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Expression Patterns of Cytokines and Chemokines Genes in Human Hepatoma Cells

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Various cytokines and chemokines play a role in carcinogenesis. However, no study has previously been undertaken to investigate comprehensively the expressions of cytokines and chemokines in hepatoma cells. In this study, we determined which cytokines and chemokines are expressed in hepatoma cells. Recently, it was reported that the expressions of several chemokines could be increased by Fas stimulus in many normal and cancer cells. Therefore, we also investigated whether chemokines expression is regulated by Fas ligation. To address this issue, we performed RNase protection assays upon 13 cytokines and 8 chemokines genes in 10 human hepatoma cell lines, comprising 8 hepatitis B virus (HBV)-associated hepatoma cell lines. Transforming growth factor-β2 (TGF-β2) was found to be expressed in 8 HBV-associated hepatoma cell lines, and to be potently expressed in 5 cell lines; however, the mRNA expressions of interleukin-10 (IL-10), IL-12, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were not detected in any cell lines examined. Among the chemokines investigated in this study, IL-8 was expressed by 8 HBV-associated hepatoma cell lines, and monocyte chemoattractant protein-1 (MCP-1) by 7 HBV-associated hepatoma cell lines. However, the mRNA expressions of macrophage inflammatory protein-1α (MIP-1α), MIP-1β, interferon-inducible protein-10 (IP-10), RANTES, lymphotactin and I-309 were either very weak or undetectable. Fas ligation did not increase chemokines expression in hepatoma cells. Conclusively, TGF-β2, IL-8 and MCP-1 were overexpressed in HBV-associated hepatoma cells, and the expressions of chemokines were not increased by Fas ligation in human hepatoma cells.

Key Words: Hepatoma, cytokine, chemokine, TGF-β, interleukin-8, MCP-1, Fas

INTRODUCTION

Cytokines are secreted regulatory proteins that control the survival, growth, differentiation and the effector functions of cells. Chemokines are family of cytokines that contain four conserved cysteines linked by two disulfide bonds, and which play a role in the recruitment of inflammatory cells.[1,2] During carcinogenesis, various cytokines and chemokines may play diverse roles, for example, they may either promote or inhibit cancer formation. Cancer cells can be influenced directly by cytokines and chemokines through the control of proliferation and apoptosis, and influenced indirectly through the regulation of antitumor immune response and neovascular angiogenesis.[1]

Cytokines and chemokines may be produced and secreted by normal cells, such as immune cells, and by cancer cells, and they can act in a paracrine or autocrine manner. For example, in the case of human hepatoma, it was reported that Hep G2 cells express cytokines and chemokines genes, such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), transforming growth factor β (TGF-β), macrophage colony-stimulating factor (M-CSF), interleukin-4 (IL-4), IL-5, IL-7, IL-10, IL-11 and IL-12.[3] Moreover, human monokine induced by IFN-γ (HuMig), IL-8, macrophage inflammatory protein-1α (MIP-1α) or MIP-1β was found to be expressed in human hepatoma tis-

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sues.[4] However, to date these studies have involved only restricted hepatoma cell lines and the examinations of limited numbers of cytokines and chemokines, and therefore, no study in which the expressions of cytokines and chemokines in human hepatoma cells have been investigated comprehensively has been undertaken.

The expressions of cytokines and chemokines can be regulated by various factors. Recently, it was reported that the cross-linking of Fas receptor in several cell types could increase the expression of chemokines. The secretion of IL-8 was induced in HT-29 colon cancer cell line,[5] rheumatoid arthritis synoviocytes[6] and bronchiolar epithelial cells[7] by cross-linking the Fas receptor, and the increased expression of IL-8, MIP-1 and MIP-2 was noticed in astrocytes.[8,9] More recently, the Fas-induced expression of IL-8 and monocyte chemoattractant protein-1 (MCP-1) in human glioma cells was also reported, and it was suggested that increased IL-8 might influence angiogenesis.[10] However, the Fas-induced expression of chemokines has never been studied in human hepatoma cells.

