Suppression of Alkali Burn-Induced Corneal Neovascularization by Dendritic Cell Vaccination Targeting VEGF Receptor 2

Hiroshi Mochimaru,1,2,3,4 Tomobiko Usui,4,5 Tomonori Yaguchi,3 Yasubara Nagabama,5 Go Hasegawa,3 Yoshibiko Usui,6 Shigeto Shimmura,2 Kazuo Tsubota,2 Shiro Amano,5 Yutaka Kawakami,3 and Susumu Ishida1,2

PURPOSE. To investigate whether the induction of cytotoxic T lymphocytes (CTLs) targeting VEGF receptor 2 inhibits corneal neovascularization caused by alkali injury.

METHODS. H-2Db–restricted peptide corresponding to amino acids 400 to 408 of VEGF receptor 2 (VEGFR2 400–408) was used as an epitope peptide. Dendritic cells (DCs) were harvested from bone marrow progenitors of C57BL/6 mice. Six-week-old C57BL/6 mice received subcutaneous injections of VEGFR2 400–408 or gp70-pulsed mature DCs three times at 6-day intervals. After the third immunization, corneal neovascularization was induced by alkali injury. Two weeks after the injury, the corneal vascularized area was evaluated by lectin angiography. To confirm the peptide-specific CTL activities in vivo, effector peptides in mice were intraperitoneally injected with an anti-CD4 or -CD8 depletion antibody.

RESULTS. Corneal neovascularization was significantly attenuated in mice immunized with VEGFR2 400–408 compared with those not immunized or immunized with gp70. VEGFR2 400–408 or gp70, but not β-gal 96–103, application led to dose-dependent induction of IFN-γ and TNF-α in the CD8+ T cells co-cultured with stimulator cells. Cytotoxicity assays showed the specific lysis of major histocompatibility complex–matched cells expressing VEGFR2, but not β-gal 96–103. In vivo depletion of CD8+ T cells significantly reversed the suppressive effect of VEGFR2 400–408 immunization on corneal neovascularization to the level observed in nonimmunized or gp70-immunized animals.

CONCLUSIONS. These results indicate the possibility of DC vaccination targeting VEGFR2 as a novel therapeutic strategy for corneal chemical injury. (Invest Ophthalmol Vis Sci. 2008;49:2172–2177) DOI:10.1167/iovs.07-1396

Extensive corneal injury from chemical burns develops conjunctivalization of the corneal epithelial surface with massive neovascularization, leading to severe reductions in corneal transparency and visual acuity. Previous studies demonstrated the involvement of inflammation with conjunctivalization and neovascularization. Of several growth factors and inflammatory cytokines, vascular endothelial growth factor (VEGF) was shown to play a central role in corneal neovascularization, suggesting the potential validity of targeting the VEGF-VEGFR receptor (VEGFR) system. Aptamer- and antibody-based VEGF blockers are now clinically used for ocular neovascular diseases, including age-related macular degeneration, whereas the inhibitory effect of these agents on corneal neovascularization has only recently been confirmed with animal models. Although corneal transplantation surgery is conventionally applied for the treatment of corneal opacity resulting from neovascularization, it is well known that the vascularized cornea substantially decreases the success rate of penetrating keratoplasty. The relatively poor outcome of the current modality has been arousing further interest for the establishment of a novel therapeutic strategy for corneal neovascularization.

As an immunologic approach to combat angiogenesis-dependent solid tumor, the regression of murine renal carcinoma was achieved by interleukin (IL)-12/pulse IL-2 combination therapy eliciting CD8+ cytotoxic T lymphocyte (CTL)-mediated apoptosis of endothelial cells. In the eye as well, we previously demonstrated CD8+ CTL-mediated regression of physiologic and pathologic retinal new vessels. In addition, our recent data using dendritic cell (DC) vaccination targeting VEGFR2 has shown the induction of CD8+ CTLs led to significant suppression of laser-induced choroidal neovascularization. VEGFR2, which plays a pivotal role in endothelial cell proliferation and migration, is upregulated in pathologic corneal vessels. Indeed, oral immunization with recombinant Salmonella typhimurium harboring VEGFR2 proved useful to reduce herpetic keratitis-associated corneal neovascularization. In tumor models, immunization with full-length cDNA or recombinant protein of VEGFR2 successfully inhibited tumor growth and angiogenesis. In particular, specific immunization with the major histocompatibility (MHC) class I–restricted epitope peptides of VEGFR2, which were recently identified in separate studies, led to significant suppression of tumor angiogenesis. In the present study, we investigated for the first time the potential usefulness and efficacy of DC vaccination against a specific VEGFR2 peptide to suppress alkali burn-induced corneal neovascularization.
**Materials and Methods**

