Identification of squamous cell carcinoma antigen-derived peptides having the capacity of inducing cancer-reactive CTLs in HLA-A24+ cancer patients

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Abstract. Squamous cell carcinoma antigen (SCCA) is a useful marker for SCCs. In this study, we attempted to identify SCCA-derived peptides that could be applied in the development of specific immunotherapy for HLA-A24+ cancer patients with SCC or non-SCC. A variety of SCC and non-SCC lines were examined for their expression of *SCCA* mRNA using quantitative PCR. SCCA protein expression in cancer tissues was investigated by immunohistochemical staining. Thereafter, SCCA-derived peptide candidates were prepared based on their binding motifs to HLA-A24 molecules. Among these peptides, SCCA-derived peptides that were frequently recognized by humoral immunity were further tested for their ability to induce cancer-reactive cytotoxic T lymphocytes (CTLs) from the peripheral blood mononuclear cells of HLA-A24+ patients with SCC or non-SCC. As a result, the majority of SCC lines and tissues were positive for SCCA both at mRNA and protein levels. By contrast, non-SCC cancer tissues hardly expressed it at the protein level, although adenocarcinoma cell lines partly expressed it at the mRNA level. Four SCCA-derived peptides were frequently recognized by immunoglobulin G of both SCC and non-SCC cancer patients. Among these peptides, both the $SCCA_{112-120}$ and $SCCA_{215-224}$ peptides were found to effectively induce peptide-specific CTLs toward HLA-A24+ SCCA+ cancer cells from the peripheral blood mononuclear cells of both SCC and non-SCC cancer patients. Two newly identified SCCA-derived peptides with the ability to induce CTL activity in both SCC and non-SCC cancer patients may be applicable to specific immunotherapy for HLA-A24+ cancer patients with SCC, but not those with non-SCC.

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Introduction

Squamous cell carcinoma antigen (SCCA) was purified from the TA-4 complex of SCCA of the uterine cervix (1). Using polyclonal antibodies, SCCA was detected in SCCs, as well as normal squamous epithelium (2). SCC was also detected in saliva, respiratory secretions, and amniotic fluid samples from healthy individuals (3). Nevertheless, SCCA has been considered as a useful marker for SCCs (4). In fact, SCCA is currently used to predict disease-free and overall survival in head and neck, lung, and vulvar SCCs (5-7). In addition, several studies have confirmed that elevated SCCA levels correlate well with the extent of disease in patients with cervical SCC (8-12). These lines of evidence suggest that SCCA could be a promising candidate in the development of anti-tumor immunotherapy.

We have identified a panel of cancer-related antigens and their epitope peptides that are recognized by cancer-reactive cytotoxic T lymphocytes (CTLs) (13-15), and we have been conducting clinical trials of peptide-based vaccines against various types of cancer (16-19). In such trials, some of the CTL-directed peptides tested exhibited the ability to elicit both cellular and humoral immune responses *in vivo*. In addition, the levels of anti-peptide antibodies in post-vaccination sera were closely correlated with the overall survival of advanced cancer patients who had received a peptide vaccination (20). These observations suggest that CTL-directed peptides that can be recognized by both the humoral and cellular immune systems could serve as potent candidates for specific immunotherapies for the treatment of cancer patients.

In this study, we attempted to identify SCCA-derived peptides that could be applied as specific immunotherapies for HLA-A24+ cancer patients; whereby the applicability of a peptide would be based on its ability to be recognized

by both the humoral and cellular immune systems. Here, we report two SCCA-derived peptides applicable as specific immunotherapies for HLA-A24$^+$ patients with SCC.

Materials and methods

Samples and cell lines. After written informed consent had been obtained from each participant, peripheral blood mononuclear cells (PBMCs) and plasma were collected from patients with esophageal, uterine cervical, lung, gastric, colon, and breast cancer at Kurume University Hospital. PBMCs and plasma were also obtained from healthy donors (HDs). All subjects were free from human immunodeficiency virus (HIV) infection. All sera and PBMCs were cryopreserved at -80˚C and -196˚C until use, respectively. The expression of HLA class I antigens on these PBMCs was serologically defined by flow cytometric analysis. YES-1 and YES-2 are esophageal SCCs. The other cancer cell lines used in this study are shown in Fig. 1A (SCCs) and B (adenocarcinomas and lung small cell carcinomas).

Quantitative PCR. Gene expression was quantified by real-time PCR using ABI PRISM 7000 (Applied Biosystems, Foster City, CA), as previously reported (21). RNAs were extracted using the RNA-Bee RNA isolation reagent (Tel-Test, Inc., Friendswood, TX) method according to the manufacturer's instructions. The cDNA of mRNA was prepared from 5 μg of total RNA using a SuperScript preamplification system (Invitrogen) according to the manufacturer's instructions. Real-time PCR of the cDNA specimens was conducted in a total volume of 12.5 μl with 1X TaqMan Master mix (Applied Biosystems), and 1.25 μl of mixture of primers and probes. The primers and TaqMan probes used in this study were purchased from Applied Biosystems (Assay ID#: Hs00199468_m1). The thermal cycler parameters included 2 min at 50˚C, 10 min at 95˚C, and 40 cycles involving degeneration at 95˚C for 15 sec and annealing extension at 60˚C for 1 min.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were deparaffinized, incubated with 0.02 U/ml α2-3,6,8-neuraminidase, Vibrio Cholerae (Calbiochem EMD Biosciences, Inc., La Jolla, CA), in phosphate-buffered saline (PBS) at room temperature for 1 h for antigen retrieval, and then treated with 3% H_2O_2 in PBS to block endogenous peroxidase activity. Immunohistochemistry was performed using 1:100-diluted rabbit polyclonal antibody against SCCA1/2 (H-390: Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HISTONE, SAB-PO® kit (Nichirei Co., Ltd., Tokyo, Japan). The peroxidase reaction was developed using 3,3-diaminobenzidine tetrahydrochloride, and nuclei were counterstained with hematoxylin.

Peptides. SCCA-derived peptides were purchased from BioSynthesis (Lewisville, TX); an HIV peptide with the HLA-A24 binding motif (RYLRQQLLGI) was also used as a negative control. All peptides were dissolved with dimethyl sulfoxide at a dose of 10 mg/ml.

