

## EXPRESSION OF FAS LIGAND IN HUMAN HEPATOMA CELL LINES: ROLE OF HEPATITIS-B VIRUS X (HBX) IN INDUCTION OF FAS LIGAND

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It has been postulated that tumor cells expressing Fas ligand (FasL) can evade immune surveillance by inducing apoptosis in T cells expressing Fas. In this study, we investigated FasL expression in 13 human hepatoma cell lines. Strong FasL expression was detected by reverse transcription-polymerase chain reaction or immunofluorescence in Hep G2.2.15, in which the hepatitis-B-virus (HBV) genome was transfected, and in SNU-354, which showed HBx transcripts. To determine the biological activity of FasL, Hep G2.2.15 was co-cultured with MOLT-4, T-cell-leukemia cells. Hep G2.2.15 induced apoptosis in MOLT-4 and this was inhibited by the antagonistic anti-Fas antibody, ZB4. For further analysis of the role of HBx in the induction of FasL, PLC/PRF/5 cells were transfected transiently with the HBV genome, or HBx, or the frameshift mutant of HBx. In PLC/PRF/5 cells transfected with the HBV genome or HBx but not in cells transfected with the frameshift mutant of HBx, FasL expression was detected. Our data suggest that HBx plays a role in the induction of FasL in hepatoma cells and in the escape from immune surveillance. *Int. J. Cancer* 82:587–591, 1999.

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Fas ligand (FasL) belongs to the tumor necrosis factor (TNF) family and induces apoptosis of Fas-bearing cells (Suda *et al.*, 1993). FasL is expressed mainly on the surface of activated T cells. Fas–FasL interaction is one of the main effector mechanisms of cytotoxic T lymphocytes (CTL) (Kagi *et al.*, 1994) and is involved in the activation-induced apoptosis of activated T cells (Dhein *et al.*, 1995). FasL is also expressed in immune-privileged sites, such as eyes and testis, and it induces apoptosis of T cells (Griffith and Ferguson, 1997). Therefore, FasL participates in the maintenance of immune privilege status (Griffith and Ferguson, 1997). It has been postulated that tumor cells expressing FasL could evade immune surveillance by inducing apoptosis in tumor-infiltrating T cells expressing Fas (Nagata, 1997; Walker *et al.*, 1998). FasL was detected in the sera of patients with malignant melanoma and FasL-expressing melanoma cells induced apoptosis in Fas-bearing cells (Hahne *et al.*, 1996). In this study, delayed tumorigenesis was shown by FasL-bearing melanoma cells in Fas-mutated *lpr* mice (Hahne *et al.*, 1996). Expression of FasL in colon cancer, lung cancer, astrocytoma, pancreatic cancer and esophageal cancer has also been reported (Bennett *et al.*, 1998; Gratas *et al.*, 1998; O'Connell *et al.*, 1996; Niehans *et al.*, 1997; Saas *et al.*, 1997; Ungefroren *et al.*, 1998). FasL-bearing astrocytoma efficiently induced apoptosis of CD4<sup>+</sup> or CD8<sup>+</sup> tumor-infiltrating lymphocytes in a co-culture assay (Walker *et al.*, 1997). These data indicate that tumor cells expressing FasL induce apoptosis of tumor-infiltrating T cells. In the liver, Fas and FasL may play an important role in hepatocarcinogenesis (Strand *et al.*, 1996). In hepatocellular carcinomas (HCCs), Fas (which is highly expressed in normal hepatocytes) was down-regulated (Shin *et al.*, 1998), and FasL (which is not expressed in normal hepatocytes) was expressed (Luo *et al.*, 1997; Strand *et al.*, 1996). Moreover, FasL was expressed in liver cirrhotic nodules (Luo *et al.*, 1997).

