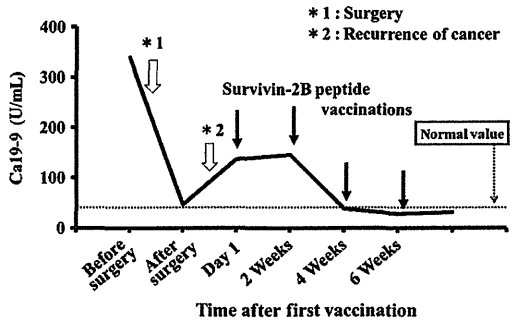

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

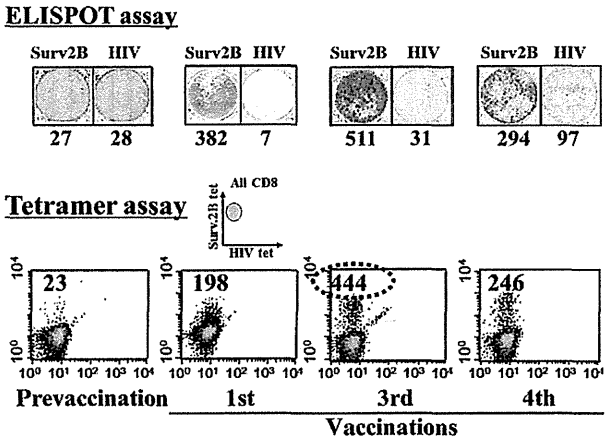

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against survivin2B80-88 or HIV peptide pulsed T2-A24 cells in $10^4\times$ $CD8^+$ T cells. Numbers in tetramer analysis indicate survivin-2B80-88 peptide-specific $CD8^+$ T cells among $10^4\times$ $CD8^+$ T cells.

with the survivin-2B80-88 peptide plus IFA and IFNα could work as a potential therapeutic regimen in pancreatic cancers. However, it remained to be clarified if IFNα alone without the peptide could function in a similar manner, at least to some extent, as this cytokine is considered to be the most potent for the activation and maturation of dendritic cells (DCs) as well as upregulation of HLA class I in tumor cells. To this end, we studied this profile in three patients with colon cancer, not pancreatic cancer, whose condition was similar to those in this study, that is, patients with unresectable advanced or recurrent cancer. This was done because patients with the latter cancer had highly advanced clinical cases, making this type of study impossible. As shown Table 2, all three patients showed no obvious increases, but rather reductions, in the frequency of survivin-2B peptide-specific T cells as assessed by tetramer analysis before and after two to four treatments with IFNα alone. Furthermore, this was also true for ELISPOT analysis. These data supported the idea that IFNα alone did not actively participate in the activation of survivin-2B peptide-specific T cells.

Discussion

Our group previously showed that the vaccination protocol of survivin-2B80-88 plus IFA and IFNα could work as a potent

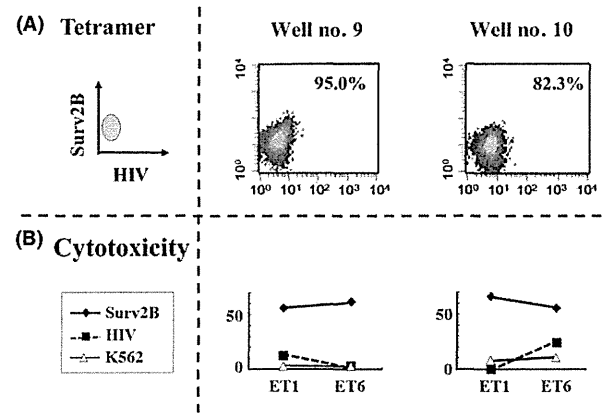

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer (Surv2B) (A) and against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide and against control K562 cells (B). ET, effector/target ratio.

immunotherapeutic regimen in colon cancers.[8] In addition to colon cancer, survivin2B protein is expressed in most tumor cells of various tissue origins, such as those in the gastrointestinal and biliary tracts and pancreas, therefore, there is a possibility that the survivin2B peptide could work as a potential therapeutic tumor vaccine in cancer patients with these neoplasms.

In this present study, we assessed whether the vaccination protocol using survivin-2B80-88 plus IFA and IFNα could be effective in pancreatic cancer patients from immunological and clinical points of views. Consequently, our data strongly suggested that this protocol was very effective and useful in immunotherapy for advanced pancreatic cancers as in colon cancers. Actually it was shown that more than 50% of patients with pancreatic cancers showed positive clinical and immunological responses in tetramer and ELISPOT analyses. In some cases, the immunological response of survivin-2B80-88 peptide-specific CTLs was elucidated at the single-cell level. Taken together, the current data implied that our vaccination protocol was very useful in immunotherapy for pancreatic cancers.

As shown in Fig. 3, the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced from the third to the fourth vaccination. We speculate that there could be various reasons for this reduction. One might be immune escape by the downregulation of HLA expression, cytokines, or regulatory T cells. Another might be an activity of the stored samples, or differences between the environment of the peripheral circulation and the tumor. In other words, the peptide-specific CTL responses were reduced in immunological monitoring in the peripheral circulation, but maintained in the local cancer environment. In this case, the clinical responses, such as tumor marker (CA19-9) level and tumor size evaluated by CT, had been maintained also after that, even though the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced between the third and fourth vaccinations. Therefore, CA19-9 levels had been kept within normal limits and new cancer lesions had not appeared.

We evaluated immunological monitoring of this clinical protocol by tetramer staining and IFNγ ELISPOT assay. Tetramer staining recognizes the structure of the T cell receptor, and

Table 2. Frequency monitoring of the number of survivin-2B80-88 peptide tetramer-positive CTLs in cancer patients treated with IFNα alone

Patient no.	Tumor	Age/sex	Number of treatment	Tetramer staining†		ELISPOT‡	
				Pre/post	% Increase	Pre/post	% Increase
1	Colon	60/M	3	1/0	0.0	111/75	67.6
2	Colon	63/M	4	11/9	81.8	44/20	45.5
3	Colon	77/F	2	13/3	23.1	26/40	153.8

†CTL frequency before and after treatment with IFNα alone in patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. An HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 CD8 T cells is shown. ‡γ-Interferon (IFNγ) secretion of pre and post IFNα treatmnet were assesed with ELISPOT assay using T2-A24 cells pulsed with survivin2B80-88 peptide. The number of spots in 5×10^3 CD8 T cells are shown. IFNα, α-interferon.

detects naive T cells, memory T cells, and activated CTLs. The ELISPOT assay detects more the functional aspects of T cells by IFNγ release, therefore, ELISPOT detects memory T cells and CTLs. In this study, the tetramer-positive cases are also positive in the ELISPOT study. Therefore, these results indicate that memory T cells and CTLs can be effectively induced by this peptide vaccination protocol.

In this present study, we also assessed evidence concerning the extent to which peptide-specific CTL responses in pancreatic cancer patients treated with peptide vaccines could occur at the single-cell level. To assess this, CTLs of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed peptide-specific in the context of the expression of HLA-A24.

Type-I interferons such as IFNα are known to work in various immunological manners to activate T cell responses.[18–25] The maturation of DCs and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Although we could not actually compare these features of patients' DCs and primary pancreatic tumor tissues before and after treatment with IFNα, the obvious enhancement of CTL responses and improvement of clinical responses in our previous and current studies favors the two main actions described above. These observations strongly suggest that the action of IFNα is remarkable from the aspect of being an immunogenic enhancer for human cancer peptide vaccines.

It is widely known that IFNα is involved in DC maturation and activation.[18,21] This particular cytokine is also potent for increasing the expression of MHC class I molecules.[26–29] Indeed, our previous study of the expression of HLA class I molecules in pancreatic cancer indicated that many tumor tissues heterogeneously expressed such molecules, with some tumor cells showing high expression, whereas others had only weak expression. Interferon-α is presumed to actually enhance their expression even in those tumor tissues with weak expression. Moreover, because tumor patients generally show overt expression of survivin protein in their tumor tissues and, although in small numbers, survivin-2B peptide-specific T cells in peripheral blood, it is considered that IFNα alone may increase the frequency of these T cells in peripheral blood as well. These features of this particular cytokine lead to the possibility that treatment with IFNα alone could result in, at least to some extent, certain immunological and clinical effects of survivin-2B peptide-specific T cells in tumor-bearing patients. However, we analyzed three colon cancer patients, and our data strongly suggested that there was no increase of these T cells as assessed by tetramer and ELISPOT analyses.

