Caspase-3 activation as a key factor for HBx-transformed cell death

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Abstract. *Objectives:* Nuclear factor-kappa B (NF- κ B) activation has been associated with the tumorigenic growth of hepatitis B virus X protein (HBx)-transformed cells. This study was aimed to find a key target for treatment of HBx-mediated cancers. *Materials and methods:* NF- κ B activation, endoplasmic reticulum-stress (ER-stress), caspase-3 activation, and cell proliferation were evaluated after Chang/HBx cells permanently expressing HBx viral protein were treated with inhibitors of NF- κ B, proteasome and DNA topoisomerase. *Results:* Inhibition of NF- κ B transcriptional activity by transient transfection with mutant plasmids encoding Akt1 and glycogen synthase kinase-3 β (GSK-3 β), or by treatment with chemical inhibitors, wortmannin and LY294002, showed little effect on the survival of Chang/HBx cells. Furthermore, I κ B α (S32/36A) mutant plasmid or other NF- κ B inhibitors, 1-pyrrolidinedithiocarbonyl acid and sulphasalazine, were also shown to have little effect on the cell proliferation. By contrast, proteasome inhibitor-1 (Pro1) and MG132 enhanced the HBx-induced ER-stress response and the subsequent activation of caspase-12, -9 and -3 and reduced cell proliferation. Camptothecin (CPT), however, triggered activation of caspase-3 without induction of caspase-12, and reduced cell proliferation. In addition, CPT-induced cell death was reversed by pre-treatment with z-DEVD, a caspase-3-specific inhibitor. *Conclusions:* Detailed exploitation of the regulators of caspase-3 activation could open the gate for finding an efficient target for development of anticancer therapeutics against HBx-transformed hepatocellular carcinoma.

INTRODUCTION

The pathogenic mechanism of hepatocellular carcinoma development (HCC) by hepatitis B virus X protein (HBx) has not been fully described, although HBx is suggested to play a crucial

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role in development of HCC (Feitelson 1992; Robinson 1994). The pleiotropic action of HBx on cellular functions may be a major obstacle for clear understanding of its mechanism and development of anticancer drugs associated with HCC. Concerning tumorigenesis, it was previously reported that the PI3K/Akt pathway is involved in metastasis of HBx-transformed cells (Chung *et al.* 2004). Furthermore, glycogen synthase kinase-3 β (GSK-3 β), commonly known as a substrate of Akt, has been reported to phosphorylate RelA/p65 *in vitro*, and is related to the regulation of nuclear factor-kappa B (NF- κ B) (Hoeflich *et al.* 2000; Schwabe & Brenner 2002), reflecting the observations that demonstrate that HBx transcriptional coactivation is linked to the antiapoptotic action of NF- κ B (Su & Schneider 1996; Lin *et al.* 1998). However, the role of GSK-3 β in NF- κ B activation and its contribution to cell proliferation in response to HBx are poorly understood, although HBx is considered to play an important role in NF- κ B activation.

In the context of NF- κ B activation, the proteasome has emerged as an excellent target for cancer therapy (Su & Schneider 1996; Zavrski *et al.* 2005). Very recently, a therapeutic implication of proteasome inhibition for differential regulation of Noxa in normal melanocytes and melanoma cells was reported (Fernandez *et al.* 2005). The proteasome inhibitor PS-341 was also demonstrated to enhance chemosensitivity to CPT-11, a topoisomerase inhibitor (Cusack *et al.* 2001). Moreover, I κ B α superrepressor mutant greatly enhanced chemosensitivity of human pancreatic carcinoma cells (Sato *et al.* 2003).

The proteasome is also required for transactivation function of HBx (Hu *et al.* 1999). In addition, it has been described that HBx interacted with the proteasome complex, acting as a substrate and thereby potentially inhibiting the complex (Zhang *et al.* 2000). Moreover, a recent report demonstrating a link between endoplasmic reticulum (ER) stress and functionality of the proteasome in the long term with gradual accumulation of misfolded proteins (Menendez-Benito *et al.* 2005). Perturbation of normal ER function by ER stress can induce expression of novel genes through activation of either the unfolded protein response (UPR) or ER overload response, or with combination of both pathways (Welihinda *et al.* 1999). For maintenance of cell homeostasis following the ER stress, UPR induces activation of three proteins in the ER, activation transcription factor-6 (ATF6), inositol-requiring endonuclease-1 α (IRE-1 α) and PKR-like ER kinase (PERK) to remove or correct unfolded proteins by expressing ER chaperones, such as GRP78/Bip and GRP94 (Yoshida *et al.* 1998; Tirasophon *et al.* 2000; Shuda *et al.* 2003). When the extent of damage exceeds a cell's ability for rescue, however, cells proceed along the apoptotic cell death programme through the IRE-1 α -mediated caspase-12/7 activation pathway, a typical ER stress-induced initiation of apoptosis (Kaufman 2002).

In this study, we found that HBx induced UPR signalling, including GRP78 expression. Moreover, ER stress-induced apoptotic proteins, caspase-12 and -9, were activated in HBx-transformed cells (Chang/HBx). Of interest, expression of the apoptotic proteins was further elevated by treating the tumour cells with proteasome inhibitors. In addition, treatment of the cells with proteasome inhibitors or activation of caspase-3 led to significant reduction in survival of Chang/HBx tumour cells, while inhibition of other pathways using chemical compounds showed only a marginal effect.

Given recent reports suggesting that the proteasome pathway might contribute to development of HBx-related HCC and be crucial for regulation of cell growth and oncogenic transformation (Hochstrasser 1996; Adams 2004; Cui *et al.* 2006), our study suggests that although the PI3K-Akt pathway might mediate HBx-induced NF- κ B activation, investigation of caspase-3 activation would lead to discovery of a promising target for development of anticancer drugs, specially for treatment of HBx-transformed cell HCC.