Hepatitis B virus (HBV), especially the HBx gene product, is suspected as being an oncogenic molecule responsible for hepatocarcinogenesis, but the molecular mechanism is not clearly elucidated. HBx protein interacts with wild-type p53 protein and inhibits its function (Wang *et al.*, 1994). Moreover, HBx protein activates protein kinase C, Ras-Raf-MAP kinase cascade, AP-1 and

NF- $\kappa$ B (Henkler and Koshy, 1996) and HBx up-regulates various host genes coding molecules of immunologic importance, such as major histocompatibility complex (MHC) class I, MHC class II, intracellular adhesion molecule (ICAM)-1 and TNF- $\alpha$  (Amaro *et al.*, 1994; Henkler and Koshy, 1996). However, the effect of HBx on the expression of FasL, which belongs to the TNF family and plays a role in the immune evasion of tumor cells, was not determined.

In this study, we investigated the expression of FasL in human hepatoma cell lines and evaluated the ability of FasL to induce apoptosis of Fas-bearing cells. Furthermore, we investigated the role of HBx in the induction of FasL in hepatoma cells to elucidate the mechanism of FasL expression in human hepatoma cell lines.

### MATERIAL AND METHODS

#### Cell lines and cell culture

Chang liver (ATCC CCL 13), SK-HEP-1 (ATCC HTB 52), Hep G2 (ATCC HB 8065), Hep 3B (ATCC HB 8064), PLC/PRF/5 (ATCC CRL 8024), and MOLT-4 (ATCC CRL 1582), a T-cell leukemia cell line, were obtained from the ATCC (Rockville, MD). Hep G2.2.15 cells, a stable transfectant of HBV genome into Hep G2 (Sells *et al.*, 1987), were also included. SNU-182, SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449 and SNU-475 were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) (Park *et al.*, 1995). It has been reported that SNU-354 and SNU-368 expressed HBx mRNA (Park *et al.*, 1995). All cell lines were grown in modified Eagle's medium (MEM) or RPMI 1640 containing 10% fetal calf serum (FCS; GIBCO BRL, Grand Island, NY), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin.

#### RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells with RNeasy kit (Qiagen, Santa Clarita, CA). cDNA was synthesized from 5  $\mu$ g of total RNA using 2  $\mu$ g of random hexamer (Pharmacia, Uppsala, Sweden), 1.25 mM dNTP (Boehringer, Mannheim, Germany) and 200 U of M-MLV reverse transcriptase (GIBCO BRL). PCR was performed using 0.25 mM dNTP, 0.25 U of *Taq* polymerase (Bioneer, Chongwon, Republic of Korea), 10 pmol of primer pairs compatible with FasL or  $\beta$ -actin, and cDNA with thermal cycler (Perkin Elmer, Branchburg, NJ). The following primer pairs were used respectively: FasL: 5'-ATGTTTCAGCTCTTCCACCTA-CAGAAGGA-3' and 5'-CAGAGAGAGCTCAGATAC GTTGAC-3';  $\beta$ -actin: 5'-CGTGGGCCCGCCCTAGGCCACCA-3' and 5'-TTGGCCTTAGGG TTCAGGGGG-3'. PCR cycling conditions were: de-naturation at 94°C for 30 sec, annealing at 56°C for 30 sec

Grant sponsor: Ministry of Health and Welfare, Republic of Korea; Grant number: HMP-98-B-3—0021 (1998 Good Health R & D Project).

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Received 18 January 1999; Revised 26 February 1999

and extension at 72°C for 1 min. Thirty-five cycles were carried out for amplification of FasL and 22 cycles for  $\beta$ -actin.

#### RNase protection assay

RNase protection assay was performed with RiboQuant multi-probe RNase protection assay kit (PharMingen, San Diego, CA) according to the manufacturer's instructions.  $^{32}$ P-labeled anti-sense riboprobes compatible to apoptosis-related genes including FasL and internal control (L32 and GAPDH) were synthesized with hAPO-3 Human Apoptosis template set, 2.75 mM ATP, GTP, CTP, 100  $\mu$ Ci [ $\alpha$ - $^{32}$ P]-UTP (3,000 Ci/mmol, NEN, Boston, MA) and 20 U T7 RNA polymerase.  $^{32}$ P-labeled anti-sense riboprobes were hybridized with 10  $\mu$ g of total RNA extracted from cultured cells at 56°C for 16 hr. After hybridization, 20 ng of RNase A and 50 U of RNase T1 were added to digest unhybridized RNA and duplex RNA hybrids were loaded onto 6% denaturing polyacrylamide gel containing 8 M urea and autoradiography was performed.

