種サイトカイン濃度測定、がん組織の抗原免疫組織染色解析、がん組織のHLA classI発現解析、がん組織におけるT細胞免疫組織染色解析、新規バイオマーカーの探索

20.4. 独立画像評価医師

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放射線科　医師　吉田　哲雄

【主な業務内容】

STEP1におけるRECISTに基づく腫瘍縮小効果を治験責任医師、治験分担医師とは独立して評価を行う。

20.5. 治験薬製造者

札幌医科大学附属病院

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責任者：第1外科　医師　水口　徹

【主な業務内容】

治験薬の原薬保管、治験薬製造に係る手順書に基づく治験薬を製造する。

20.6. モニター

株式会社アイロム

〒102-0071　東京都千代田区富士見2-14-37 富士見イースト

TEL：03-3264-3346　FAX：03-3264-3347

株式会社アイクロスジャパン

〒102-0071　東京都千代田区富士見2-14-37 富士見イースト

TEL：03-3237-3641　FAX：03-3237-3642

【主な業務内容】

被験者の人権、安全および福祉が保護されていること、治験が最新の治験実施計画書およびGCP等を遵守して実施され、治験データが正確かつ完全で、原資料等の治験関連記録に照らして検証で

きることを確認する。

20.7. 監査

監査委託会社：株式会社メディクロス

〒 143-0023　　東京都大田区山王2-5-1　大森北口ビル5F

TEL： 03-4500-4666　　FAX： 03-3775-3066

【主な業務内容】

治験の品質保証の一環として、治験が治験実施計画書、治験の依頼および管理に係わる細則、GCP標準業務手順書（GCP-SOP）、薬事法第14条第3項および第80条の2に規定する基準ならびにGCPに従って実施されているか否かを、通常のモニタリングおよび治験の品質管理業務とは独立・分離して評価する。

20.8. 治験調整医師

札幌医科大学附属病院

〒060-8543　札幌市中央区南1条西16丁目

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第1外科　医師　水口　徹

治験調整事務局

札幌医科大学

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TEL：011-611-2111（内線：2691）　　FAX：011-643-2310

責任者：病理学第一講座　医師　鳥越　俊彦

【主な業務内容】

自ら治験を実施する者が行う各種業務を調整し、支援する。

20.9. 登録事務局

〒102-0071　東京都千代田区富士見2-14-37 富士見イースト

株式会社アイクロスジャパン

TEL： 03-3237-3641　　FAX： 03-3237-3642

【主な業務内容】

Web登録システム（動的割付、治験薬の割付の機能を含む）構築、適格性確認、動的割付、治験薬の割付に責任を持つ。

20.10. データ管理

〒102-0071　東京都千代田区富士見2-14-37 富士見イースト

株式会社アイクロスジャパン

TEL： 03-3237-3641　　FAX： 03-3237-3642

【主な業務内容】

治験の品質管理のために、治験データの包括的な質の確保に努める責任を持ち、電子症例報告書（EDC）構築、EDCにより収集されたデータの固定、EDCシステムの運用保守管理等を行う。

20.11. 統計解析

〒102-0071　東京都千代田区富士見2-14-37 富士見イースト

株式会社アイクロスジャパン

TEL： 03-3237-3641　　FAX： 03-3237-3642

【主な業務内容】

治験の統計学的事項に関して責任を持ち、治験実施計画書に基づきランダム化仕様書の作成および統計解析計画書を作成し、統計解析を実施するとともにその確認を行う。

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株式会社化合物安全性研究所
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II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（特に関連の深い論文）

