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# Serine Proteinase Inhibitor 9 Can Be Recognized by Cytotoxic T Lymphocytes of Epithelial Cancer Patients

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**Serine proteinase inhibitor 9 (PI-9) inhibits granzyme B-mediated apoptosis and interleukin-1β-converting enzyme activity. In this study, we report that the *PI-9* gene encodes antigenic epitopes recognized by the HLA-A24-restricted and tumor-reactive cytotoxic T lymphocytes (CTLs) of epithelial cancer patients. Screening of an autologous cDNA library using a CTL line recognizing HLA-A24$^+$ tumor cells resulted in the isolation of a cDNA, which had an identical coding region to the previously described *PI-9* genes. *PI-9* gene was expressed in approximately three-fourths of epithelial cancer cell lines and all leukemic cell lines tested. It was also expressed in normal peripheral blood mononuclear cells (PBMCs), but not in a normal fibroblast cell line. CTL sublines contained T cells capable of recognizing the PI-9$_{292–300}$ and PI-9$_{348–356}$ peptides among 13 different peptides having the HLA-A24 binding motifs. These two peptides were recognized by the CTL line in a dose-dependent and HLA class-I-restricted manner, and also possessed the ability to induce HLA class I-restricted and tumor-reactive CTLs in PBMCs from HLA-A24$^+$ cancer patients. These results demonstrate that PI-9 is recognized by HLA class I-restricted and tumor-reactive CTLs of epithelial cancer patients.**

Key words: CTLs — PI-9 — Serpin — Peptide

Many genes encoding tumor antigens (Ags) recognized by cytotoxic T lymphocytes (CTLs) have recently been identified from melanoma cDNA.[1–8] Tumor Ags from other tumors recognized by CTLs have also been characterized, including HER-2/neu,[9, 10] prostate-specific Ag,[11, 12] mucin,[13] CEA,[14] SART1,[15] SART2,[16] SART3,[17] ART-1,[18] ART-4,[19] and cyclophilin B.[20] These Ags are for the most part not truly tumor-specific Ags, but rather non-mutated self Ags which are expressed in normal cells and tissues. Specific immunotherapy using the antigenic peptides of such Ags is an interesting possibility.

The serine proteinase inhibitors (serpins) regulate a wide variety of physiological processes that involve proteinase activity, including blood coagulation, complement activation, cell migration and differentiation, intracellular proteolysis, and tumor suppression.[21] Proteinase inhibitor 9 (PI-9) is a serpin and a potent inhibitor of the proteinase granzyme B.[22, 23] CTLs and natural killer cells use perforin and granzyme B-containing granules to destroy target cells,[24] and PI-9 is reported to inhibit apoptosis that is mediated by either granzyme B or perforin.[25] In this study, we report that the *PI-9* gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-reactive CTLs of epithelial cancer patients. Two PI-9 peptides were revealed to possess the ability to induce HLA class I-restricted and tumor-reactive CTLs in HLA-A24$^+$ cancer patients. These results demonstrate that PI-9 is an Ag recognized by CTLs of epithelial cancer patients.

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## MATERIALS AND METHODS

**Tumor cell lines and tissue samples** Tumor cell lines used in this study were 15 esophageal cancers (KE3, KE4, KE6, KE9T, KE9LN, TE-8, TE-9, TE-10, TE-11, YES-1, YES-2, YES-3, YES-4, YES-5 and YES-6), 8 colon cancers (COLO201, COLO205, COLO320, SW480, SW620, HCT116, KM12LM and WIDR), 1 lung cancer (QG-56), 9 gastric cancers (MKN-45, MKN-28, MKN-7, KWS, KATO-III, SSTW-9, AZ-521, SH-10-TC and NS-8), and 5 leukemias (RAJI, DAUDI, JURKAT, HPB-ALL, and K562). Histology and HLA-class I genotyping of the majority of these tumor cell lines were reported elsewhere.[15–20]

**HLA-A2402-restricted and tumor-reactive CTLs** An HLA-A2402-restricted and tumor-reactive CTL line designated as KE4-CTLs was established from an esophageal cancer patient (HLA-A2402/2601). The detailed characterization of this cell line has been described elsewhere.[16, 26] From the parental KE4-CTL line, many sublines and clones were established by limiting dilution, and were used in this study.

**Identification of the *PI-9* gene** The KE4-CTL line was used to identify tumor Ags. An expression-gene cloning

method was applied to identify tumor-rejection Ag genes; the detailed protocol has been described elsewhere.[15] In brief, poly(A)$^+$ RNA of the autologous KE4 tumor cells was converted to cDNA, ligated to the *Sal*I adapter, and inserted into the expression vector pSV-SPORT-1 (GIBCO BRL, Gaithersberg, MD). The cDNAs of *HLA-A2402* and *HLA-A0201* were obtained by reverse transcription-PCR, and were cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). Two hundred nanograms of cDNA library and 200 ng of the *HLA-A2402* cDNA were both mixed with 1 $\mu$l of lipofectin in 70 $\mu$l of OPTI-MEM (GIBCO BRL) for 15 min. Fifty microliters of the mixture was then added to the VA13 ($1\times10^4$) cells and incubation was conducted for 5 h. Thereafter, 200 $\mu$l of the RPMI-1640 medium containing 10% FCS was added and culture was continued for 2 days, followed by the addition of the CTLs ($1\times10^4$ cells/well). After 18 h culture, 100 $\mu$l of supernatant was collected to measure IFN-$\gamma$ by ELISA in a duplicate assay, as previously reported.[15] DNA sequencing was performed by a dideoxynucleotide sequencing method using a DNA Sequence Kit (Perkin-Elmer Corp., Foster, CA). The sequence was analyzed by the "ABI PRISM" 377 DNA Sequencer (Perkin-Elmer).

**Northern blot analysis** RNA expression of the *PI-9* gene on various tumor cell lines and normal cells was investigated by northern blot analysis with a $^{32}$P-labeled *PI-9* probe according to previously reported methods.[15, 16] Twenty micrograms of total RNA from each sample was subjected to electrophoresis.