#### Immunofluorescence staining for detection of FasL expression

Hep G2 or Hep G2.2.15 cells were fixed with acetone for 10 min on a multi-spot microscope slide, then stained with 5  $\mu$ g/ml NOK-1 anti-FasL antibody (PharMingen) in phosphate-buffered saline (PBS) for 2 hr at room temperature. After 2 washes with PBS, the cells were incubated with goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugate (Cappel, West Chester, PA) for 1 hr at room temperature in the dark. After 3 washes with PBS, cells were observed by fluorescent microscopy.

#### Staining of apoptotic cells by annexin V

Hep G2.2.15 cells were cultured at  $1 \times 10^6$  cells/well in 12-well plates. After overnight incubation, MOLT-4 cells were seeded at  $2 \times 10^5$  cells/well into a Hep G2.2.15 growing well, and co-cultured with Hep G2.2.15 for 30 hr. Then, non-adherent cells were collected carefully and stained with propidium iodide and annexin V-FITC conjugate (Clontech, Palo Alto, CA). Fluorescence intensity was measured by FACStar (Becton Dickinson, Lincoln Park, NJ). MOLT-4 cells were treated with antagonistic anti-Fas antibody, ZB4 (MBL, Watertown, MA) or agonistic anti-Fas antibody, CH11 (MBL). MOLT-4 was pre-incubated with ZB4 at 500 ng/ml for 1 hr and finally mixed into co-culture at 125 ng/ml of ZB4. MOLT-4 was incubated with CH11 at 250 ng/ml for 30 hr.

#### Co-culture DNA fragmentation assay (JAM test)

MOLT-4 cells were incubated in media containing 10  $\mu$ Ci/ml [ $^3$ H]-thymidine (20 Ci/mmol, NEN) for 4 hr. After being washed twice, labeled MOLT-4 cells were added at  $2 \times 10^4$  cells/well into a Hep G2.2.15 growing well at effector-cell-to-target-cell ratio (E:T ratio) of 1:1, 1:2, and 1:5, and co-cultured with Hep G2.2.15 for 14 hr. DNA was harvested onto a glass fiber filter by cell harvester (Skatron, Sterling, VA) and the radioactivity of intact DNA retained on each filter was measured with a beta counter. Specific cell killing was calculated using the following equation: % specific killing =  $(S - E)/S \times 100$ , where E (experimental) is counts per minute (cpm) of retained DNA in the presence of effector cells, and S (spontaneous) is cpm of retained DNA in the absence of effector cells. Four independent experiments were carried out, each experiment was performed in quintuplicate, and the median cpm was used as the representative value.

#### Transient transfection of HBV genome or HBx

PLC/PRF/5 cells were cultured at 40% confluence in a 60-mm culture dish. After overnight incubation, 10  $\mu$ l of lipofectin (GIBCO BRL) was mixed with 100  $\mu$ l of OPTI-MEM (GIBCO BRL), and incubated for 30 min at room temperature. Lipofectin-OPTI-MEM mixture was mixed with 100  $\mu$ l of OPTI-MEM containing 2  $\mu$ g of DNA construct. The DNA constructs used for transfection were HBV genome, HBx under control of SV40 promoter (pSVX) and frameshift mutated HBx under control of SV40 promoter (pSVXkb) (Spandau and Lee, 1988). After incubation for 10 min at room temperature, the mixture was added into PLC/PRF/5 cells growing in a 60-mm culture dish. After 24 hr, the

medium was replaced with MEM containing 10% fetal bovine serum, and cells were cultured for another 2 days, after which total RNA was isolated.

