Identification of a Gene Coding for a Protein Possessing Shared Tumor Epitopes Capable of Inducing HLA-A24-restricted Cytotoxic T Lymphocytes in Cancer Patients

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ABSTRACT

Genes encoding tumor epitopes that are capable of inducing CTLs against adenocarcinomas and squamous cell carcinomas, two major human cancers histologically observed in various organs, have rarely been identified. Here, we report a new gene from cDNA of esophageal cancer cells that encodes a shared tumor antigen recognized by HLA-A2402-restricted and tumor-specific CTLs. The sequence of this gene is almost identical to that of the KIAA0156 gene, which has been registered in GenBank with an unknown function. This gene encodes a Mr 140,000 protein that is expressed in the nucleus of all of the malignant tumor cell lines tested and the majority of cancer tissues with various histologies, including squamous cell carcinomas, adenocarcinomas, melanomas, and leukemia cells. However, this protein was undetectable in the nucleus of any cell lines of nonmalignant cells or normal tissues, except for the tests. Furthermore, this protein was expressed in the cytosol of all of the proliferating cells, including normal cells and malignant cells, but not in normal tissues, except for the tests and fetal liver. Two peptides of this protein were recognized by HLA-A2402-restricted CTLs and were able to induce HLA-A24-restricted and tumor-specific CTLs from peripheral blood mononuclear cells of most of HLA-A24+ cancer patients tested, but not from peripheral blood mononuclear cells of any healthy donors. These peptides may be useful in specific immunotherapy for HLA-A24+ cancer patients with various histological types.

INTRODUCTION

Many genes encoding tumor antigens and peptides that are recognized by CTLs have been identified from melanoma cDNA (1–15). Some of these peptides can induce CTLs that recognize tumor cells from PBMCs of melanoma patients (16–20). Immunotherapy with some of the peptides that are capable of inducing HLA class I-restricted CTLs has been shown to result in tumor regression in HLA-A0201 melanoma patients (21, 22). These results indicate that identification of the peptides capable of inducing CTLs may provide a new modality of cancer therapy. However, only a few tumor rejection antigen genes have been identified from the adenocarcinomas and SCCs, which are, histologically, the most frequently observed cancers in various organs (23–28). In addition, peptides capable of inducing CTLs against these cancers have not yet been fully identified. We have recently reported a SART1 gene coding for tumor antigens and peptides from the cDNA of SCCs (25). Here, we investigated new genes encoding CTL-directed antigens from SCCs, and we report a gene encoding epitopes that are capable of inducing CTLs in PBMCs of patients with SCCs and adenocarcinomas.

MATERIALS AND METHODS

Generation of HLA-A2402-restricted CTLs. HLA-A2402-restricted and tumor-specific CTLs were established from the PBMCs of an esophageal cancer patient (HLA-A2402/A2601) by the standard method of mixed lymphocyte tumor cell culture, as reported previously (29). Briefly, the patient’s PBMCs were repeatedly stimulated with the autologous tumor cell line (KE4) in the culture medium (45% RPMI 1640, 45% AIM-V medium (Life Technologies, Inc., Grand Island, NY), and 10% FCS (Equitech Bio, Ingram, TX) with 100 units/ml interleukin 2 (Shionogi Pharmaceutical Co., Osaka, Japan)). The CTLs were tested for cytotoxicity to various cancer cells, EBV-transformed B cells, and normal cells in a 6-h 51Cr release assay, as reported previously (29), at various E:T ratios.

