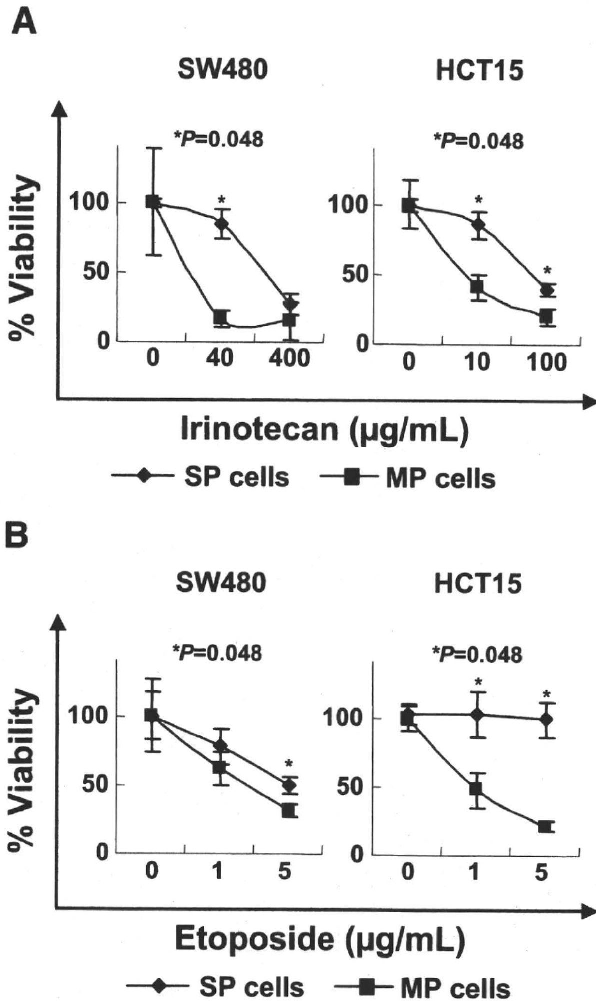

Figure 3. Sensitivity to chemotherapeutic agents. SP cells and MP cells derived from SW480 and HCT15 were incubated in the presence of irinotecan (CPT-11) (**A**) or etoposide (VP-16) (**B**) for 3 days. After incubation, the cell viabilities were measured by WST-1 assay. Data are reported as means ± SD. Differences between SP cells and MP cells were examined for statistical significance using the Mann-Whitney *U*-test.

therefore used an adoptive transfer model, as described under *Materials and Methods*. SW480 SP cells were inoculated into the back of NOD/SCID mice subcutaneously. Three weeks later, after confirmation of palpable tumors, CTLs were injected intravenously. Tumors of CTL-injected mice were significantly inhibited in growth, compared with tumors of control mice (Figure 4E). These data indicate that CTLs could recognize CSCs/TICs both *in vitro* and *in vivo*.

Discussion

In the present study, we successfully isolated colon cancer CSCs/TICs as SP cells, using Hoechst 33342 staining. Side population cells were first described by Goodell et al,[12] and CSCs/TICs of several types of malignancies were successfully isolated as SP cells in subsequent studies.[14,20–24] Haraguchi et al[13] isolated SP cells from gastrointestinal cancer cell lines; they reported the gene expression profiles and resistance to chemotherapeutic agents of SP cells derived from liver cancer cell line Huh7, but did not determine their tumorigenicity. Burkert et al[15] found that SP cells derived from gastrointestinal cancers cell lines HT29, HGT101, Caco2, and HRA19a1.1 were not enriched with a CSC/TIC population. In the present study, we were able to isolate SP cells from all six colon cancer cell lines studied (SW480, HT29, HCT15, KM12LM, Lovo, and Colo320). However, in only three of the six cell lines did the SP cells show higher tumorigenicity than MP cells, suggesting that these SP cells were enriched with CSC/TIC populations. Thus, SP cells might not be the definitive phenotype of CSCs/TICs, and confirmation of tumorigenicity in immunodeficient mice is essential for validation of SP cells as a source of CSCs/TICs. In the present study, the SP cells derived from SW480, HCT15, and HT29 cells were confirmed to be enriched with CSCs/TICs. Furthermore, these SP cells expressed stem cell markers, including SOX2, POU5F1 and LGR5, at higher levels than MP cells, suggesting correspondence with CSCs/TICs. Thus, these SP cells would be a useful tool for analysis of colon CSCs/TICs.

In the present study, we evaluated the immunogenicity of colon CSCs/TICs. Colon cancer CSCs/TICs expressed HLA class I molecules, and also CEP55, which is one of the TAAs. Furthermore, colon CSCs/TICs expressed several other TAA-encoding genes (data not shown), including BIRC5 (encoding apoptosis inhibitor survivin), BIRC7 (encoding livin), WT1, CTAG1B (alias *NY-ESO-1*), and MAGEA4. As a novel finding, colon cancer CSCs/TICs were sensitive to CTLs both *in vitro* and *in vivo*. Recently, Todaro et al[25] showed that colon CSCs/TICs were sensitive to γδT cells. Because both CTLs and γδT cells kill target cells through secretion of perforin (encoded by the *PRF1* gene) and granzyme B (encoded by *GZMB*), these observations strongly suggest that CSCs/TICs are sensitive to PRF1- and GZMB-dependent apoptosis. Todaro et al[26] had earlier reported that PROM1-positive ($CD133^+$) colon cancer CSCs/TICs secrete IL-4 in an autocrine manner and upregulate the antiapoptotic proteins CFLAR (c-FLIP), BCL2L1 (Bcl-xL), and PEA15 (PED), thereby gaining resistance to chemotherapeutic agents. Saigusa et al[27] reported that distant recurrence of rectal cancer after chemotherapy was related to the expression of CSC/TIC markers such as PROM1 (CD133), POU5F1 (Oct3/4), and SOX2. These reports support the idea that colon CSCs/TICs are resistant to apoptotic cell death. The fact that immunocytes induce apoptosis in their target cells raises the question of whether colon CSCs/TICs are also sensitive to immunotherapy.

In the present study, and in that of Todaro et al,[25] colon CSCs/TICs were sensitive to perforin- and granzyme B-dependent apoptosis. Thus, both CTLs and γδT cells can be useful tools for colon CSC/TIC targeting therapy. However, because γδT cells do not recognize target cells in an antigen-specific manner, immunotherapy using γδT cells should also recognize the non-CSC/TIC population. Because the number of γδT cells is restricted *in vivo*, it may be in doubt whether γδT cell can recognize colon

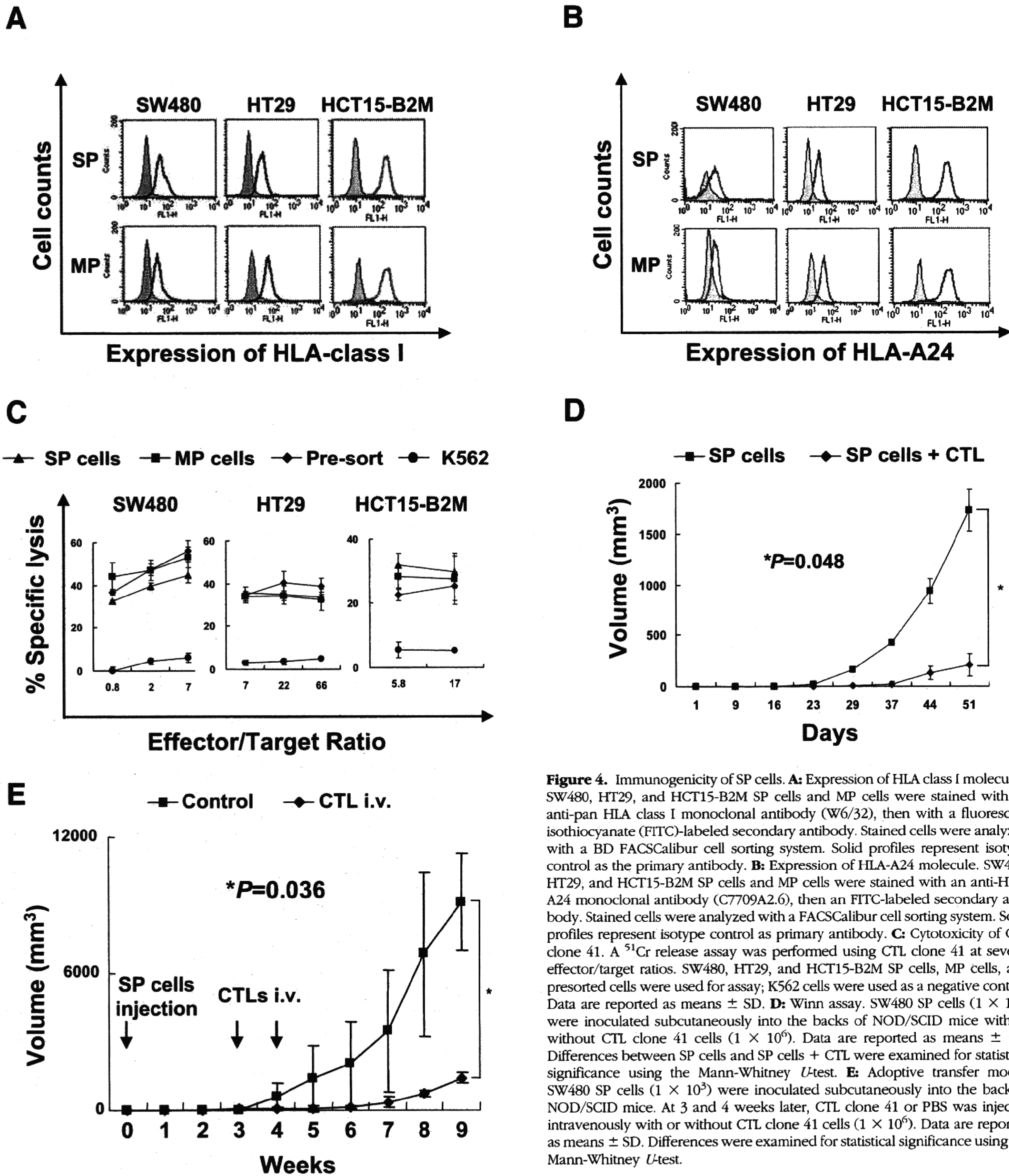

Figure 4. Immunogenicity of SP cells. **A:** Expression of HLA class I molecules. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-pan HLA class I monoclonal antibody (W6/32), then with a fluorescein isothiocyanate (FITC)-labeled secondary antibody. Stained cells were analyzed with a BD FACSCalibur cell sorting system. Solid profiles represent isotype control as the primary antibody. **B:** Expression of HLA-A24 molecule. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-HLA-A24 monoclonal antibody (C7709A2.6), then an FITC-labeled secondary antibody. Stained cells were analyzed with a FACSCalibur cell sorting system. Solid profiles represent isotype control as primary antibody. **C:** Cytotoxicity of CTL clone 41. A ^{51}Cr release assay was performed using CTL clone 41 at several effector/target ratios. SW480, HT29, and HCT15-B2M SP cells, MP cells, and presorted cells were used for assay; K562 cells were used as a negative control. Data are reported as means ± SD. **D:** Winn assay. SW480 SP cells (1×10^5) were inoculated subcutaneously into the backs of NOD/SCID mice with or without CTL clone 41 cells (1×10^6). Data are reported as means ± SD. Differences between SP cells and SP cells + CTL were examined for statistical significance using the Mann-Whitney *U*-test. **E:** Adoptive transfer model. SW480 SP cells (1×10^3) were inoculated subcutaneously into the back of NOD/SCID mice. At 3 and 4 weeks later, CTL clone 41 or PBS was injected intravenously with or without CTL clone 41 cells (1×10^6). Data are reported as means ± SD. Differences were examined for statistical significance using the Mann-Whitney *U*-test.

cancer CSCs/TICs *in vivo* efficiently. Recently, based on a large cohort study, Ogino et al[28] reported that lymphocytic reaction to tumor was associated with longer survival of colorectal cancer patients. They did not analyze the subtypes of infiltrating lymphocytes; however, the findings from this large-scale study strongly support the notion that immune reaction to tumor cells is important for control of the disease.

