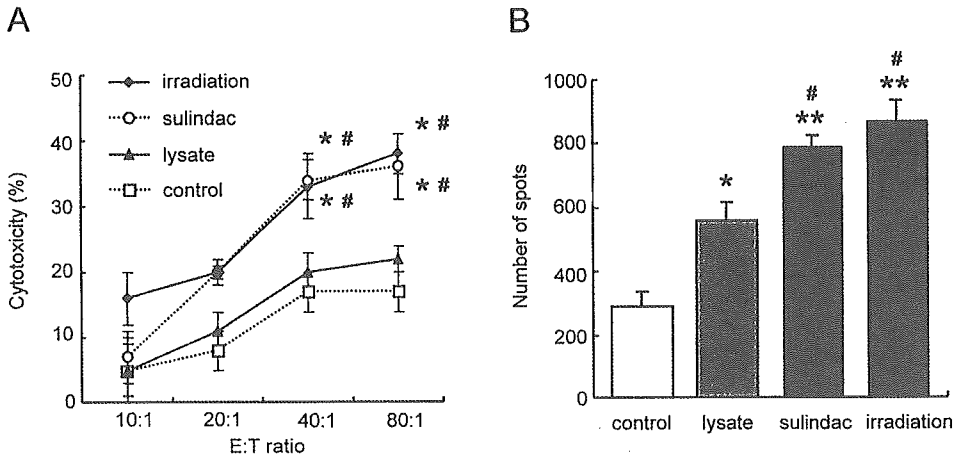

Figure 4. Antitumor cellular immunity induced by vaccination with DCs pulsed with apoptotic hepatoma cells. (A), Cytolytic activity of spleen cells against Hepa 1-6 cells. Spleen cells from vaccinated C57BL/6 mice with PBS alone (control), DCs pulsed with Hepa1-6 cell lysates, and DCs pulsed with sulindac- or irradiation-induced apoptotic Hepa1-6 cells were cocultured with 20 Gy irradiated Hepa1-6 cells. Cytotoxic effector lymphocytes were harvested after 5 days of incubation and the ^{51}Cr release assay was performed at indicated E:T ratios. Data are expressed as mean ± SD (n=6). *p<0.05 versus control; #p<0.05 versus lysate-pulsed DCs. (B), IFN-γ ELISPOT assay. Spleen cells derived from vaccinated C57BL/6 mice were cultured with irradiated Hepa1-6 cells for 48 h, and IFN-γ ELISPOT assay was performed. The spots in each well were counted under a microscope. Data are expressed as mean ± SD (n=6). *p<0.05 versus control; **p<0.01 versus control; #p<0.05 versus lysate-pulsed DCs.

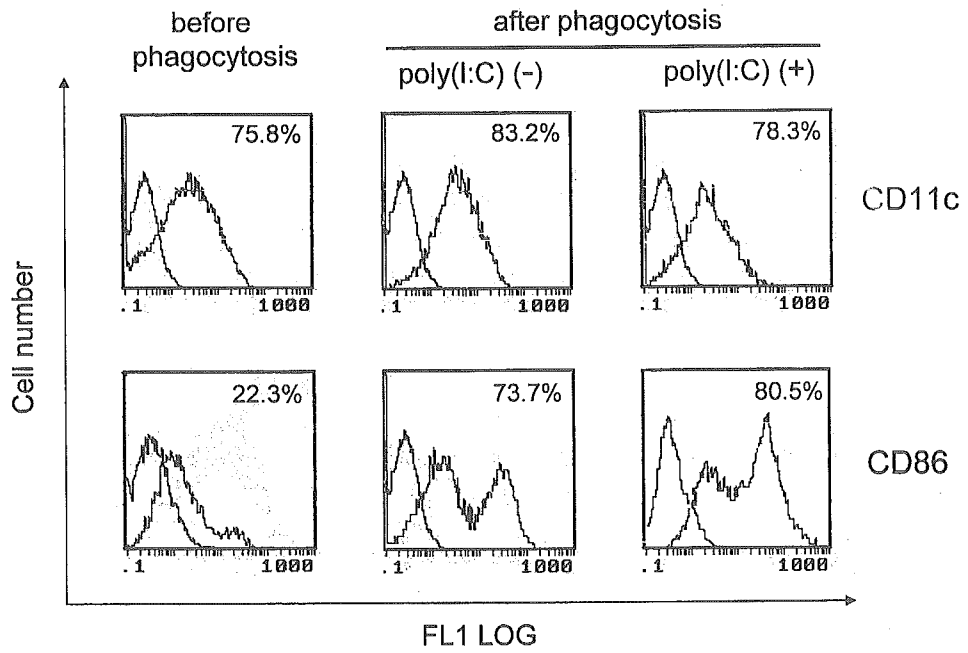

Figure 5. DC maturation after phagocytosis of apoptotic cells and poly (I:C) treatment. CD11c and CD86 expression in DCs after phagocytosis of apoptotic BNL1ME.A.7R.1 cells and poly (I:C) treatment were analyzed by a flow cytometer. The horizontal and vertical bars indicate the fluorescence intensity and the relative number of cells, respectively. Left histograms in each experiment represent negative controls. The percentages of positive cells are indicated. Results shown are from one representative experiment from a total of three performed.

Vaccination with DCs pulsed with apoptotic hepatoma cells represents antitumor effect against pre-established hepatoma. Finally, we elucidated the antitumor effect of vaccination with DCs pulsed with apoptotic BNL1ME.A.R.1 cells against pre-established BNL1ME.A.R.1 tumors. In addition, we examined whether DC maturation induced by poly (I:C) treatment influenced the antitumor effect of vaccination. CD86 expression, one of the maturation phenotypes of DCs (8,15), was upregurated after phagocytosis of apoptotic BNL1ME.A.R.1 cells, and addition of poly (I:C) further enhanced it (Fig. 5) as described previously (20). The growth of pre-established BNL1ME.A.R.1 tumors was significantly