In the present study, we investigated the mRNA expressions of 13 cytokines and 8 chemokines genes in 10 human hepatoma cell lines including 8 hepatitis B virus (HBV)-associated hepatoma cell lines. Cytokines and chemokines studied in this study were IL-1α, IL-1β, IL-6, IL-10, IL-12, IFN-β, IFN-γ, TNF-α, TNF-β, lymphotoxin-β (LT-β), TGF-β1, TGF-β2, TGF-β3, IL-8, MCP-1, MIP-1α, MIP-1β, interferon-inducible protein-10 (IP-10), regulated upon activation normal T lymphocyte expressed and secreted (RANTES), lymphotactin (Ltn) and I-309. In addition, we investigated the change in the mRNA expressions of chemokines after cross-linking the Fas receptor with the agonistic anti-Fas antibody, CH11.

MATERIALS AND METHODS

Reagents

Agonistic anti-Fas monoclonal antibody with the IgM isotype, CH11, was purchased from MBL (Watertown, MA, USA), recombinant IFN-γ was purchased from Genzyme (Cambridge, MA, USA). MEM, RPMI 1640 media and fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY, USA).

Cell lines and cell culture

Of the 10 human hepatoma cell lines included in this study, 8 hepatoma cell lines, except Hep G2 and Hep G2.2.15, were HBV-associated hepatoma cell lines. Hep G2 (ATCC HB 8065) was derived from hepatoblastoma, and Hep G2.2.15 were derived by transfecting the HBV genome into Hep G2.[11] Among the 8 HBV-associated hepatoma cell lines, Hep 3B (ATCC HB 8064) and PLC/PRF/5 (ATCC CRL 8024) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA); and SNU-182, SNU-354, SNU-368, SNU-387, SNU-398 and SNU-449 from the Korean Cell Line Bank (Seoul, Korea).[12] All cell lines were grown in MEM or RPMI 1640 containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

RNA extraction and RNase protection assay (RPA)

Total RNA was isolated from cultured hepatoma cells by using a RNeasy kit (Qiagen, Santa Clarita, CA, USA). RPA was performed with RiboQuant^{TM}multi-probe RPA kits (Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, [^{32}P]-labeled antisense riboprobes, compatible with the specific cytokines and chemokines genes and the internal controls (L32 and GAPDH), were synthesized with a hCK-2, hCK-3, or hCK-5 template set (Pharmingen), using 2.75 mM ATP, GTP, CTP, 100 μCi [^{32}P]-UTP (3000 Ci/mmol, NEN, Boston, MA, USA) and 20 U T7 RNA polymerase. [^{32}P]-labeled antisense riboprobes were hybridized with 10 μg of total RNA extracted from the cultured cells at 56℃ for 16 hr. After hybridization, 20 ng RNase A and 50 U RNase T1 were added to digest unhybridized RNA and then duplex RNA hybrids were loaded onto 6% denaturating polyacrylamide gel containing 8 M urea and autoradiography was performed.

To investigate Fas-induced chemokines expression, hepatoma cells were treated with 125 ng/ml

CH11 for 6 or 12 hr, with or without pre-treatment with 250 U/ml IFN-γ for 24 hr, and then RPA was performed.

Immunofluorescence staining and flow cytometry

In order to investigate surface Fas expression in hepatoma cells, immunofluorescence staining and flow cytometric analysis were performed. After treating cells with or without 250 U/ml IFN-γ for 36 hr, the cells were detached with 0.125% trypsin and 0.5 mM EDTA. They were then washed with PBS and re-suspended in RPMI 1640 containing 1% FCS and incubated with anti-Fas monoclonal antibody, DX2 (Calbiochem, La Jolla, CA, USA), at 4℃ for 30 min. Cells were washed twice with PBS containing 0.5% bovine serum albumin (BSA), FITC-conjugated goat anti-mouse IgG (Becton Dickinson, Lincoln Park, NJ, USA) was added and the cells were incubated at 4℃ for 30 min. They were then re-washed in PBS containing 0.5% BSA, and fixed with 1% paraformaldehyde. Flow cytometric analysis was performed using a FACScalibur (Becton Dickinson) and data was analyzed using the WinMDI program.