Taken together, our results highly suggest that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα is very effective for pancreatic and colon cancers, and that this protocol might be useful as a standard, general immunotherapy modality for human cancers. However, further clinical studies involving many patients are necessary in order to consolidate the immunotherapeutic benefit of this vaccination protocol.

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Disclosure Statement

The authors have no conflict of interest.

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Stem Cells, Tissue Engineering, and Hematopoetic Elements

Cytotoxic T Lymphocytes Efficiently Recognize Human Colon Cancer Stem-Like Cells

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Cancer stem-like cells (CSCs) and tumor-initiating cells (TICs) are a small population of cancer cells that share three properties: tumor initiating ability, self-renewal, and differentiation. These properties suggest that CSCs/TICs are essential for tumor maintenance, recurrence, and distant metastasis. Here, we show that cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigen CEP55 can efficiently recognize colon CSCs/TICs both *in vitro* and *in vivo*. Using Hoechst 33342 dye staining, we isolated CSCs/TICs as side population (SP) cells from colon cancer cell lines SW480, HT29, and HCT15. The SP cells expressed high levels of the stem cell markers SOX2, POU5F1, LGR5, and ALDH1A1 and showed resistance to chemotherapeutic agents such as irinotecan or etoposide.To evaluate the susceptibility of SP cells to CTLs, we used CTL clone 41, which is specific for the CEP55-derived antigenic peptide Cep55/c10orf3_193 (10) (VYVKGLLAKI). The SP cells expressed HLA class I and CEP55 at the same level as the main population cells. The SP cells were susceptible to CTL clone 41 at the same level as main population cells. Furthermore, adoptive transfer of CTL clone 41 inhibited tumor growth of SW480 SP cells *in vivo*. These observations suggest that Cep55/c10orf3_193(10) peptide-based cancer vaccine therapy or adoptive cell transfer of the CTL clone is a possible approach for targeting chemotherapy-resistant colon CSCs/TICs. *(Am J Pathol 2011, 178:1805–1813; DOI: 10.1016/j.ajpath.2011.01.004)*

Colon cancer is one of the most common malignancies worldwide. With recent progress in treatment, the prognosis has improved to some extent. In advanced disease, however, the prognosis remains unfavorable, because of recurrence, distant metastasis, and resistance to treatment. Thus, novel treatment modalities are needed.

Cancers contain morphologically heterogeneous populations. This fact has led to the cancer stem cell theory,[1] the idea that cancers are composed of several types of cells, and that only a small population of cancer cells that can regenerate cancer tissues, much as normal tissue can be regenerated only by a small population of stem-like cells. Recently, cancer stem-like cells and tumor-initiating cells (CSCs/TICs) have been isolated from various types of malignancies, including colon cancer.[2–6] In colon cancer, CSCs/TICs can reinitiate tumors that resemble mother colon cancer tissues morphologically when transplanted into immunodeficient mice.[3] Furthermore, these CSCs/TICs have higher tumorigenic potential than do non-CSCs/TICs. Previous reports have shown that CSCs/TICs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery, and resistance to apoptosis.[7] These reports[3–6] support the hypothesis that malignant cancers comprise heterogeneous populations that organize in a hierarchical differentiation model. The CSCs/TICs are located at the top of this hierarchy, and targeting CSCs/TICs is essential to achieve efficient effects for treatment of malignant diseases. Recently, some trials targeting CSCs/TICs have been reported for hema-

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topoietic malignancies.[8] Hedgehog signaling is essential for maintenance of myeloid leukemia stem cells, and inhibition of hedgehog signaling by cyclopamine is effective for imatinib-resistant myeloid leukemia.[9] To date, however, no such CSC/TIC targeting approach has been reported for colon cancer.

In the present study, we evaluated the efficiency of CTL-based immunotherapy targeting colon CSCs/TICs. Using Hoechst 33342 dye, we isolated colon CSCs/TICs as side population (SP) cells from six colon cancer cell lines. The SP cells derived from SW480, HT29, and HCT15 showed higher tumorigenicity than did main population (MP) cells. On the other hand, SP cells from KM12LM, Lovo, and Colo320 did not show any increase in tumorigenicity, compared with MP cells. This suggests that SW480, HT29, and HCT15 SP cells (but not KM12LM, Lovo, and Colo320 SP cells) were enriched with CSCs/TICs. In RT-PCR analysis the SW480, HT29, and HCT15 SP cells showed a stem cell-like gene expression signature, including SOX2, POU5F1, LGR5, and ALDH1A1. Furthermore, these SP cells also showed resistance to chemotherapeutic agents, including irinotecan and etoposide. These observations support the idea that these SP cells had stem cell-like features. To assess the immunogenicity of SP cells, we evaluated the expression of HLA class I and of CEP55, which is a tumor-rejection antigen of breast and colon cancer.[10,11] The SP cells expressed HLA class I (and also HLA-A24) at the same level as MP cells. The SP cells also expressed CEP55 messenger RNA (mRNA) at the same level as MP cells in RT-PCR. To confirm the susceptibility of SP cells to cytotoxic T lymphocytes (CTLs), we used CTL clone 41, which recognizes CEP55 in an HLA-A24-restricted manner.[10] CTL clone 41 killed SW480, HT29, and HCT15 SP cells at the same level as it killed MP cells and presorted cells. These observations suggest that colon CSCs/TICs are also sensitive to CTLs, as non-CSC/TIC populations are. Furthermore, adoptive transfer of CTL clone 41 inhibited the tumor growth of SW480 SP cells in immunodeficient mice. These observations suggest that CTL-based colon cancer immunotherapy is efficient for colon CSCs/TICs. To our knowledge, the present study provides the first direct evidence that colon CSCs/TICs are susceptible to CTLs and thus opens possibilities for future applications in immunotherapy using CSC/TIC-specific vaccines.

Materials and Methods

Cell Lines

Colon adenocarcinoma cell lines SW480 (HLA-A*0201/2402), HCT15 (HLA-A*0201/2402), HT29 (HLA-A1/24), Lovo, and Colo320 were kind gifts of Dr. K. Imai (Sapporo, Japan), and KM12LM was a kind gift of Dr. K. Itoh (Kurume, Japan). All cell lines except K562 were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). K562 was cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum. HCT15-B2M, a stable transfectant of HCT15 cells with B2M (β2 microglobulin) cDNA, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 μg/mL puromycin (Sigma-Aldrich).[11]

Side Population Analysis

Side population analysis was performed as described previously, with some modifications.[12] Trypsinized cultured cells were washed with PBS and were resuspended at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. After 10 minutes preincubation, the cells were labeled with Hoechst 33342 dye (Lonza, Walkersville, MD) for 90 minutes at concentrations of 3.75 μg/mL for Colo320, 5 μg/mL for SW480 and Lovo, 7.5 μg/mL for HT29 and KM12LM, and 10 μg/mL for HCT15, with or without verapamil (Sigma-Aldrich), which is an inhibitor of ABC transporters, at concentrations of 50 μmol/L for SW480, HCT15, and Colo320, 75 μmol/L for Lovo, and 100 μmol/L for HT29. Cells were counterstained with 1 μg/mL propidium iodide to label dead cells. Next, 1×10^6 viable cells were analyzed and sorted using a BD FACSAria II fluorescence-activated cell sorting system (BD Biosciences, Franklin Lakes, NJ). The Hoechst dye was excited at 355 nm, and its fluorescence was measured at two wavelengths using optical filters 405 DF20 [450/20 nm bandpass filter O (Hoechst Blue)] and 635LP [635 nm longpass edge filter (Hoechst Red)]. Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

Xenograft Model

The SP cells, MP cells, and presorted cells from colon cancer cell lines were mixed 1:1 by volume with Matrigel (BD Biosciences) and were injected subcutaneously into the backs of female 4- to 8-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size in cubic millimeters was assessed weekly with calipers and was calculated as Tumor Size = (Longest Diameter $\times$ Shortest Diameter^2)/2.