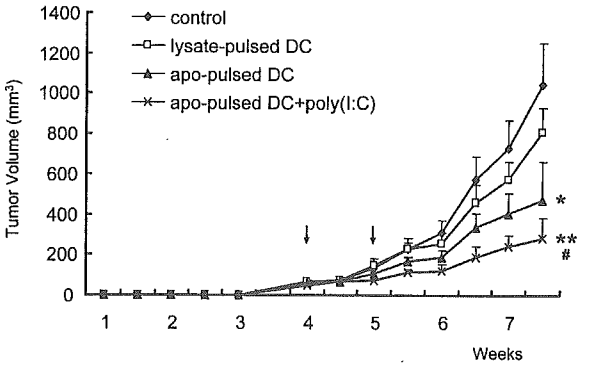

Figure 6. Antitumor effect of vaccination with DCs pulsed with apoptotic hepatoma cells on pre-established BNL1ME.A.7R.1 tumors in BALB/c mice. BNL1ME.A.7R.1 cells ($3x10^6$ cells/mouse) were subcutaneously injected into the right flank of BALB/c mice. Four weeks after tumor cells inoculation when the tumor diameter became more than 5 mm, mice underwent vaccination twice with a one week interval with the injection of PBS alone (control), lysate-pulsed DCs and DCs pulsed with irradiation-induced apoptotic cells with or without treatment of 20 μg/ml of poly (I:C), respectively. Arrows indicate the day of vaccination. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6). *p<0.05 versus control; **p<0.01 versus control; #p<0.05 versus vaccinated with lysate-pulsed DCs group.

retarded by vaccination with DCs pulsed with irradiation-induced apoptotic BNL1ME.A.R.1 cells ($p<0.05$ versus control) but not by vaccination with DCs pulsed with BNL1ME.A.R.1 cell lysates. In addition, vaccination with DCs pulsed with apoptotic cells and treated with poly (I:C) more efficiently repressed the tumor growth ($p<0.01$ versus control, $p<0.05$ versus vaccination with lysate-pulsed DCs), although rejection of tumors was not observed (Fig. 6).

Discussion

Albert *et al* have reported that DCs acquire antigens from apoptotic cells and stimulate MHC class I-restricted CTLs (21), and that immature DCs efficiently phagocytose apoptotic cells through αvß5 integrin and CD36, and cross-present antigens to CTLs (22). On the basis of their findings, several *in vitro* studies have shown that DCs pulsed with apoptotic tumor cells, which is induced by UV-B, γ-irradiation or anti-fas antibody, effectively stimulate tumor-specific CTLs (15-18,23), but DCs pulsed with tumor cell lysates cannot (15-18). In addition, it has been reported that protective antitumor immunity is induced by vaccination with DCs pulsed with apoptotic tumor cells in murine leukemia and melanoma models as compared to vaccination with unpulsed DCs (24,25). Taken together, it is likely that adequate cross-priming of CTLs with antitumor activity is achieved by DCs pulsed with apoptotic tumor cells rather than tumor cell lysates. However, there are few *in vivo* studies determining whether vaccination with DCs pulsed with apoptotic tumor cells is superior to that with DCs pulsed with tumor cell lysates to promote therapeutic antitumor immunity although DCs pulsed with tumor cell lysates are commonly used in phase I trials of DC-based cancer immunotherapy (5-7,13,14).

In the present study, we show that: i) immature DCs derived from murine bone marrow cells efficiently phagocytosed apoptotic hepatoma cells as reported previously (15-18,22), ii) vaccination with DCs pulsed with apoptotic hepatoma cells but not vaccination with DCs pulsed with hepatoma cell lysates induced protective antitumor immunity in three murine hepatoma models, iii) spleen cells from mice vaccinated with DCs pulsed with apoptotic hepatoma cells showed higher cytolytic activity and contained higher number of IFN-γ producing cells than those from mice vaccinated with DCs pulsed with hepatoma cell lysates, and moreover, iv) vaccination with DCs pulsed with apoptotic hepatoma cells but not hepatoma cell lysates significantly repressed the growth of pre-established hepatoma. These results indicate that vaccination with DCs pulsed with apoptotic tumor cells elicits more effective antitumor immunity than vaccination with DCs pulsed with tumor cell lysates in murine hepatoma models.

After capturing antigens from apoptotic cells, DCs start to mature (6,8). Matured DCs not only process antigens and present it to MHC molecules but also produce cytokines and chemokines and express costimulatory molecules, which further enhance cellular immunity (8). Therefore, maturation status of DCs could influence the efficacy of DC-based immunotherapy. Recently, it has been reported that heat-stressed apoptotic tumor cells express heat-shock proteins (HSPs), which act as danger signals, stimulate DCs maturation and elicit tumor-specific immunity (26,27). UV, γ-irradiation and certain chemotherapeutic agents are also known to induce HSPs in tumor cells (28-30). In addition, damaged DNA itself in leukemia cells, induced by alkylating agents but not by other chemotherapeutic agents, has been reported to stimulate DCs maturation and antigen presentation (31). Taken together, it is possible that apoptotic stimuli of tumor cells influence the maturation status of DCs uptaked tumor cell-derived antigens. In our study, irradiation or sulindac which is reported to induce apoptosis in human hepatoma cells (32) induced apoptosis in mouse hepatoma cells, and vaccination with apoptotic cell-pulsed DCs induced significant cytolytic activity against parental hepatoma cells. Therefore, loading of irradiated or sulindac-treated hepatoma cells could stimulate DCs maturation. In fact, after phagocytosis of irradiated hepatoma cells, expression of CD86 which is one of the maturation phenotypes of DCs was upregulated. DC maturation is also achieved by several internal stimuli such as TNF-α, IL-1ß, prostaglandins, interferons, and CD40 ligand and by foreign materials such as lipopolysaccharide, dsRNA, CpG-oligonucleotides via Toll-like receptors (6). Accordingly, we also examined the effects of poly (I:C) (dsRNA) on DC maturation and antitumor immunity in pre-established hepatoma models. Addition of poly (I:C) further enhanced the expression of CD86 in DCs, which was stimulated by phagocytosis of apoptotic cells. Vaccination with DCs both pulsed with apoptotic cells and treated with poly (I:C) showed the highest antitumor effect. These results suggest that maturation stimuli of DCs play a key role in DC-based cancer immunotherapy.