**RT-PCR** RT reactions were conducted using total RNA from cell lines and normal tissues with oligo(dT) primer. PCR reactions were conducted for 30 cycles. Amplification of two different PI-9 transcripts was carried out using either the 5′-CGTGTTCTGTTCTCCTGT-3′ (165S) and 5′-AAGCACCAGGAAATCTAT (1618AS) oligonucleotide primers, or the 5′-CCTGTGTCTGTCCAAGTTCG-3′ (1008S) and the 5′-ACAGAACAGAATGCTGTTGGC-3′ (1193AS) oligonucleotide primers. A control PCR was carried out using the β-actin 5′-CTTCGCGGGCGAC-GATGC-3′ and 5′-CGTACATGGCTGGGGTGTTG-3′ oligonucleotide primers.

**Peptide and binding assay** Thirteen different PI-9-derived peptides were prepared based on the HLA-A24-binding motifs.[27, 28] An HIV-derived peptide (RYPLTFG-WCF) capable of binding to HLA-A2402 molecules was used as a negative control for an ELISA.[29] All peptides were purchased from Research Genetics, Inc. (Huntsville, AL), and the purity was >95%. For the peptide-binding assay, a chimera gene *A2402/K$^b$* encoding the α1 and α2 domains of the HLA-A2402 molecule and the α3 transmembrane and intracellular domains of the H-2K$^b$ molecule was established. Exons 1 to 3 of *HLA-A2402* cDNA and exons 4 to 8 of *H-2K$^b$* cDNA were ligated into pCDNA3.1 (Invitrogen, CH Groningen, Netherlands). Thereafter, this gene was transfected into RMA-S cells (mouse lymphoma cell line).[16, 30] These cells expressed HLA-A2402 molecules, as was confirmed by the detection of anti-HLA-A23/24 mAb (One Lambda, Canoga Park, CA); cells were incubated at 26°C for 18 h. Thereafter, they were suspended with OPTI-MEM (GIBCO BRL) that contained 3 $\mu$g/ml human β2 microglobulin (CORTEX BIOCHEM, San Leandro, CA) and an appropriate concentration of peptide. Cells were then incubated at 26°C for 3 h and then at 37°C for 3 h. After washing, the cells were incubated with anti-HLA-A23/24 mAb, followed by phycoerythrin (PE)-conjugated rabbit anti-mouse IgG Ab (Cappel, Aurora, OH). After washing, the cells were suspended in 0.5 ml of PBS containing 1% formaldehyde, and were analyzed with FACScan (Becton Dickinson, Mountain View, CA). The HIV peptide was used as a control peptide capable of binding to the HLA-A2402 molecules.[29] SART1$_{736-745}$ (KGSGKMKTER), which is capable of binding HLA-A2601 molecules,[15] was used as a negative control peptide. To identify antigenic peptides recognized by the KE4-CTL line, C1R/A2402 cells ($1\times10^4$ cells/well) were pulsed with the indicated peptides at a final concentration of 10 $\mu M$ for 2 h. The KE4-CTLs ($1\times10^4$ cells/well) were then added, and after incubation for 18 h, 100 $\mu$l of the supernatant was collected to measure the level of IFN-$\gamma$ by ELISA in triplicate assays.

**Induction of CTLs by peptides** Two peptides (PI-9$_{292-300}$ and PI-9$_{348-356}$) were tested for the ability to induce HLA-A24-restricted CTLs in peripheral blood mononuclear cells (PBMCs) of cancer patients. Detailed methods for CTL induction with a peptide and establishment of CTL sublines were described elsewhere.[15–20] In brief, PBMCs from cancer patients were incubated with 10 $\mu M$ of the indicated peptides in a 24-well plate. At days 7 and 14 of the culture, they were restimulated with irradiated (30 Gy) autologous PBMCs as Ag-presenting cells that had been preincubated with the corresponding peptide (10 $\mu M$) for 2 h. The cultured cells were harvested at day 21 of the culture and were examined by ELISA for the ability to produce IFN-$\gamma$ in response to target cells. In some of the experiments, PBMCs were cultured using a different protocol. PBMCs ($1.5\times10^5$ cells/well) were incubated with 10 $\mu M$ of the indicated peptide and 200 $\mu$l of culture medium in U-bottom-type well (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% "AIM-V" medium (GIBCO BRL), 10% FCS with 100 U/ml of interleukin-2 (IL-2), and 0.1 $\mu M$ MEM nonessential amino acid solution (GIBCO BRL). Half of the medium was replaced every 2 days for up to 13 days with new medium containing a corresponding peptide (20 $\mu M$). After 6 stimulations, these cells were harvested and then tested for the ability to produce IFN-$\gamma$ in response to C1R/A2402 cells, which had been pulsed

with the indicated peptide. To examine their cytotoxicity, these cells were further expanded with irradiated autologous PBMCs ($2\times10^5$ cells/well) that had been pre-pulsed with a corresponding peptide, and were expanded in culture with IL-2 alone. These CTLs were examined for their responses to either peptides or tumor cell lines at around day 25 of culture. The cytotoxic activity was examined by an 18 h $^{51}$Cr-release assay at around day 28–35. CTL sublines and clones were established from the parental CTL line by incubation of cells at 1 or 10 cells/well in 96-well U-bottom micro-culture plates with the culture medium and IL-2 (100 U/ml) in the presence of irradiated autologous PBMCs as feeder cells. In some of the experiments, 10 $\mu$g/ml of either anti-CD4 (IgG1), anti-CD8 (IgG2a), anti-CD14 (IgG2a), anti-class I (W6/32, IgG2a), or anti-class II (H-DR-1, IgG2a) mAb was added to the culture. A two-tailed Student's *t* test was employed for the statistical analysis.