## RESULTS

### Expression of FasL in human hepatoma cell lines

To verify the expression of FasL in human hepatoma cell lines, RT-PCR was performed in 14 cell lines, *i.e.*, 13 hepatoma cell lines and 1 normal liver cell line. Strong FasL transcripts were detected by RT-PCR in Hep G2.2.15 and SNU-354 (Fig. 1a). SNU-475 showed weak FasL transcripts, and the other cell lines including Hep G2 showed very weak or no FasL transcripts. Hep G2.2.15 cell line was a stable transfectant of HBV genome into Hep G2 and produced infectious HBV virion particles (Sells *et al.*, 1987), and SNU-354 was one of the cell lines that showed HBx mRNA transcripts (Park *et al.*, 1995). With these results, we focused on HepG2 and Hep G2.2.15 for further study. We could also detect FasL mRNA transcripts in Hep G2.2.15, but not in Hep G2 by RNase protection assay (Fig. 1b). To confirm FasL expression in the protein level, we performed indirect-immunofluorescence staining for FasL. Strong FasL expression was detected in Hep G2.2.15, but not in HepG2 (Fig. 1c).

### FasL-expressing hepatoma cells induced apoptosis of Fas-bearing cells

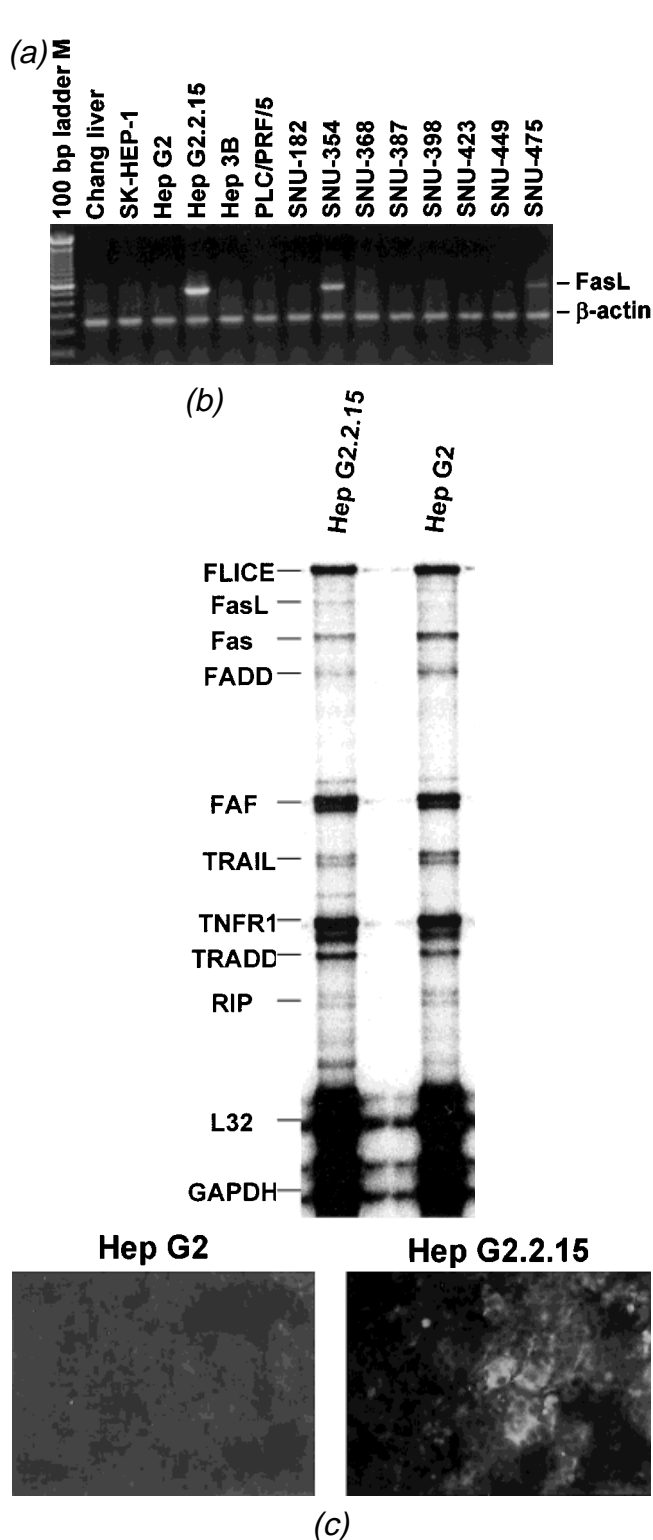
To investigate the functional activity inducing Fas-mediated apoptosis by FasL expressed on Hep G2.2.15, Hep G2.2.15 cells were co-cultured with Fas-bearing MOLT-4, a T-cell leukemic cell line known to be sensitive to Fas-mediated apoptosis. MOLT-4 cells were co-cultured with Hep G2.2.15 for 30 hr. The non-adherent cells were collected carefully and stained with propidium iodide and annexin V. These cells began to show apoptotic features (Fig. 2a). However, the antagonistic anti-Fas antibody, ZB4, blocked apoptosis in our co-culture system. MOLT-4 cells cultured without effectors or ZB4 anti-Fas antibody were not stained by annexin V and propidium iodide; however, apoptosis was induced in MOLT-4 when agonistic anti-Fas antibody, CH11, was added. Furthermore, to confirm that FasL expressed on Hep G2.2.15 induces apoptosis of MOLT-4 via Fas/FasL pathway, we performed a co-culture DNA fragmentation assay. When MOLT-4 cells were co-cultured with Hep G2.2.15, MOLT-4 cells underwent apoptosis (Fig. 2b) and this apoptosis was inhibited by ZB4 (Fig. 2c). However, Hep G2 did not induce apoptosis of MOLT-4 and the extent of apoptosis was not changed by ZB4.