**Animals**

Male C57BL/6 mice (CLEA, Tokyo, Japan) at the age of 6 weeks were purchased and maintained in the specific pathogen-free Animal Facility of the Research Park, Keio University School of Medicine. Animals were allowed free access to food and water. A 12-hour light-dark cycle was maintained. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of Corneal Neovascularization**

Corneal neovascularization was induced by alkali injury, as described previously. Briefly, after general anesthesia with xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (35 mg/kg), 2 mL of 0.15 M NaOH was applied onto the corneal surface. Subsequently, total corneal limbus and epithelium were scraped off with a surgical blade under a microscope. Erythromycin ophthalmic ointment was instilled immediately after the operation.

**Quantification of Corneal Neovascularization**

Corneal neovascularization was imaged by lectin angiography. Mice received intravenous injections of BS-1 lectin conjugated with FITC (10 μg/g; Vector, Burlingame, CA) and were killed 30 minutes later. The eyes were enucleated and fixed with 1% paraformaldehyde for 15 minutes. After fixation, the corneas were placed on glass slides and studied by a fluorescence microscopy (Leica, Deerfield, IL), as described elsewhere. Briefly, NIH Image was used for the image analysis. Neovascularization was quantified by setting a threshold level of fluorescence, above which only vessels were depicted. The vascularized area was outlined using the innermost vessel of the limbic arcade as the border.

**Epitope Peptides**

The MHC class I H-2Db–restricted peptide corresponding to amino acids 400 to 408 of murine VEGFR2 (VLTVNPSM; VEGFR2400–408)18 was used for an epitope to induce cellular immunity specific for VEGFR2 in C57BL/6 mice. The gp70 peptide, the epitope sequence of p15E (KSPWFTTL; p15E401–410),21 served as a negative control for VEGFR2-specific immunotherapy and a positive control for cytokine assays, because the gp70 peptide has a potent immunogenicity in C57BL/6 mice. The p15E is the envelope protein of an endogenous murine retrovirus of the Akv family found in the germ line of C57BL/6 mice. We also used the epitope sequence of β-galactosidase (DAPITYTNV; βgal96–103)22 as a negative control for cytokine assays and cytotoxicity assays to confirm the peptide specificity of T-cell responses. Peptides were synthesized and purified with high-performance liquid chromatography (Sigma-Aldrich, St. Louis, MO).

**Preparation of Mature DCs**

Purified DCs were obtained using previously described methods with slight modification. Briefly, marrow from tibias and femurs of C57BL/6 mice were harvested and then followed by DC enrichment with a magnetic cell sorting (MACS) kit (BD Biosciences PharMingen, San Jose, CA). Isolated precursors were cultured in the presence of OKT432 and either 5 mg/kg CD40+ cells (OKT432, 100 μCi of $^{51}$Cr release assay, as previously described. Briefly, target cells were incubated with 10 μg/mL epitope peptides (VEGFR2400–408 or gp70) and pulsed with 10 μM sodium 1,25(OH)2D3. Cytotoxic activities were tested in a 4-hour Na$^{14}$O$_2$O$_4$ ($^{14}$Cr) release assay, as previously described. Briefly, target cells were incubated with 10 μCi of $^{51}$Cr for 60 minutes. Target cells (5 × 10$^5$) were then mixed with effector cells for 4 hours at an effector-to-target ratio of 10:1, 20:1, or 40:1. The amount of $^{51}$Cr release was determined by gamma counting, and the percentage of lysis was calculated with the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.

