Rationale for Antiangiogenic Cancer Therapy with Vaccination Using Epitope Peptides Derived from Human Vascular Endothelial Growth Factor Receptor 2

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Abstract

Angiogenesis is a critical mechanism for tumor progression. Multiple studies have suggested that tumor growth can be suppressed if tumor angiogenesis can be inhibited using various types of antiangiogenic agents. Recent studies in mouse systems have shown that tumor angiogenesis can also be inhibited if cellular immune response could be induced against vascular endothelial growth factor receptor 2 (VEGFR2), which is one of the key factors in tumor angiogenesis. In this study, we examined the possibility of developing this novel immunotherapy in clinical setting. We first identified the epitope peptides of VEGFR2 and showed that stimulation using these peptides induces CTLs with potent cytotoxicity in the HLA class I–restricted fashion against not only peptide-pulsed target cells but also endothelial cells endogenously expressing VEGFR2. In A2/Kb transgenic mice that express χ1 and χ2 domains of human HLA-A*0201, vaccination using these epitope peptides in vivo was associated with significant suppression of the tumor growth and prolongation of the animal survival without fatal adverse effects. In angiogenesis assay, tumor-induced angiogenesis was significantly suppressed with the vaccination using these epitope peptides. Furthermore, CTLs specific to the epitope peptides were successfully induced in cancer patients, and the specificities of the CTLs were confirmed using functional and HLA-tetramer analysis. These results in vitro and in vivo strongly suggest that the epitope peptides derived from VEGFR2 could be used as the agents for antiangiogenic immunotherapy against cancer in clinical settings. (Cancer Res 2005; 65(11): 4939-46)

Introduction

Tumor growth is generally limited to 1 to 2 mm³ in the absence of a vascularized blood supply, and angiogenesis has a critical role in the invasion, growth, and metastasis of tumors (1–6). It has been also shown that inhibition of tumor angiogenesis is associated with suppression of tumor progression. To achieve suppression of angiogenesis, several investigators have been examining therapeutic strategies targeting vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR), which play critical roles in regulating the process of angiogenesis. These studies have shown that tumor growth can be successfully suppressed in vitro and in vivo using monoclonal antibodies (mAb), recombinant receptors, or inhibitors for signal transduction (7–11). However, these strategies require frequent or continuous administration of the reagents at relatively high dose levels, which may be associated with significant inconvenience and adverse effects. We hypothesized that induction of CTLs against receptors frequently expressed on proliferating endothelial cells may result in significant inhibition of tumor angiogenesis through the destruction of endothelial cells.

Among candidate molecules, VEGFR2 is closely related to proliferation and migration of endothelial cells and strongly expressed on endothelial cells in tumor tissue but not in normal tissue (12–15). Recent reports have shown that vaccination using cDNA or recombinant protein of mouse VEGFR2 is associated with significant antitumor effects in mouse tumor models (16, 17). However, these results cannot directly warrant clinical application of this strategy, because they used mouse homologue of human VEGFR2 in mouse systems, which are considered to be significantly different from the human counterpart.

In this study, we examined the effectiveness of this novel immunotherapy in systems closely related to clinical settings. We identified the epitope peptides of human VEGFR2 restricted to HLA-A*0201 and HLA-A*2402 (18) and showed that CTLs induced with these peptides have potent and specific cytotoxicity against not only peptide-pulsed target cells but also endothelial cells endogenously expressing VEGFR2 in the HLA class I–restricted fashion. Furthermore, we examined in vivo antitumor effects of the vaccination with these epitope peptides using a unique mouse model that may be directly translated into the clinical setting. Our model system uses A2/Kb transgenic mice, which is useful for the analysis of human CTL epitopes. There is ~71% concordance between human and A2/Kb transgenic mice in the CTL repertoire (19). To construct tumor systems, we transplanted syngenic mouse tumor cells that were chemically induced in C57BL/6 mice (H-2Kb) not expressing HLA-A*0201 molecules. This tumor system, combining A2/Kb transgenic mice and H-2Kb mouse cell line, offers a unique setting. Because endothelial cells in A2/Kb transgenic mice express HLA-A*0201 molecule, the CTLs induced by vaccination using VEGFR2 epitope peptides recognize endothelial cells that express both HLA-A*0201 and VEGFR2. Thus, in vivo antitumor effects of antiangiogenic vaccine can be evaluated in HLA-A*0201-restricted fashion. However, they do not recognize tumor cells even if they express VEGFR2. In this in vivo tumor model, vaccination using these epitope peptides was associated with significant suppression of the tumor growth without fatal adverse effects. In angiogenesis assay, tumor-induced angiogenesis was significantly suppressed with vaccination using these
epitope peptides. Furthermore, CTLs specific to the epitope peptides were successfully induced with peripheral blood mononuclear cells (PBMC) of cancer patients.

