Hepatitis B virus X protein protects against anti-Fas-mediated apoptosis in human liver cells by inducing NF-κB

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The hepatitis B virus-encoded X antigen (HBxAg) may contribute to the development of liver cancer, in part, by stimulating the growth and survival of infected cells in the face of ongoing immune responses. Given that the Fas ligand/receptor system contributes to the pathogenesis of chronic hepatitis B, experiments were designed to test the hypothesis that HBxAg mediates resistance of liver cells to anti-Fas killing. Accordingly, when HBxAg was introduced into HepG2 cells, it rendered these cells partially resistant to killing by anti-Fas. In HepG2 cells replicating virus, protection against anti-Fas killing was also observed, but to a lesser extent. Survival correlated with the activation of nuclear factor kappa B (NF-κB) by HBxAg. Sensitivity to anti-Fas was observed in control cells, and was re-established in HepG2 cells stably transfected with the dominant negative inhibitor of NF-κB, IκBα. HBxAg activation of NF-κB was also associated with decreased levels of endogenous IκBα mRNA. Hence, HBxAg stimulation of NF-κB promotes the survival of liver cells against Fas killing. This may contribute to the persistence of infected hepatocytes during chronic infection.

Introduction

Chronic hepatitis B virus (HBV) infection is strongly associated with the development of hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Tiollais et al., 1985). There is increasing evidence that the HBV-encoded X antigen, HBxAg, contributes importantly to hepatocellular transformation (Feitelson & Duan, 1997). This evidence includes findings of integrated HBV X region DNA in most chronically infected livers and HCC tissues (Wollersheim et al., 1988; Zahm et al., 1988), along with the overexpression of X mRNA (Diamantis et al., 1992; Paterlini et al., 1995) and high levels of HBxAg polypeptide expression (Wang et al., 1991a, b) in these same tissues. HBxAg also transforms nontumorigenic cell lines (Shirakata et al., 1989; Hohne et al., 1990; Seifer et al., 1991), and sustained high levels of HBxAg in transgenic mice are associated with the development of HCC (Kim et al., 1991; Koike et al., 1994). In order for HBxAg to play a significant role in hepatocarcinogenesis, there would have to be one or more mechanisms whereby HBxAg-positive hepatocytes persist in the face of ongoing antiviral immune responses.

On the molecular level, HBxAg may contribute to multistep hepatocarcinogenesis in a number of ways. For example, HBxAg trans-activates many viral and cellular promoters. Virus promoters include those in HBV (Colgrove et al., 1989; Nakatake et al., 1993) and the long-terminal repeat of human immunodeficiency virus-1 (Siddiqui et al., 1989). Cellular promoters include those for c-fos, c-jun (Natoli et al., 1994a, b), c-myc (Balsano et al., 1991), insulin-like growth factor II (Lee et al., 1998) and epidermal growth factor receptor (Menzo et al., 1993). HBxAg trans-activation is also mediated by the binding of HBxAg to a variety of transcription factors in the nucleus (Doria et al., 1995; Henkler & Koshy, 1996), and by the stimulation of several signal transduction pathways in the cytoplasm (Haskell et al., 1991; Kekule et al., 1993; Doria et al., 1995). Among the latter, many groups have shown that HBxAg stimulates the nuclear factor kappa B (NF-κB) pathway (Siddiqui et al., 1989; Mahe et al., 1991; Doria et al., 1995; Su & Schneider, 1996), which regulates a number of genes involved in the immune and inflammatory responses (Baueerle, 1991; Grilli et al., 1993; Liou & Baltimore, 1993; May & Ghosh, 1998). HBxAg may also contribute to transformation...
by functionally inactivating a number of negative growth regulatory pathways (Feitelson et al., 1993a, 1999; Lian et al., 1999), suggesting that HBxAg may participate in several steps in multistep carcinogenesis.

