Inhibition of Tumor Growth with Antiangiogenic Cancer Vaccine Using Epitope Peptides Derived from Human Vascular Endothelial Growth Factor Receptor 1

Hidenobu Ishizaki, Takuya Tsunoda, Satoshi Wada, Mai Yamauchi, Masabumi Shibuya, and Hideaki Tahara

Abstract

Purpose: Antiangiogenic therapy is now considered to be one of promising approaches to treat various types of cancer. In this study, we examined the possibility of developing antiangiogenic cancer vaccine targeting vascular endothelial growth factor receptor 1 (VEGFR1) overexpressed on endothelial cells of newly formed vessels in the tumor.

Experimental Design: Epitope-candidate peptides were predicted from the amino acid sequence of VEGFR1 based on their theoretical binding affinities to the corresponding HLAs. The A2/Kb transgenic mice, which express the α1 and α2 domains of human HLA-A*0201, were immunized with the epitope candidates to examine their effects. We also examined whether these peptides could induce human CTLs specific to the target cells in vitro.

Results: The CTL responses in A2/Kb transgenic mice were induced with vaccination using identified epitope peptides restricted to HLA-A*0201. Peptide-specific CTL clones were also induced in vitro with these identified epitope peptides from peripheral blood mononuclear cells donated by healthy volunteers with HLA-A*0201. We established CTL clones in vitro from human peripheral blood mononuclear cells with HLA-A*2402 as well. These CTL clones were shown to have potent cytotoxicities in a HLA class I restricted manner not only against peptide-pulsed target cells but also against target cells endogenously expressing VEGFR1. Furthermore, immunization of A2/Kb transgenic mice with identified epitope peptides restricted to HLA-A*0201 was associated with significant suppression of tumor-induced angiogenesis and tumor growth without showing apparent adverse effects.

Conclusions: These results strongly suggest that VEGFR1 is a promising target for antiangiogenic cancer vaccine and warrants further clinical development of this strategy.

Angiogenesis inhibition has been hypothesized to be an effective strategy to treat cancer, and clinical application of this strategy has been pursued using multiple modalities, including specific inhibitors for the signaling pathways of vascular endothelial growth factor (VEGF; ref. 1) and blocking antibodies against VEGF (2) or VEGF receptor (VEGFR; ref. 3), for more than 30 years (4). Some of these efforts led to the recent clinical discovery that the administration of anti-VEGF antibody significantly prolongs the survival of colorectal cancer patients (5). Thus, feasibility and usefulness of antiangiogenic cancer therapy are now warranted. However, these existing antiangiogenic agents have significant shortcomings, including side effects and the requirement of frequent or continuous administration. As a novel alternative therapy to overcome the shortcomings of the existing agents, we have recently reported a vaccination strategy to prevent tumor angiogenesis using epitope peptides derived from VEGFR2 (6). In such study, we have successfully shown using a mouse tumor model that this is indeed a promising strategy. We have also shown that specific CTLs could be induced in vitro from the peripheral blood mononuclear cells (PBMC) of cancer patients using these peptides.

Antiangiogenic cancer vaccination could also have an advantage over the vaccination strategy using tumor-associated antigens. The identification of tumor-associated antigens has enabled the clinical development of peptide-based cancer vaccines with multiple clinical trials (7–9). However, the rates of objective response were reported to be modest in early-phase clinical trials of cancer vaccine using tumor-associated antigens (10–12). One possible reason for this relatively low efficacy has been attributed to the loss or down-regulation of HLA class I molecules on tumor cells, which has been reported to occur frequently in solid tumors and could severely impair T cell–mediated antitumor responses (13–15). However, such HLA loss has not been reported for endothelial cells of newly formed vessels in tumors.