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III. 研究成果の刊行物・別刷

Cancer Science
The official journal of the Japanese Cancer Association JCA

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

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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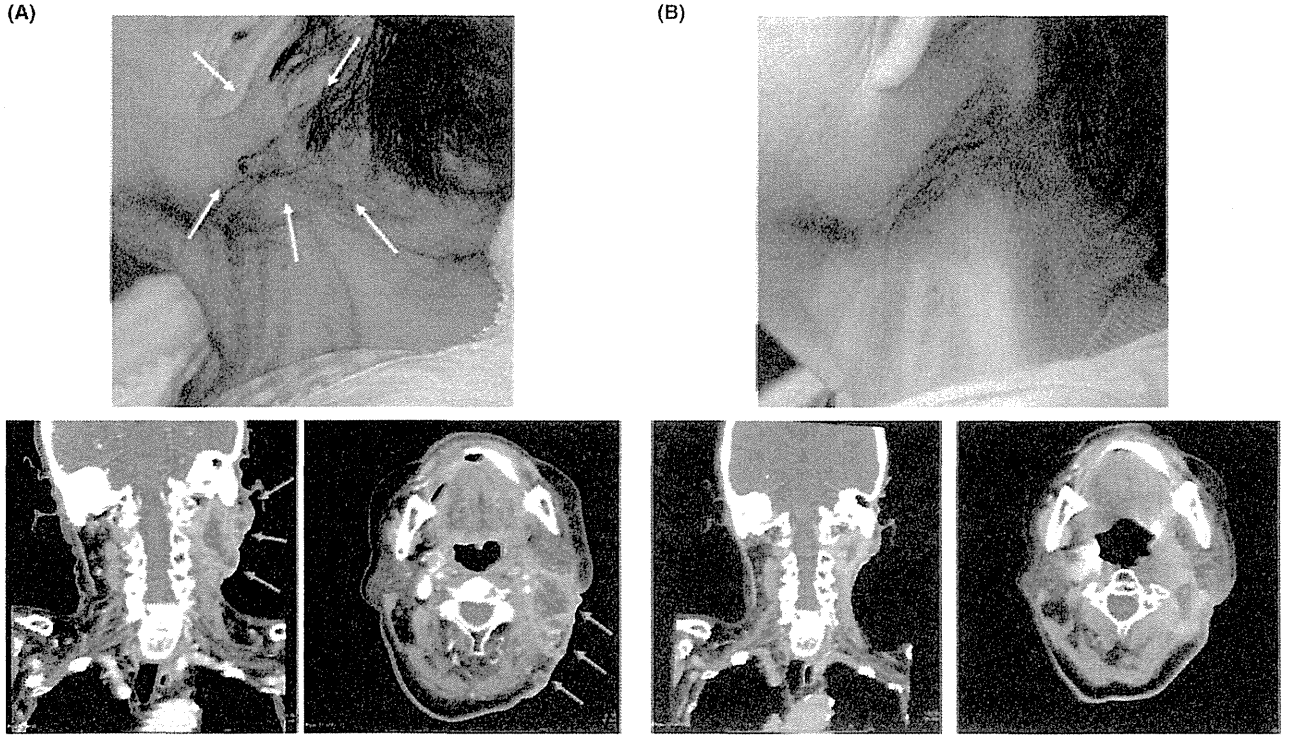

Fig. 1. Photograph of skin on the neck and computed tomography (CT) scan image of the neck showing metastatic tumors of case 10. (A) Photograph of skin on the neck and CT scan image of the neck before vaccination. Axial contrast-enhanced CT image shows multiple metastatic tumors (arrows). (B) Photograph 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 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

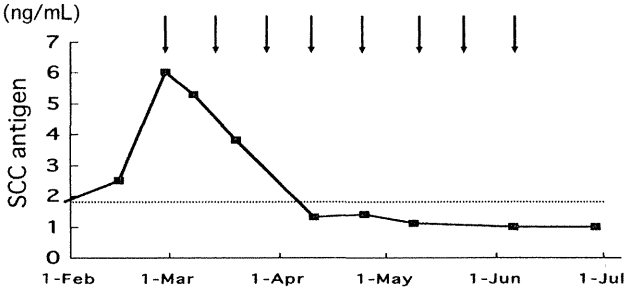

Fig. 2. Changes in the serum squamous cell carcinoma (SCC) antigen level during the vaccination in case 10. The dotted line indicates the cut-off point for the SCC antigen level. The arrows indicate the times of vaccination. The SCC antigen level significantly decreased to 0.7 ng/mL after the sixth vaccination.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