The sustained production of HBxAg during chronic infection (Wang et al., 1991a, b) is consistent with the hypothesis that it may promote the resistance of infected liver cells to immunologically mediated apoptosis. Recent studies with tissue culture cells have shown that HBxAg modulates apoptosis in a variety of settings (Wang et al., 1995; Elmore et al., 1997; Terradillos et al., 1998). In this context, it is possible that HBxAg physically or functionally interacts with components of signal transduction pathways, thereby blocking the transmission of 'death signals' to the nuclei of infected hepatocytes (Gottlob et al., 1998). The fact that HBxAg stimulates NF-κB (Doria et al., 1995; Su & Schneider, 1996), combined with the centrality of NF-κB activity to liver cell survival in vivo (Bellas et al., 1997) and in vitro (Beg et al., 1995; Li et al., 1999), suggest that HBxAg may promote hepatocellular survival, and their resistance to apoptosis, by an NF-κB-dependent pathway. Certainly, NF-κB activation blocks hepatocellular apoptosis mediated by tumour necrosis factor alpha (TNF-α) (Beg & Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996) and transforming growth factor beta (TGF-β) (Bellas et al., 1997; Arsura et al., 1997). Although these cytokines may contribute to the pathogenesis of chronic HBV infection, there is increasing evidence that the Fas ligand/receptor system also plays an important role in pathogenesis (Galle & Kramer, 1998). In particular, there is a significant correlation between levels of Fas antigen and chronic liver disease in HBV carriers (Watanabe-Fukunage et al., 1992; Galle et al., 1995; Mochizuki et al., 1996). Since activated T-cells express the Fas ligand (Rouvier et al., 1993; Suda et al., 1993), its binding to Fas receptor on hepatocytes may contribute importantly to the destruction of infected hepatocytes (Ando et al., 1994; Rouquet et al., 1995). The exquisite sensitivity of mice to anti-Fas treatment is highlighted by the massive liver cell apoptosis that accompanies such treatments (Ogasawara et al., 1993). In addition, the fact that HCC cells express little or no Fas receptor (Strand et al., 1996) may render them resistant to Fas-mediated apoptosis. Hence, experiments were designed to test the hypothesis that the HBxAg-mediated protection of liver cells against anti-Fas-triggered apoptosis is dependent upon HBxAg activation of NF-κB.

**Methods**

**Cell lines and culture.** The human hepatoblastoma cell line HepG2 was cultured on rat tail collagen as described (Lian et al., 1999). The retroviral packaging cell line PA317 (Miller & Baltimore, 1986) was maintained in Dulbecco’s MEM with 10% FCS, 2 mM l-glutamine, and antibiotics. HepG2.2.15 cells were kindly provided by George Acs (Sells et al., 1987).

**Plasmids.** The recombinant retroviral vector plasmids, pSLXCMV-FLAGX and pSLXCMV-CAT, were constructed by inserting the HBV X gene or bacterial chloramphenicol acetyltransferase (CAT) gene into the polyclinker of pSLXCMV (Duan et al., 1995), which is a murine leukaemia virus-based vector containing the neomycin-resistance gene (Miller & Rosman, 1989), as described (Lian et al., 1999). Plasmid pZeoSV-IxBz-HA was constructed by using the PCR-amplified human IxBz gene from plasmid F-IxBz (Haskill et al., 1991) (kindly provided by D. W. Ballard, Dept of Microbiology and Immunobiology, Vanderbilt University, TN, USA). The PCR product was digested with BamHI and EcoRI, and the resulting 1028 bp fragment was ligated into the polyclinker of pZeoSV2i+ (Invitrogen), which carries the zeocin-resistance gene. This recombinant produced a translation product containing the influenza virus haemagglutinin (HA) epitope at the amino terminus of IxBz. Plasmid pGL2-HIV-1-LTR contains full-length HIV-1 LTR, which drives expression of the luciferase reporter gene (a kind gift from Ed Mercer, Kimmel Cancer Center, Thomas Jefferson University, PA, USA).

**Transduction of HepG2 cells with pSLXCMV-FLAGX and pSLXCMV-CAT.** Transduction of HepG2 cells with recombinant retroviruses encoding HBxAg or CAT was performed as described (Lian et al., 1999). Cultures were selected for growth by addition of G418 to the medium for 14 days. Drug-resistant cells were trypsinized and grown in complete medium without cloning prior to analysis. These HepG2X and HepG2CAT cultures were not only used for prior work (Lian et al., 1999), but also the studies herein.

**CAT assay.** This was done essentially as described (Ausubel et al., 1991; Gorman et al., 1992) with minor modifications (Lian et al., 1999).

**Anti-Fas treatment.** Cells were added to 6-well plates (7 x 10⁵ cells per well) and incubated overnight in complete medium. Cultures were then treated with 0.5 µg/ml of anti-Fas (Ab-2, monoclonal mouse IgG; Oncogene Research, Cambridge, MA, USA) or 0.5 µg/ml of mouse IgG (Sigma) as control in the presence of actinomycin D (0.3 µg/ml) or cycloheximide (2 µg/ml). These compounds sensitize cells to anti-Fas killing by preventing de novo synthesis of cellular proteins that would otherwise diminish the effects of anti-Fas. After 0, 24, 36, 48 and 60 h treatment, all cells (adherent and floating) in each well were collected by trypsinization, stained with trypan blue, and counted in a haemocytometer. About 800 cells in each sample were counted. The percentage of live cells, as determined by trypan blue exclusion, was calculated as follows: (no. of live cells in anti-Fas or control IgG treated well/no. of total cells in each corresponding well) x 100. All tests were done in duplicate. Cell viability was independently determined using the modified tetrazolium salt (MTT) assay, as described by the manufacturers (CellTitre 96 Non-radioactive Cell Proliferation assay, Promega).