Measurement of anti-peptide antibody. The levels of anti-peptide immunoglobulin G (IgG) were measured by the Luminex™ system as previously reported (22). In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (γ chain-specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-PE into wells, and was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween-PBS into each well. Fifty μl of sample was detected using the Luminex™ system. To confirm the specificity of IgG to an SCCA peptide, sample plasma was cultured in plates coated with a corresponding SCCA peptide, or with an irrelevant SCCA peptide. Thereafter, the levels of the corresponding SCCA peptide-specific IgG in the resulting supernatant were determined by the Luminex system.

Table I. Summary of SCCA expression in various types of cancer tissue.

Types of cancer	Histology	Positive/total cases (%)
Esophageal	SCC	3/3 (100)
Cervix of uterus	SCC	4/5 (80)
Lung	SCC	4/5 (80)
	Adenocarcinoma	0/5 (0)
	Small cell carcinoma	0/3 (0)
Gastric	Adenocarcinoma	1/6 (17)
Colon	Adenocarcinoma	0/6 (0)

Induction of peptide-specific CTLs. PBMCs from HLA-A24$^+$ cancer patients and HDs were used for the CTL induction assay. For the induction of peptide-specific CTLs, PBMCs ($1.5x10^5$ cells/well) were incubated with 10 μg/ml of each peptide in four different wells of a 96-well microculture plate (Nunc) in 200 μl of culture medium containing interleukin (IL)-2, as reported previously (18). Every 3 or 4 days, half of the culture medium was removed and replaced with new medium containing the corresponding peptide (20 μg/ml) and 100 U/ml IL-2. On the 14th day, the cells from each well were independently harvested, washed, separated into 4-wells, and tested for their ability to produce interferon (IFN)-γ in response to C1R-A24 cells pulsed with a corresponding peptide or a negative control HIV peptide in the duplicate assays. C1R-A24 is an HLA-A2402-expressing subline of C1R lymphoma (Dr M. Takiguchi, Kumamoto University, Japan). After 18-h incubation, the supernatant was collected, and the level of IFN-γ was determined by ELISA.

Assay of cytotoxicity. Cultured cells in wells producing IFN-γ in response to a corresponding peptide were collected and further cultured with IL-2 alone for 10-14 days in order to obtain a sufficiently large number of cells to perform a standard 6-h ^{51}Cr-release assay. The following tumor cell lines were

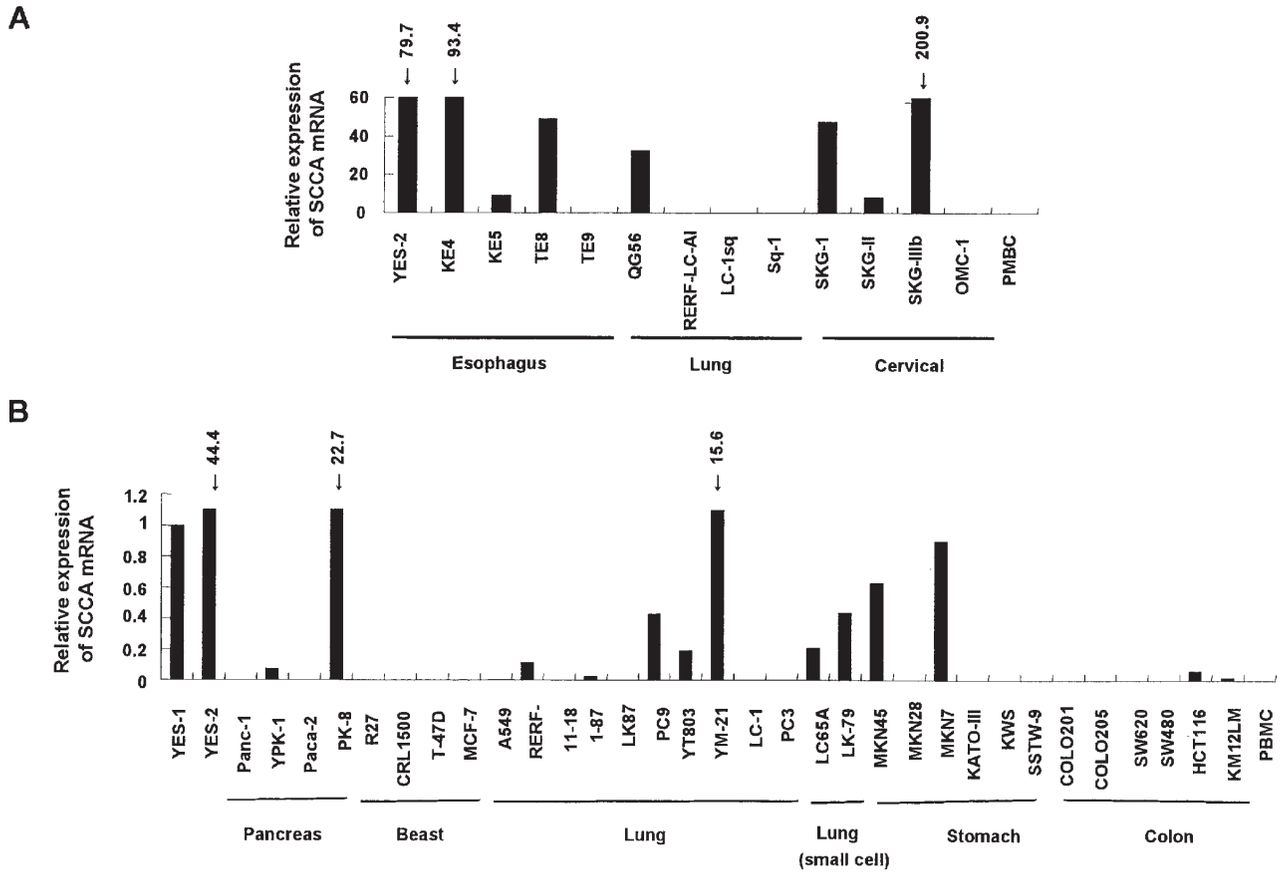

Figure 1. Expression of *SCCA* mRNA in various SCC and non-SCC lines. Quantitative real-time PCR was performed using RNA from SCC lines (A) and non-SCC lines (B). *SCCA* mRNA expression was standardized according to *β-actin* mRNA expression. The *SCCA* mRNA expression in YES-1 was considered as expression level 1, and the relative expression of the *SCCA* mRNA in other samples is shown.

