

To the Graduate Council:

I am submitting herewith a dissertation written by Bum Seok Kim entitled "HSV-1 Induced Ocular Angiogenesis: Antiangiogenic Strategies to Prevent Herpetic Stromal Keratitis Pathogenesis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Barry T. Rouse
Major Professor

We have read this dissertation
and recommend its acceptance:

Robert N. Moore

Albert T. Ichiki

Mark Y. Sangster

Accepted for the Council:

Anne Mayhew
Vice Chancellor and
Dean of Graduate Studies

(Original signatures are on file with official student records)

**HSV-1 INDUCED OCULAR ANGIOGENESIS:
ANTIANGIOGENIC STRATEGIES TO PREVENT
HERPETIC STROMAL KERATITIS PATHOGENESIS**

A Dissertation Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Bum Seok Kim

August 2005

DEDICATION

This dissertation is dedicated to my parents

Mr. In Sool Kim and Mrs. Young Hee Oh.

ACKNOWLEDGEMENTS

The deepest appreciation goes to my primary mentor, Dr. Barry T. Rouse, who has served as a father figure to me over the last four years and has given me the opportunity to achieve one of my scientific goals. Additionally, I would like to over my profound thanks to the members of my committee, Dr. Robert N. Moore, Dr. Albert T. Ichiki, and Dr. Mark Y. Sangster, for all their time and helpful guidance.

I leave as is to express my thanks to the members of my lab for all of their time and advice over the years. Specifically, I would like to thank Sujin Lee for her help and time in all studies. It has been a great pleasure to interact with the other lab members as well including: Susmit Suvas, Uday Kumaraguru, Partha Biswas, Christopher Dean Pack, Yunsang Lee, Ahmet Kursat Azkur, Daisy Vanitha, Felix Toka, Pranita Sarangi, Kaustuv Banerjee, Luiz Rodrigues, and Jason A. Burchett. The co-works with Dr. Qingquan Tang are also greatly appreciated. I also wish to thank my previous mentors, Dr. Byung Moo Rim and Dr. Chae Woong Lim for their advice and motivation.

Finally, I express my deepest gratitude to my dear parents, my sister Hyun Joo Kim, and all my family members. I am eternally grateful for their everlasting support and faith in all my undertakings.

ABSTRACT

Herpetic stromal keratitis (SK) is an immunopathological and tissue destructive corneal lesion caused by herpes simplex virus (HSV) infection, which induces an intense autoimmune inflammatory response and finally leads blindness. Accumulating evidence using the murine model has shown that Th-1 phenotype CD4⁺ T cells orchestrating the inflammation mainly contribute the immunopathological reaction in HSV-1 infected cornea. However, prior to CD4⁺ T cell infiltration into corneal lesions, various innate immune cells recruit and produce numerous inflammatory molecules into the corneal stroma. Interestingly, one prominent event early in the pathogenesis of SK is neovascularization of the usually avascular cornea. It is assumed that various angiogenic factors produced by inflammatory cells trigger the onset of pathological angiogenesis and the newly formed leaky blood vessels may assist corneal access of inflammatory cells orchestrating herpetic SK. Accordingly, inhibition of unwanted angiogenesis in an HSV infected cornea may moderate the pathologic process of SK lesions.

The first part (Part I) of this dissertation focuses on the understanding of HSV-1 induced SK pathogenesis including how abnormal neovascularization is generated after virus infection and how angiogenesis contributes the process of disease. The other parts (Part II, III, IV) explore the use of different antiangiogenic approaches as an effective therapeutic strategy for herpetic SK. Results in Part II demonstrate that the use of vascular endothelial growth factor (VEGF) pathway-specific siRNAs can efficiently knock down their target genes and consequently inhibit HSV induced angiogenesis. Results of the third section (Part III) clarify the

antiangiogenic role of IL-18 in the cornea. The data show that IL-18 down-regulates the expression of VEGF and also controls the proliferation of activated endothelial cells in inflammatory conditions. Furthermore, application of DNA encoding IL-18 into cornea diminished herpetic SK lesion severity. The final section (Part IV) explores the utilization of a VEGF receptor-2 (VEGFR-2 or FLK-1) based DNA vaccine to enhance immune response against endothelial cells expressing VEGFR-2. HSV-1 infected mice receiving attenuated *Salmonella typhimurium* harboring VEGFR-2 exhibited antiangiogenic response and reduced herpetic SK severity. These effects appeared to be due to the ability of cytotoxic CD8⁺ T cells targeting VEGFR-2 to control virus induced neovascularization.

In this study, experiments were designed to focus on controlling the pathological angiogenesis in herpetic SK model. We hope that these approaches may represent useful therapeutic strategies against neovascularization-related eye diseases.

TABLE OF CONTENTS

Part		Page
I.	Background and Overview.....	1
	Introduction.....	2
	Murine herpetic SK models.....	2
	HSV-1 induced ocular neovascularization.....	5
	Conclusion.....	19
	Specific aims and rationale.....	19
	List of references.....	21
	Appendix.....	39
 II.	 Inhibition of Ocular Angiogenesis by siRNA Targeting Vascular Endothelial Growth Factor-Pathway Genes; Therapeutic Strategy for Herpetic Stromal Keratitis.....	 46
	Abstract.....	47
	Introduction.....	48
	Materials and methods.....	49
	Results.....	58
	Discussion.....	63
	List of references.....	67
	Appendix.....	72

III. Application of Plasmid DNA Encoding IL-18 Diminishes Development of Herpetic Stromal Keratitis by Antiangiogenic Effects.....	88
Abstract.....	89
Introduction.....	90
Materials and methods.....	92
Results.....	99
Discussion.....	104
List of references.....	108
Appendix.....	114
 IV. VEGF Receptor 2 Based DNA Immunization Prevents Herpetic Stromal Keratitis by Antiangiogenic Effects.....	 126
Abstract.....	127
Introduction.....	128
Materials and methods.....	130
Results.....	136
Discussion.....	141
List of references.....	144
Appendix.....	151
VITA.....	165

LIST OF FIGURES

Part	Figure	Page
I	Figure 1. Principle cellular events in herpetic SK pathogenesis.....	40
	Figure 2. New blood vessels formation in the corneal stroma following HSV-1 infection.....	41
	Figure 3. Cellular candidates that induce angiogenesis in SK cornea.....	42
	Figure 4. Schematic representation of neovascularization process in the cornea following HSV-1 infection and its contribution to herpetic SK pathogenesis	43
	Figure 5. Role of VEGF receptor tyrosine kinases.....	45
II	Figure 1. siRNA-mediated <i>in vitro</i> knockdown of VEGF-pathway genes.....	73
	Figure 2. Local delivery of siRNAs targeting VEGF-pathway genes inhibits the CpG ODN-induced angiogenesis	75

Figure 3. Systemic delivery of siRNAs against VEGF-pathway genes inhibits the CpG ODN- induced angiogenesis	77
Figure 4. Increased efficiency of siRNAs systemic delivery by polymer and dose response experiment.....	79
Figure 5. Reduced HSK severity and angiogenic response by administration of siRNAs targeting VEGF-pathway genes	81
Figure 6. Decreased level of VEGF mRNA in the cornea that was infected and treated with siVEGFmix with either local or systemic delivery.....	83
Figure 7. Reduced levels of VEGF protein in the cornea that was infected and treated with siVEGFmix with either local or systemic delivery.....	85
Figure 8. Ocular delivery of FITC-labeled siRNA.....	86
Figure 9. Schematic structure of TargeTran.....	87
III Figure 1. Inhibition of SK lesion severity (A), incidence (B), and HSV-induced angiogenesis (C) by IL-18 DNA administration	115

	Figure 2. Reduced levels of VEGF mRNA and protein in the HSV-1 infected cornea by IL-18 treatment.....	117
	Figure 3. Inhibition of the CpG ODN-induced angiogenesis via diminished VEGF production by IL-18 pretreatment.....	119
	Figure 4. Suppression of VEGF expression and production by IL-18 incubation <i>in vitro</i>	121
	Figure 5. Inhibition of endothelial cell proliferation by IL-18.....	123
	Figure 6. Increased angiogenesis and upregulation of VEGF by IL-18 BP.....	124
IV	Figure 1. Expression of VEGFR-2 on the proliferating endothelial cells of neovessels of SK cornea.....	152
	Figure 2. Functionality of FLK-1 expression and gene transfer from attenuated <i>salmonella typhimurium</i> to Peyer's patches	154
	Figure 3. Inhibition of SK lesion severity, incidence, and HSV-induced angiogenesis by FLK-1 DNA immunization.....	156
	Figure 4. Involvement of FLK-1 specific CD8 ⁺ T cells for the	

antiangiogenic effects in SK cornea.....	158
Figure 5. Inhibition of VEGF-induced corneal angiogenesis by FLK-1 immunization.....	159
Figure 6. Specific lysis of endothelial cells by FLK-1 specific CD8 ⁺ T cells.....	161
Figure 7. Inhibition of angiogenesis by adoptive transfer of FLK-1 specific CD8 ⁺ T cells.....	163

ABBREVIATIONS

CD.....	Cluster of differentiation
CTL.....	Cytotoxic T lymphocyte
CXCR2.....	CXC chemokines receptor 2
ECM.....	Extracellular matrix
FGF.....	Fibroblast growth factor
FLK-1.....	Fetal liver kinase 1
HSV-1.....	Herpes simplex virus 1
IFN- α , γ	Interferon alpha, gamma
IL-1, 2, 6, 12, 17, 18, 23...	Interleukin 1, 2, 6, 12, 17, 18, 23
IL-18 BP.....	Interleukin 18 binding protein
IP-10.....	Interferon inducible protein 10
M-CSF.....	Macrophage colony-stimulating factor
MCP-1.....	Monocyte chemoattractant protein 1
MIP-1, 2.....	Macrophage inflammatory protein 1, 2
MMP-9.....	Matrix metalloproteinase 9
ODN.....	Oligodeoxynucleotide
PECAM.....	Platelet endothelial cell adhesion molecule
PDGF.....	Platelet-derived growth factor
PFU.....	Plaque forming unit
PF-4.....	Platelet factor 4
PMN.....	Polymorphonuclear leukocyte

RNAi.....	RNA interference
RTKs.....	Receptor tyrosine kinases
siRNA.....	small interfering RNA
SK.....	Stromal Keratitis
TGF- β	Transforming growth factor beta
TIMP.....	Tissue inhibitors of metalloproteinases
TLR.....	Toll like receptor
TNF- α	Tumor necrosis factor alpha
VCAM-1.....	Vascular cell adhesion molecule 1
VEGF.....	Vascular endothelial growth factor
VEGFR.....	Vascular endothelial growth factor receptor
VPF.....	Vascular permeability factor

Part I

Background and Overview

INTRODUCTION

Virus infections contribute to the onset of several autoimmune diseases (1-3). One of the autoimmune diseases involved in this category is herpetic stromal keratitis (SK), a lesion of the eye triggered by herpes simplex virus - 1 (HSV-1) infection (1, 3, 4). It is known that a majority of the population is sero-positive for HSV (5), but usually shows few symptoms. However, recurrent lesions on the face, or less frequently occurring eye infections following reactivation of latent virus within the trigeminal ganglion, have appeared under stressful or immunocompromised conditions (6, 7). Although facial lesions are normally mild and quickly disappear, the latter eye lesions may result in severe inflammation and ultimately vision loss (7). Numerous studies have shown that lesions due to herpetic SK appear to be mediated by infiltrating and autoreactive CD4⁺ T cells in the immune privileged cornea after day 7 post infection (8, 9). However, strong inflammation and intensive neovascularization sprouting from preexisting limbal vessels are detectable during viral replicating early phase of SK. These observations implicate that early inflammatory events and pathological angiogenesis may contribute the pathogenesis of herpetic SK (10). This review focuses on understanding and discussion about the close relationship between abnormal angiogenesis and pathogenesis of herpetic SK.

MURINE HERPETIC SK MODELS

Corneal inflammation and ulceration following ocular infection with HSV-1 is the main cause of vision loss in humans (affecting 0.15% of the population) in the developed countries (11). Primary ocular infection with HSV-1 isolated from the infected tissue of herpetic SK patients leads to the development of herpetic SK in

some mice strains (BALB/c and A/J mice) (7, 12, 13). Consequently, the animal model has proven to be highly effective for understanding the pathogenesis of human herpetic SK, since the lesions involved in murine herpetic SK are clinically and histopathologically similar to those of human patient (14). Since human herpetic SK is typically caused by infection with reactivated latent HSV-1 (15), some animal reactivation models have been used for SK studies (16). However, due to the low incidence of SK lesions, and pathologic cellular events in reactivation models similar to them in primary ocular infection models, primary infection models have recently been used more extensively (17).

Inflammatory immune response to HSV-1 ocular infection

Primary ocular infection is performed by direct application of virus into a scratched cornea epithelial layer of susceptible mouse strains. Once the virus successfully infects epithelial cells, viral replication begins and induces mild destruction of epithelial cells (18). Based on the data from tear film or eye swab assays (Fig. 1), high virus titers are detectable for a few days and finally the replication subsides by 6-7 days post infection (19). At this early period (pre-clinical phase), the infection is characterized by the infiltration of activated inflammatory cells (19). It is assumed that viral antigens and several chemoattractant molecules released from damaged corneal tissue may induce the influx of these inflammatory cells into the corneal stroma (20). The major inflammatory cell types that infiltrate into cornea within 12 h post infection are polymorphonuclear leukocytes (PMN, mainly neutrophils). Previous studies have suggested two major roles of initial influx of PMN in the pathogenesis of herpetic SK (16, 19, 21). Firstly, it has been

assumed that PMN control the viral replication and subsequently have a defense mechanism against HSV-1. The fact that depletion of PMN during early phase leads to delayed viral clearance from infected eyes and brain, and activated PMN release antiviral molecules including IFN- γ , TNF- α , and nitric oxide support the idea of advantageous functions of PMN (22, 23). However, herpetic SK is less severe in the absence of PMN (19), suggesting that PMN may contribute to the pathogenesis of herpetic SK. Since it has is known that activated PMN produce various tissue destructive enzymes (24), chemoattractive chemokines (20, 25-27), and proinflammatory cytokines (20, 28, 29), these stimulators may induce tissue destructive inflammatory conditions possibly unmasking unknown auto-antigens related to the immunopathology and subsequently increase herpetic SK severity. In addition to influx of neutrophils, infiltration of CD11b⁺ and CD11C⁺ cells, presumably macrophages (30) and dendritic cells (31, 32), respectively, are also detectable in the SK cornea within 24 h post infection. It is unclear what the role of these cell types is in SK pathogenesis, but it is assumed that the functions of macrophages may be similar to those of neutrophils (as described previously).

The cellular events in the SK cornea at clinical phase (after day 7 post infection) are characterized by huge influxes of T cells escaping from newly formed leaky blood vessels in the stroma (9, 33). Since HSV-1 infected mice without T cells do not develop herpetic SK (34), it is believed that T cells are the major cell types inducing herpetic SK pathology. Our group and others have demonstrated that herpetic SK results from Th1 type CD4⁺ T cells that secrete IFN- γ , TNF- α (35, 36), and IL-2, but not IL-4 and IL-10 (37). Accordingly, treatment of animals with neutralizing antibodies against IFN- γ or IL-2 and overexpression of Th2 type

cytokines, IL-4 or IL-10 significantly diminished herpetic SK (38). Furthermore, a recent report suggested that regulation of CD4⁺ T cells with CD4⁺CD25⁺ regulatory T cells in immunocompetent conditions controlled herpetic SK (39-41). The evidence indicates that Th1 type CD4⁺ T cell is responsible for inducing immunopathogenesis of herpetic SK. Interestingly, a huge influx of PMN is again observed in the SK cornea at this stage and is characterized by a typical biphasic response.