Identification of the Clone 13 Gene. Previously reported expression gene cloning methods were used in this study to identify a gene coding for tumor antigen recognized by CTLs (25). In brief, poly(A)+ RNA of the KE4 tumor cells was converted to cDNA, ligated to the Sfi adapter, and inserted into the expression vector pSV-SPORT-1 (Life Technologies, Inc., Gaithersburg, MD), cDNA of HLA-A2402 or HLA-A0201 was obtained by reverse transcription-PCR and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). Both 200 ng of plasmid DNA pools or clones of the KE4 cDNA library and 200 ng of the HLA-A2402 cDNA were mixed with 1 µl of Lipofectin in 70 µl of Opti-MEM (Life Technologies, Inc.) for 15 min. Thirty µl of the mixture were then added to the VA13 (2 × 106) cells and incubated for 5 h. Next, 200 µl of RPMI 1640 containing 10% FCS were added and cultured for 2 days followed by the addition of CTLs (105 cells/well). After an 18-h incubation, 100 µl of supernatant were collected and measured IFN-γ by an ELISA kit in a duplicate assay, as reported previously (25). This concentration (100 ng/ml) of HLA-A2402 cDNA was chosen based on the fact that expression levels of HLA-A24 antigens on the surface of VA13 cells transfected with 50, 100, 200, and 400 ng/ml of HLA-A2402 cDNA were 35, 42, 40, and 20% by FACSscan analysis with anti-HLA-A24 mAb, respectively. DNA sequencing was performed with dideoxynucleotide sequencing method using a DNA Sequence kit (Perkin-Elmer Corp., Foster, CA), and the sequence was analyzed by the ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

Northern Blot Analysis. Nylon membranes (Hybond-N; Amersham, Buckinghamshire, United Kingdom) with UV-fixed total RNA (5 µg/lane) from various tissues were prehybridized for 20 min and hybridized overnight at 65°C in the same solution [7% SDS, 1 mM EDTA, and 0.5 mM NaHPO4 (pH 7.2)], containing the 32P-labeled clone 13 as a probe. The membranes were washed three times at 65°C in a washing buffer [1% SDS and 40 mM NaHPO4 (pH 7.2)] and then autoradiographed. We tentatively named this full-length gene encoding a tumor antigen recognized by HLA-A2402 restricted CTLs the SART3 gene. The relative expression level of the SART3 mRNA was calculated by the following formula: index = (SART3 density of a sample/β-actin density of a sample) × (β-actin density of the KE4 tumor/SART3 density of the KE4 tumor).

Cloning of the SART3 Gene. The SART3 gene was obtained from the cDNA libraries of both KE4 and PBMCs of healthy donors by the standard colony hybridization method using the 32P-labeled clone 13 as a probe, as reported previously (25). The differences in sequence at nucleotide positions 88 and 547 of SART3 between the PBMCs and KE4 were further analyzed by treatment of the PCR products with restriction enzyme Del1 and Alul, respectively. The primers used for the amplification were: 5′-CGGAAACCTCCGCTTCGAGAC-3′ (forward) and 5′-GATGGCCTGGAACAGAGACC-3′ (reverse) and 5′-CCACCACT-3′.

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The abbreviations used are: PBMC, peripheral blood mononuclear cell; SCC, squamous cell carcinoma; SART, SCC antigen recognized by T cells; ORF, open reading frame; Ab, antibody; mAb, monoclonal Ab; PHA, phytohemagglutinin; aa, amino acid.
GAGTACTGGCC-3' (reverse), which covered the regions around positions 88 and 547, respectively. The PCR was carried out for 45 cycles of 0.5 min at 94°C and 1 min at 60°C using the AmpliTaq Gold DNA polymerase (PerkinElmer).

Preparation of the SART3-tag Fusion Protein in Expression Vector Constructs. For preparation of SART3myc, SART3 cDNA was digested with EcoRI and BstEII. The SART3 gene at positions 2390–2895 was amplified by reverse transcription-PCR using 5'-GGGTTCAGTGTCGACACCTTCC-3' (forward) and 5'-CTTCAACACTTTCCGAACGTTGGCACATC-3' (reverse). The amplified product was digested with BstEII and XhoI. The EcoRI-BstEII and BstEII-XhoI fragments were then together ligated to the EcoRI and XhoI sites of pcDNA3.1/Myc-His A vector (Invitrogen, San Diego, CA). The gene encoding a tag peptide was ligated to the 2880 position before the stop codon of the ORF, which was used as the SART3myc.

Western Blot Analysis. The SART3 obtained from the KE4 tumor cDNA library was digested with Sall and NotI at the multiple cloning site of pSV-SPORT and then ligated into the pGEX-4T-1 vector (Pharmacia Biotech AB, Uppsala, Sweden) for preparation of the SART3-glutathione S-transferase fusion protein (SART3GST) in expression vector constructs, as reported previously (25). Polyclonal anti-SART3myc Ab was prepared by the immunization of rabbits with the purified SART3GST by methods reported previously (30). Anti-myc mAb (Invitrogen) was also used for the Western blot analysis.