RT-PCR Analysis of SP and MP Cells

RT-PCR analysis was performed as described previously.[10] Total RNAs were isolated from both SP cells and MP cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen). The PCR amplification was performed in 20 μL of PCR mixture containing 1 μL of cDNA mixture, 0.5 μL of Taq DNA polymerase (Qiagen) and 4 pmol of primers. The PCR mixture was initially incubated at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The following primer pairs were used for RT-PCR analysis (forward and reverse, respectively): 5′-CATGATG-

GAGACGGAGCTGA-3′ and 5′-ACCCCGCTCGCCATGCTATT-3′ for SOX2, with an expected PCR product size of 410 bp; 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′ and 5′-GGCAGATGGTCGTTTGGCTGAATA-3′ for POU5F1, with an expected PCR product size of 163 bp; 5′-CTCTTCCTCAAACCGTCTGC-3′ and 5′-GATCGGAGGCTAAGCAACTG-3′ for LGR5, with an expected PCR product size of 181 bp; 5′-TGTTAGCTGATGCCGACTTG-3′ and 5′-TTCTTAGCCCGCTCAACACT-3′ for ALDH1A1, with an expected PCR product size of 154 bp; 5′-TGAGTTTGCCATCACAGAGC-3′ and 5′-TTGCTTGCTGGTGCATTAAC-3′ for CEP55, with an expected PCR product size of 521 bp; and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with an expected product size of 452 bp. GAPDH was used as an internal control.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Applied Biosystems). Thermal cycling was performed using 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Each experiment was done in triplicate, with normalization to the *GAPDH* gene as an internal control.

Flow Cytometric Analysis and Monoclonal Antibodies

Cells were incubated with mouse monoclonal antibodies at saturation concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with fluorescein isothiocyanate for 30 minutes. Samples were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson, Mountain View, CA). Anti-pan HLA class I (W6/32) and anti-HLA-A24 monoclonal antibodies (C7709A2.6 hybridoma, a kind gift from Dr. P.G. Coulie, Brussels, Belgium) were prepared from hybridomas.

Survival Studies for Etoposide and Irinotecan

We isolated SP and MP cells of SW480 and HCT15 and seeded them into 96-well culture plates at 1×10^4 cells per well for each population of cells. The cells in both populations were treated with etoposide (1 and 5 μg/mL) or irinotecan (40 and 400 μg/mL for SW480, 10 and 100 μg/mL for HCT15). After 72 hours of exposure to the chemotherapeutic agents, viability of the cells was determined using the SOD assay kit WST-1, which was performed according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan; Rockville, MD).

Cytotoxicity Assay for SP Cells with CTL Clone 41

We had previously established CTL clone 41, which recognizes an HLA-A24 restricted antigenic peptide (VYVKGLLAKI) termed Cep55/c10orf3_193(10), from an HLA-A24-positive breast cancer patient's peripheral blood mononuclear cells.[8] The lytic activity of CTL clone 41 for SP cells, MP cells, and presorted cells was evaluated by ^{51}Cr release assay. Briefly, SP cells, MP cells and presorted cells were labeled with 100 μCi of ^{51}Cr for 1 hour at 37°C, washed four times with PBS, and resuspended in AIM-V medium (Invitrogen). The ^{51}Cr-labeled target cells (2000 cells/well) were then incubated with various numbers of effector cells for 6 hours at 37°C in 96-well culture plates. Radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % Specific Lysis = (Experimental Release − Spontaneous Release) × 100/(Maximum Release − Spontaneous Release). Target cells were treated with 100 units/mL interferon-γ for 48 hours before the assay.

Winn Assay

SW480 SP cells were mixed with CTL clone 41 at a ratio of 1 SP cell to 10 CTL cells. The resulting mixture (200 μL with 1×10^6 CTL clone 41 and 1×10^5 SP cells) was injected subcutaneously into the backs of NOD/SCID mice. A control group of five mice was injected with SP cells alone. Tumor size was assessed weekly.

CTL Adoptive Transfer

NOD/SCID mice were inoculated subcutaneously on the back with 1×10^3 SW480 SP cells. Three weeks later, when the tumor started to be palpable, 5×10^4 Cep55/c10orf3_193(10)-specific CTL clone cells or PBS was injected intravenously. The same adoptive transfer procedure was performed 4 weeks after inoculation with SP cells. Tumor size was assessed weekly.

Statistical Analysis

In the xenograft model, survival studies using chemotherapeutic agents, cytotoxicity assay, Winn assay, and adoptive transfer model, the data were analyzed using the Mann-Whitney *U*-test, with $P < 0.05$ conferring statistical significance.

Results

Isolation of Colon CSCs/TICs as SP Cells

Several methods to isolate colon cancer CSCs/TICs has been reported, including cell surface markers such as CD44 or PROM1 (CD133), SP cells, and the Aldefluor assay.[3–6,13] In the present study, we isolated colon CSCs/TICs using SP cell analysis. Several colon cancer cell lines were dyed with Hoechst 33342 and then analyzed with a BD FACSAria II flow cytometer as

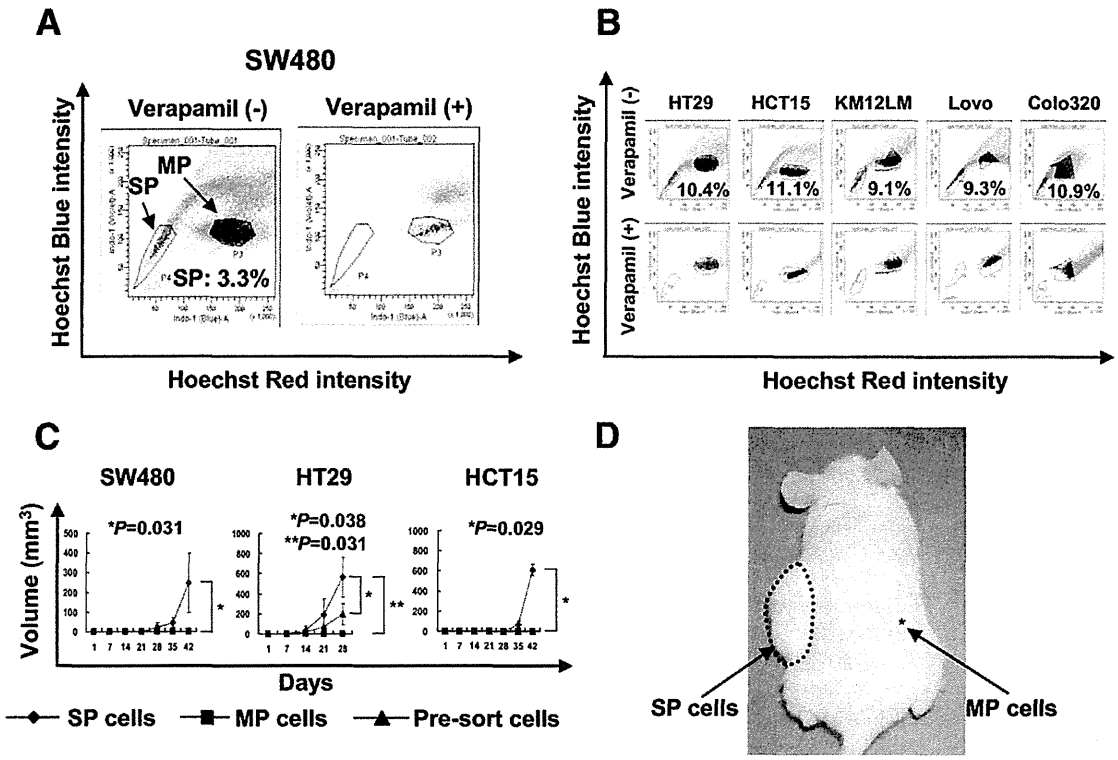

Figure 1. Isolation of colon CSCs/TICs from colon cancer cell lines and tumor growth of the SP cells. **A:** Colon cancer cell line SW480 was stained with Hoechst 33342 dye as described under *Materials and Methods*, with or without verapamil. Stained cells were analyzed using a BD FACSAria II fluorescence-activated cell sorting system. Frequency of SP cells was 3.3%. **B:** Colon cancer cell lines (HT29, HCT15, KM12LM, Lovo, and Colo320) were stained with Hoechst 33342 dye with or without verapamil. Stained cells were analyzed using a BD FACSAria II system. Frequencies of SP cells ranged from 9.1% for KM12LM cells to 11.1% for HCT15 cells. **C:** SP cells, MP cells, and presorted cells of colon cancer cell lines SW480, HT29, and HCT15 were inoculated subcutaneously into the backs of NOD/SCID mice (1×10^3 cells injected). Data are reported as means ± SD. *P* values indicate differences between cell types according to a Mann-Whitney *U*-test. **D:** Representative tumor growth in NOD/SCID mice at the SP cell injection site (1×10^3 cells injected). SP cells and MP cells were inoculated subcutaneously into the left and right side of the back, respectively.