Both VEGFR1 (also known as Flt-1; ref. 16) and VEGFR2 (17) have been shown to be overexpressed on endothelial cells of vessels in various types of primary tumor and metastases (18–22). Because VEGFR2 has been considered to be a key
regulator of the VEGF-dependent angiogenesis (23), this receptor has been a major target to date. Recent reports have shown that vaccination using the cDNA or soluble protein of mouse VEGFR2 was associated with significant antitumor effects in mouse tumor models (24, 25). However, it has been emphasized that tumor angiogenesis mediated by the VEGFR1 pathway is also important (26, 27). Recent studies have reported that VEGFR1, but not VEGFR2, is up-regulated by hypoxic conditions (28, 29), and similar patterns of distribution of both VEGFR1 and VEGFR2 are not always observed in tumors (30–33). In situ hybridization revealed that during the progression from a low-grade glioma to glioblastoma, the expression of VEGFR1 mRNA precedes that of VEGFR2 mRNA (20). Moreover, recent studies have reported that the significant inhibition of tumor growth could be achieved using a number of VEGFR1-targeting approaches (34–40). Thus, VEGFR1 is another attractive target for cancer vaccines against tumor angiogenesis.

Here, we have examined the possibility of developing an antiangiogenic cancer vaccine using epitope peptides derived from VEGFR1. Epitope-candidate peptides were predicted from the amino acid sequence of VEGFR1 based on their theoretical binding affinities to the corresponding HLA-A*0201 and HLA-A*2402 (41). These epitope peptides were used for the examination in vitro and in vivo. Animal experiments have been reported with unique models using A2/Kb transgenic mice, which express the α1 and α2 domains of human HLA-A*0201 (6). Our results suggest that an effective antiangiogenic cancer vaccine could be developed using epitope peptides derived from VEGFR1.

### Materials and Methods

**Cell lines.** The T2 cell line, which expresses HLA-A*0201, was generously provided by Dr. H. Shiku (Mie University School of Medicine, Mie, Japan). MCA205, a methylcholanthrene-induced murine fibrosarcoma cell line, was a generous gift from Dr. S.A. Rosenberg (National Cancer Institute, Bethesda, MD). MC38 murine colon carcinoma cell line, Lewis lung carcinoma, and B16F10 and B16 melanoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). We established the AG1-G1-VEGFR1 cell lines, which express high levels of VEGFR1. The AG1-G1 cell line was established from a HLA-A*2402–positive human benign hemangioma. The AG1-G1 cells were transfected with BCMS neo Flt-1 (42), a VEGFR1 expression vector, and selected for G418-resistant clones to establish the AG1-G1-VEGFR1 cell line.

**Synthetic peptides.** The epitope-candidate peptides derived from VEGFR1 restricted to HLA-A*0201 (A2) and HLA-A*2402 (A24) were selected based on the theoretical binding affinities to the corresponding HLAs. The theoretical binding affinities were estimated using the Biinformatics and Molecular Analysis Section (43) websites. To test CTL activity in our in vivo mouse model system described below, we used only the peptides that bind to HLA-A*0201 and exist both in human and mouse VEGFR1 amino acid sequences. These candidate peptides were synthesized with the standard solid-phase synthesis method and purified with reverse-phase high-performance liquid chromatography by Sawady Technology (Tokyo, Japan). 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.

**Animals.** The A2/Kb transgenic mice were generously provided by Dr. F. James Primus (Vanderbilt University Medical Center, Nashville, TN). The A2/Kb transgenic mice, which express chimeric MHC class I molecules consisting of the α1 and α2 domains of HLA-A*0201 and the α3 domain of mouse H-2Kb, were prepared as described elsewhere (44). The animals were maintained in the pathogen-free Animal Facility of Institute of Medical Science, The University of Tokyo, and all protocols for animal experiments were approved by the ethical committee of our institute.

**CTL responses of VEGFR1-derived peptides in A2/Kb transgenic mice.** Immunization was done twice using the 100-μl vaccine mixture per mouse, which contained 100 μg of candidate peptides derived from

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**Table 1. Epitope-candidate peptides derived from VEGFR1**