HSV-1 INDUCED OCULAR NEOVASCULARIZATION

HSV-1 ocular infection results in an inflammatory T cell mediated corneal lesion called herpetic SK (7). Although various inflammatory cellular events are involved in the pathogenesis of SK (18), one of the prominent early events in the process of SK is unwanted neovascularization of the normally avascular cornea (10). Newly formed neovessels with high capillary density begin sprouting from the preexisting limbal vessels within 48 h after HSV-1 infection. After this time point, sprouting blood vessels become apparent, and finally reach to the center of ulcerative cornea around day 20 post infection (Fig. 2). Although it remains unclear whether induction of ocular angiogenesis is HSV-1 specific and which multiple molecules are responsible for HSV induced angiogenesis, several recent findings indicate the close relationship between inflammatory cellular events and uncontrolled angiogenesis (42-44). Indeed, inhibiting the angiogenic process results in diminished SK lesions (24, 43, 44). Therefore, it seems likely that the angiogenic events may be an essential step in the pathogenesis of herpetic SK.

The mechanism and process of angiogenesis

Before and after birth, the formation of new blood vessels proceeds physiologically via vascularization for the growth and maintenance of organ tissue (45, 46). Blood vessels supply the oxygen and nutrients to each organ and are also responsible for wound healing (47). Vascularization requires coordinated and sequential steps. Three distinct types of vascularization have been suggested, which are vasculogenesis, arteriogenesis, and angiogenesis (46). During embryonic stages, new blood vessels are first formed by a process referred to as vasculogenesis (48). Vasculogenesis consists of the differentiation of pluripotent endothelial cell precursors to endothelial cells, which ultimately form primitive capillary plexuses (46, 49) and the cardiovascular system. Following the process of vasculogenesis, rapid proliferation of arterial collateral blood vessels occurs by a process of arteriogenesis (50). These collateral blood vessels possess more complex structures consisting of endothelial cells, smooth muscle cells and pericytes (46). Thus vasculogenesis and arteriogenesis are physiological processes that do not occur in the adult. Under the influence of pathologic stimulators, however, the distinct process of blood vessel formation from pre-existing blood vessels in the adult occurs and it is called angiogenesis (51). Angiogenesis is a complex process consisting of endothelial cell proliferation and migration, proteolysis of extracellular membrane, capillary tube formation, and remodeling of vascular wall (46, 47). Under normal or infectious conditions, the process of angiogenesis is a necessary step for wound healing (47), the female reproductive cycle (52), and ischemic restoration (53). Although almost an angiogenesis resulting from pathologic conditions is beneficial for the resolution of wounds and inflammation, there are

some instances in which unwanted angiogenesis induces detrimental effects (54-56). The typical examples of the latter case are tumor angiogenesis and neovascular related eye diseases. The hypothesis that angiogenesis is essential for tumor growth was first proposed by Dr. Judah Folkman group (57, 58) and numerous studies have demonstrated this hypothesis (59, 60). Furthermore, uncontrolled angiogenesis is found in eye diseases including macular degeneration, diabetic retinopathy and herpetic SK (61).

Release of angiogenic stimulators: angiokines

Angiogenic growth factors are produced from various cell types, such as inflammatory or tumor cells in pathologic conditions (62). Numerous angiokines have been discovered and reveal their distinct role in angiogenesis (63, 64). Almost all angiokines can be divided into two types. The first type consists of the vascular cell growth factors, including VEGFs (65), fibroblast growth factors (FGFs) (66), and angiopoietin 2 (67). In general, once these growth factors bind each receptor of the endothelial cells, signaling pathways such as MAPK and Akt are activated (68) and subsequent signaling promotes endothelial cell proliferation, migration, survival, and proteolytic activity (69). It is known that almost all vascular growth factors are produced from activated tumor cells (70), inflammatory PMN (71), macrophages (62), and activated endothelial cells (72). The second type of angiokines consists of proinflammatory molecules, including various cytokines and chemokines. Proinflammatory cytokines such as IL-1 (28, 29), IL-6 (20), IL-17 (73), M-CSF (74), TGF- β (75), and TNF- α (76) released from various cell types directly induce expression of growth factors including VEGF and FGF-2. Chemokines such as

monocyte chemoattractant factor-1 (MCP-1) (77), a potent chemoattractant of T cells and monocytes, and macrophage inflammatory protein (MIP-2, CXCL1)(78), a potent chemoattractant of neutrophils, also lead to the recruitment of inflammatory cells producing angiogenic factors.

Activation of the endothelial cells and remodeling of neovessels

Angiokines released from inflammatory origin diffuse into the nearby tissues and reach the quiescent endothelial cells of preexisting blood vessels. Once the diffused angiokines bind to their specific receptors expressed on the endothelial cells, these cells become activated and begin to produce new molecules including proteolytic enzymes and angiogenic factors by autocrine fashion for remodeling of the extracellular matrix (ECM). The enzymes, mainly of the matrix metalloproteinases (MMPs and tissue inhibitors of metalloproteinases (TIMPs)) family and plasminogen activators (such as urokinase plasminogen activator (uPA) and its inhibitor, PAI-1) (79-81), degrade the underlying basement membrane surrounding all preexisting blood vessels. Then activated endothelial cells begin to proliferate and migrate through the dissolved holes into nearby tissue stroma. During migration of endothelial cells, integrins are considered to positively regulate the remodeling process of vascular cells (82). Integrins are endothelial cell surface receptors and assist vascular cells to build new blood vessels (83, 84). The $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins have been thought to help the formation of blood vessel since antagonists against these integrins diminish pathological angiogenesis (82). Furthermore, adhesion between integrin $\alpha 4 \beta 1$ of endothelial cells and VCAM-1 of mural cells is required for blood vessel formation (85). However, one report

suggests that some integrins inhibit angiogenesis by suppressing VEGF signaling dependent endothelial cell survival (82).

Stabilization of newly formed blood vessels

To become a functional vascular network, newly formed nascent vessels require stabilization. The association of pericytes, extracellular matrix, and smooth muscle cells with newly formed vessels inhibits angiogenesis through regulation of endothelial activity and provides structural support. Platelet-derived growth factor (PDGF)-BB has been considered to have roles in the stabilization of new blood vessels, since it induces recruitment of PDGFR- β -positive mesenchymal progenitor cells (82). Another molecule to tighten blood vessels is the Tie-2 receptor, which is activated by angiopoietin-1 (82). Signaling between Tie-2 and angiopoietin-1 promotes the interaction between endothelial cells and mural cells (86). Consequently, nascent blood vessels become durable and mature vessels after stabilization.

Contribution of unwanted angiogenesis during the pathogenesis of herpetic SK

One prominent characteristic in the pathogenesis of herpetic SK involves neovascularization of the normally avascular cornea. Angiogenic sprouting with high capillary density from preexisting limbal vessels is evident by day 2 post infection and subsequently thick and durable vessels grow and reach the central cornea with ulceration by day 20 post infection. Most studies on herpetic SK have focused on the issues of autoinflammatory cellular and molecular events, but recently our group has questioned how ocular infection with HSV-1 induces

abnormal angiogenesis and how this process contributes the pathogenesis of herpetic SK. Although it remains to be more defined, evidence from several experiments demonstrates that multiple molecules are involved in HSV-1 induced angiogenesis and abnormal angiogenesis seems to be a necessary step in herpetic SK pathogenesis (10, 20, 22, 24, 28, 29).

Virus-induced diseases characterized by pathological angiogenesis have been suggested, and their pathogenesis has been revealed by numerous research efforts. HIV-1 Tat protein directly induces angiogenesis since the Tat arginine- and lysine- rich sequence is similar to that of other angiogenic factors, such as VEGF, and Tat specifically binds and activates the VEGFR-2 (87). Hepatitis B virus X protein (HBx) of the hepatitis B virus (HBV) is strongly implicated in angiogenesis during hepatocarcinogenesis through activation of hypoxia inducible factor-1 (HIF-1a) (88). The G protein-coupled receptor oncogene (vGPCR) of the Kaposi's sarcoma (KS) associated herpesvirus (KSHV) induces angiogenesis by VEGF secretion and activation of the VEGFR-2 (88). Orf virus encodes a homolog (NZ2) of the VEGF family of genes and it induces angiogenesis by binding to VEGFR-2 and neuropilin-1 (89). However, in the pathogenesis of herpetic SK, unlike some other virus related to the induction of angiogenesis, HSV-1 replication itself is not likely to directly induce angiogenesis since HSV-1 appears not to encode any angiogenic protein. Accumulating evidence indicates that innate immune cells such as neutrophils (PMN) and macrophages are responsible for inflammation and ongoing angiogenesis in the pathogenesis of herpetic SK (62, 71) (Fig. 3). As previously mentioned, these innate immune cells are observed in the subconjunctival area within 12 h post infection and quickly infiltrate into the

stromal layer within 24 h post infection. Although the antiviral role of innate inflammatory cells through IFN signaling may help for clearance of replicating virus and phagocytosis of infected cells by innate immune cells can suppress viral pathogenesis, activated neutrophils and macrophages produce enormous angiokines such as proinflammatory cytokines, chemoattractant chemokines, and endothelial cell growth factors. For example, FGF-2 (unpublished data) known as a classical angiogenic factor and MMP-9 (24), one of the major tissue destructive enzymes, are highly expressed in SK cornea and are believed to contribute HSV induced angiogenesis. Among several angiokines, high levels of VEGF production, which is a potent inducer of pathological angiogenesis, is evident in the cornea during all periods of SK (10). VEGF mRNAs and proteins are detectable by 12 h and 24 h post infection, respectively (10). Additional evidence shows that biphasic pattern of PMN recruitment reflects the similar pattern of VEGF expression in the SK corneas (10). Furthermore, corneal epithelial cells and stromal fibroblast cells including keratocytes are also considered to be sources of VEGF (18). During the clinical phase, the newly formed and highly permeable blood vessels still grow toward to the center of cornea. At this stage, by several unidentified chemoattractants, bystander CD4⁺ T cells reach the cornea by escaping from the blood stream into the stroma through gaps between vascular endothelial cells. Since CD4⁺ T cells are responsible for inducing severe autoimmune inflammatory responses in SK cornea, successful infiltration of CD4⁺ T cells into the inflammatory cornea should be crucial event in the pathogenesis of herpetic SK. Notably, new blood vessels act as a route for CD4⁺ T cells to reach into the stromal area. These observations suggest that VEGF

molecules may be a strong stimulator of HSV-1 induced angiogenesis and that neovessels contribute to herpetic SK pathogenesis (Fig. 4).

The next question is what signals induce VEGF production in the SK cornea. Infiltrated innate inflammatory cells may constitutively produce VEGF, but it is conceivable that some bystander activators may also be involved in this process. The first candidate as stimulator for VEGF is HSV-1 DNA, which was previously suggested by our group (18). Implantation of pellets including HSV-1 DNA into normal cornea induces angiogenesis by upregulation of VEGF expression. The precise mechanisms by which HSV DNA induces VEGF expression were demonstrated by *in vitro* and *in vivo* experiments. Sequence analysis of HSV-1 DNA revealed that HSV DNA has plenty of bioactive CpG motifs, and treatment of these bio-active CpG motifs into macrophage cell lines stimulates VEGF expression. Furthermore, similar to HSV-1 DNA, CpG pellet implantation induces angiogenesis (18, 90), but methylated CpG implantation abrogates the angiogenic effects of bioactive CpG motifs. Although detailed mechanisms of how CpG motifs stimulate VEGF production are yet to be elucidated, interaction of CpG and TLR9, a specific receptor for CpG, may induce a signal transduction through a Myd88 dependent or independent pathway and subsequently activate various signal transcription factors stimulating VEGF expression.

Additional stimulators for VEGF production may be various cytokines produced by innate inflammatory cells under SK conditions. Accumulating evidence suggests that some proinflammatory cytokines such as IL-1 (28, 29), IL-6 (20), IL-17 (91), and IL-23 (unpublished data) are highly overexpressed in the SK cornea. A direct injection of IL-1 or IL-6 protein into normal corneal stroma induces

angiogenesis through upregulation of the VEGF molecule and blocking of IL-1 with IL-1 receptor antagonist protein can reduce HSV-induced angiogenesis. Recently some data regarding the role of IL-17 and IL-23 in herpetic SK indicates that upregulated IL-17 by HSV infection directly induces VEGF expression (91) and IL-23 indirectly stimulates VEGF through upregulation of IL-17 (unpublished data).

Some final possible candidates that induce angiogenesis in SK cornea are chemokines that can attract innate inflammatory cells, since several chemokines having E-L-R motifs are known to induce angiogenesis (92). In SK studies, MIP-2 (CXCL1) and MCP-1 (CCL2) are upregulated in the cornea following HSV-1 ocular infection (93). Although it has not yet been clearly defined whether overexpression of MCP-1, a potent chemoattractant of T cells and monocytes, in the SK cornea highly recruits inflammatory cells expressing its receptor (CCR2) and induces angiogenesis, the role of this chemokine has been implicated in inflammatory angiogenic response in brain and retinal diseases (94). Additionally, it was reported that neutralization of MIP-2 significantly diminished the influx of innate immune cells into the SK cornea (95). Further evidence is provided by the *in vivo* study that MIP-2 induced angiogenesis is mediated by neutrophil-derived VEGF-A (78). These examples indicate that several chemokines indirectly induce angiogenesis by recruitment of inflammatory cells producing various angiokines. Since the influx of various innate and adaptive immune cells to inflammatory lesions is crucially mediated by chemoattractants, numerous uninvestigated and unidentified chemokines may be involved in the herpetic SK pathogenesis.

Role of VEGF family genes

Identification and activities of VEGF molecule

VEGF is a crucial regulator of physiological angiogenesis related to fetal embryogenesis (96, 97) and the female reproductive system (98) as well as pathological angiogenesis associated with tumor growth (99) and neovascular eye diseases, such as age-related macular degeneration (100) and diabetic retinopathy (101). In 1983, Senger *et al.* first purified a protein from a guinea pig tumor cell line, which induces vascular permeability, which they named “tumor vascular permeability factor” (VPF) (102). Six years later, Ferrara and Henzel isolated an endothelial cell-specific mitogen from bovine pituitary follicular cells, which they named “vascular endothelial growth factor” (103). However, cDNA cloning of VEGF and VPF revealed that both angiokines were the same molecule (65). The finding of this molecule was innovative since VEGF induces both endothelial cell proliferation and vascular permeability, which is unlikely in other angiokines (65). For the last decade, numerous research efforts have been focused on this molecule to define the biological and pathological role of it. It has been known that VEGF (commonly referred to VEGF-A) belongs to a gene family including placenta growth factor (PlGF), VEGF-B, C, and D (72). VEGF (VEGF-A) is considered to be the major angiogenic stimulator of blood vessels, and VEGF-C and D has been known as regulator of lymphangiogenesis. VEGF has four different isoforms, having 121, 165, 189, and 206 amino acids, with VEGF₁₆₅ being the predominant isoform (72).