Recently, it has been reported that the combined intra-tumor injection of immature DCs and systemic chemotherapy or γ-irradiation induces effective antitumor response in

tumor-bearing mice (33-36). These are possible strategies to treat unresectable cancer since several studies including ours indicate that immature DCs efficiently phagocytose apoptotic tumor cells (15-18) and certain chemotherapeutic agents and γ-irradiation not only induce apoptosis but also stimulate the production of danger signals which stimulate DCs maturation (28-31).

In conclusion, we have shown that DCs pulsed with apoptotic hepatoma cells elicit effective antitumor immunity *in vivo*, which is further enhanced by addition of a DC maturing stimulus. Our results indicate that combination of apoptotic cells as TAAs and a DC maturation signal may be a promising strategy for DC-based immunotherapy for HCC.

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• *CASE REPORT* •

An autopsy case of acute pancreatitis with a high serum IgG4 complicated by amyloidosis and rheumatoid arthritis

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Abstract

We report an autopsy case of acute pancreatitis with a high serum IgG4 concentration complicated by systemic amyloid A amyloidosis and rheumatoid arthritis (RA). The patient was a 42-year-old Japanese female with a 22-year history of rheumatoid arthritis. She was diagnosed with myasthenia gravis when she was 31-year old. At the onset of pancreatitis, the patient was anti-nuclear antibody-positive, and had high serum gamma globulin and IgG4 levels. Dexamethasone and conventional therapy induced clinical remission and significantly decreased the serum IgG4 and gamma globulin. However, despite the decreased disease parameters, the patient developed a bleeding pseudocyst and died of cardiac failure. In the autopsy examination, it was determined that pancreatitis was probably caused by ischemia due to vascular obstruction caused by amyloid deposition in the pancreas. Even though acute pancreatitis is a rare complication in RA patients, we speculate that an autoimmune pancreatitis-related mechanism and ischemia due to vascular obstruction by amyloid deposition might be attributable to a single source that leads to acute pancreatitis in our particular case.

Key words: Acute pancreatitis; Rheumatoid arthritis; IgG4; Systemic amyloidosis; Autoimmune pancreatitis

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INTRODUCTION

Acute pancreatitis is a relatively mild disease and has a low-associated morbidity. However, 4-7% of acute pancreatitis patients suffer from a severe illness that has been associated with mortality rates approaching 20-50%[1]. Etiologies of acute pancreatitis are bile duct stones, alcohol abuse, various toxins, drugs, other obstructive causes, metabolic abnormality, trauma, ischemia, infection, autoimmune disease and idiopathic causes[2]. In previous reports, pancreatitis occurring simultaneously with rheumatoid arthritis (RA) has been associated with Sjögren's syndrome (SjS)[3], anti-RA drugs[4], and amyloidosis[5]. It has been reported that a patient who developed amyloidosis following ankylosing spondylitis died of pancreatitis[6]. Occasionally, autoimmune disease is complicated by pancreatitis, which has led to the concept of an autoimmune-related pancreatitis and autoimmune pancreatitis has been correlated with other autoimmune diseases such as SjS, primary sclerosing cholangitis, ulcerative colitis, systemic lupus erythematosus[7] and myasthenia gravis[8]. Patients with autoimmune pancreatitis/sclerosing pancreatitis have several characteristic autoantibodies[7], and high serum gamma globulin and IgG4 concentrations[9]. In this paper, we report a rare case of acute pancreatitis in a patient with RA, who had a high serum IgG4 concentration and a condition complicated by systemic AA amyloidosis.

CASE REPORT

The patient was a 42-year-old Japanese female with a 22-year history of RA. In 1989, she was diagnosed with myasthenia gravis. In 1992, a stomach mucosal biopsy was carried out and amyloid deposition was confirmed. In December 1998, she had occasional epigastralgia after food intake. She had no history of habitual alcohol consumption and she had taken low-dose oral prednisolone (7.5 mg/d).

The patient presented with clinical pancreatitis with severe epigastralgia, back pain, nausea and vomiting and she was admitted to our hospital on January 7, 1999. On admission, she had bilateral joint deformity of the elbow, wrist, MP, PIP and DIP joints but no arthralgia and myasthenia. She had a fever of 38 ℃, tenderness of the epigastrium region and diminished bowel sound. Laboratory data showed the following values: 8 058 IU/L serum amylase, 1 800 ng/dL serum elastase 1, 1.946 g/dL gamma globulin, 1/160 ANA and 156 mg/dL IgG4. Ultrasound (US) examination and abdominal computed tomography (CT) scan revealed a slightly thickened gallbladder wall with a small stone and without biliary duct dilatation, and mild swelling of the pancreatic head consistent with effusion of the peripancreatic space. A left pleural effusion was identified by chest x-ray. Treatment for pancreatitis with an intravenous protease inhibitor, antibiotics and intramuscular dexamethasone

(2 mg/d) was commenced on admission. On the 9th hospital day, the patient's condition became complicated with congestive heart failure and bacterial pneumonia. From a cardiac US examination, we suspected a case of cardiac amyloidosis. On the 25th d, abdominal pseudocysts were found in hilum of the spleen and mesentery of the transverse colon by abdominal CT (Figure 1). The severity of abdominal symptoms was decreased by the treatment for pancreatitis and additionally, serum IgG4 and gamma globulin concentrations were decreased (31 mg/dL and 0.665 g/dL, respectively) on the 67th d. However, serum amylasemia continued to be severe, and we tried a new therapy using an intrasubcutaneous somatostatin analogue on the 75th d. Following this treatment, the serum amylase level gradually decreased and the patient started to eat on the 119th d.

On the 103rd d, hemorrhage occurred in a pseudocyst of the anterior spleen region. The patient complained of epigastralgia, but the hemorrhage was diminished by conservative treatment. However, re-bleeding in the same pseudocyst occurred on the 150th d, and anemia and hemorrhagic shock developed. The patient's condition recovered slightly following blood transfusion, but she then developed congestive heart failure. Several treatments for heart failure did not seem to be effective and the patient developed massive fresh blood stools and died on the 170th hospital day. We were permitted to perform an autopsy.