MATERIALS AND METHODS

Materials

Lipofectamine Plus, Dulbecco's modified Eagle's medium and foetal bovine serum were purchased from Gibco-BRL (Grand Island, NY, USA). Proteasome inhibitor-1 (Pro1), lactacystin, MG132, LY294002, wortmannin, PD98059, 1-pyrrolidinedithiocarbonyl acid (PDTTC), sulphasalazine, camptothecin (CPT) and antibody against caspase-3 were obtained from Calbiochem (San Diego, CA, USA). The antibody against GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-SAPK/JNK and caspase-9 were purchased from Cell Signalling Technology (Beverly, CA, USA), and antibodies against GRP78 and caspase-12 and the pNF- κ B-Luc plasmid were obtained from Stratagene (La Jolla, CA, USA). Cell counting kit-8 (CCK-8) was from Dogindo laboratory (Kumamoto, Japan). The luciferase assay kit was purchased from Promega (Madison, WI, USA). Sephadex G-25 columns and enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). [γ - 32 P] ATP was from NEN-DuPont (Boston, MA, USA). All the other reagents were obtained from Sigma (St. Louis, MO, USA).

Plasmids

I κ B α (S32/36A) was generated by point mutation of the wild-type plasmid. HA-tagged Akt (wild-type and kinase, dead) were kindly provided by Dr. Thomas Frank at Columbia University. HA-tagged plasmids encoding the wild-type GSK-3 β was provided by Dr. Mira Jung (Department of Radiation Medicine, Georgetown University School of Medicine, Washington DC, USA). Point mutation of plasmids GSK-3 β (GSK-3 β S9A, GSK-3 β pm) was generated using the QuickChange XL method (Stratagene) with the following sets of primers: sense strand (5'-CCCAGAACCACCGCCTTTGCGGAGAGC-3') and antisense strand (5'-GCTCTCCGCAAG-GCGGTGGTTCTGGG-3').

Mutant plasmid was verified by DNA sequencing. Based on the published sequence of the human GRP78 gene, a 378-bp fragment of the GRP78 promoter region (-371 to region; relative to the transcriptional initiation-site) was amplified by PCR from genomic DNA with the following sets of primers and cloned into the *Kpn*I and *Hind*III sites of the pGL3-basic vector (Promega), which contains the luciferase reporter gene: sense strand (5'-CGGGGTACCGTC ACTCTGCTGGA-3') and antisense strand (5'-CATCCCAAGCTTTCGACCTCACCGTCG CCTACTC-3').

Cell culture and transfection

Normal (Chang), vector control (Chang/Vc) or HBx-transformed (Chang/HBx) cells were obtained from Dr. Cheorl-Ho Kim (University of Sungkyoonkwan, Seoul, South Korea) (Chung *et al.* 2004). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, and 10% heat-inactivated foetal bovine serum, and were cultured in a humidified CO₂ incubator at 37 °C. For luciferase reporter assays, cells (2 $\times$ 10⁵/mL) were incubated in 6-well plates for 24 h. Using Lipofectamine Plus, cells were then co-transfected with 0.5 μ g of pNF- κ B-Luc, 0.5 μ g of pCMV/ β -galactosidase plasmid and 100 ng or 500 ng of a plasmid encoding the Akt1 wt, GSK-3 β wt or GSK-3 β pm. After 24 h, luciferase activity was measured with a detection kit. Alternatively, cells were co-transfected with 0.5 μ g of pGRP78-Luc, 0.5 μ g of pCMV/ β -galactosidase plasmid for 6 h or 24 h and luciferase activity was measured. The value obtained was normalized to the β -galactosidase activity measured in the corresponding cell extracts.

Immunoblot analysis

Cells were grown to subconfluency, washed four times with ice-cold phosphate-buffered saline (PBS) and then scraped on ice for incubation for 15 min in a solution containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM NaF, 0.4 mM phenylmethylsulphonyl fluoride (PMSF), leupeptin (10 $\mu g/mL$), aprotinin (10 $\mu g/mL$), 0.1 mM Na_3VO_4 , and 1 mM dithiothreitol (DTT). After the addition of Nonidet P-40 to a final concentration of 0.15%, the lysate was vigorously mixed for 15 s then centrifuged at 20 000 *g* for 1 min at 4 °C. The resulting supernatant was stored at -80 °C as the cytoplasmic extract, and the nuclear pellet was re-suspended in a solution containing 50 mM HEPES-KOH (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM NaF, leupeptin (10 $\mu g/mL$), aprotinin (10 $\mu g/mL$), 0.4 mM PMSF, 0.1 mM Na_3VO_4 , 1 mM DTT and 10% glycerol. The resulting suspension was incubated for 30 min on ice with occasional vortexing and was then centrifuged at 20 000 *g* for 30 min at 4 °C. Equal amounts of cytoplasmic or nuclear extract were subsequently fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For preparation of total cell lysate, cells washed with PBS buffer were scraped and collected as described above. They were gently re-suspended using a pipette in lysis buffer containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.4 mM PMSF, leupeptin (10 $\mu g/mL$), aprotinin (10 $\mu g/mL$), 0.1 mM Na_3VO_4 , 1 mM DTT and 1% 3-cholamidopropyl-dimethylammonio-1-propane-sulfonate (CHAPS). The mixture was placed on ice for 30 min and then was centrifuged at 24 000 *g* at 4 °C for 30 min. The supernatant was collected and preserved at -80 °C until use. Total or fractionated proteins were transferred to a polyvinylidene difluoride membrane and were subjected to immunoblot analysis with specific antibodies. Immune complexes were detected with enhanced chemiluminescence reagents.

