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Human CD8 and CD4 T cell epitopes of epithelial cancer antigens

Abstract Recent human tumor immunology research has identified several genes coding immunogenic peptides recognized by CD8 cytotoxic T lymphocytes (CTLs) in melanoma tumors. Very recently, CD4 T cell antigenic epitopes were also determined in certain melanoma tumors. The use of these peptides in conjunction with human immunotherapy could prove to be of great benefit. However, such peptides in clinically common tumors of epithelial cell origin, such as of the stomach, colon, lung, etc., have not yet been determined extensively. We describe for the first time an HLA-A31 (A*31012)-restricted natural antigenic peptide recognized by the CD8 CTL TcHST-2 of gastric signet ring cell carcinoma cell line HST-2. We also identified the HLA-DRB1*08032-restricted peptide recognized by the CD4 T cell line TcOSC-20 of squamous cell carcinoma OSC-20 derived from the oral cavity. The antigenic peptide of HST-2, designated F4.2, is composed of 10 amino acid residues with two anchor motif residues necessary for binding to HLA-A31 molecules. The synthetic F4.2 peptide enhanced the reactivity of TcHST-2 against HST-2 cells. Furthermore, introduction of an expression minigene coding F4.2 peptide to HLA-A31(+) cells conferred cytotoxic susceptibility to TcHST-2 on the cells. Some stomach cancer lines into which the HLA-A31 gene had been introduced, such as MKN28-A31-2, were lysed by TcHST-2, suggesting the presence of F4.2 peptide in at least some HLA-A31(+) stomach cancers. Furthermore, F4.2 peptide induced an F4.2 peptide-specific CTL response in at least 30–40% of HLA-A31(+) peripheral blood lymphocytes from gastric cancer patients, suggesting that F4.2 peptide could be used as a cancer vaccine for gastric tumors. The natural antigenic peptide of OSC-20 was also determined using acid extraction and biochemical separation and by mass spectrometry. Consequently, OSC-20 peptide was designated as the 6-1-5 peptide, an HLA-DRB1*08032-restricted 16-mer peptide with two possible anchor motifs. It has an amino acid sequence identical to that of human α-enolase, suggesting that it was derived from the processed parental α-enolase protein. We are presently attempting to determine the genes that code tumor rejection antigens recognized by HLA-A24- and A26-restricted T cells, including those of pulmonary and pancreatic carcinomas. The search for these antigenic peptides may lead to the identification of immunogenic peptide antigens that would be suitable for clinical use in commonly occurring epithelial cancers.

Key words Antigenic peptides · Epithelial cancer · Immunotherapy

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Introduction

Recent investigations have clarified the genes and immunobiological nature of tumor cell antigens recognized by $CD8^+$ cytotoxic T lymphocytes (CTLs). Some of these antigens have been utilized in attempts to develop clinical immunotherapy regimens, indicating that certain peptides may be effective in inhibiting not only primary

tumor growth but also metastatic foci in the lung [5, 8]. However, most of the antigens clarified so far have been studied in melanomas, whereas the characteristics of tumor antigens of epithelial origin remain largely unknown. Clinically, the incidence of tumors of epithelial origin, such as of the stomach, colon, lung, liver, etc., is higher than that of melanomas. Therefore the discovery of epithelial tumor antigens is important for the establishment of new immunological protocols in cancer treatment. In this report, we introduce an antigen recognized by HLA-A31-restricted CTLs derived from an autologous gastric signet ring cell carcinoma cell line.

It has recently become evident that major histocompatibility complex (MHC) class II-restricted tumor antigens recognized by CD4 T cells are important for the development of a potent immunotherapy protocol [2, 10]. This paper also describes an MHC class II-presenting autologous CD4 T cell epitope in squamous cancer of the oral cavity. The serial identification of genes and antigenic peptides in many epithelial tumors may lead to the development of a new modality of cancer immunotherapy.