The autopsy findings were as follows: A marked amyloid deposition (AA type) was found mainly in the vascular wall with stenotic and obstructive changes, and in stroma of the heart, kidney, gastro-intestinal tract, liver, skin, pancreas and other organs. Pancreatic pseudocysts of 5 and 10 cm in diameter retaining blood and necrotic tissue were present in the mesentery and peritonitis with yellowish turbid ascites was identified in the mesentery and retroperitonea. In the pancreas, extensive fibrosis with lymphocyte infiltration, atrophy of acinar cells, dense pancreatic juice retention and dilatation of the pancreatic duct were observed. The small arteries and arterioles in the pancreas were obstructed and narrowed by marked amyloid deposition on the artery walls (Figure 2). There was no inflammatory cell infiltration in the common bile duct of the pancreas. Heart enlargement (500 g) occurred and the right and left ventricle extension was most unsatisfactory. Histopathological examination revealed diffuse amyloid deposition in myocardium and vascular wall, and an intramural thrombus in the right atrium. Dense amyloid deposition in the stroma and vascular wall was observed throughout the gastrointestinal tract. Multiple ulceration of the rectum reached the muscle layer. These results confirmed cardiac failure with systemic amyloidosis as the cause of death of the patient.

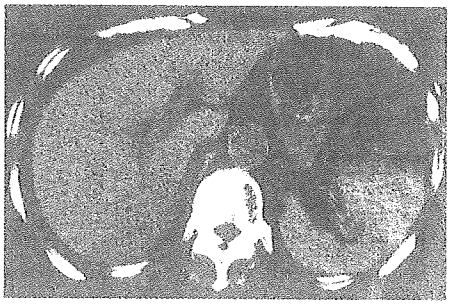

Figure 1 Enhanced abdominal CT shows the presence of pseudocysts.

DISCUSSION

We have reported an autopsy case of necrotizing pancreatitis in a RA patient. In a postmortem histopathological examination, amyloid deposition was observed on walls of the small arteries and arterioles in the pancreas. Because we did not observe inflammation in the pancreatic common bile duct, the etiology of acute pancreatitis was not bile duct stones. Several cases of pancreatitis in RA patients have previously been reported[5,10], and in one of these cases, amyloidosis was found. Oishi *et al.*[5], described that fatal pancreatitis is associated with systemic amyloidosis in RA patients. In their report, a pancreatic biopsy is performed and AA amyloid deposition is observed on the vascular wall of pancreatic arteries and arterioles. It was speculated that the impairment of microcirculation of the pancreatic vasculature reduces the blood perfusion and causes more rapid deterioration, compared to the natural course of acute pancreatitis[5]. However, acute pancreatitis is a rare complication in RA patients, and we strongly suggest that pancreatitis is likely to be caused by ischemia due to vascular obstruction by amyloid deposition in the pancreas, which is brought about by RA-induced secondary AA amyloidosis.

Occasionally, autoimmune disease has been reported to be complicated by pancreatitis, which has led to the concept of an autoimmune-related pancreatitis[7]. In our case, some of the clinical characteristics (presence of an autoantibody, increased levels of gamma globulin and IgG4, effectiveness of steroid therapy and association with another autoimmune

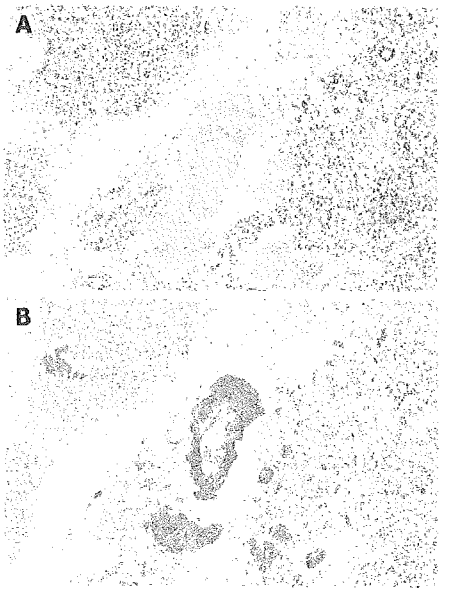

Figure 2 Histological findings of pancreas stained with hematoxylin and eosin (A) and Congo red (B).

disease, myasthenia gravis) correspond to autoimmune pancreatitis. Hamano *et al*.[9], reported that a high serum IgG4 concentration provides a useful means of distinguishing autoimmune pancreatitis/sclerosing pancreatitis from other diseases of the pancreas and biliary tract. In our case, dexamethasone and conventional therapy induced clinical remission and significantly decreased the serum IgG4 and gamma globulin levels. These results indicate that acute pancreatitis might be triggered by the same mechanism of autoimmune pancreatitis that was present in our case. However, acute pancreatitis and pancreatic cysts are unusual findings in autoimmune pancreatitis[7] and autopsy examination does not definitely indicate autoimmune pancreatitis. Hence, we speculate that an autoimmune pancreatitis-related mechanism may exist, which might be attributable to a single source that can also lead to acute pancreatitis.

In summary, acute pancreatitis is a rare complication in RA patients, but RA patients with systemic AA amyloidosis can develop acute pancreatitis from ischemia due to vascular obstruction by amyloid deposition. Such cases might be considered to be autoimmune-related pancreatitis because of the presence of autoantibodies, a high serum IgG4 concentration and an association with other autoimmune diseases.

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Cytokine gene polymorphisms in Japanese patients with hepatitis B virus infection—association between TGF-β1 polymorphisms and hepatocellular carcinoma

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***Background/Aims*: In this study, we determined the frequencies of the genotypes associated with the polymorphism of the cytokines genes, and investigated their association with the risk of hepatocellular carcinoma (HCC) in hepatitis B virus (HBV) carriers.**

***Methods*: Genetic polymorphism in the cytokines TNF-α, IFN-γ, TGF-β1, IL-6, and IL-10 were studied in 236 Japanese patients with HBV infection. The genetic polymorphisms of these cytokines were analyzed by polymerase chain reaction-sequence-specific primer (SSP).**

***Results*: There was no statistically significant difference in the genetic polymorphisms of TNF-α, IFN-γ, and IL-10 genes between HBV carriers with HCC and those without HCC. However, the TGF-β1+29 (codon 10) C/C genotype was lower in HBV carriers with HCC than in those without HCC (HCC 14.6% vs non-HCC 31.9%). The association of HCC was significantly lower in HBV carriers with C/C genotype than in those with T/C or T/T genotype in position + 29 of the TGF-β1 gene.**