Measurement of cell death

Hepatoma cells were pre-incubated with or without 250 U/ml IFN-γ for 36 hr. Media was replaced with fresh complete media with or without 250 ng/ml agonistic anti-Fas antibody, CH11, and a lactate dehydrogenase (LDH) assay was performed after 36 hr.[13] One hundred μl of this culture media was transferred into a 96 well plate, and the remaining cells were lysed with Triton X-100, and 50 μl of cell lysate was also transferred into a 96 well plate. NADH (Sigma, St. Louis, MO, USA) and pyruvate were then added to each well. Absorbance kinetics was measured on a plate reader at a wavelength of 340 nm. The percentage of cell death was calculated as [(LDH activity in 100 μl of culture media - LDH activity in 100 μl of fresh media) / (LDH activity in 50 μl of cell lysate - LDH activity in 50 μl of fresh media)] × 50.

RESULTS

The mRNA expressions of cytokines genes in human hepatoma cells

The mRNA expressions of 13 cytokines genes were determined with multi-probe RPA in human hepatoma cell lines, namely, IL-1α, IL-1β, IL-6, IL-10, IL-12, IFN-β, IFN-γ, TNF-α, TNF-β, LT-β, TGF-β1, TGF-β2 and TGF-β3. IL-6 was expressed in 3 cell lines (SNU-387, SNU-398 and SNU-449), and LT-β in 4 (Hep 3B, PLC/PRF/5, SNU-182 and SNU-368) (Fig. 1A). TGF-β2 was expressed in 8 cell lines, which were HBV- associated hepatoma cell lines, and was potently expressed in 5 cell lines (SNU-182, SNU-354, SNU-387, SNU-398 and SNU-449) (Fig. 1B). The weak expressions of TGF-β1, TGF-β3, TNF-β and IFN-β were detected in all hepatoma cell lines. The mRNA expressions of IL-10, IL-12, IFN-γ and TNF-α were undetected in all 10 hepatoma cell lines.

The mRNA expressions of chemokines genes in human hepatoma cells

The mRNA expressions of 8 chemokines genes were investigated by multi-probe RPA in human hepatoma cell lines. IL-8, MCP-1, MIP-1α, MIP-1β, IP-10, RANTES, Ltn and I-309 were included in the investigation. The expression of IL-8 was obvious in all 8 HBV-associated hepatoma cell lines, and MCP-1 expression was detected in all HBV-associated hepatoma cell lines, except Hep 3B (Fig. 2). The mRNA expressions of the other chemokines genes were either very weak or not detected.

The expression of chemokines was not induced by Fas ligation

To determine whether Fas ligation induces chemokine mRNA expression, multi-probe RPA was performed in 4 cell lines, Hep G2, Hep 3B, SNU-182 and SNU-368 with or without Fas ligation after IFN-γ pre-treatment. The effect of IFN-γ pre-treatment was determined as described previously.[13,14]