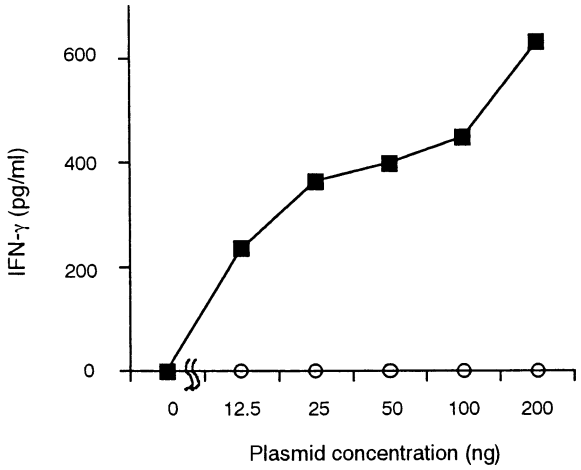


Fig. 1. HLA-2402-restricted recognition of the cDNA clone 21-encoded Ag by the KE4-CTL line. VA13 cells were co-transfected with the indicated doses of the cDNA clone 21 and either *HLA-A2402* (■) or *HLA-0201* (○) cDNA at a dose of 200 ng. Two days after the transfection, the KE4-CTL cells were co-cultured with these transfectants and the level of IFN-γ in the culture supernatants was determined by ELISA. Values represent the mean of triplicate assays. The sequence data of the cDNA clone 21 are available from EMBC/GenBank/DDBJ under Accession #AB060690.

## RESULTS

**Cloning of a cDNA that encodes an Ag recognized by the KE4-CTLs** The KE4-CTL line was established from PBMCs of an esophageal cancer patient (HLA-A2402/2601), and this line was used to identify SART1.[15] Its detailed characterization has been described elsewhere.[15–17, 26] Using this cell line, the identification of additional tumor Ags recognized by CTLs was attempted. A total of $10^5$ clones from a cDNA library of autologous KE4 tumor cells was screened for the ability to stimulate the KE4-CTLs after co-transfection with the *HLA-A2402* cDNA into VA13 cells. A 2792 bp cDNA (clone 21) was found to induce IFN-γ production by the KE4-CTL line only when VA13 cells were co-transfected with the *HLA-A2402* cDNA, but not with the *HLA-A0201* cDNA (Fig. 1).

**Expression of the PI-9 gene** Database searches were then carried out to determine if the cDNA clone 21 was similar to any previously described genes. Four entries with a similar sequence were found (Accession #: NM_Q004155, U71364, XM004390, and L40378) (Fig. 2A). The former 3 genes were registered as the *PI-9* gene, and the last one

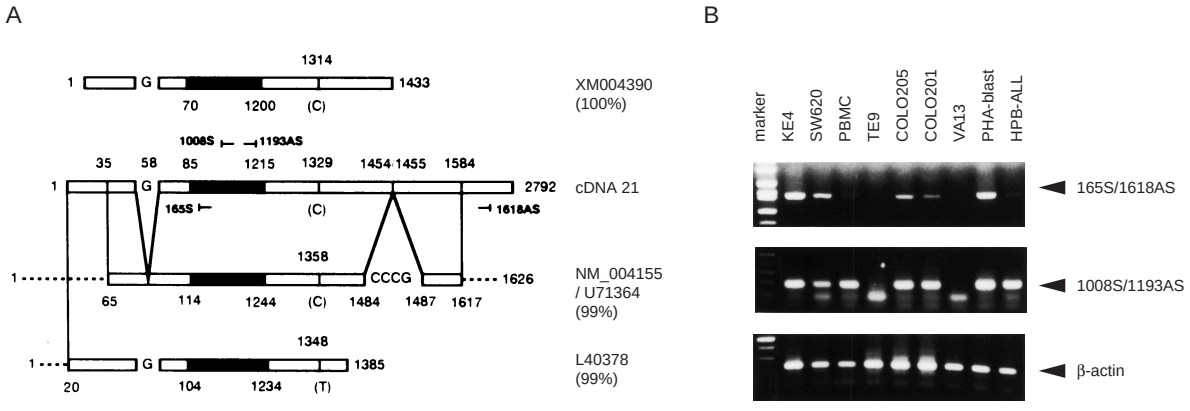


Fig. 2. Comparison of sequences and RT-PCR analysis. (A) Comparison of sequences of the cDNA clone 21 and previously described genes. The black box represents the common coding region. The numbers in the parentheses indicate the homology percentage of sequences in comparison with the cDNA clone 21. Primer pairs used to amplify two different PI-9 transcripts are also shown. (B) RT-PCR analysis on tumor cell lines and normal cells using 2 sets of primer pairs.

Table I. Expression of the *PI-9* Transcripts in Different Tumor Cell Lines and Normal Cells

| Cells | | | *PI-9* transcripts[a)] | |
|---|---|---|---|---|
| Histology | Name | Origin | Primers: 1008S/1193AS | 165S/1618AS |
| Squamous-cell carcinoma | KE3 | Esophagus | + | − |
| | KE4 | Esophagus | + | + |
| | KE5 | Esophagus | + | + |
| | KE6 | Esophagus | − | − |
| | KE9T | Esophagus | + | + |
| | KE9LN | Esophagus | + | + |
| | TE-9 | Esophagus | − | − |
| | TE-10 | Esophagus | + | + |
| | YES-1 | Esophagus | + | − |
| | YES-2 | Esophagus | + | + |
| | YES-3 | Esophagus | + | − |
| | YES-4 | Esophagus | + | − |
| | YES-5 | Esophagus | + | − |
| | YES-6 | Esophagus | − | − |
| | QG-56 | Lung | − (11/15) | − (6/15) |
| Adenocarcinoma | COLO201 | Colon | + | + |
| | COLO205 | Colon | + | + |
| | COLO320 | Colon | − | − |
| | SW620 | Colon | + | + |
| | SW480 | Colon | + | + |
| | HCT116 | Colon | + | + |
| | KM12LM | Colon | + | − |
| | WIDR | Colon | + | − |
| | MKN-45 | Stomach | + | + |
| | MKN-28 | Stomach | + | + |
| | MKN-7 | Stomach | − | − |
| | KWS | Stomach | + | − |
| | KATO-III | Stomach | + | − |
| | SSTW-9 | Stomach | − | − |
| | AZ-521 | Stomach | + | − |
| | SH-10-TC | Stomach | + | + |
| | NS-8 | Stomach | − | − |
| | PANC-1 | Pancreas | + (14/18) | + (9/18) |
| Leukemia | RAJI | B cell | + | + |
| | DAUDI | B cell | + | − |
| | JURKAT | T cell | + | − |
| | HPB-ALL | T cell | + (4/4) | + (2/4) |
| Normal cells | PBMCs | | + | + |
| | PHA-blast | | + | + |
| | VA13 | Fibroblast | − | − |