Electrophoretic mobility shift assay

In brief, cells (5×10^6 in 10 mL) grown in 100 mm dishes were lysed on ice for 15 min in a hypotonic solution containing 10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA (sodium salt), 0.2 mM NaF, 0.2 mM Na_3VO_4 , 0.4 mM PMSF, leupeptin (10 $\mu g/mL$), 1 mM DTT, and 0.15% Nonidet P-40. The lysate was centrifuged at 18 500 *g* for 1 min at 4 °C, the resulting nuclear pellet was re-suspended in ice-cold extraction buffer (50 mM HEPES-KOH pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM Na_3VO_4 , 0.4 mM PMSF, 1 mM DTT and 10% glycerol) and was incubated for 30 min at 4 °C with occasional vortexing. The nuclear lysate was then centrifuged at 15 000 r.p.m. for 30 min at 4 °C, and the resulting supernatant was stored at -70 °C or was immediately subjected to electrophoretic mobility shift assay (EMSA) analysis. An oligonucleotide containing NF- κ B binding site (3.5 pmol) was incubated for 10 min at 37 °C in 10 μL containing 10 μCi of [γ - ^{32}P] ATP, 5 U of T4 polynucleotide kinase, and 1X kinase buffer (supplied with the kinase). The labelling reaction was terminated by addition of 100 mM EDTA, after which the reaction mixture was centrifuged through a Sephadex G-25 column to remove unincorporated ^{32}P . The ^{32}P -labelled oligonucleotide was then stored at -70 °C until use. For EMSA assay, nuclear protein extract (10 μg) was incubated for 30 min at room temperature in a final volume of 10 μL containing 0.03 pmol of ^{32}P -end-labelled oligonucleotide, 40 mM HEPES-KOH (pH 7.8), 10% glycerol, 1 mM $MgCl_2$, 0.1 mM DTT and 1 μg of poly(dI-dC). The binding reaction was terminated by addition of electrophoresis sample buffer, and samples were fractionated on 5% non-denaturing polyacrylamide gels in 0.5 $\times$ Tris-boric acid-EDTA buffer. Gels were then subjected to autoradiography.

Semiquantitative RT-PCR

For RT-PCR, total RNA was harvested from cells using Trizol reagent (Gibco-BRL). First-strand cDNA synthesis was performed with the SuperScript first-strand synthesis system (Invitrogen,

Carlsbad, CA, USA). To amplify XBP-1 mRNA, PCR was performed for 35 cycles [94 °C for 30 s; 55 °C for 30 s; and 72 °C for 1 min (7 min in the final cycle)] using primers 5'-AACTCCA GCTAGAAAATCAGC-3' and 5'-CCATGGGAAGATGTTCTGGG-3' with Taq DNA polymerase (#1815105; Roche, Seoul, Korea). Three fragments, representing spliced (XBP-1 s, 215 bp), unspliced (XBP-1 μ , 241 bp) and a hybrid (XBP-1 h, 267 bp), were produced and detected by running on 2% agarose gels and staining with ethidium bromide. For HBx, the primers were: 5'-ACGGAATTCA TGGCTGCTAGGGTGT-3' and 5'-ACGGTCGACTTAGGCAGAGGTGAAA-3'. Control primers for GAPDH were 5'-TAGACGGGAAGCTCACTGGC-3' and 5'-AGGTCCACCACCCTGTTGCT-3'.

Cell counting kit-8 assay

For cell proliferation assay, semiconfluent cells in a 24-well plate were treated with various kinds of inhibitors, including Pro1. After adding 50 μ L of the CCK-8 solution to each well of the culture plate for 2 h, an aliquot (100 μ L) from each well was transferred to a 96-well microplate and absorbance was measured using a microplate reader MR700 (Dynatech Inc., Chantilly, VA, USA) at 450 nm with a reference wavelength at 650 nm.

Clonogenic assay

Cells were treated with various kinds of inhibitors for 24 h and then were trypsinized, counted and poured into 6-well plates at 1×10^2 cells/well. After 12 days incubation, colony-forming cells were stained with 0.3% crystal violet solution (dissolved in 1 : 1 mixture of methanol and H₂O), were counted and photographed using an inverted microscope.

Staining for determination of cell proliferation

Cells treated with proteasome inhibitors were washed twice with PBS buffer to remove dead cells detached from the well and were fixed in methanol for 1 min followed by dipping into 0.5% eosin Y solution in 90% ethanol containing several drops of glacial acetic acid for another 1 min, then were photographed using an inverted microscope.

DNA fragmentation assay

In 24-well plates, cells at 5×10^5 cells/well were incubated with CPT. After 24 h, cells were transferred to 1.5 mL tubes, centrifuged for 30 s at 15 000 g, and re-suspended in 20 μ L lysis buffer (20 mM EDTA, 100 mM Tris-Cl, pH 8.0, 0.8% sodium lauryl sarcosine). Lysates were treated with 10 μ L of RNase A (1 mg/mL) for 30 min at 37 °C and subsequently with 10 μ L proteinase K (20 mg/mL) for 2 h at 50 °C. Lysates were mixed with 5 μ L 10 $\times$ loading dye for agarose gel electrophoresis at 100 V for 1.5 h.