Wei et al[29] reported recently that glioma-derived CSCs/TICs suppressed T-cell proliferation and activation, and induced T-cell apoptosis through expression of co-stimulatory inhibitory molecule CD274 (B7-H1) and soluble LGALS3 (galectin-3); glioma CSCs/TICs enhance the induction of regulatory T cells. We also observed that SW480 SP cells express higher mRNA of the immunosuppressive cytokine IL-10 than MP cells (data not

shown). Thus, colon CSCs/TICs may have immunosuppressive potential and so inhibit CTL induction. However, colon CSCs/TICs are efficiently killed by CTLs, and colon CSCs/TICs have no influence on the effector phase of CTLs. Thus, adoptive cell transfer of CSC/TIC-specific CTL clones, T-cell-receptor–induced T cells, or peptide vaccination accompanied by an anti-IL-10 monoclonal antibody might be an effective approach for eliminating colon CSCs/TICs.

In the present study, we observed that both colon CSCs/TICs and non-CSCs/TICs were sensitive to CEP55-specific CTLs at the same level. This finding seems reasonable, given that CSCs/TICs express CEP55 mRNA at the same level. Huge numbers of TAAs have already been reported,[30,31] and the next challenge is to identify which TAAs would be the most suitable targets for cancer immunotherapy. According to the manner of expression in CSCs/TICs and non-CSCs/TICs, TAAs can be classified into three categories: i) CSC/TIC-specific antigens, such as SOX2 and ALDH1A1; ii) non-CSC/TIC-specific antigens; and iii) shared antigens, such as CEP55.[32] The frequencies of colon CSCs/TICs are 1% to 10%, and in the present study these cells had 10- to 100-fold higher tumorigenicity than non-CSCs/TICs. It is likely, therefore, that 1% to 10% of colon CSC/TIC populations have almost the same tumorigenic potential as 90% to 99% of the non-CSC/TIC population. To achieve a complete cure of the disease, shared antigens seem to a be reasonable candidate strategy. *In vivo*, however, CTL numbers are limited. Given that 1 L of peripheral blood contains approximately 5×10^9 lymphocytes, there are approximately 5×10^8 CD8 T cells in 1 L of peripheral blood and approximately 3×10^9 CD8 cells in the total volume of peripheral blood in a human adult. If the CTL precursor frequency reaches 0.1% of CD8 T cells in a patient receiving peptide vaccination therapy, then the total peptide-specific CTLs can be calculated as 3×10^6 cells in whole blood. This is not an inconsiderable number. Visible tumors as large as 1 cm diameter contain 1×10^9 tumor cells, and the estimated effector/target ratio (E/T) *in vivo* is 0.003. This ratio may be too low to expect an anti-tumor effect *in vivo*. However, if we focus only on CSCs/TICs, then the effector/target ratio will be improved. For targeting CSCs/TICs with 1% frequency, the effector/target ratio is correspondingly improved (E/T = 0.3). Thus, focusing only on the CSC/TIC population with CSC/TIC-specific antigens seems to be a better approach for advanced cancer cases. For prevention of disease recurrence after treatment, the target cells are likely to be limited, so shared antigens might be a reasonable choice for cancer immunotherapy.

Recently, some research groups have reported that monoclonal antibodies for insulin-like growth factor-1 receptor (IGF-1R), δ-like 4 ligand (DLL4), and CD47 efficiently eliminate colon cancer and leukemia CSCs/TICs.[33–36] These approaches are also fascinating, and a reasonable option for elimination of CSCs/TICs. An antibody is a relatively stable protein, but the half-life in peripheral blood is approximately 2 to 3 weeks, and therefore serial administration is needed to maintain the effects of the antibody. On the other hand, antigenic peptide vaccination can induce specific CTLs as memory cells *in vivo*, such that the specific immunity will last for several years. Thus, peptide vaccination therapy may also be useful for prevention of post-treatment cancer recurrence.

In conclusion, we report here the novel finding that colon cancer CSCs/TICs are as sensitive to CTLs as are non-CSCs/TICs, and that CEP55, a tumor-associated antigen, is a suitable antigen for targeting colon cancer CSCs/TICs.

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Phase I clinical trial of survivin-derived peptide vaccine therapy for patients with advanced or recurrent oral cancer

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Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is abundantly expressed in most malignancies, but is hardly detectable in normal adult tissues. Previously we have identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, survivin-2B80-88 (AYACNTSTL), recognized by CD8$^+$ cytotoxic T lymphocytes (CTL). Survivin-2B80-88-specific CTL were induced efficiently from peripheral blood mononuclear cells (PBMC) of oral cancer patients after stimulation with the peptide *in vitro*. We conducted a phase I clinical study to evaluate the safety and the efficacy of survivin-2B80-88 peptide vaccination in HLA-A24-positive patients with advanced or recurrent oral cancer. The vaccines were given subcutaneously or intratumorally six times at 14-day intervals. Eleven patients were enrolled and 10 patients completed the vaccination protocol. No adverse events were observed in any patients. In two patients, the levels of serum squamous cell carcinoma (SCC) antigen decreased transiently during the period of vaccination. Tumor regression that was compatible with a partial response (PR) was noted in one patient. The remaining nine patients experienced progressive disease (PD). Immunologically, an increase of the peptide-specific CTL frequency was detected in six of the eight patients evaluated by HLA-A24/peptide tetramer analysis. The present clinical trial revealed that survivin-2B peptide vaccination was safe and had therapeutic potential for oral cancer patients. However, subsequent clinical trials in combination with various adjuvant drugs will be required to improve the immunological and therapeutic efficacy. This trial was registered with University Hospital Medical Information Network (UMIN) number UMIN000000976. (*Cancer Sci* 2011; 102: 324–329)

Oral cancer consistently ranks as one of the 10 most frequently diagnosed cancers worldwide.[1] It encompasses a range of malignant tumors arising from various diverse and complex structures that have major physiological and aesthetic importance. For most early stage oral cancers, high cure rates are achieved with either surgery or definitive irradiation and both speech and swallowing functions can often be preserved. On the other hand, locally advanced or recurrent oral cancers are usually treated with combination therapy consisting of either surgery followed by postoperative chemoradiation or chemoradiation with surgical salvage if needed. However, most patients remain at high risk for locoregional recurrence and distant metastasis.[2] Therefore, advances in new therapeutic modalities such as tumor-specific immunotherapy for patients with locally advanced or recurrent oral cancers are urgently needed.

A large number of tumor-associated antigens have been identified from melanomas and other cancers, and clinical trials of peptide-based immunotherapy have been carried out. Melanoma antigen peptides were the first to be tested in phase I and phase II studies for active immunization of metastatic melanoma patients.[3,4] During the first stage of the studies, clinical responses were observed in Europe and the United States.[5,6] However, in 2003, Rosenberg *et al.*[7] reported that <5% of patients who received peptide vaccines such as gp100, MART-1 and tyrosinase plus IL-2 showed an overall objective response (complete response [CR] + partial response [PR]). On the other hand, investigational immunotherapy that targeted MAGE-A3 tended to reduce the risk of recurrence by 27% when used as an adjuvant therapy with surgery in stage IB/II non-small-cell lung cancer. Furthermore, enrolment in the global phase III trial of adjuvant MAGE-A3 for non-small-cell lung cancer has already started according to a certain European Union (EU)-based pharmaceutical company. This finding provides hope for current and future immunotherapies and has accelerated a variety of investigations concerned with human tumor immunology.

Survivin is a recently characterized inhibitor of apoptosis protein (IAP) that is abundantly expressed in most solid and hematological malignancies, but is barely detectable in normal adult tissues.[8] It has been shown to increase tumor resistance to apoptotic stimuli such as radiation and chemotherapy.[9,10] A number of reports have demonstrated that survivin expression in cancer cells has a prognostic value and is associated with increased tumor recurrence and a lower survival rate,[11–16] although the opposite correlation is observed in certain cancers.[17] We previously reported that survivin-2B, a splicing variant of survivin, is also expressed abundantly in various tumor cell lines and the survivin-2B80-88 (AYACNTSTL) peptide derived from the exon 2B-encoded region is recognized by CD8$^+$ cytotoxic T lymphocytes (CTL) in the context of human leukocyte antigen (HLA)-A24 molecules.[18] The CTL specific for this peptide were successfully induced from PBMC in six of seven HLA-A24-positive patients (83%) with colorectal cancers and exerted cytotoxicity against HLA-A24-positive/survivin-positive adenocarcinoma cells.[19] Furthermore, we recently demonstrated that survivin-2B peptide-specific CTL were induced in four of eight (50%) HLA-A24-positive patients with oral cancer with over stage II progression.[20] Based on these observations, a phase I clinical study of survivin-2B peptide vaccination was initiated for patients with locally advanced or recurrent oral cancer. The present clinical trial demonstrated the safety and suggested the marginal clinical effectiveness of the survivin-2B peptide vaccination alone for oral cancer patients.

Materials and Methods

Eligibility criteria. The study protocol was approved by the Clinical Institutional Ethical Review Board of the Medical

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Institute of Bioregulation, Sapporo Medical University, Japan. All patients gave their written informed consent before entry into the study. Patients enrolled in this study were required to conform to the following criteria: (i) to have histologically proven oral cancer; (ii) to be HLA-A*2402 positive; (iii) to have survivin-positive cancerous lesions by immunohistochemistry; (iv) to have HLA class I-positive cancerous lesions by immunohistochemistry using the anti-pan HLA class I mAb EMR8-5; (v) to be 20–85 years old; (vi) to have an unresectable, locally advanced or recurrent tumor; and (vii) to have an Eastern Cooperative Oncology Group (ECOG) performance status of between 0 and 3. The exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy or other immunotherapy within the previous 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection or hematopoietic failure; (v) pregnancy or breast-feeding; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Oral Surgery, Sapporo Medical University Primary Hospital from September 2003.

Peptide preparation. The survivin-2B80-88 peptide (amino acid sequence AYACNTSTL), which was derived from a splicing variant survivin-2B-specific exon 2B, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA). The identity of the peptide was confirmed by mass spectral analysis and the purity was shown to be more than 98% as assessed by high-pressure liquid chromatography analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL of physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and stored at −80°C until just before use.

Treatment protocol. Vaccinations with survivin-2B peptide were administered subcutaneously (s.c.) into the ipsilateral neck or intratumorally six times at 14-day intervals. Two incremental dose levels were planned for the peptide administration, with a starting dose of 0.1 mg. Six patients received 0.1 mg (group 1) and four patients received 1.0 mg (group 2), while each group was divided into the two different administration sites as stated above. Before proceeding to the next dose level, all previously administered patients had to have completed the trial period. Dose escalation for group 2 was allowed if no patients in group 1 experienced grade 3–4 toxicity.

If patients hoped for continuation of this peptide vaccine therapy, we conducted it in the same manner after the sixth administration.

Delayed-type hypersensitivity (DTH) skin test. The DTH skin test was performed at each vaccination. The peptide (10 μg) solution in physiological saline (0.1 mL) or physiological saline alone (0.1 mL) was separately injected intradermally (i.d.) into the forearm. A positive reaction was defined as area of erythema and induration with a diameter of more than 4 mm, 48 h after the injection.

Evaluation of toxicity and response. Patients were examined closely for signs of toxicity during and after the vaccination. The US National Cancer Institute Common Toxicity Criteria (NCI-CTC Version 2.0, Jan.30, 1998) were used to classify the toxicity grades.