described under *Materials and Methods*. Side population cells could be detected in all six colon cancer cell lines analyzed (ie, SW480, HT29, HCT15, Colo320, Lovo, and KM12LM) (Figure 1, A and B). The frequency of SP cells ranged from 3.3% for SW480 to 11.1% for HCT15 cells. All these SP cells were specifically inhibited by verapamil, as has been shown previously,[14] suggesting that these SP cells were specific for ABC transporter expression. Because previous studies showed that some colon cancer SP cells were not enriched with a CSC/TIC population,[15] it was essential to confirm the presence of CSCs/TICs in SP cells for further analysis. We inoculated these SP cells subcutaneously into the back of immunodeficient NOD/SCID mice using serial dilution. The SP cells derived from SW480, HCT15, and HT29 showed higher tumor initiating ability, compared with MP cells (Table 1). Furthermore, SW480, HT29, and HCT15 SP cells showed faster tumor growth, compared with MP cells (Figure 1, C and D), suggesting the presence of CSCs/TICs in these SP cells. In contrast, the SP cells derived from Colo320, Lovo, and KM12LM did not show any difference in tumorigenicity or tumor growth, compared with MP cells. We therefore restricted further analysis to the SW480, HT29, and HCT15 SP cells as colon cancer CSCs/TICs.

RT-PCR Analysis of Colon Cancer SP Cells

To examine the molecular properties of SP cells, we performed RT-PCR analysis. SOX2 and POU5F1 are representative markers for embryonal stem cells and CSCs/TICs.[16] The SP cells derived from SW480, HT29, and HCT15 showed higher expression of both SOX2 and POU5F1, compared with MP cells (Figure 2A). ALDH1A1, a colon CSC/TIC marker,[6] was expressed at a higher level in SP cells of HCT15 than in MP cells, but SP cells of SW480 and HT29 did not show any difference in comparison with MP cells. SW480 and HT29 SP cells also showed higher expression of LGR5, which is known as a normal colon stem cell marker.[17] To confirm the expression of stem cell markers, we also performed real-time PCR. The SW480 SP cells expressed 90 times higher SOX2, 7 times higher POU5F1, 153 times higher LGR5, and 6.1 times higher ALDH1A1, compared with MP cells (Figure 2B). These findings indicate that these SP cells had molecular properties similar to those of embryonal stem cells.

Resistance to Chemotherapeutic Reagents

Although SP cells derived from liver cancer cell line HuH7 have showed resistance to chemotherapy,[13] we know of no conclusive previous studies of such resistance in co-

Table 1. Tumor Initiating Ability of Colon Cancer SP Cells

Cell line (% SP cells)	Tumor initiating ability*		
	$1 \times 10^{4\dagger}$	$1 \times 10^{3\dagger}$	$1 \times 10^{2\dagger}$
SW480 (3.3)			
SP cells	4/4	4/6	4/4
MP cells	2/4	3/5	0/4
HT29 (10.4)			
SP cells	3/3	2/3	3/3
MP cells	3/3	0/3	0/3
HCT15 (11.1)			
SP cells	3/3	3/4	3/3
MP cells	1/3	1/4	0/3
Colo320 (10.9)			
SP cells	2/2	1/2	1/2
MP cells	2/2	2/2	1/2
Lovo (9.3)			
SP cells	0/1	1/1	0/1
MP cells	1/1	0/1	0/1
KM12LM (9.1)			
SP cells	1/2	2/2	1/1
MP cells	1/2	2/2	1/1

MP, main population; SP, side population.
*Tumor initiating ability is expressed as the ratio of tumor-initiation to injection.
†The tumor initiation abilities were evaluated at day 42 after injection of the indicated number of cells.

lon SP cells. We performed a cell survival study of colon cancer SP cells using the chemotherapeutic agents irinotecan and etoposide. The SW480 and HCT15 SP cells were more resistant to both irinotecan and etoposide than were MP cells (Figure 3, A and B). This finding is consistent with findings for CSCs/TICs derived from other organs.[22,24]

Expression of HLA and Tumor-Associated Antigens in SP Cells

Because CTLs recognize tumor-associated antigen (TAA)-derived antigenic peptides presented by HLA class I molecules, expression of HLA class I molecules is essential for activation of CTLs. Several types of malignancies have been reported to lose the expression of HLA class I molecules through various mechanisms and so escape CTL attack.[18] We therefore evaluated the expression of HLA class I molecules and TAA. We assessed the differences of HLA class I and HLA-A24 expression between SP cells and MP cells by flow cytometry. Because ELISA study has revealed that HCT15 cells lack B2M because of gene mutations of *B2M*,[19] we transduced wild-type B2M cDNA into HCT15 cells and so established HCT15-B2M cells. The SW480, HT29, and HCT15-B2M SP cells showed HLA class I and HLA-A24 expression at the same level as MP cells (Figure 4, A and B). Furthermore, we assessed the expression of one of the colon cancer TAAs, CEP55, by both RT-PCR and real-time PCR (Figure 2, A and B). Both SP cells and MP cells derived from SW480, HT29, and HCT15-B2M expressed CEP55 mRNA at the same level. These data raised the possibility that SP cells are also sensitive to CTLs specific for the CEP55-derived antigenic peptide. Because both SP cells and MP cells expressed CEP55 mRNA at the same level, this appeared to be an ideal target for comparing the susceptibilities of SP cells and MP cells to CTLs.

Susceptibility of CSCs/TICs to CTLs, in Vitro *and* in Vivo

We had previously established CTL clone 41, which is specific for the cancer-related, antigen-derived, HLA-A24-restricted peptide Cep55/c10orf3_193(10).[10] CTL clone 41 recognized CEP55-positive and HLA-A24-positive cancer cells, as described previously.[10,11] In the present study, we used a ^{51}Cr release assay to examine whether CTL clone 41 can recognize SP cells. All SP cells derived from SW480, HT29, and HCT15-B2M showed susceptibility to CTL clone 41 at the same level as the MP cells and the presorted bulk cell lines (Figure 4C). This indicates that the colon CSCs/TICs were sensitive to CTLs.

To analyze the cytotoxic activity of the CTL clone *in vivo*, we performed a Winn assay. SW480 SP cells with or without CTL clone 41 were injected into the backs of NOD/SCID mice subcutaneously. CTL clone 41 significantly inhibited the tumorigenicity of SW480 SP cells (Figure 4D). Because CTLs were injected at the same time and place as the SP cells in the Winn assay, we could not rule out the possibility that SP cells were killed *in vitro*. We

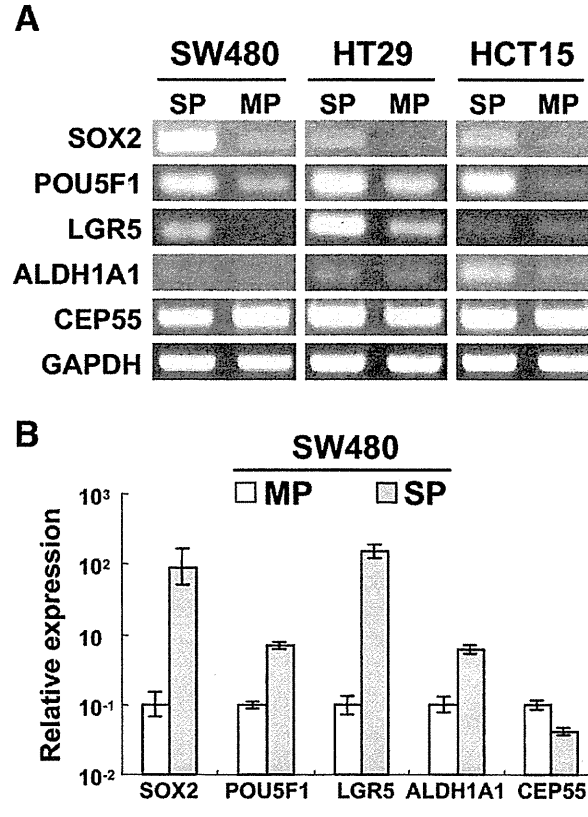

Figure 2. Expression of stem cell markers in SP and MP cells. **A:** mRNAs purified from SW480, HT29, and HCT15 SP and MP cells were analyzed by RT-PCR. **B:** mRNA purified from SW480 SP cells and MP cells were analyzed with real-time PCR. mRNA expression level is relative to MP cells. Data are reported as means ± SD.