These results strongly suggest that the vaccination using epitope peptides derived from VEGFR2 could induce antitumor immune responses in cancer patients.

Materials and Methods

Cell lines. The T2 cell line was generously provided by Dr. H. Shiku (Mie University School of Medicine, Mie, Japan). VEGFR2-expressing cells were generated by infecting the target cells with the recombinant adenoanal vector carrying VEGFR2 cDNA (20, 21). The human umbilical vein endothelial cell (HUVEC)-KT5 (HLA-A24/31) and HUVEC-P8 (HLA-A2/29) were provided by Dr. T. Takahashi (Institute of Medical Science, University of Tokyo, Tokyo, Japan). The HT29, HePG2, B16, and MC38 cell lines were purchased from American Type Culture Collection (Manassas, VA).

Synthetic peptides. The candidates of VEGFR2-derived epitope peptides restricted to HLA-A*0201 (A2) and HLA-A*2402 (A24) were selected based on the binding affinities to the corresponding HLAs. The binding affinities were predicted with the Web site of BioInformatics and Molecular Analysis Section (22, 23). These candidate peptides were synthesized by Sawady Technology (Japan) according to the standard solid-phase synthesis method and purified by reverse-phase high-performance liquid chromatography. The purity (>95%) and the identity of the peptides were determined by analytic high-performance liquid chromatography and mass spectrometry analysis, respectively. The peptides used in this study are listed in Table 1. The VEGFR2-773-2L peptide consists of the sequence containing the alteration of the second residue: methionine of VEGFR2-773 peptide to leucine. HLA-A2-binding, carcinoembryonic antigen–derived peptide (DVLYGPDTPI) and HIV peptides (HLA-A2-binding peptide: ILKEPVHG; HLA-A24-binding peptide: RYLRDQQLL) were used as negative controls (23, 24).

Animals. The A2/Kb transgenic mice, which express MHC class I molecules consisting of α1 and α2 domains of HLA-A*0201 and α3 domain of mouse H-2Kb, were prepared as described elsewhere (19). The animals were maintained in the specific pathogen-free Animal Facility of the Institute of Medical Science, University of Tokyo, and all the protocols for animal experiments were approved by the ethical committee of our institute.

Generation of CTL lines and clones. Monocyte-derived dendritic cells were used to induce CTL responses against peptides presented on HLA as described previously (25–27). In brief, the PMBCs were obtained from the healthy volunteers with corresponding HLAs and cultured in the presence of granulocyte macrophage colony-stimulating factor (provided by Kirin Brewery Co., Tokyo, Japan) and interleukin-4 (Genzyme/Techne, Minneapolis, MN). After culture for 5 days, OK-432 (Chugai Pharmaceutical Corp., Tokyo, Japan) was added to the culture to obtain mature dendritic cells (27). On day 7, generated mature dendritic cells were pulsed with each peptide for T-cell stimulation. Using these peptide-pulsed dendritic cells each time, the autologous CD8+ T cells were stimulated thrice on days 0, 7, and 14; then, the resultant lymphoid cells were tested for their cytotoxic activities on day 21. To generate CTL clones, established CTL lines were plated in 96-well plates at 0.3, 1, and 3 cells per well with allogenic PBMCs and A3-LCL as stimulator cells. Cytotoxic activities of resulting CTL clones were tested on the 14th day.