**TUNEL assay.** Apoptosis was assessed by measuring DNA fragmentation in a standard TUNEL (deoxynucleotidyltransferase-mediated dUTP nick end labelling) assay according to the instructions with the kit (TACS In situ Apoptosis Detection kit, Trevigen, Gaithersburg, MD, USA).

**Transient transfection and luciferase assay.** To measure the effects of anti-Fas treatment upon NF-κB activity, 1 x 10⁶ HepG2X or HepG2CAT cells were plated overnight, and then cotransfected, using calcium phosphate precipitates, with 10 µg of pZeoSV or pZeoSV-IxBz-HA and 2 µg of the reporter plasmid pGL2-HIV-1-LTR. Cells were incubated overnight, washed with PBS, and assayed for luciferase activity at 36 h post-transfection. To determine whether NF-κB activity is sensitive to anti-Fas treatment, cultures were transiently transfected with 2 µg of pGL2-HIV-1-LTR, and then maintained in medium containing mouse IgG (0.5 µg/ml) or anti-Fas (0.5 µg/ml) with or without either actinomycin D (0.3 µg/ml) or cycloheximide (2 µg/ml). Following
overnight incubation (17 h), the cells were washed and then lysed in 250 µl of Cell Culture Lysis Reagent from the Luciferase Assay kit (Promega). Protein concentration was determined with the Bio-Rad Protein Assay. For each tube, 30 µg of total protein was mixed with 100 µl of luciferase assay reagent and evaluated in a luminometer. All tests were done in duplicate.

Stable transfection of HepG2X cells with pZeoSV-IκBα-HA. HepG2X and HepG2CAT cells were plated at 2 x 10⁶ cells per 100 mm dish, incubated overnight, and then transfected with 15 µg of pZeoSV-IκBα-HA or pZeoSV vector in calcium phosphate precipitates (Profection Mammalian Transfection System, Promega). Cells were incubated with DNA precipitate overnight and then selected by addition of zeocin (1 mg/ml final concentration) (Invitrogen) for 3 weeks. All resistant colonies were trypsinized and grown in complete medium.

Western blot analysis. Western blotting for HBxAg was performed using a mixture of X peptide antibodies raised in rabbits (Lian et al., 1999). To detect exogenously expressed IκBα-HA in HepG2X-IκBα cells, Western blotting was performed with a mouse anti-HA monoclonal antibody (clone 12CA5, Boehringer Mannheim) at a final concentration of 2.5 µg/ml. Briefly, 5 x 10⁶ HepG2X and HepG2CAT cells were washed with ice-cold PBS and lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, phosphatase inhibitors (50 mM NaF, 1 mM Na₃VO₄), protease inhibitors (1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin) and 1% Triton X-100. Cytoplasmic extracts were isolated by centrifugation at 10000 r.p.m. for 10 min at 4 ºC. Total protein samples (150 µg, as measured with the Bio-Rad Protein Assay kit) from each cell lysate were analysed by SDS–PAGE on 12% gels. Proteins were then transferred to PVDF membranes (Millipore). To detect endogenous IκBα, rabbit anti-IκBα (Fl) polyclonal antibody (SC-847, Santa Cruz Biotechnology) was used at 1:1000. Mouse anti-β actin monoclonal antibody (clone AC-15, Sigma) was used at 1:5000 as an internal control. After incubation with horseradish peroxidase-conjugated goat anti-rabbit Ig or goat anti-mouse Ig (Accurate, Westbury, NY, USA), the results were visualized using the ECL detection system (Amersham) and quantified by gel scanning, as described (Lian et al., 1999).

