A Potent Immunogenic General Cancer Vaccine That Targets Survivin, an Inhibitor of Apoptosis Proteins

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ABSTRACT

We reported previously a HLA-A24-restricted antigenic peptide, survivin-2B80-88 (AYACNTSTL), recognized by CD8+ CTL. This peptide was derived from survivin protein, an inhibitor of apoptosis proteins, expressed in a variety of tumors, such as adenocarcinoma, squamous cell carcinoma, and malignant melanoma. In this report, we provide further evidence that survivin-2B80-88 peptide might serve as a potent immunogenic cancer vaccine for various cancer patients. Overexpression of survivin was detected in surgically resected primary tumor specimens of most breast and colorectal cancers and some gastric cancers as assessed by immunohistochemical study. HLA-A24/survivin-2B80-88 tetramer analysis revealed that there existed an increased number of CTL precursors in peripheral blood mononuclear cells (PBMC) of HLA-A24+ cancer patients, and in vitro stimulation of PBMCs from six breast cancer patients with survivin-2B80-88 peptide could lead to increases of the CTL precursor frequency. Furthermore, CTLs specific for this peptide were successfully induced from PBMCs in all 7 (100%) patients with breast cancers, 6 of 7 (83%) patients with colorectal cancers, and 4 of 7 (57%) patients with gastric cancers. These data indicate that survivin expressed in tumor tissues is antigenic in cancer patients, and survivin-2B80-88-specific CTLs are present in PBMCs of various cancer patients. Our study raises the possibility that this peptide may be applicable as a general cancer vaccine to a large proportion of HLA-A24+ cancer patients.

INTRODUCTION

In the past decade, many tumor-associated antigens, which can be recognized by CTLs, have been identified (1–4). However, such tumor-associated antigens have been expressed in certain limited tumor cells, such as melanomas. Therefore, the identification of widely expressed tumor-associated antigens is urgently required for the development of cancer immunotherapy in more common malignancies of epithelial origins like adenocarcinoma and squamous cell carcinomas.

Survivin was initially isolated as one of the inhibitors of the apoptosis protein family with only one baculovirus inhibitors of apoptosis protein (IAP) repeat domain (5). Following the first report, survivin has been proven to be one of the chromosome passenger proteins and to play an important role in mitosis and spindle checkpoint (6–8). Survivin is aberrantly expressed in various kinds of cancer cells but is undetectable in normal differentiated adult tissues, with the exception of testis, thymus, and placenta. Indeed, the accumulated reports of survivin expression in human malignant tumor tissues over the past several years are consistent with the observation that the expression of survivin was enhanced in various human cancers in comparison with adjacent normal tissues, including non-Hodgkin’s lymphoma (5) and many other malignancies (9–27). Moreover, several reports suggest that overexpression of survivin in cancer cells is associated with unfavorable clinicopathologic variables, such as poor prognosis and shorter patient survival rates.

Thus, with its high expression profile in cancer cells and function of protecting cancer cells from apoptotic stimuli, survivin is thought to be one of the most ideal immunologic target molecules for cancer treatment. With this mind, we attempted to establish an immunotherapy based on targeting the survivin molecule. In our previous report, we provided data showing that the survivin-2B80-88 peptide can be presented by HLA-A24 and that CTLs induced with this peptide could recognize survivin-positive HLA-A24+ cancer cells (28). In this report to further investigate its applicability to tumor immunotherapy, we confirmed survivin expression in a greater number of breast, colorectal, and gastric cancer specimens. Furthermore, it was possible to induce survivin peptide–specific CTLs from most of these patients’ peripheral blood mononuclear cells (PBMC). These data strongly suggest that the survivin-2B80-88 peptide is one of ideal general cancer vaccines for immunotherapy of HLA-A24+ patients.

MATERIALS AND METHODS

Patients and Samples. The surgically resected specimens used in this study were obtained from HLA-A24+ patients with breast, colorectal, and gastric cancers who underwent potentially curative resection at the Department of Surgery, Sapporo Medical University Hospital and its affiliated hospitals. Peripheral blood was obtained from patients after obtaining their informed consent.

