HLA-G as a target molecule in specific immunotherapy against renal cell carcinoma

YOSHIHIRO KOMOHARA[1,2,6], MAMORU HARADA[2,3], YUKI ISHIHARA[2], SHIGETAKA SUEKANE[4], MASANORI NOGUCHI[4], AKIRA YAMADA[1,5], KEI MATSUOKA[4] and KYOGO ITOH[2,5]

[1]Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, Kurume University School of Medicine; [2]Department of Immunology, Kurume University School of Medicine, Kurume; [3]Department of Immunology, Shimane University Faculty of Medicine, Shimane; [4]Department of Urology, Kurume University School of Medicine; [5]Center of the 21st Century COE Program for Medical Science, Kurume University, Kurume, Japan

Received July 10, 2007; Accepted August 24, 2007

Abstract. The human leukocyte antigen (HLA)-G molecule can exert immunoregulatory functions. However, its limited tissue distribution and preferential expression in a variety of malignancies suggest the possibility that it could be a target in anti-cancer immunotherapy. In the present study, we tested this possibility by focusing on renal cell carcinoma (RCC) patients, especially those with HLA-A24 alleles. Four HLA-G-derived peptides were prepared based on the binding motif to the HLA-A24 alleles. After a stabilization assay confirmed the binding of these peptides to HLA-A24 molecules, they were screened for the capacity to induce peptide-specific cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells (PBMCs) of HLA-A24$^+$ RCC cancer patients. As a result, the HLA-$G_{146-154}$ peptide was found to effectively induce peptide-specific CTLs, and HLA-$G_{146-154}$ peptide-stimulated PBMCs exhibited cytotoxic activity against HLA-G-expressing HLA-A24$^+$ RCC cells. Antibody blocking and cold inhibition experiments confirmed that the cytotoxicity was partially ascribed to peptide-specific and HLA class I-restricted CD8$^+$ T cells. These results indicate that HLA-G-associated immunoregulation can be overcome and that HLA-G peptide-based anti-cancer immunotherapy is feasible.

Introduction

Renal cell carcinoma (RCC) is resistant to conventional chemotherapy, and immunotherapy is considered a promising treatment modality because some patients with metastatic RCC have shown favorable responses to immunotherapy (1-3). Previously, a panel of cancer-associated antigens has been identified, enabling us to perform specific immunotherapy (4). We have also identified a number of cancer-associated antigens recognized by epithelial cancer-reactive cytotoxic T lymphocytes (CTLs) (5-7), and some of these antigens are applicable to RCC patients (8-10). However, cancer cells escape from the immune system through several mechanisms: loss of tumor antigen, down-regulation of human leukocyte antigen (HLA) class I antigen, defective death receptor signaling, and regulatory T cells. In addition, several immunosuppressive molecules and factors, including interleukin (IL)-10, transforming growth factor-ß, prostaglandin E2, arginase-I, and indoleamine 2,3-dioxygenase, are involved in immunosuppression in the tumor-bearing state (11).

HLA-G, a nonclassical HLA class I molecule, is known to inhibit anti-cancer immune responses. HLA-G inhibits CTL responses and protects HLA class I-deficient targets from NK-mediated lysis through interactions with killer inhibitory receptors on NK cells (12-14). On the other hand, HLA-G exhibits a limited tissue distribution (15). In addition, this molecule is preferentially expressed in a variety of malignancies, including lung cancer, breast cancer, mesothelioma, cervical cancer, choriocarcinoma, bladder cancer, and RCC (16-21). In RCC, 10 of 37 RCC samples expressed high levels of HLA-G protein, whereas normal kidney cells lack HLA-G expression (21). These lines of evidence suggest the possibility that HLA-G could be a target molecule in anti-cancer immunotherapy. Elimination of HLA-G-expressing refractory cancer cells is inevitable for successful anti-cancer therapy. With this in mind, we tested the possibility of immuno-therapy targeting HLA-G by focusing on HLA-A24$^+$ RCC patients.

