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Induction of Cytotoxic T Lymphocytes from Peripheral Blood of Human Histocompatibility Antigen (HLA)-A31$^+$ Gastric Cancer Patients by *in vitro* Stimulation with Antigenic Peptide of Signet Ring Cell Carcinoma

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Antigenic peptides have been used as a cancer vaccine in melanoma patients and have led to a drastic regression of metastatic tumors. However, few antigens have been identified in non-melanoma tumors. We recently purified a new natural antigenic peptide, designated F4.2, by biochemical elution from a human gastric signet cell carcinoma cell line and showed that it is recognized by an autologous human histocompatibility antigen (HLA)-A31-restricted cytotoxic T lymphocyte (CTL) clone. Here we describe *in vitro* induction of F4.2-specific CTLs from peripheral blood T lymphocytes of HLA-A31$^+$ gastric cancer patients. The T cells of seven HLA-A31$^+$ patients with gastric cancers were stimulated *in vitro* by F4.2-pulsed autologous dendritic cells which had been induced from peripheral blood of each patient by incubation in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4. We tested the cytotoxicity of the T cells against F4.2-loaded C1R-A*31012 by a 6-h ^{51}Cr release assay after 3 stimulations with F4.2-pulsed dendritic cells. F4.2-specific cytotoxicity was detectable in the stimulated T cells from two of the seven HLA-A31$^+$ patients. Further, both F4.2-specific CTLs also lysed the gastric cancer cell line, HST-2, from which F4.2 was derived. These results suggest that F4.2 peptide may be useful as an HLA-A31-restricted peptide vaccine in certain patients with gastric cancer.

Key words: Antigenic peptides — HLA-A31 — Human gastric carcinoma — Cytotoxic T lymphocytes

Attempts at specific immunotherapy using antigenic peptides in cancer patients are intriguing, since this therapy may lead to the development of a new modality of cancer treatment. This immunotherapy is based upon the recognition of antigenic peptides binding to human histocompatibility antigen (HLA) by the HLA-restricted cytotoxic T lymphocytes (CTLs).[1,2] Several antigenic peptides and gene-encoding antigens, derived almost exclusively from melanomas, have been identified and their effectiveness to induce regression of the tumors was observed in clinical trials using these antigenic peptides as a cancer vaccine.[3,4] Although a great deal of research has been carried out into the melanoma antigens recognized by CTLs, few antigens have been identified in epithelial carcinomas, which have a much higher rate of occurrence than melanoma. Identification of more new tumor antigens in non-melanoma tumors is required, especially in carcinomas of epithelial cell origin.

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We have recently identified a new natural antigenic peptide, F4.2, which was purified by biochemical elution from a gastric signet cell carcinoma cell line HST-2.[5] This peptide was recognized by HLA-A31-restricted autologous CTL clone TcHST-2. We have also shown that TcHST-2 lysed an allogeneic HLA-A31$^-$ gastric cancer cell line only when the cell line was transfected with HLA-A31 gene.[5,6] These findings suggest that the same antigenic peptide, F4.2, exists in allogeneic gastric cancers.[7]

In our current study we investigated F4.2 peptide-specific CTL induction *in vitro* from peripheral blood lymphocyte (PBL) of HLA-A31$^+$ gastric cancer patients. We showed that F4.2-specific CTLs were induced in certain patients and they killed F4.2-expressing tumors, suggesting that F4.2 peptide may be used as an HLA-A31-restricted peptide vaccine for gastric cancer patients.

MATERIALS AND METHODS

Screening of HLA-A31$^+$ PBL of gastric cancer patients Recruitment of patients was started by obtaining the cooperation of 21 hospitals and cancer centers from all over

Hokkaido. A total 151 patients, who either had gastric carcinoma or had just had it surgically removed, agreed to donate their peripheral blood for the screening of HLA-A31. Patients gave informed consent according to the ethical guidelines of our Medical School.

For the screening, expression of HLA-A31 molecules was analyzed by staining of each patient's PBL with anti-HLA-A31 monoclonal antibody, which was described previously.[5] Fluorescein isothiocyanate-labeled goat anti-mouse antibody was applied to allow analysis with a fluorescence activated cell sorter (FACS).

