RESULTS.

After perfusing the mice with fluorescein conjugate lectin, CNV volume was measured using a choroidal flatmount technique. The fluorescein leakage index in the control group was 18% compared with the peptide vaccination group, which was reduced to 80% and the CNV volume was measured using a choroidal flatmount after perfusing the mice with fluorescein conjugate lectin.

MATERIALS AND METHODS

The present study investigated the efficacy of an immunotherapy against age-related macular degeneration (AMD) using a choroidal neovascularization (CNV) model. Seven- to 10-week-old A2/Kb transgenic mice expressing human HLA-A*0201 were used. The mice were divided into three groups of 15 animals each: phosphate-buffered saline (PBS) treatment (control); immuand antigen (incomplete Freund adjuvant [IFA]); and antigen peptide of human VEGFR 2 and IFA (peptide vaccination group).

Immunization was given on days 0 and 11. On day 20, six CNVs were induced in both eyes of each animal using a semiconductor laser set to 75 μm, 200 mW, and 0.05 seconds. Leakage from the CNV was measured by fluorescein angiography 7 days after laser treatment. CNV volume was measured using a choroidal flatmount after perfusing the mice with fluorescein conjugate lectin.

Results. There were no significant differences in the leakage or the area of CNV in the IFA-treated group compared with controls. In contrast, the fluorescein leakage index in the peptide vaccination group was reduced to 80% and the CNV area to 18% compared with the control group (P < 0.0001 and P < 0.05, respectively).

Conclusions. This model provides a rationale for immunotherapy using the epitope peptides derived from VEGFR2 for the treatment of CNV. (Invest Ophthalmol Vis Sci. 2008;49: 2143–2147) DOI:10.1167/iovs.07-0523

E xudative age-related macular degeneration (AMD), characterized by choroidal neovascularization (CNV), is a major cause of visual loss in developed countries. Photodynamic therapy has been the main choice for CNV treatment; however, its efficacy is still limited. Molecular analysis revealed that the vascular endothelial growth factor (VEGF) signaling pathway plays a pivotal role in the development of CNV, leading to the development of anti-VEGF therapies. Clinically, inhibition of the VEGF pathway by a VEGF aptamer or an anti-VEGF antibody effectively suppresses CNV activity. However, most therapies need repetitive intravitreal injection of the drugs, which may increase the chance of local adverse effects.

Vaccination therapy targeting VEGFR2 has attracted much attention recently. VEGFR2 plays a crucial role in tumor-associated angiogenesis and vascularization. In previous studies, immunization with CD8 T-cell epitopes, identified from murine VEGFR2\(^2\) or DNA vaccine expressing mouse VEGFR2\(^2\) has been used to target tumor-associated neovascularization in mouse models. In mice, it acts by inducing cytotoxic T lymphocytes (CTLs) targeting endothelial cells, which effectively reduces angiogenesis and inhibits tumor growth. Thus, vaccination with VEGFR2 may afford a novel immunotherapy for the inhibition of tumor growth and other neovascular diseases.

Mouse homologue of human VEGFR2 is considered to be significantly different from the human counterpart. For the analysis of human CTL epitopes that may be directly translated to the clinical setting, A2/Kb transgenic mice provide a unique system. A2/Kb transgenic mice express major histocompatibility complex (MHC) class I molecules composed of the 1a and 2a domains of the HLA-A*0201 class antigen and 3a, cytoplasmic, and transmembrane domains of the mouse H-2Kb class I molecule, and they show 71% concordance with the human CTL repertoire. Thus, A2/Kb transgenic mice have been used by multiple investigators as the model to predict immune responses in humans with HLA-A0201. Published literature indicates that this mouse model provides useful information for prediction of the responses in human.

In this mouse model, vaccination using human VEGFR2 epitope peptides was shown to be associated with significant suppression of tumor-induced angiogenesis without adverse effects. Using A2/Kb transgenic mice and human VEGFR2 epitopes, the present study investigates whether vaccination therapy targeting VEGF-R2 is effective for CNV treatment.