The samples were lysed with a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO), and 0.03 trypsin inhibitor unit/ml aprotinin. They were then sonicated and centrifuged at 12,000 rpm for 20 min, and the supernatant was used as the cytosol fraction. The resulting pellet was lysed with a buffer consisting of 7.2 mM urea, 1.6% Triton X-100, 0.8% DTT, and 2% lithium dodecyl sulfate and then centrifuged, and the supernatant was used as the nuclear fraction. The lysate was separated by SDS-PAGE. The proteins in an acrylamide gel were blotted to a Hybond-polyvinylidene difluoride membrane (Amersham) and then incubated with appropriate Abs for 4 h at room temperature. The other methods used for the Western blot analysis have been described previously (30).

Construction of Deletion Mutants. The SART3pcMV-SPORT plasmid was digested with BamHI, HindIII, and SphI. The restricted sites of the three enzymes were restricted at multiple restriction sites of the pcMV-SPORT vector and were unique at positions 1060, 1234, and 2165 of the SART3 gene, respectively. The linearized SART31–1060, SART31–1234-, and SART31–2165pcMV-SPORT were separated, purified, and ligated to prepare the three deletion mutants (SART31–1060, SART31–1234-, and SART31–2165).

Peptides and CTL Assays. The SART3-derived peptides capable of binding to the HLA-A2402 molecules were searched for in the literature regarding peptides for HLA-A24-binding motifs (31), and 21 different peptides were synthesized. These peptides were kindly provided by Dr. M. Kanaoka (Research Division of Sumitomo Pharmaceutical Co., Osaka, Japan). The purity was >70% in most of the peptides, and >95% for the dose dependency assay and the induction of CTLs. For detection of antigen peptides, the HLA-A2402 or A0201 cDNA (as a control)-transfected VA13 (2 × 105) cells were pulsed with a cocktail of peptides at a final concentration of 10 μM for 2 h. The CTLs (4 × 105) were then added and incubated for 18 h, and 100 μl of supernatant were collected to measure IFN-γ by ELISA in a triplicate assay, as reported previously (25). The KE4 CTL sublines that were used to test peptide specificity were established from the parental HLA-A2402-restricted KE4 CTLs by the limiting dilution culture (10 cells/well), as reported previously (25). CIR-A*2402 cells (32) pulsed with 10 μM of SART3109–118 or SART390–118 were used as targets to test the peptide-specific reactivity of these CTLs.

Induction of CTLs by the Peptides. The method for CTL induction by a peptide has been described previously (25). Briefly, PBMCs from healthy volunteers or cancer patients were incubated with 10 μM peptide in one well of a 24-well plate. After 7, 14, and 21 days of culture, the cells were incubated with irradiated (50 Gy) autologous PBMCs acting as antigen-presenting cells that had been preincubated with the same peptide at the same dose for 3 h. Effector cells were harvested at day 21 of culture, in the case of cancer patients, and at day 21 and 28 of culture, in the case of healthy donors, and most of the cells were immediately tested for their activity to produce IFN-γ in response to various target cells by an ELISA (limit of sensitivity, 10 pg/ml). For a 51Cr release assay, the patients' PBMCs (5000 cells/well), which had been stimulated three times with the peptide, were further cultured in a 96-well U-bottomed microculture plate in the presence of feeder cells, consisting of irradiated autologous or HLA-A24* allogeneic PBMCs (2 × 103 cells/well) that had been preincubated with a corresponding peptide. Seven to 10 days later, the expanded cells were transferred to 24-well plate and incubated in the absence of either a peptide or feeder cells for 14–20 days. Their CTL activity was rechecked by an IFN-γ production assay, and the cells were then tested for their cytotoxicity by a 6-h 51Cr release assay at different E:T ratios.

