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An HLA-A24-restricted Cytotoxic T Lymphocyte Epitope of a Tumor-associated Protein, Survivin

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Abstract

To date an increasing number of T-cell epitopes derived from various tumor-associated antigens have been reported, and they proved to play significant roles for tumor rejection both in vivo and in vitro. Survivin was originally identified as a member of the inhibitor of apoptosis protein family. Expression of this gene is developmentally regulated. Although survivin is expressed during normal fetal development, the expression is barely detected in terminally differentiated adult tissues except for testis, thymus, and placenta. In contrast, it is abundantly expressed in a wide variety of malignant tissues. We examined the expression of survivin and the two splicing variants survivin-2B and survivin-ΔEx3 in various cancer cells, immortalized cells, and normal adult tissues. It was demonstrated that two splicing variants were detected in various types of cancer cells as well as survivin, and their expression was more restricted to cancer cells as compared with survivin expression. To identify HLA-A24-restricted T-cell epitopes from survivin and the variant proteins, three peptides were selected from amino acid sequence of these proteins, based on the HLA-A24-binding motif. Peptide binding assay to HLA-A24 revealed that only one peptide designated as survivin-2B80–88 (AYACNTSTL) was capable of binding to HLA-A24. By stimulating peripheral blood lymphocytes with the peptide-pulsed antigen-presenting cells, CTLs were successfully induced in vitro from five of five HLA-A24-positive cancer patients. The CTLs showed significant cytotoxicity against HLA-A24-positive survivin-2B-positive cancer cells. These data suggest that survivin-2B80–88 may be a potent T-cell epitope eliciting CTL response against a splicing variant survivin-2B, which is specifically expressed in many kinds of cancer cells.

Introduction

The scarcity of clinically significant immune responses against cancer have cast doubts for many years on whether antigen-specific immunotherapy could develop to clinical cancer therapy. However, pioneering studies in the early 1990s demonstrated the existence of tumor rejection antigens that were recognized by CTLs of melanoma patients (1, 2). Although these earlier studies focused primarily on melanoma, TAA2 have subsequently been characterized in other malignant tumors, raising the possibility that most, if not all, tumors express rejection antigens that host’s CTLs could potentially target (3, 4). Consequently, exciting clinical efforts are now underway to target these TAA in strategies, such as vaccination and adoptive T-cell immunotherapy generating antitumor CTL response (5, 6). Because most of the CTL epitopes used in these clinical trials were derived from melanoma antigens, the peptides could not be applied for tumors of nonmelanocytic malignancies. Furthermore, expression of these TAA is heterogeneous among tumors from different patients and even varies among tumors from different metastases of one patient. Therefore, it has been expected to identify TAA that are expressed in a wide variety of tumors or TAA that have critical roles in maintaining the malignant phenotype of tumors. During the last couple of years, a number of CTL epitopes of TAA that are commonly expressed in a number of different cancers have been identified, i.e., telomerase (7), SART-3 (8), and survivin (9, 10). Use of CTL epitope derived from such proteins for cancer vaccination may have the great advantage of being applicable to many cancer patients.

Apoptosis is a physiological suicide program that is critical for development and maintenance of tissue homeostasis. Cells with genetic abnormality can be eliminated by apoptosis. Therefore, diminished or disordered apoptosis results in accumulation of genetic mutation, which may lead to tumor initiation, progression, and therapeutic resistance. Survivin was identified as a member of the IAP family with single baculovirus IAP repeat domain (11). Survivin has a capability to inhibit caspase-3, -7, and -9 in cells receiving an apoptotic stimulus (12). Survivin is present during fetal development but undetectable in terminally differentiated adult tissues except for testis, thymus, and placenta. In contrast, it is abundantly expressed in a wide variety of tumors or TAAs that have critical roles in a number of different cancers have been identified, i.e., telomerase (7), SART-3 (8), and survivin (9, 10). Use of CTL epitope derived from such proteins for cancer vaccination may have the great advantage of being applicable to many cancer patients.