MATERIALS AND METHODS

Reagents

The VEGFR2-derived epitope peptide VEGFR2–773 was synthesized by Sawady Technology (Japan) using the standard solid-phase synthesis method and was purified by reverse-phase high-performance liquid chromatography. Purity (greater than 95%) and identity of the peptides were determined by analytic high-performance liquid chromatography and mass spectrometry analysis, respectively. The following antibodies were used: rat anti-mouse CD4 antibody (BD Biosciences, San Jose, CA), rat anti-mouse CD8a antibody (BD Biosciences), rat anti-mouse CD31 antibody (BD Biosciences), and goat anti-mouse VEGFR2 antibody (R&D Systems, Minneapolis, MN).
Animals
A2/Kb transgenic mice, which express MHC class I molecules with domains 1a and 2a consisting of HLA-A*0201 and domain 3a of mouse H-2Kb, were prepared as has been described elsewhere. C57bl/6 mice were purchased from CLEA Japan. The animals were maintained in the specific pathogen-free Animal Facility of the University of Tokyo, and the University of Tokyo Ethical Committee approved all the protocols for the animal experiments. All experiments were conducted in accordance with the Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

To investigate the effects of VEGFR2 vaccination, the mice were divided into three groups of 15 animals each: phosphate-buffered saline (PBS) treatment (control); immune adjuvant (complete Freund adjuvant [IFA]); and antigen peptide of human VEGFR 2 and IFA (peptide vaccination group). For immunization, IFA-conjugated peptide was subcutaneously injected into A2/Kb transgenic mice on days 0 and 11 based on a previous study. After 20 days, immunization was confirmed by ELISPOT analysis. Accordingly, experimental CNV was induced with laser photocoagulation on day 20 and was analyzed by fluorescein angiography and choroidal flatmount on days 23 and 24, respectively.

Experimental CNV
General anesthesia was induced with intraperitoneal injection (1000 L/kg) of a mixture (7:1) of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celactal; Bayer, Tokyo, Japan) or by inhalation of diethyl ether. Each pupil was dilated with one drop of 0.5% tropicamide (Mydrin M; Santen Pharmaceutical, Osaka, Japan) for photocoagulation. Experimental CNV was created as has been previously described. Laser photocoagulations were applied to each eye between the major retinal vessels around the optic disc with a diode laser photocoagulator (DC-3000; Nidek, Osaka, Japan) and a slit lamp delivery system (SL150; Topcon, Tokyo, Japan) at a spot size of 75 μm, duration of 0.05 second, and intensity of 200 mW.

Immunohistochemistry
To investigate the expression of VEGFR2 and CD31 and the expression of CD4 and CD8+ cells in the CNV lesion, eyes from the mice killed by cervical dislocation 4 days after laser photocoagulation were immediately enucleated and immersed in optimal cutting temperature compound and a slit lamp delivery system (SL150; Topcon, Tokyo, Japan) at a spot size of 75 μm, duration of 0.05 second, and intensity of 200 mW.

Reverse Transcriptase–Polymerase Chain Reaction
To investigate the expression of VEGFR2 at the mRNA level, 20 photocoagulations were applied to one eye of each of the animals, and the expression of VEGFR2 in the retina/choroid was tested 4 days after the photocoagulation. RNA for RT-PCR was isolated with an isolation kit (SV Total RNA Isolation Kit; Promega, Madison, WI) in accordance with the manufacturer’s instructions. cDNA was prepared using SuperScript III for RT-PCR (Invitrogen, Carlsbad, CA). Each PCR was carried out with the manufacturer’s instructions. The sizes of CNV lesions were measured in choroidal flatmounts, as has been described. Mice were anesthetized and perfused with 1 mL PBS containing 50 mg/mL fluorescein-labeled dextran (Sigma, St. Louis, MO), as has been described. After the eyes were enucleated and briefly fixed in 4% PFA, the anterior segment was removed and the retina was carefully dissected from the eyeball. Four to six radial cuts were made from the edge to the equator, and the eyecup was flat mounted with the sclera facing down and was examined by fluorescence microscopy (BX51; Olympus) at 40× magnification. Images were taken with a CCD camera and imported to a computer system, as has been described.