RESULTS

Establishment of HLA-A24-restricted CTLs. A HLA-A2402-restricted and tumor-specific CTL line was established from PBMCs of an esophageal cancer patient (KE4, HLA-A2402/2601) by repeated stimulation with KE4 autologous tumor cells, as reported previously (29). This CTL line began to show HLA-A2402-restricted cytotoxicity at around day 33 of culture, after being stimulated five times with the KE4 tumor cells. The CTLs lysed all of the HLA-A2402+ SCC or adenocarcinoma cell lines tested. However, this CTL line did not lyse any HLA-A2402+ SCC or adenocarcinoma cell lines or HLA-A2402+ PHA-activated T cells. Some of the representative results are shown in Fig. 1. The surface phenotype of this CTL line was CD3+ 4+ 8+, and the CTL activity was inhibited by 0.1 mg/ml anti-CD8 (IgG2a; Nichirei, Tokyo, Japan) or anti-HLA-class I (IgG2a, W6/32; American Type Culture Collection, Manassas, VA) but not by 0.1 mg/ml anti-CD4 (IgG1; Nichirei), anti-HLA-DR (IgG2a; SRL, Tokyo, Japan) mAb, or an irrelevant isotype-matched mAb [anti-CD13 (IgG1, MCS-2; developed in our laboratory) or anti-CD14 mAb (IgG2a; SRL); data not shown]. These results suggested that this CTL line recognized tumor cells in an HLA-A2402-restricted manner, and therefore, it was used in the following experiments.

Identification of the SART3 Gene. A total of 105 clones from the cDNA library of KE4 tumor cells were tested for their ability to stimulate IFN-γ production by HLA-A2402-restricted CTLs after cotransfection with HLA-A2402 into the VA13 cells. After repeated experiments with the several candidate clones, one clone (clone 13) was confirmed to encode a tumor antigen recognized by the CTLs when cotransfected with HLA-A2402 (Fig. 2A). Namely, the CTLs produced the significant amounts of IFN-γ by recognition of VA13 cells cotransfected with HLA-A2402 cDNA (100 ng/well) and 12.5 to 200 ng/ml of clone 13 cDNA with a dose-dependent manner but not with HLA-A0201 and clone 13. Reduction of IFN-γ production at the 400 ng/ml clone 13 cDNA could be due to the decreased levels of the SART3 protein expression, as measured by the Western blot analysis with the polyclonal anti-SART3 Ab (data not shown).

The sequence of clone 13 proved to be 1290 bp. Expression of this...
gene at the mRNA level was investigated by Northern blot analysis using the radiolabeled clone 13 as a probe, and a band of ~3.8 kb was observed in all of the malignant and normal cell lines and also in all 15 normal tissues tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, and colon). Some of the data are shown in Fig. 3. The relative level of mRNA expression was within the range of 1.2 ± 0.3 in tumor cell lines and 0.7 ± 0.2 in normal tissues, relative to the level of expression in the KE4 tumor cells, considered to be 1.0. In the tumor cell lines tested, relatively higher levels of expression were observed in Colo201 colon tumor (2.3), R27 breast tumor (1.9), and HSC4 oral tumor cells (1.8). In the normal tissues tested, relatively higher levels of expression were observed in the brain (1.1), placenta (1.0), pancreas (1.1), and testis (1.1), and lower expression levels were seen in skeletal muscle (0.4), thymus (0.4), colon (0.4), and PBMCs (0.2). mRNA expression of the SART3 was also observed in surgically removed cancerous lesions, nonmalignant adjacent lesions, and benign uterine myomas (data not shown). These results suggest that this gene is ubiquitously expressed at the mRNA level and that the 1290-bp cDNA was incomplete. A 3806-bp gene was then independently cloned from the cDNA libraries of KE4 tumor and PBMCs of healthy donors using clone 13 as a probe with colony hybridization methods, as reported previously (25). The nucleotide sequences of these new clones were identical, with the exception of two nucleotides: the C in KE4 versus G in PBMCs at nucleotide position 88 and the C in the KE4 versus T in PBMCs at position 547 (Fig. 4). These differences could be due to polymorphism but not to point mutations because position 88 was C in the KE4, TE8, and TE9 esophageal SCC tumor cell lines, an EBV-transformed B cell line from the KE4 patient (BEC-1), and PBMCs from a healthy donor, whereas position 88 was G in fetal liver and VA13 fibroblast cells. PBMCs from another healthy donor and a sample of testis contained both C and G. Position 547 was C in the KE4, TE8, TE9, BEC-1, VA13, and fetal liver, whereas a sample of PBMCs from a healthy donor contained both C and T. An aa translated from a codon containing nucleotide position 88 was aspartic acid (GAC) or glutamic acid (GAG), whereas the aa was identical translated from a codon containing nucleotide position 547 (GCC, GCU = alanine) in the ORF. This changed residue was not in the context of HLA-A24 antigen-binding motifs (31). The SART3 gene cloned from either the KE4 and PBMCs encoded the antigens recognized by the CTLs when cotransfected with HLA-A2402 but not with HLA-A0201 (Fig. 2B).