used as targets; YES-1 (HLA-A24$^-$ and SCCA$^+$ esophageal squamous cell carcinoma), and YES-2 (HLA-A24$^+$ and SCCA$^+$ esophageal squamous cell carcinoma). Phytohemagglutinin (PHA)-stimulated blastoid T cells were also used as a negative control. In some experiments, 20 μg/ml of anti-HLA-class I (W6/32, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), anti-CD4 (Nu-Th/i, IgG1), or anti-CD8 (Nu-Ts/c, IgG2a) monoclonal antibody (mAb) were added to the wells at the initiation of the assay. Anti-CD14 (JML-H14, IgG2a) mAb was used as a negative control. In a cold-inhibition assay, unlabeled C1R-A24 cells which were pre-pulsed with the corresponding SCCA peptide or an HIV peptide were added to the wells at a cold/hot target cell ratio of 10/1.

Statistical analyses. A two-tailed Student's t-test was employed for the statistical analysis.

Results

Expression of SCCA mRNA in various types of SCCs and non-SCCs. First, we investigated the SCCA expression in a panel of SCC lines. We investigated the expression of *SCCA* mRNA by the quantitative real-time PCR method. The expression of *SCCA* mRNA in SCC YES-1 was considered to be expression level 1, and the relative expression of *SCCA* mRNA was also shown. As a result, *SCCA* mRNA was found to be expressed in 4 of 5 esophageal cancer cell lines, 1 of 4 lung cancer cell lines, and 3 of 4 cervical cancer cell lines tested, respectively (Fig. 1A). We further determined whether or not SCCA is expressed in non-SCC lines because no report has described SCCA expression in non-SCCs. All cancer cell lines, with the exception of two lung small cell carcinoma lines, were adenocarcinoma. *SCCA* mRNA was found to be expressed in 2 of 4 pancreatic cancer cell lines, 4 of 10 lung cancer cell lines, 2 of 6 gastric cancer cell lines, and 1 of 6 colon cancer cell lines tested (Fig. 1B). Both lung small cell carcinoma cell lines were positive for *SCCA* mRNA expression, but all 4 breast adenocarcinoma cell lines were negative for it. Its expression in normal PBMCs was negative. In general, the level of *SCCA* mRNA expression was higher in SCC lines than in non-SCC lines.

Expression of SCCA in SCC and non-SCC tissues. We next examined the SCCA expression in SCC and non-SCC tissues by immunohistochemical staining. As a result, all three esophageal SCC tissues, 4 of 5 cervical cancer tissues, and 4 of 5 lung SCC tissues were positive for SCCA. SCCA expression was not detected in any of the 5 lung adenocarcinoma or the 3 small cell carcinoma tissues. One of 6 gastric cancer tissues was positive for SCCA, but all 6 colon cancer tissues were negative. Comprehensive results are summarized in Table I, and representative results are shown in Fig. 2. SCCA expression was observed in esophageal SCC (Fig. 2B) and lung SCC (Fig. 2E), and in part of the normal