Activities of VEGF are multi-functional. The main function of VEGF is to influence the fate of vascular systems. Once VEGF interacts with vascular

endothelial cells, they become activated and being the process of angiogenesis, which includes the proliferation and migration of endothelial cells. In addition, VEGF enhances the survival of endothelial cells through preventing apoptosis and expressing the anti-apoptotic molecule Bcl-2, survivin, and A1 from endothelial cells (104). Unlike other angiokines, VEGF induces vascular permeability and leakage in inflammatory circumstances. It also induces vasodilation by endothelial cell-derived nitric oxide (105). Secondly, VEGF has effects on non-endothelial cell types. VEGF attracts monocytes by chemotactic fashion and controls survival of hematopoietic stem cells, as well as bone marrow cells (106).

Interaction of VEGF with VEGF receptor tyrosine kinases

VEGF binding receptors are expressed in the cell surface of vascular endothelial cells, as well as on bone marrow-derived cells (107). Three types of receptors for VEGF were identified as VEGFR-1, VEGFR-2, and VEGFR-3 (72) (Fig. 5). VEGF binds two related receptor tyrosine kinases (RTKs), VEGFR-1 and -2 that are normally expressed on the surface of blood vessel endothelial cells (72). VEGFR-3 is not a receptor for VEGF, but it is for VEGF-C and-D and it is restrictively expressed in lymphatic endothelial cells (108). Accumulating evidence has revealed the role of each receptors signaling as a consequence of VEGF interaction. Based on *in vitro* and *in vivo* results, VEGFR-2 has an important role in pathological angiogenesis (72). Although both VEGFR-1 and -2 are expressed in the endothelial cells under the physiological conditions, activated and proliferating endothelial cells markedly upregulate the expression of VEGFR-2 (109). VEGFR-2 is the major receptor involved in the endothelial cells survival, mitogenic,

angiogenic, and permeability of blood vessels (72). In contrast, VEGFR-1 mediates the embryogenic development through release of growth factors, but is not involved in mitogenic signaling in endothelial cells (72). VEGFR-1 binds not only VEGF-A but also VEGF-B and PLGF (72). Interestingly, the soluble form of VEGFR-1 by an alternative splicing inhibits VEGF activity through prevention of VEGF binding to VEGFR-2 (110).

VEGF family genes in pathologic conditions

Several studies have implied that VEGF family genes are closely involved in the pathogenesis of diseases related to uncontrolled angiogenesis. Most extensive studies relating to angiogenesis have been done in the wide variety of tumor models (57, 58). Tumor cells need a supply of enough oxygen as well as nutrition to survive and proliferate (111). Tumor cells as well as tumor-associated stroma produce high amounts of VEGF molecules and the released VEGF spreads and binds to its receptors expressed by nearby vascular endothelial cells. Therefore, inhibition of VEGF family genes by various antiangiogenic approaches has successfully suppressed the growth and spread of tumor cells (57, 58, 111). The examples of VEGF family gene inhibitors include antibodies against VEGF and VEGFR-2 (112), antisense oligonucleotides (113), molecules inhibiting VEGFR-2 signal transduction (114), and a soluble VEGFR-1 inhibiting VEGF activities (110).

Abnormal angiogenesis and vascular leakage caused by secondary effects due to diabetes or age are a major cause of blindness worldwide. Intraocular overexpression of VEGF molecules is observed in the coroidal neovascular membrane from patients with age-related macular degeneration (100) or in the

aqueous and vitreous humor of the eyes of diabetes mellitus patients (101). In the latter case, VEGF is strongly upregulated in the ischemic retina and it may leak and accumulate in the aqueous humor of retinopathy patient.

Upregulation of VEGF molecule has been also implicated in inflammatory disorders. Although VEGF inducing angiogenesis enhances the capacity of tissue repair and wound healing, it also contributes to the tissue destructive inflammatory responses. High levels of VEGF are found in the synovial fluid of rheumatoid arthritis patients and VEGFR-1 signaling has an important role in the development of inflammatory arthritis (101). Brain adema induced by focal cerebral ischemia is also mediated by VEGF expression in the brain since hypoxia is a major stimulator for VEGF expression (115). Furthermore, *Bartonella bacilliformis* infection causing verruga peruana syndrome characterized by the development of hemangioma-like lesions induces epidermal production of VEGF (116).

Antiangiogenic strategies and therapeutic implications

As previously described, angiogenesis is mediated by complicated processes, including the involvement of various angiokines and the cells producing them. Further, pathologic angiogenesis is also often caused by the same angiokines that are responsible for physiologic angiogenesis. For example, the VEGF molecule is either beneficial for ischemic peripheral vascular disease or has detrimental effects for inflammatory areas and leads to VEGF-mediated ocular complications, depending on the tissues applied (117). Therefore, the angiokine agonist-antagonist paradox should be considered when designing therapeutic strategies against neovascular disorders.

In animal studies and human clinical trials, inhibition of angiokines including VEGF family genes by a variety of approaches has resulted in suppression of the progress of some neovascular diseases. Conventionally, application of angiogenesis inhibitors, such as TNP-470 (synthetic analogue of *fumagillin*) (118) or immunosuppressive drug rapamycin (119), and blocking antibody against angiokines and their receptors (112) efficiently inhibits primary and metastatic tumor growth by antiangiogenic effects. In addition, natural antiangiogenic molecules including endostatin (120), IFN- α (121), IL-12 (122), and TIMP-1 (123) have been shown their antiangiogenic effects in various experimental models. Recently, previously unidentified natural molecules that have antiangiogenic functions are being discovered and tested. Furthermore, knock-down of endogenous target genes by the use of siRNAs (43, 124) or antisense oligonucleotides (125) has been explored and these approaches are currently pending for future human trials. Interestingly, recent studies have shown that, using immunological concepts, immunization, targeting cells expressing angiokines and their receptors, successfully induces immune responses against self antigens and can regulate overgrowth of unwanted vessels in animal models (109, 126).

Since pathological angiogenesis occurs in lesions under complicated and complex processes of molecular and cellular events, and each neovascular disease has different stages of pathogenesis, strategies that target single molecule or that applies a single treatment may not be sufficient to control angiogenesis. Some evidence also have suggested that angiokines may be critical during the initial angiogenic stages (127). Such notions should be considered important for the design of experimental and clinical trials.

CONCLUSION

Prophylactic or therapeutic approaches have been suggested for regression of herpetic SK pathogenesis. For instance, corticosteroid (128) or antiviral treatment could reduce disease progression in animal models. However, many other cellular and molecular events that contribute to SK pathogenesis may become a future target for herpetic SK therapy. Considering one of these contributors, production of various angiokines is evident and subsequent crucial angiogenic steps are involved in the whole disease processes of murine herpetic SK. Therefore, antiangiogenic approaches may prove to be useful strategies to suppress the severity of herpetic SK. We anticipate that understanding the various antiangiogenic approaches in murine herpetic SK model may be helpful for the future design of therapeutic strategies against neovascular related ocular diseases, including herpetic SK.

SPECIFIC AIMS AND RATIONALE

Studies in mouse models have revealed that pathogenesis of herpetic SK is mediated by complex cellular and molecular events. Interestingly, inflammatory neovascularization in normally avascular cornea is prominent following HSV-1 infection. Although a large number of angiokines may be involved in SK angiogenesis, VEGF, one of the key regulators of angiogenesis, has been suggested as a critical contributor of HSV-induced angiogenesis. Therefore, to inhibit ocular angiogenesis and consequent SK lesions, the studies described here have been

aimed at evaluating the various antiangiogenic strategies which target VEGF family genes.

LIST OF REFERENCES

1. Panoutsakopoulou, V., and H. Cantor. 2001. On the relationship between viral infection and autoimmunity. *J Autoimmun* 16:341.
2. von Herrath, M. G., and M. B. Oldstone. 1996. Virus-induced autoimmune disease. *Curr Opin Immunol* 8:878.
3. Zhao, Z. S., F. Granucci, L. Yeh, P. A. Schaffer, and H. Cantor. 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science* 279:1344.
4. Rouse, B. T., and S. Deshpande. 2002. Viruses and autoimmunity: an affair but not a marriage contract. *Rev Med Virol* 12:107.
5. Christenson, B., M. Bottiger, A. Svensson, and S. Jeansson. 1992. A 15-year surveillance study of antibodies to herpes simplex virus types 1 and 2 in a cohort of young girls. *J Infect* 25:147.
6. Honda, N., N. Hato, H. Takahashi, H. Wakisaka, H. Kisaki, S. Murakami, and K. Gyo. 2002. Pathophysiology of facial nerve paralysis induced by herpes simplex virus type 1 infection. *Ann Otol Rhinol Laryngol* 111:616.
7. Streilein, J. W., M. R. Dana, and B. R. Ksander. 1997. Immunity causing blindness: five different paths to herpes stromal keratitis. *Immunol Today* 18:443.
8. Mercadal, C. M., D. M. Bouley, D. DeStephano, and B. T. Rouse. 1993. Herpetic stromal keratitis in the reconstituted scid mouse model. *J Virol* 67:3404.
9. Russell, R. G., M. P. Nasisse, H. S. Larsen, and B. T. Rouse. 1984. Role of T-lymphocytes in the pathogenesis of herpetic stromal keratitis. *Invest Ophthalmol Vis Sci* 25:938.

10. Zheng, M., S. Deshpande, S. Lee, N. Ferrara, and B. T. Rouse. 2001. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. *J Virol* 75:9828.
11. Liesegang, T. J., L. J. Melton, 3rd, P. J. Daly, and D. M. Ilstrup. 1989. Epidemiology of ocular herpes simplex. Incidence in Rochester, Minn, 1950 through 1982. *Arch Ophthalmol* 107:1155.
12. Foster, C. S., Y. Tsai, J. G. Monroe, R. Campbell, M. Cestari, R. Wetzig, D. Knipe, and M. I. Greene. 1986. Genetic studies on murine susceptibility to herpes simplex keratitis. *Clin Immunol Immunopathol* 40:313.
13. Shimeld, C., T. Hill, B. Blyth, and D. Easty. 1989. An improved model of recurrent herpetic eye disease in mice. *Curr Eye Res* 8:1193.
14. Garweg, J. G., and M. Halberstadt. 2002. [The pathogenesis of herpetic keratitis]. *Klin Monatsbl Augenheilkd* 219:477.
15. Dong, X. G., L. X. Xie, and W. Y. Shi. 1994. [Reactivation of latent virus in corneas of quiescent recurrent herpes simplex keratitis (HSK)]. *Zhonghua Yan Ke Za Zhi* 30:360.
16. Thomas, J., and B. T. Rouse. 1997. Immunopathogenesis of herpetic ocular disease. *Immunol Res* 16:375.
17. Miller, J. K., K. A. Laycock, J. A. Umphress, K. K. Hook, P. M. Stuart, and J. S. Pepose. 1996. A comparison of recurrent and primary herpes simplex keratitis in NIH inbred mice. *Cornea* 15:497.
18. Biswas, P. S., and B. T. Rouse. 2005. Early events in HSV keratitis--setting the stage for a blinding disease. *Microbes Infect* 7:799.

19. Thomas, J., S. Gangappa, S. Kanangat, and B. T. Rouse. 1997. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. *J Immunol* 158:1383.
20. Banerjee, K., P. S. Biswas, B. Kim, S. Lee, and B. T. Rouse. 2004. CXCR2-/- mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6-induced neovascularization. *J Immunol* 172:1237.
21. Thomas, J., and B. T. Rouse. 1998. Immunopathology of herpetic stromal keratitis: discordance in CD4+ T cell function between euthymic host and reconstituted SCID recipients. *J Immunol* 160:3965.
22. Daheshia, M., S. Kanangat, and B. T. Rouse. 1998. Production of key molecules by ocular neutrophils early after herpetic infection of the cornea. *Exp Eye Res* 67:619.
23. Tumpey, T. M., S. H. Chen, J. E. Oakes, and R. N. Lausch. 1996. Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. *J Virol* 70:898.
24. Lee, S., M. Zheng, B. Kim, and B. T. Rouse. 2002. Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus. *J Clin Invest* 110:1105.
25. Thomas, J., S. Kanangat, and B. T. Rouse. 1998. Herpes simplex virus replication-induced expression of chemokines and proinflammatory cytokines in the eye: implications in herpetic stromal keratitis. *J Interferon Cytokine Res* 18:681.
26. Tumpey, T. M., H. Cheng, D. N. Cook, O. Smithies, J. E. Oakes, and R. N. Lausch. 1998. Absence of macrophage inflammatory protein-1alpha

- prevents the development of blinding herpes stromal keratitis. *J Virol* 72:3705.
27. Tumpey, T. M., H. Cheng, X. T. Yan, J. E. Oakes, and R. N. Lausch. 1998. Chemokine synthesis in the HSV-1-infected cornea and its suppression by interleukin-10. *J Leukoc Biol* 63:486.
 28. Biswas, P. S., K. Banerjee, M. Zheng, and B. T. Rouse. 2004. Counteracting corneal immunoinflammatory lesion with interleukin-1 receptor antagonist protein. *J Leukoc Biol* 76:868.
 29. Biswas, P. S., K. Banerjee, B. Kim, and B. T. Rouse. 2004. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. *J Immunol* 172:3736.
 30. Bauer, D., S. Mrzyk, N. van Rooijen, K. P. Steuhl, and A. Heiligenhaus. 2000. Macrophage-depletion influences the course of murine HSV-1 keratitis. *Curr Eye Res* 20:45.
 31. Jager, M. J., D. Bradley, S. Atherton, and J. W. Streilein. 1992. Presence of Langerhans cells in the central cornea linked to the development of ocular herpes in mice. *Exp Eye Res* 54:835.
 32. Jager, M. J., S. Atherton, D. Bradley, and J. W. Streilein. 1991. Herpetic stromal keratitis in mice: less reversibility in the presence of Langerhans cells in the central cornea. *Curr Eye Res* 10 Suppl:69.
 33. Metcalf, J. F., D. S. Hamilton, and R. W. Reichert. 1979. Herpetic keratitis in athymic (nude) mice. *Infect Immun* 26:1164.

34. Gangappa, S., S. P. Deshpande, and B. T. Rouse. 1999. Bystander activation of CD4(+) T cells can represent an exclusive means of immunopathology in a virus infection. *Eur J Immunol* 29:3674.
35. Minagawa, H., K. Hashimoto, and Y. Yanagi. 2004. Absence of tumour necrosis factor facilitates primary and recurrent herpes simplex virus-1 infections. *J Gen Virol* 85:343.
36. Keadle, T. L., N. Usui, K. A. Laycock, J. K. Miller, J. S. Pepose, and P. M. Stuart. 2000. IL-1 and TNF-alpha are important factors in the pathogenesis of murine recurrent herpetic stromal keratitis. *Invest Ophthalmol Vis Sci* 41:96.
37. Stumpf, T. H., C. Shimeld, D. L. Easty, and T. J. Hill. 2001. Cytokine production in a murine model of recurrent herpetic stromal keratitis. *Invest Ophthalmol Vis Sci* 42:372.
38. Daheshia, M., N. Kuklin, E. Manickan, S. Chun, and B. T. Rouse. 1998. Immune induction and modulation by topical ocular administration of plasmid DNA encoding antigens and cytokines. *Vaccine* 16:1103.
39. Rouse, B. T., and S. Suvas. 2004. Regulatory cells and infectious agents: detentes cordiale and contraire. *J Immunol* 173:2211.
40. Suvas, S., A. K. Azkur, B. S. Kim, U. Kumaraguru, and B. T. Rouse. 2004. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* 172:4123.
41. Suvas, S., U. Kumaraguru, C. D. Pack, S. Lee, and B. T. Rouse. 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 198:889.