Materials and methods

Patients. The study protocol (Protocol # 2484) was approved by the Institutional Ethics Review Board of Kurume

Correspondence to: Dr Yoshihiro Komohara, *Present address*: [6]Department of Cell Pathology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo Kumamoto, Kumamoto 860-8556, Japan
E-mail: ycomo@kumamoto-u.ac.jp

Key words: HLA-G, renal cell carcinoma, immunotherapy, HLA-A24, peptide

Table I. HLA-G-derived peptide candidates binding to the HLA-A*2402 molecules.

Peptide	Sequence	Binding score		Stabilization assay (MFI)	
		BIMAS	SYFPEITHI	10 mM	100 mM
HLA-G 146-154	DYLALNEDL	360	23	43	48
HLA-G 194-202	RYLENGKEM	99	14	81	102
HLA-G 139-148	RYAYDGKDYL	200	20	47	65
HLA-G 141-150	AYDGKDYLAL	720	25	42	51
EBV-A24	TYGPVFMCL	403	24	80	102
EBV-A2	GLCTLVAML	5	11	5	7

The entire amino acid sequence was scanned using epitope prediction algorithm in the following web sites: BIMAS, http://wye.cit.nih.gov/molbio/hla_bind/; SYFPEITHI, http://www.syfpeithi.de/. Stabilization assay was performed as described in the Materials and methods. MFI, mean fluorescence intensity.

University. Informed written consent was obtained from all the subjects from whom peripheral blood mononuclear cells (PBMCs) were taken. PBMCs were prepared by Ficoll-Conray density gradient centrifugation and cryo-preserved until they were used for the experiments. The expression of HLA-A24 molecule on PBMCs of cancer patients was determined by flow cytometry as described previously (22).

Cell lines. All cell lines used were incubated in RPMI-1640, supplemented with 10% fetal bovine serum (FBS). MAMIYA is an HLA-A24$^+$ and HLA-G- RCC, and an HLA-G-expressing MAMIYA, designated as MAMIYA-HLAG, was established by the stable transfection with *HLA-G* gene-containing pcDNA3.1 using FuGene (Roche, Mannheim, Germany), followed by the culture with geneticin (1 mg/ml). RMA-S transfectant cells expressing the *HLA-A*2402* gene, designated as RMA-S-A*2402, were generated by the transfection with the *HLA-A*2402* gene into RMA-S cells, followed by the culture with geneticin (1 mg/ml). C1R-A24 is an HLA-A*2402-expressing C1R subline (Dr M. Takiguchi, Kumamoto University, Japan).

Immuno-staining and flow cytometry. HLA-G protein expression on MAMIYA-HLAG cells were detected by mouse anti-HLA-G antibody (MEM-G/9, Abcam). The cells were fixed in cold acetone, then incubated with MEM-G/9 or control mouse IgG. After washing in PBS, the cells were incubated with HRP-labeled goat anti-mouse antibody (Nichirei; Tokyo, Japan). The reaction was visualized by use of the DAB substrate system (Dako). Paraffin-embedded RCC samples were deparaffinized in xylene and rehydrated in ethanol, and then antigens were retrieved by microwave for 10 min in 10 mM sodium citrate (pH 6.0). The sections were reacted with mouse anti-HLA-G antibody (MEM-G/1, Abcam), and then incubated with HRP-labeled goat anti-mouse antibody (Nichirei). The reaction was visualized by use of the DAB substrate system (Dako). Slides were counterstained with hematoxylin. For flow cytometry, the cells were reacted with MEM-G/9 or control mouse IgG. The Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) was used as the secondary antibody. The cells were analyzed using EPICS (Beckman Coulter, Miami, FL).