### Transfection with HBx and induction of FasL expression in hepatoma cells

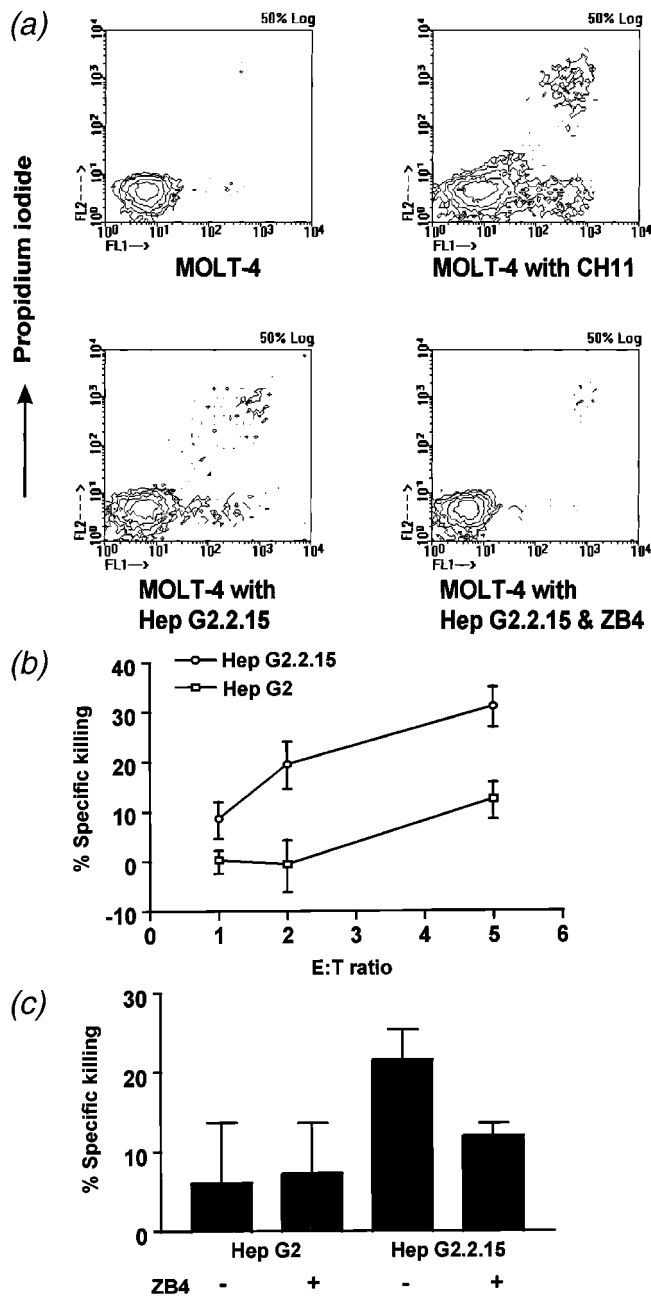
An effort was made to investigate the role of HBV or HBx in the expression of FasL in hepatoma cell lines. For this purpose, PLC/PRF/5 cells, which showed no FasL transcripts, were transfected transiently with the HBV genome, or HBx, or frameshift mutant of HBx and FasL transcripts were determined by RT-PCR. We corroborated the expression of transfected genes in PLC/PRF/5 cells through RT-PCR of the HBx gene with RNase-free DNase-treated total RNA of transfected cells. In the PLC/PRF/5 cells transfected with HBV genome or HBx, but not in cells transfected with the frameshift mutant of HBx, FasL mRNA transcripts were detected (Fig. 3).