42. Biswas, P. S., K. Banerjee, B. Kim, J. Smith, and B. T. Rouse. 2005. A novel flow cytometry based assay for quantification of corneal angiogenesis in the mouse model of herpetic stromal keratitis. *Exp Eye Res* 80:73.
43. Kim, B., Q. Tang, P. S. Biswas, J. Xu, R. M. Schiffelers, F. Y. Xie, A. M. Ansari, P. V. Scaria, M. C. Woodle, P. Lu, and B. T. Rouse. 2004. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. *Am J Pathol* 165:2177.
44. Zheng, M., M. A. Schwarz, S. Lee, U. Kumaraguru, and B. T. Rouse. 2001. Control of stromal keratitis by inhibition of neovascularization. *Am J Pathol* 159:1021.
45. Carmeliet, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6:389.
46. Buschmann, I., and W. Schaper. 1999. Arteriogenesis Versus Angiogenesis: Two Mechanisms of Vessel Growth. *News Physiol Sci* 14:121.
47. Risau, W. 1997. Mechanisms of angiogenesis. *Nature* 386:671.
48. Flamme, I., T. Frolich, and W. Risau. 1997. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J Cell Physiol* 173:206.
49. Bussolino, F., A. Mantovani, and G. Persico. 1997. Molecular mechanisms of blood vessel formation. *Trends Biochem Sci* 22:251.
50. Helisch, A., and W. Schaper. 2003. Arteriogenesis: the development and growth of collateral arteries. *Microcirculation* 10:83.

51. Yancopoulos, G. D., S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, and J. Holash. 2000. Vascular-specific growth factors and blood vessel formation. *Nature* 407:242.
52. Rogers, P. A., and C. E. Gargett. 1998. Endometrial angiogenesis. *Angiogenesis* 2:287.
53. Ibukiyama, C. 1996. Angiogenesis. Angiogenic therapy using fibroblast growth factors and vascular endothelial growth factors for ischemic vascular lesions. *Jpn Heart J* 37:285.
54. Browning, D. J., and C. M. Fraser. 2005. Ocular conditions associated with peripapillary subretinal neovascularization, their relative frequencies, and associated outcomes. *Ophthalmology* 112:1054.
55. Yamashita, A., and Y. Yonemitsu. 2005. [Angiogenic growth factors in rheumatoid arthritis]. *Nippon Rinsho* 63 Suppl 1:189.
56. Miller, K. D., C. J. Sweeney, and G. W. Sledge, Jr. 2005. Can tumor angiogenesis be inhibited without resistance? *Exs*:95.
57. Folkman, J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285:1182.
58. Folkman, J. 1974. Tumor angiogenesis: role in regulation of tumor growth. *Symp Soc Dev Biol* 30:43.
59. Langer, R., H. Conn, J. Vacanti, C. Haudenschild, and J. Folkman. 1980. Control of tumor growth in animals by infusion of an angiogenesis inhibitor. *Proc Natl Acad Sci U S A* 77:4331.

60. Shing, Y., J. Folkman, C. Haudenschild, D. Lund, R. Crum, and M. Klagsbrun. 1985. Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor. *J Cell Biochem* 29:275.
61. Adamis, A. P., L. P. Aiello, and R. A. D'Amato. 1999. Angiogenesis and ophthalmic disease. *Angiogenesis* 3:9.
62. Barbera-Guillem, E., J. K. Nyhus, C. C. Wolford, C. R. Friece, and J. W. Sampsel. 2002. Vascular endothelial growth factor secretion by tumor-infiltrating macrophages essentially supports tumor angiogenesis, and IgG immune complexes potentiate the process. *Cancer Res* 62:7042.
63. Kumar, R., and I. J. Fidler. 1998. Angiogenic molecules and cancer metastasis. *In Vivo* 12:27.
64. Tolentino, M. J., and A. P. Adamis. 1998. Angiogenic factors in the development of diabetic iris neovascularization and retinopathy. *Int Ophthalmol Clin* 38:77.
65. Ferrara, N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 25:581.
66. Ribatti, D., and M. Presta. 2002. The role of fibroblast growth factor-2 in the vascularization of the chick embryo chorioallantoic membrane. *J Cell Mol Med* 6:439.
67. Ellis, L. M., S. Ahmad, F. Fan, W. Liu, Y. D. Jung, O. Stoeltzing, N. Reinmuth, and A. A. Parikh. 2002. Angiopoietins and their role in colon cancer angiogenesis. *Oncology (Huntingt)* 16:31.
68. Gerber, H. P., A. McMurtry, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit, and N. Ferrara. 1998. Vascular endothelial growth factor regulates

- endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273:30336.
69. Mignatti, P., and D. B. Rifkin. 1996. Plasminogen activators and matrix metalloproteinases in angiogenesis. *Enzyme Protein* 49:117.
 70. Berger, D. P., L. Herbstritt, W. A. Dengler, D. Marme, R. Mertelsmann, and H. H. Fiebig. 1995. Vascular endothelial growth factor (VEGF) mRNA expression in human tumor models of different histologies. *Ann Oncol* 6:817.
 71. Jablonska, E., W. Puzewska, Z. Grabowska, J. Jablonski, and L. Talarek. 2005. VEGF, IL-18 and NO production by neutrophils and their serum levels in patients with oral cavity cancer. *Cytokine* 30:93.
 72. Ferrara, N., H. P. Gerber, and J. LeCouter. 2003. The biology of VEGF and its receptors. *Nat Med* 9:669.
 73. Takahashi, H., M. Numasaki, M. T. Lotze, and H. Sasaki. 2005. Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. *Immunol Lett* 98:189.
 74. Okazaki, T., S. Ebihara, H. Takahashi, M. Asada, A. Kanda, and H. Sasaki. 2005. Macrophage colony-stimulating factor induces vascular endothelial growth factor production in skeletal muscle and promotes tumor angiogenesis. *J Immunol* 174:7531.
 75. Asano-Kato, N., K. Fukagawa, N. Okada, T. Kawakita, Y. Takano, M. Dogru, K. Tsubota, and H. Fujishima. 2005. TGF-beta1, IL-1beta, and Th2 cytokines stimulate vascular endothelial growth factor production from conjunctival fibroblasts. *Exp Eye Res* 80:555.

76. Honorati, M. C., L. Cattini, and A. Facchini. 2004. IL-17, IL-1 β and TNF- α stimulate VEGF production by dedifferentiated chondrocytes. *Osteoarthritis Cartilage* 12:683.
77. Schwarz, E. R., D. A. Meven, N. Z. Sulemanjee, P. H. Kersting, T. Tussing, E. C. Skobel, P. Hanrath, and B. F. Uretsky. 2004. Monocyte chemoattractant protein 1-induced monocyte infiltration produces angiogenesis but not arteriogenesis in chronically infarcted myocardium. *J Cardiovasc Pharmacol Ther* 9:279.
78. Scapini, P., M. Morini, C. Tecchio, S. Minghelli, E. Di Carlo, E. Tanghetti, A. Albin, C. Lowell, G. Berton, D. M. Noonan, and M. A. Cassatella. 2004. CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. *J Immunol* 172:5034.
79. Pepper, M. S. 2001. Extracellular proteolysis and angiogenesis. *Thromb Haemost* 86:346.
80. Jackson, C. 2002. Matrix metalloproteinases and angiogenesis. *Curr Opin Nephrol Hypertens* 11:295.
81. Luttun, A., M. Dewerchin, D. Collen, and P. Carmeliet. 2000. The role of proteinases in angiogenesis, heart development, restenosis, atherosclerosis, myocardial ischemia, and stroke: insights from genetic studies. *Curr Atheroscler Rep* 2:407.
82. Carmeliet, P. 2003. Angiogenesis in health and disease. *Nat Med* 9:653.
83. Hynes, R. O. 2002. A reevaluation of integrins as regulators of angiogenesis. *Nat Med* 8:918.

84. Hood, J. D., and D. A. Cheresh. 2002. Role of integrins in cell invasion and migration. *Nat Rev Cancer* 2:91.
85. Garmony-Susini, B., H. Jin, Y. Zhu, R. J. Sung, R. Hwang, and J. Varner. 2005. Integrin $\alpha(4)\beta(1)$ -VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. *J Clin Invest* 115:1542.
86. Carlson, T. R., Y. Feng, P. C. Maisonpierre, M. Mrksich, and A. O. Morla. 2001. Direct cell adhesion to the angiopoietins mediated by integrins. *J Biol Chem* 276:26516.
87. Albini, A., R. Soldi, D. Giunciuglio, E. Giraudo, R. Benelli, L. Primo, D. Noonan, M. Salio, G. Camussi, W. Rockl, and F. Bussolino. 1996. The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nat Med* 2:1371.
88. Yoo, Y. G., and M. O. Lee. 2004. Hepatitis B virus X protein induces expression of Fas ligand gene through enhancing transcriptional activity of early growth response factor. *J Biol Chem* 279:36242.
89. Meyer, M., M. Clauss, A. Lepple-Wienhues, J. Waltenberger, H. G. Augustin, M. Ziche, C. Lanz, M. Buttner, H. J. Rziha, and C. Dehio. 1999. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *Embo J* 18:363.
90. Klinman, D. M., M. Zheng, M. Gierynska, and B. T. Rouse. 2002. DNA Containing Bioactive CpG Motifs Promote Angiogenesis. *Drug News Perspect* 15:358.

91. Maertzdorf, J., A. D. Osterhaus, and G. M. Verjans. 2002. IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts. *J Immunol* 169:5897.
92. Strieter, R. M., P. J. Polverini, D. A. Arenberg, and S. L. Kunkel. 1995. The role of CXC chemokines as regulators of angiogenesis. *Shock* 4:155.
93. Su, Y. H., X. T. Yan, J. E. Oakes, and R. N. Lausch. 1996. Protective antibody therapy is associated with reduced chemokine transcripts in herpes simplex virus type 1 corneal infection. *J Virol* 70:1277.
94. Harkness, K. A., J. D. Sussman, G. A. Davies-Jones, J. Greenwood, and M. N. Woodroffe. 2003. Cytokine regulation of MCP-1 expression in brain and retinal microvascular endothelial cells. *J Neuroimmunol* 142:1.
95. Yan, X. T., T. M. Tumpey, S. L. Kunkel, J. E. Oakes, and R. N. Lausch. 1998. Role of MIP-2 in neutrophil migration and tissue injury in the herpes simplex virus-1-infected cornea. *Invest Ophthalmol Vis Sci* 39:1854.
96. Carmeliet, P., V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau, and A. Nagy. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435.
97. Ferrara, N., K. Carver-Moore, H. Chen, M. Dowd, L. Lu, K. S. O'Shea, L. Powell-Braxton, K. J. Hillan, and M. W. Moore. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380:439.

98. Phillips, H. S., J. Hains, D. W. Leung, and N. Ferrara. 1990. Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* 127:965.
99. Stewart, R. J., D. Panigrahy, E. Flynn, and J. Folkman. 2001. Vascular endothelial growth factor expression and tumor angiogenesis are regulated by androgens in hormone responsive human prostate carcinoma: evidence for androgen dependent destabilization of vascular endothelial growth factor transcripts. *J Urol* 165:688.
100. Lopez, P. F., B. D. Sippy, H. M. Lambert, A. B. Thach, and D. R. Hinton. 1996. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci* 37:855.
101. Aiello, L. P., R. L. Avery, P. G. Arrigg, B. A. Keyt, H. D. Jampel, S. T. Shah, L. R. Pasquale, H. Thieme, M. A. Iwamoto, J. E. Park, and et al. 1994. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331:1480.
102. Senger, D. R., S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219:983.
103. Ferrara, N., and W. J. Henzel. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851.

104. Gerber, H. P., V. Dixit, and N. Ferrara. 1998. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 273:13313.
105. Janvier, A., S. Nadeau, J. Baribeau, and T. Perreault. 2005. Role of vascular endothelial growth factor receptor 1 and vascular endothelial growth factor receptor 2 in the vasodilator response to vascular endothelial growth factor in the neonatal piglet lung. *Crit Care Med* 33:860.
106. Gerber, H. P., A. K. Malik, G. P. Solar, D. Sherman, X. H. Liang, G. Meng, K. Hong, J. C. Marsters, and N. Ferrara. 2002. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. *Nature* 417:954.
107. Ferrara, N., and T. Davis-Smyth. 1997. The biology of vascular endothelial growth factor. *Endocr Rev* 18:4.
108. Karkkainen, M. J., T. Makinen, and K. Alitalo. 2002. Lymphatic endothelium: a new frontier of metastasis research. *Nat Cell Biol* 4:E2.
109. Niethammer, A. G., R. Xiang, J. C. Becker, H. Wodrich, U. Pertl, G. Karsten, B. P. Eliceiri, and R. A. Reisfeld. 2002. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med* 8:1369.
110. Kendall, R. L., and K. A. Thomas. 1993. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A* 90:10705.
111. Folkman, J. 2001. Can mosaic tumor vessels facilitate molecular diagnosis of cancer? *Proc Natl Acad Sci U S A* 98:398.

112. Holash, J., S. Davis, N. Papadopoulos, S. D. Croll, L. Ho, M. Russell, P. Boland, R. Leidich, D. Hylton, E. Burova, E. Ioffe, T. Huang, C. Radziejewski, K. Bailey, J. P. Fandl, T. Daly, S. J. Wiegand, G. D. Yancopoulos, and J. S. Rudge. 2002. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A* 99:11393.
113. Bhisitkul, R. B., G. S. Robinson, R. S. Moulton, K. P. Claffey, E. S. Gragoudas, and J. W. Miller. 2005. An antisense oligodeoxynucleotide against vascular endothelial growth factor in a nonhuman primate model of iris neovascularization. *Arch Ophthalmol* 123:214.
114. Farhadi, M. R., H. H. Capelle, R. Erber, A. Ullrich, and P. Vajkoczy. 2005. Combined inhibition of vascular endothelial growth factor and platelet-derived growth factor signaling: effects on the angiogenesis, microcirculation, and growth of orthotopic malignant gliomas. *J Neurosurg* 102:363.
115. Kovacs, Z., K. Ikezaki, K. Samoto, T. Inamura, and M. Fukui. 1996. VEGF and flt. Expression time kinetics in rat brain infarct. *Stroke* 27:1865.
116. Cerimele, F., L. F. Brown, F. Bravo, G. M. Ihler, P. Kouadio, and J. L. Arbiser. 2003. Infectious angiogenesis: Bartonella bacilliformis infection results in endothelial production of angiopoietin-2 and epidermal production of vascular endothelial growth factor. *Am J Pathol* 163:1321.
117. Aiello, L. P. 2000. Keeping in touch with angiogenesis. *Nat Med* 6:379.
118. Satchi-Fainaro, R., M. Puder, J. W. Davies, H. T. Tran, D. A. Sampson, A. K. Greene, G. Corfas, and J. Folkman. 2004. Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470. *Nat Med* 10:255.