Cell lines and minigene transfectant Tumor cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. We used C1R-A*31012, HST-2, MKN28-A31-pF4.2ss and K562 in this series. C1R-A*31012 was a gift from Dr. M. Takiguchi (Kumamoto University School of Medicine, Kumamoto) and this transfectant was introduced with a cDNA encoding an HLA-A*31012 genomic clone into the HLA-A-negative lymphoblastoid cell line C1R. HST-2 is a human gastric signet ring cell carcinoma cell line, and is recognized by HLA-A31-restricted CTL clone TcHST-2.[6] MKN28-A31-pF4.2ss is a transfectant obtained by introducing HLA-A*31012-encoding cDNA into a gastric carcinoma cell line MKN28; it expresses F4.2 peptide into its endoplasmic reticulum through an F4.2-encoding minigene attached to adenovirus E3/19 kDa protein signal sequence as described previously.[5] Briefly, we constructed an expression minigene vector, pF4.2ss, to express F4.2 peptide into the endoplasmic reticulum in endogenous form. Firstly, we inserted the oligonucleotides encoding F4.2 peptide into pcDSRα-E3 expression vector, which contains the adenovirus E3/19 kDa protein signal sequence under the control of SRα promoter. MKN28-A31-pF4.2ss is a stable transfectant line cotransfected with pF4.2ss and puromycin resistance gene, *pBabe Puro*.[8,9] C1R-A*31012 transfectant is lysed by TcHST-2 in the presence of F4.2 peptide, but not in its absence.

Synthetic peptides and peptide-specific CTL induction *in vitro* Peptide F4.2 (YSWMDISCWI) was synthesized and purified by reversed-phase, high-performance liquid chromatography (RP-HPLC); details of this peptide have been reported.[5,7] As a control peptide we also used F4.2-Arg9 (YSWMDISCRI), in which the ninth position Trp of F4.2 is substituted with Arg. It has the capability to bind to HLA-A31 molecules but not to stimulate TcHST-2, suggesting that the ninth position Trp acts as a T cell epitope.[5]

Peripheral blood mononuclear cells were separated from peripheral blood of HLA-A31$^+$ patients with gastric cancers by centrifugation on Ficoll-Conray gradients. The mononuclear cells were separated into adherent and non-adherent cells. Non-adherent cells including T lymphocytes were maintained in AIMV (Gibco BRL, New York, NY) medium without serum until antigen-presenting cells (APCs) had been prepared. To induce dendritic cells as professional APCs, we incubated a monolayer of adherent cells for 4–7 days in AIMV without serum containing 1000 U/ml GM-CSF, 10 U/ml IL-4 (Gibco BRL). GM-CSF was a kind gift from Novartis Pharmaceutical Co., Basel, Switzerland. While dendritic cells were vigorously proliferating in the presence of GM-CSF and IL-4, 10 μM F4.2 was added for 2 days. Then, 1000 U/ml IFNα (generous gift from Sumitomo Pharmaceutical Co., Ibaraki), 10 ng/ml TNFα (DAKO Japan, Kyoto), and 50 U/ml IL-12 (Gibco BRL) were added to the culture. On the following day, T lymphocytes were mixed and incubated with dendritic cells which had been washed twice and irradiated (45 Gy) beforehand. Two days later, 50 U/ml IL-2 was added. Recombinant IL-2 was kindly provided by Takeda Pharmaceutical, Osaka. After two additional peptide stimulations using the same method as for the first time, described above, these CTL lines were subjected to cytotoxicity assay.

Cytotoxicity assay To examine the induction of F4.2-specific CTLs, we measured the cytotoxic activity of the stimulated T cell lines with a conventional 6-h ^{51}Cr release assay. C1R-A*31012 cells were incubated with or without 10 μM peptide for 2 h at 37°C. One hundred millicuries of ^{51}Cr was added to each target. After incubation for 2 h at 37°C, target cells were washed 3 times and mixed with effector cells in a total of 0.2 ml of AIMV at various effector to target (E/T) ratios in triplicate in V-bottomed micro-titer plates (ICN Biomedicals, Aurora, OH). The supernatant was collected after 6-h incubation and its radioactivity was counted for 1 min in a scintillation counter. Spontaneous and maximum releases were obtained by incubating the targets alone and with 1% Nonidet P-40, respectively. The percentage of specific cytotoxicity (% cytotoxicity) was calculated with the following formula: (experimental release−spontaneous release)×100/(maximum release−spontaneous release).

RESULTS

Incidence of HLA-A31$^+$ gastric cancer in enrolled patients For the screening of HLA-A31 expression, PBLs from each patient were stained with anti-HLA-A31 monoclonal antibody 2D12[5] and analyzed by FACS. In this study, 25 (16.5%) of a total of 151 gastric cancer patients were positive for HLA-A31 by FACS analysis. Seven of the 25 HLA-A31$^+$ patients agreed to be enrolled in our CTL induction study. Table I lists the characteristics of the enrolled HLA-A31$^+$ patients with gastric cancer. All assays of CTL induction were achieved while the patients bore the tumors, or at most 3 months after tumor resection.