Initially, the surface expression of Fas was

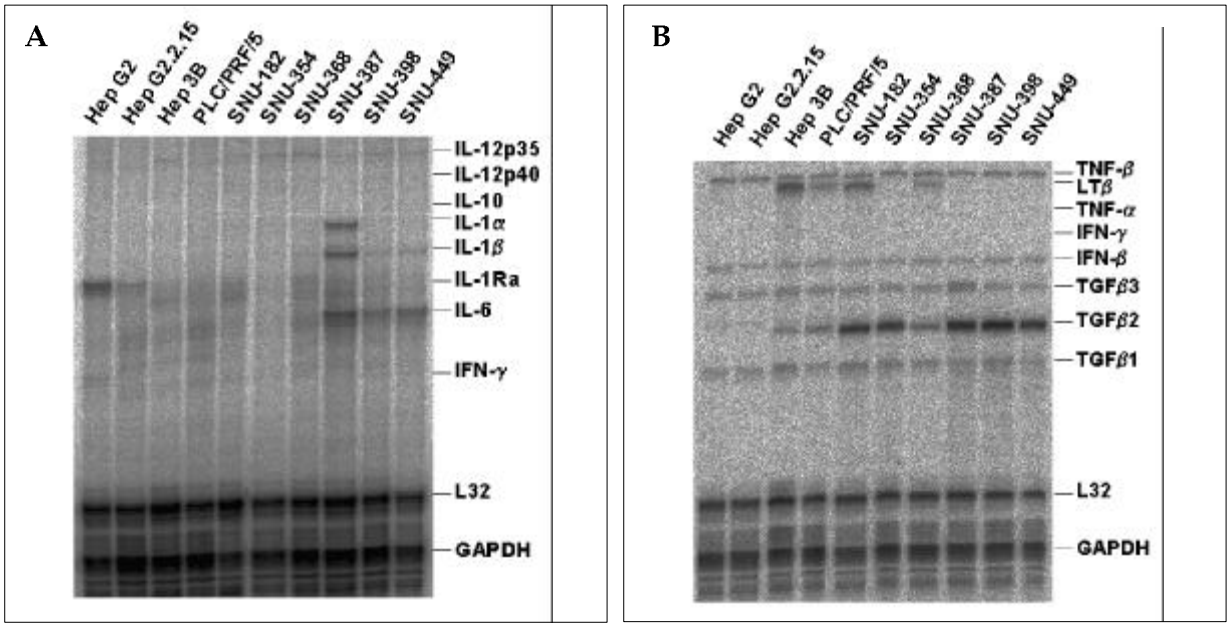

Fig. 1. The mRNA expressions of cytokines genes in hepatoma cell lines. Multi-probe RPA was performed to determine the mRNA expressions of IL-1α, IL-1β, IL-6, IL-10, IL-12 and IFN-γ (A), or IFN-β, IFN-γ, TNF-α, TNF-β, LT-β, TGF-β1, TGF-β2 and TGF-β3 (B). L32 and GAPDH were used as internal controls.

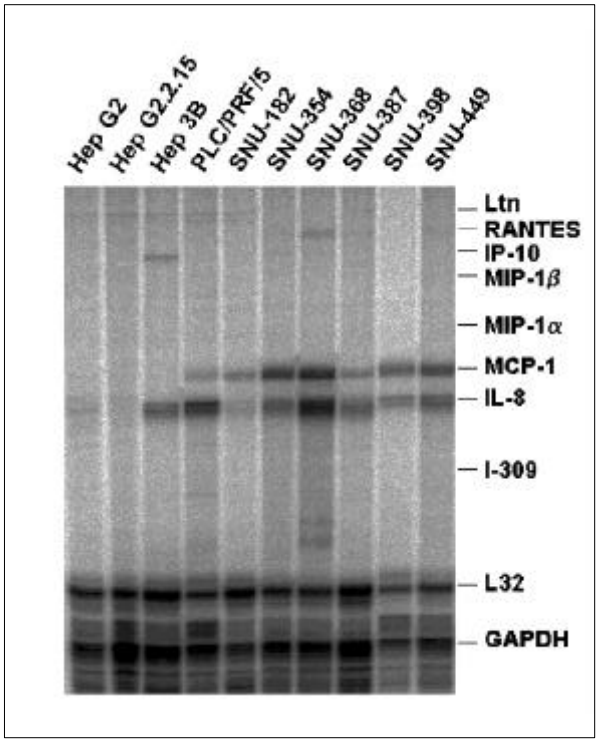

Fig. 2. The mRNA expressions of chemokines genes in hepatoma cell lines. Multi-probe RPA was performed to determine the mRNA expressions of IL-8, MCP-1, MIP-1α, MIP-1β, IP-10, RANTES, Ltn and I-309. L32 and GAPDH were used as internal controls.

examined with or without IFN-γ treatment. SNU-368 showed surface expression of Fas constitutively and this was increased by IFN-γ treatment (Fig. 3A). Hep G2 also showed constitutive Fas surface expression but no increased Fas expression by IFN-γ treatment. SNU-182 expressed basal Fas minimally and barely increased Fas expression after IFN-γ treatment. Hep 3B did not constitutively express Fas and IFN-γ treatment did not induce Fas in these cells.