***Conclusions*: Our findings suggest that the genetic polymorphism in codon 10 of the TGF-β1 gene may play a role in HCC development in patients with chronic HBV infection.**

***Keywords*: Cytokines; Hepatitis B virus; Hepatocellular carcinoma; Polymorphism; Transforming growth factor-β1**

1. Introduction

Hepatitis B virus (HBV) infection is a major cause of progressive liver disease such as chronic hepatitis and liver cirrhosis in most industrialized countries [1]. An association between HBV infection and hepatocellular carcinoma (HCC) has also been established [2]. The factors involved in the progression of chronic HBV infection to HCC require investigation. The risk for developing HCC increases with severity of inflammation and fibrosis [3]. However, the host genetic factors that affect the HCC association remain unclear. A strong genetic component determining the outcome of HBV infection has been suggested in family studies [4]. Cytokines, as the product of host responses to inflammation, play an important role in the defense against viral infections and carcinogenesis [5]. An individuals' capacity for cytokine production has a major genetic component, and the variation among individuals can be striking [6,7]. This variation has been considered to be associated with polymorphisms within the promoter lesion or signal sequence of cytokine genes [8]. For example, the promoter of the IL-10 gene contains three biallelic polymorphisms at positions −1082, −819, and −592 from the transcription start site, and these influence the capacity to produce IL-10 [9]. These genetic polymorphisms may

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affect the development of inflammation, fibrosis, and carcinogenesis. The aim of the present study was to characterize cytokine gene polymorphisms in chronic HBV infection and their associations with HCC in a Japanese population.

2. Patients and methods

2.1. Patients

Of consecutive Japanese patients with chronic HBV infection who consulted the outpatient clinic of the National Nagasaki Medical Center and Nagasaki University Hospital between 2000 and 2004, we studied 236 patients. They were regularly followed with measurements of serum ALT and HBV markers such as HBsAg, HBeAg, and anti-HBeAb using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan) every month, and ultrasonography or computed tomography of the liver every 3 months. All patients were positive for HBsAg and did not have any other types of liver diseases such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases, or metabolic liver diseases. Total 236 patients were divided into two groups; patients with HCC and without HCC. The following clinical parameters of patients were obtained at the time of whole blood collection; age, gender, serum alanine aminotransferase (ALT) levels, and platelet counts. The diagnosis of HCC was made by several imaging modalities and confirmed histologically by sonography-guided fine-needle biopsy specimens in all patients.

The study protocol was approved by the Ethics Committees of both National Nagasaki Medical Center and Nagasaki University Hospital and informed consent was obtained from each individual.

2.2. DNA extraction

Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol according to the manufacturer's instruction (Qiagen Ltd, UK).

2.3. PCR sequence-specific primer typing

Single nucleotide mutations were analyzed in five different cytokines, leading to the genotype and phenotype assignment (Table 1).

In PCR sequence-specific primer (SSP) typing, oligonucleotide primers were designed to obtain amplification of specific alleles or groups of alleles. This typing method is based on the principle that a completely matched primer will be used more efficiently in the PCR reaction than with a primer with one or more mismatches. This means that the specificity of the typing system is a part of the PCR reaction. Assignment of alleles is then based on the presence or absence of amplified product detected by agarose gel electrophoresis. In this study, PCR amplification of selected TNF-α, TGF-β1, IL-10, IL-6, and IFN-γ alleles and an internal control, the human β-globulin gene were carried out according to the manufacturer's instruction (One Lambda, VH Bio Ltd, Gateshead, Tyne and Wear, UK). Briefly, after addition of the appropriate primer pairs, salts, buffer, and Taq polymerase, the samples were subjected to PCR in a 9600 Perkin-Elmer Thermocycler. The sequences of the primer pair have been described previously [9]. Amplification conditions were 1 cycle of 130 s at 96 °C dropping at 62 °C for an additional 60 s, nine cycles of 10 s at 96 °C and 60 s at 63 °C, and then the final 20 cycles included a three-temperature ramp annealing for 10 s at 96 °C, 50 s at 59 °C and finally 30 s at 72 °C. The amplified products were then separated by agarose gel electrophoresis and visualized by staining with ethidium bromide and exposure to ultraviolet light. Interpretation of PCR results was based on the presence of the internal control band together with the presence or absence of a specific amplified fragment.

2.4. Statistical analysis

Results are expressed as mean ± SD. Comparisons were made by Student's t test, Fisher's extract probability test, and the χ^2 test. All P values were two-tailed, and P values <0.05 were considered to indicate statistical significance.

3. Results

3.1. Patient characteristics

As shown in Table 2, there was no significant difference in gender and serum ALT levels between HBV carriers with HCC and those without HCC. In HBV carriers with HCC, age and proportion of cirrhosis were higher and platelet counts were lower than those without HCC.

Table 1
Characteristics of the cytokine gene polymorphisms

Gene	Posltion of the polymorphism	Allele	Haplotype	Phenotype	Reference
TNF-α	Promoter −308	A,G	A/A	High	[9]
			G/A	High	
			G/G	Low	
TGF-β	Codon 25	C,G	G/G	High	[9,10,11]
			G/C	Intermediate	
			C/C	Low	
IL-10	Promoters −1082; −819 and −592	A,G;T,C and A,C	GCC/GCC	High	[9]
			GCC/ACC	Intermediated	
			GCC/ATA	Intermediate	
			ACC/ACC	Low	
			ACC/ATA	Low	
			ATA/ATA	Low	
IL-6	Promoter −174	C,G	G/G	High	[12]
			G/C	High	
			C/C	Low	
IFN-γ	Intron +874	T,A	T/T	High	[9]
			T/A	Intermediate	
			A/A	Low	

TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IL-10, interleukin-10;IL-6, interleukin-6; INF-γ, interferon-γ.

Table 2
Baseline characteristics of HBV carriers

	Total HBV (n=236)	Without HCC (n=188)	With HCC (n=48)	Statistical analysis
Mean age (yr)	53.7±15.2	51.5±15.6	62.5±8.9	$P<0.001$. Student's t-test
Sex (M/F)	160/76	127/67	39/9	NS, χ^2
Mean ALT (U/L)	70.4±104.2	73.6±112.2	57.8±63.8	NS, Student's t-test
Mean PLT (10^3/μl)	158.2±71.8	169.7±63.7	119.2±83.8	$P<0.001$. Student's t-test
Mean albumin (g/dl)	4.3±.6	4.4±0.5	3.6±0.6	$P<0.001$. Student's t-test
Cirrhosis (%)	45.8	34.6	91.7	$P<0.001$. χ^2

HCC, hepatocellular carcinoma; ALT, alanine aminotransferse; PLT, platelet; NS, not significant.