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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.
VEGFR1 and 100 μl of incomplete Freund's adjuvant (IFA; Sigma, St. Louis, MO). The vaccine was injected s.c. into the right flank as the first immunization on day 0, and the second immunization was injected into the left flank on day 11. The immunized mice were sacrificed on day 21 and the spleens were harvested. The lymphoid cells were prepared from the spleens and stimulated in vitro using antigen-presenting cells prepared as follows with the method described previously (45). The spleen cells from each mouse were stimulated separately with peptide-pulsed B-cell blasts at a 3:1 responder/feeder ratio. To prepare B-cell blasts as antigen-presenting cells for in vitro stimulation, spleen cells of A2/Kb transgenic mice, which received no treatment, were harvested in RPMI 1640. After rinsing with RPMI 1640 and erythrocyte removal with RBC lysing buffer (Sigma), the cells (1.5 × 10^6/μl) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.1 mM L-arginine, 0.01 mM L-mercaptoethanol in the presence of 25 μg/mL lipopolysaccharide at 37°C for 2 days. B-cell blasts were pulsed with the corresponding peptide (20 μg/mL) for 4 hours, irradiated at 20 Gy, and used for in vitro stimulation. The cells were then maintained in 25-cm² flasks for 5 days at 37°C in a 5% CO₂ incubator. The cytotoxicity activities were tested with 4-hour 51Cr release assay against T2 cells pulsed with or without peptides. Spleen cells harvested from the immunized mice were also used as responder cells in the enzyme-linked immunospot (ELISPOT) assay. The T2 cells pulsed with or without corresponding peptides were used as stimulator cells in the ELISPOT assay.

*Generation of human CTL clones.* We examined whether CTLs could be induced with the peptide from human PBMCs. Monocyte-derived dendritic cells were used to induce CTLs against peptides as previously described (46, 47). In brief, the PBMCs were obtained from healthy volunteers with corresponding HLAs and were cultured in the presence of granulocyte macrophage colony-stimulating factor (provided by Kirin Brewery Co., Tokyo, Japan) at 1,000 units/mL and interleukin-4 (Genzyme/Techne, Minneapolis, MN) at 1,000 units/mL. After 5 days of culture, OK-432 (Chugai Pharmaceutical Corp., Tokyo, Japan) was added at 10 μg/mL to the supernatant and cultured for 2 more days to obtain mature dendritic cells (47). The mature dendritic cells generated in this manner were pulsed with each peptide for T-cell stimulation. Using these peptide-pulsed dendritic cells, the autologous CD8+ T cells were stimulated thrice on days 0, 7, and 14; then, the cytotoxic activities of the resultant lymphoid cells were tested 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-LCLs as stimulator cells. The cytotoxic activities of the resulting CTL clones were tested on the 14th day of culture.

*In vitro angiogenesis assay.* Cytotoxicity activity was measured using a standard 4-hour 51Cr-release assay. T2 cells and A24-LCLs were pulsed with the candidate peptides and used as target cells. The percentage of specific lysis was calculated as follows: % specific lysis = [(experimental release − minimum release) / (maximum release − minimum release)] × 100.

*In vivo angiogenesis assay.* We examined the in vivo antiangiogenic effects of peptide vaccination using the dorsal air sac assay (6, 48). In brief, the A2/Kb transgenic mice were immunized twice, 7 and 14 days before the assay, in the upper flank using the corresponding IFA-conjugated peptides. The Millipore chambers (Millipore Corp., Bedford, MA) having filters with 0.45-μm pore size were filled with MC38 cells (1 × 10^6) suspended in 150 μL of HBSS and were implanted s.c. in the dora of anesthetized mice on day 0. The implanted chambers were removed from the s.c. fascia on day 6, and the black rings were placed at the sites exposed to a direct contact with the chamber. The angiogenic response was assessed with photographs taken with a dissecting microscope. The newly formed blood vessels induced with angiogenic factors released from tumor cells in the chamber were morphologically distinct from the preexisting background vessels, as characterized by the coiled and thin structure. The extent of angiogenesis was determined by measuring the number of newly formed blood vessels >3 mm in length within the area marked by the black ring.

*In vivo antitumor effects.* In vivo antitumor effects of peptide vaccination were examined using the system we developed (6). The A2/Kb transgenic mice were inoculated i.d. in the right flank with MCA205 cells (1 × 10^6 per mouse), MC38 cells (3 × 10^5 per mouse), or LLC cells (5 × 10^5 per mouse). When the tumors reached 3 to 4 mm in diameter in 4 or 5 days, the mice were immunized with the corresponding peptides conjugated with IFA. Second vaccination was done 7 days after the first one. Mice were injected i.v. with B16F10 cells (1 × 10^6 per mouse) to give experimental pulmonary metastasis (25). We tested in a therapeutic setting by vaccinating the animals 7 days after i.v. injection of B16F10 cells; second vaccination was done 7 days after the first one. Tumor formation was evaluated 28 days later by counting the number of tumor nodules on the lung surface and measuring wet lung weight. For experiments testing syngeneic effect targeting both VEGFR1 and VEGFR2, mice were immunized twice with IFA-conjugated VEGFR1-770, VEGFR2-773 (VIAMFFWLL; ref. 6), or a combination of both peptides. When mice were immunized with combination, VEGFR1 immunization was injected s.c. into the right upper flank and VEGFR2 immunization was injected into the left upper flank at the first immunization, and the second immunizations were done in the lower flanks.

