Expression of Tumor-rejection Antigens in Gynecologic Cancers

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We recently reported the four tumor-rejection antigens (SART1259, SART2, SART3, and ART4) that possess tumor epitopes capable of inducing HLA-A2402-restricted cytotoxic T lymphocytes (CTLs) in cancer patients. This study investigated the expression of these tumor antigens in gynecologic cancers, including 33 ovarian cancers, 38 cervical cancers, and 40 endometrial cancers. SART1 259 antigen was detected in 56%, 35%, and 30% of ovarian, cervical, and endometrial cancers, while SART2 antigen was detected in 46%, 66%, and 30% of these cancers, respectively. Both SART3 and ART4 antigens were detectable in the majority of these gynecologic cancers tested. In contrast, none of these antigens was detectable in any of the normal ovarian and uterine tissues tested. Peripheral blood mononuclear cells (PBMCs) of HLA-A24+ patients with gynecologic cancers were found to produce significant levels of interferon-γ in response to HLA-A24+ SART3+ gynecologic cancer cells after having been stimulated three times in vitro with either SART3109–118 or SART3315–323 peptide. These PBMCs lysed HLA-A24+ SART3+ gynecologic cancer cells, but not HLA-A24− SART3+ gynecologic cancer cells or HLA-A24+ normal cells. Therefore, these four antigens and their peptides, including SART3 peptides, would be appropriate molecules for use in specific immunotherapy of HLA-A24+ gynecologic cancer patients.

Key words: Tumor antigen — Gynecologic cancer — Specific immunotherapy — HLA-A24 — Cytotoxic T lymphocyte

Gynecologic cancer is a major cause of death from cancer in women. Surgery and chemotherapy are the main treatment modalities effective for patients with the early stages of such cancers. However, the presently available treatment modalities are not equally effective for recurrent or advanced stages of gynecologic cancers. Development of a new treatment modality geared toward these cancers is needed. Many genes encoding tumor-rejection antigens recognized by CTLs (cytotoxic T lymphocytes) have been identified from the cDNA of melanomas in the past 10 years.1–8 Some of the peptide antigens encoded by these genes are currently under clinical trials as cancer vaccines, and have led to major tumor regression in some melanoma patients.9–12 Specific immunotherapy with tumor-rejection antigens might also be applicable for gynecologic cancer patients. Although infiltration of CD8+ T cells in dysplastic tissues has been reported, little is known about the molecular basis of the host defense against cancer cells in gynecologic cancer patients. A search of the current literature revealed little information on tumor antigens available for specific immunotherapy of gynecologic cancers.13–16 We recently reported four tumor-rejection antigens (SART1259, SART2, SART3, and ART4) that possess tumor epitopes capable of inducing HLA-A2402-restricted CTLs in cancer patients.17–21 In this study, we have investigated the expression of these antigens in gynecologic (ovarian, cervical, endometrial) cancer tissues to elucidate appropriate molecules for use in specific immunotherapy. SART1259 and SART2 were expressed in more than 50% and 60% of ovarian and cervical cancers, respectively, while SART3 and ART4 were expressed in the majority of gynecologic cancers.