Physical examinations and hematological examinations were conducted before and after each vaccination. The serum level of squamous cell carcinoma (SCC) antigen, which is the current standard tumor marker for head and neck cancer, was examined at 14-day intervals. A SCC antigen level of 1.5 ng/mL was generally taken as the upper limit of the normal range. The tumor size was evaluated by visual inspection, computed tomography (CT) and magnetic resonance imaging (MRI) before treatment, after three vaccinations and at the end of the study period. The tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines:[21] a complete response (CR) was defined as the disappearance of all target lesions; and a partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameters of the target lesions for at least 4 weeks without the appearance of new lesions. Progressive disease (PD) was defined as at least a 20% increase in the sum of the longest diameters of the target lesions or the appearance of one or more new lesions. Stable disease (SD) was defined as neither sufficient shrinkage to qualify for a PR nor a sufficient increase to qualify for PD.

***In vitro* stimulation of PBMC.** The PBMC were isolated from blood samples by Ficoll–Conray density gradient centrifugation and then frozen and stored at −80°C. As needed, frozen PBMC were thawed and incubated in the presence of 30 μL/mL survivin-2B peptide in AIM-V medium containing 10% human serum at room temperature. Interleukin-2 (IL-2) was added at a final concentration of 50 U/mL for 1 h on days 0, 2, 4 and 6 of culture. On day 7, the PBMC were analyzed by tetramer staining.

Tetramer staining. HLA-A24/peptide tetramers were constructed according to the procedure described by Altman *et al.*[22] Briefly, recombinant HLA-A24 heavy chain[23] and human β-2-microglobulin were refolded with the survivin-2B80-88 peptide as described previously.[24] The resulting HLA-A24-peptide monomer was biotinylated by incubation with the enzyme BirA (Avidity, Denver, CO, USA) for 17 h at room temperature and purified using fast protein liquid chromatography. A tetromeric HLA-peptide complex was produced by incubating streptavidin-PE (Vector Laboratories, Burlingame, CA, USA) with the biotinylated monomer at a 1:4 molar ratio. For flow cytometric analysis, the PBMC, which were stimulated *in vitro* as above, were stained with the phycoerythrin (PE)-labeled tetramer at 37°C for 20 min, followed by staining with an FITC-conjugated anti-CD8 mAb (Becton Dickinson Biosciences, San Jose, CA, USA) at 4°C for 30 min. The cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using a FACSCalibur and the CellQuest software program (Becton Dickinson Biosciences). The frequency of the CTL precursors was calculated as the number of tetramer-positive cells over the number of CD8-positive cells. Moreover, the PBMC were stained with an FITC-labeled HLA-A*2402-restricted human immunodeficiency virus (HIV) peptide (RYLRDQQLL) tetramer and PE-labeled HLA-A*2402-survivin-2B80-88 peptide tetramer, which were purchased from MBL Co., Ltd. (Nagoya, Japan), at 37°C for 20 min, followed by staining with an FITC- or PerCP-conjugated anti-CD8 mAb (Becton Dickinson Biosciences) at 4°C for 30 min. The frequency of the CTL precursors was calculated in the same manner.

Results

Patient characteristics. Eleven patients (six males, five females) were eligible and agreed to participate in this phase I study. The patients' characteristics are summarized in Table 1. The patients' median age at enrolment was 66.5 years, with a range 38–84 years. Based on the ECOG classification, five patients were PS1, five were PS2, and one was PS3. The patients' primary tumor sites were: buccal mucosa, three; palate, two; upper or lower alveolus and gingiva, two; mandible, one; floor of mouth, one; submandibular gland, one; and tongue, one. The histological type was SCC in seven patients, adenoid cystic carcinoma (ACC) in three and alveolar soft part sarcoma (ASPS) in one. Table 2 summarize the clinical and immunological outcomes for the 11 patients. One patient discontinued the regimen after four vaccinations. She (case 8) had a growing locoregional recurrence and her general condition deteriorated. Subsequently she was removed from the study after four vaccinations because she refused to continue the protocol. None of

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Table 1. **Summary of the characteristics of patients enrolled in the present study**

Patient no.	Histology	Age/Sex	PS	Primary tumor site	Recurrent or metastatic sites
1	ASPA	38/M	1	Mandible	Local, brain, lung
2	ACC	60/M	1	Hard palate	Local, lung
3	SCC	84/F	3	Floor of mouth	Locoregional
4	ACC	50/F	2	Submandibular gland	Lung
5	SCC	83/F	2	Upper alveolus and gingiva	Locoregional
6	SCC	72/M	2	Buccal mucosa	Local
7	SCC	55/M	2	Tongue	Locoregional
8	SCC	82/F	2	Lower alveolus and gingiva	Neck
9	SCC	73/M	1	Hard palate	Lung, liver
10	SCC	82/F	1	Buccal mucosa	Neck
11	SCC	68/M	1	Buccal mucosa	Locoregional

ACC, adenoid cystic carcinoma; ASPS, alveolar soft part sarcoma; SCC, squamous cell carcinoma.

Table 2. **Profiles of the enrolled patients and clinical responses to the survivin-2B peptide vaccination**

Patient no.	Dose of peptide (mg)	Injection route	HLA class I expression	Prior therapy (washout time)	Adverse events	Tetramer staining† (pre-/post-)	Tumor marker	Clinical response	Follow up (months)	Progress
1	0.1	Intratumoral	+	S + C (1 month)	–	ND	ND	PD	43	AWD
2		Intratumoral	+	S + C (1 month)	–	121/103	ND	PD	25	DOD
3		Intratumoral	+	C + R (1 month)	–	ND	ND	PD	3	DOD
4		s.c.	+	S + C + R (6 years, 4 months)	–	1/100	ND	PD	15	DOD
5		s.c.	+	C (1 month)	–	6/16	INC	PD	6	DOD
6		s.c.	+	S + R + C (1 months)	–	65/244	INC	PD	3	DOD
7	1.0	Intratumoral	+	S + R + C (1 month)	–	96/528	ND	PD	6	DOD
8‡		Intratumoral	+	S + R (1 month)	–	ND	ND	ND	2	DOD
9		s.c.	+	S + C (2 months)	–	77/204	DEC	PD	5	DOD
10		Intratumoral	+	S + C (1 month)	–	5/20	DEC	PR	5	DOD
11		s.c.	+	S + R + C (5 months)	–	5/1	ND	PD	8	DOD

†Tetramer staining: Tetramer(+)CD8(+) in 10 000 CD8(+) cells. ‡Patient refused to continue the protocol (case 8). AWD, alive with disease; C, chemotherapy; DEC, decreased; DOD, dead of disease; HLA, human leukocyte antigen; INC, increased; ND, not determined; PD, progressive disease; post-, after the fourth vaccination; PR, partial response; pre-, before the first vaccination; R, radiotherapy; S, surgery.

the treatment interruptions were due to any adverse reactions to the vaccination. Ten patients received the complete regimen including six vaccinations and thereafter were evaluated.

Safety. The peptide vaccination was well tolerated in all 10 patients. No hematological, cardiovascular, hepatic or renal toxicity was observed during or after vaccination. Skin reactions such as induration, pain or rash were not observed in any case.

DTH skin test. A DTH skin test was performed at each vaccination and assessed 48 h later. No positive DTH reaction was observed in any patient.

Clinical responses. In two patients (cases 9 and 10) the tumor marker level (SCC antigen) transiently decreased. In two patients (cases 5 and 6) it increased and in the remainder it was not useful for monitoring. A PR was observed in one patient (case 10), who also demonstrated a remarkable decrease in the SCC antigen level (6.0 ng/mL → 0.7 ng/mL). The remaining nine patients experienced PD.

Case 9, who had multiple lung metastases, transiently showed a positive level of SCC antigen of 2.1 ng/mL that decreased after the second vaccination and was within the normal range just after the third vaccination. However, after the fourth vaccination it increased abruptly, which closely corresponded to his clinical progress. Until the fourth vaccination, CT imaging of the lung revealed virtually dormant disease, however, it revealed progressive disease after the sixth vaccination.

One responder (case 10) with PR developed multiple neck metastases and skin metastases in the left side of her neck at 3 months after surgery followed by treatment with tegafur/uracil (UFT) at a daily dose of 400 mg as oral adjuvant chemotherapy. She was judged to be impossible to treat radically because CT imaging showed that the recurrent tumor had metastasized to lymph nodes and the skin, including the parotid gland, submandibular region, posterior cervical region, occipital region of the head, posterior skull base and lower cervical region (Fig. 1A). The metastatic progressive tumor samples from her neck obtained by neck dissection previously were confirmed by immunohistochemical staining to markedly express survivin and HLA class I molecules. Survivin-2B peptide vaccine was administered intratumorally to the left side of her neck nine times at biweekly intervals. The SCC antigen level was 6.0 ng/mL before vaccination. Her skin metastatic tumor and pain disappeared transiently after the fifth vaccination, thus resulting in an improvement in her quality of life. A tumor regression rate of 70% was observed by CT imaging (Fig. 1B). The SCC antigen level decreased to 0.7 ng/mL after the sixth vaccination (Fig. 2). Nevertheless, these effects were maintained for 2 months only.

Tetramer staining assay. Peptide-specific immunological responses were evaluated in eight patients by HLA-A24/survivin-2B80-88 peptide tetramer analysis. The change of the tetramer-positive CTL frequency was evaluated by comparison with that before the first vaccination and that after each vaccination. The frequency of tetramer-positive CTL tended to increase after the vaccination in six patients (cases 4, 5, 6, 7, 9 and 10) (Table 2). In Figure 3, the peptide-specific CTL frequencies in cases 9 and 10 are indicated as the percentages of

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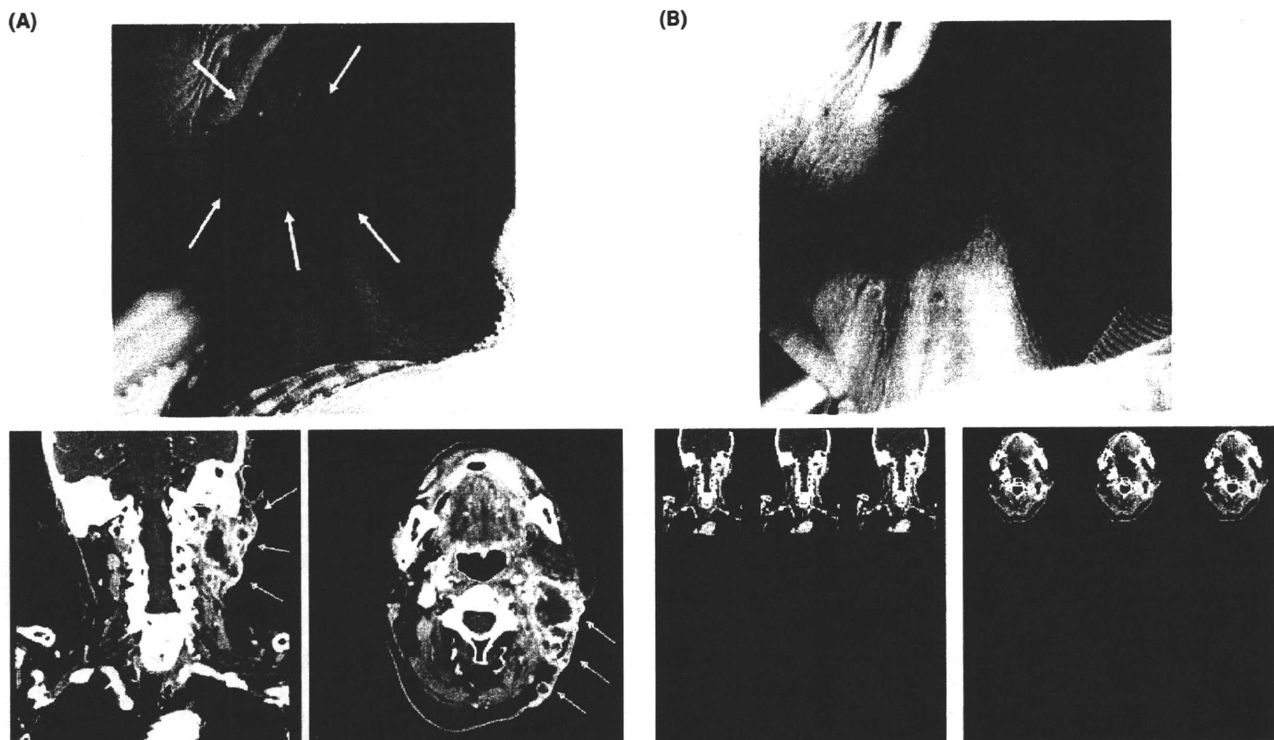

Fig. 1. Photograph of skin on the neck and computed tomography (CT) scan image of the neck showing metastatic tumors of case 10. (A) Photograph of skin on the neck and CT scan image of the neck before vaccination. Axial contrast-enhanced CT image shows multiple metastatic tumors (arrows). (B) Photograph of skin on the neck and CT scan image of the neck after the fifth vaccination. The metastatic tumors show significant remission after the fifth vaccination compared with before vaccination (70% reduction).

tetramer-positive CTL among CD8-positive T cells before and after the fourth vaccination. The frequency of tetramer-positive CTL was increased from 0.77% to 2.04% and from 0.05% to 0.20%, in cases 9 and 10 respectively.

