Table 4
Haplotype frequency distributions of IL-10 gene promoter in HBV carriers

Haplotype (−1082/−819/−592)	Without HCC	With HCC
ATA	0.660	0.594
ACC	0.303	0.333
GCC	0.037	0.073

4. Discussion

In this study, we investigated the cytokine genes polymorphisms and determined whether these genetic factors are related to the occurrence of HCC in a Japanese population infected with HBV. Our results showed that the risk of HCC was significantly lower in HBV carriers with TGF-β1 codon 10 C/C genotype than in those with T/C or T/T genotype. A previous study demonstrated that the TGF-β1 polymorphism at codon 25 is associated with the progression of fibrosis in chronic HCV infection [14]. However, no polymorphism was found at codon 25 of the TGF-β1 gene in a Japanese population. These findings raise the possibility that the polymorphism of codon 10 in the TGF-β1 gene may play a role in determining the susceptibility to HCC of HBV-infected patients.

TGF-β1 is a pluriopotent cytokine that is potentially linked with fibrosis and neoplasm in the liver [15]. It is well established that this cytokine promotes hepatic fibrosis by stimulating the synthesis of the extracellular matrix [16]. TGF-β1 induces the activation of hepatic stellate cells to myofibroblasts, which is considered to be a crucial biological step in liver fibrogenesis [17]. TGF-β1 is also implicated in carcinogenesis. In normal cells, TGF-β1 acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation and apoptosis [18]. In contrast, the expression of TGF-β1 appears to be increased in cancer cells [19]. Its impact on the initiation or progression of neoplasm is controversial. This is due to the large parts of the multiple actions of TGF-β [20]. In vitro studies have shown that the increased activity in the TGF-β1 pathway leads to tumor inhibition in most mammary cell lines [21]. Transgenic mice with a single gene deletion of TGF-β1 are more susceptible to liver tumors induced by carcinogens [22].

Table 5
Differential distribution of TGF-β1 genotype in HBV carriers

Locus	Genotype	Without HCC	With HCC	OR (95% CI)	P
TGF-β1 codon 10	T/C, T/T	128 (68.1%)	41 (85.4%)	–	
	C/C	60 (31.9%)	7 (14.6%)	0.36 (0.154–0.859)	0.028

OR, odds ratio; 95% CI, 95% confidential interval.

Table 6
Distribution of TGF-β1 genotype in HBV carriers with or without LC

Locus	Genotype	Without LC	With LC	OR (95% CI)	P
TGF-β1 codon 10	T/T	19 (14.8%)	27 (25.0%)	–	
	T/C, C/C	109 (85.2%)	81 (75.0%)	0.52 (0.27–100)	0.072

OR, odds ratio; 95% CI, 95% confidential interval.

The T to C transition at position +29 the TGF-β1 gene results in a change from leucine to proline at codon 10. The presence of proline rather than leucine in the hydrophobic region of the signal sequence is thought to affect the export efficiency of the newly synthesized protein [23]. In fact, the C/C genotype at position +29 in a Japanese population was found to be associated with higher serum levels of TGF-β1 than T/T or T/C genotype [24]. Clinical studies indicated that the C/C genotype at the +29 position of the TGF-β1 gene is associated with a reduced risk of breast cancer [25]. These findings indicate that the TGF-β1-induced suppression of oncogenesis could be augmented by the increased TGF-β1 levels resulting from these genetic factors. Our data suggest that the increased serum levels of TGF-β1 in subjects with the C/C genotype in codon 10 may contribute to the suppression of hepatic tumorigenesis and lead to the lower risk of HCC. Originally, TGF-β1 gene polymorphism at codon 10 was reported to be associated with the progression of liver fibrosis. Gewaltig et al., demonstrated that the presence of proline at TGF-β1 gene codon 10 (C/C or C/T genotype) was associated with higher stage of fibrosis in HCV-infected patients [11]. However, there was no statistically significant association between this TGF-β1 gene polymorphism and the presence of LC in our data. Our data are consistent to those of Powell et al. [14] demonstrating no association between TGF-β1 gene codon 10 polymorphism and the stage of fibrosis in HCV-infected populations. Also, this discrepancy could be attributable to the differential genetic background of investigated populations and the difference of HCV and HBV infection. More recently, Kim et al. reported that the risk of HCC was lower in Korean HVB carriers with C/C or T/C genotypes at the +29 position of the TGF-β1 gene than those with T/T genotype [26]. In our study, the presence of C/C genotype was associated with

Table 7
Distribution of IFN-γ1 genotype in HBV carriers with or without LC

		Without LC	With LC	OR (95% CI)	P
IFN-γ+ 874	T/A	18 (14.1%)	20 (18.5%)	–	
	A/A	110 (85.9%)	88 (81.5%)	0.72 (0.35–1.44)	0.35

OR, odds ratio; 95% CI, 95% confidential interval.

a reduced risk with HCC, however, the presence of C/C or T/C genotypes was not associated with the risk of HCC significantly. This discrepancy may be due to the different ethnic populations studied.

Previous results in cohort studies demonstrated that advanced age and liver function impairment have been associated with a higher HCC incidence in patient with virus hepatitis [27,28]. Consistent with these findings, the age and the presence of LC were significantly higher in HCC groups compared to those of non-HCC groups in our study. Therefore, TGF-β1 genotype could be one of the factors influencing the development of HCC in addition to these major factors.

IL-10, produced mainly by macrophages, is a potent immunosuppressive cytokine that down-regulates the Th1 cytokines [29]. The greater susceptible effects of IL-10 haplotype on chronic hepatitis B progression were demonstrated. We previously reported that the frequencies of ACC haplotype of IL-10 were higher in progressive HBV carriers than in asymptomatic carriers [30]. Shin et al. reported that the IL-10-ACC haplotype showed a strong association with the occurrence of HCC [31]. We found that the frequency of the ACC haplotype appears to be increased in HCC patients, though the statistical difference, compared with that in non-HCC patients, was not significant. Further studies are needed to determine the association between the IL-10 haplotype and the HCC occurrence.

In summary, we found that the presence of the TGF-β1 C/C genotype at codon 10 was associated with a reduced risk of HCC occurrence in patients with HBV infection. These data suggest that the TGF-β1 polymorphism is one of the genetic factors affecting hepatic carcinogenesis in patients with HBV infection.

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Parathyroid hormone-related protein as a common target molecule in specific immunotherapy for a wide variety of tumor types

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Abstract. Parathyroid hormone-related protein (PTH-rP) has been considered to be responsible for malignancy-associated hypercalcemia and is thought to participate in pathological changes in bone metastases of cancer. In this study, we determined whether or not PTH-rP could be a common target molecule in specific immunotherapy for patients with a wide variety of tumor types. Various types of tumor cell lines were examined for PTH-rP expression at the mRNA and protein levels by RT-PCR, flow cytometry, and immunocytochemistry. We also determined whether or not cancer-reactive cytotoxic T lymphocytes (CTLs) could be induced from the peripheral blood mononuclear cells (PBMCs) of HLA-A24+ patients with gastric, colon, renal, or cervical cancer by *in vitro* stimulation with two PTH-rP peptides. As a result, *PTH-rP* mRNA was expressed in the majority of gastric, breast, lung, colon, cervical, and renal cancer cell lines. Expression of the protein was confirmed by both flow cytometry and immunocytochemistry. Furthermore, PTH-rP peptide-specific and cancer-reactive CTLs were successfully generated from the PBMCs of HLA-A24+ patients with different tumor types using *in vitro* stimulation with either the $PTH\text{-}rP_{102\text{-}111}$ or $PTH\text{-}rP_{110\text{-}119}$ peptide. These findings indicate that PTH-rP could be a common target molecule in specific immunotherapy for patients with a wide variety of tumor types, particularly bone metastases.

Introduction

Surgery and chemotherapy are the main treatment modalities found to be effective for many patients with early-stage cancer. However, the presently available treatment modalities are not equally effective for recurrent or advanced stages of cancer. As the prognosis for cancer patients with distant metastases is extremely poor, a new modality of treatment is urgently needed for the treatment of such patients. One approach currently under consideration is specific immunotherapy, whereby peptide-based vaccines appear to offer a simple and attractive strategy for eliciting systemic immunity against cancer.

Parathyroid hormone-related protein (PTH-rP) was designed as such due to its structural similarity to parathyroid hormone (PTH) (1). PTH-rP is thought to be responsible for malignancy-associated hypercalcemia (2). In addition, PTH-rP is known to be expressed in 90% of primary prostate carcinomas and is a key agent in the development of bone metastases (3,4). Therefore, this molecule has been considered to be a promising target molecule for the immunotherapeutic treatment of cancer patients with bone metastases (3). We also identified PTH-rP-derived peptides that are applicable in a peptide-based immunotherapy for HLA-A24+ or HLA-A2+ prostate cancer patients (5,6). In this study, in order to evaluate the extent of the feasibility of this antigen as a target molecule in specifically designed immunotherapy, we investigated PTH-rP expression in various types of cancer cell lines and revealed that this antigen could be used as a promising target molecule in the treatment of various types of cancer.

Materials and methods

Cell lines. MKN-7, MKN-28, and MKN-45 (gastric adenocarcinomas); RERF-LC-AI and QG56 (lung squamous cell carcinomas); 11-18, 1-87, LK87, and LC-1 (lung adenocarcinomas); R-27 and CRL1500 (breast carcinomas); KUR-11, RC30-14, Caki-1, MAMIYA, and VMRC-RCW (renal cell carcinomas); and COLO201, COLO205, COLO320, and SW480 (colon adenocarcinomas) cells were cultured in RPMI-1640 supplemented with 10% FCS. MDA-MB-231 (breast carcinoma); HCT116 (colon adenocarcinoma); SKG-I, SKG-II, OMC-1, and SKG-IIIb (cervical squamous cell carcinomas); SKG-IIIa and OMC-4 (cervical adenocarcinomas); and OMC-3 (ovarian carcinoma) cells were cultured in DMEM supplemented with 10% FCS. KWS (gastric adenocarcinoma), MCF-7 and YMB-1-E (breast carcinomas),

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and SW620 (colon adenocarcinoma) cells were cultured in EMEM supplemented with 10% FCS at 37˚C in a humidified atmosphere of 5% CO_2.