**In Vivo Depletion of T-Cell Subsets**

Immune cell subsets were depleted in vivo, as previously described. Mice were injected intraperitoneally with 5 mg/kg body weight of either an anti-CD4+ or -CD8 depletion antibody (clone GK1.5 or clone 53-6.72, respectively; eBioscience, San Diego, CA) or an isotype control antibody (BD Biosciences PharMingen) 1 day before and 1 week after alkali injury. The depletion of T-cell subsets was confirmed by flow cytometry using splenocytes from immunized mice.
Suppression of Corneal Neovascularization by Vaccination with VEGFR2\textsubscript{400–408}–Pulsed DCs

The vascularized area after alkali injury was quantified to evaluate the effect of vaccination with VEGFR2\textsubscript{400–408}–pulsed or gp70-pulsed DCs on the development of corneal vascularization (Fig. 1). Interestingly, the VEGFR2\textsubscript{400–408}–immunized mice exhibited significant \((P < 0.05)\) suppression of corneal neovascularization compared with nonimmunized or gp70-immunized mice, suggesting that the observed suppression of corneal neovascularization was attributable to the VEGFR2\textsubscript{400–408} peptide-specific immunologic responses.

Specific Cytokine Responses by Peptide-Induced CD8\textsuperscript{+} T Cells

To confirm the induction of T cells specific for the immunized peptides, we used ELISA to examine the peptide-induced secretion of IFN-\(\gamma\) and TNF-\(\alpha\) in the culture medium (Fig. 2). VEGFR2\textsubscript{400–408} application to CD8\textsuperscript{+} T cells harvested from mice immunized with VEGFR2\textsubscript{400–408} led to significant \((P < 0.01)\) production of both IFN-\(\gamma\) and TNF-\(\alpha\) in a dose-dependent manner (Figs. 2A, 2B). Similarly, a significant \((P < 0.01)\) increase in the production of these cytokines in response to gp70 stimulation was detected in a dose-dependent manner with CD8\textsuperscript{+} T cells from the gp70-immunized mice (Figs. 2C, 2D), in which the vascularized area was not reduced (Fig. 1). In contrast, irrelevant \(\beta\text{-gal}_{96–103}\) application at the maximal dose in the assay (10 \(\mu\)g/mL) did not significantly \((P > 0.05)\) induce the production of these cytokines by CD8\textsuperscript{+} T cells isolated from mice that received VEGFR2\textsubscript{400–408} or gp70 immunization (Figs. 2A–D).

Specific Cell Lysis by VEGFR2\textsubscript{400–408}–Induced CD8\textsuperscript{+} T Cells

\(^{51}\text{Cr}\) release assay was conducted to determine whether T cells could recognize the presentation of H-2Db (MHC class I)-VEGFR2\textsubscript{400–408} complex, thus leading to lysis or killing of target cells (Fig. 3). VEGFR2-positive syngeneic endothelial line H5V cells (H-2Db\textsuperscript{+}, VEGFR2\textsuperscript{+}) served as target cells, whereas the VEGFR2-positive allogeneic endothelial line bEND3 (H-2Db\textsuperscript{–}, VEGFR2\textsuperscript{+}) and the VEGFR2-negative syngeneic line EL4 (H-2Db\textsuperscript{+}, VEGFR2\textsuperscript{–}) functioned as controls. The ability of VEGFR2\textsubscript{400–408}–induced CD8\textsuperscript{+} T cells (effector cells) to lyse MHC-matched H5V endothelial cells was significantly more potent than that of MHC-unmatched bEND3 endothelial cells at all the effector/target ratios examined (Fig. 3A). Importantly, the effector cells significantly killed MHC-matched H5V endothelial cells at the maximal dose, whereas irrelevant peptide \(\beta\text{-gal}_{96–103}\) failed to induce significant cytolytic activity (Figs. 3B–D).

RESULTS

Suppression of Corneal Neovascularization by Vaccination with VEGFR2\textsubscript{400–408}–Pulsed DCs

1 week after the second injection. The percentage of CD4 T cells (CD3\textsuperscript{+} CD4\textsuperscript{+}) or CD8 T cells (CD3\textsuperscript{+} CD8\textsuperscript{+}) was compared between anti-CD4 or anti-CD8 antibody-treated mice and control animals.