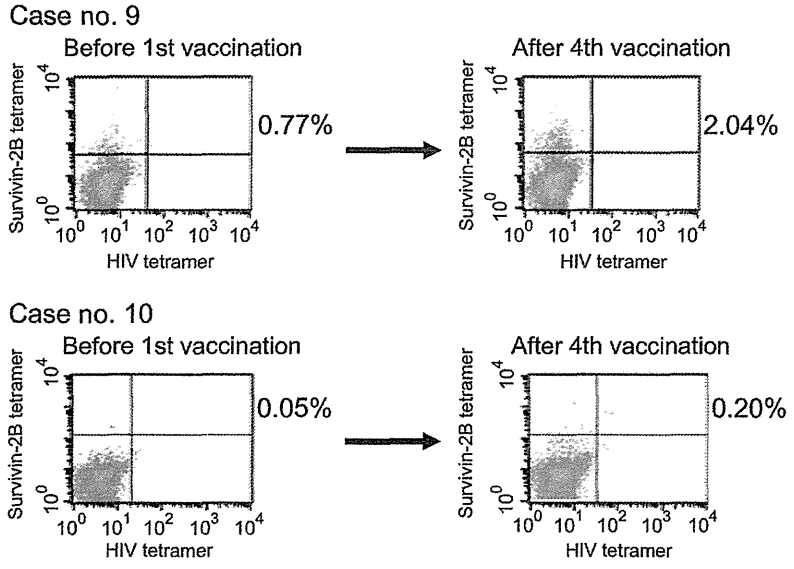

Fig. 3. Tetramer staining before the first vaccination and after the fourth vaccination in cases 9 and 10. Flow cytometric analysis was performed using a FACSCaliber and CellQuest software (Becton Dickinson Biosciences). The frequency of the cytotoxic T lymphocyte (CTL) precursors was calculated as the 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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Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients

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We previously identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, survivin-2B80-88, recognized by CD8+ cytotoxic T lymphocytes (CTL). Subsequently, we attempted clinical trials with this epitope peptide alone for some malignancies, resulting in clinical and immunological responses, although their potential was not strong enough for routine clinical use as a cancer vaccine. In the current study, to assess whether immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we performed clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with the vaccination protocol of survivin-2B80-88 plus IFA and IFNα resulted in clinical improvement and enhanced immunological responses of patients. Tetramer analysis of survivin-2B80-88 peptide-specific CTL demonstrated that such CTL were increased at least twofold after vaccination with this protocol in four of eight patients. In these patients, enzyme-linked immunosorbent spot (ELISPOT) results were also enhanced. Subsequent study of single-cell clone separation by cell sorting of peptide-specific CTL showed that each CTL clone was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules. Taken together, these results indicate that vaccination of colon cancer patients with survivin-2B80-88 plus IFA and IFNα can be considered to be a very potent immunotherapeutic regimen, and that this protocol might work for other cancers. (*Cancer Sci* 2011; 102: 1181–1187)

Human tumor immunology research has advanced since the first human melanoma tumor antigen recognized by CD8+ cytotoxic T lymphocytes (CTL) was identified in 1992,[1] and more than 20 melanoma antigens have been reported.[2–7] Some antigens and human leukocyte antigen (HLA) class I-restricted antigenic peptides underwent clinical trials, and their adverse effects and clinical and immunological responses were studied.[8–11] Rosenberg *et al.*[4] reported on a large number of melanoma patients and found that less than 5% of patients who received peptide vaccines such as gp100 and interleukin-2 (IL-2) had a complete response.

Nevertheless, a UK-based pharmaceutical company reported that a 3-year-long observation after melanoma antigen family A, 3 (MAGE-A3) vaccine inoculation indicated a 33% reduction in the post-operative recurrence of non-small-cell lung cancers when compared with a placebo group.[12] This observation gives strong hope for future cancer immunotherapy and has prompted many different investigations for the establishment of human tumor immunotherapy.

Meanwhile, human tumor antigens of non-melanoma tumors such as colon, lung, urinary tract and soft-tissue sarcomas have been analyzed extensively in various laboratories.[13–18] In our laboratory, we have identified tumor antigens using several different experimental systems.[2,3] Using reverse immunological approaches the inhibitor of apoptosis protein (IAP) family members survivin and livin were shown to be highly immunogenic tumor antigens in addition to the fact that these two antigens were selectively expressed in tumor tissues of different tissue origins but not in normal counterparts.[19–21] The HLA-A24-restricted survivin2B80-88 nonamer peptide, which was derived from the survivin splicing variant survivin 2B from cancer patients, appears to have strong immunogenicity as assessed by CTL induction efficiency, tetramer CTL frequency and enzyme-linked immunosorbent spot (ELISPOT).[19,20]

Our group began clinical trials several years ago.[8,9,11] The HLA-A24-restricted survivin2B80-88 peptide was given subcutaneously to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, urinary bladder cancers and lymphomas. There were no severe adverse effects and, clinically, certain patients with colon, lung and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed on computed tomography (CT). However, these effects were not strong enough for the clinical requirements as decided by the Response Evaluation Criteria in Solid Tumors (RECIST) for cancer chemotherapy. When assessed with the RECIST, which requires more than 30% regression of tumors on CT, only one of 15 patients with colon cancer and three of 15 with urinary bladder cancer had a positive clinical response.