RT-PCR. Total RNA was isolated from cancer cell lines using RNAzol™ B (Tel-Test Inc., Friendswood, TX). The cDNA was prepared using the SuperScript™ Preamplification System for First Strand cDNA Synthesis (Invitrogen), and it was amplified using the following primers: 5'-TCTTCCTTCACC ATCTGATCG-3' (sense) and 5'-TGTCCTTGGAAGGTCTC TGC-3' (anti-sense) for *PTH-rP*, and 5'-CTTCGCGGGCGA CGATGC-3' (sense) and 5'-CGTACATGGCTGGGGTGTTG-3' (anti-sense) for *β-actin*. PCR was performed using TaqDNA polymerase in a DNA thermal cycler (iCycler, Bio-Rad Laboratories, Hercules, CA) for 30 cycles of 95˚C for 1 min, 60˚C for 1 min, and 72˚C for 1 min. The PCR products were separated by electrophoresis on 2% agarose gel.

Immunofluorescence microscopic analysis. Cells on a 35-mm culture dish were rinsed with TBS (10 mM Tris, pH 7.4, 100 mM NaCl) and fixed with 3.7% formaldehyde for 5 min at room temperature, washed with TBS, and then blocked with 3% BSA for 15 min. The cells were stained with mouse anti-PTH-rP monoclonal antibody (mAb) (1:100 dilution, Ab-1; Oncogene Research Products) for 1 h at room temperature. After being washed with TBS, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) (1:200 dilution; Molecular Probes) and counterstained with propidium iodine (PI). The stained cells were mounted with 1, 4-diazabicyclo-[2, 2, 2]-octane/ glycerol, and observed by confocal laser-scanning microscopy (Fluoview; Olympus).

Flow cytometry analysis. To examine the protein expression of PTH-rP protein, the cells were harvested by trypsinization, resuspended with 10% FCS-RPMI, and fixed in 3% formaldehyde. After fixation, the cells were washed three times in PBS and incubated with 2.5 μg/ml of rabbit polyclonal anti-PTH-rP antibody (1:75 dilution, H-137; Santa Cruz, CA) for 1 h at room temperature. Rabbit preimmune serum was used as a control. After being washed with PBS, the cells were stained with FITC-conjugated anti-rabbit IgG (1:150 dilution; Molecular Probes) for 1 h at room temperature. Flow cytometry was carried out with an EPICS flow cytometer and the data were analyzed with EXPO32 analysis software (Beckman Coulter).

Preparation of peripheral blood mononuclear cells from patients. All of the 18 cancer patients included in this study provided informed consent before enrollment. None of these participants was infected with human immunodeficiency virus (HIV). Twenty milliliters of peripheral blood was obtained from each subject, and the peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Conray density gradient centrifugation. The expression of HLA-A24 molecules on the PBMCs of cancer patients and healthy donors was determined by flow cytometry.

Peptides. All peptides were of >90% purity and were purchased from Biologica Co., Nagoya, Japan. PTH-rP-derived (102-111: RYLTQETNKV and 110-119: KVETYKEQPL), influenza (Flu) virus-derived (RFYIQMCYEL), EBV-derived (TYGPVFMCL), and HIV-derived (RYLRQQLLGI) peptides with the HLA-A24 binding motif were used. All peptides were dissolved with DMSO at a dose of 10 mg/ml.

Assay for peptide-specific CTLs in PBMCs. The assay for the detection of peptide-specific CTLs in the PBMCs was performed according to a previously reported method (7). In brief, the PBMCs (1×10^5 cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) at a volume of 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Gibco BRL), 10% FCS, 100 U/ml of interleukin (IL)-2, and 0.1 mM MEM non-essential amino acid solution (Gibco, BRL). Half of the culture medium was removed and replaced every 3 days with new medium containing a corresponding peptide (20 μg/ml). On the 15th day of culture, the cultured cells were separated into 4 wells, two of which were used for PTH-rP peptide-pulsed C1R-A24 cells (Dr M. Takiguchi, Kumamoto University, Japan), and the other two were reserved for the HIV peptide-pulsed C1R-A24 cells. After an 18-h incubation period, the supernatants were collected, and the level of IFN-γ was determined by ELISA.

Cytotoxicity assay. After *in vitro* stimulation with the PTH-rP peptides, the peptide-stimulated PBMCs were additionally cultured with 100 U/ml IL-2 for approximately 10 days in 96 round-well plates in order to obtain a sufficient number of cells to carry out a cytotoxicity assay. Immediately before the cytotoxicity assay, $CD8^+$ T cells were positively isolated using the CD8 Positive Isolation Kit (Dynal, Oslo, Norway). Then, purified $CD8^+$ T cells were tested for cytotoxicity against tumor cells by a 6-h ^{51}Cr-release assay. Two thousand ^{51}Cr-labeled cells per well were cultured with effector cells in 96 round-well plates at the indicated effector/target ratios. In some experiments, either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a), or anti-CD14 (H14: mouse IgG2a) mAb was added to the wells at a dose of 20 μg/ml at the start of the assay.

Cold inhibition assay. The specificity of PTH-rP peptide-stimulated CTLs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labeled target cells (2×10^3 cells/well) were cultured with CTLs (4×10^4 cells/well) in 96 round-well plates with 2×10^4 cold target cells. C1R-A24 cells that were pre-pulsed with either the HIV peptide or a corresponding PTH-rP peptide were used as cold targets.

Statistical analyses. The statistical significance of the data was determined using a two-tailed Student's t-test. P-values of less than 0.05 were considered to be statistically significant.

Results

PTH-rP expression in a variety of cancer cell lines. We initially investigated the mRNA expression levels of *PTH-rP* in gastric, cervical, lung, breast, renal, and colon cancer cell lines (Fig. 1). Semi-quantitative RT-PCR analysis revealed that *PTH-rP*

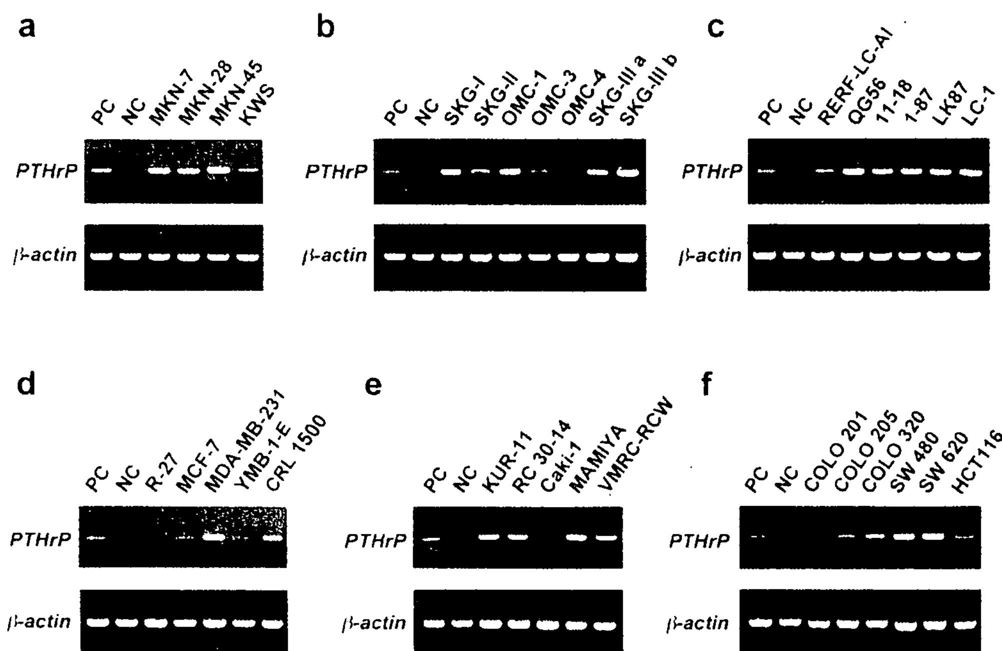

Figure 1. Expression of *PTH-rP* mRNA in a variety of cancer cell lines. *PTH-rP* mRNA expression in the following cancer cell lines was examined by semi-quantitative RT-PCR analysis: gastric (a), cervical (b), lung (c), breast (d), renal (e), and colon (f) cancer cell lines. PC, positive control; LNCaP (prostate cancer cell line) and NC, negative control: peripheral blood mononuclear cells (PBMCs, healthy donor) for *PTH-rP* mRNA. RT-PCR for *β-actin* mRNA was performed in order to assess the quality of RNA used for the analysis.

mRNA was expressed in most of the cancer cell lines tested, although the levels of expression varied among tumor cell lines. Expression was found to be higher in some cancer cell lines than in a prostate cancer cell line, LNCaP, which was used as a positive control. We next performed an immunocytochemical analysis to confirm the expression of PTH-rP (Fig. 2A). Here, PTH-rP expression was distributed in a punctate pattern throughout the cytoplasm in SKG-I (cervical squamous cell carcinoma), QG56 (lung squamous cell carcinoma), and MAMIYA (renal cell carcinoma) cells, whereas very low levels of expression were observed in R-27 (breast carcinoma) cells, which were also only faintly positive for *PTH-rP* mRNA expression in the RT-PCR analysis (Fig. 1d). In addition, we analyzed the expression of PTH-rP by flow cytometric analysis of intracellular staining (Fig. 2B). In MKN-45 (gastric adenocarcinoma), SKG-I (cervical squamous cell carcinoma), QG56 (lung squamous cell carcinoma), and KUR-11 and MAMIYA (renal cell carcinomas) cells, PTH-rP was highly expressed; however, R-27 (breast carcinoma) and COLO201 (colon adenocarcinoma) cells exhibited only low levels of PTH-rP expression and negative PTH-rP expression, respectively. The results obtained by these protein expression analyses were consistent with those obtained by RT-PCR analysis.

Induction of PTH-rP peptide-specific CTLs from HLA-A24⁺ patients with various cancer types. We previously identified two PTH-rP-derived peptides, PTH-$rP_{102-111}$ and PTH-$rP_{110-119}$, which had the potential to generate peptide-specific and prostate cancer-reactive CTLs from the PBMCs of HLA-$A24^+$ prostate cancer patients (5). In order to determine whether or not PTH-rP peptide-specific CTLs could be induced in patients with various types of cancer, their PBMCs were stimulated *in vitro* with one of the PTH-rP peptides, and the cells were then examined for IFN-γ production in response to C1R-A24 cells, which were pre-pulsed with either a corresponding PTH-rP peptide or the HIV peptide (Table I). In this series, Flu- and EBV-derived peptides were used as controls. The background IFN-γ production in response to the HIV peptide was subtracted, and the results showing the best response are shown (Table I). The successful induction of peptide-specific CTLs was judged to be positive when significant values ($P<0.05$, according to a two-tailed Student's t-test) were observed. PTH-$rP_{102-111}$ peptide was found to induce peptide-specific CTLs in two of five renal cancer patients, two of five gastric cancer patients, one of four colon cancer patients, and two of four cervical patients. The PTH-$rP_{110-119}$ peptide also induced peptide-specific CTLs in two of five renal cancer patients, two of five gastric cancer patients, two of four colon cancer patients, and three of four cervical cancer patients. These findings indicate that PTH-rP peptide-specific CTLs could be induced from the PBMCs of patients with various types of cancer.

