Expression of SART3 Tumor-rejection Antigen in Gastric Cancers

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We previously reported SART3 as a tumor-rejection antigen recognized by histocompatibility leukocyte antigen (HLA)-A24-restricted cytotoxic T lymphocytes (CTLs). In this study, we investigated the expression of the SART3 antigen in gastric cancers, as a candidate for use in specific immunotherapy. The SART3 antigen was detected in 9 of 10 (90%) gastric cancer cell lines, 35 of 52 (67.3%) gastric cancer tissues, and 0 of 20 non-tumorous gastric tissues. SART3-derived peptides corresponding to positions 109–118 and 315–323 induced HLA-A24-restricted and tumor-specific CTLs from peripheral blood mononuclear cells (PBMCs) of gastric cancer patients. These peptide-induced CTLs recognized HLA-A24+ SART3+ gastric cancer cells, but not HLA-A24− SART3− or HLA-A24− SART3+ gastric cancer cells. Therefore, the SART3 peptides could be useful in specific immunotherapy of gastric cancer patients.

Key words: Gastric cancer — SART3 antigen — Cancer vaccine — HLA — A24

Many genes encoding tumor-rejection antigens that are recognized by cytotoxic T lymphocytes (CTLs) have been identified from melanoma cDNA.1–5) The peptides encoded by these genes are currently under clinical trials as cancer vaccines, and have led to major tumor regression in some melanoma patients.6–8) Gastric cancer is the major cause of cancer death in Japan and other Asian countries, and is also one of the major cancers worldwide. The prognosis of gastric cancer is poor, and that of advanced stages is extremely poor, despite recent clinical trials with many chemotherapeutic agents.9,10) Therefore, development of new treatment modalities is needed. One of them will be specific immunotherapy. However, there is very little information on tumor epitopes of gastric cancers.11,12) We reported that tumor infiltrating lymphocytes of gastric cancer possessed histocompatibility leukocyte antigen (HLA)-class I-restricted CTLs recognizing peptide antigens on the HLA-A alleles.13) We recently reported the SART3 tumor-rejection antigen, which possesses tumor epitopes recognized by HLA-A24-restricted CTLs.14) In this study, we have investigated the expression of SART3 antigen in gastric cancer tissues, and found that the SART3 antigen is expressed in the majority of gastric cancers and its peptides can induce HLA-A24-restricted and tumor-specific CTLs in peripheral blood mononuclear cells (PBMCs) of gastric cancer patients.

**MATERIALS AND METHODS**

**Samples** Gastric cancer tissues (n=52) and non-tumorous gastric tissues (n=20) were obtained at the time of surgery in the Kurume University Hospital, Medical Institute of Bioregulation of Kyushu University, and the National Cancer Center Hospital. All the cancers were histologically adenocarcinomas. A section of each sample was minced with scissors and kept at −80°C until use. The 10 gastric cancer cell lines used in this study were MKN-7, MKN-28, MKN-45, SSTW-9, KWS, KATO-3, HGC-27, SH-10-TC, GT3TKB, NS-8. HLA-class I genotypes of some of these cells were reported elsewhere14); MKN-45, SSTW-9, KATO-3, and NS-8 are HLA-A24+, while MKN-28 and KWS are HLA-A24−. The KE-4 esophageal squamous carcinoma cell (SCC) line (HLA-A24/A26) from which SART3 was cloned14) was used as a positive control. The other cancer cell lines used in this study were KE-3 (HLA-A02/A24) and KE-5 (HLA-A11/A24) esophageal SCCs, PC-9 lung adenocarcinoma (HLA-A02/A24), QG-56 lung SCC (HLA-A24/A26). VA13 fibroblast cells (no expression of HLA-class I alleles) and phytohemagglutinin (PHA)-blast cells that were prepared by incubation of PBMCs with 10 µM PHA for 4 days were also used as target cells as negative controls.

**Detection of the SART3 antigen** Expression of the SART3 antigen in cell lines, cancer tissues, and non-tumorous gastric tissues was investigated by western blot