*Evaluation of possible adverse effects.* We examined the influence of VEGFR1 vaccination on wound healing and fertility as described elsewhere (6, 24, 25). We immunized the A2/Kb transgenic mice with the selected peptides conjugated with IFA on days 0 and 7. To evaluate wound healing, we inflicted four square wounds (6-mm diameter each) on the backs of these mice. The wound area was measured twice weekly and the time until wound closure was recorded. Fifteen days after this excision, some mice were sacrificed, and scar tissues were removed for histologic examination. To evaluate fertility, 1 week after the second immunization, immunized and nonimmunized A2/Kb transgenic mice were allowed to cohabitate with males. The number of days until parturition and the number of pups were counted. The pups were also carefully examined for signs of sickness and abnormality. To determine the effects of VEGFR1 vaccine on hematopoiesis, A2/Kb transgenic mice were immunized twice. Ten days after the last immunization, bone marrow cells were obtained from mice by flushing femoral bone, and peripheral blood was collected by retro-orbital bleeding. Bone marrow cells per femur and total WBC were counted (49, 50).

*Histology.* Lung samples and scar tissues were fixed overnight in 10% buffered formalin, embedded in paraffin, and sectioned at 5 μm. H&E staining was done. Frozen sections were fixed in acetone, and the time until wound closure was recorded. Fifteen days after this excision, some mice were sacrificed, and scar tissues were removed for histologic examination. To evaluate fertility, 1 week after the second immunization, immunized and nonimmunized A2/Kb transgenic mice were allowed to cohabitate with males. The number of days until parturition and the number of pups were counted. The pups were also carefully examined for signs of sickness and abnormality. To determine the effects of VEGFR1 vaccine on hematopoiesis, A2/Kb transgenic mice were immunized twice. Ten days after the last immunization, bone marrow cells were obtained from mice by flushing femoral bone, and peripheral blood was collected by retro-orbital bleeding. Bone marrow cells per femur and total WBC were counted (49, 50).

*Histology.* Lung samples and scar tissues were fixed overnight in 10% buffered formalin, embedded in paraffin, and sectioned at 5 μm. H&E staining was done. Frozen sections were fixed in acetone, incubated, and stained with an antibody reactive to CD31 (BD PharmMingen, San Diego, CA) as previously described (37). Sections were counterstained with hematoxylin. Vessel density was determined by counting the stained vessels per high-power field.

**Statistical analysis.** Each experiment was done thrice to confirm the reproducibility of the results, and the representative results were shown. Values of the results were expressed as means and SEs. Student's t test was used to examine the significance of the data when applicable. Comparisons with more than two groups were done using ANOVA with appropriate post hoc testing. Differences were considered to be statistically significant when P < 0.05.

**Results**

*Evaluation of CTL response against each candidate peptide derived from VEGFR1 in A2/Kb transgenic mice.* Candidate peptides for class I epitopes were selected according to the binding scores, which reflected the binding affinity of the peptide to HLA class I molecules (Table 1). We first examined the specific responses of IFN-γ of the CTLs induced with these peptides in A2/Kb transgenic mice. Each peptide conjugated with IFA was injected s.c. into A2/Kb transgenic mice.
mice on days 0 and 11. On day 21, spleen cells of the immunized mice were harvested and used as responder cells for the ELISPOT assay. Peptide-specific production of IFN-γ was observed in mice immunized with VEGFR1-1087, VEGFR1-770, and VEGFR1-417 peptides (Fig. 1A). Furthermore, we examined the cytolytic activity of CTLs induced from immunized spleen cells as described in Materials and Methods. The CTLs induced with VEGFR1-1087, VEGFR1-770, and VEGFR1-417 peptides showed specific cytotoxicity against T2 cells pulsed with the corresponding peptides (Fig. 1B). We also checked the duration of the peptide-specific CTL response after immunization. Peptide-specific CTLs could be detected in most of the mice up to 28 days after immunization. However, the CTLs were not detected in the mice immunized 45 days before.