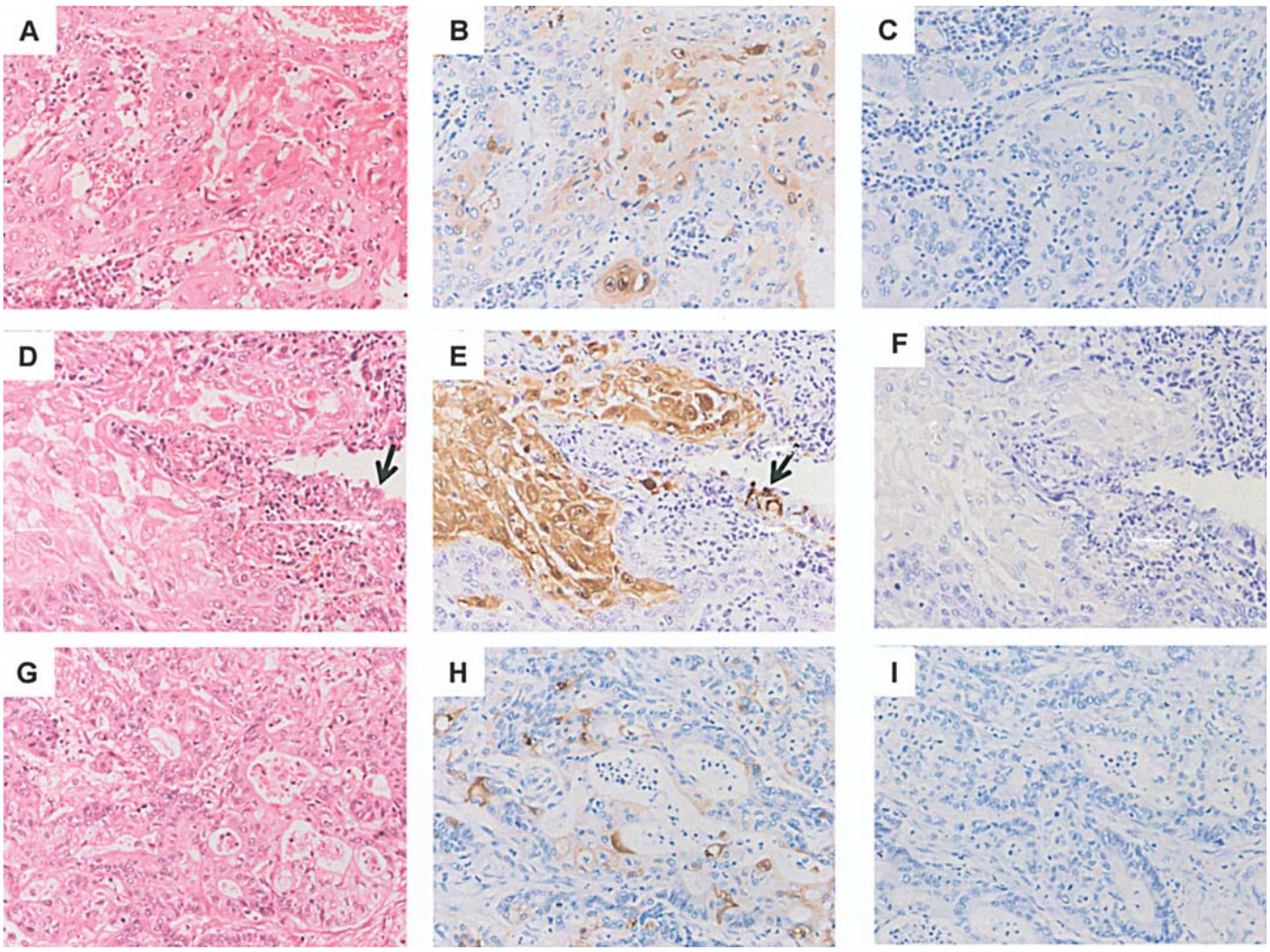

Figure 2. Immunohistochemical staining with anti-SCCA antibody. The expression of SCCA protein in different cancer tissues was evaluated by immunohistochemical staining on formalin-fixed, paraffin-embedded tissue sections with anti-SCCA antibody. Representative photographs of esophageal SCC (A-C), lung SCC (D-F), and gastric adenocarcinoma (G-I) are shown. A, D, and G are the H&E staining, and C, F, and I are the control staining with rabbit Ig. The arrows show normal bronchial epithelia. The magnification of A was x200, and that of the other 8 photographs was x100.

Table II. The SCCA-derived peptides.

Position	Sequence	Binding score[a]
10-19	KFMFDLFQQF	51.8
84-92	QFQKLLTEF	19.8
98-107	AYELKIANKL	554.4
107-116	LFGEKTYLFL	24.0
112-120	TYLFLQEYL	360.0
118-126	EYLDAIKKF	198.0
215-224	QYTSFHFASL	240.0
286-295	RFKVEESYDL	40.0
362-370	EFHCNHPFL	20.0

[a]The peptide binding score was calculated based on the predicted half-time of dissociation from HLA class I molecules as obtained from a Website (Bioinformatics and Molecular Analysis Section, Computational Bioscience and Engineering Laboratory, Division of Computor & Technology, NIH).

bronchial epithelia. SCCA expression was sparsely observed in gastric adenocarcinoma (Fig. 2H). These results indicate that most SCC tissues were positive for SCCA expression, but its expression in non-SCC tissues was rare.

SCCA peptide-specific IgG in sera of cancer patients and HDs. We next prepared 9 SCCA-derived peptides based on their binding motif to HLA-A24 molecules (Table II). The SCCA peptides used in the following experiments were identified by the starting position of the amino acid sequence. As explained in the Introduction, we screened these 9 SCCA-derived peptides based on their ability to be recognized by IgG in cancer patients. Peptide-specific IgG was measured by a novel multiplexed flow cytometric assay (Luminex) (22). The results are shown in Table III. The samples were derived from 35 uterine cervical SCC patients, 10 breast adenocarcinoma patients, 10 pancreatic adenocarcinoma patients, 10 gastric adenocarcinoma, and 6 colon adenocarcinoma patients. The level of fluorescence intensity was judged to be significant when it exceeded 100 at an 1/100 dilution. As a result, IgG reactive to the $SCCA_{107}$, $SCCA_{112}$, $SCCA_{215}$, and

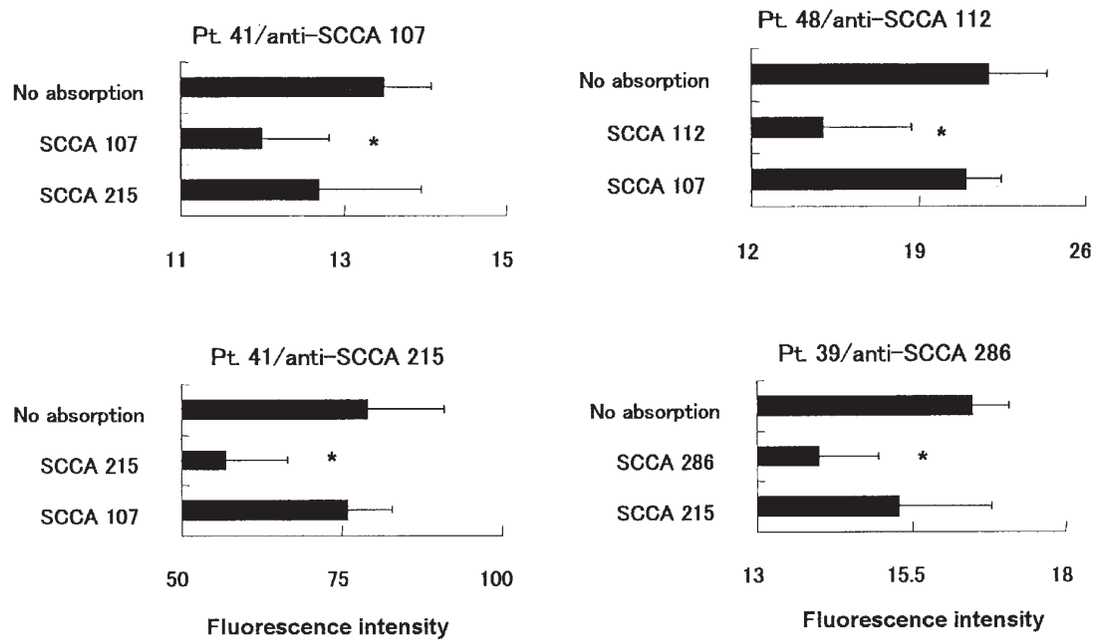

Figure 3. Peptide-specific absorption of IgG. Each sample was absorbed three times with either a corresponding SCCA peptide or an irrelevant SCCA peptide at 37˚C, followed by examination of the corresponding SCCA peptide-specific IgG using the Luminex method. The representative results of four peptides are shown. *P<0.05, as determined by a two-tailed Student's t-test.