HLA-A31-restricted natural antigenic peptide of gastric tumor recognized by CTLs

We previously established the human gastric signet ring cell carcinoma cell line HST-2 and autologous $CD8^+$ CTL clone TcHST-2 [3, 4]. This CTL clone can lyse HST-2 cells in the context of HLA-A31 (A*31012), since the cytotoxicity of TcHST-2 was selectively inhibited only by treatment with an anti-HLA-A31-specific monoclonal antibody (mAb). Subsequently, we intended to identify the natural form of the antigenic peptides in this novel target-CTL system [9]. Large-scale in vitro culture of HST-2 cells was performed, and approximately 5×10^{10} cells were obtained and treated with trifluoroacetic acid (TFA). Biochemical procedures, including gel-filtration chromatography on a Sephadex G25 column and three steps of reverse-phase high-performance liquid chromatography (RP-HPLC), were performed to separate the potential candidates for antigenic peptides, and finally a peptide candidate with antigenic activity was isolated and subjected to peptide sequencing.

As shown in the upper panel of Fig. 1, this peptide has a primary amino acid sequence of Tyr-Ser-Trp-Met-Asp-Ile-Ser-Cys-Trp-Ile and is designated as F4.2 peptide. We synthesized it and assessed its antigenic activity by enhancing TcHST-2 reactivity against HST-2 cells. As shown in the lower panel of Fig. 1, it appeared that F4.2 peptide enhanced TcHST-2 reactivity against HST-2 cells pulsed with F4.2, but not that of those pulsed with control HBA31 11-mer peptide (Ser-Thr-Leu-Pro-Glu-Thr-Thr-Val-Val-Arg-Arg), which is an HLA-A31-restricted hepatitis B virus protein-derived antigenic peptide [6].

To confirm that F4.2 peptide was immunogenic when generated endogenously, we constructed an expression

F4.2 Tyr-Ser-Trp-Met-Asp-Ile-Ser-Cys-Trp-Ile (YSWMDISCWI)

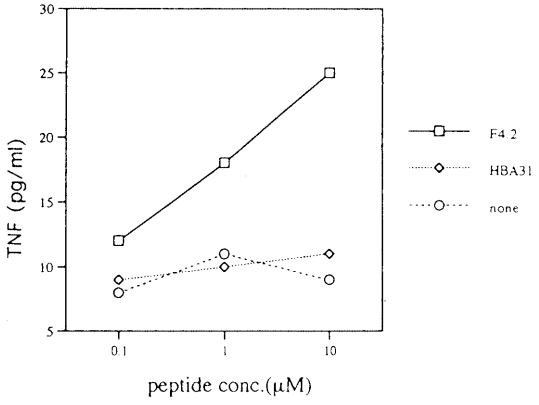


Fig. 1 Upper panel, amino acid sequence of F4.2 peptide. Underlined amino acids correspond to the peptide binding motif of HLA-A31. Lower panel, specificity of TcHST-2 response against F4.2 and HBA31 (STLPETTVVRR) peptides. HST-2 cells were pulsed with or without 0.1, 1.0, and 10 μM of these synthetic peptides, and mixed for 6 h with TcHST-2 at an E:T ratio of 3:1. TNF production by TcHST-2 was determined as described previously [9]. □ F4.2; ◇ HBA31; ○ none

minigene vector, pF4.2ss, using pCDSRa-E3. pcDSRa-E3 contains an adenovirus E3, 19-kDa protein signal sequence under the control of the SRa promotor. pF4.2 was constructed by insertion of oligonucleotides corresponding to F4.2 peptide in the *Pst*I and *Xba*I site of the pcDSRa-E3 expression vector. MKN28-A31-2 and HOBC8-A31-12 cell lines were transfectants of the gastric cancer cell line MKN28 and squamous cancer cell line HOBC8, respectively, with HLA-A*31012 expression vector pBJ-A*31012 [9].