2 The abbreviations used are: TAA, tumor-associated antigen; IAP, inhibitor of apoptosis protein; RT-PCR, reverse transcription-PCR; IL, interleukin; mAb, monoclonal antibody; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; PHA, phytohemagglutinin; hTERT, human telomerase reverse transcriptase subunit.
differentiated normal adult tissues. Importantly, survivin is abundantly expressed in transformed cell lines and in most of common cancers, including colorectal cancer, non-small cell lung cancer, gastric cancer, bladder cancer, breast cancer, melanoma, neuroblastoma, hepatocellular carcinoma, and diffuse large B-cell lymphoma (13). The overexpression of survivin in tumors correlates with resistance to a variety of apoptotic stimuli, including chemotherapeutic agents and radiation therapy, and is closely associated with poor clinical prognosis.

Mahotka et al. (14) reported that two novel splice variants of survivin were expressed in renal cell carcinoma lines. One is survivin-ΔEx3 lacking exon 3 of survivin, and the other is survivin-2B containing a part of intron 2 as a cryptic exon. In their transfection experiments, survivin-ΔEx3 conserved antiapoptotic potential, and survivin-2B also had significant antiapoptotic potential, although their physiological functions have not been clarified. Using the RT-PCR method, we show here that both survivin-ΔEx3 and survivin-2B are expressed in various types of tumor cell lines as well as survivin but not in normal tissues. On the basis of this finding, we hypothesized that survivin and its two splicing variants might become target molecules of CTLs. Three peptides were determined from survivin-2B amino acid sequence according to the binding motif for HLA-A*2402, the most common allele in the Japanese population and also frequent in Caucasians. One of these peptides, survivin-2B80–88, was capable of inducing CTL response in the context of HLA-A*2402, when pulsed with APCs. CTLs were induced in vitro from both cancer patients and normal healthy donors, and the CTLs exerted cytotoxic activity against survivin-2B-expressing tumor cell lines in an HLA-A24-restricted manner. We demonstrate for the first time the HLA-A24-restricted CTL epitope derived from the survivin splicing variant, which may serve as a widely applicable antigen for cancer immunotherapy of HLA-A24-positive patients.

Materials and Methods

Cell Lines. Lung adenocarcinoma cell lines LHK-2 (HLA-A*0207, A*2402, B*4601, B*4801, and Cw*0102) and LNY-1 (HLA-A26, A31, B51, B054, C4, and C10), oral squamous cell carcinoma cell lines OSC-20 (HLA-A1, A2, HLA-B27, and C1), and EBV-transformed B-cell lines YM-EBV, KK-EBV, and N-EBV were established in our laboratory. Gallbladder adenocarcinoma cell line KMG-A (HLA-A*2601, B*4002, and Cw*0304) and esophageal squamous cell carcinoma cell line KE-4 (HLA-A*2402, A*2601, Cw*0102, and Cw*0302) were kind gifts from Dr. Kyogo Itcho (Kurume University, Kurume, Japan). Melanoma cell lines 888MEL (HLA-A1 and HLA-A24), 1102MEL (HLA-A2 and HLA-A24), and 1335MEL (HLA-A26 and HLA-A31) were kind gifts from Dr. Francesco M. Marincola (National Cancer Institute, Bethesda, MD). Melanoma cell lines LB33-MEL (HLA-A24, A28, B13, B44, Cw6, and Cw7) and LG2-MEL (HLA-A*2402, A*3201, B35, B44, and Cw04), renal carcinoma cell line BB64-RCC (HLA-A23, A26, B44, B51, Cw1, and Cw4), bladder carcinoma cell line LBr05-BLC (HLA-A24, A26, B38, B62, Cw7, and Cw9), and EBV-transformed B-cell line LG2-EVB were kind gifts from Dr. Benoit J. Van den Eynde (Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium). All of these cells were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Filitron, Brooklyn, New South Wales, Australia) and antibiotics. CIR-A*2402, a stable transfectant of CIR cells with HLA-A*2402 cDNA (a kind gift from Dr. M. Takiguchi, Kumamoto University, Kumamoto, Japan) was cultured in RPMI 1640 supplemented with 10% FBS and 500 µg/ml of hygromycin B (WAKO Chemicals, Osaka, Japan). LNY-1-A*2402, a stable transfectant of LNY-1 cells with HLA-A*2402 cDNA, was cultured in RPMI 1640 supplemented with 10% FBS and 500 ng/ml of puromycin (Sigma).