Cytotoxicity of PTH-rP peptide-specific CTLs from cancer patients against cancer cells. We further investigated whether

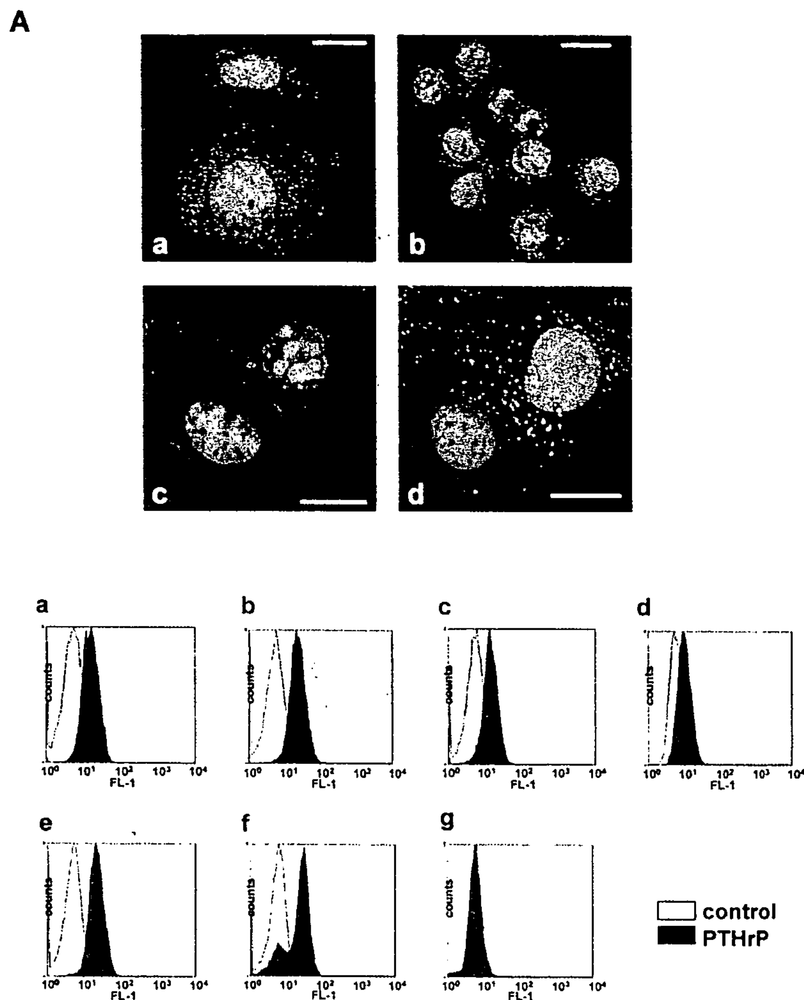

Figure 2. Expression of PTH-rP in a variety of cancer cell lines. (A) Subcellular distribution of endogenous PTH-rP (green) in SKG-I (a, cervical), QG56 (b, lung), R-27 (c, breast), and MAMIYA (d, renal) cells was determined by indirect immunofluorescence staining with the anti-PTH-rP polyclonal antibody, H-137. DNA/RNA was visualized by staining with propidium iodide (PI, red). A punctate distribution of PTH-rP was detected in the cytoplasm. (B) MKN-45 (a, gastric), SKG-I (b, cervical), QG56 (c, lung), R-27 (d, breast), KUR-11 (e, renal), MAMIYA (f, renal), and COLO201 (g, colon) cells were stained with the anti-PTH-rP rabbit polyclonal antibody H-137, and endogenous PTH-rP expression was determined by flow cytometric analysis. Rabbit preimmune serum was used for the control staining.

or not PTH-rP peptide-specific CTLs induced from cancer patients could exhibit cytotoxicity against cancer cells. Based on the RT-PCR results (Fig. 1), the following cancer cell lines were used as targets in the cytotoxicity assay: KUR-11 and RC30-14 (renal cell carcinoma), MKN-45 and MKN-28 (gastric adenocarcinoma), COLO320 and COLO205 (colon adenocarcinoma), and SKG-I and OMC-1 (cervical squamous cell carcinoma). Positive surface expression of HLA-A24 molecules was observed in the following cell lines: KUR-11, MKN-45, COLO320, and SKG-I; however, negative surface expression of HLA-A24 molecules was observed in the following cell lines: RC30-14, MKN-28, COLO205, and OMC-1 (data not shown). The PBMCs from 4 patients (renal, colon, and cervical cancer) that produced significant levels of IFN-γ (Table I) were repeatedly stimulated with the indicated PTH-rP peptide, based on the culture protocol described in Materials and methods. Then, these cells were positively isolated for $CD8^+$ T cells immediately before the CTL assay was carried out. Although no clear PTH-rP peptide-specific CTLs were induced from the PBMCs of patient 6 in the first experiment (Table I), $PTH\text{-}rP_{110\text{-}119}$ peptide-specific CTLs could be induced in the subsequent experiment. Therefore, the PBMCs from this patient were employed for cytotoxicity assay. As a result, the $PTH\text{-}rP_{102\text{-}111}$ and the $PTH\text{-}rP_{110\text{-}119}$ peptide-stimulated $CD8^+$ T cells showed higher levels of cytotoxicity against the $PTH\text{-}rP^+/HLA\text{-}A24^+$ cancer cells (KUR-11, MKN-45, COLO320, and SKG-I) than against the $PTH\text{-}rP^+/HLA\text{-}A24^-$ tumor cells (RC30-14, MKN-28, COLO205, and OMC-1) and against the $PTH\text{-}rP^-/HLA\text{-}A24^+$ PHA-induced T-cell blasts (Fig. 3). In addition, cytotoxicity against the $PTH\text{-}rP^+/HLA\text{-}A24^+$ tumor cells was significantly inhibited by the addition of anti-HLA-class I mAb, but not by

Table I. Reactivity of PTH-rP peptide-stimulated PBMCs from HLA-A24$^+$ cancer patients.

Patient	Peptide			
	PTH-rP$_{102-111}$	PTH-rP$_{110-119}$	Flu	EBV
	IFN-γ production (pg/ml)			
Renal cancer				
#1	$\underline{67}$	$\underline{122}$	$\underline{139}$	$\underline{110}$
#2	$\underline{86}$	0	43	$\underline{1588}$
#3	3	33	$\underline{167}$	$\underline{199}$
#4	20	$\underline{226}$	$\underline{318}$	$\underline{648}$
#5	0	0	$\underline{58}$	$\underline{79}$
Total	2/5	2/5	4/5	5/5
Gastric cancer				
#6	6	25	22	$\underline{74}$
#7	0	$\underline{378}$	$\underline{439}$	23
#8	32	$\underline{78}$	6	0
#9	$\underline{78}$	3	23	$\underline{4011}$
#10	$\underline{525}$	0	0	25
Total	2/5	2/5	1/5	2/5
Colon cancer				
#11	11	$\underline{92}$	6	27
#12	$\underline{184}$	44	11	$\underline{163}$
#13	7	$\underline{81}$	$\underline{232}$	0
#14	25	25	$\underline{670}$	22
Total	1/4	2/4	2/4	1/4
Cervical cancer				
#15	$\underline{160}$	$\underline{71}$	$\underline{292}$	27
#16	0	$\underline{88}$	0	0
#17	$\underline{153}$	$\underline{82}$	$\underline{432}$	$\underline{567}$
#18	0	0	37	31
Total	2/4	3/4	2/4	1/4

The PBMCs of HLA-A24$^+$ cancer patients were stimulated *in vitro* with the indicated PTH-rP peptides, as described in Materials and methods. On the 15th day, the cultured PBMCs were tested for their reactivity to C1R-A24 cells, which were prepulsed with the corresponding PTH-rP peptide or HIV peptide. The values represent the mean of two wells, and the background IFN-γ production in response to the HIV peptide was subtracted. Significant values ($P<0.05$ by two-tailed Student's t-test) are underlined.

the addition of anti-HLA-class II or anti-CD14 mAb, which were used as controls (Fig. 4A). Furthermore, the cytotoxicity against PTH-rP$^+$/HLA-A24$^+$ tumor cells was significantly suppressed by the addition of the corresponding PTH-rP peptide-pulsed C1R-A24 cells, used as a cold target, but this type of suppression was not observed with the addition of HIV peptide-pulsed C1R-A24 cells (Fig. 4B). Taken together, these results suggest that PTH-rP peptide-stimulated PBMCs from cancer patients are able to facilitate the lysis of corresponding cancer cells; in addition, the cytotoxicity observed here could be largely ascribed to HLA class I-restricted and PTH-rP peptide-specific CD8$^+$ T cells.