119. Guba, M., P. von Breitenbuch, M. Steinbauer, G. Koehl, S. Flegel, M. Hornung, C. J. Bruns, C. Zuelke, S. Farkas, M. Anthuber, K. W. Jauch, and E. K. Geissler. 2002. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* 8:128.
120. Abdollahi, A., L. Hlatky, and P. E. Huber. 2005. Endostatin: The logic of antiangiogenic therapy. *Drug Resist Updat* 8:59.
121. Folkman, J., and D. Ingber. 1992. Inhibition of angiogenesis. *Semin Cancer Biol* 3:89.
122. Masiero, L., W. D. Figg, and E. C. Kohn. 1997. New anti-angiogenesis agents: review of the clinical experience with carboxyamido-triazole (CAI), thalidomide, TNP-470 and interleukin-12. *Angiogenesis* 1:23.
123. Rhee, J. S., R. Diaz, L. Korets, J. G. Hodgson, and L. M. Coussens. 2004. TIMP-1 alters susceptibility to carcinogenesis. *Cancer Res* 64:952.
124. Nogawa, M., T. Yuasa, S. Kimura, M. Tanaka, J. Kuroda, K. Sato, A. Yokota, H. Segawa, Y. Toda, S. Kageyama, T. Yoshiki, Y. Okada, and T. Maekawa. 2005. Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. *J Clin Invest* 115:978.
125. Wasmuth, S., D. Bauer, Y. Yang, K. P. Steuhl, and A. Heiligenhaus. 2003. Topical treatment with antisense oligonucleotides targeting tumor necrosis factor-alpha in herpetic stromal keratitis. *Invest Ophthalmol Vis Sci* 44:5228.

126. Nair, S., D. Boczkowski, B. Moeller, M. Dewhirst, J. Vieweg, and E. Gilboa. 2003. Synergy between tumor immunotherapy and antiangiogenic therapy. *Blood* 102:964.
127. Kerbel, R., and J. Folkman. 2002. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2:727.
128. Tullo, A. 2003. Pathogenesis and management of herpes simplex virus keratitis. *Eye* 17:919.

APPENDIX

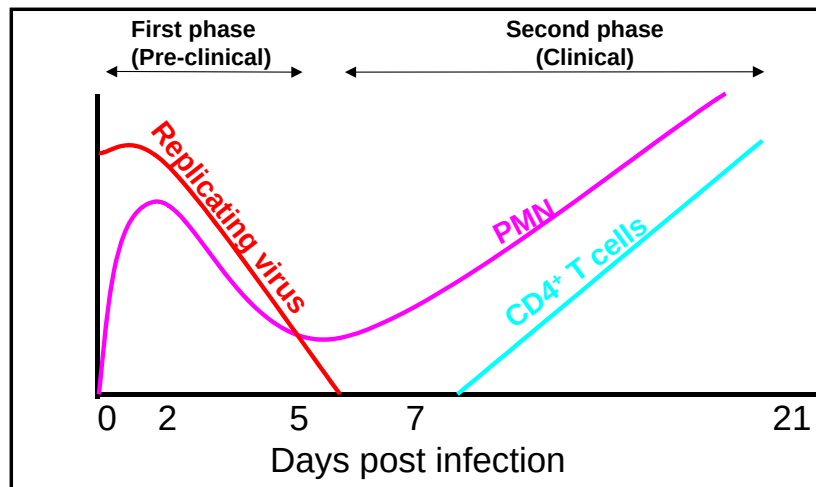


Figure 1. Principle cellular events in herpetic SK pathogenesis.

After primary HSV-1 ocular infection, highly replicating virus are detectable for a few days and finally the replication subsides by 6-7 days post infection. At this early period (pre-clinical phase), the infection is characterized by the infiltration of activated inflammatory cells mainly PMN (neutrophils). The cellular events in the SK cornea at clinical phase (after day 7 post infection) are characterized by a huge influx of T cells escaping from newly formed leaky blood vessels in the stroma. Interestingly, a huge influx of PMN is again observed in the SK cornea at this stage. Biphasic invasion of PMN in the cornea appears to be the crucial event for herpetic SK pathogenesis.

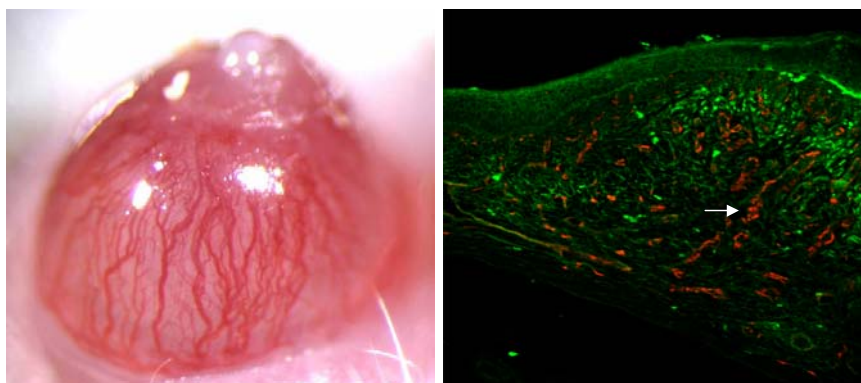
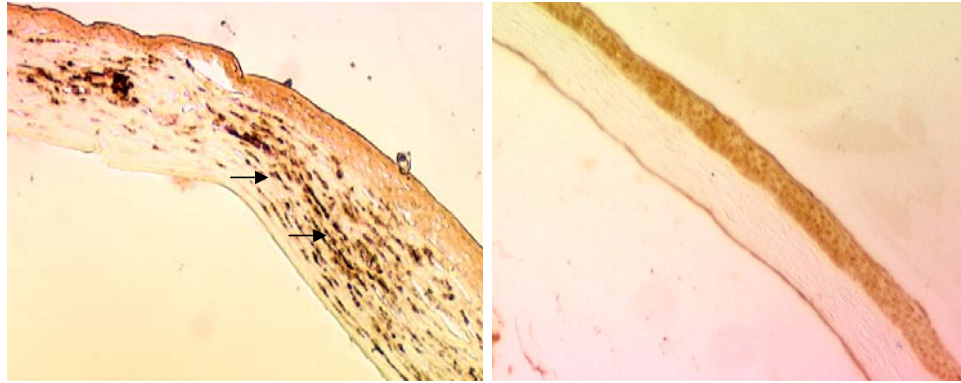


Figure 2. New blood vessels formation in the corneal stroma following HSV-1 infection.

One of prominent events early in the process of SK is uncontrolled neovascularization of the normally avascular cornea. Newly formed neovessels with high capillary density begin sprouting from the preexisting limbal vessels within 48 h after HSV-1 infection. After this time point, sprouting blood vessels become apparent, and they ultimately reach the center of ulcerative cornea around day 20 after virus infection. The neovessels (arrow) express CD31 (PECAM-1), which is a typical molecule expressed in blood vessel endothelial cells.



Day 2 after HSV-1 infection

Naïve cornea

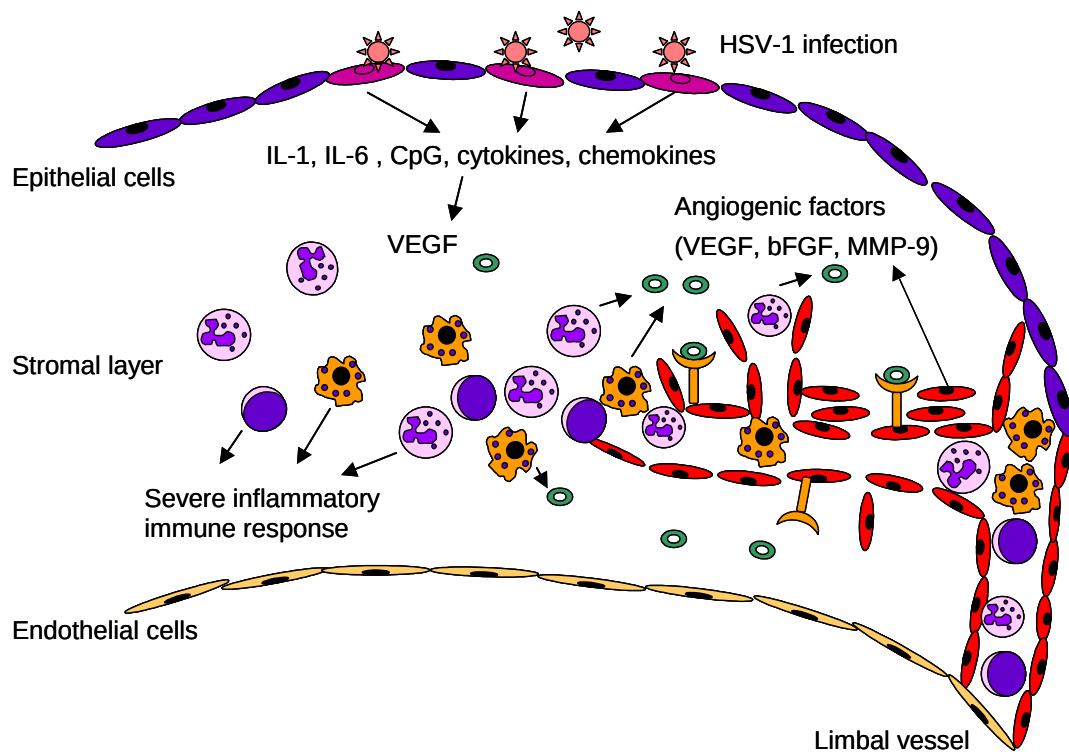
Figure 3. Cellular candidates that induce angiogenesis in SK cornea.

Following HSV-1 ocular infection, inflammatory cells with various subtypes infiltrate into the SK cornea. Among them, PMNs, mainly neutrophils, are the predominant cells during both preclinical and clinical phases of herpetic SK. Gr-1 positive inflammatory cells (arrow) are observed in the SK cornea at day 2 after HSV-1 infection.

Figure 4. Schematic representation of neovascularization process in the cornea following HSV-1 infection and its contribution to herpetic SK pathogenesis.

HSV-1 infection and replication in the corneal epithelial cell resulted in the production of various cytokines and chemokines. These proinflammatory molecules subsequently lead to recruitment of innate immune inflammatory cells within 24 h post infection and also directly induce VEGF expression from the SK cornea. Infiltrating inflammatory cells such as neutrophils and macrophages produce enormous numbers of angiokines such as proinflammatory cytokines, chemoattractant chemokines, and endothelial cell growth factors, mainly VEGF. Once the released angiokines reach the nearby preexisting blood vessels, it leads to the activation and proliferation of endothelial cells, which build up the new blood vessels. In the clinical phase, new blood vessels supply a route for CD4⁺ T cells, which are responsible for inducing severe autoimmune responses, to infiltrate into the stromal area.

Abbreviations: HSV-1, Herpes Simplex Virus-1; CpG, Cytosine phosphate Glycine; IL, Interleukin; VEGF, Vascular Endothelial cell Growth Factor; bFGF, basic Fibroblast Growth Factor; MMP-9, Matrix Metallo Proteinase 9



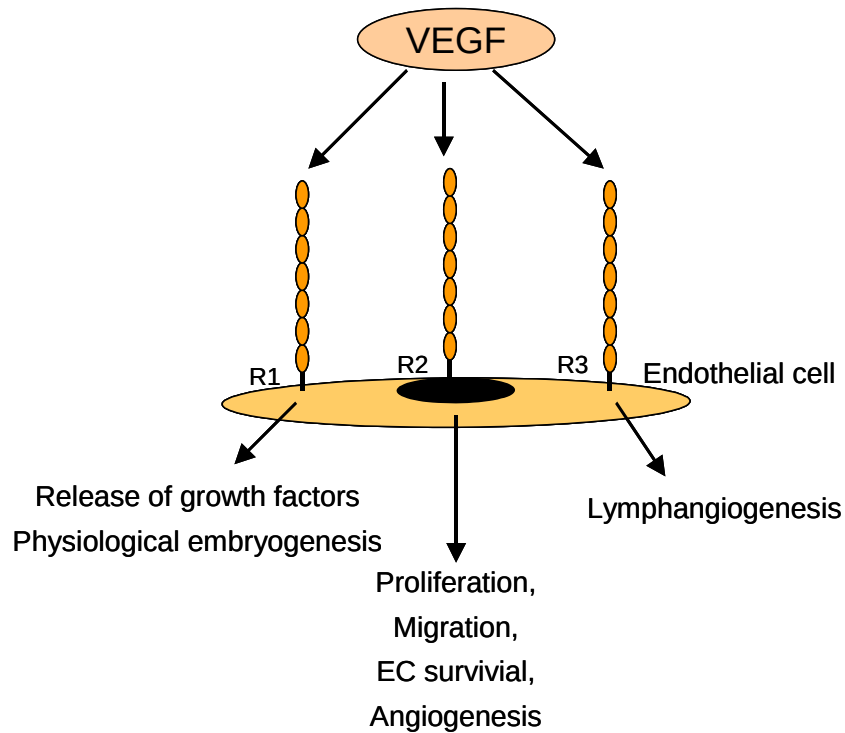


Figure 5. Role of VEGF receptor tyrosine kinases.

VEGF receptors have seven immunoglobulin-like domains in the extracellular domain and a single transmembrane region. VEGFR-1 and -2 are expressed on the surface of vascular endothelial cells and VEGFR-3 is expressed in lymphatic endothelial cells. Once VEGF binds to each receptor, the signaling roles of each receptor tyrosine kinase work differently.

Part II

Inhibition of Ocular Angiogenesis by siRNA Targeting Vascular Endothelial Growth Factor- Pathway Genes; Therapeutic Strategy for Herpetic Stromal Keratitis

Research described in this chapter is a slightly modified version of an article published in 2004 in *The American Journal of Pathology* by Bumseok Kim, Qingquan Tang, Partha S. Biswas, Jun Xu, Raymond M. Schiffelers, Frank Y. Xie, Aslam M. Ansari, Puthupparampil V. Scaria, Martin C. Woodle, Patrick Lu, and Barry T. Rouse

Kim, B., Q. Tang, P. S. Biswas, J. Xu, R. M. Schiffelers, F. Y. Xie, A. M. Ansari, P. V. Scaria, M. C. Woodle, P. Lu, and B. T. Rouse. 2004. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. *Am. J. Pathol.* 165(6): 2177-85. Copyright 2004. The American Association of Pathologist, Inc.

In this chapter “we” and “our” refers to co-authors and me. My contributions in the paper include (1) selection of the topic (2) data analysis and interpretation (3) planning experiments (4) compiling and interpretation of the literature (5) understanding how results fit with the literature (7) compilation of contributions into one paper (8) providing structure to the paper (9) making graphs, figures and tables (10) writing and editing.

ABSTRACT

Ocular neovascularization often results in vision impairment. Frequently vascular endothelial cell growth factors (VEGF) are mainly responsible for the pathological neovascularization as in the case in neovascularization induced by CpG oligodeoxynucleotides (ODN) and herpes simplex virus (HSV) infection in this report. siRNAs targeting either VEGFA, VEGFR1, 2 or a mix of the three were

shown to significantly inhibit neovascularization induced by CpG when given locally or systemically. The efficacy of systemic administration was facilitated by the use of a polymer delivery vehicle. Additional experiments showed a significant inhibitory effect of the siRNAs mix when given either locally or systemically in vehicle against HSV induced angiogenesis as well as against lesions of stromal keratitis (SK). These results indicate that the use of VEGF pathway-specific siRNAs represents a useful therapy against neovascularization-related eye diseases.

INTRODUCTION

New and uncontrolled blood vessel development in the eye is a pivotal process in the pathogenesis of several ocular neovascularization diseases, including herpes simplex virus (HSV)-induced stromal keratitis (SK), diabetic retinopathy, and age-related macular degeneration (1). These ocular diseases may finally cause blindness and managing them therapeutically is problematic. With HSV, the commonest infectious cause of vision impairment and blindness in the western world (2), neovascularization of the avascular cornea represents an essential step in its pathogenesis (3). Multiple molecules may be responsible for the HSV-induced angiogenesis and it remains unclear how the virus infection results in the induction of angiogenic factors (4). Recently, we demonstrated that HSV DNA which contains abundant potentially bioactive CpG-containing motifs (5, 6), can induce the potent angiogenesis factor vascular endothelial growth factor (VEGF) and that neutralization of VEGF with antibody minimized HSV-induced angiogenesis (3). A convenient model was also established in which bioactive CpG-containing oligodeoxynucleotides (ODN) were also shown to induce

neovascularization via the induction of VEGF (7, 8). This model is employed in the present study to evaluate the therapeutic potential of RNA interference (RNAi) to suppress VEGF expression and responsiveness.