Northern blot hybridization. Whole cell RNA from HepG2X-pZeoSV, HepG2X-IκBα and HepG2CAT-pZeoSV cells was isolated using the RNeasy Mini kit (Qiagen) according to the enclosed instructions. Total RNA (10 µg) isolated from each cell lysate was then analysed by denaturing agarose gel electrophoresis using formaldehyde. Samples were transferred to a Nytran nylon membrane (Schleicher & Schuell), and Northern blot hybridization was carried out using an IκBα-specific probe. Briefly, pZeoSV-IκBα-HA was digested with BamHI and EcoRI, and the insert isolated by agarose gel electrophoresis followed by extraction. This probe was then radio-labelled with [α-³²P]dCTP using the Prime-a-Gene Labelling System (Promega). Hybridization and washing were done under stringent conditions. RNA levels on the resulting autoradiograms were semiquantified by gel scanning. A β-actin probe was used to normalize for loading of the cellular RNA in each sample.

Statistical analysis. All data points represent the mean ± two standard deviations of duplicate determinations. Statistical analysis was done with Student’s t-test.

Results

Expression of HBxAg and CAT in transduced HepG2X and HepG2CAT cells

pSLXCMV-FLAGX and pSLXCMV-CAT were stably introduced into independent cultures of HepG2X cells by replication-defective, recombinant retroviruses. Preliminary characterization of HepG2X cells verified the presence of p17X by Western blotting using anti-HBX while no HBxAg was observed in HepG2CAT cells (Lian et al., 1999). Likewise, CAT activity was only detectable in HepG2CAT but not in HepG2X cells (Lian et al., 1999). These results show that the corresponding recombinant genes were expressed in HepG2 cells. HBxAg polypeptide and CAT activity were undiminished for more than 3 months of passage, suggesting stable expression (data not shown). In contrast, all attempts to detect HBxAg in HepG2.2.15 cell lysates by Western blotting were unsuccessful, even though the clone replicates in tissue culture cells and is infectious in chimpanzees (Sells et al., 1988).

HBxAg partially protects HepG2 cells from killing with anti-Fas

HepG2X, HepG2CAT and HepG2.2.15 cells were treated with anti-Fas or an equivalent amount of normal mouse IgG in the presence of cycloheximide or actinomycin D, and cell viability was determined by trypan blue staining 0, 24, 36, 48 and 60 h later. The results for cultures treated with cycloheximide are shown (Fig. 1) and are similar to those obtained in parallel cultures treated with actinomycin D (data not shown). In both cases, anti-Fas killed greater than 80% of HepG2CAT cells within 60 h. In contrast, the fraction of HepG2X cells killed by anti-Fas was significantly less than that of HepG2CAT cells after 24 h (P < 0.006) 36 h (P > 0.008), 48 h (P > 0.006) and 60 h (P > 0.02) of treatment. When HepG2X.2.15 cells were treated with anti-Fas, protection was observed at 36 h (P < 0.05), 48 h (P < 0.03) and 60 h (P > 0.03) compared to HepG2CAT cells (Fig. 1a). No killing was observed when HepG2X, HepG2CAT or HepG2.2.15 cells were treated in parallel experiments with an equivalent amount of normal IgG (Fig. 1a). A representative experiment, presented in Fig. 1 (b–e), shows confluent cultures of HepG2X and HepG2CAT cultures after 48 h treatment with normal mouse IgG, suggesting that under the conditions used cycloheximide was not toxic (Fig. 1b and c, respectively). Likewise, cycloheximide was not toxic to HepG2.2.15 cells (data not shown). Treatment of parallel cultures with cycloheximide plus anti-Fas resulted in about 30–45% killing of HepG2X cells (Fig. 1d) and > 80% killing of HepG2CAT cells after 48 h treatment (Fig. 1e). Although these results were obtained using trypan blue staining, very similar observations were made when cell viability was independently determined using the MTT assay (data not shown). Hence, HBxAg partially protected HepG2 cells from anti-Fas killing. Partial protection was also observed in HepG2.2.15 cells compared to HepG2CAT cells, although at lower levels.

To determine whether anti-Fas treatment resulted in the degradation of cellular DNA, cells treated with anti-Fas or an equivalent amount of control IgG were analysed for in situ nicking of cellular DNA by a standard TUNEL assay. The
results shown (Fig. 2) are after 24 h treatment, which was prior to loss of most cells from the anti-Fas-treated cultures. Widespread staining was also observed after 36 and 48 h anti-Fas treatment (data not shown). After 24 h treatment, no staining was observed in HepG2CAT or HepG2X cells treated with normal IgG (Fig. 2a and c, respectively). In contrast, anti-Fas treatment of HepG2CAT (Fig. 2b) or HepG2X (Fig. 2d) cells resulted in the appearance of strong nuclear staining in about 60% and 20% of the cells, respectively. Agarose gel electrophoresis of whole cell DNA isolated from anti-Fas-treated, but not normal IgG-treated cells 36 and 48 h after the beginning of treatment showed ethidium bromide-stained smears (Fig. 2e). These results, which are similar to those in other systems (Ramachandra & Studzinski, 1995), suggest that cell death was due to apoptosis.