$SCCA_{286}$ peptides was more frequently detected in the patients' sera than was IgG reactive to the other 5 peptide. Their frequencies were 23/53, 20/53, 16/53, and 13/53, respectively. The sera from 10 HDs also showed similar results. As illustrated in Fig. 3, the specificity of IgG reactive to each of the $SCCA_{107}$, $SCCA_{112}$, $SCCA_{215}$, and $SCCA_{286}$ peptides was confirmed by absorption assay. Namely, the level of IgG reactive to each of the 4 SCCA peptides was absorbed by culturing the sample in corresponding SCCA peptide-coated wells, but not in irrelevant SCCA peptide-coated wells. Based on these findings, we focused on the following peptides in the subsequent experiments: $SCCA_{107}$, $SCCA_{112}$, $SCCA_{215}$, and $SCCA_{286}$.

Induction of peptide-specific CTLs from the PBMCs of cancer patients. We next investigated whether or not the $SCCA_{107}$, $SCCA_{112}$, $SCCA_{215}$, and $SCCA_{286}$ peptides had the potential to induce peptide-specific CTLs from the PBMCs of 20 HLA-A24$^+$ cancer patients and 8 HLA-A24$^+$ healthy donors (Table IV). The group of twenty cancer patients consisted of 4 esophageal SCC patients, 4 cervical SCC patients, 4 lung cancer patients (2 adenocarcinomas, one small cell carcinoma, and one squamous cell carcinoma), 4 gastric adenocarcinoma patients, and 4 colon adenocarcinoma patients. The assay was carried out in quadruplicate. The cultured cells in one well were separated into 4 wells, and 2 wells were used for the SCCA peptide-pulsed C1R-A24 cells, and the other two were used for the HIV peptide-pulsed C1R-A24 cells. The successful induction of peptide-specific CTLs was judged to be positive when the supernatant of at least one well showed more than 50 pg/ml IFN-γ production with a statistically significant difference ($P<0.05$). The results which showed the best response are shown, and the background IFN-γ production in response to an HIV peptide was subtracted. It was found that peptide-specific CTLs were induced from the PBMCs of 12 of 20 cancer patients when either the $SCCA_{112}$ or the $SCCA_{215}$ peptide was used for *in vitro* stimulation. Each of the $SCCA_{107}$ and $SCCA_{286}$ peptides induced peptide-specific CTLs in 3 and 2 of 20 cancer patients, respectively. On the other hand, these 4 SCCA peptides were less efficient at inducing peptide-specific CTLs from the PBMCs of 8 healthy donors. These results indicate that both the $SCCA_{112}$ and $SCCA_{215}$ peptides can efficiently induce peptide-specific CTLs in HLA-A24$^+$ patients with SCC or non-SCC.

SCCA peptide-specific and CD8$^+$ T cell-dependent cytotoxicity against cancer cells. We further investigated whether or not $SCCA_{112}$ peptide- or $SCCA_{215}$ peptide-stimulated CTLs from SCC patients could exhibit cytotoxicity against cancer cells. As shown in Fig. 4A, although both YES-1 and YES-2 were SCCs, only the YES-2 was positive for HLA-A24 molecules. Therefore, YES-2 was used as a positive target expressing both the SCCA and the HLA-A24 molecules. Here, SCCA peptide-stimulated CTLs from 4 SCC cancer patients (pts. 2, 5, 7, and 8) showed a higher level of cytotoxicity against YES-2 than against YES-1 and HLA-A24$^+$ PHA-stimulated T-cell blasts (Fig. 4B). The PBMCs stimulated with the HIV peptide, used as a negative control, did not show any cytotoxicity (data not shown).

Which cell types were responsible for the cytotoxicity were then determined. The cytotoxicity of SCCA peptide-stimulated PBMCs against YES-2 was significantly inhibited by the addition of anti-HLA-class I or anti-CD8 mAb, but not by the addition of other mAbs (Fig. 5A). In a cold-inhibition assay, the cytotoxicity was diminished by the addition of the corresponding SCCA peptide-pulsed unlabeled C1R-A24 cells but not by the addition of HIV peptide-pulsed unlabeled

Table III. Humoral responses to the SCCA peptides.

Serum derived from pts. with	Peptide								
	SCCA 10	SCCA 84	SCCA 98	SCCA 107	SCCA 112	SCCA 118	SCCA 215	SCCA 286	SCCA 362
	Fluorescence intesity								
Uterine cervical Ca.									
#1	25	20	11	20	3301	0	37	75	0
#2	21	2	0	106	66	0	15	0	0
#3	11	0	0	51	575	0	25	21	0
#4	5	29	0	109	3138	0	68	229	0
#5	14	15	0	55	76	0	28	58	3
#6	786	922	1226	925	428	507	738	869	611
#7	14	12	26	15	14	11	34	15	11
#8	38	28	26	30	40	24	55	30	49
#9	76	67	58	84	94	82	106	93	90
#10	16	15	14	14	19	13	26	14	15
#11	21	23	33	25	19	20	105	23	28
#12	41	35	33	41	42	37	68	44	37
#13	22	23	397	33	26	20	60	29	25
#14	22	20	26	22	20	16	42	19	17
#15	154	116	136	134	82	87	152	129	106
#16	225	182	145	193	206	179	198	147	191
#17	50	56	72	65	53	45	87	63	56
Breast Ca.									
#18	66	95	44	211	26	5	281	292	10
#19	0	0	18	242	0	23	47	54	24
#20	27	31	12	146	0	1	142	95	8
#21	38	34	20	149	0	1	102	117	11
#22	0	5	10	146	567	0	30	32	7
#23	201	182	214	269	113	123	236	321	215
#24	0	11	12	219	62	14	76	47	19
#25	44	26	12	136	5924	7	81	58	10
#26	18	20	0	146	0	0	131	155	0
#27	55	39	18	272	0	18	123	80	20
Pancreas Ca.									
#28	0	0	0	0	0	0	0	0	0
#29	12	3	6	115	0	0	27	37	1
#30	206	344	369	431	137	404	468	946	339
#31	0	0	0	30	0	0	174	97	0
#32	311	0	0	29	280	0	10	0	0
#33	0	0	0	2	0	0	0	0	0
#34	172	108	100	286	185	79	198	275	104
#35	0	0	0	43	1150	0	50	41	0
#36	11	13	0	126	0	0	36	48	6
#37	0	0	0	0	0	0	6	24	0
Gastric Ca.									
#38	38	18	2	38	11506	0	152	243	0
#39	11	7	0	114	0	0	8	9	0

Table III. Continued.