**Establishment of human CTL clones using epitope candidates found with in vivo screening.** We then examined whether these three candidates could induce CTLs from PBMCs to confirm the responses in human immune system as described in Materials and Methods. We successfully generated CTLs only with VEGFR1-1087 and VEGFR1-770 peptides using PBMCs from healthy volunteers with HLA-A*0201. These CTL clones showed specific cytotoxicity against target cells pulsed with the corresponding peptides (Fig. 2A). However, we could not generate CTLs with VEGFR1-417 peptide using human PBMCs.

**Identification of class I epitope restricted to HLA-A*2402 derived from VEGFR1.** Because we do not have access to A24/Kb transgenic mice, we searched for class I epitopes restricted to HLA-A*2402 using only in vitro assays with human PBMCs as previously described (46). Epitope-candidate peptides were selected in the order of the binding scores, which reflected the binding affinity of the peptide to HLA class I molecules (Table 1). We successfully established CTL clones with the VEGFR1-1084 peptide using PBMCs from healthy volunteers bearing HLA-A*2402. The CTL clones exhibited potent cytotoxicities against target cells pulsed with the corresponding peptides (Fig. 2B, left). Furthermore, the CTL clones induced with VEGFR1-1084 peptide showed significantly more potent cytotoxic activity against AG1-G1-VEGFR1 cells expressing VEGFR1 when compared with those against AG1-G1-Neo cells expressing no VEGFR1 (P < 0.01; Fig. 2B, right). This cytotoxicity was significantly reduced with monoclonal antibodies against CD8 and HLA class I antigen but was not blocked with monoclonal antibodies against CD4, nor with HLA class II antigen (data not shown). The CTL clone could not be induced from healthy volunteer with HLA-A*2402 using the rest of peptides binding to HLA-A*2402.

**Inhibition of tumor-induced angiogenesis by immunization with candidate peptides.** To determine whether the anti-VEGFR1 immune responses could inhibit tumor-induced angiogenesis in vivo, we conducted a dorsal air sac assay that enables the visualization of the extent of neovascularization as described in Materials and Methods. Implantation of a chamber containing murine colon carcinoma MC38 cells, which produce VEGF in the dorsal air sac, resulted in the
development of microvessels (indicated by arrows) exhibiting a coiled, thin structure along with the preexisting vessels (Fig. 3A). A significant inhibition of tumor-induced angiogenesis was observed in mice immunized with the VEGFR1-1087 and VEGFR1-770 peptides (Fig. 3B).