Peptides and stabilization assay. All peptides used were purchased from Genenet (Fukuoka, Japan), and their amino acid sequences are shown in Table I. Peptide binding to HLA-A*2402 molecules was examined using the stabilization assay according to a previously reported method with several modifications (23). Briefly, RMA-S-A*2402 cells ($5x10^5$ cells per well in a 24-well plate) were cultured at 26˚C for 20 h in RPMI-1640 supplemented with 20% FBS, followed by incubation with Opti-MEM (Invitrogen) containing 10 or 100 μM peptide and human ß$_2$ microglobulin (2 ng/ml) at 26˚C for 2 h, and then cultured at 37˚C for 3 h. After washing with PBS, these cells were put on ice for 30 min with anti-MHC class I mAb, PT85A (VMRD, Pullman, WA). After washing twice with PBS, these cells were put on ice for 30 min with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). An EBV-derived peptide binding to HLA-A24 molecules, designated as EBV-A24, was used as a positive control, and an EBV-derived peptide binding to HLA-A2 molecules, designated as EBV-A2, was used as a negative control.

Induction of peptide-specific CTLs from PBMCs. Influenza (Flu) virus, EB virus, and HIV-derived peptides were used as controls binding to HLA-A24 alleles, as described previously (8-10). *In vitro* induction of peptide-specific CTLs was performed according to a previously reported method with several modifications (24). Briefly, PBMCs were incubated with 10 μg/ml of each peptide in quadruplicate in a 96-well microplate (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Life Technologies, Gaithersburg, MD), 10% FBS, 100 units/ml IL-2, and 0.1 mM MEM non-essential amino acid solution (Life Technologies). On the 15th day of culture, cells were separated into four wells, and then cultured with the corresponding peptide-pulsed C1R-A24 cells. The HIV peptide was used a negative control. After an 18-h incubation, the IFN-γ production was determined by ELISA.

Table II. Induction of peptide-specific CTLs from the PBMCs of HLA-A24+ RCC patients.

	IFN-γ (pg/ml)						
	HLA-G peptide						
Patient	146-154	194-202	139-148	141-150	Flu	EBV	HLA-G expression in cancer tissue
Pt. 1	83	8	64	39	109	186	+
Pt. 2	17	7	16	17	108	125	+
Pt. 3	143	3	0	0	59	22	+
Pt. 4	0	46	26	162	89	20	+
Pt. 5	93	47	20	14	199	298	-
Pt. 6	29	15	0	9	142	19	-
Pt. 7	115	30	177	55	79	109	-
Pt. 8	0	37	9	15	N.D.	N.D.	-
Pt. 9	0	0	65	1	67	23	N.E.
Pt. 10	51	7	70	63	108	50	-
Total	(5/10)	(0/10)	(3/10)	(2/10)	(7/9)	(4/9)	(4/9)

The PBMCs were stimulated *in vitro* with each of the indicated peptides, and peptide-specific reactivity was examined. The results were evaluated by the two-tailed Student's t-test ($P<0.05$), and the positive results are underlined. N.D., not done. HLA-G expression in RCC tissues were examined by immunohistochemistry. Samples were judged to be positive if >10% of the tumor cells showed moderate or strong immunoreaction. NE, not examined.

Cytotoxicity assay. After the repeated stimulation with a corresponding peptide for 15 days, these cultured cells were cultured in the presence of 100 units/ml IL-2 alone for further 10-14 days. Then, the cultured cells were tested for their cytotoxicity against MAMIYA and MAMIYA-HLAG cells by a standard 6-h ^{51}Cr-release assay. Phytohemagglutinin (PHA)-activated T cells from HLA-A24+ healthy donors were used as a control. Peptide-stimulated PBMCs or purified CD8+ T cells were used as effector cells. CD8+ T cells were positively isolated using a CD8-positive isolation kit (Dynal, Oslo, Norway). Two thousand ^{51}Cr-labeled cells per well were cultured with effector cells in 96-round-well plates, and then the specific ^{51}Cr-release was calculated as described previously (22). When peptide-stimulated PBMCs were used as effector cells, K562 cells were added into wells to prevent non-specific cytotoxicity of NK cells. In an experiment, either anti-HLA class I mAb (W6/32) or anti-CD14 mAb, as an isotype-matched control, was added into wells before the ^{51}Cr-release assay to confirm HLA class I restriction. The specificity of peptide-stimulated PBMCs was confirmed by cold inhibition assay. Twenty thousand unlabeled C1R-A24 cells, which were pre-pulsed with either a corresponding HLA-G peptide or the HIV peptide, were used as cold target cells (22).