Serum derived from pts. with	Peptide								
	SCCA 10	SCCA 84	SCCA 98	SCCA 107	SCCA 112	SCCA 118	SCCA 215	SCCA 286	SCCA 362
	Fluorescence intesity								
#40	0	0	0	133	0	0	9	27	6
#41	177	118	15	237	2549	21	1453	258	10
#42	4	0	1	24	1371	0	17	79	0
#43	81	102	8	282	0	5	47	286	1
#44	0	0	0	37	195	0	0	0	0
#45	0	0	0	29	0	0	0	7	0
#46	9	7	0	67	0	2	44	40	0
#47	0	0	0	72	1312	0	10	37	0
Colon Ca.									
#48	5	0	0	40	257	2	16	4	0
#49	79	47	0	49	0	0	74	35	0
#50	0	0	0	0	0	0	0	0	0
#51	2	0	0	20	317	0	41	8	0
#52	17	0	0	91	105	0	59	16	1
#53	6	8	26	70	0	17	23	45	19
Total	8/53	8/53	7/53	23/53	20/53	4/53	16/53	13/53	6/53
Healthy donors									
#1	0	0	0	53	607	0	0	21	0
#2	68	79	35	297	52	47	164	219	37
#3	0	0	0	67	0	0	14	9	0
#4	0	0	0	0	0	0	0	0	0
#5	8	0	0	331	387	0	87	239	0
#6	0	0	0	0	0	0	0	2	0
#7	0	0	0	0	990	0	0	0	0
#8	345	332	361	454	496	324	427	516	283
#9	34	33	39	246	1396	37	41	100	29
#10	0	0	0	106	0	0	134	138	0
Total	1/10	1/10	1/10	5/10	5/10	1/10	3/10	4/10	1/10

IgG reactive to a corresponding peptide was judged to be significant when the fluoresence intensity at a 1:100-diluted sample was >100. The significant values are underlined.

C1R-A24 cells (Fig. 5B). Collectively, these results suggest that the cytotoxicity of SCCA peptide-stimulated PBMCs against YES-2 could largely be due to HLA class I-restricted and SCCA peptide-specific $CD8^+$ T cells. As is the case with SCC patients, $SCCA_{112}$ peptide- or $SCCA_{215}$ peptide-stimulated CTLs from adenocarcinoma patients (pts. 10, 15, and 20) exhibited cytotoxicity against YES-2 cells in an HLA class I-restricted manner (data not shown).

Discussion

In this study, we identified two SCCA-derived epitope peptides that could be recognized by both the humoral and cellular immune systems. We first screened 9 CTL-directed SCCA peptides by their susceptibility to the humoral immune system, as we had previously observed that some CTL-directed peptides possessed the ability to elicit both cellular as well as humoral immune responses *in vivo* (16-19). Furthermore, we had already demonstrated that the levels of anti-peptide antibodies in post-vaccination sera correlated well with the overall survival of advanced cancer patients who had received peptide vaccinations (20). Here, IgGs reactive to $SCCA_{112\text{-}120}$ and $SCCA_{215\text{-}224}$ peptides were detected in the sera of 20 and 16 of 53 cancer patients, respectively. Reactive IgG was also detected in similar percentages among healthy donors. This finding indicates that the immunogenicity of these SCCA peptides in terms of

Table IV. Reactivity of SCCA peptide-stimulated PBMCs from HLA-A24$^+$ healthy donors and cancer patients.

PBMCs derived from	Types of cancer	Peptide				
		SCCA 107	SCCA 112	SCCA 215	SCCA 286	EBV
		IFN-γ production (pg/ml)				
Patient's no.						
1	Esophagus	-	-	-	-	748
2	Esophagus	84	106	85	-	491
3	Esophagus	-	-	-	-	879
4	Esophagus	-	-	-	97	-
5	Cervix of uterus	-	678	117	-	-
6	Cervix of uterus	105	-	96	-	-
7	Cervix of uterus	-	-	210	169	-
8	Cervix of uterus	-	61	541	-	67
9	Lung (small cell)	-	321	-	-	-
10	Lung (adeno)	-	837	362	-	-
11	Lung (SCC)	-	75	83	-	-
12	Lung (adeno)	-	319	-	-	62
13	Stomach	500	148	-	-	-
14	Stomach	-	-	129	-	109
15	Stomach	-	958	340	-	291
16	Stomach	-	-	-	-	61
17	Colon	-	-	-	-	-
18	Colon	-	171	121	-	-
19	Colon	-	115	371	-	-
20	Colon	-	430	118	-	70
Total		3/20	12/20	12/20	2/20	9/20
Healthy donor's no.						
1		-	-	-	-	-
2		-	-	-	-	-
3		-	-	-	-	-
4		-	-	-	-	635
5		-	1239	-	-	51
6		-	-	-	-	-
7		259	-	-	-	-
8		-	-	-	-	73
Total		1/8	1/8	0/8	0/8	3/8

The PBMCs from HLA-A24$^+$ cancer patients and healthy donors were stimulated *in vitro* with the indicated SCCA peptides as described in Materials and methods. On day 15, the cultured PBMCs were tested for their reactivity to C1R-A24 cells, which were pre-pulsed with a corresponding peptide in quadruplicate. Values represent the means of IFN-γ production. Background IFN-γ production in response to the HIV peptide was subtracted. Significant values ($P<0.05$ by two-tailed Student's t-test) are shown.

humoral immunity was almost equal in the cancer patients and healthy donors. On the other hand, although these SCCA peptides effectively induced peptide-specific CTLs from the PBMCs of SCC and non-SCC patients, they rarely induced such CTLs from the PBMCs of healthy donors. This result suggests that the immunogenicity in terms of cellular immunity differed markedly between cancer patients and healthy donors. SCCA is known to be expressed in certain normally squamous epithelial tissues (2); we confirmed this observation in the present study. It is possible that aberrant expression of SCCA in malignant cells might disrupt the immunological tolerance of CTLs to non-mutated self-antigen SCCA. In this study,