Specific Cytokine Responses by Peptide-Induced CD8\textsuperscript{+} T Cells

To confirm the induction of T cells specific for the immunized peptides, we used ELISA to examine the peptide-induced secretion of IFN-\(\gamma\) and TNF-\(\alpha\) in the culture medium (Fig. 2). VEGFR2\textsubscript{400–408} application to CD8\textsuperscript{+} T cells harvested from mice immunized with VEGFR2\textsubscript{400–408} led to significant \((P < 0.01)\) production of both IFN-\(\gamma\) and TNF-\(\alpha\) in a dose-dependent manner (Figs. 2A, 2B). Similarly, a significant \((P < 0.01)\) increase in the production of these cytokines in response to gp70 stimulation was detected in a dose-dependent manner with CD8\textsuperscript{+} T cells from the gp70-immunized mice (Figs. 2C, 2D), in which the vascularized area was not reduced (Fig. 1). In contrast, irrelevant \(\beta\text{-gal}_{96–103}\) application at the maximal dose in the assay (10 \(\mu\)g/mL) did not significantly \((P > 0.05)\) induce the production of these cytokines by CD8\textsuperscript{+} T cells isolated from mice that received VEGFR2\textsubscript{400–408} or gp70 immunization (Figs. 2A–D).
Suppression of Corneal NV by Immunization against VEGFR2

To confirm the role of CD8+ T cells as in vivo effectors in the vaccination-induced suppression of corneal neovascularization (Fig. 1), in vivo depletion of immune cell subsets using an anti-CD4 or -CD8 antibody was performed to mice immunized with VEGFR2 400–408 (Fig. 4). Compared with treatment with an isotype-matched control antibody, antibody-based depletion of CD8+, but not of CD4+, T cells significantly reversed the VEGFR2 400–408-induced reduction of the vascularized area to the level in nonimmunized mice (Fig. 4A). The depletion of T cells in immunized mice was confirmed by flow cytometry 1 week after the second treatment with each depletion antibody (Fig. 4B). The percentage of CD4+ T cells out of splenocytes was 22% with the isotype-matched control compared with 3% with the anti-CD4 antibody. Similarly, the percentage of CD8+ T cells was 12.3% with the isotype-matched control compared with 4.9% with the anti-CD8 antibody.

**DISCUSSION**

The present study reveals, for the first time to our knowledge, that the induction of VEGFR2 peptide-specific CTLs targeting endothelial cells inhibits corneal neovascularization caused by alkali injury (Fig. 1). Vaccination of DCs pulsed with the epitope VEGFR2 400–408 elicited CD8+ T cells (effector cells) effectively killing MHC-matched H5V endothelial cells, but not MHC-unmatched bEND3 cells, at all the effector/target ratios examined (A). Effector cells exhibited the substantial ability to lyse EL4 cells pulsed with VEGFR2 400–408 but not those pulsed with βgal96–103 (B).

**CD8+ T Cells as In Vivo Effector Cells to Inhibit Corneal Neovascularization**

In our peptide-pulsed DC vaccination, VEGFR2 400–408 immunization led to significant suppression of corneal neovascularization compared with no immunization in mice with corneal alkali injury (Fig. 1). Although peptide-specific CTL responses were induced by VEGFR2 400–408 and gp70 (Fig. 2), gp70 immunization did not reduce the area of corneal neovascularization (Fig. 1), suggesting that the suppressive effect observed in the present study resulted from VEGFR2 400–408-specific induction of T-cell response. In the recent report showing that VEGFR2 400–408 immunization inhibited tumor growth and angiogenesis, the adjuvant mixture of IFA, GM-CSF, and an anti-CD40 activating antibody was applied to enhance the specific immunoreaction. Because GM-CSF signaling and CD40 ligation proved to be proangiogenic, these were not used as the vaccine adjuvant in the present study. Instead, we determined the use of DCs, the most potent antigen-presenting cells, validated as effective adjuvant therapy in our recent report showing that the vaccination of VEGFR2 400–408-pulsed DCs led to significant suppression of laser-induced choroidal neovascularization.

Peptide-specific T-cell responses were confirmed by cytokine assays (Fig. 2), indicating that our peptide-pulsed DC vaccination broke immunotolerance against the self-antigen...
VEGFR2400–408. In these assays, IFN-γ and TNF-α were used as indicators for the peptide-specific induction of CTLs. The direction of acquired immunity is regulated by the Th1/Th2 balance, in which Th1 and Th2 CD4+ T cells promote cellular and humoral immunity, respectively, through cytokines inhibitory to each other. IFN-γ, produced by Th1 cells and CTLs, is one of the most important cytokines for the induction of cellular immunity. Moreover, IFN-γ was shown to induce endothelial cell apoptosis and to contribute to CTL-mediated tumor rejection, suggesting its role in the observed suppression of corneal neovascularization in this study. TNF-α is known to be capable of activating cell survival and death. Recent data demonstrated that genetic ablation of TNF-α led to significant suppression of cautery-induced corneal neovascularization, suggesting the antiangiogenic role of TNF-α in the cornea. TNF-α stimulates the nuclear factor-kB pathway leading to cell proliferation, whereas endothelial cells undergo apoptosis through the TNF-α-induced activation of caspase 8. Accordingly, CTL-derived TNF-α is thought to contribute to cytotoxicity, together with perforin and Fas ligand (FasL), each of which triggers key distinct pathways responsible for CTL-mediated apoptosis.