The nucleotide sequence of this 3806-bp SART3 gene was almost identical to that of the KIAA0156 gene already registered in the GenBank (accession no. D63879). The KIAA0156 gene was isolated from a human myeloid cell line (KG-1) in a human gene cloning project (33). Except for its sequence and ubiquitous mRNA expression by Northern blot analysis, nothing is known about KIAA0156 according to a search at the literature level. KIAA0156 is 3660 bp, which is identical to the region of the SART3 located between nucleotide positions 20 and 3679, except for positions 118 and 3449 (in which G and A are replaced by A and C, respectively; Fig. 4). The SART3 gene cloned from KE4 encodes 963 aa in the second frame if its first ATG at nucleotide positions 20–92 is used as a start codon, with the aa sequence being completely identical to the translated aa sequence of KIAA0156 if its ATG at positions 1–3 is used as a start codon (Fig. 4). These results suggest that 3660 bp of the reported KIAA0156 is a truncated form of the 3806 bp of the SART3 gene. The sequence of SART3 cloned from the KE4 cDNA is available from European Molecular Biology Laboratory/GenBank/DDBJ (accession no. AB020880).

![Fig. 4. Homology between SART3 and KIAA0156 cDNA. The indicated nucleotide (nt) and aa positions refer to the SART3 sequence. The sequences of SART3 cloned from KE4 and KIAA0156 are almost identical except at positions 137 and 3468, and at the ends of 5' and 3' noncoding regions. The difference at position 137 causes a synonymous change. The shaded areas represent the ORFs of clone 13, SART3, and KIAA0156 cDNAs. Arrows, positions of the two antigenic peptides. The segments of the SART3 longer than KIAA0156 cDNA at both the 5' and 3' ends. The sequence of segment in the 5' region (19 bp) is CCGGCCGCGGCGCCGAGA, whereas that in the 3' region (129 bp) is AAAACCTTTCTGTAAGTTGACTCAGATGTGCATTCACATACAGATGTGT. The sequences of the SART3 cloned from the KE4 is available from European Molecular Biology Laboratory/GenBank/DNA Data Bank of Japan (accession no. AB020880).](image-url)
Expression of the SART3 Protein. Expression of SART3 at the protein level in various cells and tissues was studied by Western blot analysis with the polyclonal anti-SART3GST Ab. It recognized a Mr ≈ 140,000 band of a recombinant SART3 protein after cleavage of glutathione S-transferase with thrombin (data not shown). When SART3 was transfected to VA13 cells, an intensive Mr ≈ 143,000 band was observed in the cytosol fraction but not in the nuclear fraction (Fig. 5A). Furthermore, both this polyclonal Ab and anti-myc mAb recognized an Mr ≈ 143,000 band in the cytosol but not in the nucleus of VA13 cells transfected with the SART3 gene of positions 1–2880 (containing 953 aa) in conjunction with the pcDNA3.1/myc-His vector (SART3myc; Fig. 5A). The different migration of these bands (Mr ≈ 140,000 and 143,000) is probably due to a tag peptide (Mr ≈ 5000). In contrast to VA13 cells, a Mr ≈ 143,000 band was detected in both the cytosol and nuclear fractions of the KE4 or TE9 tumor cells when the SART3myc was transfected to the KE4 (Fig. 5A) or TE9 tumor cells (data not shown).