Gene silencing by RNAi represents a potential important approach for therapy as well as a rapid and reliable tool for gene discovery or gene validation (9). Currently, small interfering RNA (siRNA) has received modest use *in vivo* (10). To achieve high efficacy in siRNA-mediated therapy, it is critical not only to choose the correct gene target and to design appropriate siRNAs, but more importantly also to efficiently deliver siRNAs into specific tissues or cells *in vivo* (11). A previous report from our group demonstrated that targeting the VEGF protein and its receptor was a successful means of inhibiting neovascularization associated with tumor growth (12). Other reports have also shown success using siRNA to MMP-9 against tumor angiogenesis (13). In the present study, we have used siRNA targeting the VEGF pathway and shown that this system significantly inhibits ocular angiogenesis induced by bioactive CpG and HSV infection. The results of these studies have implication for the control of HSV induced vision loss.

MATERIALS AND METHODS

Reagents

Phosphorothioate ODNs were kindly provided by Dennis M. Klinman (Biologics Evaluation and Research, FDA, U.S.A.). The sequences of stimulatory ODNs used in this study were: 1466, TCAACGTTGA, and 1555, GCTAGACGTTAGCGT. Subsequent studies were performed using an equimolar mixture of ODNs 1466 and 1555.

Molecular design of gene targets and siRNAs

Three mVEGF pathway factors, mVEGF A and two mVEGF receptors (mVEGFR1 and 2), were targeted by RNAi. For each gene target, two target sequences were assigned at different locations on the same mRNA. siRNAs were designed correspondent to the above target sequences. These siRNAs were designed according to the guideline proposed by Tuschl's group (14, 15). The designed siRNAs (duplexes of sense and antisense strands) were synthesized by Qiagen (Valencia, CA). All siRNAs were 21-nt long double stranded RNA oligos with 2-nt (TT) overhang at 3' end. The targeted sequences of mVEGFA were (a) AAGCCGTCCTGTGTGCCGCTG and (b) AACGATGAAGCCCTGGAGTGC. The targeted sequences of mVEGFR1 were (a) AAGTTAAAAGTGCCTGAAC-TG and (b) AAGCAGGCCAGACTCTCTTTC. The targeted sequences of mVEGFR2 were (a) AAGCTCAGCACACAGAAAGAC and (b) AATGCGGC-GGTGGTGACAGTA. The synthesis of unrelated siRNA controls, two target sequences each for LacZ and firefly luciferase were used. They were LacZ (a) AACAGTTGCGCAGCCTGAATG and (b) AACTTAATCGCCTTGCAGCAC, Luc (a) AAGCTATGAAACGATATGGGC and (b) AACCGCTGGAGAGCAA-CTGCA. Subsequent studies were conducted using an equimolar mixture of (a) and (b) for individual siRNA.

Mice

Female BALB/c mice (H-2^d), 5 to 6 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.) and housed conventionally. All investigations

followed guidelines of the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee (Knoxville) are fully accredited by the American Association of Laboratory Animal Care.

Virus

HSV-1 strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile) was used in all the procedures. Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA; Cat. no. CCL81), titrated, and stored in aliquots at -80°C until used.

***In vitro* efficacy of siRNA**

To test the efficacy of RNAi *in vitro*, the following cell lines were used. RAW264.7 gamma NO (-) cells (ATCC, CRL-2278) were used to test the efficiency of siVEGFA specific knockdown of VEGFA gene that is spontaneously expressed in these cells. The cells were plated in a 6 well plate in RPMI with 10% FBS overnight at 37°C in 5% CO₂. One day after cell plating, the cells were transfected with different concentrations of siVEGFA or siLuc (at 0, 0.1, 0.5, 1.0, or 2.0 µg/2 ml/well, respectively) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty four hours later RNA from these cells was extracted for RT-PCR to detect VEGF (see *RNA Extraction and RT-PCR*).

SVR cells (ATCC, CRL-2280) were used to test the efficiency of siVEGFR1 specific knockdown of VEGFR1 gene that is constitutively expressed on these cells. The cells were plated in a 6 well plate in DMEM with 5% FBS

overnight at 37°C in 5% CO₂. One day after cell plating, the cells were transfected with different concentrations of siVEGFR1 or siLuc (at 0, 0.1, 0.5, or 1.0 µg/2 ml/well, respectively) using Lipofectamine 2000. Forty eight hours later RNA from these cells was extracted for RS-PCR to detect VEGFR1 (see *RNA Extraction and RS-PCR*).

293 cells (ATCC, CRL-1573) were used to transfect with mVEGFR2-expressing plasmid for the detection of knockdown of exogenous mVEGFR2. The cells were plated in a 6 well plate in DMEM with 5% FBS overnight at 37°C in 5% CO₂. One day after cell plating, the cells were cotransfected with plasmid pCI-VEGFR2 (0.2 µg/2 ml/well) and siVEGFR2 [a, b, a+b], or siLuc (0, 0.1, 0.5, or 1.0 µg/well, respectively) using Lipofectamine 2000. Forty eight hours later RNA from these cells was extracted for RS-PCR to detect VEGFR2 (see *RNA Extraction and RS-PCR*).

RNA Extraction and RT or RS-PCR

At various time points following siRNA treatment, the cells were harvested and RNA was extracted by using RNeasy protect mini kit (Qiagen, Valencia, CA). In case of ocular infection, 2 corneas/time point were excised and transferred to RNAlaterTM. Briefly, cells were lysed in RLT buffer (Qiagen, catalog no. 79216) and RNA was purified according to manufacturer's instructions. RNase-free Dnase set (Qiagen) was used to remove any contaminating genomic DNA. Total cellular RNA (10 µg/ml) was reversed-transcribed using oligo (dT) primers and reverse transcriptase (Promega, Madison, WI), according to protocols described previously (16). The cDNA was made by the reverse-transcription reaction incubated at 42°C

for 90 min. The primer sequences for mVEGF RT-PCR were: 5'-GCGGGCTGCCTCGCAGTC-3' (sense) and 5'-TCACCGCCTTGGCTTGTCAC-3' (antisense). RT-PCR products were 644 bp for mVEGF164 and 512 bp for mVEGF120. The amplification profile was 94°C for 1 min, 65°C for 1 min, and 72°C for 15 min for 30 cycles. The PCR products were separated by 1% agarose gel electrophoresis.

The RS-PCR was also performed for detection of mRNA knockdown by siRNAs in vitro. Cytoplasmic RNA was isolated by RNAwiz (Ambion, #9736) according to manufacturer's instruction with additional DNase treatment, and subjected to RS-PCR with specially designed primers. The mRNA-specific reverse primers for the RT reaction were all 47-mer oligos with the 5'-end 30-mer of unique sequence (called "TS1" sequence, indicated in uppercase below) linked to a 17-mer sequence unique for each mRNA molecule (in lower case below). They were (from 5' to 3'): 1) mVEGFR1 dn: GAACATCGATGACAAGCTTAGGTATCGATAtagattgaagattccgc; 2) mVEGFR2dn: GAACATCGATGACAA-GCTTAGGTATCGATAggtcactgacagaggcg. The PCR assays for all the tested genes, that follow the RT assay, used a same reverse primer, TS1: GAACATCGATGACAAGCTTAGGTATCGATA. The forward primers for PCR were all 30-mer oligos, unique for each gene: mVEGFR1up: GTCAGCTGCTGGGACACCGCGGTCTTGCCT and mVEGFR2up: GGCGCTGCTAGCTGTCGCTCTGTGGTTCTG. The RT-PCR of housekeeping gene GAPDH was used as control for RNA amount used in RS-PCR. An oligo dT primer (19-mer) was used for RT assay of GAPDH. The primers used for the followed PCR were 20-mer

oligos: 1) GAPDHup: CCTGGTCACCAGGGCTGCTT; 2) GAPDHdn: CCAG-CCTTCTCCATGGTGGT.

Corneal micropocket Assay

The corneal micropocket assay used in this study observed the general protocol of Kenyon et al. (17). Pellets for insertion into the cornea were made by combining known amounts of CpG ODNs, sucralfate (10 mg, Bulch Meditec), and hydron polymer in ethanol (120 mg/1ml ethanol, Interferon Sciences), and applying the mixture to a 15×15 mm² piece of synthetic mesh (Tekto). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 1 µg of CpG ODNs. The micropocket was made around 1 mm from the limbus under a stereomicroscope (Leica Microsystems, Wetzlar, Germany) (four eyes per group) and pellets containing CpG ODNs were inserted into the micropocket.

Angiogenesis was evaluated at day 4 and 7 after pellet implantation by using calipers (Biomedical research instruments, Rockville, Maryland) with a stereomicroscope. The length of the neovessels originated from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours were measured (4). Each clock hours is equal to 30° at the circumference. The angiogenic area was calculated according to the formula for an ellipse. $A = [(clock\ hours) \times 0.4 \times (vessel\ length\ in\ mm) \times \pi] / 2$.

***In vivo* delivery of siRNA**

For local delivery, siRNA (10 µg/10 µl per eye) was diluted in PBS and delivered subconjunctivally. The subconjunctival injections were given by a 32-gauge hamilton syringe (Hamilton company, Reno, Nevada) at 6 and 24 h after CpG pellets implantation or day 1 and 3 after virus infection under deep anesthesia induced by Avertin (Pittman Moore, Mondelein, IL). siRNA was injected 2 mm behind the limbus. For systemic injection, siRNA (40 µg/100 µl per mice) was mixed with polymer (TargeTran) and delivered intravenously. The tail vein injections were given at 6 and 24 h after CpG pellets implantation or day 1 and 3 after virus infection using a 32-gauge syringe.

Corneal HSV-1 infection

Corneal infections of all mouse groups were conducted under deep anesthesia induced by Avertin. The mice were scarified lightly on their corneas with a 30-gauge needle, and a 2µl drop containing 1×10^5 plaque-forming units (PFU) of HSV-1 RE was applied to the eye and gently massaged with the eyelids (six mice per group).

Clinical observations (HSK severity and angiogenic scoring)

The eyes were examined on different days after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Company, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4,

opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing stromal keratitis. The severity of angiogenesis was recorded as described previously (4). Briefly, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovascularization index (range 0–16) for each eye at a given time point (4).

Quantitative real-time PCR

Total cellular RNA was isolated from two corneas at day 7 p.i. using RNeasy RNA extraction kit (Qiagen, Valencia, CA) according to manufacturer's protocol. DNase treatment (Qiagen, CA) was done to remove any contaminating genomic DNA. To generate cDNA, 1 µg of total RNA was reverse transcribed using murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Bethesda, MD) with oligo(dT) as primers (Invitrogen, CA). All cDNA samples were aliquoted and stored at -20°C until further use.

Real-time PCR was performed using a DNA Engine Opticon (MJ Research Inc.). PCR was performed using SYBR Green I reagent (Qiagen, Valencia, CA), according to manufacturer's protocol. During the optimization procedures of the primers, 1% agarose gel analysis verified the amplification of one product of the predicted size with no primer-dimer bands. The absence of primer-dimer formation for each oligonucleotide set was also validated by establishing the melting curve profile. The semiquantitative comparison between samples was calculated as follows: the data were normalized by subtracting the difference of the threshold cycles (C_T) between the gene of interest's C_T and the "housekeeping" gene

GAPDH's C_T (gene of interest C_T – GAPDH $C_T = \Delta C_T$) for each sample. The ΔC_T was then compared to the expression levels of the vector control sample (sample ΔC_T – vector ΔC_T). To determine the relative enhanced expression of the gene of interest, the following calculation was made: fold change = $2^{(-\text{sample } \Delta C_T - \text{vector } \Delta C_T)}$. The primers used were murine GAPDH sense, CATCCTGCACCACCAACTGCTTAG and antisense, GCCTGCTTCACCACCTTCTTGATG, and murine VEGF-164 sense, GCCAGCACATAGAGAGAATGAGC and antisense, CAAGGCTCAGTGATTTTCTGG.

VEGF quantification of corneal lysates by ELISA

The level of corneal VEGF protein expression was measured by a standard sandwich enzyme-linked immunosorbent assay (ELISA) protocol. For preparation of corneal lysates, two corneas/time point (n = 4) were collected and minced. Minced pieces were collected in 1 ml of DMEM without FCS and homogenized using ultra sonicator (Heat systems-Ultrasonics, NY, U.S.A.). The lysate was then clarified by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was collected and stored at -80°C till further use.

The ELISA plate was coated with anti-mouse VEGF capture antibody (100 µl/well of the capture antibody at 0.4 µg/ml) and incubated at 4°C overnight. The plate was washed with 0.05% tween20/PBS and blocked with 3% BSA for 2 h at room temperature. After washing, serially diluted corneal lysates were added to the plate and incubated at 4°C overnight. The plate was washed followed by anti-VEGF biotinylated detection Ab (R&D system) for 2 h. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory, PA, U.S.A.) was

added. The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Statistical analysis

Significant differences between groups were evaluated by using the Student's t test. $P < 0.05$ was regarded as significant difference between the two groups.

RESULTS

Knockdown of VEGF-pathway genes by siRNA *in vitro*

Three individual siRNAs were developed. These were siVEGFA, siVEGFR1, and siVEGFR2. Each was tested in a different cell system *in vitro* to measure its gene silencing efficiency. The siVEGFA was tested in RAW NO (-) macrophage cells, that produce VEGFA endogenously (18). Cells were transfected with siVEGFA, or control siLuc siRNA at various doses, and RT-PCR was performed 24 h after transfection. As shown in Fig. 1A, expression of both the 120 and 164 isoforms of VEGF was reduced by siVEGFA, in a dose dependent manner. The efficacy of the siVEGFR1 reagent was evaluated in SVR cells that endogenously express the VEGF receptor 1 (19). As shown in Fig. 1B, VEGFR1 expression measured by RS-PCR 48 h post transfection was diminished in a dose dependent manner whereas all concentrations of the control siLuc siRNA resulted in similar levels of VEGFR1 signal. Finally the siVEGFR2 reagent was tested in 293 cells that were exogenously transfected with a plasmid encoding VEGFR2. To measure the

silencing effect of siVEGFR2, a cotransfection approach using siVEGFR2 and VEGFR2 DNA at various doses was performed. 293 cells were cotransfected with various concentrations of siRNA targeting mVEGFR2 (0.1, 0.5, 1.0 µg/well) and plasmid expressing mVEGFR2 (0.2 µg/well). RNA was isolated 48 h post-cotransfection and exogenous expression of mVEGFR2 was measured by RS-PCR. Fig. 1C shows that VEGFR2 expression was reduced by siVEGFR2, but not by the control siRNA molecule. These results indicate that all the tested siRNAs were able to suppress the targeted genes *in vitro*.