3.2. Polymorphism in the TNF-α and IFN-γ genes

There was no polymorphism at the position +74 (codon 25) of the TGF-β1 gene and the position of -174 of the IL-6 gene in Japanese population studied. In the genotype frequencies at position −308 of the TNF-α genes and position of +874 of the IFN-γ gene, no statistically significant difference was found between HBV carriers with HCC and those without HCC (Table 3).

3.3. IL-10 gene promoter polymorphisms in hepatitis B virus carriers

We examined the three biallelic polymorphisms in the IL-10 gene promoter, at positions −1082, −819, and −592 from the transcription start site, respectively, which produce three different haplotypes: GCC, ACC, and ATA. The genotype frequencies are shown in Table 3, and the haplotype frequencies in Table 4. The frequencies of the ACC haplotype were increased in HBV carriers with HCC (33.3%) compared to those without HCC (30.3%), though the difference was not statistically significant.

3.4. TGF-β1 gene codon 10 polymorphisms

The distributions of the genotype of the polymorphism at the position +29 (codon 10) in HBV carriers with or without HCC are shown in Table 3. The genotype distributions were different in HBV carriers with HCC and those without HCC. In HBV carriers without HCC, the genotype frequencies were 18.6% for T/T, 49.5% for T/C, and 31.9% for C/C. In HBV carriers with HCC, on the other hand, the genotype frequencies were 22.9% for T/T, 62.5% for T/C, and 14.6% for C/C. As shown in Table 5, the association with HCC was significantly lower in HBV carriers with C/C genotype than in those with T/T or T/C genotype (P=0.028, odds=0.36, 95% CI; 0.154–0.859). Associations between cytokine gene polymorphism and the development of virus hepatitis were also reported. Ben-Air et al. reported that A/A genotype in the position +874 of IFN-γ gene was associated the development of HBV infection [13]. Gewaltig et al., reported that the presence of proline at TGF-β1gene codon 10 (C/C or C/T genotype) was associated with higher stage of fibrosis in HCV-infected patients [11]. Therefore, we assessed the genetic polymorphism of TGF-β1gene codon10 and the position +874 of IFN-γ gene in HBV carriers with or without liver cirrhosis (LC). As shown in Table 6 and 7, there was no significant association between these genes polymorphisms and the presence of LC.

Table 3
Genotype frequencies in HBV carriers

Variables	Patients with HBV		
	Total (n=236) (%)	Without HCC (n=188) (%)	With HCC (n=48) (%)
TNF-α			
G/G	230(97.5)	183(97.3)	47(97.9)
G/A	6(2.5)	5(2.7)	1(2.1)
A/A	0	0	0
TGF-β1-10			
C/C	67(28.4)	60(31.9)	7(14.6)
T/C	123(52.1)	93(49.5)	30(62.5)
T/T	46(19.5)	35(18.6)	11(22.9)
TGF-β1-25			
G/G	236(100)	188(100)	48(100)
G/C	0	0	0
C/C	0	0	0
IL-10			
ATA/ATA	102(43.2)	85(45.2)	17(35.4)
ACC/ATA	91(38.6)	72(38.3)	19(39.6)
ACC/ACC	25(10.6)	19(10.1)	6(12.5)
GCC/ATA	10(4.2)	6(3.2)	4(8.3)
GCC/ACC	5(2.1)	4(2.1)	1(2.1)
GCC/GCC	3(1.3)	2(1.1)	1(2.1)
IL-6			
G/G	236(100)	188(100)	48(100)
G/C	0	0	0
C/C	0	0	0
IFN-γ			
A/A	198(83.9)	157(83.5)	41(85.4)
T/A	38(16.1)	31(16.5)	7(14.6)
T/T	0	0	0

Note. The genotypes are shown as frequency (percentage). Abbreviations: HBV; hepatitis B virus, HCC; hepatocellular carcinoma, TNF-α; tumor necrosis factor-α, TGF-β; transforming growth factor-β, IL-6; Interleukin-6, IL-10; interleukin-10, IFN-γ; interferon-γ.

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-rP$_{102-111}$ or PTH-rP$_{110-119}$ 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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Key words: parathyroid hormone-related protein, immunotherapy, peptide, cytotoxic T lymphocytes, HLA-A24

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 *ß-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 ($1x10^5$ 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 ($2x10^3$ cells/well) were cultured with CTLs ($4x10^4$ cells/well) in 96 round-well plates with $2x10^4$ 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*