Discussion

PTH-rP is well known as a partly responsible factor in malignancy-associated hypercalcemia (2,8-10). In addition, PTH-rP is involved in pathological changes in bone metastases of several types of cancer (2,11-13). PTH-rP has been reported to be a useful prognostic factor of patients with cancer and hypercalcemia (14). These lines of evidence have thus suggested that this molecule could be a promising target in the treatment of cancer, especially in cases involving bone metastases. As regards the expression of this molecule in cancer tissues, PTH-rP has been reported to be detectable in 90% of primary prostate and lung spindle cell carcinomas and in 50% of primary breast cancers (15-18). In addition, PTH-rP was suggested to be expressed in other types of cancer, including colon, renal cell carcinoma, and cervical cancer (19-21). In this study, we comprehensively showed that PTH-rP mRNA is detectable in gastric, cervical, lung, breast, renal, and colon cancer cell lines. In addition, the expression of PTH-rP was further confirmed by flow cytometry and immunocytochemistry. Furthermore, PTH-rP was also detected

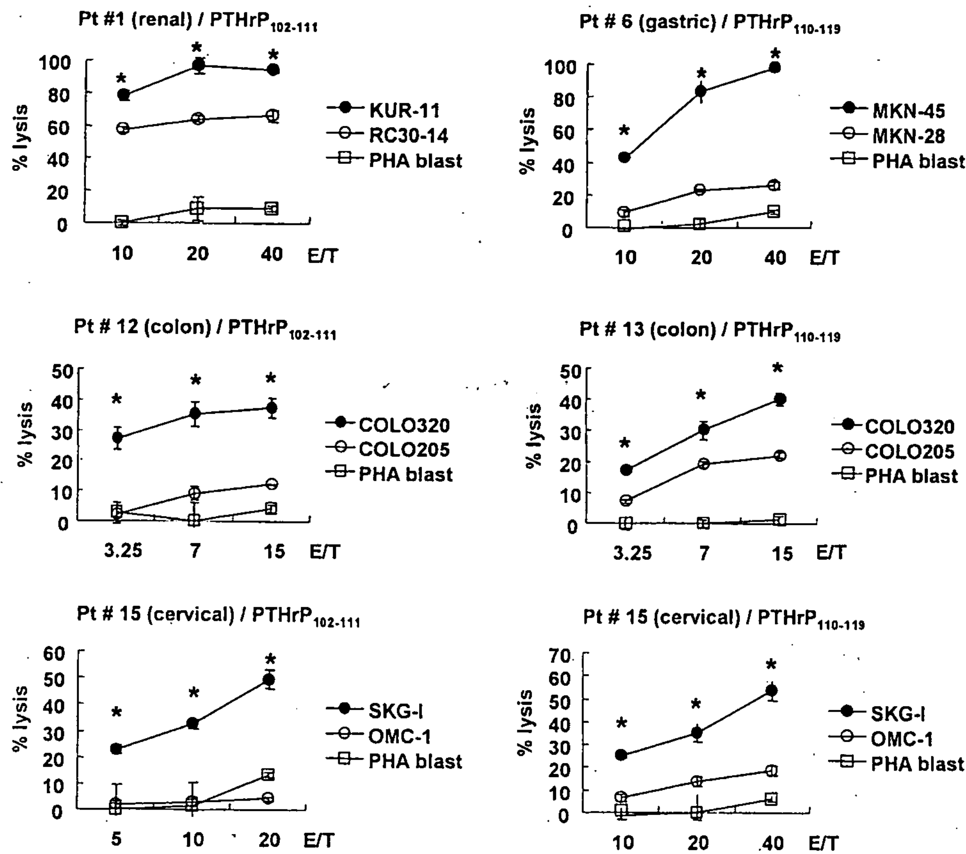

Figure 3. Induction of cancer-reactive CTLs from the PBMCs of patients with a variety of cancer types. PBMCs from five HLA-A24+ cancer patients (Pt #1, renal cancer; Pt #6, gastric cancer; Pt #12, colon cancer; Pt #13, colon cancer; and Pt #15, cervical cancer) were stimulated *in vitro* with the indicated PTH-rP peptide, as described in Materials and methods. Purified CD8+ T cells were examined for their cytotoxicity against the following targets: KUR-11 (PTH-rP+/HLA-A24+) and RC30-14 (PTH-rP+/HLA-A24-) for Pt #1, MKN-45 (PTH-rP+/HLA-A24+) and MKN-28 (PTH-rP+/HLA-A24-) for Pt #6, COLO320 (PTH-rP+/HLA-A24+) and COLO205 (PTH-rP+/HLA-A24-) for Pt #12 and Pt #13, SKG-I (PTH-rP+/HLA-A24+) and OMC-1 (PTH-rP+/HLA-A24-) for Pt #15, and PHA-blastoid T cells (PTH-rP-/HLA-A24+). A 6-h ^{51}Cr-release assay was performed. Values represent the mean of triplicate assays. *$P<0.05$ was considered statistically significant.

in several glioma cell lines (Yajima *et al*, unpublished data), and pancreatic adenocarcinoma cell lines (unpublished data). Although PTH-rP has been reported to be expressed in fetal tissue and to be involved in bone tissue differentiation (17), this molecule could be a promising target in the context of a specific immunotherapy as it shows preferential expression in a wide variety of tumor types. Immunohistochemical analysis is currently being performed to confirm that PTH-rP is expressed in various clinical tumor samples as well.

We have been conducting trials of a peptide-based anticancer vaccine against hormone-refractory prostate cancer; to date, no autoimmune symptoms have been observed in any of the patients who received a vaccination of CTL-directed peptides, including PTH-rP-derived CTL-directed peptides (unpublished data). However, these patients will continue to be carefully monitored for autoimmune symptoms.

Several PTH-rP-derived peptides with the potential to induce cancer-reactive CTLs have been reported (3,22). We have also identified CTL-directed PTH-rP-derived peptides applicable for the treatment of HLA-A24+ and HLA-A2+ prostate cancer patients (5,6). In the present study, we demonstrated that PTH-rP peptide-specific and cancer-reactive CTLs could be successfully induced from the PBMCs of patients with renal, gastric, colon, or cervical cancer by *in vitro* stimulation with either the PTH-rP$_{102-111}$ or the PTH-rP$_{110-119}$ peptide. In addition, these CTLs failed to lyse PTH-rP+/HLA-A24- tumor cells and PTH-rP-/HLA-A24+ T-cell blast cells. These results indicate the presence of CTL precursors reacting to PTH-rP peptides in the circulation of patients with gastric, renal, colon, or cervical cancer.

In an assay of peptide-specific CTLs, the reactivity of PBMCs that were stimulated *in vitro* with the Flu or EBV peptide was relatively low in colon, gastric, or cervical cancer patients compared to that in renal cancer patients. Although at present we have no clear explanation for this finding, pre-trial chemotherapy might in part account for any differences in immune reactivity. Namely, all of these patients, with the exception of the renal cancer patients, had received prior chemotherapy (along with radiotherapy in the case of the cervical cancer patients); such a treatment protocol might in turn suppress CTL activity against the Flu and/or EBV peptide.

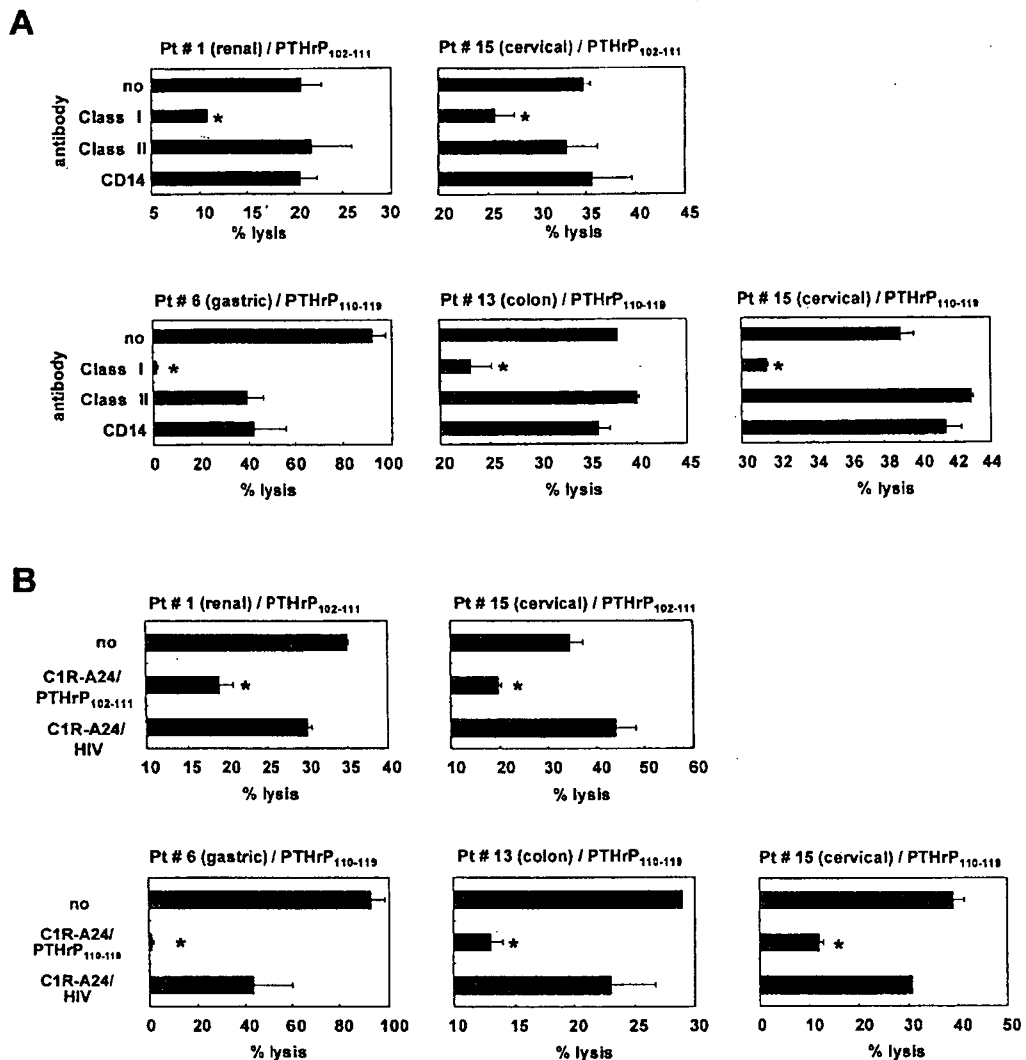

Figure 4. CD8+ T cell-dependent and PTH-rP peptide-specific cytotoxicity against PTH-rP+/HLA-A24+ cancer cells. (A) Purified CD8+ T cells from the PTH-rP peptide-stimulated PBMCs were examined for their cytotoxicity against PTH-rP+/HLA-A24+ tumor cell lines with or without anti-HLA class I, anti-HLA class II, or anti-CD14 mAb at a dose of 20 µg/ml. The values represent the mean of triplicate assays. *P<0.05 was considered statistically significant. (B) Cytotoxicity against the PTH-rP+/HLA-A24+ tumor cell line was also examined in the presence of unlabeled C1R-A24 cells, which were pre-pulsed with HIV peptide or with a corresponding PTH-rP peptide. The values represent the mean of triplicate assays. *P<0.05 was considered statistically significant. In this assay, cancer type-matched tumor cell lines were used as the target.

The HLA-A24 allele is found in 60% of Japanese, 20% of Caucasians, and 12% of Africans, and the HLA-A2 allele is found in 40% of Japanese, and 50% of Caucasians (23). Both the expression of PTH-rP in a wide variety of tumor types and the identification of CTL-directed PTH-rP-derived peptides for both HLA-A24+ and HLA-A2+ patients may enable us to design a peptide-based anti-cancer vaccine for the vast majority of cancer patients throughout the world.