## DISCUSSION

It has been suggested that tumor cells expressing FasL could evade immune surveillance by inducing apoptosis in tumor-infiltrating T cells expressing Fas (Nagata, 1997; Walker *et al.*, 1998), and this process was designated the Fas counterattack (O'Connell *et al.*, 1996). Expression of FasL has been reported in many types of tumor including malignant melanoma, hepatoma, colon cancer, lung cancer, astrocytoma, pancreatic cancer and esophageal cancer (Bennett *et al.*, 1998; Gratas *et al.*, 1998; Hahne



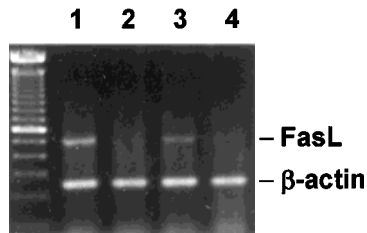
**FIGURE 1** – Expression of FasL in a panel of human hepatoma cell lines. (a) Detection of FasL mRNA transcript by reverse transcription-polymerase chain reaction. Strong FasL transcripts were detected in Hep G2.2.15 and SNU-354. (b) Detection of FasL-mRNA transcript by RNase protection assay. FasL transcripts in Hep G2.2.15 were observed. (c) Immunofluorescence staining with anti-FasL antibody showed protein expression of FasL in Hep G2.2.15 (right), but not in Hep G2 (left).



**FIGURE 2** – Induction of apoptosis in MOLT-4 by Hep G2.2.15 via Fas/FasL pathway. (a) MOLT-4 was treated with agonistic anti-Fas antibody, CH11 or co-cultured with Hep G2.2.15 and stained with annexin V. Apoptosis of MOLT-4 was induced by CH11 or Hep G2.2.15, and Hep G2.2.15-induced apoptosis was blocked by ZB4. (b) MOLT-4 cells labeled with [ $^3$ H]-thymidine were co-cultured with Hep G2 or Hep G2.2.15, and DNA fragmentation of MOLT-4 was measured and calculated as a percentage of specific killing. Each dot and line represent the mean and the standard error of the mean (SEM) of 3 independent experiments. E:T ratio represents ratio of effector cells to target cells. (c) Antagonistic anti-Fas antibody, ZB4 was added into co-culture system. Hep G2.2.15 induced-apoptosis was blocked by ZB4. Each bar and line represent the mean and the SEM of 4 independent experiments.

*et al.*, 1996; Niehans *et al.*, 1997; O’Connell *et al.*, 1996; Saas *et al.*, 1997; Strand *et al.*, 1996; Ungefroren *et al.*, 1998).