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analysis with polyclonal anti-SART3 antibody, as reported previously. Expression of HLA-class I or HLA-A24 antigens on tumor cells was studied by staining the cells with anti-class I (w6/32) monoclonal antibody (mAb) recognizing a monomorphic region of class I molecule or anti-HLA-A24 mAb recognizing a polymorphic region of HLA-A24 (One Lambda, Inc., Canoga Park, CA), and the expression was measured by FACSscan (Becton Dickinson, San Jose, CA) as reported previously.14) Peptides and CTL induction The SART3 109–118 (VYDY-NCHVDL) and SART3 315–323 (AYIDFEMK) peptides were used for this study. The method for CTL induction with the peptides was described elsewhere.14) Briefly, PBMCs from gastric cancer patients were incubated with 10 µM peptide in one well of a 24-well plate containing 2 ml of culture medium (45% RPMI-1640 medium, 45% AIM-V medium [GIBCO BRL, Walkersville, MA], 10% fetal calf serum (FCS) [EQUITech BIO, Ingram, TX]) with 100 units/ml of interleukin (IL)-2 [Shionogi Pharm. Co., Osaka]). At days 7, 14, and 21 of culture, the cells were harvested, washed, and re-incubated with irradiated (30 grays) autologous PBMCs acting as antigen-presenting cells that had been pre-incubated with the same peptide at the same dose for 3h. Effector cells were harvested at day 22 or 28 of culture, and were immediately tested for both surface phenotypes and CTL activity to produce interferon (IFN)-γ in response to various target cells by ELISA at various effector-to-target cell (E/T) ratios in triplicate assays. The limit of sensitivity of the ELISA was 10 pg/ml. For a 6-h 51Cr-release assay, these peptide-stimulated PBMCs were further cultured in the presence of irradiated autologous PBMCs that had been pre-pulsed with a corresponding peptide in order to obtain large numbers of effector cells for an additional 21–35 days. The surface phenotypes and CTL activity of these cells were re-checked by an IFN-γ production assay, and then the cells were tested for cytotoxicity by a 6-h 51Cr-release assay at different E/T ratios. The surface phenotype of effector cells was investigated by an immunofluorescence assay with fluorescein isothiocyanate (FITC)-conjugated anti-CD3, -CD4 or -CD8 mAb. For inhibition of CTL activity, 10 µg/ml of anti-class I (W6/32, IgG2a) or anti-CD8 (IgG2a), anti-class II (H-DR-1, IgG2a), anti-CD4 (IgG1) mAb was used as reported previously. Anti-CD14 mAb (IgG2a) served as a control mAb. Two-tailed Student’s t test was employed for statistical analysis in this study.

RESULTS
Expression of SART3 antigen The 140 kD band of SART3 antigen was expressed in both the cytosol and nuclear fractions of all 10 gastric cell lines tested, except for NS-8 cells. SART3 was detectable in both cytosol and nuclear fractions of 29 (56%) of 52 gastric cancer tissues, in the cytosol fraction alone in 6 cancer tissues, and in nuclear fraction alone in 6 cancers. Overall, SART3 was detectable in the cytosol fraction of 35 of 52 (67%) gastric cancer tissues and in the nuclear fraction of 35 of 52 (67%) cancer tissues. It was undetectable in either the cytosol or nuclear fraction of the remaining 17 cancer tissues (33%), and also undetectable in all of 20 non-tumorous gastric tissues tested. Representative results are shown in Fig. 1, and a summary is given in Table I.

Table I. Expression of SART3 Proteins (Summary)

| Samples                  | Numbers | Cytosol (%) | Nucleus (%) |
|--------------------------|---------|-------------|-------------|
| Gastric cancer cell lines| 10      | 9 (90)      | 9 (90)      |
| Gastric cancer tissues   | 52      | 35 (67.3)   | 35 (67.3)   |
| Non-tumorous gastric tissues | 20      | 0 (0)       | 0 (0)       |
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Table II. Induction of HLA-A24-restricted CTL Activities in PBMC by SART3-derived Peptides

| Donor cells                  | Peptide       | IFN-γ production (pg/ml) in response to |
|-----------------------------|---------------|----------------------------------------|
|                             |               | KE4 (A24/26) | PC-9 (A2/24) | KE-5 (A11/11) | QG-56 (A26/46) |
| Gastric cancer patient      | SART3<sub>109–118</sub> | 1025<sup>a</sup> | 1087 | 0 | 0 |
| (Patient 1)                 | SART3<sub>315–323</sub> | 247 | 476 | 0 | 0 |
|                             | no peptide    | 0 | 0 | 0 | 0 |
| Gastric cancer patient      | SART3<sub>109–118</sub> | 214 | 40 | 14 | 8 |
| (Patient 2)                 | SART3<sub>315–323</sub> | 200 | 38 | 18 | 0 |
|                             | no peptide    | 85 | 0 | 0 | 0 |
| Gastric cancer patient      | SART3<sub>109–118</sub> | 1732 | 143 | 24 | 0 |
| (Patient 3)                 | SART3<sub>315–323</sub> | 1233 | 256 | 15 | 0 |
|                             | no peptide    | 130 | 0 | 0 | 0 |
| Gastric cancer patient      | SART3<sub>109–118</sub> | 287 | 73 | 0 | 0 |
| (Patient 4)                 | SART3<sub>315–323</sub> | 434 | 252 | 40 | 88 |
|                             | no peptide    | 0 | 0 | 0 | 0 |
| Gastric cancer patient      | SART3<sub>109–118</sub> | 193 | 152 | 0 | 0 |
| (Patient 5)                 | SART3<sub>315–323</sub> | 718 | ND<sup>c</sup> | 0 | 0 |
|                             | no peptide    | 10 | 217 | 0 | 0 |

<sup>a</sup>) PBMCs from 5 HLA-A24+ gastric cancer patients were stimulated in vitro with 10 µM peptide, and tested for the ability to produce IFN-γ by recognition of various target cells at an E/T ratio of 2.