**HBxAg-mediated resistance of HepG2 cells to anti-Fas killing is NF-κB dependent**

The fact that HBxAg stimulates NF-κB (Siddiqui et al., 1989; Mahe et al., 1991; Doria et al., 1995; Su & Schneider, 1996), and that NF-κB protects liver cells from apoptosis (Beg & Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996;
HBx-stimulated NF-κB blocks anti-Fas killing

Fig. 2. Degradation of cellular DNA by anti-Fas treatment. HepG2CAT (a, b) and HepG2X (c, d) cells were treated with cycloheximide and normal IgG (a, c) or anti-Fas (b, d) and then stained for nicked DNA (TUNEL assay) 24 h later. Brown-coloured nuclei are indicative of nicked cellular DNA. The bar in the lower left-hand corner of (a) represents 100 nm (for a–d). In (e), whole cell DNA was extracted from anti-Fas-treated HepG2CAT (lane 1) or HepG2X cells (lane 2) 36 h after the beginning of treatment, or from identically treated HepG2CAT (lane 3) or HepG2X (lane 4) at 48 h. HepG2CAT cells were treated with an equivalent amount of normal IgG for 36 (lane 5) or 48 h (lane 7). HepG2X cells were also treated with normal IgG for 36 (lane 6) or 48 h (lane 8). The gel was stained with ethidium bromide and photographed.

Wang et al., 1996; Arsura et al., 1997; Bellas et al., 1997), suggests that the mechanism of protection against anti-Fas-mediated killing in HepG2X cells may be NF-κB dependent. To test this hypothesis, HepG2X and HepG2CAT cells were transiently transfected with 2 µg of pGL2-HIV-1-LTR, which contains NF-κB binding sites within the HIV-LTR sequences just upstream from the luciferase reporter gene. Cotransfection was done with 10 µg of pZeoSV or 10 µg of pZeoSV-IκBα-HA; the latter expresses the dominant negative NF-κB inhibitor, IκBα. In all experiments, luciferase activity was
assayed 36 h after transfection. Preliminary experiments showed that pZeoSV-IκBα-HA expressed the expected 38 kDa IκBα in transfected HepG2X cells, as determined by Western blot analysis using anti-HA as primary antibody (Fig. 3a). This permitted endogenous and exogenous IκBα to be detected separately. As shown in Fig. 3(b), luciferase activity was up to 8-fold higher in HepG2X cells (lane 1) compared to HepG2X cells expressing exogenous IκBα (lane 2) or HepG2CAT cells expressing a baseline level of luciferase activity (lane 3). These results show that the exogenous IκBα is functional, and that it inhibits HBxAg-mediated stimulation of luciferase activity.

To examine the relationship between NF-κB activation and cell survival, HepG2X cells were stably transfected with pZeoSV-IκBα-HA and selected with zeocin. Exogenous IκBα expression was again assessed by Western blotting (data not shown but similar to Fig. 3a). For controls, HepG2X and HepG2CAT cells were also stably transfected with the pZeoSV vector in the presence of zeocin. Anti-Fas killing (in the presence of cycloheximide) was then assessed over 60 h. When HepG2CAT cells were stably transfected with pZeoSV, > 95 % killing was observed within 48 h of anti-Fas treatment (Fig. 4b, c). In contrast, < 30 % of HepG2X cells stably transfected with the pZeoSV vector were dead by 48 h of treatment (Fig. 4a, d) (P < 0.005), suggesting that HBxAg provides some protection against anti-Fas killing, and that this protection was NF-κB dependent (Fig. 4a, d and e). However, by 60 h nearly 100 % of HepG2X-IκBα and HepG2CAT cells were killed in the presence of anti-Fas. When HepG2X cells were stably transfected with pZeoSV-IκBα-HA, however, HepG2X cells became as sensitive to anti-Fas killing as the control HepG2CAT cells (Fig. 4a to 4b, and 4c to 4e). In contrast, when pZeoSV-IκBα-HA was transfected into HepG2CAT cells, no colonies were recovered following zeocin selection. Addition of an equivalent amount of IgG in place of anti-Fas in parallel experiments resulted in < 5 % killing of HepG2X and HepG2CAT cells (P < 0.001), suggesting that killing was associated with anti-Fas and not some other component of serum (Fig. 4a, b). Hence, anti-Fas-mediated killing appears to be, at least in part, NF-κB dependent.