Results

*Four HLA-G peptides binding to the HLA-A*2402 molecules*. First, we prepared four HLA-G peptides based on the binding motifs using the BIMAS and SYFPEITHI web sites (Table I). We then conducted the HLA stabilization assay using RMA-S-A*2402 cells to directly confirm that these four peptides can bind to the HLA-A*2402 molecules. RMA-S is a mouse mutant cell line that is deficient in antigen processing (25), and its HLA class I transfectant is useful for the stabilization assay (23). RMA-S-A*2402 is a subline that was stably transfected with the *HLA-A*2402* gene. EBV-A24 and EBV-A2 peptides were used as positive and negative controls, respectively. As shown in Table I, the expression levels of HLA-A24 molecules when pulsed with HLA-G-derived peptides at a dose of either 10 or 100 μM were higher than those when pulsed with the negative control EBV-A2 peptide. Their levels were comparable to those of the positive control EBV-A24 peptide. These results indicate that four HLA-G peptides can bind to the HLA-A*2402 molecules and have the potential to induce peptide-specific CTLs.

Induction of HLA-G peptide-specific CTLs from the PBMCs of HLA-A24+ RCC patients and healthy donors. We next determined whether or not the four HLA-G peptides could induce peptide-specific CTLs from the PBMCs of HLA-A24+ RCC patients or healthy donors. The PBMCs were stimulated *in vitro* with each HLA-G peptide or control peptide, and were examined for their IFN-γ production in response to corresponding peptide-pulsed C1R-A24 cells. As a result, the HLA-$G_{146-154}$, HLA-$G_{194-202}$, HLA-$G_{139-148}$, and HLA-$G_{141-150}$ peptides induced corresponding peptide-reactive CTLs from the PBMCs of 5, 0, 3, and 2 of 10 HLA-A24+ RCC patients, respectively (Table II). The Flu and EBV peptides induced peptide-specific CTLs from the PBMCs of 7 and 4 of 9 HLA-A24+ RCC patients, respectively. We also examined the HLA-G expression in RCC tissues of 9 patients to see whether or not there is a correlation between the induction of HLA-G peptide-specific CTLs and HLA-G expression in

Table III. Induction of peptide-specific CTLs from the PBMCs of HLA-A24+ healthy donors.

	IFN-γ (pg/ml)					
	HLA-G peptide					
Healthy donors	146-154	194-202	139-148	141-150	Flu	EBV
HD 1	0	39	92	26	740	20
HD 2	78	0	0	19	20	22
HD 3	0	13	6	0	82	17
HD 4	6	0	0	0	47	9
HD 5	0	0	106	62	40	56
Total	(1/5)	(0/5)	(2/5)	(1/5)	(2/5)	(1/5)

The PBMCs were stimulated *in vitro* with each of the indicated peptides, and peptide-specific reactivity was examined. The results were evaluated by the two-tailed Student's t-test ($P<0.05$), and the positive results are underlined.