**In vivo antitumor effects of immunization with VEGFR1 epitope peptides on multiple tumor cell lines.** We examined the in vivo antitumor effects of immunization with VEGFR1 epitope peptides using a tumor system with A2/Kb transgenic mice. The MCA205 fibrosarcoma cells, MC38 colon carcinoma cells, and Lewis lung carcinoma cells were injected i.d. into A2/Kb transgenic mice on day 0. When the tumors reached 3 to 4 mm in diameter, the mice were immunized twice with VEGFR1 epitope peptides conjugated with IFA. Significant inhibition of tumor growth of all these cell lines was observed in the mice treated with the VEGFR1-1087 and VEGFR1-770 peptides (Fig. 4A, D, and E). With histologic sections of the MCA205 tumors, the effect of immunization with VEGFR1 epitope peptides on tumor angiogenesis was verified by immunohistochemical analysis with an endothelial cell-specific surface marker (CD31). As determined by the number of CD31-stained microvessels, tumor angiogenesis was significantly inhibited in the mice immunized with VEGFR1 epitope peptides (Fig. 4B and C). Because these tumor cells do not express HLA-A*0201, these results strongly suggest that these antitumor effects were mediated by the antangiogenic effects induced by immunization with VEGFR1-derived peptides. Tumor angiogenesis is also critical for tumor metastasis. To determine whether immunization with VEGFR1 epitope peptides could inhibit tumor metastasis, we examined the effects of the vaccination in an experimental metastasis model of B16F10 melanoma in a therapeutic setting. Tumor formation was evaluated 28 days later by counting the number of tumor nodules on the lung surface and measuring lung weight. As shown in Fig. 4F and G, VEGFR1 immunization significantly inhibited lung metastasis when compared with the IFA control group. We clearly showed that VEGFR1 immunization was effective against various kinds of tumors in the s.c. and metastatic tumors. To obtain more potent antitumor effects with angiogenic cancer vaccine using epitope peptide derived from VEGFR1, we tested VEGFR1 immunization in combination with an epitope peptide derived from VEGFR2, VEGFR2-773 (VIAMFFWLL), which was previously reported (6). For experiments testing synergic effect targeting both VEGFR1 and VEGFR2 in a therapeutic setting, mice were immunized twice with IFA-conjugated VEGFR1-770, VEGFR2-773, or a combination of both peptides. As shown in Fig. 4H, there was significant inhibition on B16 tumor growth in mice immunized with each single peptide. The combination therapy with both peptides showed significantly more potent inhibition on tumor growth when compared with single-peptide vaccination ($P < 0.05$). The effect of combination therapy with both peptides on tumor angiogenesis was also analyzed by immunohistochemical analysis with CD31 (Fig. 4I and J). There were significantly less vessels stained with CD31 in the B16 tumor samples obtained from the animals vaccinated with VEGFR1 when compared with those treated with IFA. Regarding the effects of combination therapy, significantly fewer CD31-positive vessels were observed in the B16 tumor samples obtained from the animals receiving both peptides when compared with those treated with IFA or VEGFR1 alone. However, the difference was not significant when compared with those treated with VEGFR2 alone.

**Evaluation of possible adverse effects.** All the mice immunized with VEGFR1-derived peptides seemed to be generally healthy and showed no obvious signs of toxicity. To evaluate whether VEGFR1 immunization is associated with previously reported specific adverse effects, we did experiments to examine the effects on wound healing and pregnancy. To test wound healing, wounding was done as described in Materials and Methods. Full-thickness wounds were created 1 week after two immunizations on the dorsa of mice immunized with IFA only, VEGFR1-1087 conjugated with IFA, or VEGFR1-770 conjugated with IFA. The wound areas were measured twice weekly until the wounds had completely healed. No significant delay of wound healing was observed in anti-VEGFR1–immunized mice when compared with control mice (Fig. 5A). Tissue samples of the wound in the peptide-treated animals were not

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![Image](image-url)
Fig. 4. VEGFR1 immunization inhibits the growth and metastasis of various types of tumors in a therapeutic setting. A to E, A2/Kb transgenic mice were inoculated i.d. with MCA205 cells, MC38 cells, and LLC cells. The mice were immunized twice (arrows) with PBS (○), IFA only (▲), VEGFR1-1087 conjugated with IFA (●), and VEGFR1-770 conjugated with IFA (■).

A. inhibition of MCA205 fibrosarcoma in a therapeutic setting. *, P < 0.02; **, P < 0.002. B. histologic examination of MCA205 tumor tissues from each group. Top, H&E staining; original magnification, ×100. Bottom, immunohistochemistry of tumor angiogenesis; original magnification, ×200. Tumor vessels were stained with anti-CD31 antibody (brown). C. vessel density was determined by counting the number of the microvessels per high-power field. *, P < 0.001; **, P < 0.005. D. inhibition of MC38 colon cancer in a therapeutic setting. *, P < 0.05; **, P < 0.005.

E. inhibition of LLC lung cancer in a therapeutic setting. *, P < 0.05; **, P < 0.01. Significant suppression of tumor growth was observed in mice immunized with VEGFR1-1087 and VEGFR1-770 peptides.

F and G. inhibition of experimental lung metastasis in a therapeutic setting. The B16F10 cells (10⁵) were injected i.v. and mice were immunized twice 7 days after the tumor injection. On the 28th day, the lungs were removed and assessed for tumor formation. F, top, pictures of representative lung samples from each group. Bottom, H&E staining; original magnification, ×100. G, assessment for tumor formation of experimental lung metastasis. Left, average number of metastatic nodules. Right, average lung weight. *, P < 0.001; **, P < 0.005; ***, P < 0.001; ****, P < 0.001.