In this study, we investigated the expression of FasL in human hepatoma cell lines and evaluated the ability of FasL to induce



**FIGURE 3**—Induction of FasL by transfection of *HBx* or HBV genome in PLC/PRF/5. PLC/PRF/5 cells were transfected transiently with pSVX (*HBx* gene) or pSVXkB (frameshifted *HBx* gene) or HBV genome. On the 3rd day, RNA was isolated and RT-PCR for FasL was performed. Expression of FasL was induced by transfection of *HBx* gene or HBV genome, but not induced by frameshift mutant of *HBx* gene. Lane 1, transfection with pSVX (*HBx* gene); lane 2, transfection with pSVXkB (frameshifted *HBx* gene); lane 3, transfection with construct containing HBV genome; lane 4, no transfection.

apoptosis of Fas-bearing cells. We also investigated the role of HBx in the induction of FasL in hepatoma cells to elucidate the mechanism of FasL expression in human hepatoma cell lines. As a result, strong FasL expression was detected by RT-PCR in Hep G2.2.15 in which HBV genome was transfected, and in SNU-354, which showed *HBx* transcripts. The hypothesis that HBV gene products including HBx may play an important role in the expression of FasL was derived from these observations. FasL expression was also confirmed by RNase protection assay or immunofluorescence in Hep G2.2.15. To determine the biological activity of FasL expressed on Hep G2.2.15 cells, Hep G2.2.15 was co-cultured with MOLT-4, a T-cell-leukemia cell line. Hep G2.2.15 induced apoptosis in MOLT-4 and this was inhibited by the antagonistic anti-Fas antibody, ZB4. These results imply that Hep G2.2.15 induced apoptosis of MOLT-4 via Fas/FasL interaction. To prove our hypothesis that HBx may play a role in induction of FasL expression in hepatoma cell lines, PLC/PRF/5 cells were transfected transiently with the HBV genome, or *HBx*, or the frameshift mutant of *HBx*. In PLC/PRF/5 cells transfected with the HBV genome or *HBx* but not in cells transfected with the frameshift mutant of *HBx*, FasL expression was detected. These results indicate that HBx might play an important role in activating the expression of FasL in hepatoma cells. A similar result was reported

in the viral gene product of simian immunodeficiency virus. *Nef* of simian immunodeficiency virus induced FasL in virus-infected cells, and virus-infected cells induced apoptosis of CTLs to evade the immune system (Xu *et al.*, 1997).

It is known that HBx acts as a transcriptional transactivator and up-regulates various host genes including, MHC class I, MHC class II, ICAM-1 and TNF- $\alpha$  (Amaro *et al.*, 1994; Henkler and Koshy, 1996). HBx also activates various cellular transcription factors such as AP-1 and NF- $\kappa$ B (Henkler and Koshy, 1996). In addition, HBx may induce FasL expression through activation of cellular transcription factors. Although there is no confirmative report of transcription factor regulating FasL expression, the enhancer region of the FasL gene has a putative binding site of NF- $\kappa$ B (Takahashi *et al.*, 1994) and the important role of NF- $\kappa$ B has been reported in FasL gene activation (Kasibhatla *et al.*, 1999; Matsui *et al.*, 1997). Because HBx activates NF- $\kappa$ B, it is possible that HBx may induce FasL expression through NF- $\kappa$ B activation. The FasL expression in hepatoma cells appears to be regulated by multiple factors, because there was no strict correlation between HBx expression and FasL expression in the hepatoma cell lines. It is known that mRNA expression of *HBx* was observed not only in SNU-354, but also in SNU-368 (Park *et al.*, 1995). However, SNU-368 did not express FasL. In opposition to SNU-368, SNU-475, which did not show *HBx* transcripts (Park *et al.*, 1995), expressed weak FasL transcripts. These results imply that HBx is not the only factor inducing FasL expression in hepatoma cell lines. There have been many reports of factors capable of inducing FasL expression on various tumor cells. They include cytokines (Ohtsuki *et al.*, 1997), anti-cancer chemotherapeutic drugs (Strand *et al.*, 1996), oxidative stress (Hug *et al.*, 1997) and some viral gene products (Chlichlia *et al.*, 1997; Xu *et al.*, 1997). It is unknown whether these factors also induce FasL expression in various hepatoma cell lines. More investigations are needed on the regulation of FasL expression in hepatoma cells and transcription factors regulating FasL expression. Moreover, the role of HBx in transcription regulation of the FasL gene should be examined further regarding its mechanism and responsible transcription factor.

In conclusion, we propose that HBx plays a role in the induction of FasL in hepatoma cells and in the escape from immune surveillance through the induction of apoptosis of activated T cells expressing Fas.

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