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Immunohistochemical Staining of Survivin, Cell Lines, and Culture Media. For immunohistochemical detection of survivin protein, the standard avidin-biotin-peroxidase complex technique was done with simple stain MAX-PO (MULTI) methods (Nichirei, Tokyo, Japan).

K562 cells were purchased from American Type Culture Collection (Manassas, VA). Lymphoblastoid cell lines transfected with HLA-A*2402 (C1R-A*2402) and HLA-A*31012 (C1R-A*31012) were kind gifts from Dr. M. Takiguchi (Kumamoto University School of Medicine, Kumamoto, Japan). Cells were cultured in RPMI 1640 supplemented with 2 mmol/L l-glutamine, 10% FCS, 100 units/mL penicillin G, and 100 µg/mL streptomycin and were maintained at 37°C in a humidified 5% CO₂ atmosphere. Hygromycin B (0.5 mg/mL, WAKO Chemicals, Osaka, Japan) was continuously added to the culture medium for C1R-A*2402 and C1R-A*31012 cells.

Peptides and Cytokines. We used a HLA-A24-restricted survivin-derived antigenic peptide (AYACNTSTL), designated as 2B80-88 peptide, and a HLA-A24-restricted SYT-SSX SS393 peptide (GYDQIMPKK) that was derived from (X18) chromosomal translocation in synovial sarcomas (29). Peptides were kindly provided by Sumitomo Pharmaceutical (Osaka, Japan). They were resolved in DMSO at the concentration of 5 mg/mL and stored at −80°C. Human recombinant interleukin (IL)-2 and IL-4 were a kind gift from Takeda Pharmaceutical Co. (Osaka, Japan) and Ono Pharmaceutical Co. (Osaka, Japan), respectively. Human recombinant granulocyte-macrophage colony-stimulating factor was a kind gift from Kirin (Tokyo, Japan) and Novartis Pharmaceutical (Basel, Switzerland). Human recombinant IL-7 was purchased from Invitrogen (San Diego, CA).

Fluorescence-Activated Cell Sorting Analysis and Tetramer Construction. Fluorescence-activated cell sorting analysis was done for the expression of HLA-A24 and peptide-specific tetramer analysis. To detect the cell surface expression of HLA-A24 molecule, after washing with PBS, the cells were incubated with anti-HLA-A24 antibody (c7709A2.6, a kind gift from Dr. P.G. Coulie, Brussels, Belgium; ref. 30) for 1 hour at 4°C. After washing with PBS, the cells were incubated with diluted FITC-labeled goat anti-mouse IgG + IgM antibody (KPL, Guildford, United Kingdom) and, after washing again with PBS, were fixed with formaldehyde and analyzed with FACSscan (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences).

HLA-A24/peptide tetramers were constructed according to the procedure described by Altman et al. (31). A soluble form of mutated HLA-A24 heavy chain (32) and human β₂-microgloblin were refolded with the synthesized survivin-derived antigenic peptide (AYACNTSTL), designated as 2B80-88 peptide, and a HLA-A24-restricted SYT-SSX SS393 peptide (GYDQIMPKK) for 1 hour and washed with AIM-V thrice and medium supplemented with HEPES (10 mmol/L), 2-mercaptoethanol (50 µmol/L), granulocyte-macrophage colony-stimulating factor (1,000 units/mL), and IL-4 (1,000 units/mL) for 7 days. CD8⁺ cells were isolated from plastic flask nonadherent cells of PBMCs with magnetic affinity cell sorting separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-CD8 monoclonal antibody coupled with magnetic microbeads according to manufacturer’s instruction. PHA-blasts were derived from CD8⁺ cells by culturing in AIM-V supplemented with HEPES (10 mmol/L), 2-mercaptoethanol (50 µmol/L), IL-2 (100 units/mL), and PHA (1 µg/mL) for 3 days followed by culture in AIM-V medium without PHA for 4 days. APCs (dendritic cells and PHA-blasts) were cultured in AIM-V medium supplemented with 50 µmol/L peptide at room temperature for 1 hour and washed with AIM-V thrice and then irradiated 100 Gy and used for stimulation of CTLs. CTL induction procedure was initiated by stimulating 2 × 10⁶ CD8⁺ cells with peptide-pulsed autologous dendritic cells at a 20:1 effector/APC ratio in AIM-V supplemented with HEPES, 2-mercaptoethanol, and IL-2 (10 ng/mL) for 7 days at 37°C. The following stimulation was done with peptide-pulsed PHA-blasts at a 5:1 effector/APC ratio. On the next day of the second stimulation, IL-2 was added to the culture at a concentration of 50 units/mL. The same CTL stimulation cycle with PHA-blasts was then done twice more over the period of 2 weeks. One week after the fourth stimulation, the cytotoxic activity of the CTLs was measured by ⁵¹Cr release assay.

