF-β1 expression levels by HBxAg during chronic infection may be another pathway whereby HBxAg contributes to hepatocarcinogenesis on the molecular level.
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REFERENCES