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Expression of matrix metalloproteinases (MMPs) in cultured hepatocellular carcinoma (HCC) cells and surgically resected HCC tissues

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Abstract. Matrix metalloproteinases (MMPs) relate to the growth and infiltration of cancer cells, but the frequency and amount of their expression are not yet fully examined in hepatocellular carcinoma. Expression of MMPs (MMP-2, MMP-7, MMP-9, MT1-MMP, MT2-MMP, MT3-MMP) and tissue inhibitors of metalloproteinase (TIMP: TIMP-1, TIMP-2) was investigated on cultured hepatocellular carcinoma (HCC) cells and surgically resected HCC tissues. The cultured cells and tissues expressed MMPs and TIMPs at various degrees, and high expression was observed for MMP-2, MMP-9, MT1-MMP and TIMP-2. Expression of MMP-7, MT2-MMP and TIMP-1 was found at a low frequency and a low amount in both the cells and the tissues. MMP-2 was expressed in various cells: HCC cells, vascular wall and sinusoidal endothelial cells in the cancer area of surgically resected tissues; and hepatocytes, bile duct cells, vascular wall, macrophages and Kupffer cells in the non-cancerous area. MMPs and TIMPs were expressed at a relatively high frequency in hepatocytes of the cancerous area and surrounding non-cancerous area as well as in the other cells and tissues. MMPs and TIMPs may be involved in the progression of hepatocellular carcinoma including the infiltration of cancer cells.

Introduction

Tumor cells during their progression obtain high capability of growth, invasion and metastasis (1), and they change into highly malignant cells. Recent studies demonstrated that tumor cells require protease activities that resolve extracellular matrix in order to infiltrate into normal tissues, and this fact attracted attention to a family of endoproteinases, i.e. matrix metalloproteinases (MMPs) whose enzymatic activity is directed against components of the extracellular matrix. Invasive spread or infiltration of cancer cells needs resolution of extracellular matrix, and this is the result of the impaired balance between several MMPs and proteinase inhibitors called the tissue inhibitor of metalloproteinases (TIMP) (2). MMPs are produced not only by cancer cells but also stromal fibroblasts, infiltrating macrophages and granulocytes. MMP-2 and MMP-9 are known to be directly involved in degradation of extracellular matrix and of specific basement membrane molecules. In fact, various malignant tumors have abnormal MMP expression on their stroma. When tumor cells are unable to express secretory-type MMPs, they induce MMP production on the host cells. Tumor cells could specifically activate catalytic function of certain MMPs secreted by host cells by using proteases and other MMPs (3) and they utilize the activated MMPs for their invasion. MT-MMPs and MMP-3 on the other hand activate movement and angiogenesis of cancer cells, and this then induces effective invasion to the tissues. Therefore, clarification of the production and activation of MMPs is expected to provide a novel means that would prevent cancer growth and metastasis. We investigated the expression of MMPs and their inhibitors TIMPs in hepatocellular carcinoma (HCC) cell lines and surgically resected HCC tissues.

Materials and methods

Cell lines and cell cultures. This study utilized 11 human HCC cell lines that were originally established in our laboratory, i.e. KIM-1 (4), KYN-1 (5), KYN-2 (6), KYN-3 (Murukami T, *et al*, Jpn J Cancer Res 292: abs. 1988), HAK-1A, HAK-1B (7), HAK-2 (8), HAK-3 (9), HAK-4, HAK-5 and HAK-6. These cell lines were previously confirmed to retain morphological and functional characteristics of the original HCC. KIM-1, KYN-1, HAK-2 and HAK-3 were established from surgically resected moderately differentiated HCC nodules; KYN-2 and HAK-6, from surgically resected moderately to poorly differentiated HCC nodules; and KYN-3, HAK-4 and

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Key words: matrix metalloproteinase, tissue inhibitor of metalloproteinase, hepatocellular carcinoma, cancer growth, infiltration

Table I. Results of flow cytometric analyses on the expressions of MMPs and TIMPs in HCC cell lines.

Cell	MMP-2	MMP-7	MMP-9	MT1-MMP	MT2-MMP	MT3-MMP	TIMP-1	TIMP-2
KIM-1	+++	±	+++	±	-	-	-	±
KYN-1	++	-	++	±	-	-	-	+
KYN-2	+++	-	+++	±	-	-	-	±
KYN-3	+++	-	+++	±	-	-	-	++
HAK-1A	+++	-	+++	+	-	-	-	+
HAK-1B	+++	-	+++	±	-	-	-	±
HAK-2	++	-	++	+++	-	-	-	+
HAK-3	+++	-	+++	+++	±	-	-	±
HAK-4	+++	±	+++	++	±	-	-	+
HAK-5	+++	±	+++	±	-	-	±	+
HAK-6	+++	-	+++	±	-	-	-	+

-, negative, positive cells accounted for <5% in total area; ±, weakly positive, 5-<25%; +, moderately positive, 25-<50%; ++, strong positive, 50-<75%; +++, very strong positive, ≥75%.

HAK-5, from peritoneal effusion of HCC patients with moderately to poorly differentiated HCC, poorly differentiated HCC, and sarcomatous HCC, respectively. HAK-1A and HAK-1B were 2 clonally related HCC cell lines established from a single HCC nodule showing a 3-layered structure with a different histological grade in each layer. HAK-1A is morphologically a well-differentiated HCC cell line, while HAK-1B is a poorly differentiated HCC cell line and biologically more malignant than HAK-1A, and is presumed to be derived from HAK-1A through its clonal dedifferentiation.

Tissue samples of HCC and non-HCC were obtained from 21 surgically treated HCC patients, and all specimens were obtained at Kurume University Hospital between 1996 and 2000. Informed consent was obtained from each patient who enrolled in the study. Tissue samples were used for immunohistochemistry, enzyme linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) analyses for MMPs and TIMPs. Each cell line was examined with flow cytometry and RT-PCR for MMPs and TIMPs.

Immunohistochemistry. Immunohistochemical staining of MMPs (MMP-2, MMP-7, MMP-9, MT1-MMP, MT2-MMP and MT3-MMP) and TIMPs (TIMP-1 and TIMP-2) were conducted by using catalyzed signal amplification system kits (Dako, CA) on paraffin sections of HCC tissues and their surrounding non-HCC tissues obtained from 18 cases (or 15 cases for MMP-7 examination). Primary antibodies were monoclonal antibody against each of the above-mentioned 6 human MMPs and 2 TIMPs (final dilution: 1/100-1/200, Daiichi Fine Chemical Co., Ltd., Tokyo, Japan). Peroxidase reaction was developed by using 3,3-diaminobenzidine tetrahydrochloride and the cells were counterstained with hematoxylin. The specimen was evaluated as having either: i) equivalent expression, i.e. HCC cells or non-neoplastic hepatocytes were stained as intensively as that of the positive internal control, ii) weak expression, i.e. the staining was less intensive, iii) high expression, i.e. they were more intensive or iv) negative expression, i.e. they were not stained at all.

ELISA. Portions of the surgically obtained HCC and non-HCC tissues were cut into pieces, and an appropriate amount was homogenized in 500 µl of ice-cold Ca^{++}-and Mg^{++}-free phosphate-buffered saline containing 100 µl/ml phenymethylsulfonyl fluoride by using a pellet pestle. The mixture was centrifuged for 10 min (12,000 rpm, 4°C), and the supernatant was stored at -20°C until use. The amount of tissue protein was determined by using the BCA protein assay reagent (Pierce Rockord, IL). The amount of active-MMP-2 and of pro-MMP-2 plus active-MMP-2 in supernatant was measured by using ELISA kits (Amersham International plc, Buckinghamshire, England). The amount of active-MMP and of pro-plus active-MMPs was assessed by making a correction of the measured level for the amount of protein.

Sixteen of the 18 cases whose tissues were examined in ELISA were also examined in the immunohistochemical staining, and they consisted of 3 cases of well + moderately differentiated HCC, 9 of moderately differentiated HCC and 4 of moderately + poorly differentiated HCC.

Flow cytometric analysis. Flow cytometric analyses for the MMPs and TIMPs were performed as described in our previous report (10). Antibodies used in the current study were monoclonal anti-MMP antibodies (final dilution, 1/50), anti-TIMP antibodies (final dilution, 1/50), and FITC conjugated goat anti-mouse IgG (Becton Dickinson Immuno-cytometry System USA, San Jose, CA), and analyses were done by using a FACScan (Becton Dickinson Immuno-cytometry System USA).

Analyses of MMP and TIMP mRNAs with reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA of cultured cells and tissues were extracted by using RNA-Bee™ (TEL-TEST, Inc., Friendswood, TX). RT-PCR for MMPs and TIMPs was performed as described in our previous report (10). PCR reaction was made with a primer specific to either MMP-2, MMP-7 (11), MMP-9 (12), MT1-MMP, MT2-MMP, MT3-MMP (13), TIMP-1, TIMP-2 (12)

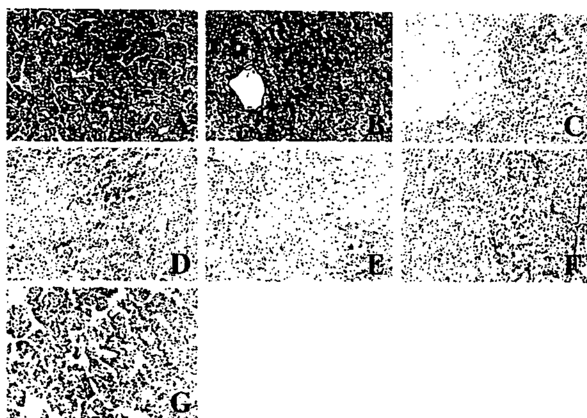

Figure 1. Immunohistochemical staining of MMPs and TIMPs. (A) MMP-2 was stained in the cytoplasms of neoplastic hepatocyte and sinusoidal endothelial cells in HCC. (B) MMT-2 was stained in the cytoplasm of non-neoplastic hepatocytes and in macrophages and Kupffer cells. (C) MMT-2 was stained predominantly on the cell surface of neoplastic hepatocytes at the boundary between HCC and non-HCC areas. (D) MMP-9 was stained in the cytoplasm of neoplastic hepatocytes in HCC. (E) MMP-9 was stained in the cytoplasms of non-neoplastic hepatocytes and sinusoidal lining cell in HCC. (F) MT3-MMP was stained in the cytoplasm of neoplastic hepatocytes in HCC. (G) TIMP-2 was stained in the cytoplasm of neoplastic hepatocytes in HCC. Counterstained with Mayer's hematoxylin. Original magnification, x100.

or ß-actin (14). PCR reaction was repeated 30-40 cycles by using iCycler (Bio-Rad Laboratories, CA), and one cycle consisted of denaturation, annealing and extension. PCR product (5 µl) was electrophorased with a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) which contained 0.5% ethidium bromide, and specific DNA bands were examined under a UV transilluminator.