RT-PCR Analysis of the Expression of Survivin and Its Splicing Variants. A set of total RNA from normal human adult tissues was purchased from Clontech (human total RNA master panel). Total RNA was isolated from cultured cells by using ISOGEN reagent (Nippon Gene, Tokyo, Japan). The cDNA mixture was synthesized from 1 µg of total RNA by reverse transcription using Superscript II and oligo(dT) primer (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocols. PCR amplification was performed in 50 µl of PCR mixture containing 1 µl from the cDNA mixture, KOD Plus DNA polymerase (Toyobo, Osaka, Japan), and 50 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 57°C (or 64°C for specific detection of survivin-2B or survivin-ΔEx3) for 30 s, and extension at 68°C for 1 min. Primer pairs used for RT-PCR analysis were 5’TCAAGGACCCAGCATCTCTAC-3’ and 5’GCACTTCTTCGCGATTTCCTC-3’ as a forward and a reverse primer, respectively. Expected sizes of PCR products for survivin, survivin-ΔEx3, and survivin-2B were 355, 236, and 424 bp, respectively. For specific detection of survivin-2B and survivin-ΔEx3 expression, the following reverse primers were used: 5’TGCTGTTATTACAGCCGTAAAG-3’ for survivin-2B with an expected PCR product of 221 bp; and 5’TGGTTTCTTGTGATGCGG-3’ for survivin-ΔEx3 with an expected PCR product of 195 bp. As an internal control glyceraldehyde-3-phosphate dehydrogenase was detected by using a forward primer 5’ACCAAGTCTGCAATCGTC-3’ and a reverse primer 5’GCCACCCCTGTTATCTA-3’ with an expected PCR product of 452 bp. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.0% agarose gel. Nucleotide sequence of the PCR products was confirmed by direct sequencing using ABI Genetic analyzer PRIM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

Cloning of HLA-A*2402 cDNA. Total RNA from LHK-2 cells was extracted and cDNA mixture was synthesized from the RNA by reverse transcription reaction using Superscript II and oligo(dT) primer as described previously. PCR was performed using 1 µl of the reverse transcription mixture, Pfu DNA polymerase (Stratagene, La Jolla, CA), and 50 pmol of forward and reverse primers in a total reaction volume of 50 µl. Nucleotide sequences of the forward and reverse primers were 5’GACTCAGTGATATCCAGAGCCGGAGATTGCGCGTATG-3’ and 5’CGCGGATCCGGGGCCGCGAAGGACACAGTCTGATGCGGTGGAA-3’, which contain HLA-A gene-specific sequence flanked by EcoRV or BamHI restriction sequence, respectively. The amplification protocol consisted of denaturation for 15 s at
98°C, annealing for 45 s at 58°C, and extension for 4 min at 72°C for a total of 30 cycles, using a GeneAmp PCR system model 2400 (Perkin-Elmer). The PCR product was purified and cloned into pIRESpuro mammalian expression vector (Clontech, Palo Alto, CA). Nucleotide sequence of the insert was analyzed by ABI Genetic analyzer PRIM 310 using AmpliCycle sequencing kit (Perkin-Elmer).

**Synthetic Peptides.** Three peptides survivin85–93 (AFLS|VKKQF), survivin92–101 (QFEEL|TLGEF), and survivin-2B80–88 (AYACNTSTL) were derived from the amino acid sequence of survivin or survivin-2B (11, 14), based on the HLA-A24-binding motifs. Three peptides used as control peptides were EBV LMP2-derived peptide (TYGPVFMSL) and HIV env-derived peptide (RYLDRQQLLGI), which have been shown to become CTL epitopes in the context of HLA-A*2402 previously (15, 16), and F4.2 peptide (YSWMDISCWI) that was identified as HLA-A31-bound gastric cancer peptide (4). All of the peptides were synthesized and purchased from Sigma Genosys (Ishikari City, Japan). Peptides were dissolved in DMSO and stored at −80°C before use.

**Cytokines.** Human recombinant IL-2 and IL-4 were kind gifts from Takeda Pharmaceutical (Osaka, Japan) and Ono Pharmaceutical (Osaka, Japan), respectively. Human recombinant granulocyte/macrophage-colony stimulating factor was a kind gift from Novartis Pharmaceuticals (Basel, Switzerland). Human recombinant IL-7 was purchased from Life Technologies, Inc.