We further confirmed the peptide-specific cytotoxic activity of CD8+ T cells induced by VEGFR2400–408–pulsed DC vaccination (Fig. 3). The substantial ability of VEGFR2400–408–induced CD8+ T cells to kill HSV endothelial cells (H-2Db+, VEGFR2+), but not MHC-unmatched bEND3 endothelial cells (H-2Db+; VEGFR2+), demonstrated that the VEGFR2400–408 epitope peptide was naturally processed and was presented with MHC class I H-2Db by endothelial cells. Moreover, the CTL-induced killing of MHC-matched EL4 nonendothelial cells (H-2Db+; VEGFR2+) pulsed with VEGFR2400–408 but not with the irrelevant peptide βgal46–105, indicated that CD8+ T cells of VEGFR2400–408–immunized C57BL/6 mice recognized the H-2Db-VEGFR2400–408 complex presented on the cell surface, thus leading to effective and selective cytotoxicity. In accordance with the in vitro killing assay (Fig. 3), the in vivo depletion experiments (Fig. 4) indicated CD8+ T cells as the major effector cells for the suppression of corneal neovascularization in our immunotherapy. This is compatible with the previous data showing that CD8+ CTLs as negative regulators of tumor, retinal, and choroidal neovascularization. Given the in vitro results confirming the specific lysis of endothelial cells by VEGFR2400–408–induced CTLs (Fig. 3), the suppressive effect on corneal neovascularization observed in the present study is thought to depend mainly on the specific CTL–endothelial cell interaction.

In the present study, we used the murine model of conjunctivalization of the cornea from alkali burn, a relevant model to reflect chemical injury in human ocular surface leading to vision loss because of corneal scarring and neovascularization. Considering immunotherapy in clinical practice, highly purified peptides for vaccination have several advantages over full-length proteins. Peptides are more easily synthesized, and they lack the potential dangers of infection by recombinant viruses or exposure to exogenous allergens. Clinically, DC vaccination is already established in the cancer field. The sustained effect of immunotherapy may theoretically benefit patients with chemical burn-induced corneal neovascularization; this angiogenic activity lasts at least several months. Long-term attention should be paid, however, to systemic adverse events potentially caused by the antiangiogenic action of VEGFR2-targeting CTLs. Additionally, VEGF signaling through VEGFR2, weakly expressed on normal vascular and nonvascular cells in various organs including the eye, is suggested to play physiologic roles in cell survival and tissue maintenance. Importantly, CTLs induced by active immunization against VEGFR2 in the present and previous studies do not target functional VEGF to block its downstream signaling but induce apoptosis exclusively in endothelial cells that present the epitope peptide(s) naturally processed from VEGF2 protein (VEGFR2400–408) in the present study) with the MHC class I molecule. In previous data on mice receiving anti-VEGFR2 immunotherapy, vaccination of S. typhimurium transfected with a VEGFR2-containing plasmid led to delayed wound healing and negligible impact on fertility. In contrast, vaccination of DCs pulsed with VEGFR2 full-length protein did not affect wound healing. Similarly, mice immunized with VEGFR2400–408, which we used in the present study, exhibited no obvious side effects. In the eye as well, VEGF2400–408 immunization did not affect retinal vasculature or leukocyte recruitment in our study (data not shown). Minimal side effects observed in these series of immunotherapy suggest that the MHC-mediated presentation of the VEGFR2 epitope peptide(s) is preferentially limited to proliferating endothelial cells during tumor growth and corneal chemical injury. However, delayed wound healing, conceivably resulting from cytotoxicity for proliferating endothelial cells, has raised safety concerns as a potential adverse event in future clinical settings.

In summary, the present data are the first to show that VEGF2-specific CTL induction leads to significant suppression of corneal neovascularization caused by chemical injury. These findings indicate the possibility of active immunization as a novel therapeutic strategy to inhibit corneal neovascularization.

References