MATERIALS AND METHODS

Samples Ovarian cancer tissues (n=33) and non-tumorous ovarian tissues (n=4) were obtained at the time of surgery in the Kurume University Hospital. A total of 33 ovarian cancer tissue samples histologically consisted of 9 serous cystadenocarcinomas, 5 mucinous cystadenocarcinomas, 3 clear cell carcinomas, 4 endometrioid cell carcinomas, 5 borderline malignancies, and 7 cancers with other histological types. Uterine cancer tissues (n=78), benign uterine tumor (myoma) tissues (n=7), and non-tumorous uterine tissues (n=5) were obtained at the time of surgery in the Kurume University Hospital or in the Kyoundo Hospital. A total of 78 uterine cancer tissue samples consisted of 38 cervical cancers and 40 endometrial cancers. The 38 cervical cancers consisted of 29 squamous
cell carcinomas (SCCs), and 9 cancers with other histological types. The 40 endometrial cancers consisted of 38 adenocarcinomas, and 2 cancers with other histological types. A section of each sample was minced with scissors and kept at −80°C until use. The HLA-A2402+ uterine tumor cell lines used for this study were SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, TCS, HCS, OMC-4, KCC-Ia, and KCC-Ib and the HLA-A2402+ uterine tumor cell line was OMC-I. The HLA-A2402+ ovarian cancer cell lines were RMG-I, RMG-II, MCA, KOC-2S, KOC-3S, KOC-5C, RMUG-I, RTSG, and the HLA-A2402+ ovarian cancer cell lines were RMUG-S, TYK-nu, KOC-7C, and RKN. The KE4 esophageal SCC line (HLA-A2402/A2601) from which the SART1, SART2, and SART3 genes were cloned was used as a positive control.13) The HT1376 bladder carcinoma cell line (HLA-A2402/) from which the ART4 gene was cloned was used as a positive control. The other cancer cell lines used in this study included QG56 lung SCCs (HLA-A2601/), VA13 fibroblast cells (no expression of HLA-class I alleles), and PHA-blastoid T cells that were prepared by incubation of HLA-A24+ PBMCs (peripheral blood mononuclear cells) with 10 µM PHA for 4 days.

**Detection of tumor antigens** Expression of these tumor antigens in cell lines, cancer tissues, benign tumor tissues, and non-tumorous tissues was investigated by western blot analysis with polyclonal antibodies as reported previously.17–21) Expression of HLA-class I or HLA-A24 antigens on tumor cells was studied by staining of the cells with anti-HLA-class I (W6/32) mAb recognizing a polymorphic region of the HLA-class I molecule or anti-HLA-A24 mAb recognizing a polymorphic region of the HLA-A24 molecule (One Lambda, Inc., Canoga Park, CA), respectively. The expression was measured by FACSscan (Becton Dickinson, San Jose, CA) as reported previously.20)

**Peptides and CTL induction** The peptides used in this study were as follows: SART1690–698 (EYRGFTQDF), SART2693–699 (DYSARWNEI), SART2696–700 (AYDFLYNKL), SART2699–703 (SYTRFLFIL), SART3109–118 (VYDYНCH-VDL), SART3115–123 (AYIDFEMKИ), ART413–23 ( AFLRHAAAL), ART215–44 (DYPVSЛAСTDI) peptides. These peptides were able to induce HLA-A24-restricted CTLs as reported previously.16–21) HIV peptide (RYLRDQQLLG] capable of binding to HLA-A24 was used as a negative control. These peptides were kindly provided by Dr. Kanaoka (Sumitomo, Osaka) and the purity was >95%. The method for CTL induction by the peptide was described elsewhere.20) In brief, PBMCs from gynecologic 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 (Life Technologies, Inc., Rockville, MD), 10% fetal calf serum (FCS) (EQUITECH BIO, Ingram, TX) with 100 units/ml of interleukin-2 (Shionogi Pharm. Co., Osaka)]. At days 7 and 14 of culture, the cells were harvested, washed, and re-incubated with the irradiated (30 grays) autologous PBMCs acting as antigen-presenting cells, which had been pre-incubated with the same peptide at the same dose for 2 h. Effector cells were harvested at day 21 of culture, and were immediately tested for both surface phenotypes and the ability to produce interferon-γ in response to various target cells by an ELISA at various effector-to-target cell (E/T) ratios in triplicate assays. The limit of sensitivity of the ELISA was 10 pg/ml. The peptide specificity of effector cells was examined by incubation of these cells for 18 h with C1R-2402 cells that were pre-loaded with 10 µM peptide, followed by harvesting of the supernatant for measurement of interferon-γ production, as reported previously.19, 20) For a 6-h 51Cr-release assay, these peptide-stimulated PBMCs (5000 cells/well) were further cultured in a 96-well U-bottomed microculture plate in the presence of feeder cells (irradiated autologous PBMCs) that had been pre-pulsed with the corresponding peptide in order to obtain large numbers of effector cells, as reported previously.15–20) Briefly, effector cells (1000 cells/well) were incubated with the above culture medium in the presence of both irradiated autologous PBMCs (1×106 cells/well) and 10 µM corresponding peptide. Seven to 10 days later, the expanded cells were transferred to wells of a 24-well plate and incubated in the absence of either a peptide or feeder cells for an additional 21–35 days. The surface phenotypes (CD3, CD4 and CD8) and the ability to produce interferon-γ of these cells were re-checked and then the cells were tested for their cytotoxicity against 51Cr-labeled target cells at different E/T ratios in triplicate assay.