A Mr ≈ 140,000 band of SART3 protein was undetectable in either the cytosol or nuclear fractions of any of the normal tissues tested except for testis and fetal liver under the used condition. In fetal liver, the Mr ≈ 140,000 band was detected in only the cytosol fraction, and it was detectable in both the cytosol and nuclear fractions of testis. Part of these results are shown in Fig. 5B. The Mr ≈ 140,000 of SART3 protein was not observed in unstimulated PBMCs, but it did become detectable in the cytosol fraction of activated PBMCs after stimulation with 10 μg/ml PHA for 48 h (data not shown). The Mr ≈ 140,000 band was detected in both the cytosol and nucleus of all of the malignant tumor cell lines tested, including SCC or adenocarcinoma, leukemia, and melanoma cell lines as well as in more than half of fresh cancer tissues from various organs, including head and neck SCCs, esophageal SCCs, lung SCCs, lung adenocarcinomas, melanomas, and fresh leukemia cells. Some of these results are shown in Fig. 5B, and a summary is shown in Table 1. The SART3 protein was undetectable in surgically removed nonmalignant adjacent lesions or benign uterine myomas, regardless of the expression at the mRNA level.4

Identification of Antigenic Peptides. To identify the SART3-derived epitopes recognized by HLA-A2402-restricted CTLs, we initially investigated the capability of the deletion mutants of the SART3 to stimulate IFN-γ production by the CTLs. All three mutants, including the shortest one, retained the ability to stimulate IFN-γ production by the CTLs (Fig. 6A), suggesting that the antigenic epitopes were located within the first 1060 bp. Meanwhile, each of the 21 different synthesized peptides with HLA-A2402 antigen-binding motifs among the 963 aa of the SART3 was loaded onto the HLA-A2402-transfected VA13 cells and tested for its ability to induce IFN-γ production by the CTLs. High levels of IFN-γ production were induced by the SART3109–118 (VYDYNCHVDL) and SART3315–323 peptide (AYIDFEMKIL; Fig. 6B), both of which are located in the region encoded by the shortest deletion mutant (Fig. 6A). A dose-dependent reaction was observed in these two peptides (Fig. 6C). Significant levels of IFN-γ production were observed at a concentration of 0.1 μM each peptide. Low but significant levels of IFN-γ production were also induced by the SART3141–150, SART3172–181, and SART3284–292 peptide (Fig. 6B), but consistent results were not obtained with these three peptides.

To confirm the presence of SART3109–118 and SART3315–323 peptide-specific CTLs, we tested 40 different KE4 CTL sublines for their reactivity to either of the peptides. Four and 5 of the 40 different sublines showed the SART3109–118 and SART3315–323 peptide-specific reactivity, respectively. The IFN-γ production by these CTL sublines in response to a peptide was inhibited by 0.1 mg/ml anti-CD8

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or anti-HLA class I (W6/32) mAb but not by anti-CD4 or anti-HLA-class II mAb. The representative results are shown in Fig. 7. One subline (10-31) reacted to the two peptides, and the remaining 30 sublines did not react to either (data not shown).

**Induction of CTLs by the SART3 Peptides.** The SART3 109–118 and SART3 315–323 peptides were tested for their ability to induce CTLs from the PBMCs of seven HLA-A241 patients with epithelial cancer (four patients with head and neck SCCs, two with esophageal SCCs, and one with lung adenocarcinoma) and from five healthy donors. None of the PBMCs from five healthy donors produced significant levels of IFN-γ in an HLA-A24-restricted fashion when stimulated three times (data not shown) or even four times (Table 2) in vitro with either SART3 109–118 or SART3 325–323. In contrast, PBMCs from five of seven patients produced significant levels of IFN-γ in response to the HLA-A241 tumor cells but not to the HLA-A242 tumor cells when stimulated three times in vitro with SART3 109–118 (Table 2). Similarly, PBMCs from six of seven cancer patients produced significant levels of IFN-γ in response to the HLA-A241 tumor cells but not to the HLA-A242 tumor cells, the HLA-A241 EBV-transformed B cell line, or the HLA-A242 PHA-blastoid normal cells. Results of the three patients are shown in Fig. 8. The peptide-induced CTL activity tested in patients 2 and 5 was inhibited by 1 mg/ml anti-CD8 and anti-HLA-
of the mechanism because there are also multiple As located in identical regions of the KIAA0156 genes that immediately flank 3′ end of the SART3 gene or positions 1–3 of the SART3 and KIAA0156 are probably due to the processes by which the cDNA libraries are constructed. There are several As in the 3′ unique segment of SART3 immediately flanking the site corresponding to the 3′ end of KIAA0156, and the oligo(dT) primer might anneal to this region during the construction of cDNA libraries. In addition, clone 13 likely originates from the same mechanism because there are also multiple As located in identical regions of the SART3 and KIAA0156 genes that immediately flank 3′ end of the clone 13 (data not shown). Furthermore, a band of 3.8 kb was observed in the Northern blot analysis. These results suggest that the 3660 bp of the reported KIAA0156 gene is a truncated form of the 3806 bp of the SART3 gene.