To examine the apoptosis-inducing capability of Fas expressed in hepatoma cells, Fas-mediated cell death was investigated with or without IFN-γ pre-treatment. SNU-368 showed marked Fas-mediated cell death after IFN-γ pre-treatment, however, Hep G2, Hep 3B and SNU-182 were resistant to Fas-mediated cell death, even after IFN-γ pre-treatment (Fig. 3B).

Finally, we investigated the mRNA expression of chemokines after Fas ligation, and found that chemokines were not induced by Fas ligation, even though Fas surface expression was increased by IFN-γ pre-treatment (Fig. 3C). Incidentally, increased expression of IP-10, a known IFN-γ-responsive and anti-tumor chemokine, was well observed in SNU-182 and SNU-368 cells after IFN-γ pre-treatment.

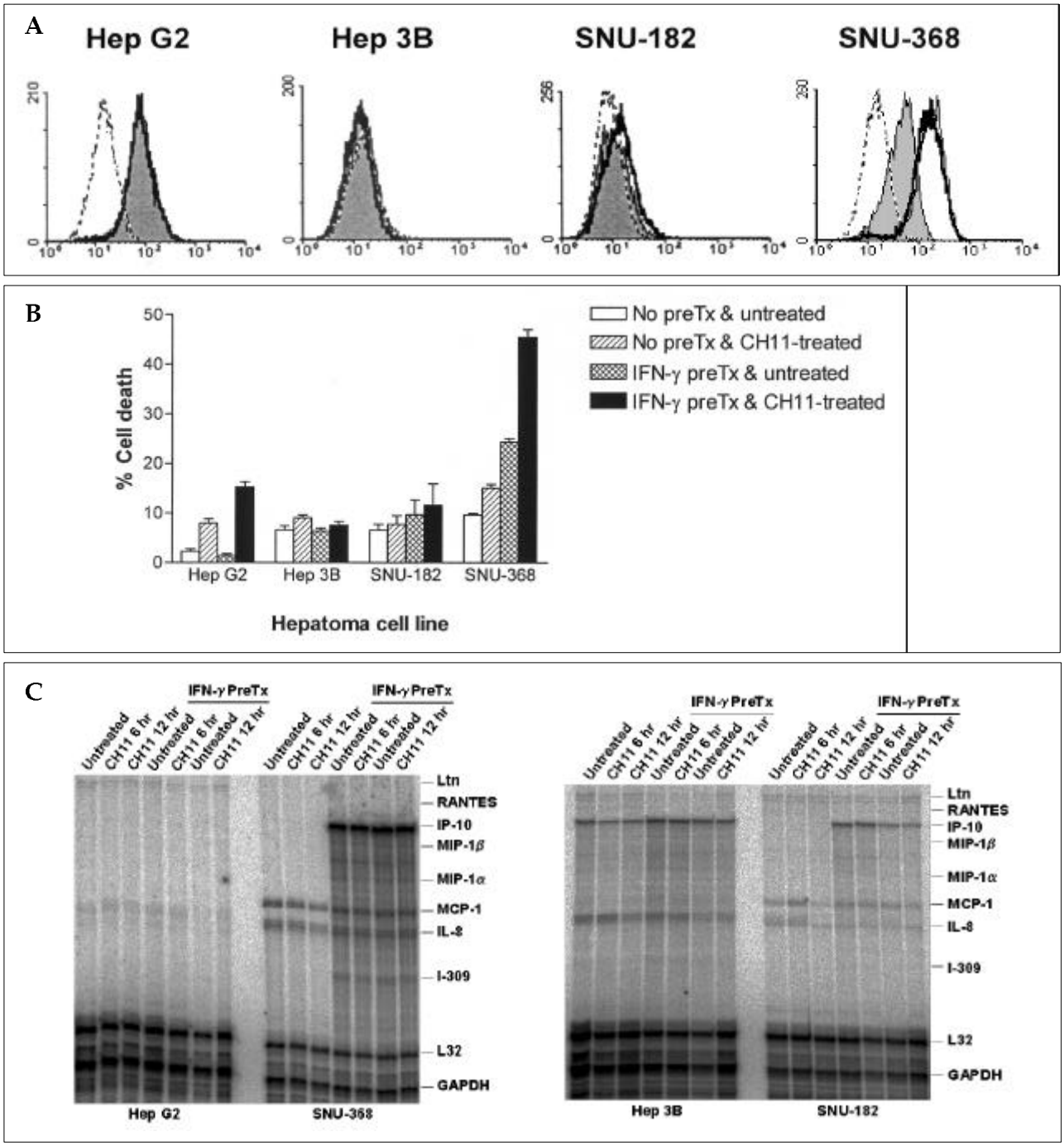

Fig. 3. The effect of Fas ligation on the mRNA expressions of chemokines genes in hepatoma cells. (A) The effect of IFN-γ on the surface expressions of Fas. The cell lines, Hep G2, Hep 3B, SNU-182 and SNU-368, were treated with 250 U/ml IFN-γ for 36 hr, cell surface Fas was then stained with DX2 anti-Fas antibody, and fluorescence intensity was measured by flow cytometry. In each cell line, the dashed line represents the negative control with no anti-Fas antibody, the filled area represents the basal level of Fas without IFN-γ treatment, and the solid line represents the level of Fas with IFN-γ treatment. (B) The effect of IFN-γ on Fas-mediated cell death in hepatoma cell lines. Hep G2, Hep 3B, SNU-182 and SNU-368 were pre-treated with 250 U/ml IFN-γ for 36 hr, and then treated with 250 ng/ml CH11 for another 36 hr. The extent of cell death was measured by LDH assay. Each bar represents the mean ± standard error of 4 