**RESULTS**

**Expression of SART1 antigen** The 43-kD of SART1 antigen in the cytosol fraction was detected in 8 of 12 (67%) ovarian cancer cell lines, and in 15 of 27 (56%) ovarian cancer tissues. Representative results are shown in Fig. 1A, and a summary is given in Table I. The SART1 antigen was detectable in many different histological types of ovarian cancers, including cystadenocarcinoma, clear cell carcinoma and endometrioid cell carcinoma. In contrast, it was not detectable in the cytosol fraction of any of the normal ovarian tissues tested (Fig. 1A and Table I). Its expression in cervical and endometrial cancers has been reported elsewhere,6) although the same results are shown in Table I to facilitate better understanding.

**Expression of SART2 antigen** The 100-kD SART2 antigen in the cytosol fraction was detected in 12 of 12 (100%) ovarian cancer cell lines, in 13 of 28 (46%) ovarian cancer tissues, in 6 of 7 (85%) cervical cancer cell lines, in 25 of 38 (66%) cervical cancer tissues, in 0 of 2 endometrial cancer cell lines, and in 12 of 40 (30%) endo-
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metrial cancer tissues. Representative results are shown in Fig. 1B, and a summary is given in Table I. In contrast, it was not detectable in the cytosol fraction of any of the normal uterine or ovarian tissues tested (Fig. 1 and Table I). However, it was detectable in 6 of 7 (86%) benign tumors (myoma) tested (Table I).

**Expression of SART3 antigen** The 140-kD SART3 antigen in the cytosol fraction was detected in 10 of 10 (100%) ovarian cancer cell lines, in 19 of 23 (83%) ovarian cancer tissues, in 8 of 8 (100%) cervical cancer cell lines, in 25 of 37 (68%) cervical cancer tissues, in 2 of 2 endometrial cancer cell lines, and in 28 of 38 (74%) endometrial cancer tissues. Representative results are shown in Fig. 1C, and a summary is given in Table I. In the nuclear fraction, the SART3 antigen was detected in 10 of 10 (100%) ovarian cancer cell lines, in 19 of 23 (83%) ovarian cancer tissues, in 7 of 8 (88%) cervical cancer cell lines, in 27 of 38 (71%) cervical cancer tissues, in 2 of 2 endometrial cancer cell lines, and in 22 of 25 (88%) endometrial cancer tissues (Table I). In contrast, it was not detectable in either the cytosol or the nuclear fraction of any of the normal uterine or ovarian tissues or in the benign tumors tested (Fig. 1C and Table I).

**Expression of ART4 antigen** The 46-kD ART4 antigen in the cytosol fraction was detected in 10 of 12 (83%) ovarian cancer cell lines, in 11 of 14 (79%) ovarian cancer tissues, in 5 of 5 (100%) cervical cancer cell lines, in 7 of 8 (88%) cervical cancer tissues, in 2 of 2 endometrial cancer cell lines, and in 6 of 8 (75%) endometrial cancer tissues. Representative results are shown in Fig. 1D, and a summary is given in Table I. In the nuclear fraction, the ART4 antigen was detected in 9 of 12 (75%) ovarian cancer cell lines, in 10 of 14 (71%) ovarian cancer tissues, in 5 of 5 (100%) cervical cancer cell lines, in 5 of 8 (63%) cervical cancer tissues, in 2 of 2 endometrial cancer cell lines, and in 6 of 8 (75%) endometrial cancer tissues (Table I). In contrast, it was not detectable in either the cytosol or the nuclear fraction of any of the normal ovarian tissues tested (Fig. 1D and Table I). A part of the results on ART4 expression in cervical and endometrial