RESULTS

Nuclear factor-kappa B activation in HBx-expressing cells

Hepatitis B virus X protein is associated with both lacks of apoptosis plus increased chemosensitivity of cells depending on the surrounding environment (Shih *et al.* 2000; Miao *et al.* 2006). Of the key cellular molecules involved in carcinogenesis, NF- κ B and Akt have been targets for increased sensitization for cancer cell death. To discover crucial factors for efficient treatment of HCC, we have examined cell survival of Chang liver cells transformed by HBx (Chang/HBx), using several chemical compounds against NF- κ B and Akt. First, transcriptional activation of NF- κ B was determined by transfecting both Chang and Chang/HBx cells with a plasmid

Figure 1. NF-κB activation in HBx-overexpressing cells. (a) Luciferase reporter gene assay. Chang or Chang/HBx cells in 6-well plates were cotransfected with 0.5 μg of pNF-κB-Luc and the same amount of pCMV/β-galactosidase for 4 h and then were refreshed with a new serum-free medium for 24 h. Cells were washed once with cold PBS and lysed for measurement of luciferase activity. Vc; EGFP vector control, HBx; HBx-EGFP transformed. Bars represent means ± SE from a representative triplicate experiment. (b) EMSA analysis for HBx-induced NF-κB activation. Serum-starved Chang or Chang/HBx cells were lysed and fractionated to cytosolic or nuclear fractions (10 μg) for application to EMSA analysis with or without specific antibody (2 μg) for supershift. (c) NF-κB expression in HBx-transformed cells. Equal amount (30 μg) of cytoplasmic or nuclear fraction obtained as above was applied to SDS-PAGE for immunoblotting with an antibody specific to p50 or p65.

containing six tandem repeats of the promoter region of NF-κB binding site (pNF-κB-Luc). Cells permanently expressing HBx showed a huge increase in NF-κB activation when compared to vector control cells, as shown in Fig. 1a. Consistent with this transcriptional activation by HBx, DNA-binding activity of NF-κB was also found to be greatly augmented when determined

by EMSA analysis (Fig. 1b, lanes 1–3). NF- κ B proteins constitute a large family of transcription factors, whose members can homo- and hetero-dimerize with each other (Grilli *et al.* 1993). We found that p65/RelA is the main component of the HBx-inducible NF- κ B complex as determined by antibody supershift EMSA analysis (Fig. 1b, lane 6). Absence of p50 supershift (Fig. 1b, lane 4) could be further confirmed by Western blot analysis, where, unexpectedly, p50 could not be found in HBx-transformed cells although abundant in the control cells (Fig. 1c), although this is in accordance with a further recent report (Hayashi & Faustman 2000). Although HBx induced the mobility shift of two bands, however, the identity of the lower one is not yet known.

GSK-3 β -mediated NF- κ B transcriptional activation by HBx

Hepatitis B virus X protein activates both PI3K-Akt/PKB and Ras-Raf-MAPK signalling pathways (Benn & Schneider 1994). Akt was also implicated in HBx-induced NF- κ B activation (Chung *et al.* 2004). In determination of the involvement of Akt and GSK-3 in NF- κ B activation in response to HBx, increased phosphorylation at the serine residue of Akt1 and its substrate GSK-3 β could be detected in Chang/HBx cells (Fig. 2a). On the other hand, treatment of Chang/HBx liver cells with inhibitors of PI3K/Akt, wortmannin (wort) and LY294002 (LY) at concentrations generally used for inhibition of cellular enzyme activity, significantly reduced the NF- κ B activation but without effect on expression of HBx itself (Fig. 2b). Thus, GSK-3 phosphorylation by activated Akt was expected to be involved in HBx-induced NF- κ B activation. Hence, to clarify the contribution of Akt and GSK-3 to HBx-induced NF- κ B activation, Chang/HBx cells transiently transfected with a plasmid containing Akt1 or GSK-3 β , either wild-type or mutant (Fig. 2c, insert), were processed for measurement of NF- κ B transcriptional activity by a luciferase reporter gene assay. As shown in Fig. 2c, while wild-type Akt1 and GSK-3 β significantly increased NF- κ B transcriptional activity, the dominant-negative kinase-dead Akt1 (kDa) and phosphorylation-deficient mutant GSK-3 β (pm) having a change from Ser9 to Ala9 did not show any enhancing effect on NF- κ B activation, demonstrating the crucial role of phosphorylation of Akt1 and GSK-3 β for NF- κ B activation in Chang/HBx cells. On the other hand, either of these vectors showed no significant effect on other transcription factors, such as activator protein-1, cAMP response element and serum response factor, indicating their specificity to NF- κ B regulation (Fig. 2d,e).

Proteasome inhibitor-1 inhibits HBx-transformed cell proliferation

Nuclear factor-kappa B activation is one of the main contributors to carcinogenesis, and tumour cells have a tendency to grow and form clones. Thus, in order to determine the effect of NF- κ B inhibition on HBx-induced cell survival, Chang/HBx cells were either treated with NF- κ B inhibitory compounds or were transiently transfected with dominant-negative Akt1/GSK-3 β mutant plasmids. Unexpectedly, none of the treatments with wortmannin, LY294002 or mutant plasmids showed inhibition of colony formation of Chang/HBx cells, except the NF- κ B inhibitor Pro1 (Fig. 3a). Furthermore, two other NF- κ B inhibitors, sulphasalazine and PDTC did not affect cell survival. Even increasing amounts of wortmannin and GSK-3 β , the point mutant did not show inhibition (data not shown). Unexpectedly, transient transfection with a plasmid DNA coding for the mutant form of I κ B α (pCMV4–3HA/I κ B α S32/36A) could not reduce cell survival (Fig. 3b). Given the observation that among NF- κ B inhibitors Pro1 only had the inhibitory effect on cell death (Fig. 3a), this result suggested that NF- κ B activation might have little association with malignant growth of HBx-transformed cell populations.