independent experiments. (C) The mRNA expressions of chemokines genes after Fas ligation in hepatoma cell lines. Hepatoma cells were treated with 125 ng/ml CH11 for 6 hr or 12 hr, with or without being pre-treated with 250 U/ml IFN-γ for 24 hr. Multi-probe RPA was then performed to determine the mRNA expressions of the chemokines genes. L32 and GAPDH were used as internal controls.

DISCUSSION

In the present study, we investigated the expressions of the cytokines, IL-1α, IL-1β, IL-6, IL-10, IL-12, IFN-β, IFN-γ, TNF-α, TNF-β, LT-β, TGF-β1, TGF-β2, TGF-β3, and the chemokines, IL-8, MCP-1, MIP-1α, MIP-1β, IP-10, RANTES, Ltn and I-309 in human hepatoma cell lines. We found that the expressions of TGF-β2, IL-8 and MCP-1 were increased in HBV-associated HCC cells, and the expressions of chemokines, such as IL-8 and MCP-1, were not increased by Fas ligation in human HCC cells.

Of these cytokines and chemokines, TGF-β2 was found to be expressed in 8 cell lines, all HBV-associated hepatoma cell lines, and was potently expressed in 5 of these cell lines. The expression of IL-8 was obvious in the same 8 HBV-associated hepatoma cell lines, and MCP-1 was expressed in the HBV-associated hepatoma cell lines, except one, Hep 3B. It was interesting to note that TGF-β, IL-8 and MCP-1 were overexpressed in HBV-associated hepatoma cell lines, in which the HBV genome is integrated into the cellular genome. Remarkably, it was previously reported that the HBx protein of HBV induces the expression of various cytokines, such as, IL-6, IL-8, TNF-α and TGF-β.[15-18] It has also been reported that patients with HCV-related hepatoma exhibit TGF-β overexpression in terms of mRNA tissue transcripts[19] and serum protein.[20]