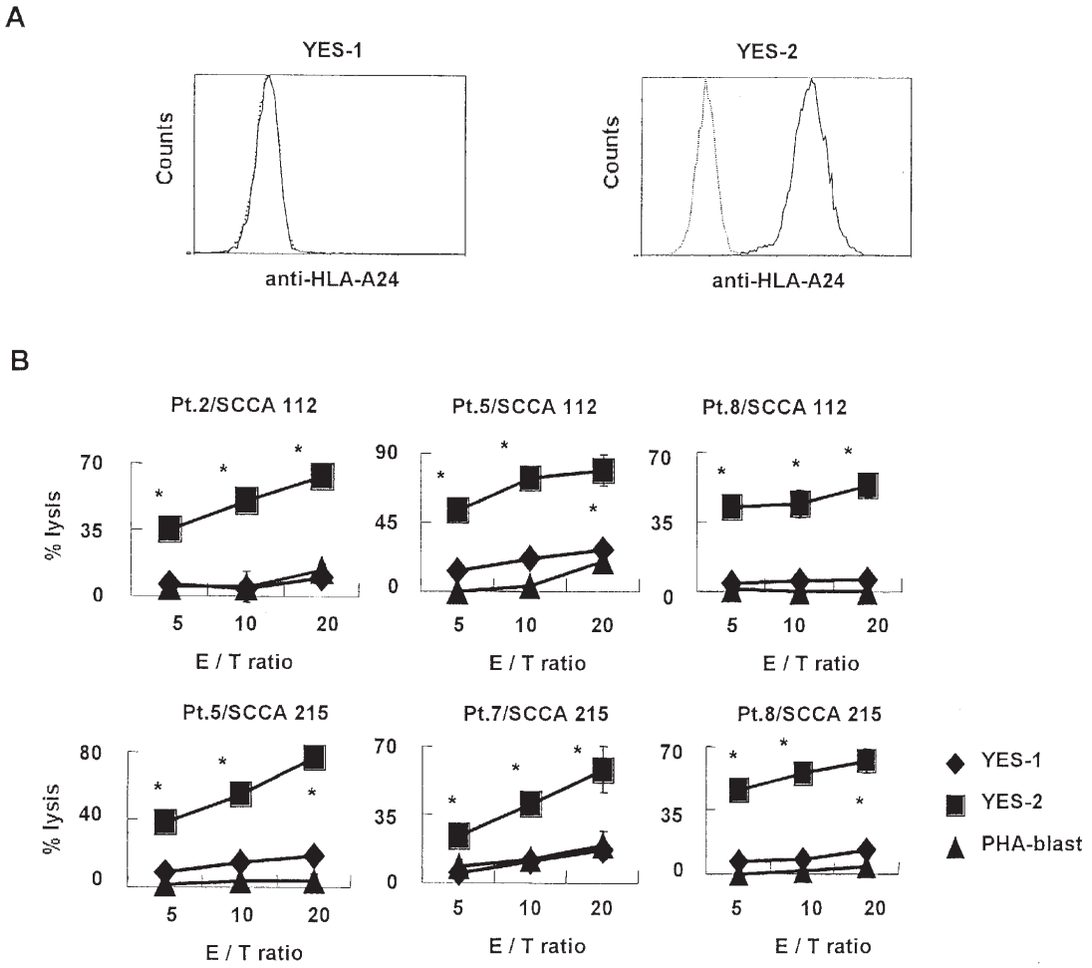

Figure 4. Cytotoxicity of SCCA peptide-induced CTLs. (A) Two esophageal SCC cell lines, YES-1 and YES-2, were examined for their expression of HLA-A24 molecules by flow cytometry. These cells were first stained with anti-HLA-A24 mAb, followed by FITC-conjugated anti-mouse IgG mAb. The dotted lines represent staining without the first mAb. (B) A standard 6-h ^{51}Cr-release assay was carried out to test SCCA peptide-induced CTLs for their cytotoxicity against three targets: YES-1 (HLA-A24$^-$, SCCA$^+$), YES-2 (HLA-A24$^+$, SCCA$^+$), and PHA-stimulated T-cell blasts (HLA-A24$^+$, SCCA$^-$). The representative results of four cancer patients (pts. 2, 5, 7, and 8) are shown. *P<0.05, as determined by a two-tailed Student's t-test.

SCCA peptide-specific CTLs were efficiently induced from the PBMCs of non-SCC patients, despite the fact that the SCCA expression in non-SCC tissues was rare. Information about MUC-1 might provide a clue to understanding this observation. In the case of MUC-1 antigen, its expression is confined to the apical surface, which is inaccessible to immune system cells due to its ductal architecture, whereas transformation of ductal epithelial cells results in a loss of polarized expression of mucin and increases susceptibility to the immune system (23,24). Because SCCA expression in normal cells seems to be limited to normal squamous, columnar, or bronchial epithelia, this antigen in normal epithelia might be neglected by the immune system. However, transformation of normal epithelia might make the SCCA susceptible to the immune system. Although SCCA was rarely detected in non-SCC tissues, the development of non-SCC in epithelia might break the architecture and expose the SCCA on normal epithelia to the immune system. Further studies are needed to elucidate this issue.

There was no difference in humoral and cellular immune responses to either the $SCCA_{112-120}$ or $SCCA_{215-224}$ peptide between SCCs and non-SCCs. The expression of *SCCA* mRNA was detected in both SCCs and non-SCCs, but the level of expression in non-SCCs was relatively low. More importantly, although the majority of SCC tissues were positive for the SCCA protein, non-SCC tissues hardly expressed the protein. These lines of evidence clearly indicate that SCCA can be a target molecule in specific immunotherapy only against SCCs.

Thus far, a number of tumor-related antigens recognized by the immune system have been identified, and some of them are non-mutated self-antigens (25). We have also identified tumor-related antigens and peptides that are recognized by tumor-reactive CTLs, and most of them are non-mutated self-antigens (13-15). We have conducted several clinical trials using these peptides, but no severe adverse events except for local redness and swelling of injection sites have been observed (16-19). However, careful observation is needed to confirm the safety of vaccination with these SCCA peptides.