**Peptide Binding Assay.** Peptide binding affinity to HLA-A24 was assessed by HLA-A24 stabilization assay as described previously (16), based on the findings that MHC class I molecules could be stabilized on the cell surface in the presence of binding peptides. RMA-S A*2402/Kb cells are TAP-deficient mouse RMA-S cells transfected with a chimeric MHC class I cDNA consisting of α1 and α2 domains derived from human HLA-A*2402 molecule and α3, transmembrane, and intracellular domains derived from mouse H-2Kb molecule (kindly provided from Dr. H. Takasu, Sumitomo Pharmaceutical, Osaka, Japan). After incubation of cells in culture medium at 26°C for 18 h, cells (2 × 10⁶) were washed with PBS and suspended with 1 ml of Opti-MEM (Life Technologies, Inc.) containing 3 μg/ml of α2-microglobulin with or without 100 μg of peptide, followed by incubation at 26°C for 3 h and then at 37°C for 3 h. After washing with PBS, the cells were incubated with anti-HLA-A24 mAb (c7709A2.6, kindly provided by Dr. P. G. Coulie, Ludwig Institute for Cancer Research, Brussels Branch) at 4°C for 30 min, followed by incubation with FITC-conjugated rabbit antimouse IgG at 4°C for 30 min. The cells were then suspended with 1 ml of PBS containing 1% formaldehyde and analyzed by FACSscan (Becton Dickinson, Mountain View, CA). Binding affinity was evaluated by comparing mean fluorescence intensity of HLA-A24 expression in the presence of peptide pulsation to mean fluorescence intensity in the absence of the peptide.

**Preparation of APCs and CD8-positive T Cells from PBMCs.** PBMCs were isolated from four healthy volunteers and five cancer patients (patient 1, colon cancer; patient 2, breast cancer; patient 3, esophagus cancer; patients 4 and 5, gastric cancer) by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). PBMCs were incubated in AIM-V medium (Life Technologies, Inc.) supplemented with 2-mercaptoethanol (50 μM) and HEPES (10 mM) for 2 h at 37°C in a culture flask to separate adherent cells and nonadherent cells. Adherent cells were then cultured in the presence of IL-4 (1000 units/ml) and granulocyte/macrophage-colony stimulating factor (1000 units/ml) in AIM-V medium for 7 days to generate monocytes-derived DCs. The adherent cells containing DCs were used for APCs. CD8-positive T lymphocytes were isolated from nonadherent cells by the MACS separation system (Miltenyi Boitech, Bergish Blabach, Germany) using anti-CD8 mAb coupled with magnetic microbeads according to the manufacturer’s instruction. To obtain PHA-stimulated blasts, CD8-negative nonadherent PBMCs were cultured in AIM-V medium containing 1 μg/ml of PHA (WAKO Chemicals, Osaka, Japan) and 100 units/ml of IL-2 for 3 days, followed by washing and culturing in the presence of IL-2 (100 units/ml) for 4 days.

**CTL Induction Using Autologous DCs and PHA-Blasts.** CTL induction was performed according to a procedure described previously with a slight modification (17, 18). Briefly, autologous DCs were incubated at room temperature for 2 h in AIM-V with β2-microglobulin (2.5 μg/ml) and peptide (50 μg/ml), DCs were then irradiated (100 Gy) and washed with AIM-V medium. On day 1, 1 × 10⁵ peptide-pulsed DCs/well were plated on 24-well plates and cultured with 1 × 10⁶ CD8+ T cells in 2 ml of AIM-V supplemented with recombinant IL-7 (10 ng/ml). On day 7, 5 × 10⁵ PHA-blasts were pulsed with 50 μg/ml of peptides, irradiated (100 Gy), washed once, and then added to each well. On day 8, IL-2 was added to each well at a concentration of 50 units/ml. The peptide stimulation using PHA-blasts as APCs was repeated every 7 days. During CTL induction, cells were fed with fresh AIM-V medium supplemented with IL-2 (50 units/ml) every 3–4 days. On day 28, the cytotoxic activity of T cells was assessed by conventional 6-h ⁵¹Cr release assay.