CTL induction by F4.2 peptide We stimulated T lym-

Table I. Characteristics of HLA-A31$^+$ Gastric Cancer Patients

Patient no.	Sex/Age in year	Pathological diagnosis	TNM stage	Chemotherapy	State of tumor bearing[a)]
1	M/42	adenocarcinoma	1B	done[b)]	after resection
2	F/79	adenocarcinoma	3A	not done	after resection
3	M/73	adenocarcinoma	0	not done	before resection
4	F/85	adenocarcinoma	0	not done	after resection
5	M/47	signet ring cell carcinoma	4	done[c)]	peritonitis carcinomatosa
6	M/76	adenocarcinoma	1B	not done	after resection
7	F/78	signet ring cell carcinoma	4	not done	peritonitis carcinomatosa

a) At the time of assay.
b) Epirubicin hydrochloride and cisplatin were injected 1 month before assay, fluorouracil and mitomycin C were used during assay.
c) Cisplatin and methotrexate were injected 1 month before assay.

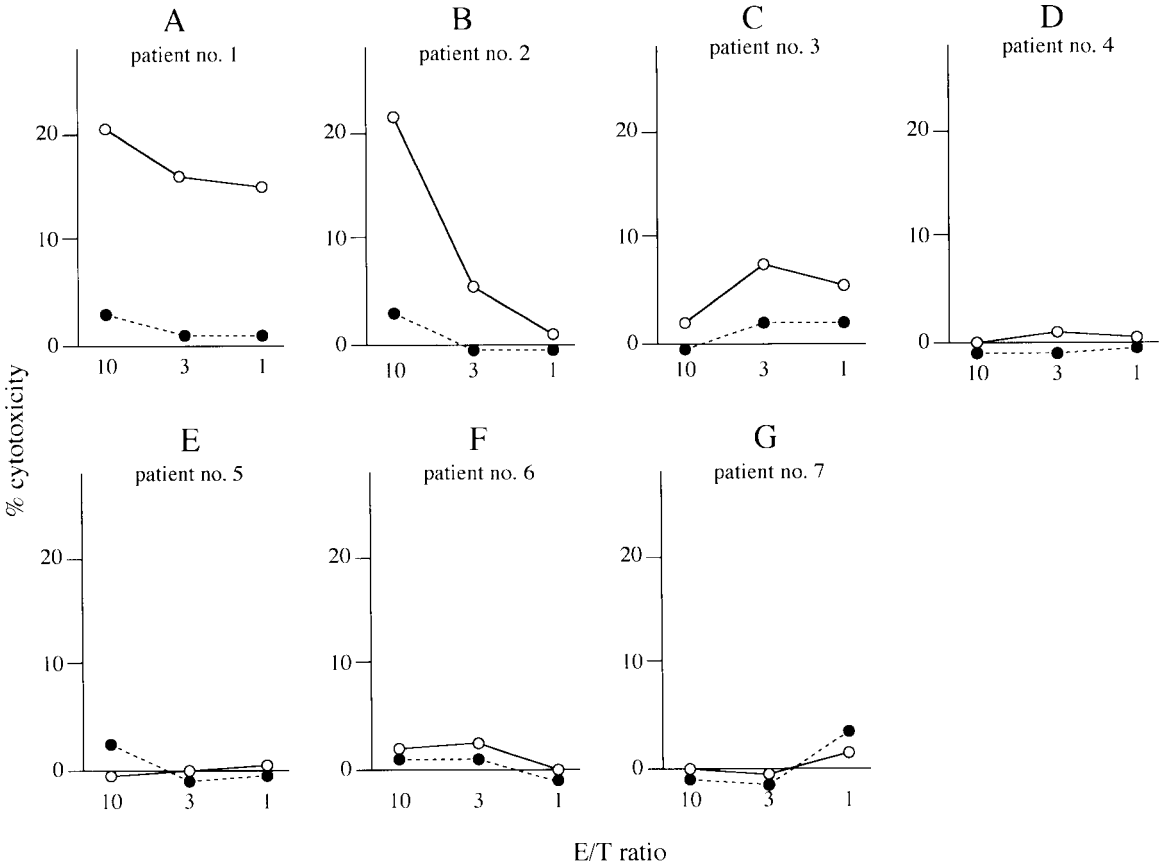

Fig. 1. Percent cytotoxicity of each patient's PBL after 3 stimulations with F4.2-pulsed dendritic cells against F4.2-pulsed C1R-A*31012 (open circles) and non-pulsed C1R-A*31012 (closed circles) at E/T ratios of 10:1, 3:1, and 1:1.