In a supportive experiment with a variety of inhibitors of enzymes known to be involved in metastasis of HBx-transformed cells (Chung *et al.* 2004), it was revealed that inhibitors of NF- κ B (PDTC and sulphasalazine), PI3K (wortmannin and LY294002) or MEK (PD98059) did

Figure 2. Involvement of Akt1 and GSK-3 β in NF- κ B transcriptional transactivation in Chang/HBx cells. (a) HBx increases phosphorylation of Akt1 and GSK-3 β . Serum-starved Chang, Chang/EGFP (Vc) or Chang/HBx (HBx) cells were lysed, and 60 μ g of cytoplasmic and 70 μ g of nuclear fraction were subjected to immunoblotting analysis with antibodies to phospho-Akt1 (Ser473) and phospho-GSK-3 β (Ser9), respectively. The membrane was washed and re-probed with an antibody to GAPDH or PCNA as been described previously. (b) GSK-3 β -dependent NF- κ B transcriptional transactivation. Cells were transfected with 0.5 μ g of pNF- κ B-Luc for 4 h and refreshed with serum-free medium with or without the inhibitors (wortmannin and LY294002, 20 and 200 nm). After 24 h, cells were lysed and luciferase activity was measured as described above. Bars represent means $\pm$ SE from a representative triplicate experiment of two independent experiments. Expression of HBx was not shown to be affected by the compounds to which the cells were exposed for 24 h at varying concentrations and RT-PCR was performed for measurement of HBx. Alternatively, immunoblot with anti-HBx rabbit polyclonal antibody was performed (lower panel). (c) Regulation of NF- κ B transcriptional activity by Akt1 and GSK-3 β . Cells were incubated in 6-well plates at 80% confluent and then transfected for 4 h with 0.5 μ g of pNF- κ B-Luc with or without 50, 200 or 500 ng of HA-tagged plasmids encoding wild-type Akt1 or GSK-3 β (wt) or point mutant GSK-3 β (pm). Fresh medium was added for 24 h and luciferase activity was measured. All the bars represent means $\pm$ SE from a representative triplicate experiment of two independent experiments. Transfection of plasmids was evaluated by Western blot analysis with a HA antibody as depicted in the insert. The upper arrow indicates HA-Akt1 and the lower one, HA-GSK-3 β . All the bars represent means $\pm$ SE from a representative triplicate experiment of two independent experiments. (d and e) Effect of Akt1 and GSK-3 β on activator protein-1 (AP-1), cAMP response element (CRE) and serum response factor (SRF) transcription factors. Cells were co-transfected for 4 h with 50, 200 or 500 ng of HA-tagged plasmids encoding wild-type Akt1 or GSK-3 β (wt) or point mutant GSK-3 β (pm) together with 0.5 μ g of pAP1-Luc, pCRE-Luc or pSRF-Luc. All other procedures were performed as in (c), results shown as means $\pm$ SE from a representative triplicate experiment.

Figure 2. *Continued*

not show any significant effect on cell proliferation (data not shown), although PDTC and sulphasalazine dose-dependently reduced NF- κ B transcriptional transactivation (Fig. 3c). Thus, it was presumable that inhibition of the proteasome but not NF- κ B itself, could be efficiently used for treatment of HBx-transformed hepatocarcinoma cells. In this respect, the proteasome has been reported to be one of the promising therapeutic targets for anticancer drug development (Mitchell 2003; Lenz 2003). Accordingly, when Chang/HBx cells were treated with Pro1, a reversible inhibitor, there appeared to be dose-dependent reduction in cell proliferation (Fig. 3d) and in colony formation (Fig. 3e). As expected, Pro1 was also found to reduce HBx-induced NF- κ B activation (Fig. 3f). Altogether, these results suggested that proteasome inhibitors could be promising anticancer drugs against HBx-induced carcinoma cells.

Figure 3. Increased death of HBx-transformed cells by proteasome inhibitor-1 (Pro1). (a) Effect of inhibitors against Akt, GSK-3 β and NF- κ B on cell survival. Cells were treated with wortmannin (100 nM), LY294002 (500 nM), PDTC (100 μ M), sulphasalazine (Sz) (100 μ M) or Pro1 (50 nM) for 10–12 days for measurement of cell survival as described in Materials and Methods. Otherwise, cells were transfected with plasmids encoding Akt1 or GSK-3 β , either wild-type (wt) or mutant (kd, pm) and incubated for 24 h. After refreshment with a new complete medium, cells proceeded to cell survival assay. (b) Effect of I κ B α (S32/36A) mutant on cell proliferation. Cells in a 60-mm dish were transfected with 5 μ g I κ B α (S32/36A) mutant for 2 days and stained with eosin Y as described in Materials and Methods. Efficient transfection of the mutant plasmid and the endogenous I κ B α were confirmed by an immunoblotting analysis with an antibody to I κ B α (insert). (c) PDTC and Sz inhibit transcriptional activation of NF- κ B in Chang/HBx cells. Cells in 6-well plates were transfected with 0.5 μ g of pNF- κ B-Luc for 4 h and were incubated in fresh serum-free medium containing PDTC or Sz at various concentrations for 24 h. Cells were washed with cold PBS and lysed for the measurement of luciferase activity. The bars represent means $\pm$ SE from a representative triplicate experiment. (d) Effect of Pro1 on cell proliferation. Pro1 at 0.05 μ M or 0.2 μ M was treated to Chang/HBx cells in 24-well plates for various times, and the cell proliferation was measured with CCK-8 kit at appropriate days (NT, not treated). Bars represent means $\pm$ SE from a representative triplicate experiment. (e) Clonogenic assay. Cells treated with Pro1 as in (d) in 60 mm dishes for 24 h were trypsinized, counted, reseeded into 6-well plates at 1×10^2 cells/well and further incubated for 12 days. Following colony formation, cells were stained with crystal violet solution and counted. Bars represent means $\pm$ SE from a representative duplicate experiment of two independent experiments. (f) Pro1 inhibits transcriptional activation of NF- κ B Chang/HBx cells. Cells in 6-well plates were transfected with 0.5 μ g of pNF- κ B-Luc for 4 h and incubated in a fresh serum-free medium containing Pro1 at various concentrations for 24 h. Cells were washed with cold PBS and lysed for the measurement of luciferase activity. The bars represent means $\pm$ SE from a representative triplicate experiment.