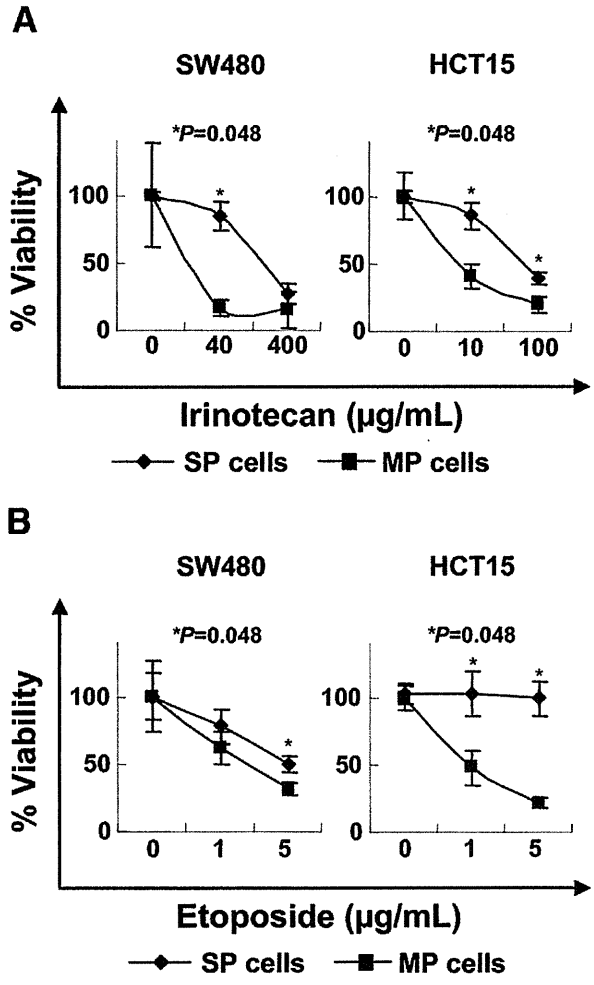

Figure 3. Sensitivity to chemotherapeutic agents. SP cells and MP cells derived from SW480 and HCT15 were incubated in the presence of irinotecan (CPT-11) (**A**) or etoposide (VP-16) (**B**) for 3 days. After incubation, the cell viabilities were measured by WST-1 assay. Data are reported as means ±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

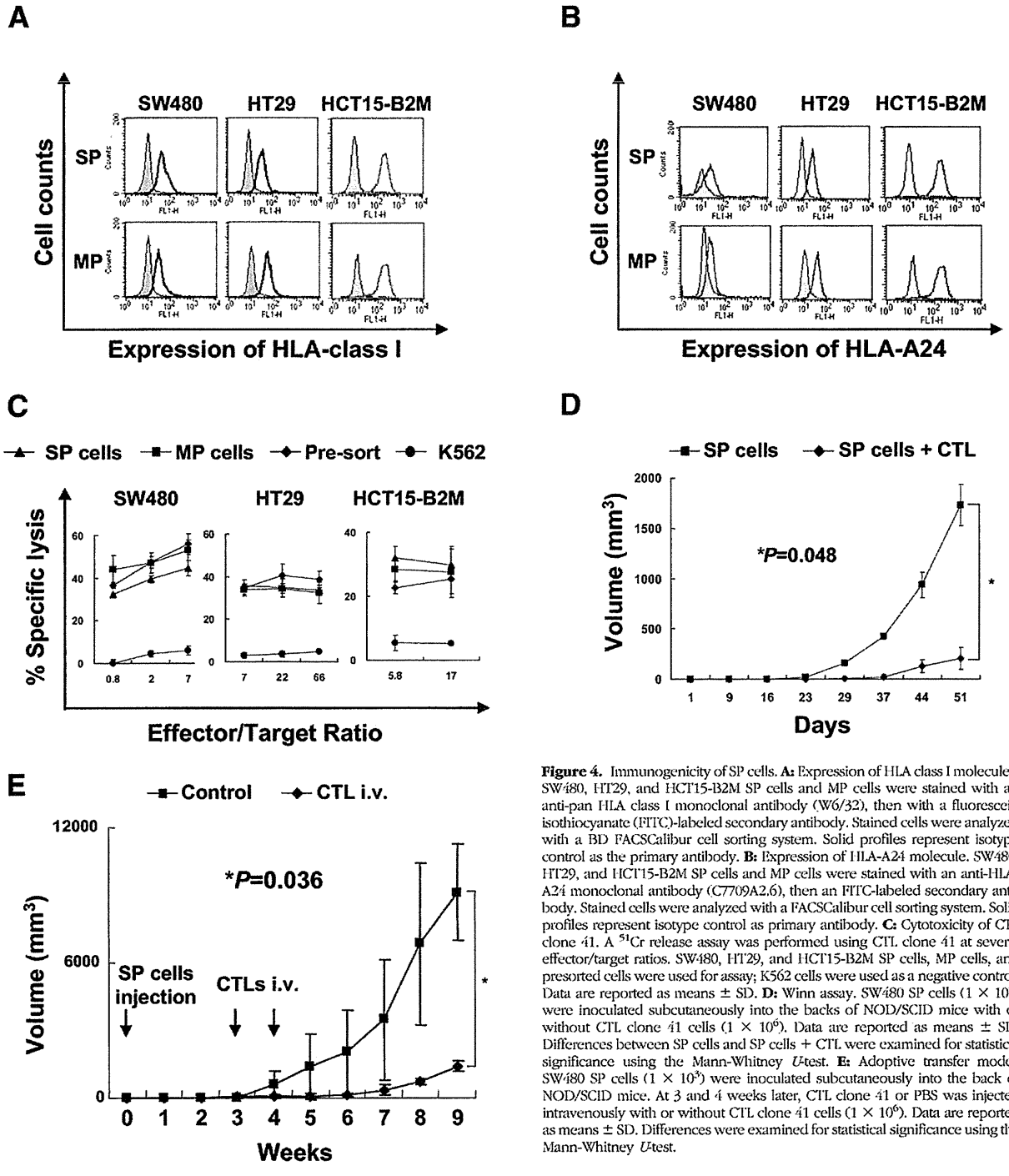

Figure 4. Immunogenicity of SP cells. **A:** Expression of HLA class I molecules. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-pan HLA class I monoclonal antibody (W6/32), then with a fluorescein isothiocyanate (FITC)-labeled secondary antibody. Stained cells were analyzed 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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がん治療の新たな展開

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がん治療の新たな展開

4．癌ワクチン・免疫療法の実際と展望

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要　旨

最近の約４分の１世紀における腫瘍免疫学の進歩は目覚ましく、免疫療法の臨床応用に広く工夫がみられている。その代表的な背景として、免疫細胞の分離・培養・増殖・活性化などによるin vitroでの修飾化技術の向上、腫瘍細胞上の標的分子の特定とその抗体による細胞傷害作用・MHCクラスⅠあるいはⅡ上に結合するがん抗原の特定とそれを認識するCTLの活性化および抗腫瘍作用の確認、などについては飛躍的な進歩がみられるに至っている。その過程で免疫細胞療法が実臨床の場へ提案され、1980年代初めにNIHがCTLを用いた免疫細胞療法の臨床研究を認可して以来、同法を応用した臨床研究の爆発的普及がみられている。

日本でも1980年代半ばよりLAK T細胞を用いた養子免疫療法の普及がみられ、さらに樹状細胞を用いた免疫細胞療法が加わり、各種固型がんや血液腫瘍に対する臨床試験が高度先進医療として実施されてきた。結果的には、際立った効果を得たという十分な結果は得られていない。本邦での免疫学的治療法については上記の動きが主流で先行し、がん特異的ワクチン療法を目的としたがん抗原の探究研究の動きはごく少数の研究者に限られた時期があった。しかし、近年では、次々に数多くのがん特異的抗原の同定が進み、それにともないphase Ⅰ臨床研究が数多く成されている。また同法における免疫逃避機構と、その機構に対する解決策が徐々に明らかになり、それを試みる臨床研究も行われるに至っている。

今後は手術後の補助療法としての再発・転移予防策への応用に大きな期待が寄せられている。また、免疫細胞療法、ワクチン療法に共通して言えることは毒性や有害事象については、さほど重篤な事例が発生しないことである。今後の一層の研究展開に期待がもたれている領域といえよう。