*a*) Two kinds of *PI-9* transcripts were examined by RT-PCR.

was registered as cytoplasmic antiproteinase 3. The gene (XM004390) consisted of 1433 bp and was 100% identical to the partial sequence of clone 21. Sequences of two genes (NM_Q004155 and U71364) were identical, and their sequence from nucleotide 65 to 1617 was almost the same as that of the cDNA clone 21 nucleotides 35 to 1584, with the exception of a 5 nucleotide difference. The gene (L40378) was the same as that the region from nucleotides 1 to 1366 of the cDNA clone 21, with the exception that C at nucleotide 1348 was substituted with a T. There was no difference in the coding region among these genes, implying that all of them encode a 377 amino acid PI-9 protein. These results indicate that, although half of the C-terminal region of the cDNA clone 21 remains function-

ally unknown, the *PI-9* gene encoded an Ag that was recognized by the KE4-CTL line.

To examine the mRNA expression of PI-9, RT-PCR was carried out. As shown in Fig. 2A, two sets of primer pairs were designed to detect either the transcript that is specific to the common coding region or the transcript that is specific to the cDNA clone 21. A representative result is shown in Fig. 2B. The mRNA expression of PI-9, which could be detected by either the primer pair 165S/1618AS or the primer pair 1008S/1193AS, was observed in all samples tested except TE-9 and VA13. The mRNA expression of PI-9 detected by the primer pair 165S/1618AS in PBMC and HPB-ALL leukemia was faint. Overall, the expression pattern was similar between the 2 sets of primer pairs, but the expression level appears to be higher when examined with the primer pair 1008S/1193AS. The results are summarized in Table I. The PI-9 transcript detected by the primer pair 1008S/1193AS was expressed in 73%, 78%, and 100% of 15 squamous-cell carcinomas, 18 adnocarcinomas, and 4 leukemias, respectively. Among them, some tumor cell lines did not show the PI-9 transcript when the primer pair 165S/1618AS was used. Normal PBMC and phytohemagglutinin (PHA)-blast expressed the PI-9 transcript, but no expression was detected in VA13 fibroblast cells.

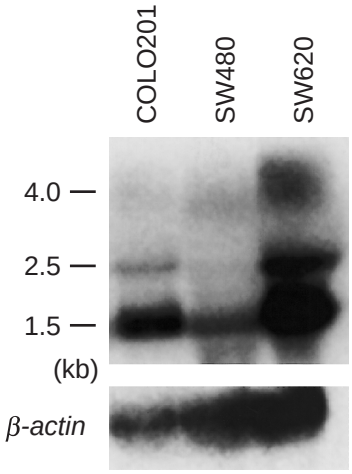


Fig. 3. Northern blot analysis. Three tumor cell lines were analyzed by northern blot analysis using a full length cDNA clone 21 probe. Twenty micrograms of total RNA from each sample was subjected to electrophoresis.

It has been reported that the *PI-9* gene can generate 3 different transcripts (4.0, 2.5, and 1.5 kb) in leukemic cell lines.[22] Therefore, RNA expression of the *PI-9* gene in tumor cell lines was further examined by northern blot analysis using a $^{32}$P-labeled full length cDNA 21 probe. Representative results are shown in Fig. 3. As previously reported, 3 different transcripts (approximately 4.0, 2.5, and 1.5 kb) of the *PI-9* gene were observed in 3 different epithelial tumor cell lines. In all cases, expression of the 1.5 kb transcript was highest among the 3 different transcripts and the expression of the 2.5 or 4.0 kb transcript was relatively low.

**Identification of PI-9 T cell epitopes** To identify epitopes recognized by the KE4-CTLs, 13 different PI-9-derived peptides were prepared based on HLA-A24-binding motifs.[27, 28] We examined whether or not these peptides could be recognized by 2 different KE4-CTL sublines (Fig. 4). It was discovered that the PI-$9_{10-19}$, PI-$9_{292-300}$,

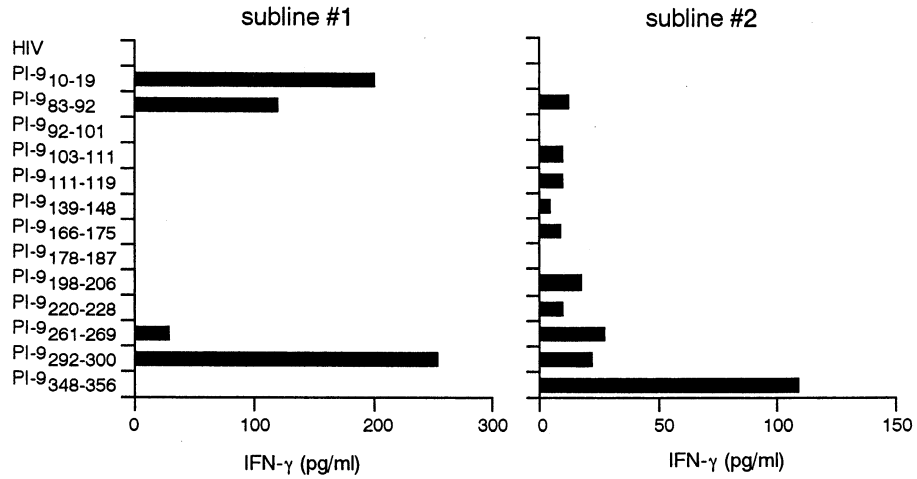


Fig. 4. Recognition of PI-9-derived peptides by KE4-CTL sublines. (A) Thirteen PI-9-derived peptides were loaded onto C1R/A2402 cells at a concentration of 10 $\mu M$ for 2 h, and subsequently co-cultured with the indicated KE4-CTL sublines. As a negative control, the HIV peptide, which has the ability to bind to HLA-A2402 molecules, was used. After 18 h of culture, the level of IFN-γ in the culture supernatant was determined by ELISA. Values represent the mean of triplicate assays.