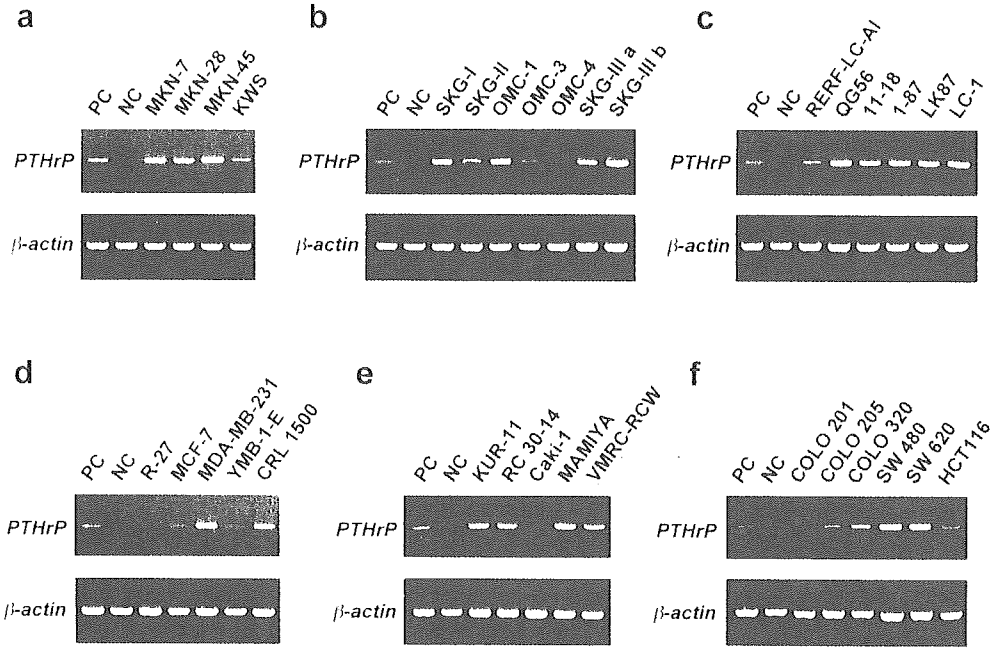

Figure 1. Expression of *PTH-rP* mRNA in a variety of cancer cell lines. *PTH-rP* mRNA expression in the following cancer cell lines was examined by semi-quantitative RT-PCR analysis: gastric (a), cervical (b), lung (c), breast (d), renal (e), and colon (f) cancer cell lines. PC, positive control; LNCaP (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

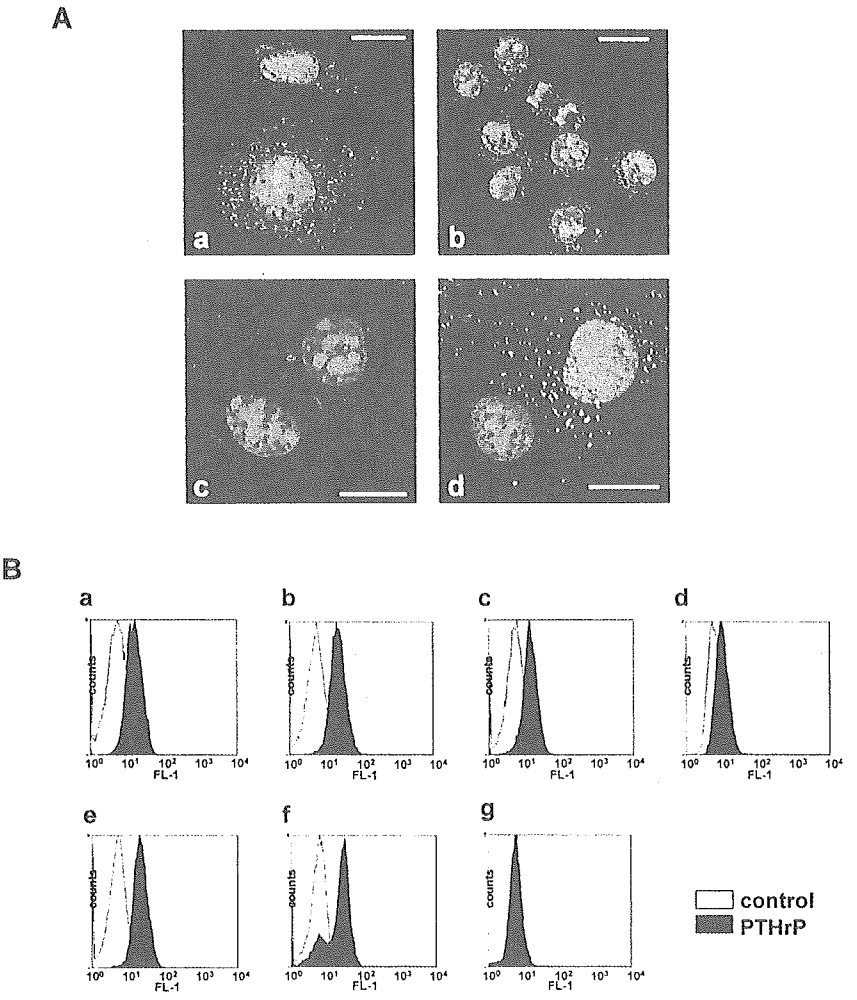

Figure 2. Expression of PTH-rP in a variety of cancer cell lines. (A) Subcellular distribution of endogenous PTH-rP (green) in SKG-I (a, cervical), QG56 (b, lung), R-27 (c, breast), and MAMIYA (d, renal) cells was determined by indirect immunofluorescence staining with the anti-PTH-rP polyclonal antibody, 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 1) 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-$rP_{110-119}$ 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-$rP_{102-111}$ and the PTH-$rP_{110-119}$ peptide-stimulated $CD8^+$ T cells showed higher levels of cytotoxicity against the PTH-rP^+/HLA-$A24^+$ cancer cells (KUR-11, MKN-45, COLO320, and SKG-I) than against the PTH-rP^+/HLA-$A24^-$ tumor cells (RC30-14, MKN-28, COLO205, and OMC-1) and against the PTH-rP^-/HLA-$A24^+$ PHA-induced T-cell blasts (Fig. 3). In addition, cytotoxicity against the PTH-rP^+/HLA-$A24^+$ 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	67	122	139	110
#2	86	0	43	1588
#3	3	33	167	199
#4	20	226	318	648
#5	0	0	58	79
Total	2/5	2/5	4/5	5/5
Gastric cancer				
#6	6	25	22	74
#7	0	378	439	23
#8	32	78	6	0
#9	78	3	23	4011
#10	525	0	0	25
Total	2/5	2/5	1/5	2/5
Colon cancer				
#11	11	92	6	27
#12	184	44	11	163
#13	7	81	232	0
#14	25	25	670	22
Total	1/4	2/4	2/4	1/4
Cervical cancer				
#15	160	71	292	27
#16	0	88	0	0
#17	153	82	432	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