Cytotoxicity assay. Cytotoxic activities were measured using a standard 4-hour 51Cr-release assay. Percent specific lysis was calculated as follows:

\[
\text{% Specific lysis} = \left( \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \right) \times 100
\]

Immunogenicity of epitope peptides in A2/Kb transgenic mice. For priming the peptide-specific CTLs, immunization was given using 200 μL vaccine mixture, which contains 100 μg HLA-A2-restricted peptide and 100 μL IFA per mouse. The vaccine was injected i.d. in the right flank for the

Table 1. Epitope candidate peptides from VEGFR2

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NOTE: These epitope candidate peptides were selected with binding prediction software described in Materials and Methods and shown in the order of binding scores reflecting binding affinity of the peptide to the HLAs.
first immunization on day 0 and in the other flank for the second on day 11. On day 21, splenocytes of the vaccinated mice were used as the responder cells, and T2 cells pulsed with or without peptides were used as the stimulator cells for ELISPOT assay.

Semi-quantitative reverse transcription-PCR analysis. For reverse transcription-PCR (RT-PCR) analysis, total RNA was extracted from tumor cells or tumor tissue using isogen (Nippon Gene, Tokyo, Japan). Reverse transcription of total RNA into cDNA was done by using SuperScript II, 1 µg mRNA, pdN (N), primer, and DTT solution for 60 minutes at 37°C. The cDNA mixture (1 µl) was subjected to PCR amplification with 2.5 units AmpliTaq (5 units/µL, Perkin-Elmer, Wellesley, MA), 1.5 µmol/L PCR buffer (MgCl₂), 10 µmol/L deoxynucleotide triphosphate, and 25 pmol of each of two oligonucleotide primers targeting VEGFR2 (sense VEGFR2 5’-GGGACTTG-CAAAACAGTAGCC-3’ and antisense VEGFR2 5’-CGTCTTTTACGATCCACCGGAG-3’). The thermocycling was at 94°C for 30 seconds, 59°C for 1 minute, and 72°C for 2 minutes, and 40 cycles were employed.

In vivo antitumor effects. We examined the antitumor effects of this vaccination with a therapeutic model. MC38 cells (3 × 10⁵ per mouse) or B16 cells (1 × 10⁵ per mouse) were injected i.d. in the right flank on day 0, and vaccination was done on days 4 and 14 using the corresponding IFA-conjugated peptides.

In vivo angiogenesis assay. We examined the effects of peptide vaccination using dorsal air sac assay, which was designed to measure in vivo angiogenesis induced by tumor cells as described previously (28). In brief, the A2/Kb transgenic mice were vaccinated twice with 1-week interval in the left flank using 5 × 10⁵ dendritic cells with or without pulsing peptides as described previously with some modification (29–31). Millipore chamber (Bedford, MA) was filled with PBS containing B16 cells (1 × 10⁵) and implanted in the dorsum of anesthetized mice on day 0. The implanted chambers were removed from s.c. fascia on day 6. The angiogenic response was assessed with photographs taken using a dissecting microscope. The extent of angiogenesis was determined with the number of newly formed blood vessels of >3 mm in length and scored semiquantitatively using an index ranging from 0 (none) to 5 (many).

Detection of CTL precursors from cancer patients. Peptide-specific CTLs were induced from PBMCs of cancer patients using the method described previously (32). In brief, PBMCs (1 × 10⁶) were incubated with the peptides in the 96-well plates at a final concentration of 10 µmol/L. Half of the medium was removed and replaced with the new medium containing a corresponding peptide every 3 days. After the culture for 15 days, these cells were harvested and then tested for their ability to produce IFN-γ in response to each peptide. Existence of CTL precursors was predicted using the value of IFN-γ and DTT solution in the supernatant of the peptide-stimulated PBMCs, considering IFN-γ level of the PBMCs stimulated with HIV peptide as a negative control. If the IFN-γ level of the tested sample was fold higher than the negative control, the lymphoid cells in the tested wells were cultured further to grow CTLs and tested for cytotoxicity.

Statistical analysis. Each experiment was done in triplicate to confirm reproducibility of the results, and representative results are shown. Student’s t test was used to examine the significance of the data when applicable. The difference was considered to be statistically significant when P < 0.05.