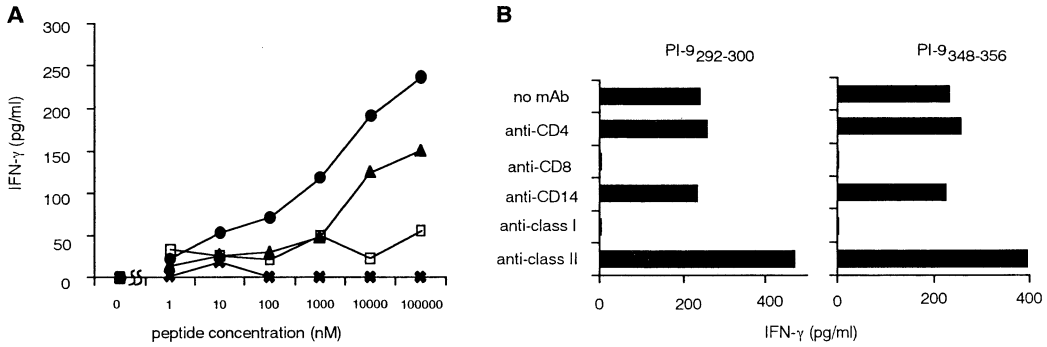


Fig. 5. HLA class-I-restricted recognition of the PI-$9_{292-300}$ and PI-$9_{348-356}$ peptides by the KE4-CTL line. (A) The indicated PI-9 peptides were loaded on C1R/A2402 cells at the indicated doses for 2 h, and subsequently were co-cultured with the parental KE4-CTL line. After 18 h of culture, the level of IFN-γ in the culture supernatant was determined by ELISA. Values represent the mean of triplicate assays. The following peptides were used; ● PI-$9_{348-356}$, ▲ PI-$9_{292-300}$, □ PI-$9_{10-19}$, and × PI-$9_{139-148}$. (B) The PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide was loaded on C1R/A2402 cells at a concentration of 10 $\mu M$ for 2 h, and subsequently was co-cultured with the KE4-CTL line. The mAbs were added at the initiation of the culture at a concentration of 10 $\mu$g/ml.

Table II. Binding Affinity of the PI-9 Peptides to HLA-A2402 Molecules

| Peptide | Sequence | HLA-A alleles | Score[a)] | MFI[b)] |
|---|---|---|---|---|
| PI-$9_{292-300}$ | AFQQGKADL | HLA-A2402 | 30.00 | 22.8 |
| PI-$9_{348-356}$ | RFCADHPFL | HLA-A2402 | 40.00 | 10.3 |
| PI-$9_{10-19}$ | TFAIRLLKIL | HLA-A2402 | 24.00 | 10.4 |
| PI-$9_{83-92}$ | QYLLRTANRL | HLA-A2402 | 360.00 | 17.1 |
| PI-$9_{92-101}$ | LFGEKTCQFL | HLA-A2402 | 28.80 | 11.2 |
| PI-$9_{103-111}$ | TFKESCLQF | HLA-A2402 | 12.00 | 12.2 |
| PI-$9_{111-119}$ | FYHAELKEL | HLA-A2402 | 220.00 | 14.2 |
| PI-$9_{139-148}$ | KTEGKIEELL | HLA-A2402 | 16.80 | 10.3 |
| PI-$9_{166-175}$ | YFKGKWNEPF | HLA-A2402 | 10.00 | 15.3 |
| PI-$9_{178-187}$ | TYTREMPFKI | HLA-A2402 | 55.00 | 17.1 |
| PI-$9_{198-206}$ | MYQEATFKL | HLA-A2402 | 396.00 | 22.1 |
| PI-$9_{220-228}$ | PYARKELSL | HLA-A2402 | 20.00 | 13.2 |
| PI-$9_{261-269}$ | KSTEVEVLL | HLA-A2402 | 13.44 | 10.8 |
| HIV[c)] | RYRQQLLGI | HLA-A2402 | | 13.0 |
| SART1[d)] | KGSGKMKTE | HLA-A2601 | | 4.3 |

*a*) Score represents an estimated half-time of dissociation of the PI-9 peptide binding to HLA-A24 molecules.[28)]
*b*) The binding affinity of the PI-9 peptides to HLA-A2402 molecules was evaluated by the mean fluorescence intensity (MFI) of the HLA-A24 molecules on the RMS-S-A2402/$K^b$ cells that were pulsed with the corresponding peptide.
*c*) The HIV peptide was used as a positive control that binds to HLA-A2402 molecules.
*d*) The SART1$_{736-744}$ peptide, capable of binding to HLA-A2601 molecules, was used as a negative control.