**Fig. 3.** Expression and function of exogenous IκBα-HA in HepG2X cells. (a) Total protein was extracted from 1 × 10⁶ cells and IκBα-HA was detected by Western blotting using mouse anti-HA (2.5 µg/ml final concentration). HepG2X cells were transfected with pZeoSV-IκBα-HA (lane 1) or pZeoSV (lane 2). The arrow indicates the position of the exogenous IκBα. (b) Inhibition of HBxAg-stimulated NF-κB activity by IκBα. Transfections of HepG2X and HepG2CAT cells with the indicated plasmids were performed in three independent experiments, each done in duplicate.

**Anti-Fas treatment is associated with a decrease in NF-κB activity**

The above results suggest that NF-κB may play an important role in mediating resistance to anti-Fas killing. If this is true, then cells treated with anti-Fas should have lower endogenous NF-κB activity compared to control cells. To test this possibility, HepG2X or HepG2CAT cells were transiently transfected with the reporter plasmid pGL2-HIV-LTR and then treated with IgG or anti-Fas in the presence or absence of either actinomycin D or cycloheximide. In the presence of mouse IgG, HepG2X cells stimulated endogenous NF-κB activity by an average of almost 6-fold compared to HepG2CAT cells (Fig. 5, compare lanes 1 and 2) (P < 0.008), as expected. When parallel cultures were treated with an equivalent amount of anti-Fas alone (without actinomycin D or cycloheximide), similar results were observed for the NF-κB levels in HepG2X cells (lanes 3 and 4) (P < 0.01). This indicates that in the absence of a block in de novo transcription or translation, anti-Fas treatment does not have a significant impact upon NF-κB activity (compare lanes 1 and 3, 2 and 4). Similar results were obtained when the experiment was repeated with normal IgG in the presence of actinomycin D (lanes 7 and 8) or cycloheximide (lanes 11 and 12), showing that actinomycin D and cycloheximide did not inhibit NF-κB activity. However, treatment of cells with anti-Fas in the presence of actinomycin D (lanes 5 and 6) or cycloheximide (lanes 9 and 10) resulted in statistically significant decreases in NF-κB activity (P < 0.004 and P < 0.002, respectively). These results suggest that anti-Fas treatment depresses NF-κB activity in both HepG2X and HepG2CAT cells. However, NF-κB activity was always higher in HepG2X compared to HepG2CAT cells independent of treatment, suggesting that there was always some degree of NF-κB activation as long as HBxAg was present. Although cell viability was only 70–80 % in cells treated with anti-Fas plus actinomycin D or cycloheximide, compared to other
HBx-stimulated NF-κB blocks anti-Fas killing

Fig. 4. Anti-Fas treatment of HepG2X and HepG2CAT cells in the presence and absence of exogenous IκBz. (a) HepG2X cells were stably transfected with pZeoSV (■) or pZeoSV-IκBα (▲), and then treated with anti-Fas. As a control, HepG2X cells transfected with pZeoSV (□) were treated with normal IgG. (b) In parallel experiments, HepG2CAT cells were transfected with pZeoSV and then treated with anti-Fas (●) or an equivalent amount of normal IgG (○). The data shown are means from four independent experiments, each done in duplicate. (c)–(e) Photographs of HepG2CAT cells transfected with pZeoSV (c), HepG2X cells transfected with pZeoSV (d) and HepG2X cells transfected with pZeoSV-IκBα (e). The photographs were taken 48 h after the beginning of treatment. The black bar in the bottom left-hand corner of (c) represents 100 nm (for c–e).
HepG2CAT cells (Luciferase activity was then measured and recorded in lysates from Fas in the presence or absence of actinomycin D or cycloheximide. treated with anti-Fas. HepG2X and HepG2CAT cells were transiently of HepG2X (lane 1) and HepG2CAT cells (lane 2). I α
Differential expression of endogenous I α
B in HepG2X and HepG2CAT cells was determined in HepG2X compared to HepG2CAT cell extracts, the levels were up to five times lower in HepG2X cells compared to HepG2CAT cells (Fig. 6a). When the relative levels of IκBα mRNAs were determined in these two cell lines by Northern blot analysis, there was up to 12-fold less transcript in HepG2X compared to HepG2CAT cells (Fig. 6b, lanes 1 and 2). Hence, the lower steady-state levels of IκBα in HepG2X compared to HepG2CAT cells may be due partially to a decrease in the steady-state levels of IκBα mRNA. This observation may be associated with the recently documented trans-suppressor activity associated with HBxAg (Lee et al., 1998).