Results

Flow cytometric analyses. All cell lines expressed MMP-2 and MMP-9 at a high amount and frequency, i.e. ≥75% cells were positive. TIMP-2 and MT1-MMP were expressed but generally at a low amount and frequency, i.e. ≤50% cells were positive. Expression of MMP-7, TIMP-1, MT2-MMP and MT3-MMP as detected in 1-3 cell lines and the positive rate was also low (Table I).

Immunohistochemistry of surgically resected tissues. In the non-HCC tissues, MMPs and TIMPs were expressed on the hepatocytes, bile duct, vascular wall and metaplastic hepatocytes, showing ductular structures at the periphery of hepatic lobules; MMP-2, TIMP-2 and MMP-9 were also expressed on the Kupffer cells and macrophages; and MMP-2 was also expressed on the stromal connective tissues in the cases with abundant fibrosis. In the HCC tissues, MMP-2 was expressed on the HCC cells, sinusoidal endothelial cells and other lining cells, and fibrous capsule; and MMP-2, MMP-7, MMP-9 and MT3-MMP expression in some cases was more apparent on the HCC cells at the boundary between HCC and non-HCC areas. In the case of MMP-2, the expression was sometimes clearer on the cell surface than in the cytoplasm (Fig. 1).

In the 18 cases of HCC tissues, the highest frequency was noted for TIMP-2 (18/18, 100%), and the lowest frequency was noted for TIMP-1 (8/18, 44%). In the non-HCC tissues, the highest frequency was obtained also for TIMP-2 (17/18, 94%), and the lowest was also TIMP-1 (8/18, 44%). TIMP-2 and MMP-2 were expressed at a high frequency and a high

Table II. Immunohistochemical staining for MMTs and TIMPs in cancerous and non-cancerous tissues according to histological grade.

	Well + Mod (n=4)		Mod (n=9)		Mod + Poor (n=5)		Total (n=18)	
	C (%)	N (%)	C (%)	N (%)	C (%)	N (%)	C (%)	N (%)
MMP-2	4 (100)	4 (100)	8 (89)	8 (89)	5 (100)	4 (80)	17 (94)	16 (89)
MMP-7*	3 (75)	3 (75)	4 (44)	6 (67)	1 (20)	2 (40)	8 (53)	11 (73)
MMP-9	3 (75)	2 (50)	7 (78)	6 (67)	3 (60)	4 (80)	13 (72)	12 (67)
MT1-MMP	3 (75)	3 (75)	6 (67)	6 (67)	3 (60)	5 (100)	12 (67)	14 (78)
MT 2-MMP	2 (50)	2 (50)	5 (56)	5 (56)	2 (40)	3 (60)	9 (50)	10 (56)
MT 3-MMP	2 (50)	2 (50)	6 (67)	6 (67)	4 (80)	5 (100)	12 (67)	13 (72)
TIMP-1	2 (50)	2 (50)	5 (56)	4 (44)	1 (20)	2 (40)	8 (44)	8 (44)
TIMP-2	4 (100)	4 (100)	9 (100)	8 (89)	5 (100)	5 (100)	18 (100)	17 (94)

Well: well-differentiated HCC. Mod: moderately-differentiated HCC. Poor: poorly-differentiated HCC. n: number of HCC tissues examined. C: cancerous tissues. N: non-cancerous tissues. *Total number was 15 (4 Well+Mod, 7 Mod and 4 Mod+Poor).

Table III. Active or total MMP-2 protein levels in surgically resected HCC and its continuous areas: Results of ELISA.

Histologic grade	MMP-2 protein	HCC tissue	Non-HCC tissue
Well + Mod (n=3)	Total (pro + active)	0.92 ± 0.15	0.48 ± 0.05
	Active	0.00	0.00
Mod (n=10)	Total (pro + active)	0.69 ± 0.52	0.87 ± 0.55
	Active	0.01 ± 0.01	0.03 ± 0.03
Mod + Poor (n=4)	Total (pro + active)	0.66 ± 0.63	1.13 ± 0.43
	Active	0.02 ± 0.02	0.06 ± 0.60
Poor (n=1)	Total (pro + active)	4.09	0.47
	Active	0.00	0.00
Total (n=18)	Total (pro + active)	0.91 ± 0.92	0.84 ± 0.50
	Active	0.01 ± 0.02	0.03 ± 0.04

Data are expressed as ng/20 μg protein. Well: well-differentiated HCC. Mod: moderately-differentiated HCC. Poor: poorly-differentiated HCC.

amount, and there were no remarkable differences between HCC and non-HCC tissues. On the other hand, the expression of TIMP-1 and MT2-MMP was low in frequency and amount. In the comparison of MMP and TIMP expression between neoplastic and non-neoplastic hepatocytes, frequencies of the expression were almost the same except MMP-2 that expression was higher in the neoplastic hepatocytes (Table II).

ELISA: Measurement of active and total (pro-active) MMP-2 proteins. The mean amount of total MMP-2 in the 18 cases was 0.91 ng/20 μg protein in the HCC area and 0.84 ng/20 μg protein in the non-HCC area. Amount of total MMP-2 protein was higher in the non-HCC area than in HCC area of the following 9 cases (50%), i.e. 6/10 (60%) moderately differentiated HCC cases and 3/4 (75%) moderately + poorly differentiated HCC cases. The remaining 9 cases (50%) had higher total MMP-2 in the non-HCC area than in HCC area, and the highest amount of total MMP-2 was found in the HCC area (4.09 ng/20 μg protein) of one poorly differentiated HCC (Table III). Active-MMP-2 was detected in the HCC and/or non-HCC areas of 8/18 cases. In the 9 cases whose total MMP-2 was higher in the non-HCC area, mean active-MMP-2 in the HCC area was 0.02 ng/20 μg protein and that in the non-HCC area was higher, i.e. 0.05 ng/20 μg protein.

RT-PCR. All cell lines clearly expressed MMP-2, MMP-9 and MT1-MMP mRNAs, and mRNAs of the other MMPs and TIMPs at various degrees. MMP-7 and MT3-MMP mRNAs were detected at a low frequency. All cases expressed MMP-2, MMP-7, MMP-9 and MT1-MMP mRNAs in both HCC and non-HCC tissues. Frequencies of MT2-MMP and TIMP-1 mRNA expression were low (Fig. 2).

Discussion

Our *in vitro* study showed that HCC cell itself expresses MMPs and TIMPs at various degrees. The expression of MMP-2 and MMP-9 was high in terms of frequency and

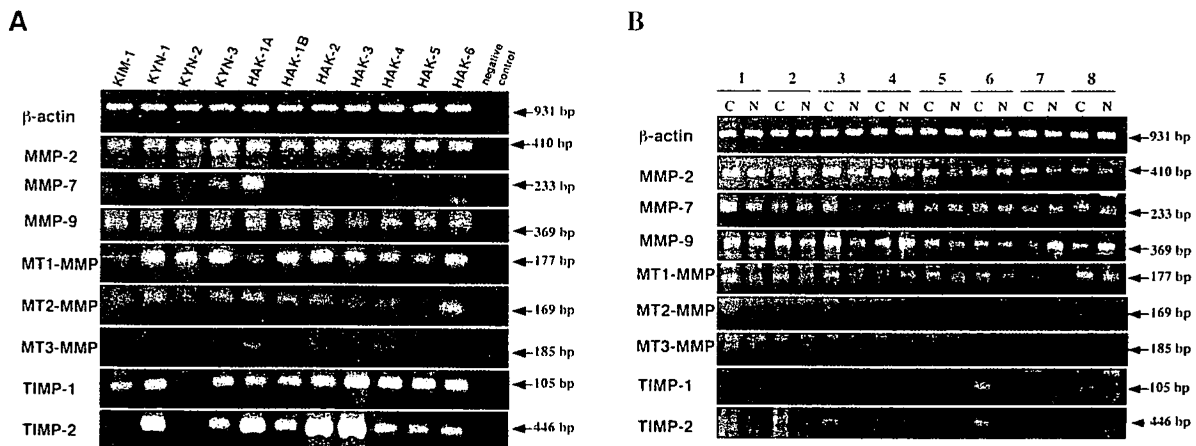

Figure 2. Expression of mRNAs of the MMPs and TIMPs in the HCC cell lines (A) and HCC tissues (B) as measured with RT-PCR technique. The PCR products were electrophoresed in a 2% NuSieve agarose gel and stained with ethidium bromide. In (B), case number is shown on the top. C: cancerous tissue. N: non-cancerous tissue. PCR reaction using ß-actin-specific primers showed that equal amounts of cDNA had been used and similarly amplified.

amount, and this was followed by MT1-MMP and TIMP-2. On surgically resected HCC tissues, the expression of MMP-2, MMP-9, MT1-MMP and TIMP-2 was high, and the *in vivo* and *in vitro* results corresponded well.

MMP-2 produces a complex with TIMP-2 as well as with MT1-MMP, and activation of pro-MMP-2 is usually inhibited by TIMP-2. MT1-MMP activates MMP-2 (15,16) and then the active-MMP-2 activates pro-MMP-9 (17). MMP-9 is also partly activated with MMP-7 (18). MMP-2 and MT1-MMP degrade the extracellular matrix and relate to invasion and metastasis of the cancer cells (19). MMP-2 in particular is the key enzyme that takes an important role in the invasion to basement membrane. It is shown that cancerous tissues with a high expression level of active-MMP have a high risk of metastasis. Therefore, the activation rate of pro-MMP-2 is used as an indicator of cancer metastasis (20). In regard to HCC, there are reports showing the involvement of MMP-2 and MMP-9 to capsular invasion (1,2), involvement of MMP-9 to portal vein invasion (21), high expression of MT1-MMP in poorly differentiated HCCs, and poor prognosis of the cases with MT1-MMP expression (17). In our immunohistochemistry, the 5 cases that contained poorly differentiated HCC (i.e. 4 moderately + poorly differentiated HCCs and 1 poorly differentiated HCC) expressed MT1-MMP in either or both of the cancerous and non-cancerous areas. The 8 cases in which ELISA showed the expression of active-MMP-2 did not contain well-differentiated HCC. These findings indicate that, in HCC, poorly differentiated HCC expresses TIMP-2 but this expression interacts with MT1-MMP and this makes an activation system for MMP-2.