Cytotoxicity Assay. The cytotoxic activities of CTLs were measured by ⁵¹Cr release assay as described previously (34). Briefly, target cells were labeled with 100 µCi ⁵¹Cr for 1 hour at 37°C and washed with RPMI 1640 thrice. Then, 2 × 10³ ⁵¹Cr-labeled target cells were incubated with effector cells at various effector/target (E:T) ratios at 37°C for 6 hours in V-bottomed 96-well microtiter plates. Then, supernatants were collected and the radioactivity was measured with a gamma counter. % Specific lysis was calculated as % Specific lysis = (Experiment release – Spontaneous release) × 100 / (Maximum release – Spontaneous release). For preparation of peptide-pulsed target cells, C1R-A*2402 cells, C1R-A*31012, or PHA-blast T cells were incubated with 1 µg/mL peptide at room temperature for 1 hour before the assay.
RESULTS

Survivin Expression Profiles in Breast, Colon, and Gastric Cancer Specimens. Several research groups have shown that the expression of survivin was up-regulated in various types of cancer specimens. In our current study, we focused on breast, colorectal, and gastric cancers because of higher incidence among malignancies in Japanese. Using reverse transcription-PCR method, we showed previously that the survivin mRNA level was elevated in several cancer cell lines, including adenocarcinoma cells, squamous cell carcinoma cells, renal cell carcinoma cells, and melanoma cells (28). In this report, immunohistochemical staining was done to study the expression profiles of survivin protein in surgically resected primary tumor specimens.

Because survivin is suggested to be involved in the process of nuclear chromatin organization, we examined if survivin was detected in the nucleus. In concurrence with previous reports (9, 10, 16), our data indicated that survivin was overexpressed in breast, colorectal, and gastric cancer tissues (Fig. 1A–C; Tables 1, 2, and 3). Survivin overexpression was detected in all the cases of breast invasive ductal carcinomas (Table 1) and colon adenocarcinomas (Table 2). In gastric cancer, six of nine cases were survivin positive (Table 3). It was noted that survivin was detected in the nucleus as well as in the cytoplasm in most of breast cancers (Fig. 1A; Table 1), whereas survivin was detected mainly in the nucleus of colon cancer tissues (Fig. 1B) and gastric cancer tissues (Tables 2 and 3).

Immunogenicity and CTL Precursor Induction with Survivin-2B80-88 Peptide in Cancer Patients. Because the expression of survivin was up-regulated in most cases of breast, colorectal, and gastric cancers, we next determined whether this protein could be an antigenic target recognized by CTL. To confirm this point, we first studied the CTLp frequency in several survivin-positive cancer patients’ PBMCs by using HLA-A24/survivin-2B80-88 peptide tetramer as described previously (31). As shown in Table 4, CTLp frequencies were >0.1% in 8 of 13 survivin-positive cancer patients, whereas they were <0.1% in 5 healthy individuals. There did not seem to be obvious correlation between survivin-2B80-88 peptide-specific CTLp frequency and clinical stages, but the data strongly implied that survivin protein overexpressed in cancer tissues might be antigenic and survivin-2B80-88 peptide could be recognized by T cells in the context of HLA-A*2402 molecule in these cancer patients.