phocytes of the seven HLA-A31$^+$ patients with peptide-pulsed autologous dendritic cells *in vitro*, as mentioned above. To determine if F4.2-specific CTLs were being generated from the seven patients' T cells, we measured the cytotoxic activity of each PBL against F4.2-pulsed C1R-A*31012 in a ^{51}Cr-release assay. Fig. 1 shows that F4.2-specific cytotoxic activity against F4.2-pulsed C1R-A*31012 was detectable in the peptide-stimulated T cell lines from patient no. 1 and no. 2. When F4.2 was not pulsed to C1R-A*31012, these CTL lines did not lyse the

target cells (Fig. 1, A and B). In the other five patients, F4.2-specific cytotoxicity was not detected (Fig. 1, C–G).

To examine whether CTLs have F4.2-specific cytotoxic activity, we further analyzed the cytotoxicity of CTL lines from patient no. 1 and no. 2 against additional targets. As targets, we used C1R-A*31012 with or without F4.2 and a control non-immunogenic peptide, F4.2-Arg9. This F4.2-Arg9 peptide contained Arg at the 9th N-terminal position in place of Trp of F4.2 wild-type peptide. As previously reported, F4.2-specific CTL, TcHST-2, could not respond to F4.2-Arg9. As another target, we used HST-2, speculating that the F4.2-specific CTL lines might lyse gastric cancer cells which have F4.2 on the HLA-A31 molecules. As shown in Fig. 2, both CTL lines lysed F4.2-pulsed C1R-A*31012, whereas neither lysed non-pulsed C1R-A*31012, F4.2-Arg9-pulsed C1R-A*31012 or K562, showing that F4.2-specific cytotoxicity was induced. In addition, these F4.2-specific CTL lines induced from patient no. 1 and no. 2 both lysed HST-2, showing that the F4.2-specific CTLs also have the potential to kill gastric cancer cells. Further, the CTL of patient no. 1 also lysed MKN28-A31-pF4.2ss, which is a minigene (pF4.2ss)-transfectant expressing HLA-A31 and the minigene-encoded F4.2 peptide. That is to say, these CTLs were acting as tumor-specific CTLs against these gastric cancer lines, which express F4.2 binding to HLA-A31 molecules.

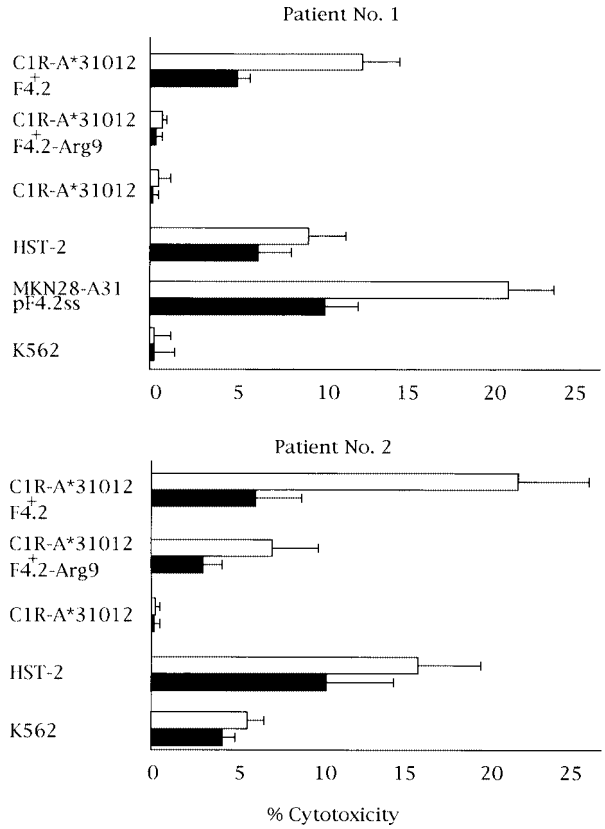

Fig. 2. Cytotoxicity of F4.2-specific CTL lines from no. 1 and no. 2 patients against additional targets, such as control peptide (F4.2-Arg9)-pulsed C1R-A*31012, C1R-A*31012 alone, F4.2-pulsed C1R-A*31012, HST-2, MKN28-A31-pF4.2ss, and K562. HST-2 and MKN28-A31-pF4.2ss are both gastric cancer cell lines expressing F4.2 on HLA-A31. Bars represent mean±SD. Open column, E/T ratio=10; closed column, E/T ratio=1.