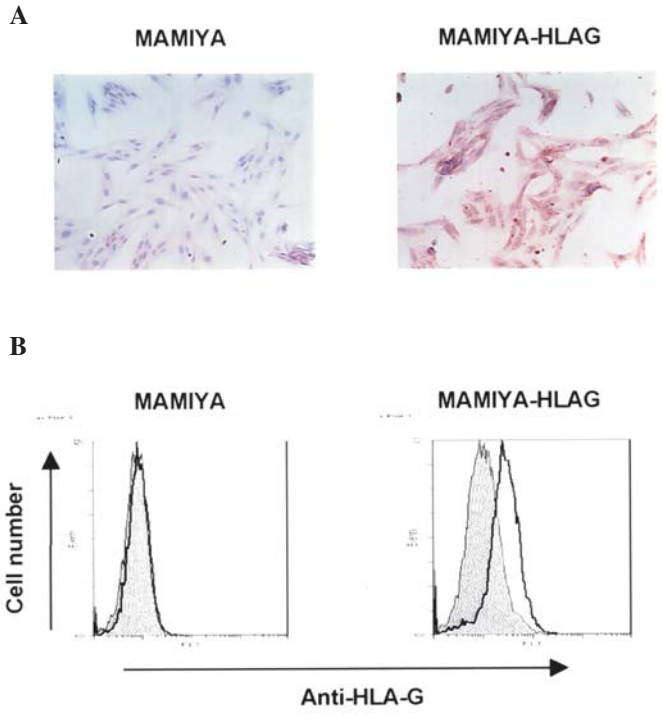

Figure 1. HLA-G-expressing RCC cell line. (A) Immunocytostaining was performed on MAMIYA and MAMIYA-HLAG. (B) These two cell lines were stained with anti-HLA-G mAb followed by Alexa Fluor 488-conjugated anti-mouse IgG, and were then analyzed by flow cytometry.

cancer tissues. We detected HLA-G expression in cancer tissues of 4 patients, but the induction of HLA-G peptide-specific CTLs was possible even from the PBMCs of RCC patients whose cancer tissues were negative for HLA-G protein. We also determined whether or not HLA-G peptide-specific CTLs could be induced from the PBMCs of HLA-A24+ healthy donors, and found that these four HLA-G peptides induced corresponding peptide-reactive CTLs from the PBMCs of 1, 0, 2, 1, and 0 of 5 HLA-A24+ healthy donors, respectively (Table III). In contrast to the cases with RCC patients, the HLA-$G_{146-154}$ peptide was not the best candidate to induce peptide-specific CTLs from the PBMCs of HLA-A24+ healthy donors. These results indicate that the HLA-$G_{146-154}$ peptide is useful for inducing peptide-specific CTLs in HLA-A24+ RCC patients.

Cytotoxicity of HLA-$G_{146-154}$ peptide-stimulated PBMCs against HLA-G-expressing RCC cells. To examine whether or not HLA-G peptide-stimulated PBMCs could exhibit cytotoxicity against RCC cells expressing HLA-G protein, the *HLA-G1* gene was stably transfected into HLA-A24+ MAMIYA cells, designated MAMIYA-HLAG. The expression of HLA-G protein was confirmed by both immunocytostaining and flow cytometry (Fig. 1A and B).

We next determined whether or not HLA-$G_{146-154}$ peptide-stimulated PBMCs induced from the PBMCs of HLA-A24+ RCC patients could show cytotoxicity against MAMIYA-HLAG cells (Fig. 2A). HLA-$G_{146-154}$ peptide-stimulated PBMCs of patients 5 and 7 showed higher levels of cytotoxicity against MAMIYA-HLAG cells than against MAMIYA cells or PHA-stimulated T-cell blasts. These results indicate that HLA-$G_{146-154}$ peptide-specific CTLs exhibited cytotoxicity against HLA-G-expressing HLA-A24+ RCC cells.

CD8+ T-cell-dependent and peptide-specific cytotoxicity against MAMIYA-HLAG cells. We further attempted to identify cells responsible for the cytotoxicity of HLA-$G_{146-154}$ peptide-stimulated PBMCs against HLA-G-expressing RCC cells. HLA-$G_{146-154}$ peptide-stimulated cells were positively isolated for CD8+ T cells before the cytotoxicity assay. Anti-pan HLA class I mAb (W6/32) or control anti-CD14 mAb was added before the cytotoxic assay. Fig. 2B shows that the cytotoxicity of HLA-$G_{146-154}$ peptide-stimulated CD8+ T cells against MAMIYA-HLAG cells was significantly inhibited by the addition of anti-HLA class I mAb, but not by the addition of control anti-CD14 mAb. In addition, cytotoxicity was significantly suppressed by the addition of HLA-$G_{146-154}$ peptide-pulsed unlabeled C1R-A24 cells, but not by the addition of control HIV peptide-pulsed unlabeled C1R-A24