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**Fig. 1.** Expression of the SART1\(_{259}\), SART2, SART3, and ART4 antigens. Expression of SART1\(_{259}\), SART2, SART3, and ART4 antigens in various samples of uterine and ovarian cancer cell lines, uterine and ovarian cancer tissues, non-tumorous ovarian tissues and non-tumorous uterine tissues was investigated by western blot analysis with polyclonal antibodies by the method reported previously.\(^{16-21}\) A: Representative results on SART1\(_{259}\) antigen expression in the cytosol fraction are shown. Samples were KE4 (a positive control), PBMCs (a negative control), ovarian cancer cell line (KOC-2S and RMG-II), ovarian cancer tissue (OC3 and OC35), and non-tumorous ovarian tissue (OT4). B: Representative results on SART2 antigen expression in the cytosol fraction are shown. Samples were KE4 (a positive control), MKN45 (a negative control), ovarian cancer cell line (KOC-2S), cervical cancer cell line (SKG-IIIb), ovarian cancer tissue (OC7), cervical cancer tissue (CC8), non-tumorous ovarian tissue (OT4), and non-tumorous uterine tissue (UT3). C: Representative results on SART3 antigen expression in the cytosol fraction are shown. Samples were KE4 (a positive control), PBMCs (a negative control), ovarian cancer cell line (RMG-II), cervical cancer cell line (SKG-IIIb), ovarian cancer tissue (OC29), cervical cancer tissue (CC6), non-tumorous ovarian tissue (OT7), and non-tumorous uterine tissue (UT1). D: Representative results on ART4 antigen expression in the cytosol fraction are shown. Samples were HT1376 (a positive control), PBMC (a negative control), ovarian cancer cell line (KOC-2S), cervical cancer cell line (SKG-IIIb), ovarian cancer tissues (OC7), cervical cancer tissue (CC6), non-tumorous ovarian tissue (OT4), and non-tumorous uterine tissue (UT5).
Table I. Expression of Tumor-rejection Antigens in Gynecologic Cancers

| Tissue Type                      | SART1259 | SART2 | SART3 | ART4 |
|----------------------------------|----------|-------|-------|------|
|                                  | (Cytosol)| (Cytosol) | (Cytosol) | (Cytosol) | (Nucleus) | (Nucleus) | (Cytosol) | (Nucleus) |
| Ovarian cancers                  | Cell line | Tissue | Cell line | Tissue | Cell line | Tissue | Cell line | Tissue | Cell line | Tissue | Cell line | Tissue |
| Serous cystadenocarcinoma         | 1/2      | 3/7    | 2/2     | 6/9    | 2/2       | 7/7    | 2/2       | 7/8    | 1/2       | 3/4    | 1/2       | 2/4    |
| Mucinous cystadenocarcinoma       | 2/3      | 4/5    | 3/3     | 1/2    | 2/2       | 1/2    | 2/2       | 0/2    | 2/3       | 1/1    | 1/3       | 1/1    |
| Clear cell carcinoma             | 3/4      | 0/1    | 4/4     | 1/3    | 3/3       | 2/2    | 3/3       | 2/2    | 4/4       | 1/1    | 4/1       | 0/1    |
| Endometrioid cell carcinoma      | 2/2      | 1/4    | 3/3     | 3/3    | 0/1       | 0/1    | 0/1       | 0/1    | 0/1       | 0/1    | 0/1       | 0/1    |
| Borderline malignancy            | 4/5      | 1/3    | 3/3     | 2/2    | 1/2       | 1/2    | 1/2       | 1/2    | 1/2       | 1/2    | 1/2       | 1/2    |
| Other types                      | 2/3      | 2/7    | 3/3     | 3/7    | 3/3       | 3/6    | 3/3       | 5/6    | 3/3       | 5/5    | 3/3       | 5/5    |
| Non-tumorous ovarian tissues     | 0/4      | 0/2    | 0/2     | 0/2    | 0/2       | 0/2    | 0/2       | 0/2    | 0/2       | 0/2    | 0/2       | 0/2    |
| Cervical cancers                 | 4/8c)    | 13/37 (35%) | 6/7 (85%) | 25/38 (66%) | 8/8 (100%) | 25/37 (68%) | 7/8 (88%) | 27/38 (71%) | 5/5d) | 7/15 (88%) | 5/5d) | 5/8 (63%) |
| Other types                      | 4/5      | 9/28   | 4/5     | 20/29  | 6/6       | 20/29  | 6/6       | 20/29  | 3/3       | 7/8    | 3/3       | 5/8    |
| Endometrioid cancers             | 0/3      | 4/9    | 2/5     | 5/9    | 2/2       | 5/8    | 1/2       | 7/9    | 2/2       | 2/2    | 2/2       | 2/2    |
| Adenocarcinoma                   | 11/37    | 0/2    | 12/40   | 2/2    | 28/38     | 2/2    | 22/25     | 2/2    | 2/2       | 6/8    | 2/2       | 6/8    |
| Other types                      | 10/36    | 0/1    | 11/38   | 1/1    | 26/36     | 1/1    | 20/23     | 1/1    | 1/1       | 5/6    | 1/1       | 4/6    |
| Myoma (Benign)                   | 1/1      | 0/1    | 1/2     | 1/1    | 2/2       | 1/1    | 2/2       | 1/2    | 1/1       | 1/1    | 2/2       | 1/1    |
| Non-tumorous uterine tissues     | 0/7      | 0/7    | 0/7     | 0/7    | 0/7       | 0/7    | 0/7       | 0/7    | 0/7       | 0/7    | 0/7       | 0/7    |