H to J, enhanced inhibition of tumor growth in combination with epitope peptide derived from VEGFR2. A2/Kb transgenic mice were inoculated i.d. with B16 melanoma cells. The mice were immunized twice (arrows) with IFA only (▲), IFA-conjugated VEGFR1-770 (●), VEGFR2-773 (VIAMFWLL; ▲), or combination of VEGFR1-770 and VEGFR2-773 (■).

H. inhibition of B16 melanoma in a therapeutic setting. Enhanced antitumor effect was observed in mice immunized with both VEGFR1-770 and VEGFR2-773 peptides. *, P < 0.02; **, P < 0.05. I. histologic examination of B16 tumor tissues from each group. Top, H&E staining; original magnification, ×100. Bottom, immunohistochemistry of tumor angiogenesis using anti-CD31 antibody (brown); original magnification, ×200. J. vessel density was determined by counting the number of the microvessels per high-power field. *, P < 0.05; **, P < 0.001; ***, P < 0.001. Bars, SE.
morphologically different from those in the control animals. To further examine whether anti-VEGFR1 immunization has an effect on pregnancy, we did a pregnancy experiment as described in Materials and Methods. In these experiments, no significant influence was found in the fertility of the mice immunized with VEGFR1-1087 or VEGFR1-770, as based on the time elapsed from the initiation of cohabitation until parturition. Furthermore, no significant influence was found in the number of pups born of immunized females (Fig. 5B). All females in each experimental group gave birth. Because VEGFR1 is expressed on hematopoietic stem cells and has functional role on the recruitment of hematopoietic stem cells and reconstitution of hematopoiesis (49), we did bone marrow and peripheral blood analyses (refs. 49, 50; Fig. 5C). Ten days after the last immunization, bone marrow cells per femur and total WBC were counted. There was no significant difference of cell counts of bone marrow cells and total WBC between anti-VEGFR1–immunized mice and control mice.

**Discussion**

We developed a novel antiangiogenic cancer vaccine that targets VEGFR1. We selected epitope candidates derived from VEGFR1 based on theoretical binding affinities to HLA-A*0201 and immunized A2/Kb transgenic mice that express the α1 and α2 domains of human HLA-A*0201, a useful animal model for the analysis of human CTL epitopes (6, 44), with epitope candidates. CTL responses, such as increased frequencies of IFN-γ response and potent cytotoxicity, were observed with immunization with the VEGFR1-1087, VEGFR1-770, and VEGFR1-417 peptides. We then showed that peptide-specific CTL clones were successfully established from PBMCs of healthy HLA-A*0201 volunteers with *in vitro* stimulation using VEGFR1-1087 or VEGFR1-770 peptides. However, we could not generate CTLs using VEGFR1-417 peptide. The reason for this discrepancy may be due to the difference of CTL repertoire between human and A2/Kb transgenic mice. It has been shown that there is 29% discordance in the CTL repertoire between human and A2/Kb transgenic mice (44). On the other hand, because we do not have access to A24/Kb transgenic mice, we searched for class I epitopes restricted to HLA-A*2402 using only *in vitro* assays with PBMCs from healthy volunteers bearing HLA-A*2402. We successfully established CTL clones specific to VEGFR1 *in vitro*, and we showed that CTL clones exert potent and specific cytotoxicities against peptide-pulsed target cells and target cells that endogenously express VEGFR1. These findings clearly show that VEGFR1 is immunogenic in humans.

To confirm the *in vivo* effects of immunization with selected peptide restricted to HLA-A*0201 from *in vivo* screening and human CTL generation, we examined whether immunization of A2/Kb transgenic mice with these peptides could suppress tumor-induced angiogenesis and tumor growth. Significant inhibition of tumor-induced angiogenesis was observed with immunization using these peptides in an *in vivo* dorsal air sac assay. These results confirmed that the immunization with VEGFR1-1087 or VEGFR1-770 leads to the inhibition of tumor angiogenesis. Furthermore, significant *in vivo* antitumor effects associated with the immunization of VEGFR1-1087 and VEGFR1-770 peptides have also been observed against various types of mouse tumors including fibrosarcoma, colon carcinoma, and lung carcinoma. We further confirmed significant inhibition of tumor angiogenesis using anti-CD31 staining in the tumor tissue samples. Because tumor angiogenesis has essential role in tumor progression and tumor metastasis, we examined experimental lung metastasis in a therapeutic setting. In the experiment with these tumor models, antitumor effects of immunization with epitope peptide derived from VEGFR1 were shown not only against s.c. tumors but also against experimental lung metastasis.