Discussion

Many tumor-associated antigens have been identified and clinical trials utilizing them have been conducted.[3–6] However, most such clinical trials were aimed at the treatment of advanced melanoma and there are few reports on the treatment of patients with solid cancers. Although the immunogenicity of these non-melanoma-associated antigens is relatively weak, a specific number of tumor antigens were determined. The HLA-A24-restricted CTL epitope survivin-2B80-88 derived from survivin-2B has high potency for CTL induction in various cancer patients, including those with breast cancer, colorectal cancer, gastric cancer and oral cancer.[10,18–20] Based on the findings of these studies *in vitro*, a phase I clinical study of survivin-2B peptide vaccine therapy began in September 2003 for patients with advanced or recurrent oral cancer, following those for colorectal cancer and breast cancer. In many clinical trials, patients received the peptide in combination with certain adjuvants such as incomplete Freund's adjuvant (IFA) and cytokines for the purpose of enhancing the immune responses against cancer. In the present study, patients received the survivin-2B peptide dissolved in physiological saline without any adjuvant in order to strictly evaluate the clinical effect of the peptide alone.

A dose-escalation trial was chosen to estimate the safe and optimal doses. Dosage groups of 0.1 and 1.0 mg were set up, consisting of six and four patients, respectively. None of the patients had any sign of toxicity. Therefore, the survivin-2B

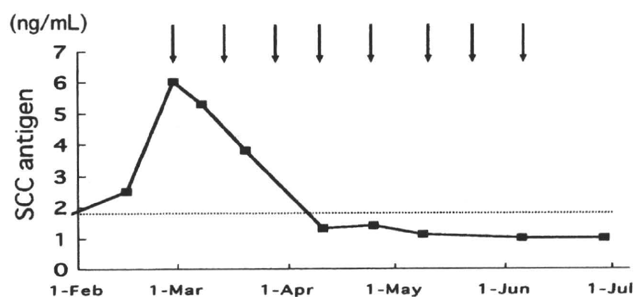

Fig. 2. Changes in the serum squamous cell carcinoma (SCC) antigen level during the vaccination in case 10. The dotted line indicates the cut-off point for the SCC antigen level. The arrows indicate the times of vaccination. The SCC antigen level significantly decreased to 0.7 ng/mL after the sixth vaccination. The cut-off value was 1.5 ng/mL.

peptide vaccine was safe and could be repeatedly injected into patients without serious side-effects. In terms of the clinical responses, the levels of tumor markers were temporarily decreased in comparison with the pretreatment status in two patients in the 1.0 mg dosage group. No patients in the 0.1 mg dosage group experienced a decrease in tumor markers. A PR was observed in one patient who was administered 1.0 mg of peptide. Therefore, the 1.0 mg dosage group appeared to have a better clinical outcome than the 0.1 mg dosage group. Based on these results, the recommended survivin-2B vaccine dose was 1.0 mg. Furthermore, we set up two distinct injection routes, s.c. into the ipsilateral neck or intratumorally. Intratumoral injection was concretely done by

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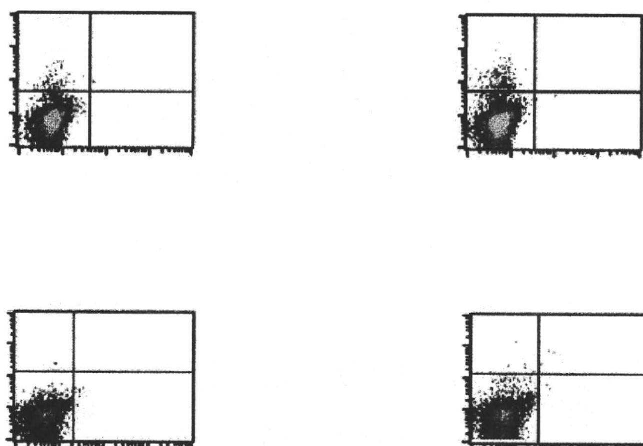

Fig. 3. Tetramer staining before the first vaccination and after the fourth vaccination in cases 9 and 10. Flow cytometric analysis was performed using a FACSCaliber and CellQuest software (Becton Dickinson Biosciences). The frequency of the cytotoxic T lymphocyte (CTL) precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells. The peptide-specific CTL frequency is indicated as the percentage of tetramer-positive CTL among CD8-positive T cells before the first vaccination and after the fourth vaccination. In cases 9 and 10, the frequency of tetramer-positive CTL was increased from 0.77% to 2.04% and from 0.05% to 0.20%, respectively. HIV, human immunodeficiency virus.

submucosal or subcutaneous vaccination into the peripheral parts of tumors, avoiding necrotic areas and vessels, for intraoral tumors and neck tumors, respectively. However, no significantly different findings as a whole were noted for the clinical and immunological responses.

In the present study, one patient (case 10) achieved a clinical PR. This demonstrated that the survivin-2B vaccination could yield an excellent response in oral cancer. The patient had received tegafur/uracil (UFT) as oral adjuvant chemotherapy and limited systemic chemotherapy for a few months prior to the vaccine treatment. She was judged to have PS1 in the ECOG classification. It is possible that peptide-based immunotherapy might be more effective in patients with reduced immune suppression as a result of recent intensive chemotherapy, as suggested by the previous clinical study of survivin-2B vaccination for colon cancer, although the study consisted of only a limited number of patients.[25] The results of the present trial were mostly compatible with the colon cancer studies in terms of the chemotherapeutic background. Furthermore, by immunohistochemistry, we preliminarily examined the infiltration of local immune cells in metastatic progressive tumor samples from her neck obtained before the first vaccination. Infiltration of CD8 T-cells into the peripheral parts of the tumor was markedly observed. On the other hand, a large number of tumor cells with strong survivin and HLA class I expression were observed. It was presumed that these findings indicated good conditions for immune responses in the tumor microenvironment. However, we failed to obtain a specimen during or after vaccination to evaluate the frequency of these cells (data not shown). Further studies to elucidate the immunoregulatory mechanisms of the immune escape by analyzing the infiltrating immune cells in local tumor sites will be necessary.

Although analysis of peripheral blood lymphocytes using HLA-A24/peptide tetramers actually revealed a slight increase in the peptide-specific CTL frequency in six patients, the immune responses had no relevance to the clinical responses in this study. It seems reasonable to conclude that the number of CTL induced by the vaccine was insufficient to induce tumor regression in patients with advanced or recurrent oral cancer, as vaccine-specific CTL might not be recruited into the tumor site, and the cytotoxic function of CTL might be suppressed in the tumor site by certain mechanisms such as regulatory T cells and immunosuppressive cytokines in the tumor microenvironment.

Overall, the survivin-2B peptide vaccination was well tolerated, but it is suggested that this vaccination protocol might provide only marginal immunological and clinical responses in most advanced or recurrent oral cancer patients. It is possible that advanced protocols such as a more intense immunization schedule and delivery in combination with a specific adjuvant and/or an immune-stimulatory cytokine might improve the efficacy of the survivin-2B peptide vaccine against oral cancer. Indeed, vaccination of the survivin-2B peptide mixed with IFA increased the frequency of peptide-specific CTL more than vaccination with the peptide alone in a phase I clinical trial for patients with advanced or recurrent breast cancer.[26] Based on the results of the present study and the other trials, a second clinical study of survivin-2B peptide vaccine has recently been started in combination with IFA and interferon-alpha.

Disclosure Statement

The authors have no conflict of interest.

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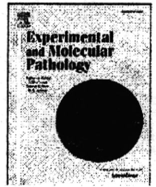

The feasibility of Cep55/c10orf3 derived peptide vaccine therapy for colorectal carcinoma

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ABSTRACT

In our previous study, we demonstrated that a peptide derived from the novel centrosome residing protein Cep55/c10orf3 can be targeted by the cytotoxic T lymphocytes (CTLs) in peripheral blood mononuclear cells (PBMCs) of breast carcinoma patients. In this report, we evaluated the feasibility of cancer immunotherapy using Cep55/c10orf3 peptide for colorectal carcinoma (CRC). To evaluate the expression of Cep55/c10orf3 in CRC tissues, we performed immunohistochemical staining of using anti-Cep55/c10orf3 monoclonal antibody. Sixty-three percent cases showed weak positive for Cep55/c10orf3 in total 70 CRC cases. The Cep55/c10orf3 expression intention was collated with high histological grade of CRC. Thus, we hypothesized that Cep55/c10orf3 can also be the target of CTLs in CRC cases. We generated CTLs from PBMCs of human leukocyte antigen (HLA)-A24-positive colorectal carcinoma patients using HLA-A24-restricted Cep55/c10orf3 peptides. Two of 6 colorectal cancer patients were reactive for the Cep55/c10orf3_193(10) peptide, which was the only immunogenic peptide in breast carcinoma patients. CTL clone specific for Cep55/c10orf3_193(10) recognized and lysed HLA-A24 (+) and Cep55/c10orf3 (+) colorectal carcinoma cell lines. In addition, 1 of 6 colorectal carcinoma patients was reactive for the Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) peptides, but not for Cep55/c10orf3_193(10) with the ELISPOT assay. These observations suggest that the antigenic peptide repertoire presented by HLA-A24 in colorectal carcinoma might be different from that in breast carcinoma. Thus, these peptide vaccination peptide mixture of Cep55/c10orf3_193(10), Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) might be more effective than a single peptide in the treatment of colorectal carcinoma patients.

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Introduction

Colorectal carcinoma is one of the major deadly malignancies in the world. Despite recent progress in the treatment of colorectal carcinoma, recurrence, metastasis or resistance to treatment remain the biggest challenges to control disease and contribute to unfavorable prognosis. In the advanced cases, it is very difficult to treat and control the disease, and new therapeutics are urgently needed. Immunotherapy might represent an effective novel treatment for colorectal carcinoma.

Previously, Cep55/c10orf3 was reported to be overexpressed in colorectal carcinomas (Inoda et al., 2009; Sakai et al., 2006). Cep55/c10orf3 plays essential role in G2/M cell cycle phase (Fabbro et al., 2005; Zhao et al., 2006) and is presumably expressed in all cancer cells. In contrast, Cep55/c10orf3 expression in normal tissues is restricted to testis and thymus (Inoda et al., 2009; Sakai et al., 2006). This makes Cep55/c10orf3 a suitable target for cancer immunotherapy (Hirohashi et al., 2009).

Abbreviations: CTLs, cytotoxic T lymphocytes; TAAs, tumor associated antigens; mAb, monoclonal antibody; PBMCs, peripheral blood mono-nuclear cells; IFN, interferon; B2M, beta-2 microglobulin.

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Table 1
Summary of the Cep55/c10orf3 peptides carrying HLA-A24 binding motief.