Image Analysis
The area of CNV in the choroidal flatmounts was outlined with the computer mouse and measured using the public domain NIH Image program (developed at the NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/). Image analysis was performed with the observer masked as to treatment.

Statistical Analysis
Signal intensities obtained from fluorescein angiography and CNV areas of the choroidal flatmount were averaged from 12 spots from both eyes, and one value for one animal was used for statistical analysis. The Mann-Whitney U test was used. Values of $P < 0.05$ were considered statistically significant.

RESULTS
VEGFR2 Overexpression in the CNV Lesion
To determine whether VEGFR2 was expressed primarily in the endothelial cells in the CNV model, VEGFR2 expression was confirmed by immunohistologic analysis 4 days after the CNV lesion was induced by laser photocoagulation in C57bl/6 mice. As expected, the results demonstrated that the endothelial cells of the newly grown blood vessels expressed CD31, and such cells expressed VEGFR2 (Figs. 1A, 1B). VEGFR2 was strongly detected in the CNV lesion (Fig. 1B), and real-time PCR analysis demonstrated the upregulation of VEGFR2 4 days after photocoagulation (Fig. 1C). These results demonstrated that VEGFR2 was overexpressed in proliferating endothelial cells under pathologic conditions, similar to neovascularization in a tumor.
**CD8⁺ T Cells Are Responsible for Antiangiogenic Effects**

It has been reported that immunization with recombinant VEGFR2 induces a cytotoxic CD8⁺ T-cell response against the recombinant protein. In vivo effects of vaccination with VEGFR2 epitope peptides on CD8⁺ T cells were investigated using A2/Kb transgenic mice with or without VEGFR2 immunization. Although there was no apparent morphologic difference between the control group and the vaccinated group, immunohistologic analysis demonstrated that the CD8⁺ T cells were located primarily with neovessels in the CNV of mice treated with the VEGFR2 peptide conjugated with IFA, whereas few CD8⁺ T cells were observed in the CNV treated with the IFA alone (Figs. 2B, 2D) Quantitative analysis demonstrated the induction of CD8⁺ T cells only in the CNV lesion of the vaccinated animals (Fig. 2E). In sharp contrast, there was no difference in the presence of CD4⁺ T cells in the CNV lesion between the VEGFR2 immunized mice and the control mice (Figs. 2A, 2C). These results indicate that immunization against VEGFR2 induced the infiltration of CD8⁺ T cells into the CNV lesion.

**In Vivo Antiangiogenic Effects Associated with Vaccination Using Human VEGFR2 Epitope Peptides**

To clarify whether vaccination with epitope peptides derived from VEGFR2 affects the growth of the CNV through the effects on the VEGFR2-expressing endothelial cells of the vessels, the effects of the immunization were analyzed by fluorescein angiography and choroidal flatmount analysis. Leakage from the fluorescein angiography was analyzed as previously described, which demonstrated that a significant inhibition of the CNV leakage was observed in A2/Kb transgenic mice treated with the VEGFR2 peptide conjugated with IFA (954 ± 87 arb), whereas no significant suppression was observed for the CNVs in the A2/Kb mice treated with IFA alone (1237 ± 101 arb) compared with PBS-treated control (1169 ± 95 arb) (Fig. 3). Similarly, choroidal flatmount analysis was used to quantify the CNV area and demonstrated that the CNV area was smaller in the mice treated with the VEGFR2 peptide conjugated with IFA (651 ± 170 mm²) than in the mice treated with IFA alone (852 ± 162 mm²) compared with PBS-treated control (813 ± 145 mm²), in which no significant change was observed (Fig. 4). These results strongly suggest that the cytotoxic effects induced with vaccination using the peptides derived from VEGFR2 are effective in suppressing CNV leakage and growth.