Figure 3. Continued

Effect of proteasome inhibitor MG132 on HBx-transformed cell proliferation

To support Prol inhibition of HBx-transformed cell proliferation, Chang/HBx cells were dose-dependently treated with a further potent proteasome inhibitor, MG132, and cell proliferation was measured at appropriate times. It was found that MG132 had a dose-dependent inhibitory effect on cell proliferation as determined by MTT assay (Fig. 4a). Inhibition was significant even at 24 h after treatment. A clonogenic cell survival assay was also performed in accordance with the cell proliferation results (Fig. 4b). Moreover, this compound had a strong inhibitory effect on NF-κB activation in Chang/HBx cells (Fig. 4c). Hence, it was necessary to determine the factor that is influenced by proteasome inhibitors.

ER stress-induced cell survival and death signalling in HBx-transformed cells

A major function of the ubiquitin-proteasome system is to identify and degrade aberrant proteins (Sherman & Goldberg 2001; Ciechanover & Brundin 2003). Substrates of this proteasomic degradation system include either misfolded proteins or normal short-lived proteins, such as p53, cyclins and IκBα. Although ubiquitin-proteasome-mediated proteolysis is exclusively executed in the nucleus, one recent report has demonstrated that the endoplasmic reticulum is another

Figure 4. Effect of proteasome inhibitor MG132 on Chang/HBx cell growth. (a) Effect of MG132 on cell proliferation. MG132 at indicated concentrations were treated to Chang/HBx cells in 24-well plates for various times, and the cell proliferation was measured with CCK-8 kit (NT, not treated). The bars represent means $\pm$ SE from a representative triplicate experiment. (b) Confluent growth of Chang/HBx cells in 24-well plates was treated with MG132 at various concentrations for 2 days. Cells were stained with eosin Y as described in Materials and Methods. (c) MG132 inhibits transcriptional activation of NF- κ B in Chang/HBx cells. Cells in 6-well plates were transfected with 0.5 μ g of pNF- κ B-Luc for 4 h and were incubated in fresh serum-free medium containing MG132 at various concentrations for 24 h. Cells were washed with cold PBS and were lysed for measurement of luciferase activity. Bars represent means $\pm$ SE from a representative triplicate experiment.

place where stress conditions (ER stress) could invoke activation of the proteasome system (Menendez-Benito *et al.* 2005). Accumulation of viral or misfolded proteins induces a UPR for correction or elimination of newly synthesized proteins. Using this UPR system, the ER can maintain homeostasis of the cells by making several chaperone proteins, like GRP78, the expression of which in a large part depends on splicing X-box binding protein-1 (XBP-1) mRNA. However, under certain circumstances using chemical compounds, stresses are beyond

the threshold level for correction, and cells begin to go along the apoptotic pathway to remove the stressed cells.

Thus, it was proposed that increased death of Chang/HBx cells on exposure to proteasome inhibitors, as described above, should have some relation with ER stress-induced apoptosis of the cells. First, we determined whether overexpression of viral protein HBx could trigger activation of the UPR system in our cell type. To examine XBP-1 mRNA splicing by HBx, cells were transfected with a plasmid containing GRP78 promoter (pGRP78-Luc) and were lysed for measurement of luciferase reporter activity. As shown in Fig. 5a, GRP78 promoter driven transcription was dramatically increased in Chang/HBx cells, irrespective of incubation time, after transfection. In accordance with this transcriptional activation, XBP-1 mRNA splicing was also evident in Chang/HBx cells when determined by RT-PCR analysis (Fig. 5b), and expression of the ER stress marker chaperone, GRP78, was enhanced in transformed cells compared to controls (Vc) (Fig. 5c). Interestingly, however, slight activation of a few proteins responsible for ER stress-induced apoptosis, caspase-12, caspase-9, phospho-SAPK/JNK but not caspase-3 was also detected in Chang/HBx cells (Fig. 5c). Hence, our data suggest that HBx-induced UPR activates responses of both survival (GRP78 and GPR94) and apoptotic death (caspase-12, caspase-9 and pSAPK/JNK), maintaining homeostasis of the cells.

Proteasome inhibitors could enhance cell death through ER stress apoptosis

Proteasome inhibition has been reported to sensitize chemo-resistant cancer cells to apoptotic death (Kwon *et al.* 2006). To determine whether they have any enhancing effect on ER stress-induced apoptosis, Chang/HBx cells were treated with Pro1 and the level of caspases was evaluated by Western blot analysis. It was revealed that caspase-12, a well-known marker for ER stress-induced apoptosis, was evidently activated from 24 h after Pro1 treatment (Fig. 5d). Downstream apoptotic proteins, caspase-9 and caspase-3, were also activated in a similar patterns. However, phosphorylation of SAPK/JNK, another apoptotic protein in the ER, did not change. Thus, homeostasis of HBx-transformed cells by a simultaneous increase in expression of cell maintenance proteins GRP78/GRP94 and in activation of apoptotic proteins, caspase-12 and caspase-9, seemed to be shifted towards the apoptotic cell death programme by activating caspase-3 proteins in response to Pro1, while activation of caspase-3 by HBx does not seem to be obvious. Likewise, another reversible proteasome inhibitor MG132 was also shown to activate the apoptotic proteins, caspase-12 and caspase-3 (Fig. 5e). However, activation of caspase-8 by proteasome inhibitors could not be seen (data not shown), suggesting that the apoptotic pathway indicated by the compounds might be intrinsic but not extrinsic, occurring from the ER or mitochondria. On the other hand, treatment of proteasome inhibitors, Pro1 and MG132, in Chang cells expressing only the EGFP mock vector did not induce ER stress associated activation of caspase-12 and its downstream caspase-9, while significantly elevating amounts of activated caspase-3 (Fig. 5f). From these results, it could be possible that proteasome inhibitors promote the HBx-induced ER stress responses, leading directly or indirectly to caspase-3 activation.