Discussion

It is well known that HBxAg stimulates NF-κB (Siddiqui et al., 1989; Mahe et al., 1991; Doria et al., 1995; Su & Schneider, 1996) and that NF-κB activity is important for hepatocellular growth and survival (Beg et al., 1995; Bellas et al., 1997; Li et al., 1999). Given that the Fas ligand–receptor system appears to play a central role in the pathogenesis of chronic HBV infection in man (Galle et al., 1995; Galle & Krammer, 1998), and to the development of inflammatory liver disease in transgenic mice (Nakamoto et al., 1997), one way HBxAg expression may contribute to chronicity is by increasing the resistance of infected hepatocytes to Fas-mediated killing. This is established in Figs 1 and 2, which show that HBxAg partially protects HepG2 cells from anti-Fas-mediated apoptosis. This would permit the accumulation of HBxAg-positive cells during chronic infection, which has been observed (Wang et al., 1991a, b) and is consistent with the central role of HBxAg in the development of HCC (Tiollais et al., 1985; Feitelson & Duan, 1997). The finding that HBxAg-mediated resistance to anti-Fas-triggered apoptosis is blocked by the introduction of exogenous IκBα (Fig. 4) suggests a link between HBxAg-mediated stimulation of NF-κB and resistance to anti-Fas-mediated killing for the first time. The generality of these observations is underscored by recent observations that elevated NF-κB activity protects T-cells from anti-Fas-mediated apoptosis (Zong et al., 1998; Dudley et al., 1999). In addition, it has been shown that hepatitis C virus core protein protects cells against anti-Fas-triggered cell death by NF-κB activation (Marusawa et al., 1999). Hence, the protection of infected hepatocytes by virus gene products may contribute importantly to the establishment and persistence of chronic infections.

The finding that HBxAg may directly complex to IκBα (Weil et al., 1999) and/or stimulate IκBα phosphorylation (Su & Schneider, 1996) suggests mechanisms whereby HBxAg may stimulate NF-κB activity. In this context, the observation of decreased IκBα in HepG2X compared to HepG2CAT cells (Fig.

experiments in this series (Fig. 5), where viability was near 100%, luciferase assays were normalized to a consistent amount of protein extracted from viable cells in each culture. Hence, HBxAg stimulates endogenous NF-κB, while anti-Fas treatment results in decreased endogenous activity.

Steady-state levels of IκBα in HepG2X and HepG2CAT cells

Previous work has shown that HBxAg stimulates NF-κB by promoting the degradation of IκBα from NF-κB–IκBα com-plexes (Chirillo et al., 1996; Su & Schneider, 1996), and by preventing the association of newly synthesized IκBα with nuclear NF-κB (Weil et al., 1999). When the steady-state levels of IκBα were determined in HepG2X compared to HepG2CAT cell extracts, the levels were up to five times lower in HepG2X cells compared to HepG2CAT cells (Fig. 6a). When the relative levels of IκBα mRNAs were determined in these two cell lines by Northern blot analysis, there was up to 12-fold less transcript in HepG2X compared to HepG2CAT cells (Fig. 6b, lanes 1 and 2). Hence, the lower steady-state levels of IκBα in HepG2X compared to HepG2CAT cells may be due partially to a decrease in the steady-state levels of IκBα mRNA. This observation may be associated with the recently documented trans-suppressor activity associated with HBxAg (Lee et al., 1998).

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The finding that HBxAg may directly complex to IκBα (Weil et al., 1999) and/or stimulate IκBα phosphorylation (Su & Schneider, 1996) suggests mechanisms whereby HBxAg may stimulate NF-κB activity. In this context, the observation of decreased IκBα in HepG2X compared to HepG2CAT cells (Fig.
HBx-stimulated NF-κB blocks anti-Fas killing