The SART3 protein was undetectable in normal tissues except for the testis and fetal liver under the used condition with the polyclonal Ab, regardless of its ubiquitous expression at the mRNA level. We have also reported a similar expression pattern in the SART1 protein, the other tumor rejection antigen isolated from the KE4 tumor cells (25), and the mechanisms involved in this discrepancy are presently unclear. The SART3 protein was preferentially expressed in the cytosol of the majority of proliferating cells, including normal cells and malignant cells. Furthermore, it was expressed in the nucleus of all of the malignant cell lines and the majority of cancer tissues, regardless of their histology and origin, but not in the nucleus of any of the normal or virus-transformed proliferating cell lines or normal tissues, except for tests. We have recently developed anti-SART3 mAb useful for immunohistochemical staining of the SART3 protein in cells. The experiments with this mAb indicated that the SART3 protein was expressed in both the cytosol and nuclear fractions of the KE4 tumor cells and spermatogonia of the testis. In contrast, it was not expressed in the spermatids or Sertoli cells.5 Further studies are needed to clarify the location and distribution of the SART3 protein.

There are several motifs in the sequence of the SART3 gene, described as follows, that suggest its biological function: nuclear localization signals around positions 612–615, and the mechanisms involved in this discrepancy are presently unclear. The SART3 protein was preferentially expressed in the cytosol of the majority of proliferating cells, including normal cells and malignant cells. Furthermore, it was expressed in the nucleus of all of the malignant cell lines and the majority of cancer tissues, regardless of their histology and origin, but not in the nucleus of any of the normal or virus-transformed proliferating cell lines or normal tissues, except for tests. We have recently developed anti-SART3 mAb useful for immunohistochemical staining of the SART3 protein in cells. The experiments with this mAb indicated that the SART3 protein was expressed in both the cytosol and nuclear fractions of the KE4 tumor cells and spermatogonia of the testis. In contrast, it was not expressed in the spermatids or Sertoli cells.5 Further studies are needed to clarify the location and distribution of the SART3 protein.

DISCUSSION

The nucleotide sequence of the SART3 gene is almost identical to that of a gene with unknown function already registered in GenBank under the name KIAA0156 (33). The SART3 contains a segment that is 19 bp longer than the 5′ end of KIAA0156 cDNA. This segment contains the context of Kozak motif (AAGATGG; Refs. 34 and 35) for effective initiation of translation because the first ATG codon contains the context of Kozak motif (AAGATGG; Refs. 34 and 35).

Table 2: Induction of HLA-A24-restricted CTL activity in HLA-A24+ PBMCs by SART3-derived peptides }

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<th>Donors (disease)</th>
<th>Peptide</th>
<th>KE3 (HLA-A24)</th>
<th>KE4 (HLA-A24/26)</th>
<th>PC-9 (HLA-A24/24)</th>
<th>KES (HLA-A11/11)</th>
<th>QG56 (HLA-A26/46)</th>
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* PBMCs from five HLA-A24+ healthy donors and seven HLA-A24+ cancer patients [head and neck SCCs (n = 4), esophageal SCCs (n = 2), and lung adenocarcinoma (n = 1)] were stimulated in vitro with 10 μM peptide and tested for their ability to produce IFN-γ by recognition of various target cells at an E:T ratio of 2.