**Cytotoxicity Assay.** The lytic activity of CTLs was tested by a ⁵¹Cr release assay as described previously (19). Briefly, target cells were labeled with 100 μCi of ⁵¹Cr for 1 h at 37°C, washed three times, and resuspended in AIM-V medium. Then ⁵¹Cr-labeled target cells (2000 cells/well) were incubated with various numbers of effector cells for 6 h at 37°C in 96-well microtiter plates. Radioactivity of the culture supernatant was measured by gamma counter. The percentage of cytotoxicity was calculated as following: % cytotoxicity = (experimental release − spontaneous release)/ (maximum release − spontaneous release) × 100. In some cases, target cells (LHK-2, LNY-1, 888-mel, and 1353-mel) were treated with 100 units/ml of IFN-γ for 48–72 h before the assay. For peptide-pulsed target cells, C1R-A*2402 or C1R-A*31012 cells were incubated with 1 μg/ml of peptide at room temperature for 1 h before the assay.

**Results**

**Expression of Survivin and Two Splicing Variants Detected by RT-PCR.** We first defined an expression profile of survivin and its two splicing variants by RT-PCR analysis using specific primers for each of these genes. The exon-intron structure of the survivin gene and mRNA are shown, and the primer sets designed for this study are also indicated in Fig. 1A.
A variety of tumor cell lines and immortalized cells were tested, including two adenocarcinoma lines, four squamous cell carcinoma lines, one melanoma line, one renal cell carcinoma line, one bladder carcinoma line, and four EBV-transformed B-cells. Our study demonstrated the expression of survivin in all of the cancer cell lines and EBV-transformed B-cells tested (Fig. 2A), which was consistent with previous reports. A low level of survivin expression was also detected in PBMCs. For detection of two splicing variants of survivin (survivin-2B and survivin-ΔEx3), RT-PCR analysis was performed using the variant-specific primer sets. It was shown that both variant forms of survivin were detected in all of the tumor cell lines and EBV-transformed B-cells but not detected in PBMCs (Fig. 2A). Expression of survivin and the splicing variants was also assessed in normal adult tissues including stomach, small intestine, large intestine, spleen, lung, kidney, prostate, pancreas, heart, and thymus. As shown in Fig. 2B, low levels of survivin expression were detected in all of these normal tissues. However, no expression of the two splicing variants was detected, except in the thymus. Thus, our data indicate that the two splicing variants, as compared with survivin, have a more restricted expression profile to cancer cells.

**Binding Analysis of Synthetic Peptides to HLA-A*2402.** Amino acid sequence of survivin, survivin-2B, and survivin-ΔEx3 was screened for possible HLA-A24-bound nona- or decamer peptide, based on the HLA-A24-specific anchor motif (20). The motif consists of Y, F, M, or W at position 2 and F, L, I, W, or M at the COOH-terminal residue. As shown in Fig. 1B, two peptides, survivin85–93 (AFLSVKKQF) and survivin92–101 (QFEELTLLG) from the exon 3-encoded region, and one peptide, survivin-2B80–88 (AYACNTSTL) from exon 2B-encoded region, were determined as possible CTL epitopes presented by HLA-A24. Then the peptides were tested for binding affinity to HLA-A*2402 by using RMA-S cells transfected with HLA-A*2402/Kb chimera gene. It is known that MHC class I molecules become unstable in the absence of binding peptides. Because RMA-S cells lack TAP molecules, which transport peptides into the endoplasmic reticulum, the MHC class I level on the cell surface of RMA-S cells is low as compared with wild-type RMA cells. In the presence of binding peptides, MHC class I is stabilized, leading to up-regulation on the cell surface. As shown in Fig. 3, within the three survivin peptides only survivin-2B-derived peptide survivin-2B80–88 was capable of increasing the HLA-A24 level on RMA-A*2402/Kb cells. EBV (LMP2) peptide and HIV (env) peptide, which have been reported to be HLA-A24-presentable CTL epitopes, increased the HLA-A24 level as well, whereas F4.2 peptide, an HLA-A31-bound peptide, failed. These data indicate that survivin-2B80–88, but not survivin85–93 or survivin92–101, has a significant binding affinity to HLA-A*2402 that is comparable with that of HIV (env) peptide.