a) Expression of SART1259, SART2, SART3 and ART4 protein in gynecologic cancer cells and tissues and normal cells was investigated by western blot analyses.
b) Values represent number of positive samples/total number of samples tested (%).
c) These results were already reported.20)
d) A part of these results was reported elsewhere.21)
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Fig. 2. Cytotoxicity of the SART3-peptide-induced CTLs in an ovarian cancer patient. PBMCs from an HLA-A24+ ovarian cancer patient were stimulated with no peptide, SART3309–323, or SART3315–323, by the methods described in “Materials and Methods.” Representative results (from an HLA-A24+ ovarian cancer patient) are shown. These PBMCs were tested for their activity to produce interferon-γ at three different E/T ratios in response to HLA-A24+SART3+ ovarian cancer cells (KOC-2S and RMG-II) and esophageal SCC cells (KE4) used as a positive control, HLA-A24+SART3+ ovarian cancer cells (RMUG-S) and fibroblast cells (VA13) used as negative controls. The supernatant was collected to measure interferon-γ by means of ELISA (Otsuka Pharm. Co., Tokyo) in triplicate assay (Fig. 2, A-1 and B-1). These PBMCs were also tested for their cytotoxicity against these tumor cells and the PHA-blastoid cells of an HLA-A24+ healthy donor, and against the HLA-A24− target cells (K562 and VA13) by a 6-h 51Cr-release assay at three different E/T ratios (Fig. 2, A-2 and B-2), respectively. * P<at least 0.05. ■ KOC-2S (2402/), × RMUG-2 (0207/3302), × K562, ◆ VA13 (−/−), ▲ PHA blast.