はじめに

免疫療法において、細胞障害性T細胞（cytotoxic T lymphocyte:CTL）の高い認識システムを確立するにあたって、がんペプチドワクチンはその中核を成す分子である。すなわち、がんペプチドはCTLの機能を有効に発揮させる役割を果たす。また腫瘍組織内やがん性胸水中の腫瘍浸潤リンパ球（tumor infiltrating lymphocyte: TIL）の局所での増殖を可能にすることで、免疫作用機序がin situにおける自然な形で増幅可能となった[1,2]。すなわち抗原とCTLを制御・修飾することにより、強い腫瘍排除機構の構築を可能にしうることが明らかにされつつある。

分子生物学的制御法とその臨床応用が広く研究されている今日、大きな期待の寄せられている抗原分子は数多く、続々と臨床試験が行なわれている。多くのワクチンの抗原分子の構造が明らかにされ、そのkeyともなるペプチド形成にかかわる遺伝子構造も把握されるに至っており、人工的にペプチド分子を造り出すことが可能となり[3]、実践的ながん治療法としての発展に期待が寄せられている。

1．がんペプチドワクチンの作用機構

がんペプチドワクチン療法は、T細胞による免疫学的制御法のひとつとして能動免疫制御法のひとつとして存在する。T細胞はその機能によって分類され、なかでもヒトがん細胞が提示するがん抗原認識リセプターを有するT細胞をCTLと総称し、CD8陽性細胞として捉えられ、ヘルパーT細胞であるCD4陽性細胞からのサイトカインなどの刺激によってその機能が促進されることが知られている[4]。前者は

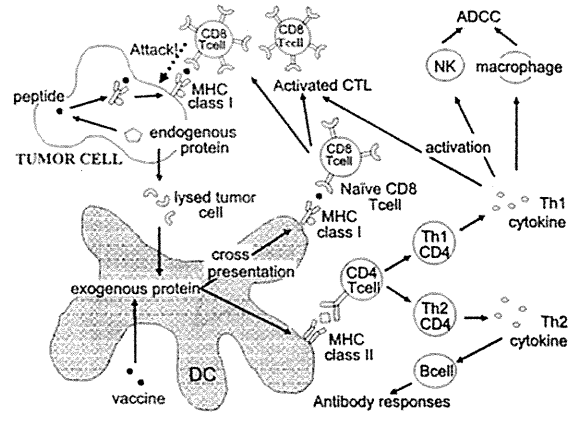


図１　腫瘍細胞の認識・排除機構

human lymphocyte antigen（HLA）クラスⅠ拘束性に、後者はクラスⅡ拘束性にがん抗原を認識することが明らかになっている[5]。

がん抗原については、がん細胞が一定の配列の蛋白あるいはミスフォールディングにて誤った配列の蛋白を過剰に産生し、その蛋白がプロテオソーム内でペプチドレベル（アミノ酸数として８～10個）にまで分解され、そのがん抗原が小胞体に輸送され、そこでHLAクラスⅠと複合体を形成し、細胞表面上に表出する（図１）[6]。このようにがん細胞膜表面上のHLAクラスはペプチド（がん抗原を含む）によって占拠される状態にあると言え、その抗原をCD8T細胞（CTL）が認識するわけである（図１）[7]。この８～10個のペプチドがT細胞ワクチンとも言えるものである。

このようにペプチドは細胞内蛋白に由来することから、それらを産生する細胞に対する認識・識別機構が生体に存在し、その中にがん固有のペプチド（がん抗原）が存在しうるのである。このようながん治療は、人体にとっては生理的機序を応用するもので、在り方としては望ましい治療手段と考えられる。

CD8陽性T細胞はT細胞受容体（TCR）を介して、MHCクラスⅠ・抗原ペプチド複合体からの第１シグナルと、CD28/CD80, 86, CD40L/CD40などの相互作用による第２シグナルによって活性化CTLとなる（図２）[8]。MHCクラスⅡ・抗原ペプチド複合体から抗原刺激を受けたCD4陽性T細胞は、サイトカイン刺激によってTh1細胞とTh2細胞に分化する[9]。Th1細胞はIL-2などのサイトカインを産生し、CTL活性化に関与する。以上のように、DC上のMHCクラスⅠ・抗原ペプチド複合体からペプチド刺激を認識したCD8陽性T細胞は、ヘルパーT細胞の作用も加わり活性化CTLとなり、腫瘍抗原特異的に腫瘍細胞を攻撃・破壊する。

がんペプチドワクチン療法においては、ワクチンとして体内投与されたペプチドはDCに取り込まれ、

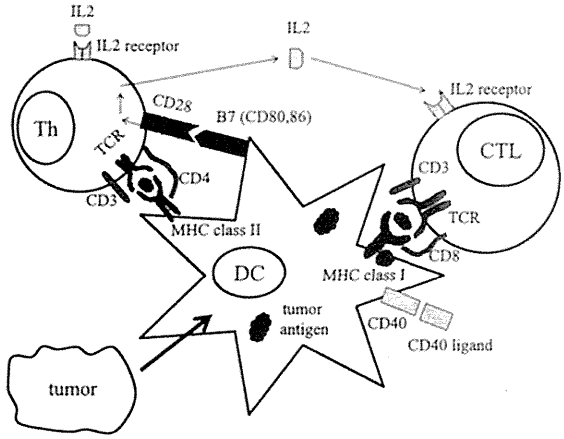


図２　DC機能への影響因子

表１　腫瘍免疫エスケープ機構

A. 腫瘍側の因子	
	MHC重鎖欠失
	β2ミクログロブリン欠失
	抗原分子の欠失
	TAP欠失
	LMP欠失
	Fasリガンド発現
	免疫制御分子（TGFβなど）の放出
B. CTL側の因子	
	T細胞受容体シグナル伝達異常
	Th1/Th2バランスの喪失
	T細胞欠失
	キラー細胞抑制受容体の発現
	TregによるT細胞制御

加工をほとんど受けることなく高率にMHCクラスⅠ分子上に抗原提示され、ペプチド特異的なCTLを活性化する[10]。ワクチンとして投与されたペプチドは、標的とするがん細胞がMHCクラスⅠ分子上に提示しているがん抗原と同一であるので、ペプチドにより活性化されたCTLはがん細胞上のがん抗原（ペプチド）同様に認識し、がん細胞傷害作用により、抗腫瘍効果を誘導する。

２．がんペプチドワクチン療法におけるエスケープ機序

そもそもがん細胞は、個体の有する自然および特異的免疫機構の監視機構を逃れて生育しているものとして捉えられる。この監視機構から逃れる多くの機序が確認されており、エスケープ機構としてその機序の解明と排除のための対応策についての研究が盛んに行なわれている。これらについては「腫瘍側の因子」と「T細胞側の因子」の双方での存在が知られている（表１）。担がん生体にこれらの現象のすべてが存在しているのではなく、その種類や程度はさまざまである。エスケープ機序に対する克服策は、がんワクチン療法の実施上、避けて通れぬ重要な課題対象である。個々のがんペプチドワクチン療

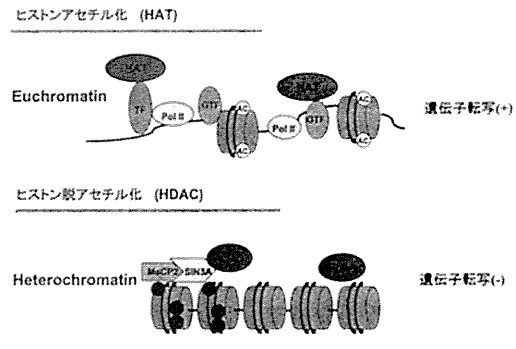


図３　ヒストンアセチル化と脱アセチル化と遺伝子転写機構

表2　抗原提示分子の発現低下と発現回復を目的とした薬剤（文献7、表2の一部を紹介）

発現抑制遺伝子	研究対象がん腫	発現回復薬剤	
		DNMT阻害剤	HDAC阻害剤
Ⅰ．MHC class Ⅰ			
	神経芽細胞腫		TSA
	前立腺がん		TSA
	巨核球性白血病		SB
	メラノーマ	5-AZA・DC	
Ⅱ．MHC class Ⅱ			
	神経芽細胞腫		TSA
	HeLa細胞		TSA
	骨髄単球性白血病	SB	
	B細胞リンパ腫		TSA
Ⅲ．β2-microglobulin			
	前立腺がん		TSA
Ⅳ．CIITA			
	扁平上皮がん		TSA