and PI-$9_{348-356}$ peptides possessed the ability to stimulate IFN-γ production by the KE4-CTLs. The dose-dependency of these PI-9 peptides was then examined further (Fig. 5A). The results demonstrated that both the PI-$9_{292-300}$ and PI-$9_{348-356}$ peptides showed a clear dose-dependency, but not the PI-$9_{10-19}$ peptide. The PI-$9_{139-148}$ peptide was used as a negative peptide. The ability of the PI-$9_{348-356}$ peptide to stimulate IFN-γ production by the CTLs was observed at concentrations as low as 10 n$M$ in a dose-dependent fashion, and that of the PI-$9_{292-300}$ peptide was observed at concentrations higher than 1 $\mu M$. Binding affinity of these PI-9 peptides was also examined (Table II). Both the PI-$9_{292-300}$ and PI-$9_{348-356}$ peptides bound to HLA-A2402 molecules with an intermediate affinity. Their score, the esti-

mated half-time of dissociation of the peptides from HLA-A24 molecules,[28] was also at an intermediate level. IFN-γ production was brought about by the KE4-CTL line in response to the C1R/A2402 cells pulsed with either the PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide. IFN-γ production was inhibited by the addition of either anti-CD8 or anti-HLA class I mAb, but not by either anti-CD4, anti-MHC class II, or anti-CD14 mAbs (Fig. 5B). These results suggest that the KE4-CTLs recognized these two PI-9 peptides in an HLA-A24-restricted manner.

**CTL induction by PI-9 peptides** To determine whether or not the PI-9 peptides have the ability to induce tumor-reactive CTLs in cancer patients, PBMCs from 5 HLA-$A24^+$ epithelial cancer patients were stimulated with either the PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide and the responses of these cells to tumor cells were examined (Table III). PBMCs from esophageal cancer patients #1 and #2 were stimulated *in vitro* with the PI-$9_{348-356}$ and PI-$9_{292-300}$ peptides, respectively. These PBMCs produced IFN-γ in response to HLA-$A24^+$ KE4 cells. PBMCs from colon cancer patient #1 also produced IFN-γ in response to HLA-$A24^+$ KE4 cells when stimulated *in vitro* with either the PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide. The CTL activity of *in vitro*-sensitized PBMCs from esophageal cancer patient #1 was further examined. As shown in Fig. 6A, these PBMCs showed a higher level of cytotoxicity against HLA-$A24^+$ KE4 tumor cells than against HLA-$A24^-$ QG-56 tumor cells. In addition, among the many clones established from the *in vitro*-sensitized PBMCs, six clones recognized C1R/A2402 cells pulsed with either the PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide, but they did not recognize the HIV-derived peptide that was used as a negative control (data not shown). Representative results concerning clones #68 and #79 are given in Fig. 6B.

We have recently established a simple culture system to induce peptide-reactive CTLs from PBMCs of cancer patients (Hida *et al.*, submitted for publication); this method was applied in the present study. After 6 cycles of stimulation by the PI-9 peptide, the reactivity of sensitized PBMCs from the HLA-$A24^+$ cancer patients or from

Table III. PI-9 Peptide Induction of Tumor-reactive CTLs in PBMCs of HLA-$A24^+$ Cancer Patients

| Patients | Peptides | IFN-γ production (pg/ml) in response to KE4 (HLA-A2402/2601) | IFN-γ production (pg/ml) in response to QG-56 (HLA-A2601/) |
|---|---|---|---|
| Esophageal cancer | | | |
| #1 | PI-$9_{292-300}$ | 29 | 0 |
| | PI-$9_{348-356}$ | <u>63</u> | 0 |
| #2 | PI-$9_{292-300}$ | <u>207</u> | 0 |
| | PI-$9_{348-356}$ | 24 | 0 |
| #3 | PI-$9_{292-300}$ | 7 | 0 |
| | PI-$9_{348-356}$ | 25 | 0 |
| Colon cancer | | | |
| #1 | PI-$9_{292-300}$ | <u>84</u> | 0 |
| | PI-$9_{348-356}$ | <u>120</u> | 0 |
| Gastric cancer | | | |
| #1 | PI-$9_{292-300}$ | 11 | 0 |
| | PI-$9_{348-356}$ | 30 | 0 |

Values represent the mean of triplicate assays of IFN-γ production. PBMCs of HLA-$A24^+$ cancer patients were stimulated with the peptides and examined for IFN-γ production in response to target cells at an E/T ratio of 8/1. IFN-γ production by effector cells alone (<100 pg/ml) was subtracted from the values. Values of >50 pg/ml are underlined.

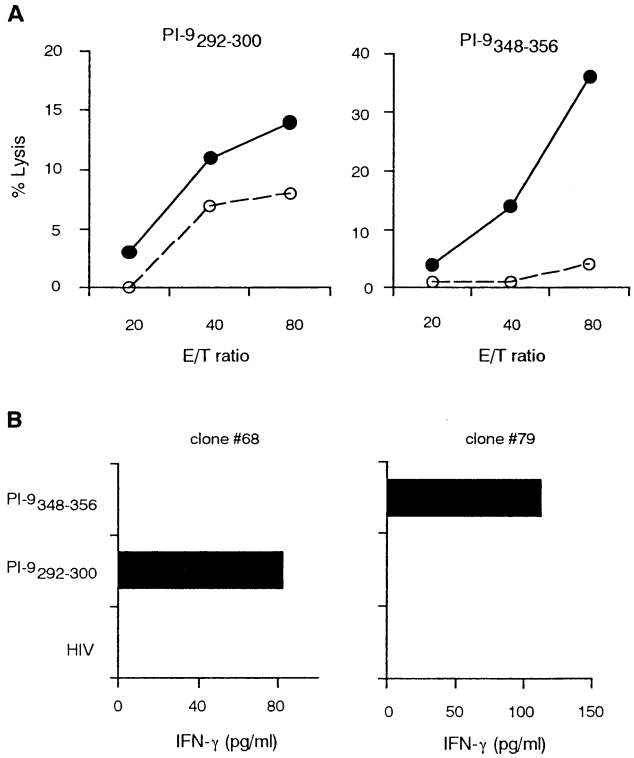


Fig. 6. Tumor-reactive and peptide-recognizing CTLs in *in vitro*-sensitized PBMCs from an esophageal cancer patient. (A) PBMCs from esophageal cancer patient #1 were stimulated *in vitro* with the PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide, as described in "Materials and Methods." The cultured cells were examined for cytotoxicity against HLA-$A24^+$ KE4 (● HLA-A2402/A2601) or HLA-$A24^-$ QG-56 (○ HLA-A2601/) tumor cells. (B) After 3 cycles of stimulation, several CTL clones were established by limiting dilution, and clones #68 and #79 were examined for reactivity against C1R/A2402 cells pulsed with the indicated peptides. As a negative control, the HIV peptide, which has the ability to bind to HLA-A2402 molecules, was used. After 18 h of culture, the level of IFN-γ in the culture supernatant was determined by ELISA. Values represent the mean of triplicate assays.