Several studies have addressed the expressions of cytokines and chemokines in human hepatoma,[3,4] including the present study, which leads us to ask: What is the role of the cytokines and chemokines expressed by hepatoma cells? It is conceivable that these cytokines and chemokines act as promoters of carcinogenesis, for example, they may activate cancer cell proliferation, inhibit cancer cell apoptosis, or act as immunosuppressors, or angiogenic factors. In the case of TGF-β, its immunosuppressive and anti-inflammatory effects are well known.[1] It has been reported that IL-8 functions as an autocrine growth factor in hepatoma,[21] and that IL-8 is a well known angiogenic factor.[22] In fact, the tumor growth promoting effect of IL-8 has been demonstrated in non-small cell lung cancer and melanoma.[23,24] However, the neutrophil chemoattractive effect of IL-8, which may be threaten to cancer cells, is also well known.[1] It is tempting to speculate, therefore, that the pro-inflammatory effect of IL-8 may be blocked by another factor. TGF-βcould potentially fill this role, because TGF-β is able to abolish the pro-inflammatory roles of chemokines,[1] and is expressed in human hepatoma cells, as shown by the present study. In contrast to IL-8, TGF-β has a hazardous effect on cancer cells, and TGF-β has been reported to induce apoptosis in human hepatoma cells.[25] However, mutations of the TGF-β receptor and the Smad family genes in human HCC have been reported and it was suggested that these mutations may cause the abrogation of the function of TGF-β and the alteration of TGF-β pathway in human HCC.[26,27] Furthermore, IL-6, which was found to be expressed in 3 cell lines in the present study, is known to inhibit TGF-β-induced apoptosis through the phosphatidyl inositol 3-kinase/Akt pathway in hepatoma cells.[28]

Recently, it was reported that the expression of chemokines could be increased by cross-linking of Fas receptor in several cell types. The secretion of IL-8 was found to be induced in the HT-29 colon cancer cell line,[5] rheumatoid arthritis synoviocytes[6] and bronchiolar epithelial cells[7] by Fas receptor ligation, and increased expression of IL-8, MIP-1 and MIP-2 was reported in astrocytes.[8,9] More recently, the Fas-induced expression of IL-8 and MCP-1 in human glioma cells was reported.[10] The increased expression of these chemokines by the cross-linking of Fas receptor coincides with the neutrophil infiltration induced by the implantation of FasL-overexpressed tissue and the subsequent Fas stimulus.[29] In the present study, however, Fas ligation did not induce the expression of chemokines in human hepatoma cells, even after treating with IFN-γ to increase the surface Fas expression. Moreover, it was found that extracellular signal-regulated kinase and p38 mitogen-activated protein kinase are involved in the Fas-induced expression of IL-8 and MCP-1. The over-expression of FADD, which is a proximal component in the Fas-mediated signal pathway, induced the expression of IL-8 and MCP-1, and macrophages infiltration in vascular smooth muscle cells.[30] The possible involvement of daxx, ceramide, and caspase in the activation of Fas downstream signaling cascades leading to chemo-

kines induction has been previously mentioned.[10] However, the components of such Fas downstream signaling cascades are poorly defined. In addition to the downregulation of Fas, our previous findings showed that the Fas downstream molecules, FADD, and caspase 8, were frequently downregulated and that FAP was upregulated in HBV-associated HCC cell lines and tissues.[31] Thus, it might be speculated that the Fas-induced chemokines expression pathway is interrupted by the downregulation of Fas downstream molecules in human hepatoma cells, however, this suggestion awaits further experimental confirmation.

Incidentally, the expression of IP-10 was very potently increased by IFN-γ pre-treatment in SNU-182 and SNU-368 cells. The anti-tumor effect of IP-10 is well known,[32,33] and IP-10 has been reported to be a specific chemoattractant for T lymphocytes in the liver.[34] These facts lead to the hypothesis that IP-10 may act as a major effector molecule of IFN-γ anti-tumor activity in hepatoma.

In conclusion, in this study we found that TGF-β2, IL-8 and MCP-1 are overexpressed in HBV-associated hepatoma cells, and that the expressions of chemokines, such as, IL-8 and MCP-1 are not increased by Fas ligation in human hepatoma cells, contrary to that observed other tumors, such as, in colon cancer and glioma.

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