Although it is a model system with some limitations as described above, A2/Kb transgenic mice are a good model for...
the evaluation of human immune responses against tumor cells with low or no HLA class I expression. To construct tumor systems closely related to the clinical setting, we transplanted tumor cells that were chemically induced in native C57BL/6 mice (H-2Kb) that do not express HLA-A*0201 molecules. Because endothelial cells in A2/Kb transgenic mice express the HLA-A*0201 molecule, the CTLs induced by immunization with HLA-A*0201–restricted VEGFR1 epitope peptides are able to recognize endothelial cells expressing both HLA-A*0201 and VEGFR1. However, these CTLs are unable to recognize tumor cells lacking “human” MHC class I molecules, even if the tumor cells express VEGFR1. Thus, the in vitro antitumor effects of an antiangiogenic vaccine using class I epitopes could be evaluated in an HLA-A*0201–restricted manner. Thus, the results in this tumor system support the notion that the present approach could be effective, even for the patients with tumors having HLA deficits, which is considered to be one of the escape mechanisms employed by malignant tumors. Because this strategy was effective for multiple tumor cell lines, it is now confirmed that VEGFR1 vaccine could be applied to treat multiple types of cancer. Furthermore, tumor endothelial cells are readily accessed by lymphocytes in the bloodstream, and CTLs can directly damage endothelial cells without the penetration of any other tissue type. In addition, the lysis of even a small number of endothelial cells within the tumor vasculature may result in the destruction of vessel integrity, thus leading to the inhibition of numerous tumor cells (51). Therefore, endothelial cells could be a good target for cancer immunotherapy. Because tumor endothelial cells highly express both VEGFR1 and VEGFR2, we further examined for more potent inhibition of tumor growth targeting tumor endothelial cells. As a result, we clearly showed significant inhibition of tumor growth at a greater extent using combination with VEGFR1 epitope peptide and VEGFR2 epitope peptide.

On the other hand, our previous report on antiangiogenic vaccine using VEGFR2-derived peptide has revealed that there are minor but certain adverse effects related to wound healing (6). In our study, no obvious adverse effects have been observed with the immunization with VEGFR1-1087 and VEGFR1-770 peptides, at least in our experiment systems identical to such study. VEGFR1 has multiple functions not only in angiogenesis but also in hematopoiesis. Recent studies have reported that VEGFR1 is expressed on hematopoietic stem cells and it has functional roles on the recruitment of hematopoietic stem cells and reconstitution of hematopoiesis (49). However, our results showed that bone marrow hematopoiesis was not affected in mice immunized with VEGFR1-1087 and VEGFR1-770 peptides. Furthermore, recent studies have reported that bone marrow–derived myeloid cells expressing VEGFR1 (52) and monocytes or pericytes expressing Tie-2 (53) contribute to tumor angiogenesis. We investigated the effect of anti-VEGFR1 immunization using F4/80 antibody or CD11b to stake macrophages in the tumor tissue sample as shown in other investigations (34, 52). However, we could not obtain significant information with immunohistochemistry (data not shown). It is puzzling that this peptide-based vaccination has significant effect on tumor angiogenesis without causing obvious toxicity on VEGFR1-expressing normal cell types. It is possible that there might be some differences how VEGFR1 is involved in pathologic angiogenesis when compared with that in physiologic angiogenesis. Further investigation could clear up this issue. These in vitro and in vivo results strongly suggest that VEGFR1 is a promising target for T-cell–mediated immunotherapy and provide evidence to support the clinical development of this strategy to treat multiple types of cancer.

**Acknowledgments**

We thank Dr. F. James Primus for supplying A2/Kb transgenic mice.

**References**

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