Results

Establishment of CTL clones using epitope candidates derived from vascular endothelial growth factor receptor 2. We first tested the immunogenicity of VEGFR2 to determine the epitope peptides. Epitope candidate peptides were selected in the order of the binding scores, reflecting binding affinity of the peptide to the HLA class I molecules (Table 1). We generated CTLs using these peptides and PBMCs given from healthy volunteers with HLA-A*0201 as described in Materials and Methods, and CTL clones were successfully established with five peptides. These CTL clones showed specific cytotoxicity against the target cells pulsed with corresponding peptides. We also examined the ability of established CTL clones induced with these five peptides to lyse the target cells endogenously expressing VEGFR2 as well. The CTL clones induced with three peptides showed potent cytotoxic activity against target cells transfected with adenovirus carrying VEGFR2-cDNA (HePG2-VEGFR2) but not against target cells transfected with adenovirus carrying GFP-cDNA (HePG2-EGFP) as a control (Fig. 1A). We also established CTL clones from PBMCs of healthy volunteers with HLA-A*2402 using five peptides, and these CTL clones showed strong cytotoxicity against target cells transfected with adenovirus carrying VEGFR2-cDNA (HT29-VEGFR2; Fig. 1B). The cytotoxicity was significantly blocked with mAbs against CD8 and HLA class I antigen but was not blocked using mAbs against CD4 nor HLA class I antigen (data not shown). These CTL clones also showed potent cytotoxic activities in HLA class I-restricted fashion against HUVECs (HUVEC-P8, HLA-A24; HUVEC-KT5, HLA-A24*), which were endothermal cells endogenously expressing VEGFR2 (Fig. 1C and D). Furthermore, these CTL clones showed strong cytotoxicity against proliferating endothelial cells but showed low cytotoxicity against nonproliferating or slowly proliferating endothelial cells (data not shown).

In vivo antitumor effects associated with the vaccination using vascular endothelial growth factor receptor 2 epitope peptides. We then tested in vivo antitumor effects of vaccination with VEGFR2 epitope peptides using A2/Kb transgenic mice. At first, we evaluated the immunogenicity of the epitope peptides for A2/Kb transgenic mice to examine the specific production of IFN-γ of the CTLs induced with these peptides by ELISPOT assay. IFA-conjugated peptide was injected s.c. into A2/Kb transgenic mice on days 0 and 11. On day 21, splenocytes of the vaccinated mice were harvested and used as the responder cells for ELISPOT assay. In this ELISPOT assay using A2/Kb transgenic mouse system, positive results were shown for the epitope peptides identified using human PBMCs (Fig. 2A). We also investigated whether VEGFR2 is expressed in the tumor tissue or not using RT-PCR (Fig. 2B). Although the VEGFR2 expression was not detected in tumor cells in culture (lane 1), significant level of the VEGFR2 expression was detected in tumor tissue harvested from the mice (lane 2). Although we cannot completely exclude the possibility that the tumor cells themselves express VEGFR2 only in situ, endothelial cells of the vessels in the tumor tissue appear to express significant level of VEGFR2 mRNAs in this model. Thus, vaccination with epitope peptides derived from VEGFR2 could affect the growth of the tumor cells through the effects on the VEGFR2-expressing endothelial cells of the vessels, which support the tumor growth in vivo in this A2/Kb transgenic mice-tumor system. The vaccination using the epitope peptide showed strong antitumor effect in therapeutic model. The MC38 colon carcinoma cells or the B16 melanoma cells were injected i.d. into A2/Kb transgenic mice and C57BL/6 mice on day 0 and vaccination was done on these mice 4 and 14 days after the tumor challenge using VEGFR2-773 peptide conjugated with IFA. Although the significant inhibition of tumor growth was observed in A2/Kb transgenic mice treated with VEGFR2-773 peptide conjugated with IFA, no significant suppression was observed for the tumors in C57BL/6 mice treated in the same manner (Fig. 2C and D). From these results, it was indicated that the in vivo antitumor effect was clearly HLA-A2 restricted but not mouse class I restricted.