**CTL Induction from HLA-A24 (+) Cancer Patients and Healthy Donors.** Because survivin-2B was expressed in various types of cancer cells but not in normal adult tissues except for thymus, we hypothesized that survivin-2B-derived peptide might be immunogenic, particularly in cancer patients. We attempted to induce CTLs recognizing survivin-2B-derived peptide in the context of HLA-A24, which was the most frequent allele in the Japanese population. PBMCs collected from five of the HLA-A24-positive cancer patients and four of the HLA-A24-positive healthy donors were used for induction of CTLs. CD8+ T cells sorted out from PBMCs were incubated with peptide-pulsed autologous monocyte-derived DCs, followed by additional stimulation with peptide-pulsed autologous PHA-blasts. After four times stimulation, cytotoxic activity against peptide-pulsed target cells was examined by 51Cr release assay. As shown in Fig. 4, CTLs recognizing survivin-2B80–88 peptide were induced from PBMCs of all of the five HLA-A24-positive cancer patients. Increased E/T ratio resulted in increased cytotoxic activity when 1 µg/ml of peptide was pulsed onto C1R-A*2402 target cells. However, the CTLs could not show cytotoxic activity against peptide-pulsed C1R-A*31012 cells or K562 cells, indicating that CTLs recognized the peptide in the context of HLA-A*2402. It was also possible...
to induce CTLs from two of four HLA-A24-positive healthy volunteers (Fig. 5).

To confirm that CTLs induced from cancer patients can recognize survivin-2B-expressing cancer cells in the context of HLA-A*2402, we tested the cytotoxic activity of CTLs against LNY-1 lung adenocarcinoma cells (HLA-A24-negative) and its HLA-A*2402-transfectant, LNY-1-A24 cells. LNY-1 has expression of survivin and survivin-2B detected by RT-PCR (data not shown). As shown in Fig. 6A, all of the CTLs induced from PBMCs of five cancer patients exerted significant cytotoxic activity against LNY-1-A24 cells but not against HLA-A24-negative LNY-1 cells. These data indicate that CTLs induced by survivin-2B80–88 expressed in cancer cells become good targets for such an approach, including Bcr-Abl protein in chronic myeloid leukemia (21), SYT-SSX protein in synovial sarcoma (22), and hTERT protein (7). In contrast to the fusion proteins derived from the tumor-specific chromosomal translocation, the latter two proteins are expressed in a wide variety of tumor cells. Therefore, identification of potent CTL epitopes derived from such proteins is expected to contribute much more to cancer-specific immunotherapy for a variety of cancers.

Discussion

Clinically successful cancer-specific immunotherapy depends on the identification of tumor-specific rejection antigens and potent CTL epitopes. Initially, antigenic peptides were identified by constructing MHC class I-bound peptides advanced, a reverse immunological approach, amino acid sequence of an antigen protein is screened for the possible binding capacity to a specific HLA molecule. Proteins that are selectively expressed in cancer cells become good targets for such an approach, including Bcr-Abl protein in chronic myeloid leukemia (21), SYT-SSX protein in synovial sarcoma (22), and hTERT protein (7). In contrast to the fusion proteins derived from the tumor-specific chromosomal translocation, the latter two proteins are expressed in a wide variety of tumor cells. Therefore, identification of potent CTL epitopes derived from such proteins is expected to contribute much more to cancer-specific immunotherapy for a variety of cancers.

In the present study, we focused on an IAP family protein survivin that is preferentially expressed in many

\[ \text{Analysis of survivin expression by RT-PCR:} \]

\[ \begin{align*}
\text{HLA-A*2402 molecule was evaluated by mean fluorescent intensity (M.F.I.) of cell surface HLA-A*2402 molecule on RMA-S-A*2402/Kb cells that were pulsed with peptides. HLA-A*2402-restricted EBV LMP2-derived peptide (TYGPVMSL) and HIV env-derived peptide (RYLRDQQLGI) were used as positive controls. HLA-A*31012-restricted F4.2 peptide (YSWMDISCWI) was used as a negative control.}
\end{align*} \]
Fig. 4 Induction of HLA-A24-restricted CTLs from PBMCs of five HLA-A24-positive cancer patients (Patient 1, colon cancer; Patient 2, breast cancer; Patient 3, esophageal cancer; Patient 4 and Patient 5, gastric cancers). CTLs were induced by stimulating PBMCs with survivin-2B80–88 peptide and APCs. Cytotoxicity of the CTLs against the peptide-pulsed C1R-A2402 cells or C1R-A*31012 cells and K562 cells were analyzed by 51Cr release assay at various E:T ratios.