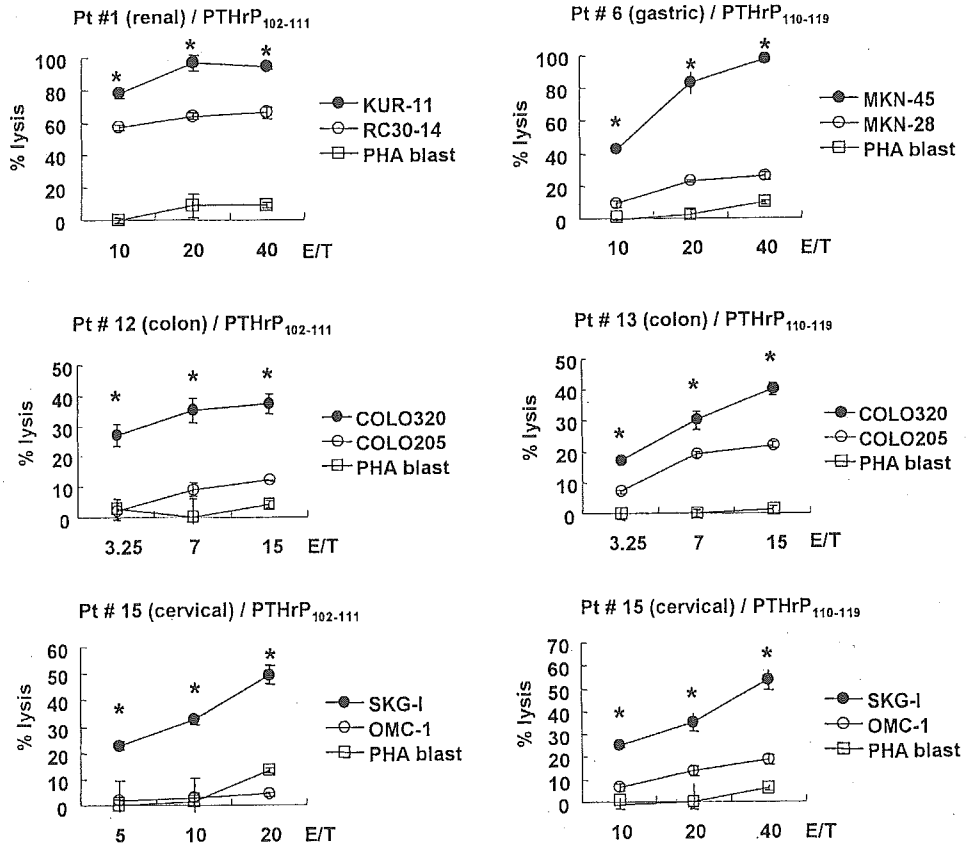

Figure 3. Induction of cancer-reactive CTLs from the PBMCs of patients with a variety of cancer types. PBMCs from five HLA-A24$^+$ cancer patients (Pt #1, renal cancer; Pt #6, gastric cancer; Pt #12, colon cancer; Pt #13, colon cancer; and Pt #15, cervical cancer) were stimulated *in vitro* with the 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 anti-cancer 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.

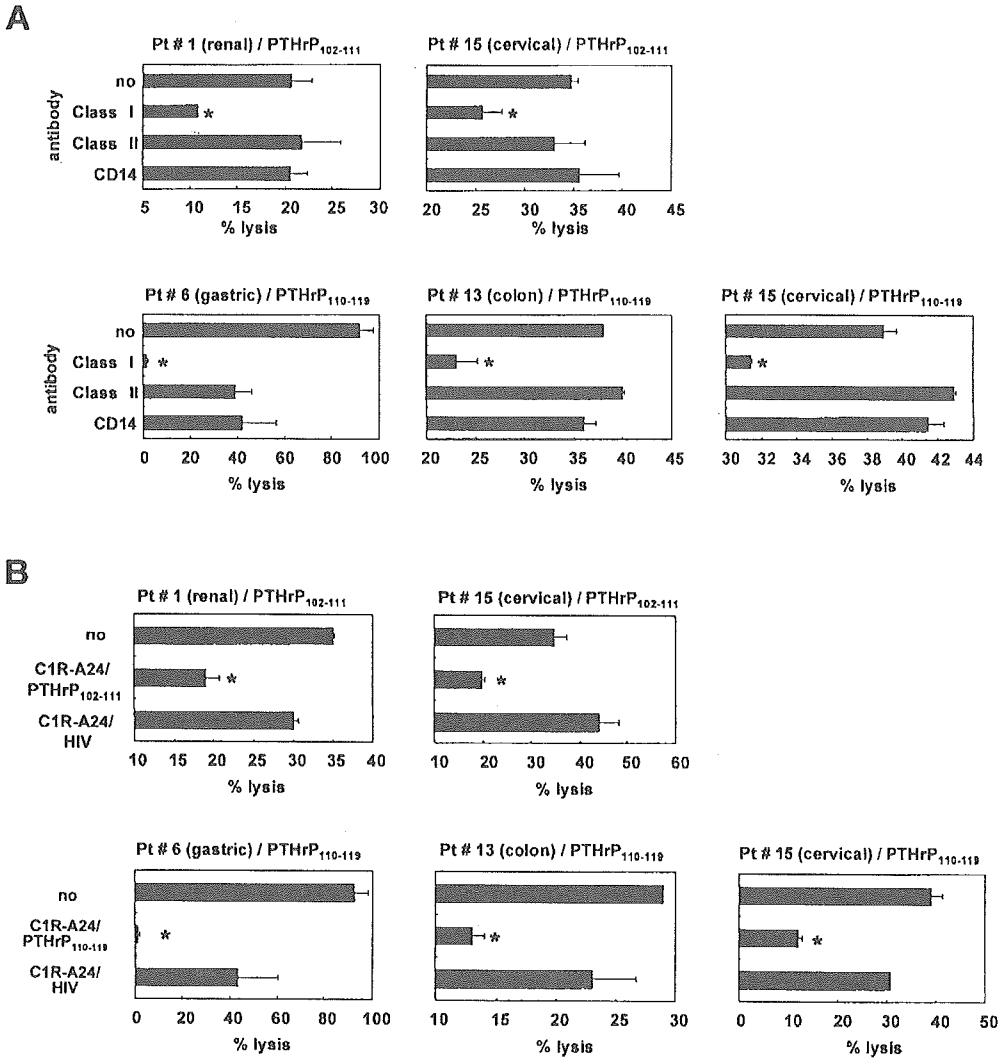

Figure 4. CD8+ T cell-dependent and PTH-rP peptide-specific cytotoxicity against PTH-rP+/HLA-A24+ cancer cells. (A) Purified CD8+ T cells from the PTH-rP peptide-stimulated PBMCs were examined for their cytotoxicity against PTH-rP+/HLA-A24+ tumor cell lines with or without anti-HLA class I, anti-HLA 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.

Acknowledgments

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