† Values represent the means of triplicate determinations. The background IFN-γ production in response to VAl3 cells (50–100 pg/ml) has been subtracted from the experimental values shown in the table.

ND, not determined.


fore, the phosphorylated SART3 protein might play a role in the metabolism of nuclear RNA. Further studies of the biological function of the SART3 protein are also needed.

The two SART3-derived peptides (SART3109–118 and SART3315–323) among the 21 peptides with HLA-A24 antigen-binding motifs tested in this study were consistently recognized by HLA-A24-restricted CTLs in repeated experiments. Because of the presence of CTL sublines reacting to either of the peptides, the parental KE4 CTLs would consist of the mixtures of these peptide-specific clones. A dose dependency was observed in these two peptides. The SART3109–118 was a peptide capable of binding to HLA-A24 antigens located in the overlapping region of the clone 13 and the shortest SART31060 mutant, which retained the ability to stimulate IFN-γ production by the CTLs. The SART3109–118 was also found in the shortest SART31060 mutant. Both peptides were able to induce HLA-A24-restricted CTLs from PBMCs of most of the HLA-A24+ cancer patients after being stimulated three times, but this induction did not occur in response to either peptide in any of the HLA-A24+ healthy donors tested, even after being stimulated four or five times. These peptides were also able to induce the CTLs from the PBMCs of a patient with gastric signet ring cell carcinoma (data not shown). Therefore, this difference may be due to the exclusive expression of SART3 in the nucleus of malignant cells. Recent results have shown that the dendritic cells-mediated cross-presentation of apoptotic tumor cells could, in part, account for the initial priming of antigen-specific CTLs in cancer patients (37, 38). Tumor cells may undergo apoptosis for many reasons in vivo, such as part of a host response to tumor cells, nutritional reasons, or the outcome of chemotherapy or radiotherapy. Indeed, the SART-3 gene product itself can induce apoptosis of TE9 tumor cells but not VA13 cells when transplanted to these cells.7 In addition, there is a RGD motif in the SART3 at positions 742–744 that might be involved in the caspase-3-mediated apoptosis (39). Dendritic cells may then acquire apoptotic tumor cells and effectively cross-present the SART3 antigens to T cells.

It is presently unclear why the KE4 CTL line or the CTLs induced by the SART3 peptides were not cytotoxic to the nonmalignant proliferating cells, in which the SART3 is also expressed in their cytosol. One possible explanation is that these SART3-derived epitopes are expressed preferentially on the HLA class I groove of malignant cells but not of nonmalignant cells. The nuclear SART3 might be an activated form and, thus, be ubiquitinized, processed, and loaded to the groove of HLA class I molecules. This assumption is partly based on the fact that a tyrosine at position 316 of the SART3 is phosphorylated.6 Another possibility can be attributed to the different posttranslational modification of the epitopes between normal and cancer cells. Some of the previously identified CTL epitopes have posttranslational modifications, and the modifications have had a significant impact on the ability of the CTLs to recognize those peptides (40–42). Further studies are needed to clarify this issue.

The HLA-A24 allele is found in ~60% of Japanese (95% of them are genotypically A2402), 20% of Caucasians, and 12% of Africans (43). The two SART3-derived peptides were able to induce HLA-A24-restricted and tumor-specific CTLs in most of the cancer patients tested but none of the healthy donors. The SART3 protein and these peptides may be appropriate molecules for use in specific immunotherapy of HLA-A24+ cancer patients with various histological types.

ACKNOWLEDGMENTS

We thank Dr. M. Takiguchi at Kumamoto University (Kumamoto, Japan) for providing an CIR-A*2402 cell line and Dr. Kunzo Orita at Hayashibara Biochemical Lab. Inc. (Okayama, Japan), for providing natural IFN-γ for ELISA.

7 D. Yang, N. Tsuda, S. Shichijo, et al., unpublished results.
REFERENCES


Identification of a Gene Coding for a Protein Possessing Shared Tumor Epitopes Capable of Inducing HLA-A24-restricted Cytotoxic T Lymphocytes in Cancer Patients

Damu Yang, Masanobu Nakao, Shigeki Shichijo, et al.


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