Table IV. Induction of the PI-9 Peptide-specific CTLs in PBMCs of HLA-A24$^+$ Cancer Patients

| Patients or healthy donors | IFN-γ production (pg/ml) in response to | |
|---|---|---|
| | PI-$9_{292-300}$ | PI-$9_{348-356}$ |
| Colon cancer | | |
| #2 | 0 | 315.6 |
| #3 | 5.6 | 24.5 |
| #4 | 0 | 0 |
| Gastric cancer | | |
| #2 | 48.5 | 122.9 |
| #3 | 52.7 | 95.2 |
| #4 | 130.2 | 95.4 |
| Healthy donor | | |
| #1 | 53.0 | 19.2 |
| #2 | 0 | 0 |
| #3 | 40.6 | 0 |

PBMCs of 6 cancer patients and 3 healthy donors were stimulated with *in vitro* with the indicated peptides (10 $\mu M$), and half of the medium was changed every 2 days for medium containing 20 $\mu M$ peptide. After 6 cycles of stimulation, the ability of these PBMCs to produce IFN-γ in response to C1R/A2402 cells which were pulsed with the corresponding peptide was examined. Values represent the mean of triplicate assays. The background IFN-γ production to unpulsed C1R/A2402 cells (<100 pg/ml) was subtracted from the values. Values of >50 pg/ml are underlined.

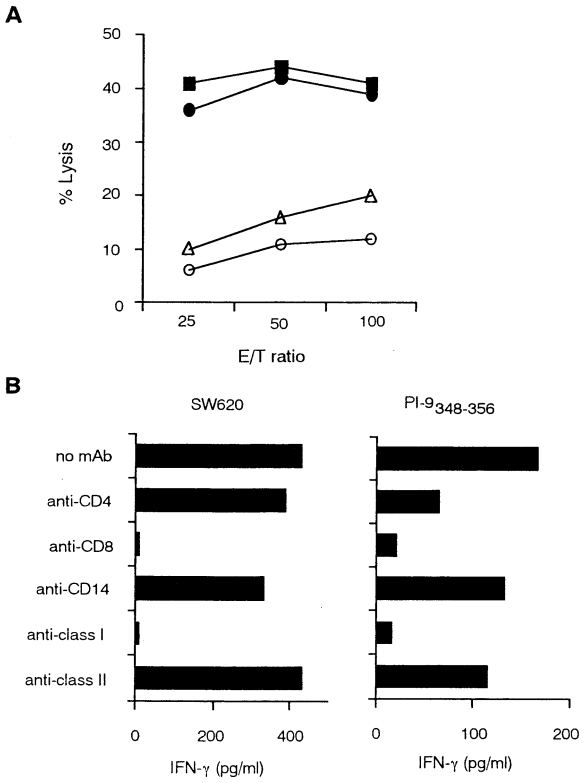


Fig. 7. Tumor-reactive and peptide-recognizing CTLs in *in vitro*-sensitized PBMCs from a colon cancer patient. (A) PBMCs from colon cancer patient #2 were stimulated *in vitro* with the PI-$9_{348-356}$ peptide, as described in the legend included in Table IV. After 6 cycles of stimulation, the cultured cells were examined for cytotoxicity (18 h) against the indicated tumor cell lines. The following target cell lines were used; ● KE4 (HLA-A2402/A2601), ■ SW620 (HLA-A2402/0201), △ COLO201 (HLA-A0201/A0101), and ○ QG-56 (HLA-A2601/). (B) The cultured cells were examined for reactivity against SW620 and C1R/A2402 cells, which were pulsed with the PI-$9_{348-356}$ peptide. The mAbs were added at the initiation of the culture at a dose of 10 $\mu$g/ml. After 18 h of culture, the level of IFN-γ in the culture supernatant was determined by ELISA. Values represent the mean of triplicate assays.

HLA-A24$^+$ healthy donors against C1R/A2402 cells loaded with the indicated PI-9 peptides was evaluated (Table IV). In one (patient #2) out of three colon cancer patients, PI-$9_{348-356}$ peptide-specific IFN-γ production was observed. PBMCs from 3 gastric cancer patients produced a significant level of IFN-γ in response to the C1R/A2402 cells which were pulsed with either the PI-$9_{292-300}$ or PI-$9_{348-356}$ peptide. Anti-PI-$9_{292-300}$-specific CTLs were also induced in PBMCs from healthy donor #1. The cytotoxicity of the *in vitro*-sensitized PBMCs from colon cancer patient #2 was further examined. Because no significant difference in CTL activity was detected in a 6 h CTL assay (data not shown), the results of an 18 h CTL assay are given (Fig. 7). The PI-$9_{348-356}$-sensitized PBMCs of the colon cancer patient #2 exhibited a higher cytotoxicity against the HLA-A24$^+$ KE4 and SW620 tumor cells, but showed a lower cytotoxicity against either HLA-A24$^-$ COLO201 or QG-56 tumor cells (Fig. 7A). This low cytotoxicity against HLA-A24$^-$ targets was probably due to the presence of non-specific effector cells or tumor necrosis factor (TNF)-α produced during the 18 h assay. In addition, the peptide-stimulated PBMCs showed a very low cytotoxicity (<5%) against PHA-activated PBMCs (data not shown). Furthermore, IFN-γ production in response to either the HLA-A24$^+$ SW620 tumor cells or C1R/A2402 cells pulsed with the PI-$9_{348-356}$ peptide was inhibited by the addition of anti-CD8 or anti-class I mAb, but not by the addition of anti-CD4, anti-CD14, or anti-class II mAb (Fig. 7B).