Thus, the therapeutic potential was not strong enough for routine clinical use as a cancer treatment.[2,3] In the current study, to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we performed and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect with survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA

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and IFNα resulted in clinical improvement and enhanced immunological responses of patients. We also analyzed CTL of these patients by single-cell sorting, finding that each CTL clone from the vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Materials and Methods

Patient selection. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan.[8–11] All patients gave informed consent before being enrolled. Patients enrolled in the present study were required to conform to the following criteria: (i) to have histologically confirmed colon cancer; (ii) to be HLA-A*2402 positive; (iii) to have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) to be between 20 and 85 years old; (v) to have unresectable advanced cancer or recurrent cancer; and (vi) to have Eastern Cooperative Oncology Group (ECOG) performance status between 0 and 2. 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) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, Sapporo Medical University Primary Hospital from December 2005 to November 2009.

Peptide preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8,9,11] The identity of the peptide was confirmed by mass spectrometry 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.

IFA and IFNα preparation. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical Co. (Osaka, Japan).

Patient treatment. Two protocols were used in the current clinical study, as illustrated in Figure 1. One was a basic protocol with the survivin-2B80-88 peptide plus IFA, and the other was the survivin-2B80-88 peptide plus IFA and a type-I IFN, IFNα. In this trial, the primary end-point was safety. The second end-point was investigations about anti-tumor effects and clinical and immunological monitoring.

In the first protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated subcutaneously (s.c.) four times at 14-day intervals. This group included five patients. If patients whose disease was not far advanced hoped for continuation of this peptide vaccination therapy, we vaccinated them in the same manner after the fourth vaccination.

In the second protocol, survivin-2B80-88 plus IFA was vaccinated in a similar manner to the first protocol. In addition, in this protocol, IFNα at a dose of 3 000 000 IU was administered (s.c.) twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and administered at the time of the peptide and IFA biweekly vaccination.

Toxicity evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria (NCI-CTC).[8,9]

Clinical response evaluation. Physical examinations and hematological examinations were conducted before and after each vaccination.[8,9] A tumor marker (carcinoembryonic antigen [CEA]) was examined. Changes in tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary colon cancer tissues was done with anti-HLA class I heavy chain monoclonal antibody EMR-8-5 (Funakoshi Co., Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response (PR) was defined as a ≥30% decrease from baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for CR, PR or PD.[8,9] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC.** The PBMC were isolated from blood samples by Ficoll-Conray density gradient centrifugation.

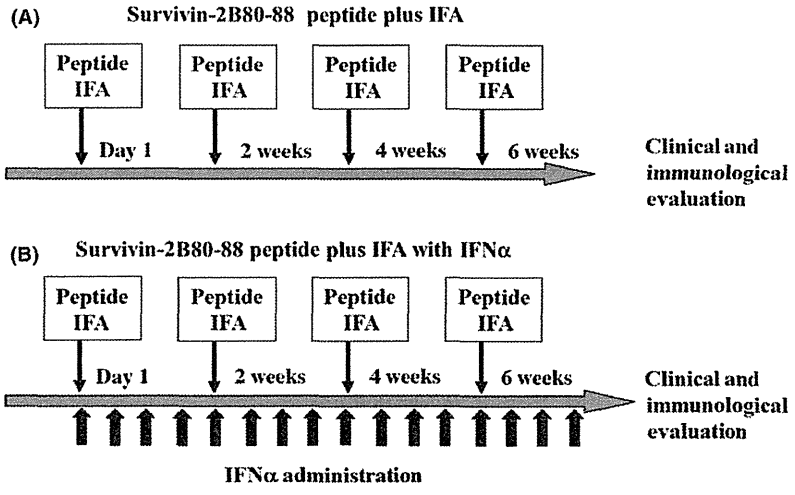

Fig. 1. Protocols of the clinical study. In the current study two protocols were used: (A) survivin-2B80-88 plus IFA and (B) survivin-2B80-88 plus IFA with IFNα. IFA, incomplete Freund's adjuvant; IFN, interferon.