6) is consistent with the post-translational degradation of IκB by HBxAg, as indicated in earlier work (Chirillo et al., 1996; Su & Schneider, 1996). However, the decreased levels of endogenous IκB mRNA in HepG2X compared to HepG2CAT cells (Fig. 6) imply that HBxAg transcriptionally downregulates the expression of IκB. This finding is another unique aspect of this work. Recently, transcriptional suppression has been suggested as a mechanism whereby HBxAg may regulate cellular gene expression (Lian et al., 1999). In this context, it is proposed that low levels of HBxAg may trigger NF-κB, but not fully suppress IκB levels. If this occurs, then infected cells with low levels of HBxAg may have limited resistance to apoptosis compared to uninfected control (HBxAg-negative) cells. On the other hand, in the presence of high levels of HBxAg, IκB would be fully inactivated, resulting in an HBxAg-associated increased resistance to Fas-mediated apoptosis. The finding that HepG2X cells (with high levels of HBxAg) are considerably more resistant to anti-Fas than HepG2.2.15 cells (with lower levels of HBxAg), and that the latter are more resistant than HepG2CAT cells (with no HBxAg), is consistent with this idea. In this model, levels of HBxAg are low early in the course of chronic infection; when HBV DNA integrates into the host chromosomal DNA during the regeneration that follows a bout of hepatitis, the intracellular levels of HBxAg slowly rise. However, further work must be done in order to determine whether IκB levels are depleted in chronically infected livers, whether the IκB gene is transcriptionally downregulated by HBxAg in natural infection, and whether this correlates with the levels of intrahepatic HBxAg.

The findings that anti-Fas inhibits NF-κB function (Fig. 5), and that HBxAg stimulates NF-κB activity, suggest that HBxAg may modulate the effects of Fas-mediated killing during chronic infection. Further, the fact that apoptosis is commonly observed in chronic viral hepatitis (Galle & Krammer, 1998; Lau et al., 1998), and that HBxAg is frequently detected in chronically infected liver (Wang et al., 1991a, b), may explain the close correlation between X antigen expression and chronic liver disease (Feitelson et al., 1993a). HBxAg has been shown to promote apoptosis in some systems (Kim et al., 1998; Terradillos et al., 1998), while providing resistance to apoptosis in others (Wang et al., 1995), suggesting that HBxAg may modulate the response of infected cells to different types of apoptotic stimuli (Elmore et al., 1997). The ability of HBxAg to promote or inhibit apoptosis may also depend upon the state of cell differentiation and/or whether hepatocytes are quiescent or regenerating. For example, NF-κB may be antiapoptotic in thymocytes but proapoptotic in mature peripheral T-cells (Lin et al., 1999). Although not addressed in this study, HBxAg also appears to stimulate the expression of Fas ligand (Shin et al., 1999), which is a natural effector of NF-κB (Kasibhatla et al., 1999). If this occurs in vivo, it would promote the lysis of effector T-cells expressing Fas receptor, resulting in enhanced survival of HBxAg-positive hepatocytes. The fact that NF-κB stimulates multiple immune response genes, and that it also protects a variety of cell types against apoptosis, provides a potential link between inflammation and the survival of HBxAg-positive cells.

In chronic hepatitis B, apoptosis is mediated by activated T-cells that have increased expression of Fas ligand upon hepatocytes that constitutively express Fas receptor (Mochizuki et al., 1996). These observations suggest that activated T-cells kill HBV antigen-expressing hepatocytes by Fas ligand–receptor interaction, thereby mediating virus clearance. The ability of HBxAg to prevent Fas-mediated killing, at least in part, would provide some protection to infected cells replicating virus, thereby promoting the development and persistence of the chronic carrier state. This idea is supported by the results in Fig. 1(a), showing that HepG2.2.15 cells are partially resistant to anti-Fas-mediated killing. However, the fact that the levels of resistance of HepG2.2.15 cells to anti-Fas killing are considerably lower than that of HepG2X cells may be due to the relative levels of HBxAg in both cell types, with low, undetectable levels in HepG2.2.15 cells, and easily detectable levels in HepG2X cells. Although the protection afforded by HBxAg in HepG2.2.15 cells is not impressive, small differences in protection over the course of many years (or decades) of chronic infection may significantly contribute to the persistence of virus-infected cells. After the clearance of virus from blood and liver, the increased resistance of HBxAg-positive hepatocytes to Fas-mediated apoptosis would contribute to the persistence of such cells in the chronically infected host. These cells would then be ripe for additional steps in multitstep carcinogenesis, such as the HBxAg-mediated inactivation of p53 (Feitelson et al., 1993b; Truant et al., 1995) its stimulation of the cell cycle (Benn & Schneider, 1995) and its putative interruption of DNA repair machinery (Lee et al., 1995). Hence, the HBxAg-mediated resistance to Fas killing may serve to sustain virus replication by promoting the survival of infected cells, as well as contribute to the pathogenesis of HCC.

This work was supported by NIH grants CA48656 and CA66971 to M.F. We would like to acknowledge Dr. Zhaoqi Tian for his advice and help in performing this work.

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Received 5 June 2000; Accepted 20 September 2000
Published ahead of print (6 October 2000) in JGV Direct as DOI 10.1099/vir.0.17187-0