Cytokines such as TNF-α are reported to activate MMP-2 and MMP-9 (22,23). TNF-α is also reported to enhance MT1-MMP expression (24). We therefore examined the effects of various cytokines on MMP-2 expression by using the HCC cell lines, but there were no noteworthy findings (data not shown).

In normal conditions, pro-MMP-2 and TIMP-2, and pro-MMP-9 and TIMP-1 produce a complex. MMP activity is inhibited by TIMP in 1:1 mol basis, and the activation into active-MMP-2 and active-MMP-9 is limited. In our surgically resected tissues and cultured HCC cell lines, the expressions of MMP-2 and TIMP-2 were equally observed, while expression of MMP-9 was higher than TIMP-1, causing an imbalance between MMP-9 and TIMP-1. Lichtinghagen *et al* (12) reported that TIMP-1 expression was higher than TIMP-2 in patients with hepatitis or liver cirrhosis. Hayashi *et al* showed MMP-9 expression elevated with aggravation of inflammation from hepatitis, liver cirrhosis to liver cancer (21). Sakamoto *et al* demonstrated the involvement of MMP-9 to HCC since its very early stage (25). MMP-9 is also known to be up-regulated with angiopoietin-2 and relate to angiogenesis (26,27). Mechanism of MMPs action on carcinogenesis and tumor growth is thought to be different according to the origin of the tumor, e.g. TIMP-1 was expressed but TIMP-2 was rarely expressed in thyroid carcinoma (28) and a hamster model of pancreatic duct carcinoma expressed only a low amount of TIMP-1 (29). In our findings, MMP-9 expression was obvious but TIMP-1 expression was low. Therefore, the balance between MMP-9 and TIMP-1 would be distorted in the carcinogenesis process of the liver, and MMP-9 could affect angiogenesis at the early stage of HCC and then the growth and infiltration of HCC cells.

The HCC cell lines expressed MT1-MMP but rarely expressed MT2-MMP and MT3-MMP. On the HCC tissues, MT1-MMP was expressed on the bile duct and metaplastic hepatocytes that showed ductular structures at the periphery of hepatic lobules, stromal cells and HCC cells. However, MT2-MMP expression on the tissue was low in its frequency and amount. MT3-MMP expression on the tissues was relatively high, and found on HCC cells, metaplastic hepatocytes that showed ductular structures, bile duct and vascular wall. The findings on MT1-MMP and MT2-MMP in the tissues agreed with our *in vitro* findings, but MT3-MMP results did not.

Inducers and suppressors of MT3-MMP are not yet well-known, but Lafleur *et al* (30) reported that MT-MMP expression is affected by the expression of MMP inhibitors, growth factors that relate to angiogenesis, and cytokines. In our findings, the results on MT3-MMP expression did not agree, between cultured HCC cells and surgically obtained HCC specimens, but this disagreement suggested that MT3-MMP expression in the tissue was the result of interaction between various cells including HCC cells on paracrine and autocrine bases. MT1-MMP and MT3-MMP are known to enhance fibrin-invasive activity, and MT1-MMP, MT2-MMP and MT3-MMP are related to endothelial tubulogenesis (30). In our tissue examination, endothelial cells were positive to these 3 MMPs, suggesting that these MMPs most probably take part in tubulogenesis as well as directly participate in cell proliferation and invasion (31,32).

Yamamoto *et al* (33) reported that MMP-7 relates to cancer progression, its expression in gastric cancer is higher in well-differentiated adenocarcinoma, and it plays an important role in the infiltration of cancer cells through its resolving activity on extracellular matrix. In our cases, MMP-7 expression was lower in frequency and amount in the tissues containing poorly differentiated HCC than those containing well-differentiated HCC. The expression was also low in the HCC cell lines. This suggests that MMP-7 relates to cancer progression in the early stage. MMP-7 is also reported to take part in the invasion of gastric cancer and the metastasis of lymph nodes, and it could be used as a predictive factor of prognosis (34). Cholangiocellular carcinoma expressing MMP-7 was reported to have high malignancy and short survival period (35-37). Expression of MMP-7 needs to be investigated further.

MMPs and TIMPs were expressed in cancerous and non-cancerous areas of HCC in various degrees. MMPs were thought to be activated as a result of quantitative and qualitative imbalance of these expressions, and then to take part in proliferation, invasion and metastasis of HCC. Our findings indicate that blockage of the mechanism of MMP action would be an efficacious means to prevent or suppress development and proliferation of HCC.

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Expression and activation of apoptosis-related molecules involved in interferon-α-mediated apoptosis in human liver cancer cells

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Abstract. Interferon (IFN)-α directly inhibits proliferation of liver cancer cells by inducing apoptosis, but the molecular mechanisms by which IFN-α induces apoptosis in these cells are not fully understood. We examined the effect of broad spectrum caspase inhibitor, Z-VAD-fmk, and the caspase activation in IFN-α-mediated apoptosis by using 4 liver cancer cell lines that were sensitive or resistant to IFN-α-mediated apoptosis. Involvement of apoptosis-related mitochondrial proteins and Bcl-2 family proteins in IFN-α-mediated apoptosis was further examined in 1 sensitive cell line (KIM-1). The Z-VAD-fmk completely or moderately inhibited IFN-α-mediated apoptosis in the sensitive cells. IFN-α induced time-dependent activation of caspase-3 in the sensitive cells, while the resistant cells showed mild or no activation. Activation of caspase-9, caspase-8, and caspase-7, and the cleavage of poly(ADP-ribose)polymerase were identified in either or both of the sensitive cell lines, but not in the resistant cells. In KIM-1 cells, the release of cytochrome c and Smac/DIABLO from mitochondria to cytosole was confirmed. Meanwhile, Bcl-x_L was upregulated, and Bid activation or translocation, or conformational changes of Bax were not identified. In conclusion, our results suggest IFN-α-mediated apoptosis in liver cancer cells involves the mitochondrial apoptotic pathway and is induced by activating various caspases.

Introduction

Interferon (IFN)-α has various biological actions such as antivirus, antiproliferative, immunomodulatory, anti-telomerase and anti-angiogenesis effects (1,2). Their antivirus effects are utilized in a treatment for chronic hepatitis C; and the antiproliferative effects, for malignant diseases such as leukemia and renal cancer (3). *In vitro* experiments of the direct antiproliferative effects of IFN-α demonstrated the induction of apoptosis and cell-cycle arrest at G_1/S, S or G_2/M phases in many cell lines (4-10).

Induction of apoptosis mechanism would be initiated through 2 major signaling pathways, i.e., death receptor pathway (11) and a mitochondrial pathway (12-16). Recent studies (17-20) suggest the involvement of the mitochondrial pathway in IFN-α or -ß-mediated apoptosis, however, not all the findings are consistent. The mitochondrial pathway is initiated at the mitochondria by the release of apoptogenic factors, such as cytochrome c, and they trigger activation of effector caspases such as caspase-3 through the formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, and eventual apoptosis with the cleavage of diverse cellular substrates, such as poly(ADP-ribose)polymerase (PARP) (13-15).

We previously reported that human natural IFN-α induced apoptosis *in vitro* at a relatively high frequency (77%, 10 of the 13 cell lines), and this apoptosis was not closely related to: i) the expression of Bcl-2 family members such as Bax, Bak, Bcl-2 and Bcl-x_L, ii) condition of the gene p53, and iii) the Fas/Fas ligand (10). More recently, we reported that IFN-α-mediated suppression of hepatocellular carcinoma (HCC) cell proliferation *in vivo* is related to the induction of apoptosis and the suppression of angiogenesis (21). In the present study, we investigated the involvement of apoptosis-related molecules, including mitochondrial proteins and caspases, by using liver cancer cell lines that were sensitive or resistant to IFN-α-mediated apoptosis.

Materials and methods

Cell lines and cell culture. This study utilized 3 HCC cell lines [KIM-1 (22), KYN-3 (Murakami *et al*, Jpn J Cancer Res Proceedings of the Japanese Cancer Association: abs. 292, 1988), HAK-1B (23)] and 1 human combined hepatocellular and cholangiocarcinoma cell line [KMCH-2 (24)]. In our previous study (10), we found that KIM-1 and HAK-1B were sensitive to IFN-α-mediated apoptosis, while the other 2 cell lines (KYN-3 and KMCH-2) were resistant to this apoptosis.

Each cell line was grown in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Co., Japan) supplemented with 2.5% heat-inactivated (56˚C, 30 min) fetal bovine serum (Bioserum, Victoria, Australia), 100 U/ml penicillin, 100 μg/ml

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Key words: caspase, cytochrome c, hepatocellular carcinoma, mitochondria

streptomycin (Gibco BRL/Life Technologies, Inc., Gaithersburg, MD) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% CO_2 in air at 37°C.

Cytokines, antibodies, and reagents. Natural human IFN-α (OIF) was kindly provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Monoclonal anti-human/mouse cytochrome c antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Products from Cell Signaling Technology, Inc. (Beverly, MA) were: a biotinylated protein marker detection pack, Chaps Cell Extract buffer, Cell Lysis buffer, rabbit polyclonal antibodies to cleaved caspase-3, caspase-3, cleaved caspase-7, caspase-7, cleaved caspase-9, caspase-9, cleaved poly(ADP-ribose)polymerase (PARP), PARP, BID and Smac/DIABLO, and monoclonal antibody to caspase-8. Anti-human cytochrome oxidase subunit II mouse monoclonal 12C4-F12 antibody was purchased from Molecular Probes, Inc. (Eugene, OR); rabbit polyclonal antibodies to human Bax, N-termina, from Upstate Biotechnology (Lake Placid, NY); rabbit polyclonal anti-human Bax antibody (clone 4F11), from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan); rabbit polyclonal anti-human Bcl-x_L antibody, from Transduction Laboratories (Lexington, KY); monoclonal anti-ß-actin antibody, from Sigma Chemical Co. (Saint Louis, MO); Jurkat cell extract, non-induced and induced immunoblotting standards, from BioMol Research Laboratories Inc. (Plymouth Meeting, PA); and irreversible, cell permeable, broad spectrum caspase inhibitor, Z-Val-Ala-Asp (OMe)-Fluoromethylketone (Z-VAD-fmk), from Kamiya Biomedical Co. (Seattle, WA). Antibodies were used at the concentrations recommended by the manufacturers. The amount of protein in the samples was measured by using the BCA protein assay reagent (Pierce, Rockford, IL).