In the tumor necrosis factor (TNF) production assays, HOBC8-A31-12 cells did not stimulate TcHST-2, whereas our preliminary data indicated that MKN28-A31-2 cross-reacted with TcHST-2, suggesting that the MKN28-A31-2 gastric tumor line, but not HOBC8-A31-12, may have endogenous F4.2 peptide on the cell surface. Both HOBC8-A31-12 and MKN28-A31-2 cells were cotransfected with pF4.2ss plus pBabe at a 20:1 molar DNA ratio, then selected with puromycin, and stable transfectant lines were obtained. These cells were then assessed in a cytotoxicity experiment using TcHST-2. The data showed that introduction of the pF4.2ss minigene into HOBC8-A31-12 cells clearly conferred cytotoxic susceptibility to TcHST-2 (Fig. 2A). Furthermore, the cytotoxic susceptibility of MKN28-A31-2 cells transfected with the pF4.2ss minigene to TcHST-2 was enhanced compared with that of MKN28-A31-2 cells transfected with pBabe Puro and selected with puromycin (Fig. 2B). Taken together, these data suggest that F4.2 is the peptide antigen to TcHST-2 in the context of HLA-A31 molecules.

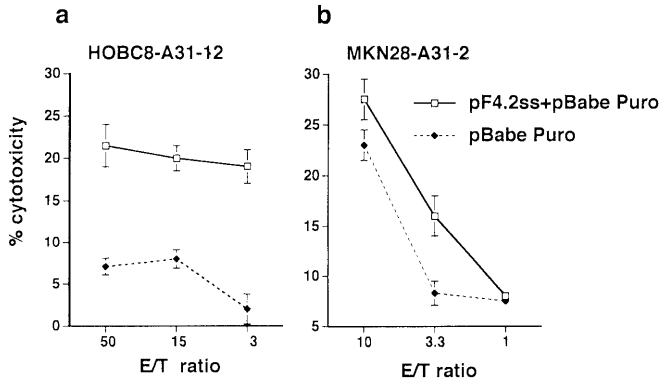


Fig. 2 Cytotoxicity of TcHST-2 against stable transfectants of HOBC8-A31-12 and MKN28-A31-2 cells with the pF4.2ss minigene. HOBC8-A31-12 (a) and MKN28-A31-2 (b) cells were transfected with pF4.2ss and pBabe Puro at a DNA molar ratio of 20:1 or with pBabe Puro alone. The cells were selected in the presence of puromycin 1.0 μg/mL, and stable transfectant lines were obtained.Then the cells were labeled with ^{51}Cr and mixed with TcHST-2 at various E:T ratios. Bars represent mean ± SE. □ pF4.2ss + pBabe Puro; ◆ pBabe Puro

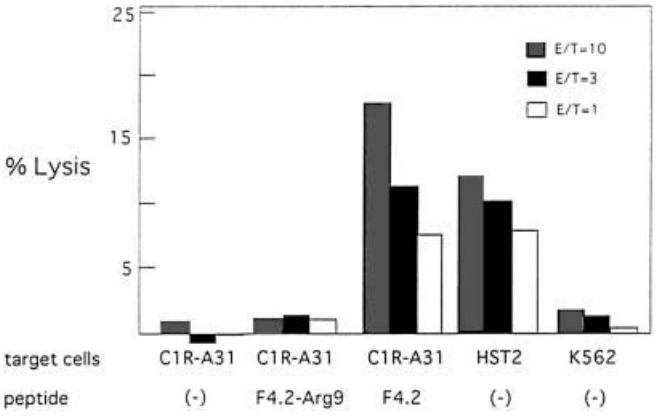


Fig. 3 T cells from HLA-A31$^+$ PBLs from gastric cancer patients were stimulated with F4.2 synthetic peptide in the presence of autologous antigen-presenting cells including dendritic cells. T cells were then tested for cytotoxic activity against ^{51}Cr-labeled target cells such as peptide (F4.2, YSWMDISCWI, or variant peptide F4.2-Arg9, YSWMDISCRI)-pulsed or nonpulsed C1R-A31, HST-2, and K562 cells at various E:T ratios. ■ E:T = 10; ■ E:T = 3; □ E:T = 1

Ability of F4.2 to induce peptide-specific CTLs from HLA-A31$^+$ gastric cancer patients in vitro