Inhibition of tumor-induced angiogenesis with the vaccination using vascular endothelial growth factor receptor 2 epitope peptides. We examined the mechanism of tumor growth inhibition associated with the vaccination using the peptides derived from VEGFR2. To directly examine the effects of the
Figure 1. Establishment of CTL clones. CTL clones were established from human PBMCs as described in Materials and Methods. A, cytotoxicity of each CTL clone against T2 cells pulsed with VEGFR2-derived, HLA-A*0201-binding peptides. T2 cells were used for CTL responses in the presence of corresponding peptide (●), HIV peptide (△), or no peptide (□). CTL clones from five peptides showed specific cytotoxicities against the target cells pulsed with corresponding peptides (top). Furthermore, each CTL clone was examined for cytotoxicity against HLA-A*0201+ VEGFR2-expressing cells (HePG2-VEGFR2; ●) or control (HePG2-EGFP; □) with a 4-hour ^51^Cr-release assay. Three of five CTL clones showed potent cytotoxic activity against HePG2-VEGFR2 but not against HePG2-EGFP (bottom). B, A24-LCL cells were used for CTL responses restricted to HLA-A*2402 in the presence (●) or absence (□) of each HLA-A*2402-binding peptide. CTL clones from five peptides showed specific cytotoxicities against the target cells pulsed with corresponding peptides (top). Each CTL clone was also examined for the cytotoxicity against VEGFR2-expressing cells (HT29-VEGFR2; ●) and control (HT29-EGFP; □) with a 4-hour ^51^Cr-release assay. All these CTL clones showed the strong cytotoxicities against HT29-VEGFR2 but not against HT29-EGFP (bottom). C, VEGFR2-169-C29 was examined for their cytotoxicities against HUVEC-KT5 (●) or HUVEC-P8 (□), which were endothelial cells endogenously expressing VEGFR2 as described in Materials and Methods. It showed potent cytotoxic activity only against HUVEC-KT5. D, expression of VEGFR2 and HLA class I antigen was examined for HUVEC-KT5 (left) and HUVEC-P8 (right) with fluorescence-activated cell sorting analysis. Both HUVEC cells expressed VEGFR2 and HLA class I antigen at similar levels. E:T ratio, effector/target cell ratio.
Figure 2. In vivo antitumor effect of vaccination using epitope peptides derived from VEGFR2. A, IFA-conjugated peptides were injected i.d. into A2/Kb transgenic mice on days 0 and 11. On day 21, 2 \times 10^6 splenocytes of the vaccinated mice were used as the responder cells and 1 \times 10^6 T2 cells pulsed with corresponding peptide (■), carcinoembryonic antigen peptide (■), or no peptide (□) were used as the stimulator cells for ELISPOT assay. Specific production of IFN-γ for the corresponding peptide was observed in the mice vaccinated with VEGFR2-190, -772, -773, -775, and -1,084 peptides. B, VEGFR2 mRNAs were analyzed in tumor cells and the tumor tissues with RT-PCR using primers for VEGFR2. Tumor tissues were harvested from the mice 2 weeks after i.d. injection of tumor cells. The expression of VEGFR2 was shown in the tumor tissue but not in tumor cells in culture. C, inhibition of colon cancer in a therapeutic setting. A2/Kb transgenic mice and C57BL/6 mice were inoculated i.d. with MC38 cells, and HBSS (□), IFA only (■), or IFA-conjugated VEGFR2-773 peptide (▲) was given 4 and 14 days later (arrow). Significant suppression of tumor growth was observed with the vaccination using VEGFR2-773 peptide conjugated with IFA in A2/Kb transgenic mice but not in C57BL/6 mice. *, \( P < 0.01 \); **, \( P < 0.001 \). D, inhibition of melanoma in a therapeutic setting. A2/Kb transgenic mice and C57BL/6 mice were inoculated i.d. with B16 cells, and HBSS (□), IFA only (■), or IFA-conjugated VEGFR2-773 peptide (▲) was given 4 and 14 days later (arrow). Significant suppression of tumor growth was observed with the vaccination using VEGFR2-773 peptide conjugated with IFA in A2/Kb transgenic mice but not in C57BL/6 mice. *, \( P < 0.05 \); **, \( P < 0.04 \).
peptide vaccination on angiogenesis induced by tumor cells, we employed dorsal air sac assay that visualizes the extent of neovascularization in vivo (Fig. 3A). In this semiquantitative assay, significant inhibition on angiogenesis was observed in the mice vaccinated with VEGFR2-772, -773, -773-2L, -775, and -1,084 peptides (Fig. 3B). These results strongly suggest that the antitumor effects induced with the vaccination using the peptides derived from VEGFR2 might be mediated by the inhibition of tumor angiogenesis.