In general, the roles played by peptide-specific IgG *in vivo* remain unknown. We previously reported that IgG reactive to CTL-directed peptides was often detected in the sera not only of cancer patients, but also of healthy donors (26). In contrast, IgG reactive to these CTL peptides has been reported to be

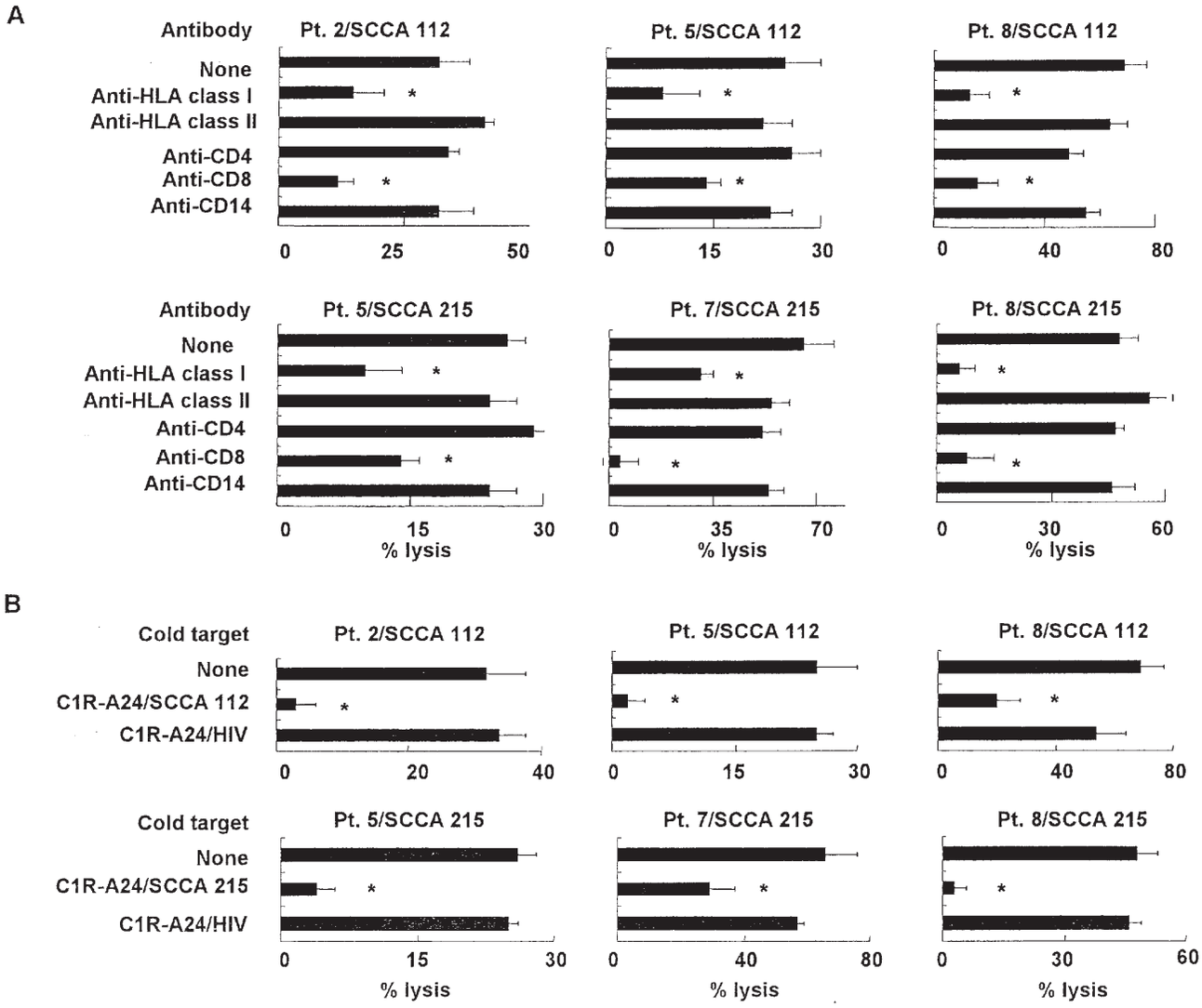

Figure 5. SCCA peptide-specific and $CD8^+$ T cell-dependent cytotoxicity. The cytotoxicity of SCCA peptide-induced CTLs was examined in the presence of blocking antibodies or cold-inhibition targets. In both assays, YES-2 (HLA-$A24^+$, $SCCA^+$) was used as a target. (A) Twenty μg/ml of anti-HLA-class I (W6/32, mouse IgG2a), anti-HLA-class II (H-DR-1, mouse IgG2a), anti-CD8 (Nu-Ts/c, mouse IgG2a), or anti-CD4 (Nu-Th/i, mouse IgG1) mAb was added to the wells. Anti-CD14 (JML-H14, mouse IgG2a) mAb was used as a negative control. $^*P<0.05$, as determined by a two-tailed Student's t-test. (B) Unlabeled C1R-A24 cells which had been pre-pulsed with a corresponding SCCA peptide or with an irrelevant HIV peptide were added to the wells at a cold/hot target cell ratio of 10/1. The 6-h ^{51}Cr-release assay was performed at an E/T ratio of 10/1. $^*P<0.05$, as determined by a two-tailed Student's t-test.

either lacking or unbalanced in the sera of patients with atopic disease (27). Although peptide-specific IgG might play a role in several immunological events, its function in anti-tumor immune responses remains unclear at present. Interestingly, we recently observed significant levels of inflammatory responses in the proximity of prostate cancer upon the surgical treatment of patients who had been vaccinated with peptides to which IgG was detected in the sera prior to undergoing radical prostatectomy (Noguchi *et al*, unpublished data). It is well known that T cells in the circulation rarely infiltrate into tumor sites, but IgG can easily reach peri-tumoral and/or intra-tumoral sites. Therefore, one possible explanation for the present findings would be that peptide-specific IgG easily reached the peri-tumoral and/or intra-tumoral sites, which in turn would facilitate local inflammatory responses. Our group has focused on gaining a better understanding of these issues.

In conclusion, this study provides new insights into the development of an SCCA-targeting, specific immunotherapy applicable for a substantial number of cancer patients, as SCC accounts for the majority of epithelium-derived neoplastic cells. Based on our findings, SCCA can be a target molecule in specific immunotherapy only against SCCs. Although further studies are necessary to clarify the roles and mechanisms of action associated with anti-peptide antibodies, we have successfully identified two SCCA peptides that are recognized by both cellular and humoral immune responses. In the future, these agents could serve as peptide-based anti-cancer vaccines that might be more effective than vaccines recognized by the cellular immune response alone.

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