We next determined in vitro whether F4.2 peptide could induce F4.2-specific CTLs from peripheral blood lymphocytes (PBLs) of HLA-A31$^+$ gastric cancer patients. Patient T cells were stimulated for 2 days in vitro in the presence of synthetic F4.2 peptide 10 μmol with antigen-presenting cells including autologous peripheral dendritic cells and macrophages that had been precultured for 24 h with granulocyte-macrophage colony-stimulating factor (GM-CSF) 1000 U/mL (Novartis Pharmaceutical Co., Basel, Switzerland). T cells were then cultured for 2–5 days in the presence of interleukin (IL)-2 100 U/mL (Takeda Pharmaceutical Industries, Osaka, Japan). These T cells were further treated with mixed lymphocyte-peptide culture (MLPC). T cells were subjected to an additional two cycles of MLPC and then assessed for cytotoxic activity against ^{51}Cr-labeled C1R-A31 cells that had been pulsed with F4.2 and control peptides. As controls, the cytotoxic activity of T cells against ^{51}Cr-labeled HST-2 and K562 cells was also determined. We conducted these CTL induction experiments using PBLs from seven HLA-A31$^+$ gastric cancer patients.

Figure 3 shows that F4.2 peptide-specific CTLs were generated by PBLs from one patient. T cells in this patient were cytotoxic against not only F4.2 peptide-pulsed C1R-A31 cells but also HST-2 cells. However, they were not cytotoxic against C1R-A31 or K562 cells. Furthermore, T cells were not cytotoxic against C1R-A31 cells pulsed with F4.2 variant peptide (F4.2-Arg9, Tyr-Ser-Trp-Met-Asp-Ile-Ser-Cys-Arg-Ile), which has an Arg substitution at the ninth-position Trp of F4.2 peptide. This position of F4.2 peptide appeared to be the T cell epitope in the TcHST-2-HST-2 interaction [9]. Further experiments indicated that T cells from the PBLs of two of the seven HLA-A31$^+$ gastric cancer patients induced such F4.2 peptide-specific CTLs. Some of these patients had received chemotherapy, suggesting that the precursors of F4.2 peptide-specific T cells in peripheral lymphoid tissue may be deleted from the peripheral circulation. Therefore we concluded that F4.2 peptide may be useful as immunotherapy for certain HLA-A31$^+$ gastric cancer patients.

DR8-restricted squamous cell cancer antigen recognized by CD4$^+$ CTLs

We previously reported that the CD4$^+$ T cell line TcOSC-20 could be cytotoxic against autologous OSC-20 squamous cancer lines in the context of HLA-DR8 (DRB1*08032) [7]. As shown in Fig. 4, TcOSC-20 lysed OSC-20 but not the autologous Epstein-Barr (EB)-virus-

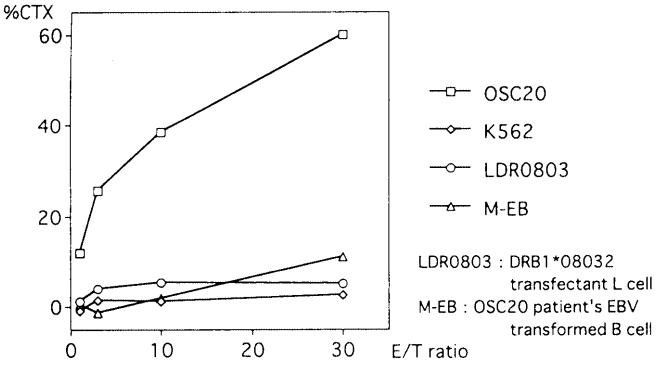


Fig. 4 Cytotoxicity of TcOSC20 against ^{51}Cr-labeled OSC20, M-EB, LDR0803, and K562 cells at various E:T ratios. □ OSC20; ◇ K562; ○ LDR0803; △ M-EB

transformed B cell line M-EB. Because this line did not react with LDR0803, which is a mouse L cell line transfected with an expression vector of the HLA-DRB1*08032 gene, we utilized this L cell tranfectant to determine the antigenic peptide of OSC-20 cells. Approximately 5×10^9 OSC-20 cells were harvested and treated with TFA, followed by the biochemical separation of peptides by two steps of RP-HPLC. In each RP-HPLC step, the antigenic activity of peptides to TcOSC-20 was assessed by pulsing fractions to LDR0803. As shown in Fig. 5, in the second step of RP-HPLC we obtained a single fraction (fraction 6) with peptide antigenicity to TcOSC20 in the cytotoxicity assays.