**CTL precursors in peripheral blood mononuclear cells of cancer patients.** We examined whether VEGFR2-specific CTLs could be also induced in cancer patients. The PBMCs given from six cancer patients with HLA-A*0201 or HLA-A*2402 were stimulated in vitro with each peptide derived from VEGFR2 and tested for their ability to produce IFN-γ in response to each peptide (Table 2). In every patient, significant IFN-γ responses were detected against at least one peptide included in the test. Furthermore, CTL lines were successfully induced using the VEGFR2 epitope peptides and showed potent cytotoxicity against target cells pulsed with the corresponding peptide (Fig. 4). These results strongly suggest that CTLs specific to VEGFR2 can be induced in cancer patients as well. However, HLA-A2-restricted lysis by the CTL lines could not be observed in these limited sets of experiments. Further analysis on the cytotoxicity of HLA-A2-restricted CTLs would be needed.

**Discussion**

Identification of the tumor-associated antigens has enabled the clinical development of peptide-based cancer vaccine, which could induce CTLs and lyse tumor cells in a HLA class I–restricted fashion (33–36). Until now, multiple clinical trials using tumor-associated antigen peptides have reported that tumor regressions were observed in ~20% of the melanoma patients. However, complete response has rarely been reported (35, 37–40). One of the possible reasons of modest clinical efficacy could be loss or down-regulation of HLA class I molecules on the tumor cells (41–44). The frequency of tumors showing some alteration in expression of HLA class I molecules has been estimated to be >40% (42, 44). Thus, significant portion of tumor cells could escape from the CTLs specific to the class I epitope, even if CTLs could be successfully induced by cancer vaccine targeting tumor cells themselves. These problems could be overcome with the development of effective vaccines.

![Figure 3. In vivo inhibition of tumor-induced angiogenesis.](image-url)
vaccine against tumor angiogenesis, because endothelial cells are genetically stable, do not show down-regulation of HLA class I molecules, and are critically involved in the progression of a variety of tumor. Furthermore, the CTLs could directly cause damage to the endothelial cells without penetrating any other tissue, and lysis of even low numbers of endothelial cells within tumor vasculature may result in destruction of vessel integrity leading to inhibition of many tumor cells (45). Therefore, endothelial cells could be a good target for cancer immunotherapy. To specifically and efficiently prevent tumor angiogenesis with CTL response, the appropriate target needs to be selected among the molecules related to angiogenesis. VEGFR2 has been reported to be closely related to proliferation and migration of endothelial cells and strongly expressed on endothelial cells in tumor tissue but not within normal tissue (12–14). Furthermore, it became clear that VEGFR2 has important antitumor effects through VEGF-VEGFR2 cross-linkage, including neutralizing anti-VEGFR2 mAb, recombinant receptors, or VEGFR2 kinase inhibitors (7–11).

In this study using our novel model systems in vitro and in vivo, we examined whether we could develop a novel immunotherapy targeting tumor-induced angiogenesis. At first, we identified the epitope peptides of VEGFR2 restricted to HLA-A*0201 and HLA-A*2402, which are frequently recognized HLA alleles (18). The CTLs were successfully induced with these peptides and showed potent cytotoxicities against not only peptide-pulsed target cells but also the endothelial cells endogenously expressing VEGFR2. Our findings clearly showed that human VEGFR2 is immunogenic in human system.