DISCUSSION

We recently reported a new gastric cancer antigenic peptide, F4.2, recognized by HLA-A31-restricted CD8 CTL.[5] In this study, the antigenicity of F4.2 to patients with gastric cancers was examined. We showed the existence of F4.2-specific CTL precursors in PBL in at least two of seven HLA-A31$^+$ patients with gastric cancers. These findings suggest that F4.2 peptide may be applicable for treatment of HLA-A31$^+$ patients with cancers expressing F4.2. However, the fact that F4.2-specific CTLs were induced more easily in the two patients than in the other five may indicate that T cells had been primed *in vivo* in the two patients. Although it is unknown whether the same antigen exists in each individual tumor, F4.2 has the potential to induce CTLs that kill tumors expressing F4.2. Thus, F4.2 might be useful for cancer immunotherapy, such as adoptive transfer of CTLs induced *in vitro* with this antigenic peptide or *in vivo* immunization with peptides or peptide-loaded APCs as a 'cancer vaccine.' We are now studying the F4.2-encoding gene, its function, and its expression in various tumors and normal tissues. Identification of the gene will not only allow us to determine whether it is expressed in each individual tumor, but also may provide us with information as to whether F4.2 can be safely used as a vaccine for cancer patients.

F4.2-specific cytotoxicity was detectable against F4.2-pulsed C1R-A*31012, HST-2, and MKN28-A31-pF4.2ss at levels ten times greater than the cytotoxicity against C1R-A*31012 without F4.2 in both CTLs. However, the values of % cytotoxicity of the CTLs induced from patients no. 1 and 2 in this series were a little low at about 20% (Fig. 2). One of the main reasons for their low specific cytotoxicity may be that these CTL lines did not contain a high population of CD8$^+$ T cells. Indeed, each CTL line contained about 40% CD8$^+$ T cells in a FACS analysis (data not shown). These CTLs might have recognized the epitope peptide binding to HLA-A31 because C1R-A*31012 expresses no HLA-A other than HLA-A31 and F4.2 peptide has a high binding affinity to the HLA-A31 molecule, as shown previously.[5] To confirm the HLA-A31-restriction we inhibited the recognition of CTLs with anti-HLA-A31 monoclonal antibody. The cytotoxicity of both CTLs to HST-2 was inhibited by anti-HLA-A31 monoclonal antibody (data not shown).

Although the present study did not detect F4.2-specific CTLs in the five patients other than no. 1 and no. 2, this may not mean that there are no peptide-specific precursors in those patients, since it is well-known that antigen-specific CTL can be established even from normal donors. One possibility is that insufficient *in vivo* priming by early cancer causes only a low frequency of F4.2-specific T cells *in vivo* in patients such as no. 3 and 4 (Table I). Another possible cause of failure to induce CTLs from most of the patients may be our method, in which we stimulated T cells only three times. Persistent, repetitious stimulation might make it possible to detect CTL activity from such low-frequency precursors. Likewise, tetramer analysis of peptide-specific CTL, recently reported, might enable us to analyze more precisely the CTL frequency.[10–12)]

In vitro stimulation of PBL with peptide-pulsed dendritic cells has made it possible to induce peptide-specific CTLs that can lyse HST-2. As APCs we used mature dendritic cells, induced and activated with GM-CSF and IL-4, then matured with IFNα, TNFα, and IL-12.[13)] Mature dendritic cells would stimulate PBL more effectively than immature ones.[14)] In our early study, we tried several times to induce CTL from PBL of patient no. 1 during stimulation with F4.2-pulsed monocytes which had been induced by GM-CSF alone,[15)] but no peptide-specific cytotoxicity was detectable. So we improved our CTL-inducing method by changing to using mature dendritic cells, which can stimulate T cells as professional antigen-presenting cells.[16)]

Recent reports have indicated that immunotherapy for metastatic melanoma patients using HLA class I-bound tumor antigenic peptide results in regression of the tumor.[3, 4)] Previous reports described CTL induction by *in vitro* stimulation with melanoma-associated peptides from patients and normal donors using a similar technique to that of the present study.[17)] Such studies have provided a basis for the good clinical results reported today. Because effective immunotherapy, such as exists for melanoma, is unavailable for other malignant tumors, identification of antigenic peptides is a priority in cancer research. We recently reported F4.2, the only antigenic peptide of human gastric cancer so far known. In order to examine the feasibility of clinical use of F4.2 as a cancer vaccine, we examined the antigenicity of F4.2 to patients in this study, using the same approach as employed in the research and development of immunotherapy for melanoma. We have proved that F4.2 is immunogenic to at least some HLA-A31$^+$ patients with gastric tumors, and has potential to induce CTLs for specific cancer immunotherapy.

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