Inhibition of CpG-induced angiogenesis by local delivery of siRNAs targeting VEGF-pathway genes

A previous study demonstrated that CpG containing ODN encapsulated in hydropolymer pellets induce VEGF mediated angiogenesis when inserted into corneal micropockets (7). This system was used to measure the inhibitory effect of local administration of siRNA preparations designed to target VEGF as well as two of its receptors (VEGFR1 and 2). A single dose of 10 µg siRNA in PBS was used in all cases. This was administered by subconjunctival injection 24 h after the establishment of micropockets containing CpG ODN. The siRNAs were tested individually as well as a 1:1:1 mixture of all three (siVEGFA, siVEGFR1 and siVEGFR2). New blood vessel formation in the corneal limbus was monitored at both day 4 and 7 after pellet implantation. As shown in Fig. 2, significant inhibition of corneal neovascularization resulted with all three test siRNAs compared to those given control siLacZ at day 4 after pellet implantation ($p <$

0.05). The combination of the three tested siRNAs was the most effective inhibitor, providing an approximately 60% reduction in neovascularization ($p < 0.01$).

Inhibition of CpG-induced neovascularization by systemic delivery of siRNAs targeting VEGF-pathway genes

To test the anti-angiogenic effect of targeted individual siRNA and the efficiency of systemic siRNA delivery, mice with CpG ODN-containing micropockets were given a single dose i.v. of 40 μ g siRNAs containing either siVEGFA, siVEGFR1, siVEGFR2, a mix of the three, or control siLacZ 6 and 24 h post pellet implantation. In these experiments a polymer (“Targetran”) was used that was shown in previous studies on tumor angiogenesis to facilitate extravascular delivery of siRNA (12). At day 4 and 7 after pellet implantation, the extent of angiogenesis was measured. As shown in Fig. 3, all reagents used individually induced significant inhibition of neovascularization compared to the siLacZ treated group at day 4 after pellet implantation ($p < 0.05$). As observed with local administration, the mix of the three test reagents provided the most effective inhibition (average 40% inhibition, $p < 0.01$). In additional experiments, the function of the polymer vehicle was evaluated by comparing the anti-neovascularization activity of the test mix suspended in polymer or given in PBS. These experiments revealed that the use of the polymer vehicle resulted in more effective anti-neovascularization than was evident when the PBS vehicle was used although result was only significant at the early test period ($p < 0.05$) (Fig. 4A). The results demonstrate that ocular neovascularization can be controlled by the i.v.

administration of siRNA that target the VEGF system genes and that the use of the “TargeTran” vehicle enhanced the efficacy of the therapeutic effect.

To determine the efficient anti-angiogenic dose of siRNA in systemic delivery, mice with CpG ODN-containing micropockets were given a single dose i.v. of 10, 20, 40, 80 µg siRNAs containing a mix of the siVEGFA, siVEGFR1 and siVEGFR2, or control siLuc with TargeTran vehicle at 6 and 24 h post pellet implantation. As shown in Fig 4B, administration of siRNAs inhibited CpG induced angiogenesis in a dose dependent manner.

Therapeutic application of siRNAs against VEGF-pathway genes in the HSK model

Previous studies have shown that VEGF is the critical angiogenic factor for induction of HSV specific angiogenesis in the HSK model (3). To evaluate whether administration of siRNAs targeting VEGF-pathway genes inhibits the development of HSK, the corneas of mice were scarified and infected with 1×10^5 HSV-1 RE. Then mice were given a single dose of 10 µg (subconjunctival injection for local delivery) or 40 µg (tail vein injection for systemic delivery) mix of siRNAs (an equimolar mixture of siVEGFA, siVEGFR1, and siVEGFR2) with polymer vehicle at day 1 and 3 after virus infection. As shown in Fig. 5, the angiogenesis and severity of HSK was significantly reduced in mice treated with siRNAs targeting VEGF-pathway genes either locally or systemically compared to animals treated with siLuc control ($p < 0.05$). Whilst 80% of siLuc control treated eyes developed clinically evident lesions (score 2 or greater at day 10 p.i.), only 42% (local delivery) or 50% (systemic delivery) of eyes treated with siRNAs

targeting VEGF-pathway genes developed such lesions. In addition by day 10 p.i., the angiogenesis score was greater than 6 in 9 of 12 control eyes, but only in 5 of 12 eyes of mice treated with siRNAs against VEGF-pathway genes by either local or systemic delivery. Taken together these results show that administration of siRNAs against VEGF-pathway genes reduced development of HSK via inhibition of angiogenesis.

Decreased level of VEGF mRNA following treatment of siRNAs targeting VEGF-pathway genes in HSV-1 infected cornea

To address whether treatment of siRNAs against VEGF-pathway genes reduces the level of VEGF mRNA, corneas were collected at day 4 or 7 p.i. from mice that were infected with 1×10^5 pfu HSV-1 RE and were treated with siRNAs targeting VEGF-pathway genes at day 1 and 3 after viral infection. The VEGF mRNA level was measured by RT-PCR or quantitative real-time PCR. As shown in Fig. 6A, the expression of VEGF mRNA was reduced in the cornea treated with siRNAs against VEGF-pathway genes compared to control eye at day 4 and 7 post infection. In addition, similar to what is found in RT-PCR, cornea treated with siRNAs against VEGF-pathway genes showed the significant reduction in VEGF gene expression in comparison to cornea treated with siLuc control at 7 day p.i.(Fig. 6B).

Decreased VEGF protein levels following application of siRNAs against VEGF-pathway genes in HSV-1 infected cornea

To evaluate whether treatment of siRNAs targeting VEGF-pathway genes diminishes the production of VEGF protein, we measured VEGF protein in HSV-1 infected and siRNAs treated cornea at day 7 after infection. As shown in Fig.7, VEGF protein levels were lower in those that received siRNAs targeting VEGF-pathway genes compared to controls given siLuc with polymer ($p < 0.05$). Once again administration of siRNA targeting VEGF-pathway with polymer inhibited the production of its target gene in the HSV cornea.

DISCUSSION

This study was undertaken to determine if siRNAs targeting VEGF and VEGF receptors gene expression could effectively inhibit ocular vascularization. To measure the effect, angiogenesis was induced by inserting bioactive CpG into corneal micropockets, a system that may model the mechanism by which HSV-1 induces neovascularization and ultimately blindness (2, 7, 17). Our results show that siRNA administered either locally or administered systemically had significant inhibitory effects on the neovascularization. Whereas individual siRNA targeted against VEGF or either of its two receptors had activity, maximal effects were observed when a 1:1:1 mix of the three siRNAs was used. Local administration to the conjunctiva was observed to result in the best therapeutic effect, but i.v. administration, especially when administered in the polymer vehicle “TargeTran”, was almost as effective. Additional experiments showed that the siRNA mixture given i.v. diminished VEGF protein levels in the HSV-1 infected

cornea. The approach holds promise as a means to control ocular neovascularization, a pathological event in several ocular diseases.

Gene silencing by RNA interference has been well demonstrated to efficiently knockdown targeted genes *in vitro*, but few reports describe similar effects *in vivo*. One of the important factors for its reliability *in vivo* may be the delivery and transfection efficiency of siRNAs. However the efficiency of RNAi delivery systems in mammalian cells is still low (20, 21). Based on previous reports, systemic delivery of siRNAs by tail vein injection could not efficiently deliver their siRNAs to their target areas because siRNA molecules are easily trapped in the non-specific organs including liver, lungs, and spleen (11, 22). The use of the polymer “TargeTran” may facilitate the delivery of contained reagents at inflammatory neovascular sites. Using a FITC-labeled siRNA system we could demonstrate signals in the neovascular bed that were appreciably greater than observed when the labeled siRNA was administered in PBS (Fig. 8). This polymer is composed of b-polyethylene imine (bPEi), polyethylene glycol (PEG), and arginine-glycine- aspartate peptide sequences (RGD) (23, 24) (Fig. 9). bPEi binds to negative charges in phosphates of the siRNA. RGD motif has been identified as an integrin ligand of activated endothelial cells (25). Endothelial cells express a number of different integrins and integrin $\alpha v\beta 3$ and $\alpha 5\beta 1$ have been shown to be important during angiogenesis (25, 26). Both integrins are the receptor for matrix proteins with an exposed RGD tripeptide moiety and are most prominent on cytokine-activated endothelial cells during angiogenesis (25). Therefore in this study, we targeted the RGD specific integrins on activated and proliferating endothelial cells for siRNA delivery. Our systemic delivery study showed that the

angiogenic inhibitory efficiency of siRNAs with polymer was higher than that of siRNAs without polymer. In addition, we did not observe any abnormalities in mice that experienced systemic delivery of siRNAs with either polymer or PBS at least until the end of the experiment at 21 days after virus infection and siRNA treatment although VEGF pathway genes are necessary for physiological events. Because the half-life of siRNA molecules is short in serum, we believe not only that our siRNAs with polymer can specifically reach in the inflammatory area within short time, but also that the dosage of our siRNAs is not toxic concentration in the mouse treated with siRNAs systemically.

Ocular neovascularization is abnormal proliferation and migration of new blood vessels from preexisting vessel in the eye. Cornea is the avascular organ in normal eye (27). However, if the cornea is infected or stimulated by angiogenic factors, the development of new blood vessels starts from the vessel of limbal area and new formed blood vessels reach on the top of the cornea (28). This neovascularization in the cornea supports the recruitment of inflammatory cells to the corneal tissue. These recruited inflammatory cells, especially neutrophils, produce many angiogenic factors such as VEGF and then these angiogenic factors induce angiogenesis and finally cause blindness. One of the diseases that show severe angiogenesis in the cornea is the herpetic stromal keratitis (HSK) induced by herpes simplex virus 1 (HSV-1) (3). Recent several studies show that potentially bioactive CpG-containing motifs of HSV DNA could stimulate cells to produce VEGF and induce angiogenesis in the eye (7, 8). In addition, strong induction of VEGF expression was found in several neovascularized eye diseases. For these reasons, we designed our experiment using CpG ODN as angiogenic

stimulator and siRNA against VEGF and its receptors as angiogenic inhibitor for the eye disease model.

In conclusion, using nanoparticle polymer systems, we delivered siRNA duplexes targeting VEGF pathway genes to inhibit the CpG ODN induced corneal neovascularization with a mouse model. The significant inhibition of the CpG-induced angiogenesis observed in the study strongly suggested that siRNA-mediated anti-angiogenesis effect represents a potential therapeutic for several uncontrolled angiogenesis diseases. In addition administration of siRNA duplexes targeting VEGF pathway significantly inhibits development of HSK. This present observation demonstrates that the use of VEGF pathway-specific siRNAs with clinically feasible delivery system holds great potential as a novel therapeutic for neovascularized eye diseases. It may also apply to other neovascularization-related eye diseases, including diabetic retinopathy and age-related macular degeneration.

LIST OF REFERENCES

1. Deshpande, S. P., M. Zheng, S. Lee, and B. T. Rouse. 2002. Mechanisms of pathogenesis in herpetic immunoinflammatory ocular lesions. *Vet Microbiol* 86:17.
2. Thomas, J., and B. T. Rouse. 1997. Immunopathogenesis of herpetic ocular disease. *Immunol Res* 16:375.
3. Zheng, M., S. Deshpande, S. Lee, N. Ferrara, and B. T. Rouse. 2001. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. *J Virol* 75:9828.
4. Zheng, M., M. A. Schwarz, S. Lee, U. Kumaraguru, and B. T. Rouse. 2001. Control of stromal keratitis by inhibition of neovascularization. *Am J Pathol* 159:1021.
5. Klinman, D. M., A. K. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg. 1996. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A* 93:2879.
6. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546.
7. Zheng, M., D. M. Klinman, M. Gierynska, and B. T. Rouse. 2002. DNA containing CpG motifs induces angiogenesis. *Proc Natl Acad Sci U S A* 99:8944.

8. Klinman, D. M., M. Zheng, M. Gierynska, and B. T. Rouse. 2002. DNA Containing Bioactive CpG Motifs Promote Angiogenesis. *Drug News Perspect* 15:358.
9. Sioud, M. 2004. Therapeutic siRNAs. *Trends Pharmacol Sci* 25:22.
10. Verma, U. N., R. M. Surabhi, A. Schmaltieg, C. Becerra, and R. B. Gaynor. 2003. Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. *Clin Cancer Res* 9:1291.
11. Sorensen, D. R., M. Leirdal, and M. Sioud. 2003. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J Mol Biol* 327:761.
12. Lu, P. Y., F. Y. Xie, and M. C. Woodle. 2003. siRNA-mediated antitumorigenesis for drug target validation and therapeutics. *Curr Opin Mol Ther* 5:225.
13. Sanceau, J., S. Truchet, and B. Bauvois. 2003. Matrix metalloproteinase-9 silencing by RNA interference triggers the migratory-adhesive switch in Ewing's sarcoma cells. *J Biol Chem* 278:36537.
14. Tuschl, T. 2001. RNA interference and small interfering RNAs. *Chembiochem* 2:239.
15. Tuschl, T. 2002. Expanding small RNA interference. *Nat Biotechnol* 20:446.
16. Babu, J. S., S. Kanangat, and B. T. Rouse. 1993. Limitations and modifications of quantitative polymerase chain reaction. Application to measurement of multiple mRNAs present in small amounts of sample RNA. *J Immunol Methods* 165:207.

17. Kenyon, B. M., E. E. Voest, C. C. Chen, E. Flynn, J. Folkman, and R. J. D'Amato. 1996. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci* 37:1625.
18. Ramanathan, M., A. Giladi, and S. J. Leibovich. 2003. Regulation of vascular endothelial growth factor gene expression in murine macrophages by nitric oxide and hypoxia. *Exp Biol Med (Maywood)* 228:697.
19. Autiero, M., A. Luttun, M. Tjwa, and P. Carmeliet. 2003. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost* 1:1356.
20. Bushman, F. 2003. RNA interference: applications in vertebrates. *Mol Ther* 7:9.
21. Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494.
22. Sioud, M., and D. R. Sorensen. 2003. Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem Biophys Res Commun* 312:1220.
23. Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science* 238:491.
24. Godbey, W. T., K. K. Wu, and A. G. Mikos. 1999. Poly(ethylenimine) and its role in gene delivery. *J Control Release* 60:149.
25. Collo, G., and M. S. Pepper. 1999. Endothelial cell integrin $\alpha 5 \beta 1$ expression is modulated by cytokines and during migration in vitro. *J Cell Sci* 112 (Pt 4):569.

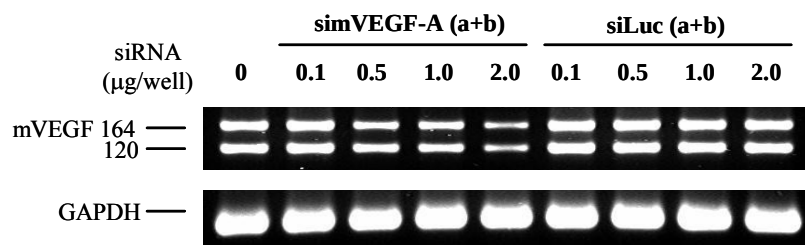
26. Sudhakar, A., H. Sugimoto, C. Yang, J. Lively, M. Zeisberg, and R. Kalluri. 2003. Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. *Proc Natl Acad Sci U S A* 100:4766.
27. Lee, S., M. Zheng, B. Kim, and B. T. Rouse. 2002. Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus. *J Clin Invest* 110:1105.
28. Banerjee, K., P. S. Biswas, B. Kim, S. Lee, and B. T. Rouse. 2004. CXCR2^{-/-} mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6-induced neovascularization. *J Immunol* 172:1237.