## DISCUSSION

The serine proteinase inhibitors (serpins) regulate a wide variety of physiological processes that involve proteinase activity, including blood coagulation, complement activation, cell migration and differentiation, intracellular proteolysis, and tumor suppression.[23] PI-9 is a member of the serpin superfamily.[21–23] PI-9 shares several biochemical characteristics with the endogenous caspase-1 inhibi-

tory activity, which was previously identified in vascular smooth muscle cells.[31] The human serpin PI-9 protein shares 54% identity with residues found in the reactive center loop of the cowpox virus cytokine response modifier A (CrmA) serpin [22, 31] and inhibits granzyme B-mediated apoptosis as well as caspase-1-mediated cleavage of synthetic substrates.[25, 31, 32] Both CTLs and natural killer cells use perforin and granzyme B-containing granules to destroy target cells that are neoplastic or infected with intracellular pathogens,[24] and PI-9 is reported to inhibit apoptosis mediated by either granzyme B or perforin.[21–24, 31]

In this study, a 2792 bp cDNA (clone 21) was found to encode an Ag recognized by the HLA-A24-restricted and tumor-reactive KE4-CTLs. A sequence alignment search revealed that the partial sequence of cDNA clone 21 shared 99–100% homology with 4 genes resigtered in the GenBank, namely, with homo sapiens serine proteinase inhibitor, clade B (ovalbumin), member 9 (SERPINB9) (NM_004155: 1626 bp); human serine proteinase inhibitor (PI-9) (U7164: 1626 bp); homo sapiens serine proteinase inhibitor, clade B (ovalbumin), member 9 (SERPINB9) (XM-004390: 1433 bp); and homo sapiens cytoplasmic antiproteinase 3 (CAP3) (L40378: 1385 bp). Almost half of the nucleotide sequence of clone 21 was identical to that of the *PI-9* gene. No information was obtained about half of the C-terminal region of clone 21. However, it is of note that the PI-9 protein was encoded from nucleotide 85 to 1215 of clone 21, and that the other 4 genes had a 100% identical coding region (Fig. 2A). These findings suggest that all genes encode the PI-9 antigen which can be recognized by the KE4-CTLs.

The expression of the PI-9 transcript was previously investigated by other researchers.[22] The highest levels of PI-9 mRNA and protein were observed in natural killer cell leukemia cell lines and in IL-2-stimulated PBMCs, which also produce granzyme B and perforin. The tissue distribution of PI-9 mRNA revealed by northern blot analysis showed that PI-9 is present in low-level transcripts in the lung, spleen, thymus, peripheral blood leukocytes, testis, and placenta.[22, 24, 33] In addition, it was recently reported that PI-9 is expressed by dendritic cells and at immune-privileged sites.[34] In this study, we examined the transcript of this gene in several kinds of tumor cell lines and in normal cells by RT-PCR. As shown in Table I, the PI-9 transcript detected by the primer pair for the common coding region could be observed in approximately 75% of epithelial tumor cell lines. Hybridization using the clone 21 probe resulted in identification of three different transcripts (approximately 4.0, 2.5, and 1.5 kb), which had previously been reported by other researchers.[22] Since the cDNA clone 21 consisted of 2792 bp, its transcript might correspond to the 2.5 kb transcript. On the other hand, the 1.5 kb transcript might correspond to the other 4 genes registered in the GenBank, as each of their lengths was approximately 1.5 kb. In addition, RT-PCR revealed a discrepancy of the expression pattern observed with the two sets of primers (Fig. 2 and Table I). We suppose that the *PI-9* gene might be alternatively spliced, or perhaps it belongs to a multigene family. Further study is needed.

Among 13 peptides tested, the PI-$9_{292-300}$ and PI-$9_{348-356}$ peptides with intermediate affinity were found to be recognized by HLA-A24-restricted and tumor-reactive CTLs. Two (PI-$9_{292-300}$ and PI-$9_{348-356}$) of 13 different PI-9-derived peptides with HLA-A24 binding motifs were recognized in a dose-dependent manner by the HLA-A24-restricted CTLs used in this study. These peptides were revealed to possess the ability to induce HLA-A24-restricted tumor-reactive CTLs in HLA-$A24^+$ PBMCs from epithelial cancer patients.

Several molecules in activated T cells, including a family of serpins, were thought to be responsible for resistance to CTL-mediated lysis.[24, 25] Recently, we reported that CTLs showed cytotoxicity to C1R/A2402 cells pulsed with a peptide, but that they failed to lyse HLA-$A24^+$ (autologous as well as allogeneic) PHA-activated T cells pulsed with the same peptide.[35] Because PI-9 is a member of the serpin family, it is reasonable to think that higher expression of PI-9 makes cells more resistant to cytolysis. In support of this idea, PI-9 recognizing CTLs needed 18 h to show CTL activity toward PI-9-expressing tumor cells (Fig. 7). In addition, the cytotoxicity of the PI-9 peptide-stimulated PBMCs against PHA-activated PBMCs was very low. Similarly, CTLs recognizing survivin, an apoptosis inhibitor protein, were reported to produce IFN-γ in response to survivin-derived peptides, whereas there was no apparent cytolytic activity against survivin-expressing tumor cells.[36, 37] Further study is needed on these matters.

In conclusion, we showed that PI-9 can be recognized by HLA class I-restricted and tumor-reactive CTLs. However, PI-9 might be an inappropriate molecule for specific immunotherapy of cancer patients because high expression of PI-9 could result in resistance to cytolysis. Nevertheless, it is of note that PI-9-recognizing CTLs can produce IFN-γ in an Ag-specific manner. PI-9-reactive CTLs may play some role in anti-tumor immune response in cancer patients.

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