Increased cell death through caspase-3 activation

Camptothecin, a DNA topoisomerase inhibitor (Wang *et al.* 1999), is a potent inducer of apoptosis through caspase-9 and caspase-3 activation without association with ER stress. To determine whether ER stress induced caspase activation would be required for apoptotic death of HBx-transformed cells, Chang/HBx cells were treated with CPT and the extent of apoptotic events was analysed. DNA laddering, a classic feature of apoptotic cell death, could be observed by 24 h of CPT treatment (Fig. 6a). Moreover, caspase-3 activation was accordingly increased

Figure 5. ER stress induction by HBx and enhancement of ER stress-induced apoptosis by proteasome inhibitors in Chang/HBx cells. (a) GRP78 promoter-driven transcriptional activation in Chang/HBx cells. Cells in 6-well plates were transfected with 0.5 μ g of pGRP78-Luc for 4 h and then incubated in a serum-free medium for 6 h or 24 h. Cells were washed with cold PBS and lysed for measurement of luciferase activity. Bars represent means $\pm$ SE from a representative triplicate experiment of three independent experiments. (b) XBP-1 mRNA splicing by HBx. Total RNA isolated from serum-starved cells was subjected to RT-PCR for measuring the extent of XBP-1 mRNA splicing. Total RNA (2.5 μ g) was extracted from vector control or HBx-transformed cells using Trizol reagent. RNA (2.5 μ g) was transcribed with random primer. One-tenth of the reverse-transcribed RNA was amplified with Taq polymerase using a specific primer as the described in Materials and Methods. (c) Western blot analysis for ER stress induced protein expressions. Serum-starved cells grown in 60 mm dishes were lysed and cytosolic fractions were immunoblotted with the specific antibodies as the described. (d) Enhanced expression of ER stress-induced apoptotic proteins by Pro1 in HBx-transformed cells. Cells grown 100 mm dishes were treated with Pro1 at 0.1 μ M or 0.2 μ M for various times as described. Cells were lysed at appropriate times and the lysates were subjected to Western blot analysis. (e) Enhanced expression of apoptotic proteins by MG132 in HBx-transformed cells. Cells grown 100 mm dishes were treated with MG132 at 2.5 μ M or 5 μ M for various times as indicated. Cells were lysed at appropriate times and the lysates were subjected to Western blot analysis. (f) Proteasome-induced caspase-3 activation could occur without caspase-12 activation in the absence of HBx. Chang cells harbouring the EGFP mock vector were treated with Pro1 (0.1 μ M) or MG132 (5 μ M) for the times indicated. Western blot analysis with antibodies against the caspases showed only the unfragmented forms of caspase-12 and caspase-9. Both the compounds, however, could induce the activation of caspase-3.

Figure 5. *Continued*

by CPT treatment (Fig. 6b). However, caspase-12 activation, a hallmark of ER stress-induced apoptosis, did not occur. Cell proliferation was also significantly reduced by exposing the cells to CPT (Fig. 6c). These results suggested caspase-3 or its associated factor as a key molecule for induction of apoptotic death of HBx-transformed cells. In support of this, z-DEVD, a specific caspase-3 inhibitor, suppressed CPT-induced death of Chang/HBx cells (Fig. 6d).

DISCUSSION

Hepatitis B virus X protein induces apoptotic cell death at early time points after hepatitis B virus viral infection; however, this eventually leads to HCC (Feitelson 1992; Robinson 1994). The main reason for occurrence of HCC is associated with HBx-induced NF- κ B activation (Wang *et al.* 2004). Recently, much effort has been made for enhancing sensitization of tumour

Figure 6. Enhanced cell death by CPT through caspase-3 activation in Chang/HBx cells. (a) DNA fragmentation by CPT. Cells were treated with CPT at 10 μ M for 24 h. Cells were lysed and lysates were subjected to DNA fragmentation analysis on 2% agarose gel electrophoresis. (b) Enhanced expression of apoptotic proteins by CPT in HBx-transformed cells. Cells grown 100 mm dishes were treated with CPT at 10 μ M for 24 h. Cells were lysed and the lysates were subjected to Western blot analysis. (c) Effect of CPT on cell proliferation. CPT at 10 μ M concentration was treated to Chang/HBx cells in 24-well plates for various times, and cell proliferation was measured with CCK-8 kit (NT, not treated). Bars represent means $\pm$ SE from a representative triplicate experiment of two independent experiments. (d) Effect of caspase inhibitors on CPT-induced cell death. Cells pre-treated or not with 100 μ M z-DEVD 1 h were exposed to 10 μ M CPT. Cells were stained with eosin Y as described in Materials and Methods.

cells to anticancer drugs through inhibition of NF- κ B activation (Mayo & Baldwin 2000). The exact target for improved chemotherapy of HBx-transformed cells, however, is not yet clearly defined, although NF- κ B activation by HBx has been extensively exploited. Thus, we examined whether NF- κ B could be perceived as an excellent target against HBx-transformed cell proliferation. In this respect, Akt and GSK-3 have also been recognized as multifunctional enzymes involved in cell survival and apoptotic death (Brazil & Hemmings 2001; Frame & Cohen 2001).

Wortmannin and LY294002 significantly inhibited transcriptional activation of NF- κ B in Chang/HBx cells (Fig. 2b). The critical role of Akt and GSK-3 β in HBx-induced NF- κ B activation was further confirmed when the cells were exogenously transfected with Akt1 and GSK-3 β . Both wild-type plasmids significantly increased NF- κ B activity while the dominant-negative kinase-dead form of Akt1 and the point mutant GSK-3 β which can not be phosphorylated at Ser9, showed no stimulatory effect (Fig. 2c). These results indicate that GSK-3 β phosphorylation is crucial for HBx-induced NF- κ B transcriptional activation.