Because survivin was frequently overexpressed in breast cancers, we next examined if stimulation of PBMCs in vitro with survivin-2B80-88 peptide was capable of increasing the

<table>
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<th>Survivin expression*</th>
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*Negative (−) or positive (+) staining in contrast with normal tissue as background.
†, ≥15% specific lytic activity at E:T ratio = 3 by 51Cr release assay.
frequency of peptide-specific CTL using breast cancer patients’ PBMCs. The peptide-specific CTLs were induced by mixed lymphocyte peptide culture (MLPC) in the same manner as described in our previous reports (28). CTL frequencies analyzed by HLA-A24/survivin-2B80-88 tetramer were compared between pre-MLPC and post-MLPC. Figure 2A shows a representative data of breast cancer patient 3. In vitro stimulation of PBMCs with survivin-2B80-88 peptide-pulsed APCs increased the CTL frequency from 0.07% to 0.89%. We did the similar MLPC experiments using a total of six cases of breast cancer. As shown in Fig. 2B, MLPC with survivin-2B80-88 peptide could lead to increases in the frequency of the peptide-specific CTLs in most cases of breast cancer, although the efficiency was distinct in each case.

**HLA-A24-Restricted Survivin-2B80-88 Peptide–Specific Cytotoxicity in Cancer Patients.** We evaluated the cytotoxic activity of CTLs that were induced with survivin-2B80-88 peptide from breast, colorectal, or gastric cancer patients. CTLs were induced from HLA-A24-positive cancer patients’ PBMCs by MLPC using autologous monocyte-derived dendritic cells and PHA-blasts as APCs. Then, HLA-A24-restricted peptide-specific cytotoxic activity was assessed by 51Cr release assay using survivin-2B80-88 peptide-pulsed or nonpulsed K562 cells. As shown representatively in Fig. 3, stimulation of PBMCs from breast cancer patient 4 (Fig. 3A) and patient 7 (Fig. 3B) with survivin-2B80-88 peptide resulted in the induction of CTLs that were preferentially cytotoxic against survivin-2B80-88 peptide-pulsed C1R-A*2402 cells. However, these CTLs were less cytotoxic to SYT-SSX peptide-pulsed C1R-A*2402 or nonpulsed C1R-A*2402 cells, although they showed some extent of background killing activity against C1R-A*2402 cells. These CTLs did not exert cytotoxicity against survivin-2B80-88 peptide-pulsed C1R-A*31012 or K562 cells. These data indicated that CTLs induced by MLPC with survivin-2B80-88 peptide had HLA-A*2402-restricted peptide-specific cytotoxicity. To know whether survivin-2B80-88-specific CTLs could exert cytotoxicity against autologous activated T cells that expressed survivin, we tested the cytotoxicity of the CTL against autologous PHA-blast cells. CTLs were induced from PBMCs of gastric cancer patient 9. Peptide-pulsed or nonpulsed autologous activated T cells were resistant to the CTLs, whereas the peptide-pulsed C1R-A*2402 cells were killed (Fig. 3C). Therefore, it is probable that normal activated lymphocytes might have a protective mechanism against the cytotoxicity of CTLs even if they express survivin.

Finally, we examined the cytotoxic activity of CTLs induced by survivin-2B80-88 peptide in various cancer patients. As shown in Fig. 4A, survivin-2B80-88 peptide-specific CTLs could be successfully induced from all seven cases of breast cancer patients (summarized in Table 1). In colorectal cancer patients, specific CTLs could be induced from six (patients 1-6) of seven cases (Fig. 4B; Table 2). Meanwhile, although

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**Table 2** Survivin expression and CTL induction in colorectal cancer cases

<table>
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<th>Case no.</th>
<th>Age</th>
<th>Sex</th>
<th>Stage (tumor-node-metastasis classification)</th>
<th>Histology</th>
<th>Survivin expression*</th>
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<td>Nucleus Cytoplasm CTL induction†</td>
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*Negative (−) or positive (+) staining in contrast with normal tissue as background.
†+, ≥15% specific lytic activity at E:T ratio = 3 by 51Cr release assay.