法を実施する中で、in vivo, in vitroでのエスケープ機構の詳細な分析が、治療成績向上のための重要な基礎知見となりうるであろう。

近年、がん細胞クロマチンにおいて、過剰なDNAメチル化[11]あるいはヒストン脱アセチル化[12]によって、ヒストンのN末端が陽性荷電となり陰性荷電のDNAと結合し転写が抑えられることが知られている。Methyl-CpG-binding protein (MBP)はヒストンメチル化酵素と結合し、N末端はメチル化し、その部分を認識するヘテロクロスチン蛋白（HPI）が結合し、HPI同士が重合することによりその領域が凝縮される。増殖抑制遺伝子やアポトーシス誘導遺伝子の発現のための転写は抑制され（図3）、がんのエスケープ機序の重要なひとつとして幅広く関与していることが明らかとなっている[13,14]。

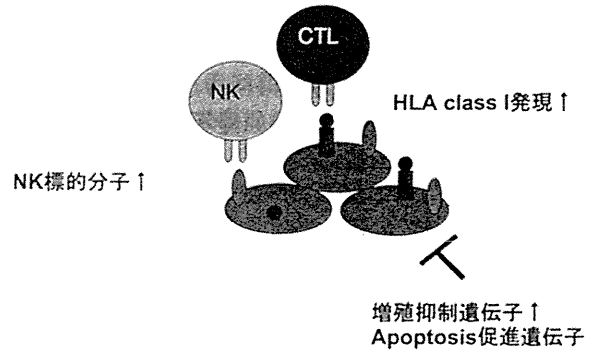


図4　想定されるHDAC阻害剤の抗腫瘍効果

3．エピジェネティクス制御による免疫逃避機構の解除は可能か

前項で述べたようなエピジェネティクス免疫逃避機構が証明された結果、がん抗原遺伝子の増幅やHLAクラスⅠ発現機構の修飾化、すなわちがん細胞におけるDNAメチ化現象あるいはヒストン脱アセチル化現象の阻害、そして細胞性免疫のCTL賦活化、などが治療法のひとつとして想定される。

HLAクラスⅠにおけるエスケープ機構に焦点を当てると、DNAメチル化酵素阻害剤[15]、ヒストン脱アセチル化酵素阻害剤[16]の有用性が想定される。すでに今日までにDNA methyltransferase (DMNT)阻害剤として5-aza-2'-deoxycytidine (5-AZA/DC)、histone deacetylase inhibitor (HDAC)阻害剤としてvalproic acid (VPA)、trichostatin A (TSA)、sodium

表3　進行・再発大腸癌に対するサバイビン2Bペプチド単剤による第一相臨床試験結果

症例	性/年	stage	投与量	投与回数	副作用	腫瘍マーカー	画像評価	DTH
1	M/63	Ⅲb	0.1mg	10	貧血	低下	PR	−
2	M/53	Ⅱ	0.1mg	10	無	増加	PD	−
3	M/42	Ⅳ	0.1mg	17	無	増加（一時低下）	SD	−
4	F/54	Ⅲb	0.1mg	2				
5	M/65	Ⅰ	0.1mg	14	無	増加（一時低下）	PD	−
6	F/64	Ⅳ	0.1mg	6	全身倦怠感	増加（一時低下）	PD	+
7	F/48	Ⅳ	1.0mg	7	無	低下	PD	−
8	F/62	Ⅱ	1.0mg	13	無	増加	PD	−
9	M/64	Ⅲb	1.0mg	18	無	増加（一時低下）	PD	−
10	M/52	Ⅲb	1.0mg	6	無	増加	PD	+
11	M/58	Ⅳ	1.0mg	14	無	増加	SD	+
12	F/58	Ⅱ	10.0mg	12	発熱	増加	PD	−
13	F/62	Ⅱ	10.0mg	6	無	増加	PD	+
14	M/72	Ⅳ	10.0mg	10	無	増加	PD	+
15	M/71	Ⅳ	10.0mg	4				
16	M/67	Ⅳ	10.0mg	7	無	増加	PD	−
17	F/59	Ⅳ	10.0mg	9	無	増加	SD	+

butyrate(SB)は、すでに適応疾患を異にして市販されている。他に標的分子の特徴と、その特異的選択性を目指した阻害作用を示す薬剤開発研究も行われており、それらの臨床応用に期待が寄せられる[17]。これまでに明らかにされた抗原提示関連分子の発現抑制遺伝子と発現回復薬剤を表2に要約した。これら薬剤の作用は図4に示したような抗腫瘍効果が期待される。

4．第Ⅰ相臨床治験成績

(1)進行・再発大腸がんにおける臨床試験結果[18]

(a)ペプチド単剤投与

17症例が臨床試験に登録され(表3)、ワクチン投与を開始したが、2症例(症例4、症例15)では本治験を途中で中止した。症例4は脳転移の増悪にてステロイド治療のため本治験を中止、症例15は肺転移の増悪にて治験終了となった。いずれも病状増悪による本治験中止であり、ワクチンの副作用による理由ではなかった。

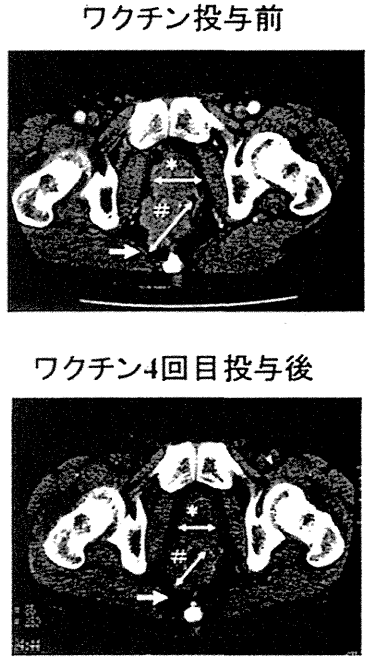
有害事象は、貧血、全身倦怠感、発熱であったが、いずれもgrade2以下であり、このペプチドの安全性は評価しうると考えられた。

臨床効果としては、2例に腫瘍マーカーの低下を認めた。そのうち、臨床試験期間内に一時的にでも腫瘍マーカーが低下した症例は6例(症例1、3、5、6、7、9)あった。CTによる画像的評価では、3例にSD(stable disease)を認めた。また、直腸がんの吻合部再発である症例1においては、ペプチドワクチン投与開始前のCTと比較し、4回目のワクチン投与後のCTでは32%の腫瘍縮小を認めPR(partial response)と判定した。また、腫瘍縮小に伴って腫瘍マーカーも低下した(図5)。

6回のワクチン投与による臨床試験期間終了後は、腫瘍からの出血も消失し、全身状態もきわめて良好に改善されたため、その後、ワクチン投与を中断していた。すると、腫瘍マーカーは再上昇し始めた。そこで、再度ワクチン投与を再開すると、再び腫瘍マーカーは減少し始めた。このことから、ワクチ

a. CTにおける腫瘍径の変化

投与後に縮小化を生じている。



b. CEA値の推移

再投与で高値化抑制がみられる。

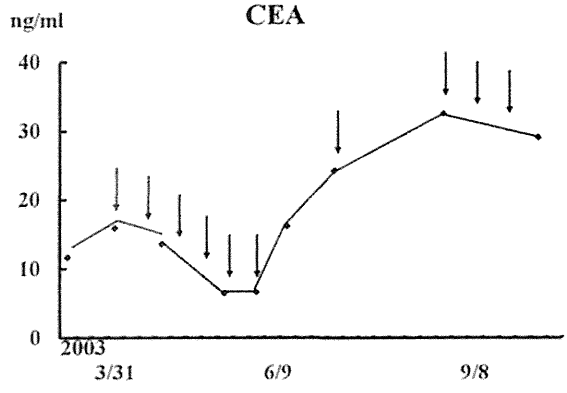


図5　ワクチン投与経過時の検査結果

表4　再発進行性乳癌に対するSurvivin-2B単独投与の効果

患者	ペプチド投与量	有害事象	腫瘍マーカー値推移			CT上の効果	DTH skin test	Tetramer 染色	ELISPOT assay
			マーカー	投与前	投与後*				
1	0.1	(−)	ICTP**	7.2	5.5	PD	(+)	(−)	(−)
2	0.1	(−)	CEA**	6.1	14.9	PD	(−)	(−)	(−)
3	0.1	(−)	CA15-3***	323.7	412.1	SD	(−)	(−)	(−)
4	0.1	(−)	CEA	10.3	28.6	PD	(−)	(−)	(−)
5	0.1	(−)	ICTP	7.8	10.5	SD	(+)	(−)	(−)
6	1.0	(−)	CA15-3	53.4	179.8	PD	(−)	(−)	(−)
7	1.0	(−)	CEA	72.5	15.2	PD	(−)	(+)	(+)
8	1.0	(−)		WNR			(−)	(−)	(−)
9	1.0	(−)	CEA	22.5	45.2	PD	(−)	(+)	(−)
10	1.0	(−)	CEA	47.4	81.3	PD	(−)	(+)	(−)