<sup>b</sup>) Values represent the mean of triplicate determinations. Background IFN-γ production in response to VA13 cells (about 100 pg/ml) was subtracted from the values in the table.

<sup>c</sup>) ND: not determined.

DISCUSSION

This study showed that the SART3 antigen was detectable in 67% of gastric cancers tested. It was undetectable in non-tumorous gastric tissues. SART3 antigen was also expressed in other cancers tested. The SART3 antigen was also expressed in other cancers tested. The SART3-negative gastric cancer tissues might contain too few proliferating cells for detection with western blot analysis under the conditions employed, because the pathological features and growth pattern of gastric cancer are different in each gastric cancer tissue. A study to identify molecular mechanisms involved in the lack of expression of SART3 on NS-8 gastric cancer cells is in progress.

Although we previously reported that the SART3 antigen was expressed in 60–70% of head and neck cancers and esophageal cancers, a study of the expression of SART3 antigen on gastric cancers is needed to provide a scientific basis for specific immunotherapy of gastric cancer.

Antigenic levels of IFN-γ by recognition of HLA-A24+ cancer cells (KE-4 and/or PC-9) when stimulated in vitro with either SART3<sub>109–118</sub> or SART3<sub>315–323</sub> peptide (Table II). In contrast, they failed to react to HLA-A24+ cancer cells (KE-5 or QG-56). These effector cells consisted of 20 to 30% of CD3−CD4+CD8+ T cells and 70 to 80% of CD3+CD4−CD8− T cells (data not shown). CTL activity in the PBMCs from three patients was confirmed by a 6-h 51Cr-release assay after further expansion in vitro. Similar results were obtained in all three cases tested, and representative results are shown in Figs. 2 and 3. The peptide-stimulated PBMCs lysed the HLA-A24+ tumor cells (KE-3, KE-4, PC-9, and SSTW-9), but not HLA-A24+ tumor cells (KE-5, QG-56, and KWS) or HLA-A24+ PHA-blastoïd cells (Fig. 2, A and B). They also failed to lyse HLA-A24+SART3+ NS-8 gastric cancer cells (Fig. 2B). HLA-A24 molecules were expressed on the surface of NS-8 cells (data not shown). The CTL activity was inhibited by anti-class I (W6/32) or anti-CD8, but not by anti-CD4, anti-class II (DR) or anti-CD14 mAb taken as a negative control in both IFN-γ production and 51Cr-release assays (Fig. 3, A and B). These effector cells consisted of 30 to 40% of CD3+CD4−CD8+ T cells and 60 to 70% of CD3+CD4+CD8− T cells (data not shown). All of these results suggest that PBMCs stimulated with the SART3-derived peptides showed HLA-A24-restricted and tumor-specific cytotoxicity against SART3+ cancer cells.
cancer patients. The prognosis of gastric cancer at the advanced stages is extremely poor, despite recent clinical trials with many chemotherapeutic agents. Therefore, development of new treatment modalities is needed. The present study also showed that the SART3\textsubscript{109-118} and SART3\textsubscript{315-323} peptides induced HLA-A24-restricted and tumor-specific CTLs recognizing SART3\textsuperscript{+} tumor cells, including gastric cancer cells, in PBMCs of all 5 HLA-
A24+ gastric cancer patients tested. These CTLs failed to lyse HLA-A24+ SART3+ gastric cancer cells, HLA-A24+ SART3- NS-8 tumor cells and HLA-A24+ SART3+ PHA-blastoid cells. These results suggest the presence of CTL precursors reacting to SART3 epitopes on gastric cancer cells in the circulation of the majority of gastric cancer patients. The HLA-A24 allele is found in 60% of Japanese, 20% of Caucasians, and 12% of Africans.15) We have recently identified tumor-epitopes of SART3 antigen on HLA-A0207 molecules which are able to induce HLA-A2-restricted and tumor-specific CTLs in PBMCs of epithelial cancer patients (Ito et al., unpublished results). The HLA-A2 allele is found in 40% of Japanese, and 50% of Caucasians.15) All these results suggest that the SART3 peptides could be appropriate molecules for use in specific immunotherapy of HLA-A24+ or -A2+ gastric cancer patients. This might allow the development of specific immunotherapy of relatively large numbers of gastric cancer patients throughout the world.

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