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**Table 3** Survivin expression and CTL induction in gastric cancer cases

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*Negative (−) or positive (+) staining in contrast with normal tissue as background.
†+, ≥15% specific lytic activity at E:T ratio = 3 by 51Cr release assay.
survivin-2B80-88 peptide-specific CTL was efficiently induced from most cancer patients, the CTL induction efficiency did not correlate with clinical stage or with subcellular staining pattern of survivin expression.

In gastric cancer patients, however, only four (patient 1-3 and 9) of the seven cases showed survivin-2B80-88 peptide-specific CTL induction (Fig. 4C; Table 3). Although the number of gastric cancer patients examined in this study was not enough to know the accurate immunogenic potential of this peptide or correlation with clinical variables, it should be noted that survivin-2B80-88 peptide-specific CTL was successfully induced in PBMCs from patients (1-3 and 9) with survivin-positive gastric tumors but not from patients (5 and 6) with survivin-negative gastric tumors (Table 3).

**Cytotoxicity of the Survivin-2B80-88 Peptide-Specific CTL against HLA-A2402-Positive Adenocarcinoma Cells.** To confirm that CTLs induced with survivin-2B80-88 peptide can react with survivin-expressing cancer cells in the context of HLA-A*2402, we examined cytotoxic activity against lung adenocarcinoma LNY-1-A*2402 cells that express both endogenous survivin and gene-transfected HLA-A*2402. As shown in Fig. 5, CTLs induced from PBMCs of HLA-A24-positive breast (Fig. 5A), colorectal (Fig. 5B), and gastric (Fig. 5C) cancer patients by MLPC with survivin-2B80-88 peptide exerted significant cytotoxicity against LNY-1-A*2402 cells but not against LNY-1 lacking HLA-A24 expression or K562 cells. These data indicate that survivin-2B80-88 peptide-specific CTLs were capable of recognizing endogenously processed survivin peptide in a HLA-A24-restricted manner.

Taken together, it was strongly indicated that survivin protein expressed in cancer cells was antigenic in HLA-A2402-positive cancer patients, and survivin-2B80-88 peptide might be suitable for the general cancer vaccine.

**DISCUSSION**

Among various types of malignancy, the incidence of breast, colorectal, and gastric cancers are the most frequent in Japanese population. In this study, to assess the immunogenic potency of survivin protein in these cancer patients, its expression and the frequency of survivin-specific CTLs were examined using surgically resected specimens and PBMCs of the patients.

*Patient number corresponding to that in Tables 1, 2, and 3.*
Because survivin is considered to be a chromosomal passenger protein and to play an important role in the mitotic process, we studied the nuclear expression as well as cytoplasmic expression of this protein. Survivin expression was detected in the nucleus of many cases of breast, colorectal, and gastric cancers, and it was also detected in the cytoplasm in some of these cancer tissues. These results were consistent with previous reports (9, 10, 16). An earlier study also reported that survivin was a cell cycle–regulated protein with a robust expression in the G2-M phase (6). Immunofluorescence and confocal microscopy techniques showed that survivin was associated with the microtubule organization center interphases, centrosomes, and mitotic spindles at metaphase and relocated to midbodies in later telophase (6, 35). Mahotka et al. showed that survivin-δ Ex3 splicing variant had a potential nuclear localization signal and was localized in the nucleus, whereas survivin and survivin-2B splicing products were in the cytoplasm (36). Because there is no antibody available that can detect the splicing variants specifically, it is hard to detect the subcellular localization of the variants. Besides the subcellular localization, we have shown previously that the mRNA levels of three survivin isoforms are comparable among them in most tumor cells (28). Therefore, immunohistologic survivin detection even in the nucleus or in the cytosol should indicate the comparable production of survivin-derived antigenic peptides in cancer cells.