Peptides	Position number	Sequence
Cep55/c10or3_169(10)	169-178	EMEIQLKDAL
Cep55/c10orf3_193(10)	193-202	VYVKGLLAKI
Cep55/c10orf3_355(9)	355-363	QMQACTLDF
Cep55/c10orf3_446(10)	446-455	QYPATEHRDL
Cep55/c10orf3_74(11)	74-84	AYQLTEKDKEI
Cep55/c10orf3_94(11)	94-104	RYSTTALLEQL
Cep55/c10orf3_402(11)	402-412	EFAITEPLVTF
Cep55/c10orf3_186(13)	186-198	VYDQQREVYVKGL
Cep55/c10orf3_227(14)	227-240	GYLQEEKQKCYNDL
Cep55/c10orf3_268(12)	268-279	KYEETQKEVHNL
Cep55/c10orf3_283(12)	283-294	LYSQRRADVQHL
HIV-A24		RYLRDQQLLGI

In our previous study, we found that the centrosomal protein Cep55/c10orf3 was overexpressed in breast, colorectal and lung carcinoma tissues. We isolated an HLA-A24-restricited Cep55/c10orf3 peptide [Cep55/c10orf3_193(10)], which has been shown to be the target of CTLs from PBMCs of HLA-A24-positive breast carcinoma patients (**Inoda et al., 2009**). We established that Cep55/c10orf3 may be used for the cancer immunotherapy of breast carcinoma patients. However, the immunogenicity in colorectal carcinomas is still elusive.

Here we extended our studies to colorectal carcinoma. We evaluated the feasibility of using Cep55/c10orf3 derived peptides for HLA-A24 positive, which is a frequent allele in Japanese and also other populations, colorectal carcinoma immunotherapy. To investigate the immunogenicities of Cep55/c10orf3 derived peptides, PBMCs of colorectal carcinoma patients were stimulated several times using Cep55/c10orf3 peptides, and the CTL reactivity was assessed with the ELISPOT assay and the ^{51}Cr release assay. Two of the 6 colorectal carcinoma patients were positive for the Cep55/c10orf3_193(10) peptide, which is also immunogenic in breast carcinoma patients. The Cep55/c10orf3-specific CTL clone recognized HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinoma cell lines, suggesting that colorectal carcinoma express endogenously processed Cep55/c10orf3 peptide. Furthermore, one of the six colorectal carcinoma patient showed immune reactivity for other peptides, including Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) by the ELISPOT assay. Unfortunately, we could not further analyze the cytotoxic

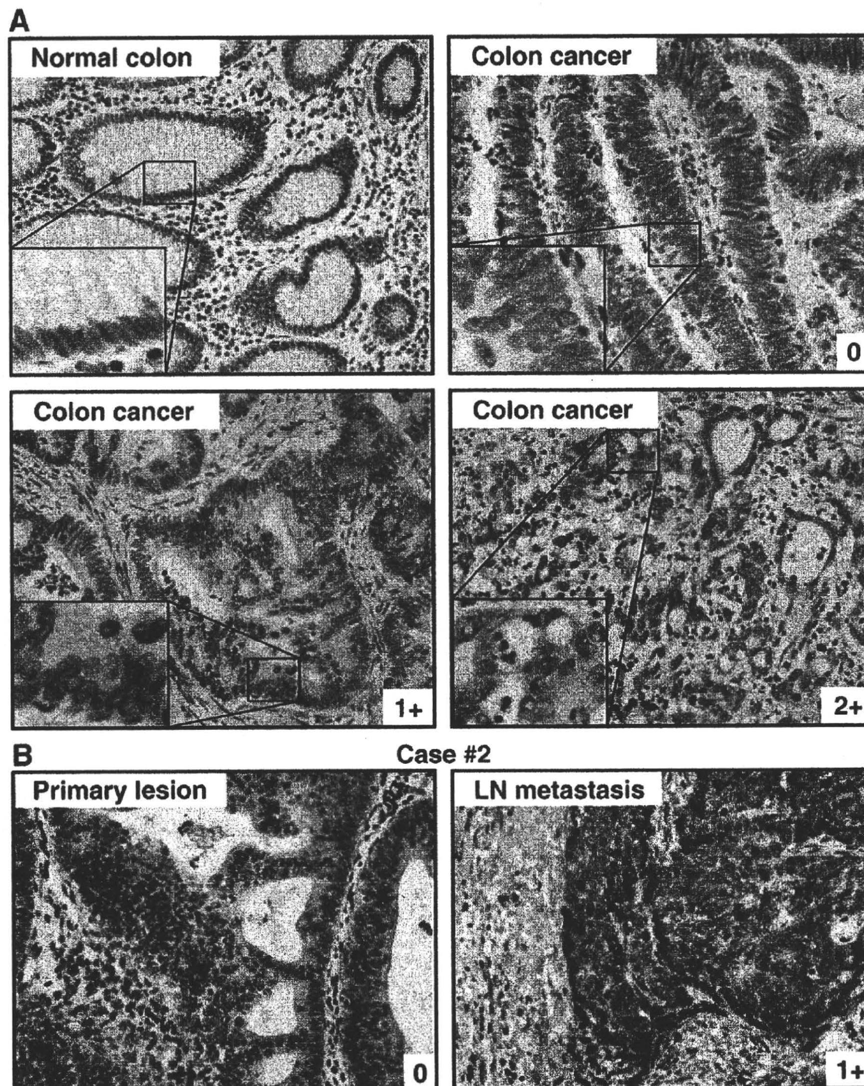

Fig. 1. Immunohistochemical staining of normal colon mucosa and colon cancers. A. Normal colon tissue and colon cancer tissues (summarized in Table 2) were stained with mAb #11-55 (magnification: ×200). Immunoreactivities were classified as follows: 0 as negative staining; 1+, weak positive staining; 2+, strong positive staining. B. Immunohistochemical staining of case #2 primary lesion and lymphnode metastasis lesion (magnification: ×200).

Table 2
Cep55/c10orf3 protein expression in colorectal carcinomas.

Histology*	N	Expression of Cep55/c10orf3		
		0 (Negative) (%)	1+ (Weak positive) (%)	2+ (Strong positive) (%)
Total	70	26 (37%)	36 (51%)	8 (12%)
Well differentiated adenocarcinoma	19	10 (53%)	9 (47%)	0 (0%)
Moderately differentiated adenocarcinoma	44	15 (34%)	25 (57%)	4 (9%)
Poorly differentiated adenocarcinoma	7	1 (14%)	2 (29%)	4 (57%)

*Histology of the colorectal cancer cases were classified according to 'WHO classification Tumors of the Digestive System'. N, case numbers.

activity for these peptides; however, the immune reactivities were specific and vigorous, suggesting that these peptides specific CTL precursors (CTLp) might be activated and expanded clonally *in vivo*. These observations indicate that Cep55/c10orf3 derived peptides, including Cep55/c10orf3_193(10), Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12), may be useful for HLA-A24-positive colorectal carcinoma immunotherapy.

Materials and methods

Immunohistochemical staining of tissue sections

Immunohistochememical staining was done with formalin-fixed, paraffin-embedded sections of 64 CRC, including tissue microarray and 6 biopsy CRC specimens. Sections (4–5-μm thick) were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by boiling sections in 120 °C for 5 min in a microwave oven in preheated 0.01-mol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by 3%hydrogen peroxide in ethanol for 10 min. After blocking with 1%nonfat dry milk in PBS (pH 7.4), the sections were reacted with monoclonal anti-Cep55/c10orf3 antibody #11-55 for 1 hour, followed by incubation with biotinylated anti-mouse IgG (Nichirei) for 30 min. Subsequently, the sections were stained with the streptavidin–biotin complex (Nichirei), followed by incubation, with 3,3'-diaminobenzidine used as the chromogen and counterstaining with hematoxylin. Cytosolic staining was considered positive. We graded the immunoreactivity as follows: 0 as negative staining; 1+, weak positive staining; 2+, strong positive staining. Breast cancer cases were used as positive controls.

Patients

The PBMCs used in this study were obtained from HLA-A24-positive colorectal carcinoma patients who were hospitalized at Higashi Sapporo hospital (Sapporo, Japan) after obtaining their informed consent.

CTL induction, ELISPOT assay and ^{51}Cr release assay

CTLs were generated from PBMCs of HLA-A24 positive colorectal carcinoma as described previously (Hirohashi et al., 2002; Inoda et al., 2009). In brief, dendritic cells (DCs) and PHA-blasts were generated from the PBMCs of HLA-A24-positive colorectal carcinoma patients. CD8-positive T cells were isolated using the MACS separation system (Miltenyi Boitech, Bergish Blabach, Germany) according to the manufacturer's instructions. The CD8-positive T cells were stimulated for three times using DC for the 1st time of stimulation and PHA-blast for the 2nd and 3 rd times of stimulations as antigen presenting cells (APCs) with a mixture of eleven Cep55/c10orf3 peptides carrying HLA-A24 binding motif, as summarized in Table 1, (Inoda et al., 2009) in AIM-V medium (Invitrogen) supplemented with 10% of human serum.

One week later after the last stimulation, the CD8-positive T cells were evaluated the reactivity for peptides with the ELISPOT assay as described previously (Inoda et al., 2009). ELISPOT plates were coated sterilely overnight with an IFN-gamma capture antibody (Beckton Dickinson Biosciences) at 4 °C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 hours at room temperature. Then, twenty thousand CTLs were incubated with 5×10^4/well T2-A24 (kind gift from Dr. K. Kuzushima, Nagoya, Japan) cells pulsed with Cep55/c10orf3 specific peptide or control peptides (5 μg/mL) or K562 cells. After incubation in a 5% CO2 humidified chamber at 37 °C for 24 hours, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-gamma antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Germany).

The lytic activity of CTLs was tested by the ^{51}Cr release assay as described previously (Sato et al., 1986). Briefly, ^{51}Cr-labeled target cells (2000 cells/well) were incubated with various numbers of effector cells for 6 hours at 37 °C in 96-well microtiter plates. The radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release) X100. Colorectal carcinoma cell lines Sw480, HT29 and HCT15 (kind gift from Dr. K. Imai, Sapporo, Japan) were used as target cells. The beta 2 microglobulin (*B2M*) transformant of HCT15 cells (HCT15-B2M) were obtained by the infection of retrovirus coding beta 2 microglobulin (*B2M*) cDNA. The retrovirus was generated as described previously using the retrovirus vector pMXs-puro encoding *B2M* cDNA and PLAT-A packaging cells (kind gift from Dr. T. Kitamura, Tokyo, Japan) (Morita et al., 2000), and

Table 3
Summary of the colorectal carcinoma cases.

Case #	Stage	Histology	Expression of Cep55/c10orf3	Reactive peptides	ELISPOT assay	^{51}Cr release assay
1	IIIb	Adenocarcinoma (mod. diff.)	Primary (1+)	193(10)	+	+
2	IV	Adenocarcinoma (mod. diff.)	Primary (0), LN (1+)	193(10)	-	+
3	IV	Adenocarcinoma (mod. diff.)	Primary (1+)	402(11),283(12)	+	ND
4	IV	Adenocarcinoma (mod. diff.)	Primary (1+)	-	-	-
5	II	Adenocarcinoma (well diff.)	Primary (1+)	-	-	-
6	II	Adenocarcinoma (well diff.)	Primary (0)	-	-	-

Abbreviations: mod. diff., moderatry differentiated; well diff., well differentiated; Primary, Primary lesion; LN, lymph node metastasis lesion; ND, not detected.