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They were then frozen and stored at −80°C. As needed, frozen PBMC were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM-V medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days and 6 days after the addition of the peptide. On day 7 of culture, the PBMC were analyzed by tetramer staining and ELISPOT assay.

Tetramer staining. FITC-labeled HLA-A*2402-human immunodeficiency virus (HIV) peptide (RYLRDQQLL) and PE-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from MBL, Inc. (Nagoya, Japan). For flow cytometric analysis, PBMC, which were stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a FITC-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (Beckton Dickinson Biosciences, San Jose, CA, USA). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,9,11]

ELISPOT assay. ELISPOT plates were coated sterilely overnight with an IFN-γ 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 h at room temperature. CD8-positive T cells separated from patients' PBMC (5×10^3 cells/well), which were stimulated *in vitro* as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) (5×10^4 cells/well), which had been preincubated with or without survivin-2B80-88 (10 mg/mL) or with a HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-γ antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany). In the present study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) represents a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTL. Survivin-2B80-88 peptide tetramer-positive CTL were sorted and subsequently cloned to single cells using FACS (Aria II Special Order, BD, Houston, TX, USA). The peptide-specific cytotoxicity of each of these CTL was determined by pulsing T2A24 cells[8,9,11,20] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides. These CTL were also assessed for live tumor cell cytotoxicity against LK79 (survivin-2B positive and HLA-A24 positive), A549 (survivin-2B positive, HLA-A24 negative) and K562 (survivin-2B negative and HLA-A24 negative) target cells.

Results

Patient profiles and safety. In the first protocol with the survivin-2B80-88 peptide plus IFA, five patients were enrolled in the study (Table 1). None of the treatment interruptions was due to adverse effects of the vaccination. These five patients received the complete regimen including four vaccinations and were evaluated (Fig. 1). They consisted of three men and two women, whose age range was 50–76 years.

In the second protocol with the survivin-2B80-88 peptide plus IFA and IFNα, eight patients were enrolled in the study (Table 2). In this protocol, there were no patients who dropped out because of adverse events due to the vaccination. They consisted of four men and four women, whose age range was 33–76 years.

With respect to safety, the vaccination was well tolerated in all patients in both vaccination protocols. In patients vaccinated with the survivin-2B80-88 peptide plus IFA, no adverse events were observed during or after vaccination except for induration at the injection site. In the second protocol, the survivin-2B80-88 peptide plus IFA and IFNα, approximately half of the patients had a fever reaching almost 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination.

Clinical responses. As shown in Table 1, vaccination with survivin-2B80-88 plus IFA was given to five colorectal cancer patients. The post-vaccination CEA values of four patients were increased compared with the pre-vaccination values. In the other patient (No. 5), the CEA value remained almost the same, although it was beyond the upper limit during the vaccination. As for tumor size, only one patient was considered to have SD, whereas the other four patients were considered to have PD. These outcomes suggested that vaccination with this first protocol was ineffective for clinical responses.

Table 2 summarizes the clinical outcomes for the eight patients in the second protocol with survivin-2B80-88 plus IFA and IFNα. In some patients, particularly No. 6, the post-vaccination CEA value was clearly decreased compared with the pre-vaccination value, and was within the normal limit. Other patients such as Nos 2 and 3 also had decreased post-vaccination levels of CEA, although not so large. As for tumor size evaluated by CT, four patients (Nos 1, 2, 3 and 6) were considered to have SD, but the other four patients (Nos 4, 5, 7 and 8) had PD.

Table 1. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide and IFA

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOT§
					Pre-/post-	% increase	
1	76/M	No	13/20	PD	192/103	53.6	−
2	59/M	No	369/463	SD	13/16	123.1	−
3	60/F	No	685/1010	PD	60/80	133.3	−
4	72/M	No	55/64	PD	11/4	36.4	−
5	50/F	No	8/7	PD	127/97	76.4	+

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among 10^4 CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNg-positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.