Then, we showed in vivo antitumor effects using multiple tumor cell lines and A2/Kb transgenic mice, a good model system to evaluate immune responses in human against tumor cells with the loss of HLA class I expression. It has been shown that there is ~71% concordance between the CTL repertoire of human and A2/Kb transgenic mice (19). Thus, CTLs induced by vaccination using epitope peptides could recognize endothelial cells, which are derived from A2/Kb transgenic mice and express VEGFR2 and HLA-A*0201, but do not recognize the tumor cells that have no "human" MHC class I molecules. Using this unique tumor model system, significant inhibition of the tumor growth was observed with vaccination using these epitope peptides. This peptide-based vaccine was also associated with significant suppression of tumors before the vaccination as well. These results support that our vaccination strategy would be effective even for the tumors with HLA deficit, which is considered to be one of the escape mechanism of tumors.

We also showed in dorsal air sac assay that tumor-induced angiogenesis was significantly inhibited with vaccination using these epitope peptides. This result suggests that the inhibition of Table 2. Specific IFN-γ response of stimulated PBMC harvested from cancer patients

<table>
<thead>
<tr>
<th>HLA-A*2402</th>
<th>Patient A</th>
<th>Patient B</th>
<th>Patient C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive wells</td>
<td>Total wells</td>
<td>Positive wells</td>
<td>Total wells</td>
</tr>
<tr>
<td>VEGFR2-169</td>
<td>12</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>VEGFR2-189</td>
<td>3</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>VEGFR2-220</td>
<td>0</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>VEGFR2-826</td>
<td>11</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>VEGFR2-1318</td>
<td>0</td>
<td>32</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HLA-A*0201</th>
<th>Patient D</th>
<th>Patient E</th>
<th>Patient F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive wells</td>
<td>Total wells</td>
<td>Positive wells</td>
<td>Total wells</td>
</tr>
<tr>
<td>VEGFR2-773</td>
<td>1</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>VEGFR2-773-2L</td>
<td>—</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>VEGFR2-775</td>
<td>2</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>VEGFR2-1328</td>
<td>0</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Specific IFN-γ responses to VEGFR2 epitope peptides were examined after the stimulation with each peptide on PBMCs of cancer patients. The wells with the detectable levels of IFN-γ were judged to contain specific IFN-γ responding to the corresponding peptides using HIV peptide as a control. Patients A and D were colon cancer patients. Patients B, C, E, and F were melanoma patients.

[Figure 4. Functional analysis of CTLs induced from PBMCs of cancer patients. The CTLs were induced with the stimulations using epitope peptides for five times from PBMCs of cancer patients. Then, CTL lines were successfully induced from PBMC of cancer patients with VEGFR2-169, -189, or -220. A24-LCL cells were used to test CTL responses in the presence (●) or absence (○) of each peptide. The CTL lines showed specific cytotoxocities against the target cells pulsed with corresponding peptides in a standard 4-hour 51Cr-release assay.]
tumor angiogenesis could be achieved with peptide vaccination targeting the molecule expressed on proliferating endothelial cells. Before a clinical application, it is important to confirm whether CTLs could be induced with the epitope peptides derived from VEGFR2 in cancer patients. In functional and HLA-tetramer analysis (data not shown) using PBMCs of cancer patients, we confirmed that there are CTL precursors for epitope peptide of VEGFR2. Interestingly, the frequencies of CTL precursors against each epitope peptide were different from patient to patient.

Because VEGFR2-specific CTLs had strong cytotoxicity against proliferating endothelial cells, they could suppress proliferating endothelial cells in physiologic angiogenesis. Adverse effects in wound healing and fertility have been reported with the vaccination using whole VEGFR2 protein or cDNA (16, 17). The same types of adverse effects were observed with some of the epitope peptides we used. However, we observed no other significant side effects with the treatment (data not shown). Thus, this strategy could be applied to the patients with some restriction.

These results in vitro and in vivo strongly suggest that VEGFR2 could be a promising target of immunologic therapy using cellular immunity and support the definitive rationale of the clinical development of this strategy against a broad range of cancers. Vaccination using these epitope peptides derived from VEGFR2 is now in the process of phase I clinical application in our institute.

Acknowledgments
Received 10/19/2004; revised 2/23/2005; accepted 3/24/2005.

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References
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