In this report, we also showed elevation of CTLp frequencies in HLA-A24-positive cancer patients’ PBMCs by using HLA-A24/survivin-2B80-88 peptide tetramer. CTLp could be expanded in vitro by MLPC and showed peptide-specific cytotoxicity. These CTLs were also cytotoxic against HLA-A24-positive and survivin-positive adenocarcinoma cell lines (Fig. 5). These data strongly indicate that survivin is one of the immunogenic tumor antigens in HLA-A24-positive cancer patients. It is noteworthy that there was a clear correlation between the survivin expression detected by immunohistochemistry and the CTL induction capability. Immunohistochemical detection of survivin expression might become one of the predictive markers in the survivin targeting immunotherapy of cancer.

It is likely that survivin is expressed in some normal tissues like thymus, testis, and placenta. Testis and placenta are immunoprivileged organs and rarely disclose classic HLA class I molecules to the immune system. Therefore, CTLs cannot recognize HLA class I–restricted antigens expressed in testis or placenta. However, because survivin is expressed in normal activated lymphocytes or stem cells in bone marrow, it is anticipated that those normal cells might become targets of survivin-specific CTLs. In this study, we showed that survivin-2B80-88-specific CTLs could not kill autologous activated T cells either in the peptide-pulsed condition or in nonpulsed condition. Our data are consistent with previous reports showing that immunocompetent cells, including CD4+ T cells, B cells, natural killer cells, and dendritic cells, are resistant to CTL activity (37). Schmidt et al. showed that HLA-A2-restricted survivin peptide–specific CTLs could not recognize activated B cells, activated T cells, immature dendritic cells, or mature dendritic cells, which express survivin (38). Meanwhile, granzyme B is one of the most potent cytotoxic enzymes that are released from CTLs and activate cell death program inside the target cells. The resistance mechanism of normal lymphocytes and dendritic cells against CTLs has been explained at least in part by the presence of a potent granzyme B inhibitor, proteinase inhibitor-9, in those cells (38). These findings suggest that although survivin may be expressed in some sorts of normal tissues it does not become an obvious drawback in the survivin targeting cancer immunotherapy.

In addition to the antiapoptotic effect, survivin is involved in the tumor angiogenesis (39). Pathologic angiogenesis is essential for tumor outgrowth in vivo, and various tumor cells were reported to express angiogenic factors like vascular endothelial growth factor, basic fibroblast growth factor, and transforming growth factor-β. Treatment with angiogenic reagents was effective in the suppression of tumor growth and metastasis (40). O’Connor et al. showed that vascular endothelial growth factor, one of the angiogenic factors, could induce 16-fold up-regulation of survivin expression in vascular endothelial cells, leading to the
resistance against proapoptotic stimuli. Hence, survivin targeting immunotherapy of cancer should be advantageous not only to the anti–cell survival effect but also to the anti-angiogenesis effect.

In conclusion, we provided evidence that survivin was overexpressed in a large proportion of breast, colorectal, and gastric cancer tissues. Survivin-2B80-88-specific CTLs could be induced efficiently from PBMCs of HLA-A24-positive patients.

Fig. 4 Induction of HLA-A24-restricted CTL from PBMCs of HLA-A24+ breast, colorectal, and gastric cancer patients. CTLs were induced from PBMCs of HLA-A24-positive breast (A), colorectal (B), and gastric (C) cancer patients by MLPC with survivin-2B80-88 peptide as described in Materials and Methods. Cytotoxic activity of the CTL against survivin-2B80-88 peptide-pulsed C1R-A*2402 cells or C1R-A*31012 cells and nonpulsed K562 cells was analyzed by ³¹Cr release assay at various E:T ratios.
survivin-positive cancer patients. Our overall data indicate that survivin targeting immunotherapy with survivin-2B80-88 peptide should be suitable for HLA-A24+ breast, colorectal, or gastric cancer. Currently, a phase I clinical trial of survivin-2B80-88 peptide vaccine immunotherapy has been undertaken (41).

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