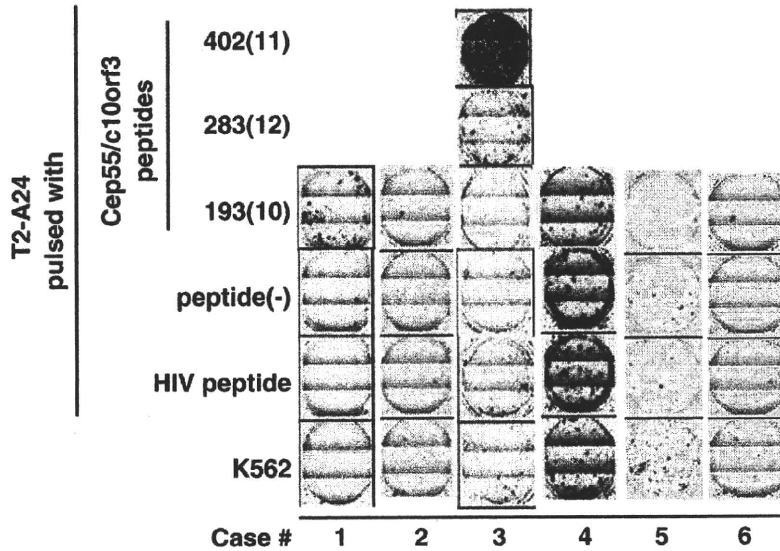

Fig. 2. ELISpot assay for Cep55/c10orf3 peptides in colorectal carcinoma patients. The Cep55/c10orf3 peptides specific CTLs were induced from HLA-A24 (+) colorectal carcinoma patients' PBMCs. The PBMCs were stimulated with the Cpe55/c10orf3 peptide cocktail for 3 times, and the peptide-specific reactivities were evaluated with the IFN-gamma ELISPOT assay using Cep55/c10orf3 peptide pulsed T2-A24 cells. The HIV derived peptide was used as a negative control. K562 is used as a negative control.

after 2days of post-infection the HCT15 cells were selected by a medium containing puromycin (5 μg/ml). The expression of *B2M* mRNA was confirmed by RT–PCR.

Results and discussion

We described that Cep55/c10orf3 was expressed in 25% (6/25) colorectal carcinoma cases previously (Inoda et al., 2009). The protein expression frequencies were relative low compared with breast carcinoma. In this study, to evaluate the protein expression in more detail, we improved the method of immunohistochemical staining. With this protocol, the stained specimens showed lower background, and more suitable for monoclonal antibody #11–55. With this protocol, we re-stained 70 colorectal carcinoma tissue specimens. As shown in Fig. 1A and Table 2, 44 of 70 colon cancer tissues showed positive staining for monoclonal antibody (#11–55), while normal colon epithelial cells showed negative for #11–55. The positivities for #11–55 in well differentiated adenocarcinoma was 47% (47% of weak positive and 0% of strong positive); in moderately differentiated adenocarcinoma, 66% (57% of weak positive and 9% of strong positive); and in poorly differentiated adenocarcinoma, 86% (29% of weak positive and 57% strong positive). Thus, we hypothesized that Cep55/c10orf3 might also be the immunological target of CTLs in CRC patients. In this study, we evaluated 6 CRC patients for immunological assays (Table 3). Fig. 1B shows the representative Cep55/c10orf3 protein staining patterns used for the CTL assays. In the colorectal

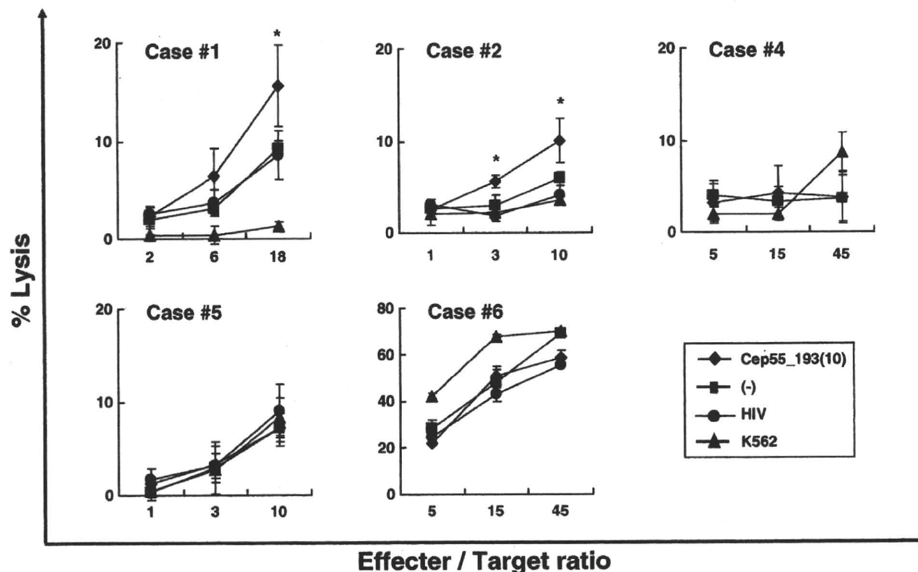

Fig. 3. Cytotoxic activity for Cep55/c10orf3 peptide in colorectal carcinoma patients. The cytotoxicity of the CTLs from colorectal carcinoma patients' PBMCs against K562 cells and T2-A24 cells pulsed with the Cep55/c10orf3_193(10) peptide, HIV peptide, or no peptide were analyzed using the ^{51}Cr release assay at various effector/target (E/T) ratios. Asterisks denote statistically significant differences from negative control peptide and no peptide pulsed T2-A24 cells. Each point represents the mean ± SD. Asterisks indicate statistically significant differences from the highest negative control the Cep55/c10orf3_193(10) peptide pulsed T2-A24 and negative controls by the Student's *t*-test (*, $p<0.05$).

carcinoma of patient #2, the primary colon carcinoma lesion did not show any Cep55/c10orf3 protein expression. In contrast, the regional lymph node metastasis lesion showed Cep55/c10orf3 protein expression. Because Cep55/c10orf3 is essential for the G2/M phase of the cell cycle (Fabbro et al., 2005), the protein is presumably expressed in mitotic cells. In our previous study, we could detect Cep55/c10orf3 mRNA expression in all of colorectal carcinoma cell lines (Inoda et al., 2009), suggesting that the proliferating colorectal carcinoma cells express Cep55/c10orf3 mRNA and protein.

To evaluate the feasibility of colorectal carcinoma immunotherapy using Cep55/c10orf3 peptides, we generated CTLs specific for Cep55/c10orf3 peptides in 6 colorectal carcinoma patients. To determine which Cep55/c10orf3 peptide is immunogenic in the PBMCs of colorectal carcinoma patients, we stimulated CD8-positive T cells of HLA-A24-positive colorectal carcinoma patients using an 11 Cep55/c10orf3 peptide cocktail. As shown in Fig. 2, case #1 showed Cep55/c10orf3_193(10) specific reactivity with the ELISPOT assay, which is the only immunogenic peptide for HLA-A24-positive breast carcinoma patients, as described before (Inoda et al., 2009). On the other hand, case #3 showed vigorous reactivity for T2-A24 cells pulsed with Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) compared to control peptides, which showed also high HLA-A24 binding affinity before (Inoda et al., 2009). Interestingly, these peptides are 11- and 12-mer, which are longer than the standard HLA-A24-restricted peptides (Kondo et al., 1995), and these peptides were not immunogenic for breast carcinoma patients. These observations suggest that not only the Cep55/c10orf3_193(10) peptide but also the Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) peptides can be presented by HLA-A24-positive colorectal carcinoma cells and caused the clonal expansion of the CTL precursor (CTLp) *in vivo*.

To assess the cytotoxic activity of the CTLs, we performed the ^{51}Cr release assay. Unfortunately, the CTLs of case #3 were used up in the ELISPOT assay, so we could not perform the ^{51}Cr release assay. As shown in Fig. 3, case #1 and case #2 showed specific reactivity for T2-A24 cells pulsed with the Cep55/c10orf3_193(10) peptide compared to the control peptides. The CTL of case #1 showed specific reactivity for the Sw480 colorectal carcinoma cell line, which is Cep55/c10orf3 positive and HLA-A24 positive, suggesting this CTL recognized the endogenously expressed Cep55/c10orf3_193(10) peptide (data not shown). To analyze further, we characterized Cep55/c10orf3_193(10) CTL clone #41, which was previously isolated from the PBMCs of HLA-A24-positive breast carcinoma patients (Inoda et al., 2009). As shown in Fig. 4B, Cep55/c10orf3_193(10) specific CTL clone #41 showed a specific lytic activity for HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinoma cell lines, including Sw480 and HT29. The beta 2 microglobulin (*B2M*) gene of the HCT15 cell line is known to be mutated, and the B2M protein is downregulated, which causes the low expression of the HLA-A24 molecule on the cell surface. We therefore established the wild type *B2M* gene stably transduced subline (HCT15-B2M). HCT15-B2M

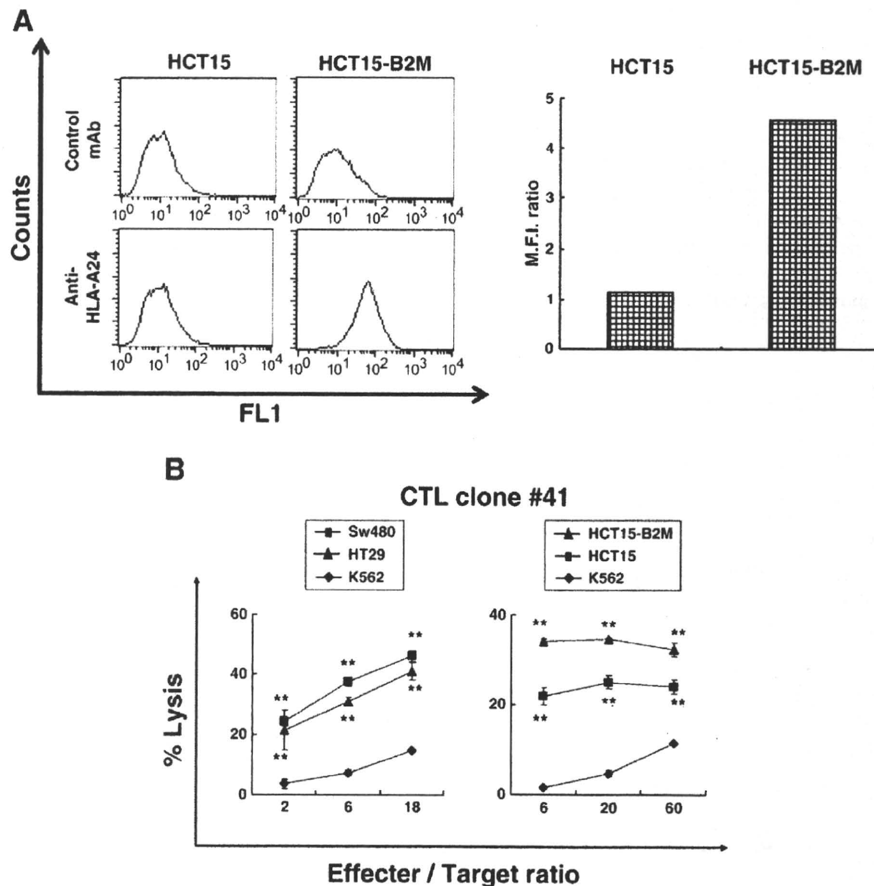

Fig. 4. Cytotoxicity for HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinomas. A. Expression of HLA-A24 on HCT15 and HCT15-B2M cells. The expression of HLA-A24 on the HCT15 and HCT15-B2M cells were evaluated by the FACS analysis. The HCT15 and HCT15-B2M cells were stained by the control antibody and anti-HLA-A24 antibody and detected by FACS Caliber. The right bar graph indicates the ratio of mean fluorescent intensity (M.F.I.), which is calculated as follows: M.F.I. ratio = (M.F.I. with the anti-HLA-A24 antibody)/(M.F.I. with control antibody) B. Cep55/c10orf3_193(10) specific CTL clone (#41) was assessed for the cytotoxicity for HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinoma cell lines (Sw480, HT29 and HCT15) with the ^{51}Cr release assay. HCT15-B2M is a beta-2 microglobulin gene transduced cell line. HLA-A24 (−) and Cep55/c10orf3 (+) K562 were used as negative controls. Each point represents the mean ± SD. Asterisks indicate statistically significant differences from the K562 negative control cell by the Student's *t*-test (**, $p<0.01$).

showed the enhanced HLA-A24 expression with the FACS analysis (Fig. 4A). CTL clone #41 recognized HCT15 cells compared with K562 cells, and the lytic activity was enhanced with the ectopic expression of the *B2M* gene (Fig. 4B). These observations suggest that the Cep55/c10orf3_193(10) peptide is endogenously expressed in a number of colorectal carcinoma cell lines and that the Cep55/c10orf3_193(10) peptide is suitable for colorectal carcinoma immunotherapy.