Effects of caspase inhibitor on IFN-a-mediated apoptosis. KIM-1 or HAK-1B cells (2.0×10^5 cells/well) were treated or untreated with 1,000 IU/ml IFN-α for 72 h and processed for assessment of apoptosis. Broad spectrum caspase inhibitor Z-VAD-fmk was used at a final concentration of 8, 20, or 50 µM and it was added to the culture medium 1.5 h prior to IFN-α treatment. For the control, vehicle (acetone) alone was added to the culture. For KIM-1 cells, an additional experiment was conducted, in which cultured cells were pretreated with Z-VAD-fmk at a final concentration of 40 or 100 µM, 1.5 h prior to the addition of IFN-α, cultured with IFN-α for 24 h, retreated with the same amount of a fresh inhibitor, cultured for 24 h, and processed for assessment of apoptosis. Assessment of apoptosis was conducted by observation under a phase-contrast microscope (Nikon, Tokyo, Japan), and by terminal deoxynucleotidyl transferase (tdt)-mediated dUTP nick end-labeling (TUNEL) method. MEBSTAIN Apoptosis Kit Direct (Medical & Biological Laboratories Co., Ltd.) was used according to the manufacturer's recommendation. The stained cells were analyzed by using a FACScan (Becton Dickinson Immunocytometry Systems USA, San Jose, CA).

Assessment of caspase-3 and caspase-9 activities by fluorometric protease assay. Quantitative analysis on the activation of caspase-3 and -9 was conducted by using Fluorometric Protease Assay Kits (Medical & Biological Laboratories Co., Ltd.) according to the manufacturer's protocol. Briefly, the cellular protein (100 µg) obtained from the cells cultured with or without 1,000 IU/ml IFN-α for several intervals was incubated with appropriate caspase substrate in the reaction buffer at 37°C for 2 h. DEVD-AFC and LEHD-AFC substrates were used for the activity measurement of caspase-3 and -9, respectively. Caspase activities were estimated by measuring a yellow-green fluorescence with a Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) by setting the excitation filter at 400 nm and the emission filter at 505 nm.

Sodium dodecyl sulfate-polyacrylamide slab gel (SDS-PAGE) electrophoresis and Western blotting. We investigated the activation of caspase-3, -7, -8 and -9, and cleavage of PARP in the 4 cell lines cultured with or without 1,000 IU/ml IFN-α for 72 h. Cells were solubilized in Chaps Cell Extract buffer or Cell Lysis buffer for the detection of caspase-3, -7 and -9, and PARP according to the manufacturer's protocol. For caspase-8 detection, cultured cells were lysed by adding Laemmli's sample buffer. We also investigated the expression of cytochrome c, cytochrome oxidase subunit II, Bax, BID, Bcl-x_L, and Smac/DIABLO in mitochondrial and cytosolic fractions of KIM-1 cells cultured with or without 1,000 IU/ml IFN-α for 24, 48, or 72 h. The mitochondrial and cytosolic fractions were isolated from KIM-1 cells using the method described by Wang *et al* (25). The cellular proteins were subjected to electrophoresis in the 12.5% SDS-PAGE and transferred to polyvinylidine difluoride transfer membrane (Immobilon-P, Millipore Corporation, Bedford, MA) using the Trans-blot SD semi-dry transfer cell (Bio-Rad, Richmond, CA). Immunoblotting was performed using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK) as previously described (10). The intensities of the bands were quantified densitometrically using the NIH Image 1.61 software program. The protein levels were normalized against ß-actin levels used as an internal control.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction and RT-PCR reaction were performed by using a technique described previously (10) with slight modification. After RT reaction, phenol (pH 8.0) extraction was performed twice, and purified cDNA was used as the template of DNA synthesis. PCR reaction of Apaf-1 and caspase-2, -3, -4, -5, -6, -7, -8, -9 and -10 was performed with Human Apoptosis Genes Set-5 and Set-6 MPCR Amplification Kits (Maxim Biotech. Inc., San Francisco, CA) according to the manufacturer's protocol. PCR reaction was made through 2 cycles of 94°C (1 min) and 62°C (4 min), followed by 30 cycles of 94°C (1 min) and 62°C (2.5 min), and 1 cycle of 70°C (3 min), by using a Thermocycler (Perkin-Elmer Cetus Corp., Norwalk, CT). PCR product (5 µl) was electrophoresed with a 4% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). The gel was stained with 0.1% ethidium bromide, and then specific DNA bands were examined under an ultraviolet transilluminator.

Results

Effects of caspase inhibitor on IFN-a-mediated apoptosis. Addition of 1,000 IU/ml of IFN-α to the cultures of KIM-1

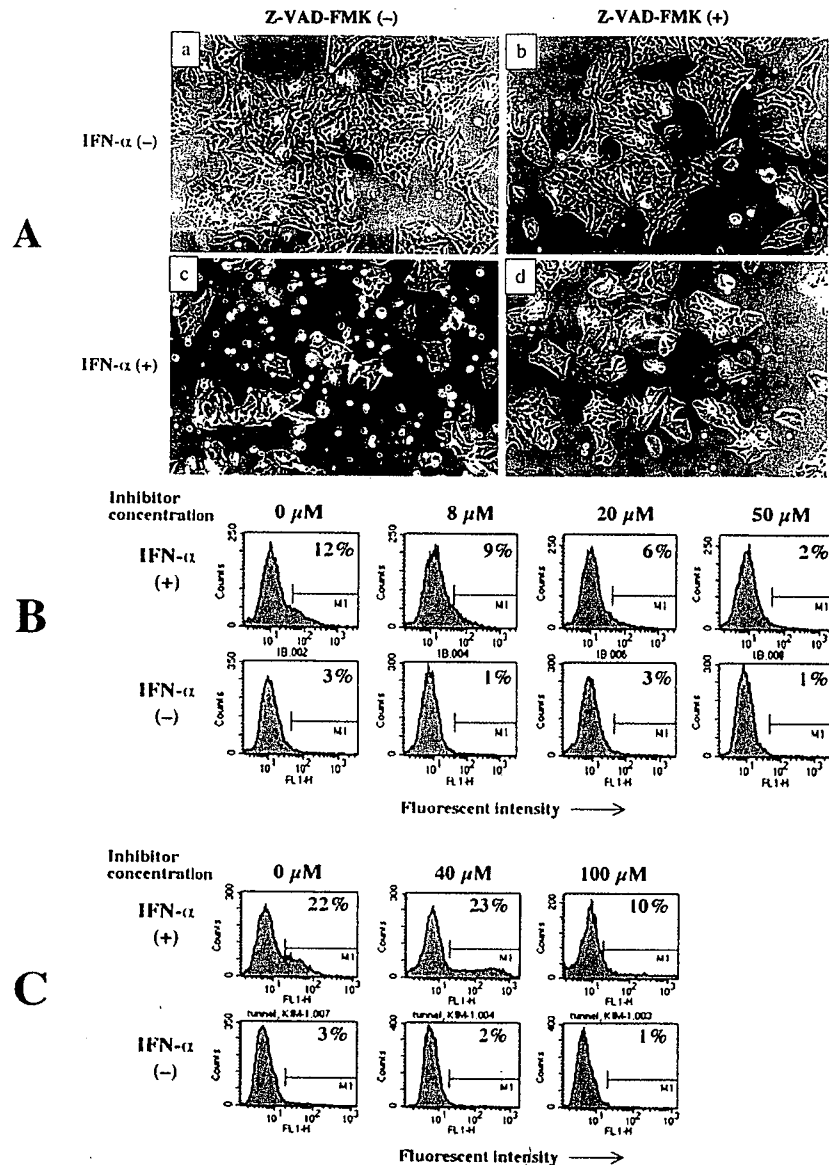

Figure 1. Effects of the broad spectrum caspase inhibitor Z-VAD-fmk on IFN-α-mediated apoptosis in HAK-1B cells (A and B) or KIM-1 cells (C) were assessed under a phase-contrast microscope (A) or by TUNEL method (B and C). In all experiments, Z-VAD-fmk was added to the medium 1.5 h before the addition of IFN-α. (A), HAK-1B cells that were pretreated with (b and d) or without (a and c) 50 μM Z-VAD-fmk were cultured with (c and d) or without (a and b) 1,000 IU/ml IFN-α for 72 h. (B), HAK-1B cells that were pretreated with 0, 8, 20 or 50 μM Z-VAD-fmk were cultured with or without 1,000 IU/ml IFN-α for 72 h, and TUNEL-positive cell rates were assessed by flow cytometry. The value shown with each histogram represents the percentage of TUNEL-positive apoptotic cells. (C), KIM-1 cells that were pretreated with 0, 40 or 100 μM Z-VAD-fmk were cultured with IFN-α for 24 h, re-treated with the same amount of a fresh inhibitor, cultured for another 24 h, and processed for assessment of apoptosis by TUNEL method. The value shown with each histogram represents the percentage of TUNEL-positive apoptotic cells.

and HAK-1B cells resulted in the induction of apoptosis from 48 h later, and the number of apoptotic cells increased over time. These findings agree with our previous results (10). In HAK-1B cells, the broad spectrum caspase inhibitor Z-VAD-fmk showed dose-dependent decrease of IFN-α-mediated apoptotic cells until 72 h after the IFN-α addition under a phase-contrast microscope (Fig. 1A). In addition, TUNEL-positive apoptotic cells acounted for 12% of the cells in the cultures without addition of the inhibitor, and the ratio decreased along with the dose-increase of the inhibitor, e.g. to 2% with 50 μM (Fig. 1B). The same experiment was performed for KIM-1 cells, but suppression of apoptosis was not observed under both assays (data not shown). We then used 100 μM of the inhibitor and the cells were re-challenged with fresh inhibitor after 24 h of the culture. Under phase-contrast microscope (data not shown), suppression of apoptosis was observed, and the TUNEL examination also showed that the rate of positive cells decreased from 22 to 10% (Fig. 1C).

Activation of caspases by IFN-α. In the fluorometric protease assay, activation of caspase-3 and -9 did not occur or occurred at a low level 24 h after the addition of IFN-α in the 2 cell lines