Fig. 3. Cytotoxicity of the SART3-peptide-induced CTLs in a cervical cancer patient. PBMCs from an HLA-A24+ cervical cancer patient were stimulated with no peptide, SART3309–323, or SART3315–323, by the methods described in “Materials and Methods.” Representative results from an HLA-A24+ cervical cancer patient are shown. These PBMCs were tested for their activity to produce interferon-γ at three different E/T ratios in response to HLA-A24+SART3+ cervical cancer cells (SKG-IIIb) and esophageal SCC cells (KE4) used as positive controls, HLA-A24− SART3+ cervical cancer cells (OMC-1), fibroblast cells (VA13) and lung adenocarcinoma cells (QG56) used as negative controls. The supernatant was collected to measure interferon-γ by means of ELISA (Otsuka Pharm. Co.) in triplicate assays (Fig. 3, A-1 and B-1). These PBMCs were also tested for their cytotoxicity against these tumor cells and the PHA-blastoid cells of an HLA-A24+ healthy donor, and against the HLA-A24− target cells (VA13) by a 6-h 51Cr-release assay at three different E/T ratios (Fig. 3, A-2 and B-2), respectively. * P<at least 0.05. ■ KE-4 (HLA-A24+), ● SKG-IIIb (HLA-A24+), ■ VA13 (−/−), * OMC-1 (HLA-A24−), ▲ PHA-blast (HLA-A24+).
after having been stimulated with the SART1690, SART2125 would be more appropriate for use in specific immunotherapy of cervical cancer patients than in ovarian or endometrial cancer patients. The SART3 and ART4 antigens were detectable in both the cytosol and nuclear fractions of the majority of gynecologic cancer cell lines and tissues, but undetectable in non-tumorous uterine or ovarian tissues. These results are consistent with our findings on other types of cancer.20,21 There was no obvious correlation between the expression of any of these antigens and the clinical stage or histological grade of the tumors (data not shown).

Our findings in the present study showed that the SART3109–118 and SART3315–323 peptides induced HLA-A24-restricted and tumor-specific CTLs recognizing SART3 tumor cells, including ovarian and cervical cancer cells, in PBMCs from ovarian and cervical cancer patients. These CTLs failed to lyse either HLA-A24+ SART3+ cancer cells or HLA-A24+ PHA-blastoid normal cells. These results indicate the presence of CTL precursors reacting to SART3 epitopes in gynecologic cancer cells in the circulation of these cancer patients. We previously reported that these SART3-derived peptides were able to induce HLA-A24-restricted and tumor-specific CTLs in most of the cancer patients tested, but in none of the healthy donors.20 Taken together, these results suggest that the SART3 antigen and peptides could be useful in specific immunotherapy of HLA-A24+ gynecologic cancer patients. Further, this study showed that the PBMCs of gynecologic cancer patients produced significant amounts of interferon-γ when stimulated with the SART11690–698, SART2161–169, and ART413–21 peptides, but not with the other peptides (Table II).

or ART413–21 peptide. PBMCs from an HLA-A24+ cervical cancer patient produced significant amounts of interferon-γ in response to KE4 and SKG-IIIb tumor cells after having been stimulated with the SART11690–698, SART2161–169, and ART413–21 peptides, but not with the other peptides (Table II).

**DISCUSSION**

This study investigated the expression of four tumor-rejection antigens in gynecologic cancers. SART1259 was detected in 56% of ovarian cancers investigated in this study, in 35% of cervical cancers, and in 30% of endometrial cancers as reported previously.16 It was not detectable in the cytosol fraction of any of the normal uterine or ovarian tissues or in the benign tumors tested. Therefore, SART1259 would be more appropriate for use in specific immunotherapy of ovarian cancer patients than in cervical or endometrial cancer patients. SART2 was detected in 66% of cervical cancers, in 46% of ovarian cancers and in 30% of endometrial cancers. In the previous study, we reported that SART2 was detectable in the majority of SCCs from various organs (head and neck, esophagus, and lung) tested.19 Although detectable in the majority of benign tumors (myoma), it was undetectable in all non-tumorous uterine tissues and non-tumorous ovarian tissues tested. Consequently, SART2 would be more appropriate for use in specific immunotherapy of cervical cancer patients than in ovarian or endometrial cancer patients. The SART3 and ART4 antigens were detectable in both the cytosol and nuclear fractions of the majority of gynecologic cancer cell lines and tissues, but undetectable in non-tumorous uterine or ovarian tissues. These results are consistent with our findings on other types of cancer.20,21 There was no obvious correlation between the expression of any of these antigens and the clinical stage or histological grade of the tumors (data not shown).

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