Fig. 5 Induction of HLA-A24-restricted CTLs from PBMCs of four HLA-A24-positive healthy donors. CTLs were induced by stimulating PBMCs with survivin-2B80–88 peptide and APCs. Cytotoxicity of the CTLs against the peptide-pulsed C1R-A2402 or C1R-A*31012 cells and K562 cells were analyzed by 51Cr release assay at various E:T ratios.
cancer cells and tissues. Thus far, there have been several reports showing that survivin expressed in tumor cells could elicit both humoral and cellular immune responses in cancer patients. Anti-survivin antibodies were detected in sera of lung cancer patients and colon cancer patients (23, 24). HLA-A2-restricted CTL responses against survivin-derived peptides were shown (9) in melanoma patients and chronic lymphocytic leukemia patients by ELISPOT assay (10). Tumor-infiltrating T cells reactive to survivin-derived peptides were also detected in situ by immunohistochemical staining of tumor tissues using FITC-conjugated multimeric peptide/MHC complexes (25). These findings clearly demonstrate that survivin protein expressed in tumor cells is immunogenic to cancer patients.

Initially, it was reported that survivin was expressed specifically in cancer cells but not in normal tissues. However, it was revealed that survivin was a crucial element for mitosis of cells, and even noncancerous cells expressed survivin during mitosis (13). Our study actually demonstrated the low level of survivin expression in PBMCs and most of normal tissues (Fig. 2). These findings raise a possible limitation as to cancer-specific immunotherapy using survivin-derived peptides, because CTLs may injure normal proliferating cells as well as cancer cells. A similar drawback is also pointed out for hTERT-
derived peptides, because hTERT expression is required for the maintenance of stem cells. Intriguingly, it was shown that two splicing variants were produced from a survivin gene (14). Survivin-ΔEx3 lacks exon 3 of survivin and contains frame-shift exon 4 and an extra-open reading frame consisting of the 3′-untranslated region. Survivin-2B contains a cryptic exon named exon 2B between exon 2 and exon 3 (Fig. 1A). Therefore, the variant proteins encoded by these splicing variants should contain a unique amino acid sequence to each variant protein, which may serve as CTL epitopes eliciting antitumor immune responses. Our study showed that both splicing variants were expressed in a variety of cancer cells as well as survivin. Importantly, the expression of splicing variants was more specific to cancer cells except for thymus (Fig. 2) than survivin expression. In addition, it is possible that immune escape of tumor cells by loss of the expression of these variants may bring a disadvantage to tumor cells, because the splicing variant proteins conserve antiapoptotic potential in tumor cells as well as survivin. Taken together, it is speculated that the survivin variants may have the capability to become ideal tumor rejection antigens.

We demonstrated HLA-A24-restricted CTL responses against survivin-2B-derived peptide for the first time. The CTLs induced by the mixed lymphocytes peptide culture in the presence of DCs exerted a significant cytotoxic activity against survivin-2B-expressing cancer cells in the context of HLA-A24, indicating that survivin-2B80–88 (AYACNTSTL) might become a good candidate of peptide vaccines for HLA-A24-positive patients.

In the current study, CTLs were induced from PBMCs of two of four HLA-A24-positive healthy volunteers as well as cancer patients. Because survivin-2B is expressed in EBV-transformed B cells (Fig. 2A) and PHA-blasts (data not shown), it is possible to speculate that previous viral infection might trigger off the sensitization of T cells, leading to increased CTL precursor frequency.

In general, CTLs are supposed to exert cytotoxicity on target cells by inducing apoptotic cell death. Because survivin is a member of IAP that inhibits caspase activation, one may ask how cancer cells with high survivin expression are sensitive to CTL-mediated cytotoxicity. Recently, it was reported that granzyme B, a component of cytotoxic granules, could directly cleave DFF45/ICAD protein, causing apoptosis of target cells without activation of caspase-3 (26). Hence, overexpression of survivin may not be able to protect tumor cells from CTL-mediated apoptosis. In contrast, overexpression of survivin is correlated with resistance to chemotherapeutics or radiation therapy (13). In this context, immunotherapy targeting survivin and its splicing variants may be advantageous to patients who suffer chemoresistant or radiation-resistant cancers.

In conclusion, we demonstrated the expression of survivin and its two splicing variants survivin-2B and survivin-ΔEx3 in a wide variety of cancer cells. Survivin-2B-derived peptide could induce CTL response in the context of HLA-A*2402. This peptide may serve as a widely applicable cancer antigen for HLA-A24-positive patients.

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