**DISCUSSION**

Vaccination therapy breaking of immune tolerance of self-antigen is a relatively new concept and is an attractive approach that has been studied in the field of cancer therapy. Through the induction of T-cell-mediated autoimmunity against VEGFR2-expressing cells, the current vaccination therapy aims at inducing endothelial cell death and inhibiting pathologic neovascularization. The major advantages of VEGFR2 vaccination are that the prolonged therapeutic effect on the vascular endothelial cells is genetically stable. Similar to the concept in the present study, a recent study investigated the effect of VEGFR2 immunization therapy and demonstrated...
that with the use of dendritic cells immunized with VEGFR2, choroidal neovascularization was inhibited in a mouse model.\textsuperscript{12} However, the results cannot be directly applied in a clinical setting because the mouse homologue of human VEGFR2 in mouse systems is considered to be different from the human counterpart.

A previous study demonstrated that the VEGFR2 peptide used in the present study induces CTLs in the HLA class I-restricted manner.\textsuperscript{9} Importantly, the study used a unique system to examine the effectiveness in systems closely related to clinical settings, such as those using the A2/Kb transgenic mouse system, which is useful for the analysis of human CTL epitopes. It has been demonstrated that potent cytotoxicity has been induced against endothelial cells endogenously expressing VEGFR2 and has been blocked by monoclonal antibodies against CD8 but not against CD4. Using the same VEGFR2

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Representative fluorescein angiograms. (A) Fluorescein angiogram from the control eye. (B) Fluorescein angiogram of the IFA-treated eye. (C) Fluorescein angiogram for the VEGFR2 peptide-treated eye. Leakage from the fluorescein angiography was analyzed, and the results of quantitative analysis are shown in (D). Data are plotted as means; error bars represent the SD. *$P < 0.05$ versus control.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Representative CNV lesion. Representative figures of the CNV are shown for the control eye (A), the IFA-treated eye (B), and the VEGFR2 peptide-treated eye (C). Scale bar, 200 $\mu$m. CNV areas in the choroidal flatmounts were measured, and the results of quantitative analysis are shown in (D). Data are plotted as means; error bars represent the SD. *$P < 0.05$ versus control.}
\end{figure}
peptide and A2/Kb transgenic mice, the present study demonstrated that CD8+ T cells, but not CD4+ T cells, were recruited in the CNV lesion of mice immunized against VEGFR2. The immunization reduced the leakage from and decreased the size of the CNV lesion. Thus, findings from the present study and a previous study7 suggest that immunization against VEGFR2 induces CD8+ T-cell response against vascular endothelial cells and suppresses angiogenesis, which leads to the decrease in the leakage from the CNV lesion and the suppression of CNV growth. The present study shows that immunization with human VEGFR2 inhibits CNV in a model closely related to clinical settings, suggesting that vaccination using epitope peptides derived from human VEGFR2 is a promising tool in CNV treatment.

Anti-VEGF therapy has become a main choice for the treatment of CNV caused by AMD.4-5 Anti-VEGF therapy has been shown to achieve better treatment efficacy than photodynamic therapy for a certain types of exudative AMD. However, all currently available anti-VEGF drugs require repetitive intravitreal drug injection, which exposes patients to the repeated (albeit low) risk for severe complications. Additionally, there is the concern of possible adverse effects such as myocardial infarction and cardiovascular events, though no clinical studies have confirmed the increase of such severe systemic events thus far. Endothelial cells are genetically stable and do not show the downregulation of HLA class I molecules. Thus, one of the major advantages of the immunization therapy against VEGFR2 is that it harbors a long-lasting effect, which is difficult to achieve with other approaches.7

It is assumed that this treatment strategy may be ineffective in already established vessels because VEGFR2 is upregulated only in newly formed vessels. However, recent immunohistochemical analysis of human CNV has demonstrated the overexpression of VEGFR2 colocalizing with CD31+ endothelial cells,13 raising the possibility that this strategy might work on established CNVs. The side effects of this vaccination therapy have been examined in a previous study; and though minor adverse events related to the delay of the wound healing were sporadically seen, no serious side effects were observed. Although further studies are necessary for clinical application, these results provide a strong experimental basis for immunization therapy using the VEGFR2 peptide for CNV treatment.

References