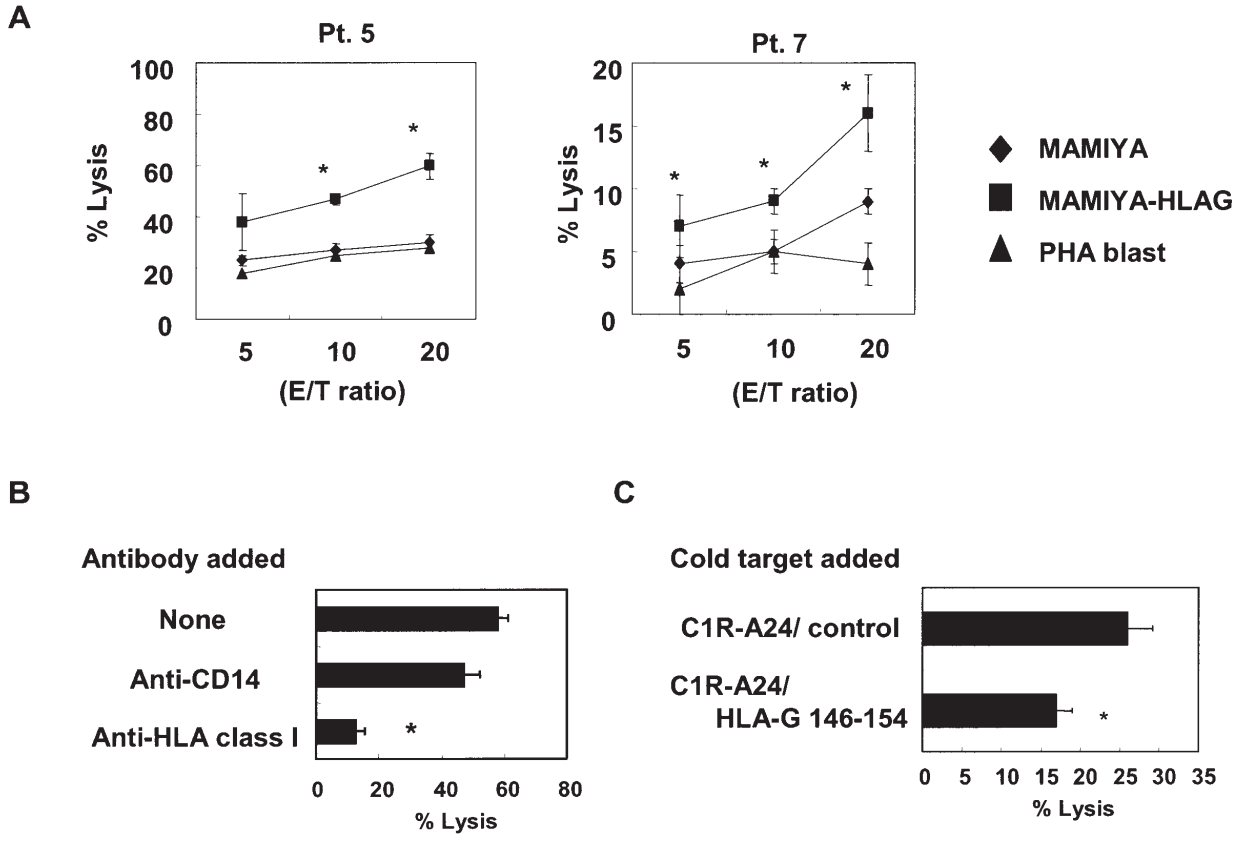

Figure 2. Cytotoxicity of peptide-stimulated PBMCs against HLA-G-expressing RCC cells. (A) PBMCs of 2 HLA-A24$^+$ RCC patients were *in vitro* stimulated with the HLA-G peptide and examined for their cytotoxicity against MAMIYA, MAMIYA-HLAG, and PHA-activated T-cell blasts. (B) Indicated anti-HLA class I and control (anti-CD14) monoclonal antibodies were added into wells before the cytotoxic assay. The cytotoxicity of peptide-stimulated CD8$^+$ T cells from patient 5 was significantly inhibited by the addition of anti-HLA class I mAb, but not by that of control mAb. (C) Cytotoxicity of peptide-stimulated CD8$^+$ T cells from patient 5 against MAMIYA-HLAG cells was examined in the presence of HLA-$G_{146\text{-}154}$ peptide-pulsed unlabeled C1R-A24 cells (cold target) or control HIV peptide-pulsed unlabeled C1R-A24 cells. $^*P<0.05$ statistically significant (Student's t-test).

cells (Fig. 2C). These results indicate that the cytotoxicity against HLA-A24$^+$ and HLA-G-expressing RCC cells was partially dependent on HLA class I-restricted and peptide-specific CD8$^+$ T cells.