On the other hand, when Chang/HBx cells were exposed to some chemical inhibitors or plasmid vectors, Pro1 showed strong inhibition of cell survival only (Fig. 3a). Although the I κ B α (S323/36A) mutant has been shown to be efficient in enhancing cancer cell death (Sato *et al.* 2003), it was not effective against Chang/HBx cells in our study (Fig. 3b). Moreover, other NF- κ B inhibitors, sulphasalazine and PDTTC, did not reduce cell survival (Fig. 3a) and proliferation (data not shown) despite their effectiveness in inhibiting HBx-induced NF- κ B activation (Fig. 3c). Similar to Pro1, a potent proteasome inhibitor MG132 significantly reduced cell survival (Fig. 4a,b). Hence, together these results have suggested that proteasome inhibitors, regardless of their effect on NF- κ B, might have a strong connection with cell proliferation.

Endoplasmic reticulum stress-induced UPR regulates three ER transmembrane proteins, IRE-1 α , ATF6 and PERK (Zhang & Kaufman 2004), activation of IRE-1 α eliciting two pathways, cell survival and death. Because various stimuli can invoke a cascade of apoptotic cell death activators through ER stress, and the proteasome has been reported to be directly or indirectly associated with HBx and ER stress (Hu *et al.* 1999; Zhang *et al.* 2000; Menendez-Benito *et al.* 2005), it was expected that pronounced cell death by Pro1 or MG132 in Chang/HBx cells was associated with ER stress. For apoptotic cell death, ER stress induces caspase-12 activation and subsequently downstream activation of caspase-9 and caspase-3 (Nakagawa *et al.* 2000; Kaufman *et al.* 2002). It was revealed that survival factors, XBP-1 mRNA splicing and expression of genes *GRP78* and *GRP94*, were significantly enhanced in HBx-transformed cells (Fig. 5b,c). Unexpectedly, however, amounts of activated forms of ER stress-induced apoptotic proteins, such as caspase-12 and caspase-9 but not caspase-3, were also elevated in Chang/HBx cells (Fig. 5c). Given that HBx or phosphorylated GSK-3 β inhibited caspase-3 activation (Gottlob *et al.* 1998; Song *et al.* 2002), our data suggested that HBx-induced ER stress triggers UPR signalling both for cell survival and for apoptosis, maintaining homeostasis of the cells. Thus, it was probable that proteasome inhibitors could shift the ER stress balance towards apoptosis through caspase-3 activation. In this respect, treatment of Chang/HBx cells with Pro1 enhanced the HBx-induced caspase-3 activation and caspase-12 and caspase-9 (Fig. 5d). Besides, the other proteasome inhibitor MG132 showed strong enhancement of caspase-3 activation although caspase-12 was only slightly activated (Fig. 5e). On the other hand, CPT treatment increased both caspase-3 activation and apoptotic death without effect on caspase-12, indicating that caspase-12 is not necessarily required for increased apoptosis of HBx-transformed cells in response to the anticancer drug CPT (Fig. 6b,c). Instead, caspase-3 activation seems to play a central role in chemotherapeutic-induced apoptosis in HBx-transformed hepatocarcinoma cells. Support for the importance of caspase-3 activation in cell death comes from the observation that z-DEVD, a specific inhibitor of caspase-3, prevented the CPT-enhanced death (Fig. 6d). At present, caspase-3 could feature a converging point for chemotherapeutic-induced apoptosis; however, other pathways are also expected to take part in this process.

In conclusion, our study suggests that although NF- κ B might be activated by viral HBx, its inhibition could not be effective for the death of HBx-transformed carcinoma cells. HBx seems to induce both cell survival proteins, including GRP78/94 and apoptotic proteins caspase-12/9. Caspase-3, which is inactive in HBx-transformed cells, could be activated by proteasome

inhibitors likely Pro1 and MG132, which leads to the enhanced death of the cancer cells. Hence, it could be suggested that one of the factors involved in the regulation of caspase-3 activity could be a promising target for the development of anticancer therapeutics.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of tunicamycin and thapsigargin on ER stress-associated proteins in Chang cells. Cells were treated with tunicamycin (5 and 10 $\mu\text{g/mL}$) or thapsigargin (0.3 and 0.6 μM) for 24 h and assessed for the determination of the expression (GRP94) or activation (caspases) of proteins. No activation of caspase-12, -9 and -3 could be found in response to the ER stress inducers.

Figure S2. Effect of a proteasome inhibitor on Akt1 expression in Chang/HBx cells. Cells were treated with Pro1 at 0.1, 1 and 10 mM for 24 h to be determined for the amount of Akt1 in the cells. Equal amounts of the total cell lysate (50 μg) were subjected to immunoblotting analysis with a specific antibody to Akt1.

Figure S3. Effect of HBx on the expression of p50 and p105 in HBx-transformed cells. The result is the same as that in Fig. 1c, describing the expression of p50/NF- κB 1 and its precursor p105. Equal amounts (30 μg) of cytoplasmic or nuclear lysate obtained from Chang, Chang/EFGP or Chang/HBx were applied to SDS-PAGE for immunoblotting with an antibody to p50 detecting both p50 and p105.

Figure S4. ER stress signalling in HepG2 cells expressing low level of HBV genome. HepG2 cells were transfected with a plasmid pHBV1.2X, or pHBV1.2X (–X) which contains ochre termination signal (CAA to UAA) after codon 7 in the HBx open reading frame. After incubation for 4 h, Pro1 was added to cells for the times indicated. Cell lysates were subjected to Western blot analysis with antibodies concerned in ER stress signalling (a). Alternatively, RT-PCR was performed to determine the transcription level of GRP78 chaperone and HBx (b).

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