Fraction 6 was then analyzed by mass spectrometry. The data indicated that there were nine different peptides in fraction 6. Since three of these nine had a peptide binding motif to HLA-DR8 molecules, we synthesized and purified these three peptides for use in the cytotoxicity assays using TcOSC-20. As shown in Fig. 6, the amino acid sequences of peptide OSC-20 F6-1-1, F6-1-4, and F6-1-5 were VLLPKKTESHHKAK, KVLKQV-HPDTGISS, and TVTNPKRIAKAVNEKS, respectively. It is known that F6-1-1 and F6-1-4 have

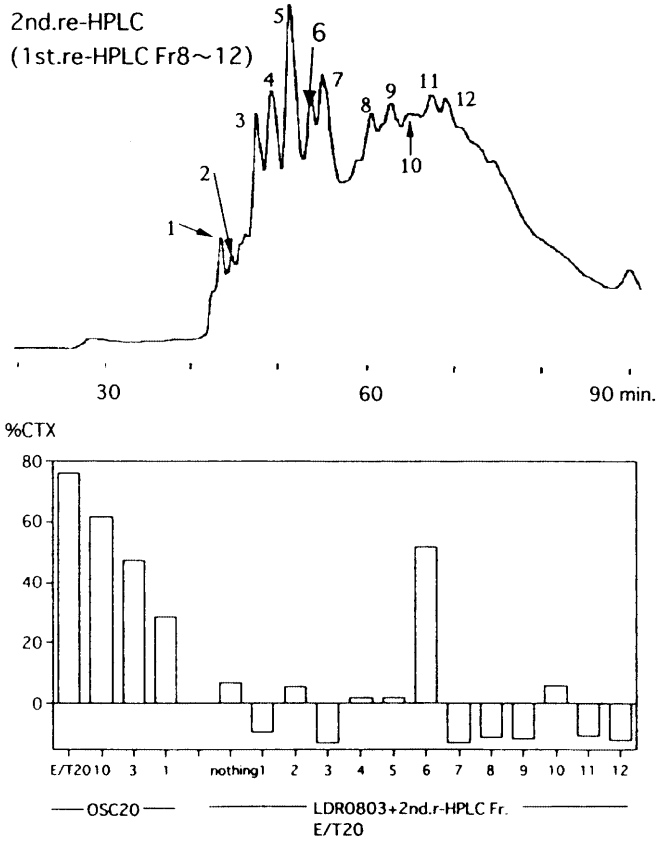
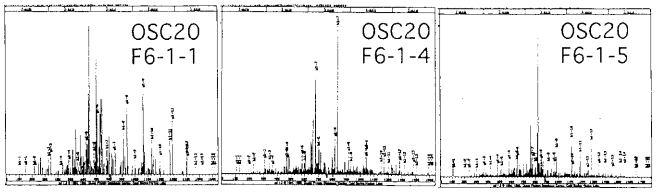


Fig. 5 Elution pattern of the second step of RP-HPLC of TFA-extracted OSC20 antigens and the antigenic activity of each fraction in cytotoxicity assays with TcOSC20. ^{51}Cr-labeled LDR0803 cells were treated with 50 μL of each fraction, admixed with TcHST-2 at an E:T ratio of 20:1, and the cytotoxic activity of each fraction was determined



OSC20 F6-1-1	VLLPKKTESHHKAK	human histon H2A.1.
OSC20 F6-1-4	KVLKQVHPDTGISS	human histon H2B.1.
OSC20 F6-1-5	TVTNPKRIAKAVNEKS	human alpha enolase

Fig. 6 Upper panel, profiles of mass spectrometry analysis of peptides F6-1-1, F6-1-4, and F6-1-5. Lower panel, amino acid sequence of peptides. Underlined peptides correspond to the peptide binding motif of HLA-DR8 molecules

sequences identical to human histons H2 A.1 and H2B.1, respectively, on the SEQUST database. In contrast, F6-1-5 is identical to the amino acid sequence of human α-enolase. Figure 7 shows the peptide antigenicity. The data indicate that F6-1-5 peptide, but not F6-1-1 or F6-1-4 peptide, can confer cytotoxic susceptibility on ^{51}Cr-labeled LDR0803 L cells, suggesting that the α-enolase-derived F6-1-5 peptide is the natural antigenic peptide of OSC-20 cells.