Discussion

Although molecular targeting therapies have been developed, immunotherapy is a promising alternative modality for the treatment of RCC (3). Nevertheless, effective specific immunotherapy against RCC has not been established yet, and one plausible reason is the limited information on target antigens of RCC. In this study, we tested the possibility that HLA-G could be a target molecule in specific immunotherapy against RCC in spite of its immunoregulatory functions.

Certain cancer cells resist anti-tumor immune responses by expressing immunoregulatory molecules or anti-apoptotic genes. However, from a therapeutic standpoint, elimination of these therapy-resistant cells is inevitable for successful anti-cancer therapy. Previous studies have revealed that anti-apoptotic or immunoregulatory molecules can be targets in specific immunotherapy. A cancer vaccine targeting survivin, an inhibitor of apoptosis protein, has been developed (26), and an HLA-A2-restricted Bcl2-derived epitope on tumors recognized by CTLs has been identified (27). We have also reported that serine protein inhibitor 9 can be recognized by CTLs of epithelial cancer patients (28), and that immediate early response gene X-1, a stress-inducible anti-apoptotic gene, encodes CTL epitopes capable of inducing HLA-A33-restricted and tumor-reactive CTLs in gastric cancer patients (30). These lines of evidence indicate that immunoregulatory molecule-derived epitope peptides could be recognized by CTLs and might be a target in specific immunotherapy when their tissue distribution is limited and preferentially expressed in malignancies. We therefore undertook this study. As a result, we identified that the HLA-$G_{146\text{-}154}$ peptide can efficiently induce peptide-specific and RCC-reactive CTLs from PBMCs of HLA-24$^+$ RCC patients. We suppose that the HLA-$G_{146\text{-}154}$ peptide is a promising candidate in peptide-based immunotherapy for HLA-A24$^+$ RCC patients.

HLA-G is known to inhibit the cytotoxic activity of not only NK cells but also antigen-specific T lymphocytes. Le Gal *et al* demonstrated the decreased cytotoxicity of influenza-virus-specific CD8$^+$ T lymphocytes against HLA-G-expressing cells, and that the cytotoxicity was reversed by the addition of anti-HLA-G antibody (13). In the present study, however, HLA-G molecules expressed on MAMIYA-HLAG cells did not inhibit the CTL function and were recognized by peptide-specific CTLs. One explanation for this observation is that the level of surface HLA-G was too low to inhibit T-cell function.

As shown in Fig. 1A, HLA-G expression in MAMIYA-HLAG cells was prominent in the cytoplasm and less expressed on the cell surface. This idea was also supported by Malmberg *et al*, who showed that a lower level of HLA-G expression did not impair peptide-specific $CD8^+$ T cells, but that up-regulation of HLA-G protein induced by IFN-γ resulted in the inhibition of cytotoxic activity of peptide-specific $CD8^+$ T cells (26). However, further analysis is needed to elucidate the matter.

In conclusion, we revealed that HLA-G could be a target antigen in specific immunotherapy against HLA-G-expressing RCC. Since HLA-G is preferentially expressed in many malignancies (14-21), our finding might open up the possibility of HLA-G-targeting immunotherapy against a variety of HLA-G-expressing cancers.

Acknowledgements

This study was supported in part by Grant-in-Aids from the Ministry of Education, Science, Sports and Culture of Japan (KAKENHI: no. 16591628 to K. Matsuoka, no. 16790933 to S. Suekane, no. 18591449 to M. Harada, and no. 12213134 to K. Itoh), and from the Ministry of Health, Labor and Welfare, Japan (no. H14-trans-002, 11-16, and H12-cancer-004 to K. Itoh, and no. 15-17 to M. Harada).

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