*ワクチン投与後4週経過時測定、　**単位：ng/ml、　***単位：U/ml

ン投与による抗腫瘍効果が実証されたと考えられる。

(b)アジュバント併用投与

IFAを併用投与するとほぼ全例に投与部位に硬結を認めた。IFAとIFN-aを併用すると、硬結以外に発熱を2症例に認めた。しかしながら、IFA併用、IFAとIFN-a併用、いずれにおいてもgrade3以上の重篤な副作用を認めず、アジュバント併用サバイビン2Bペプチド投与においても安全性は評価された。

臨床効果としては、ペプチドワクチンにIFAを併用しても5例中1例(20%)にSDを認めたのみで、他症例ではすべてPR (progressive disease) であった。しかし、IFAにIFN-aを併用したところ5例中3例(60%)にSDを認め、IFN-aの有用なアジュバント効果を示したと考えられた。このことより、より的確なアジュバント設定がペプチドワクチン療法には重要であると考えられた。

(2)進行・再発乳がんに対するペプチド単剤投与による臨床試験[19]

進行・再発乳がんに対するペプチド単剤投与による臨床試験(表4)では、ペプチド投与量を2段階(0.1, 1.0mg) に設定し、それぞれ5例ずつに投与した。有害事象はいずれの症例にも認めなかった。

臨床効果においては、初回ペプチド投与前と4回目のペプチド投与後の腫瘍マーカー値を比較したところ2症例以外で上昇した。CT画像においては2症例にSDを認めたが、他症例ではPDであった。

免疫学的反応では、3症例でペプチド特異的CTLの頻度が増大、またそのうちの1症例ではELISPOT assayにてペプチド投与後にスポット数の増加を認めた。

次に、進行・再発乳がんに対するペプチドおよびIFA併用投与による臨床試験を試みた。本臨床試験では、ペプチド投与量を1.0mgに設定し、IFAとエマルジョン化し4症例に投与した。有害事象は2症例に投与部位の皮膚の硬結、1症例に全身倦怠感、1症例に全身倦怠感と発熱を認めた。しかしいずれも軽度で、ペプチドにIFAを併用しても安全性は確認された。

臨床反応としては、4症例いずれでも腫瘍マーカーは高値化し、CT評価においてもPDであった。しかし、免疫学的反応においてはすべての症例でペプチド特異的CTLの上昇を認め、そのうち1症例においてはELISPOT assayにおいてもスポット数の増大を認めた。

5．臨床試験からの知見と新しい課題解決そして今後の展望

上述の臨床試験結果より、サバイビン2Bペプチドの安全性においては単剤投与、アジュバント併用投与のいずれにおいても問題はないと評価された。

サバイビン2Bペプチドによる抗腫瘍効果に関しては、乳がんでの結果よりペプチド単剤投与においては10症例中3症例 (30%) にペプチド特異的CTLの増加を認めたが、IFAを併用すると4症例すべての症例 (100%) においてペプチド特異的CTLの増加を認めた。しかし4症例すべてでPDで、ペプチド投与によりペプチド特異的CTL誘導が可能であったが、腫瘍縮小を導くには至らなかった。

本稿においては提示していないが、ペプチドとIFAおよびIFN-α併用の臨床試験では、再発膵臓がんにおいて臨床試験開始後、腫瘍マーカーが正常域へ低下し、画像上もcancer freeな状態を長期にわたり維持している症例を経験しており、的確なアジュバントを併用することにより、満足すべき抗腫瘍臨床効果誘導が可能と思われた。

上記のように乳癌症例においては、ペプチド特異的CTL誘導が可能であったにもかかわらず、それが臨床効果に至らなかった原因の一つには、がん細胞の免疫エスケープ現象の関与が考えられた。特異的CTLはがん細胞表面に発現する抗原提示分子HLAクラスⅠ分子発現を免疫染色にて解析したところ、ワクチン療法にてPRを認めた症例および腫瘍マーカーが正常値に低下した症例の多くで、がん原発巣でのHLAクラスⅠ分子が強発現していた[20]。

このことより、ペプチドワクチン療法において高い抗腫瘍効果を誘導するには、がん病巣部でのHLAクラスⅠ分子発現が低下していないか否かを確認する必要性がある。つまり、より的確な症例の絞り込みが必要であると考えられた。また、がん細胞上のHLAクラスⅠ分子発現低下には、エピジェネティック機構の介在が確認されている。われわれの研究グループの成果により、このような変化がヒストン脱アセチル阻害剤によって回復することもin vitroにおいて実証されている。

したがって、HLAクラスⅠ分子がすでに発現低下、消失している症例においても、HLAクラスⅠ分子の発現レベルを回復させることができるならば、多くの症例において高い抗腫瘍効果を誘導でき得ると考えられ、発現低下したHLAクラスⅠ分子の発現回復方法の確立に向けて、早々に臨床試験が開始される予定である。

おわりに

2003年4月より、サバイビン2Bペプチドによる第Ⅰ相臨床試験を展開してきている。確かに単独投与でも有用例がみられた他、すべてのがん腫に臨床応用可能な基礎的知見が得られている。一方、十分な抗腫瘍効果誘導には至っていない症例に対する臨床解決を示し得ていないことも事実である。現在までに画期的な抗腫瘍効果を認めた症例が経験されており、今後のアジュバントの選択やエピジェネティクス修飾的確など、的確な薬剤投与設定が臨床適用に対し定まりつつある。

これまでの臨床試験において満足すべき抗腫瘍効果を誘導できない最大原因の一つに、各種の治療を

踏み終えた進行・再発状態を対象にしていることが考えられる。したがって、今後は再発治療のfirst-lineとして化学療法との併用、あるいは術後補助療法としてのペプチドワクチン療法、臨床試験が予定されるべきである。今後、ペプチドワクチン療法が、手術、化学療法あるいは放射線療法とともに、がん集学的治療の一つとして確立されることが期待されている。

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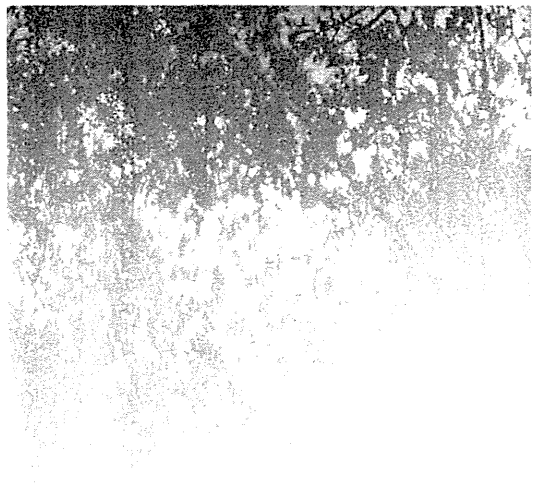

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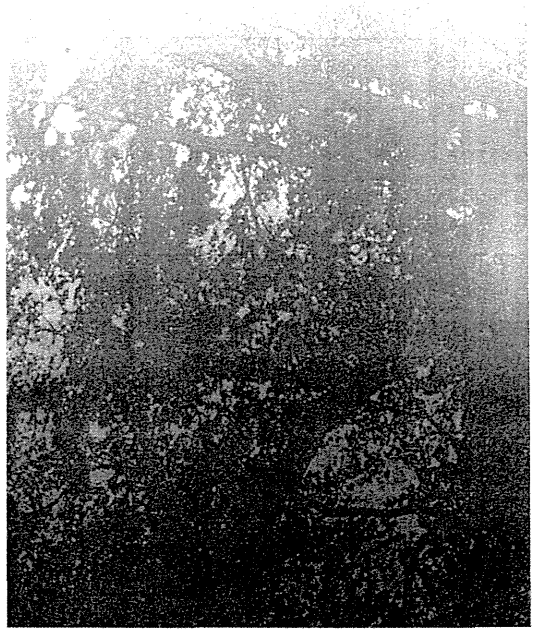

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