α-Enolase is a very common enzyme in the cells [1], and our preliminary RT-PCR analysis of α-enolase mRNA expression showed almost the same level in OSC-20 and autologous EB virus-transformed M-EB cells, although in OSC-20 cells the PCR products appeared to be of different sizes. Therefore we do not yet know why F6-1-5 peptide can act as an antigenic peptide in the TcOSC20-OSC2 system. Our preliminary experiments suggest that OSC-20, but not M-EB, cells expresses the Fas antigen on the cell surface. Since TcOSC-20 expresses the Fas ligand, the different levels of Fas expression between OSC20 and M-EB cell lines may be related to the difference in cytotoxic susceptibility.

Conclusions

In this paper, we introduced CD8 and CD4 T cell epitopes of epithelial cancer cells. Although an increasing number of tumor antigens and peptides has been clarified in melanomas, such antigens in epithelial cancers remain to be elucidated. It is critically important to identify more tumor antigens and genes from various epithelial cancers for the establishment of peptide-based immunotherapy. We are also analyzing tumor antigens and genes in pancreatic and pulmonary cancers. The search for these antigens may lead to the identification of immunogenic peptides suitable for the clinical treatment of epithelial cancer patients. The reason why CTLs do not react with certain target tumor cells irrespective of their tumor antigen expression is another important question that must be resolved. An investigation into this unresponsiveness, or tumor escape mechanism, in the CTL-tumor cell interaction is

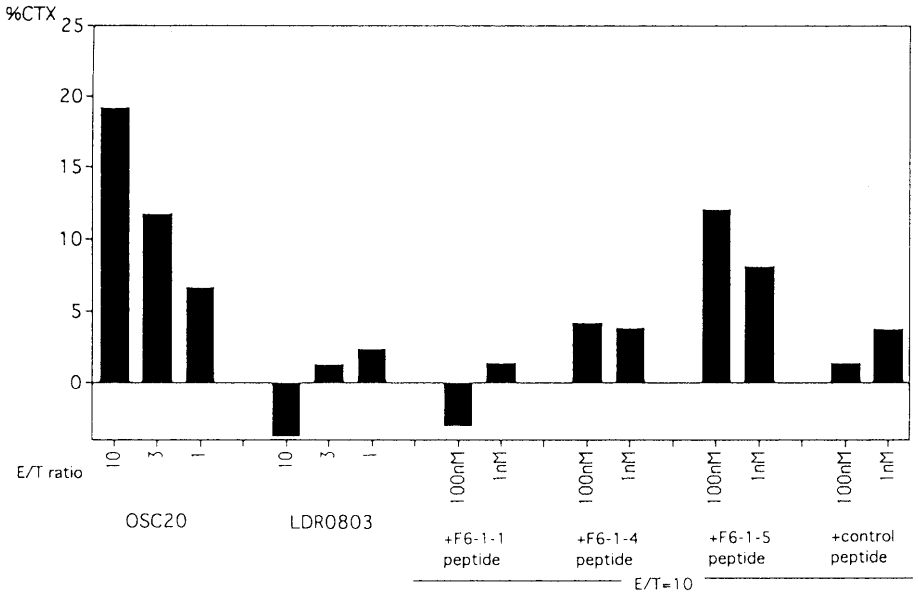


Fig. 7 Antigenic activity of F6-1-1, F6-1-4, and F6-1-5 synthetic peptides in cytotoxic assays with TcOSC20. ^{51}Cr-labeled LDR0803 cells were treated with synthetic peptides 10 μmol and mixed with TcOSC20 at an E:T ratio of 10:1

required before an effective peptide-based immunotherapy can be established.

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