Cep55/c10orf3 is expressed in a wide variety of cancer cells (Inoda et al., 2009; Sakai et al., 2006), which makes it a reasonable target for cancer immunotherapy. Cep55/c10orf3_193(10) is a high immunogenic peptide in breast carcinoma patients. However, the immunoreactivity for Cep55/c10orf3_193(10) in colorectal carcinoma is less frequent than that of breast carcinoma. Very interestingly, the Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) peptides are only immunogenic for colorectal carcinoma patient case #3. This may result from the difference in the peptide presentation profiles *in vivo* between colorectal carcinoma and breast carcinoma. In the previous study, we have shown that only the Cep55/c10orf3_193(10) peptide is suitable for breast carcinoma immunotherapy. However, the results of the current study indicate that not only Cep55/c10orf3_193(10) but also Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) may be useful for colorectal carcinoma immunotherapy.

The centrosome is the principal microtubule organizing center of the mammalian cell, consisting of a pair of barrel-shaped microtubule assemblies that are non-identical and are referred to as the mother and daughter centrioles. Defects in the number, structure or function of centrosomes can generate monopolar or multipolar mitotic spindles and cytokinesis defects, resulting in aneuploidy and chromosome instability, which are common characteristics of tumor cells. Thus, abnormal centrosome constituents may be exploited as therapeutic targets for malignantly transformed or dysplastic cells (Wang et al., 2004). In addition to Cep55/c10orf3 other centrosome residing molecules have been reported to be the target of CTLs, such as survivin (Hirohashi et al., 2002) and Aurora-A kinase (Ochi et al., 2009). These centrosome antigens are essential for G2/M phase transition and are expressed universally in malignant cells (Hirohashi et al., 2009). Thus targeting centrosome antigens is a reasonable approach for cancer immunotherapy, and more centrosome related antigens might be identified in the future.

In summary, we were able to generate CTLs specific for Cep55/c10orf3_193(10), Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) in HLA-A24-positive colorectal carcinoma patients. This suggests that these peptides may be useful for HLA-A24-positive colorectal carcinoma immunotherapy.

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Immunopathology and Infectious Diseases

Arachidonate 5-Lipoxygenase Establishes Adaptive Humoral Immunity by Controlling Primary B Cells and Their Cognate T-Cell Help

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In this study, we report the unique role of arachidonate 5-lipoxygenase (Alox5) in the regulation of specific humoral immune responses. We previously reported an L22 monoclonal antibody with which human primary resting B cells in the mantle zones of lymphoid follicles are well-defined. Proteomics analyses enabled identification of an L22 antigen as Alox5, which was highly expressed by naive and memory B cells surrounding germinal centers. Cellular growth of mantle cell lymphoma cells also seemed to depend on Alox5. *Alox5*$^{-/-}$ mice exhibited weak antibody responses specific to foreign antigens at the initial and recall phases. This was probably attributable to the low number of follicular and memory B cells and the functional loss of interleukin-21–mediated responses of follicular B cells. Moreover, *Alox5*$^{-/-}$ mice could not fully foster the development of follicular B helper T (Tfh) cells even after immunization with foreign antigens. Further experiments indicated that Alox5 affected mortality in experimentally induced enterocolitis in germ-prone circumstances, indicating that Alox5 would endow immunologic milieu. Our results illustrate the novel role of Alox5 in adaptive humoral immunity by managing primary B cells and Tfh cells *in vivo*. ***(Am J Pathol 2011, 178: 222–232; DOI: 10.1016/j.ajpath.2010.11.033)***

Regulation of specific humoral responses directs a center of adaptive immunity to prevent infection, autoimmune disease, and cancer.[1,2] Mechanisms underlying antibody production wholly depend on the cellular function of B cells. After the somatic mutation of genes encoding B-cell receptor (BCR) and its editing in the bone marrow, B cells are distributed to the peripheral lymphoid tissues, where they reside as primary resting B cells.[3,4] On stimulation of the surface BCRs and with cognate help from CD4^{+} T cells, primary B cells differentiate into germinal center B cells, where somatic hypermutations and class switch recombination occur, resulting in further differentiation into plasma cells or memory B cells. It is considered that for the sake of efficient production of specific antibodies, primary resting B cells around germinal centers keep BCR repertories as a major anatomical source of lymphoid follicles.[5] By the function of antiapoptotic factors, such as Bcl-2, it is thought that primary B cells would survive and eventually maintain BCR repertoires in individuals.[6] However, the mechanism underlying primary B-cell regulation has not yet been elucidated.

Previously, we created an L-series of monoclonal antibodies (mAbs) for use in the identification of immune cells by means of immunohistochemical analysis or flow cytometry.[7–9] The L-series panel includes L26 mAbs, which specifically bind to the cytoplasmic domain of CD20. This mAb is widely used for immunopathologic examinations of lymphoid tissue to decipher B-cell–related disorders, including malignancies and immune-associated diseases.[7,10] L22 mAbs have exhibited a unique distribution profile: L22 antigen (Ag) is specifically expressed in the cytoplasm of primary resting B cells in the mantle zones of lymphoid follicles but not in activated B cells of germinal centers and in T cells.[8] Therefore, it is of great interest to investigate the nature of L22 Ag because we consider that its functional features may provide new fundamental insights into the regulatory mechanism of adaptive humoral immune responses.

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Herein, we first identified an L22 Ag as arachidonate 5-lipoxygenase (Alox5) and elucidated its unique role of coordinating specific antibody responses. Alox5 oxidizes arachidonic acid as a substrate initially to produce 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid, which is an intermediate for various leukotrienes of lipid mediators.[11] We observed that naive ($CD19^+CD23^+CD27^-CD38^-$) and memory ($CD19^+CD23^-CD27^+CD38^-$) phenotypes of human primary B cells in the mantle zones preferentially expressed Alox5.[12] Studies of *Alox5*$^{-/-}$ mice revealed that this enzyme was required for the humoral response against T-cell–dependent foreign antigens at the initial and recall phases. This was probably because of the loss of follicular B cells ($B220^+CD21^{int}CD23^+$) and memory B cells ($B220^+IgG1^+NP^+$) due to cell death in *Alox5*$^{-/-}$ mice.[13] Furthermore, mantle cell lymphoma (MCL) would depend on the growth activities of Alox5, implying a growth regulatory role of Alox5 in normal primary B cells and their derived tumor cells. Moreover, note that *Alox5*$^{-/-}$ mice showed fewer germinal center cells after immunization of the foreign antigens and developmental insufficiency of follicular B helper T (Tfh) cells ($CD3^+CD4^+CD25^-CXCR5^+ICOS^+$).[14,15] Experimental evidence has revealed that *Alox5*$^{-/-}$ and recombination activating gene $1^{-/-}$ (*Rag1*$^{-/-}$) chimera mice were fairly susceptible to the condition of enterocolitis, especially in conventional facilities; this reiterates the pivotal role of Alox5 in the regulation of specific antibody responses. In addition to the evidence indicating the possible role of Alox5 in MCL, these and further investigations may shed light on the association of lipid metabolism of arachidonic acid by Alox5 with the regulation of adaptive humoral immunity and the nature of MCLs.[16–18]

Materials and Methods

Tissues and Cell Lines

All tissues were obtained after receiving informed consent from the subjects and with the approval of the Institutional Review Board. Tonsillar tissues were obtained from patients undergoing tonsillectomy for the treatment of recurrent tonsillitis, and lymphoma tissues were obtained by means of biopsy. Portions of the tissues were stored at −80°C as frozen tissue sections. Human embryonic kidney (HEK) 293 and P1.4 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μg/ml of streptomycin, and 100 U/ml of penicillin. Cell lines derived from MCL, including G519, HBL2, JEKO1, MINO, and REC1, as well as Jurkat, Molt4, and Daudi cells, were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mmol/L l-glutamine, 50 μg/ml of streptomycin, and 100 U/ml of penicillin. All cells were cultured at 37°C in a humidified atmosphere with 5% carbon dioxide.

Mice and Immunization

All the experiments were performed in accordance with the institutional guidelines for the care and use of animals. *Alox5*$^{-/-}$ mice[19] and C57BL/6-SV129S F1 hybrid mice used as a control were purchased from The Jackson Laboratory (Bar Harbor, ME). All these mice were maintained in specific pathogen-free or conventional animal facilities of Sapporo Medical University School of Medicine. Age- (6 to 12 weeks) and sex-matched mice from each group, which were simultaneously raised in the same environment at the facilities, were used for the experiments. Lin^-ScaI^+ bone marrow stem cells of *Alox5*$^{+/+}$ or *Alox5*$^{-/-}$ mice were transferred into recombination activating gene 1–deficient mice (C57BL/6 background; The Jackson Laboratory) to generate *Rag1*$^{-/-}$ bone marrow chimera mice. Eight weeks after stem cell transplantation, levels of serum Igs of *Rag1*$^{-/-}$ chimera mice were examined before being used for experiments. Mice were immunized by means of i.p. administration of 200 μL of 5% sheep red blood cells (SRBCs) or 100 μg of (4-hydroxy-3-nitrophenylacetyl)36 coupled to chicken gamma globulin (NP36-CGG) (with alum at initial immunization).

Reagents

L22 (clone TB1-2C3) and TE4 mAbs were purified from mouse ascitic fluid and were used for immunoprecipitation and immunohistochemical analysis. The following antihuman mAbs were purchased from BD Biosciences (San Jose, CA): fluorescein isothiocyanate (FITC)–anti-CD3 (clone SK7), FITC–anti-CD4 (RPA-T4), FITC–anti-CD8 (clone RPA-T8), antigen-presenting cell (APC)–anti-CD19 (clone HIB19), phycoerythrin (PE) -Cy7–anti-CD25 (clone MA251), PE–anti-CD27 (clone MT271), FITC–anti-CD38 (clone HIT2), PE–anti-CD278 (ICOS; clone DX29), biotin–anti-CXCR5 (clone RF8B2), FITC–anti-IgD (clone IA6-2), FITC–anti-IgM (clone G20-127), FITC–anti-IgG (clone G18-145), and Ig isotype control mAbs. Rabbit antihuman-CD23 mAbs (clone SP23), antihuman-Bcl-2 polyclonal antibodies (pAbs), and antihuman-Alox5 pAbs were obtained from Nichirei Bioscience Inc. (Tokyo, Japan), Neomarkers Inc. (Fremont, CA), and Cayman Chemical Co (Ann Arbor, MI), respectively. The following antimouse mAbs were purchased from BD Biosciences, Affinity BioReagents Inc. (Golden, CO), eBioscience Inc. (San Diego, CA), and Genetex Inc. (Irvine, CA): biotin–anti-CD1 days (clone 1B1), anti-CD3 (hamster clone 500A2), APC–anti-CD3 (clone 145-2C11), FITC–anti-CD4 (clone L3T4), PE–anti-CD8 (clone 53-6.7), anti-CD19 mAb (clone 1D3), FITC–anti-CD21 (clone 7G6), PE–anti-CD23 (clone B3B4), PE-Cy7–anti-CD25 (clone PC61), FITC– or APC–anti-CD45R/B220 (clone RA3–6B2), PE–anti-CD278 (ICOS; clone 7E17G9), biotin–anti-CXCR5 (clone 2G8), FITC–anti-IgG1 (clone LO-MG 1-2), biotin–anti–major histocompatibility complex class II (clone M5/114.15.2), and isotype control mAbs. Horseradish peroxidase–conjugated rabbit pAbs against isotypes were purchased from Southern Biotechnology (Birmingham, AL). Mouse anti–enhanced green fluorescent protein (EGFP) mAbs (clone JL-8) and biotin-conjugated peanut agglutinin were obtained from Vector Laboratories (Burlingame, CA) and Invitrogen (Carlsbad, CA), respectively. NP-CGG, 4-hydroxy-3-nitrophenylacetyl coupled to bovine serum albumin (NP-BSA), and NP-BSA-biotin were purchased from Bioresearch Technologies Inc. (Novato, CA).

Immunohistochemical Analysis

Procedures for tissue sections were conducted as described previously.[20] In brief, tissue sections were stained with primary mAbs at 4°C overnight and then were reacted with secondary pAbs conjugated to Alexa Fluor (Molecular Probes Inc., Eugene, OR). Signals were detected using an immunofluorescence microscope (IX71; Olympus, Center Valley, PA) or a laser scanning confocal microscope (R2100AG2; Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitation and Immunoblotting

Cells were treated in a lysis buffer solution consisting of 150 mmol/L sodium chloride, 50 mmol/L Tris-chloride (pH 7.5), 1 mmol/L EDTA, 0.3% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), and protease inhibitors (Roche Diagnostics GmbH) and then were subjected to immunoprecipitation or immunoblotting as described elsewhere.[21] After washing the beads, the specimens were separated by 5% to 20% gradient SDS–polyacrylamide gel electrophoresis and were stained using a silver staining MS kit (Wako Chemicals USA Inc., Richmond, VA) or blotted onto an Immobilon-P membrane (Millipore, Billerica, MA) for immunoblotting.[21]

Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry

Trypsin was used for in-gel digestion of the specimens, after which they were analyzed using tandem mass spectrometry (Gene World, Tokyo, Japan). The obtained peptides were further investigated through a Mascot search to predict an original protein.

Flow Cytometry and Cell Sorting

Lymphocytes were purified by means of centrifugation with Lymphoprep (human; Axis-Shield, Oslo, Norway) or Lympholyte-M (mouse; Cedarlane Laboratories Ltd, Burlington, NC) and then were stained using standard flow cytometry. After preparation of cell specimens, cells were analyzed using a FACSCanto II flow cytometer with DiVA software (BD Biosciences). A FACSAria II cell sorter was used to select and analyze follicular B cells and Tfh cells. AutoMACS (Miltenyi Biotec Inc., Auburn, CA) was also used for magnetically purifying lymphocytes from human tonsils or primitive hematopoietic stem cells ($Lin^{-}Scal^{+}$) from murine bone marrow. In each experiment, the purity of cells reached 95%. Apoptotic cells were stained by annexin V and were detected using an APC dye according to the manufacturer's protocol (BD Biosciences).

cDNA Transduction

EGFP-tagged expression plasmids carrying human Alox5, sorting nexin 5, sorting nexin 6, or autoimmune regulator were used in this study.[21,22] Plasmid DNAs were transfected into cells using LF2000 (Invitrogen).

Enzyme-Linked Immunosorbent Assay

A 96-well microtiter plate coated with SRBCs or NP-BSA was used to determine anti-SRBC and anti-NP Ig titers, as described previously.[13,23] Ig isotypes of mice were determined using isotype-specific enzyme-linked immunosorbent assay (Southern Biotechnology).

RT-PCR and Quantitative PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) and were reverse transcribed using oligo(dT) primers (Applied Biosystems, Foster City, CA). The RT-PCR primer pairs used in this study were designed as described previously and are summarized in Tables 1 and 2.[20] Real-time PCR was performed using an ABI-PRISM 7900 according to the manufacturer's protocol (Applied Biosystems). Target gene expression was calculated using ΔΔCT and comparative methods after normalization to 18S RNA or glyceraldehyde-3-phosphate dehydrogenase expression.

Induction of Enterocolitis

Experimental enterocolitis was induced in mice by supplementing drinking water with 1.5% dextran sodium sulfate (DSS; ICN Biomedicals, Irvine, CA), and the mice were monitored daily for survival as described previously.[24] For evaluating histologic findings, the middle parts of the colon were removed and fixed with 10% formalin containing PBS. Then, paraffin-embedded tissue sections were stained with H&E to examine histologic changes. The histologic score was examined as follows: epithelium (E), 0

Table 1. Mouse PCR Primer Pairs Used in This Study

Mouse primers	Forward primer	Genomic region	Reverse primer	Genomic region	Expected length (bp)
IL-6	5'-AGTTGCCTTCTTGGGACTGA-3'	Exon 2	5'-CAGAATTGCCATTGCACAA-3'	Exon 3	191
IFN-γ	5'-ACTGGCAAAAGGATGGTGAC-3'	Exon 2–3	5'-TGAGCTCATTGAATGCTTGG-3'	Exon 4	237
IL-4Rα	5'-CCTCACACTCCACACCAATG-3'	Exon 6	5'-AGCCTGGGTTCCTTGTAGGT-3'	Exon 7	168
IL-21R	5'-TGTCAATGTGACGGACCAGT-3'	Exon 4	5'-CACGTAGTTGGAGGGTTCGT-3'	Exon 5	163
IFN-γR1	5'-TCCTGCACCAACATTTCTGA-3'	Exon 3	5'-TACGAGGACGGAGAGCTGTT-3'	Exon 4	213
IFN-γR2	5'-GCTTCACCCTGTTCCTCAAA-3'	Exon 6	5'-AGCACATCATCTCGCTCCTT-3'	Exon 7	205
TLR4	5'-GCTTTCACCTCTGCCTTCAC-3'	Exon 3	5'-GAAACTGCCATGTTTGAGCA-3'	Exon 3	174
TLR7	5'-CCTGTTCTACTGGGGTCCAA-3'	Exon 3	5'-GCCTCAAGGCTCAGAAGATG-3'	Exon 3	167
TLR9	5'-GCTTTGGCCTTTCACTCTTG-3'	Exon 2	5'-AACTGCGCTCTGTGCCTTAT-3'	Exon 2	194
Alox5	5'-CTACGATGTCACCGTGGATG-3'	Exon 2	5'-GTGCTGCTTGAGGATGTGAA-3'	Exon 3	235

Table 2. Human PCR Primer Pairs Used in This Study

Human primers	Forward primer	Genomic region	Reverse primer	Genomic region	Expected length (bp)
BLT1	5′-TTGCTCACTGCTCCCTTTTT-3′	Exon 2	5′-AAAGGACAACACCCAGATGC-3′	Exon 2	237
BLT2	5′-GAGACTCTGACCGCTTTCGT-3′	Exon 1	5′-AAGGTTGACTGCGTGGTAGG-3′	Exon 1	183
CysLTR1	5′-TGATGACTTCCGCAATCAAG-3′	Exon 3	5′-AGCCAAATGCCTTTGTGAAC-3′	Exon 3	216
CysLTR2	5′-TCCACTTGACGACATGGAAA-3′	Exon 1	5′-GGCCTTTTCTGAGTGCAGAC-3′	Exon 1	165
MAF	5′-TGGAGTCGGAGAAGAACCAG-3′	Exon 1	5′-GCTTCCAAAATGTGGCGTAT-3′	Exon 2	228
BCL-6	5′-AACCTGAAAACCCACACTCG-3′	Exon 8	5′-TGACGGAAATGCAGGTTACA-3′	Exon 9	245
CXCR5	5′-CTCCCAAGAGAACCAAGCAG-3′	Exon 2	5′-CCAGCAGAGGAAGAAGATGC-3′	Exon 2	205
IL-21	5′-TTCTGCCAGCTCCAGAAGAT-3′	Exon 2	5′-TTGTGGAAGGTGGTTTCCTC-3′	Exon 3	153

= normal morphologic features; 1 = loss of goblet cells; 2 = loss of goblet cells in large areas; 3 = loss of crypts; and 4 = in large areas; infiltration (I), 0 = no infiltrate; 1 = infiltrate around the crypt basis; 2 = infiltrate reaching the lamina muscularis mucosae; 3 = extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; and 4 = infiltration of the lamina submucosa. The total histologic score was obtained by E + I.

Cell Growth Assay

Growth activities of cultured cells were investigated using a premix WST-1 cell proliferation assay system as per the manufacturer's instructions (Takara Bio Inc., Shiga, Japan).

Statistical Analysis

Results are expressed as mean and SE; the unpaired *t*-test was used to compare experimental groups unless otherwise stated, with $P < 0.05$ considered significant.

Results

L22 Ags in Primary B Cells Are Identical to Alox5

Mouse L22 mAbs were originally established by immunizing mice with human tonsillar lymphocytes per standard procedures.[8] Immunohistochemical analysis of tonsillar tissues demonstrated preferential distribution of L22 Ags in the cytoplasm of primary resting B cells in the mantle zones of germinal centers in lymphoid tissues (Figure 1, A and B).[25] We further examined the colocalization of L22 Ags with CD23, which was restricted to the IgM$^+$ or IgD$^+$ naive B-cell phenotype and were subsequently lost in germinal center and memory B cells.[5,26,27] Primary B cells of the mantle zones were found to contain a mixed population of L22$^+$CD23$^+$ and L22$^+$CD23$^-$ cells, indicating that primary B cells around germinal centers consisted of CD23$^+$ naive B cells and CD23$^-$ memory B cells, both of which presented L22 Ags (Figure 1C). Follicular dendritic cells of germinal centers also present CD23; however, L22 Ags were not expressed in CD23$^+$ follicular dendritic cells within germinal centers.[28] Collectively, L22 Ags were expressed by primary B cells with naive and memory phenotypes but not in follicular dendritic cells.

To identify the molecular nature of L22 Ags, we initially used L22 mAbs to perform immunoprecipitation on tonsillar lymphocytes and cell lines. After trials with lysis buffers containing different types of detergents and under different experimental conditions, a clear band was detected at approximately 78 kDa (Figure 1D). Such a band was also detected in Daudi B cells but not in Jurkat T cells and P1.4 thymic epithelial cells; these results concur with the tissue distribution of L22 Ags in human lymphoid tissues of the tonsils and thymus. Proteomics analysis of the protein band derived from tonsillar lymphocytes revealed the presence of at least four different peptides, all of which were completely matched to a core protein sequence of Alox5 (Figure 1E). Further immunoprecipitation and immunostaining experiments in which a plasmid DNA encoding EGFP-tagged Alox5 was introduced into HEK 293 cells indicated the binding specificity of L22 mAbs to Alox5 (Figure 1, F and G).

Primary B Cells Are Susceptible to Functional Loss of Alox5

To investigate the tissue distribution of L22 Ags, primary resting naive (CD19$^+$CD27$^-$CD38$^-$) and memory (CD19$^+$CD27$^+$CD38$^-$) B cells were sorted and analyzed (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).[27,29] Quantitative PCR and immunoblot analyses demonstrated high Alox5 expression in naive and memory B cells, which were also noted to be with or without CD23, respectively (Figure 2, A and B). Meanwhile, germinal center B cells (CD19$^+$CD27$^-$IgD$^+$) expressed very low levels of Alox5; these results are in accordance with those shown in Figure 1, A–C. However, Alox5 was not detected in CD4$^+$ and CD8$^+$ T cells.

To further address the functional role of Alox5 in B cells, we cultured tonsillar B cells (CD19$^+$) with or without Alox5 inhibitors of MK886 or AA861 and studied the B-cell population therein. We observed that primary B cells easily perished in the presence of MK886 or AA861 (Figure 2C). Taking this result into account, we considered Alox5 to be a potential regulator of the maintenance of human naive and memory B cells.

By means of two different types of receptors, namely, BCRs and Toll-like receptors (TLRs), B cells sense various antigens.[2–6,30] When BCRs of tonsil-derived B cells were stimulated, the cells could secrete leukotrienes (data not shown).[11,31] This indicates that leukotrienes derived from naive and memory B cells, by affecting B cells themselves or surrounding immune cells in lymphoid tissues, might act as a prompt dispatch mode to the subsequent antibody response.