APPENDIX

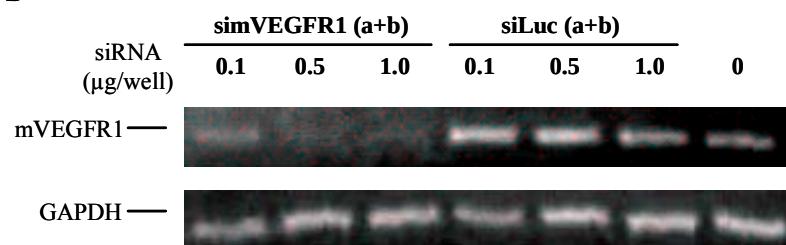
Figure 1. siRNA-mediated in vitro knockdown of VEGF-pathway genes.

RAW264.7 NO (-) cells (A) and SVR cells (B) in 35mm wells were transfected with siRNA (equimolar mixture of two targeted sequences a + b) targeting mVEGFA and mVEGFR1, respectively, at the amount indicated. 293 cells (C) were cotransfected with siRNA targeting mVEGFR2 (equimolar mixture of two targeted sequences a+b was used in subsequent experiments) and plasmid expressing mVEGFR2 at the amount indicated. Cellular RNA was isolated 24h (RT-PCR) or 48 h (RS-PCR) post-transfection, and the knockdown of endogenous expression of mVEGFA or mVEGFR1, or exogenous expression of mVEGFR2 was measured by RT-PCR for mVEGFA, or RS-PCR for mVEGFR1 and mVEGFR2.

A



B



C

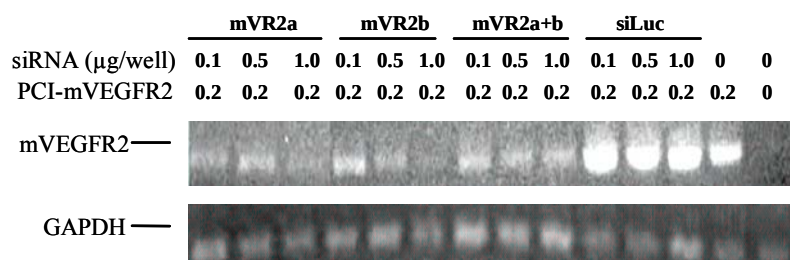


Figure 2. Local delivery of siRNAs targeting VEGF-pathway genes inhibits the CpG ODN- induced angiogenesis.

24 h after implantation with CpG ODN (1 µg) into the micropocket in mouse cornea, mouse was given 10 µg per eye of siLacZ, siVEGFA, siVEGFR1, siVEGFR2, or siVEGFmix (an equimolar mixture of total siRNAs targeting VEGF-pathway genes) by subconjunctival injection. The angiogenesis area was measured on day 4 and 7 after the CpG pellets implantation (four mice per group). Statistically significant differences in angiogenic areas (* $p < 0.05$, ** $p < 0.01$) were observed between the groups (A). Images were taken by stereomicroscopic imaging system at days 7 after CpG pellets implantation (original magnification, x40)(B).

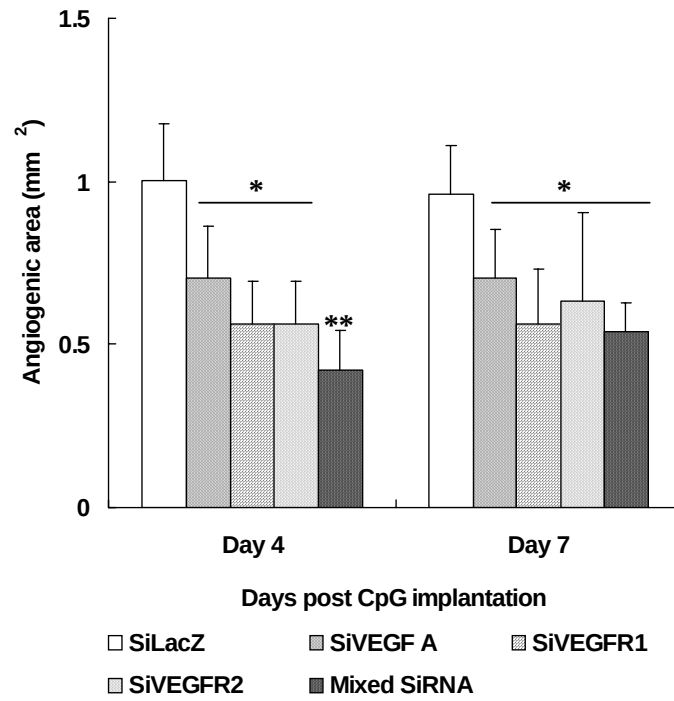
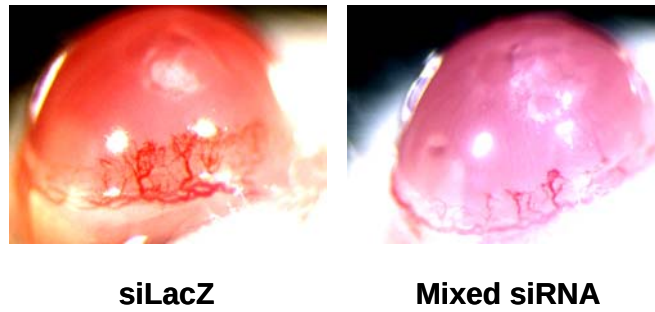
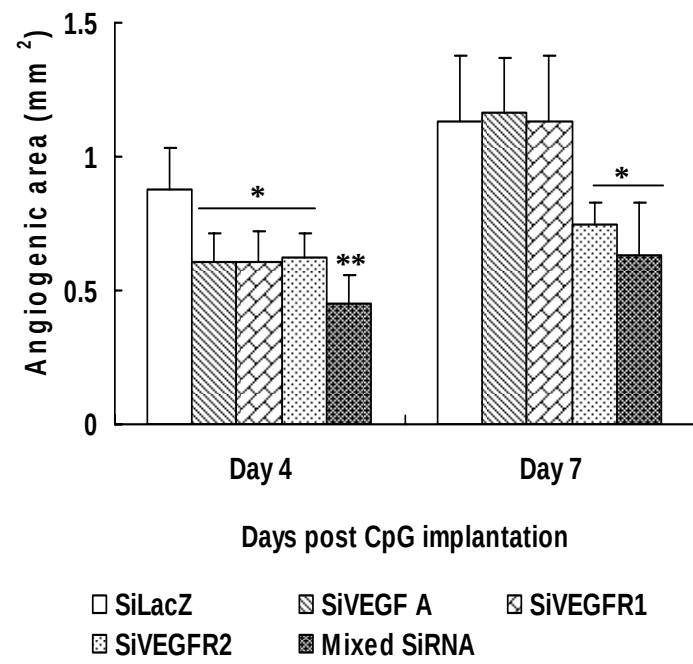
A**B**

Figure 3. Systemic delivery of siRNAs against VEGF-pathway genes inhibits the CpG ODN- induced angiogenesis.

Individual siRNAs or a mixture of total siRNAs against VEGF-pathway genes were delivered with TargeTran, 6 and 24 h after the CpG ODN induction by tail vein injection. The angiogenesis area was measured on day 4 and 7 after the CpG pellets implantation (four mice per group). Statistically significant differences in angiogenic areas (* $p < 0.05$, ** $p < 0.01$) were observed between the groups (A). Images were taken by stereomicroscopic imaging system at days 7 after CpG pellets implantation (original magnification, x 40) (B).

A



B

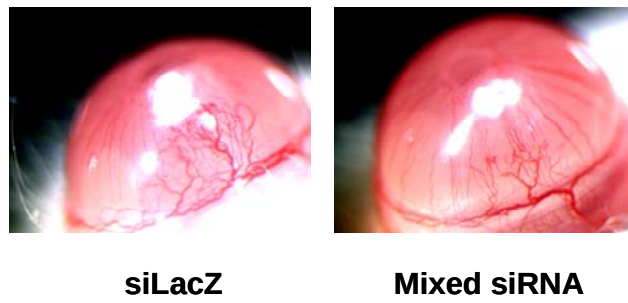
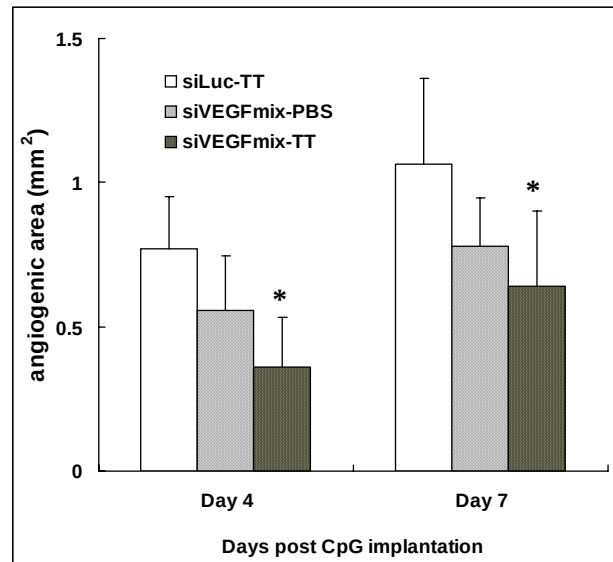


Figure 4. Increased efficiency of siRNAs systemic delivery by polymer and dose response experiment.

A mixture of siVEGF-pathway genes or siLuc control (40 µg) with polymer or PBS control was delivered 6 and 24 h after the CpG ODN induction. The angiogenesis area was measured on day 4 and 7 after the CpG pellets implantation (four mice per group). Statistically significant differences in angiogenic areas (* $p < 0.05$) were observed between the groups (A). Dose response (10, 20, 40, 80 µg siRNAs) experiment with mixed siRNAs targeting VEGF-pathway genes with polymer in the systemic delivery system was performed. The angiogenesis area in each of the four mice for each group was measured on day 4 and 7 post implantation of CpG ODN, and the anti-angiogenic efficiency was compared between different siRNA dosages (B).

A



B

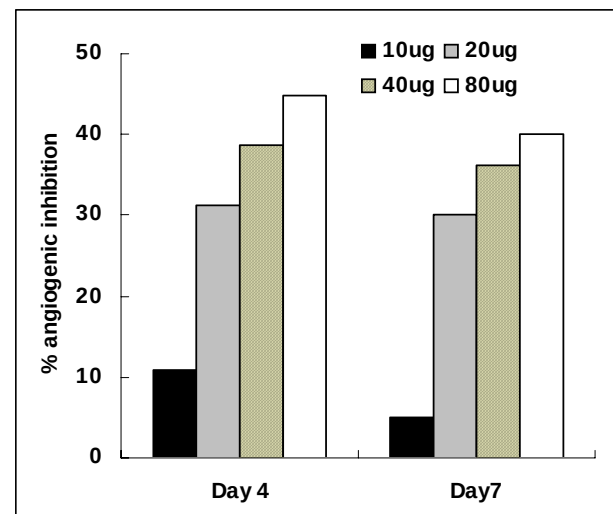
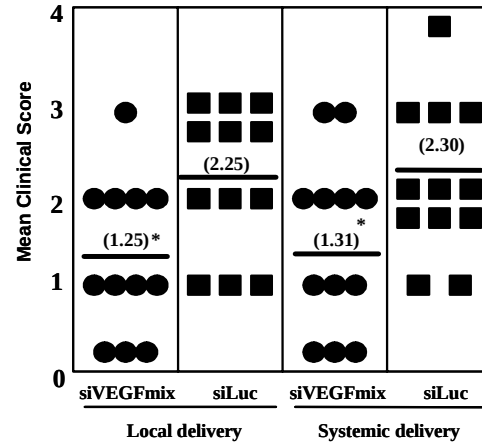


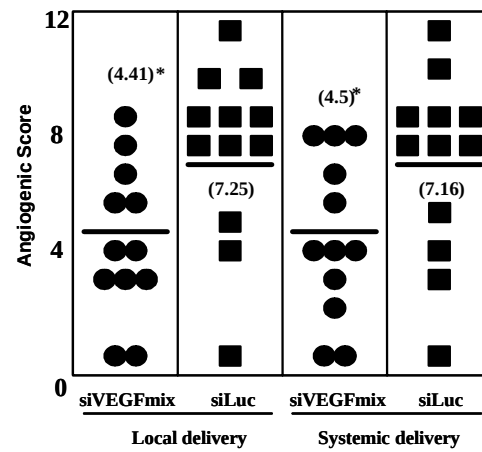
Figure 5. Reduced HSK severity and angiogenic response by administration of siRNAs targeting VEGF-pathway genes.

Mice were infected with 1×10^5 PFU HSV-1 RE per eye, and at day 1 and 3 p.i., were treated with siVEGF mix or siLuc with polymer either local or systemically. Mean lesion HSK score (A) and angiogenic score (B) were calculated at day 10 after viral infection. Each dot represents the clinical score for one eye. Horizontal bars and figures in the parenthesis indicate the mean for each group. Data is compiled from two separate experiments consisting of 6 eyes in each group. * Statistically significant differences in HSK or angiogenesis score ($p < 0.05$) were observed between the groups. At day 14 p.i. extensive growth of blood vessels and ulceration were seen in the infected cornea of siLuc-treated mice. siVEGF mix-treated mice showed less neovascularization near the limbal area (C).

A



B



C

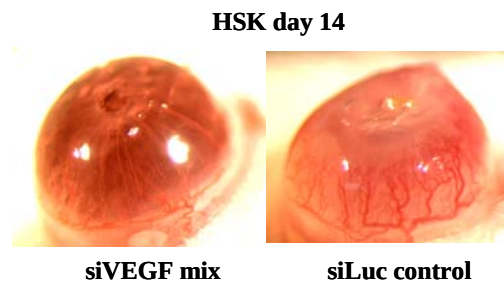
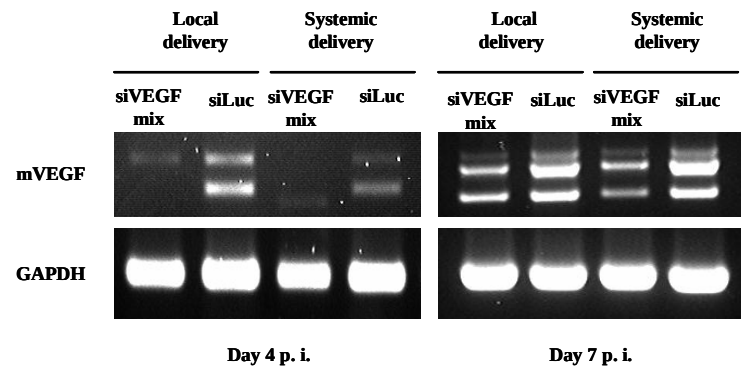


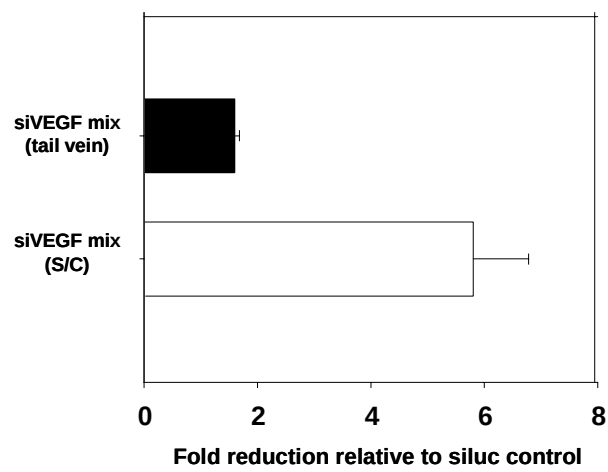
Figure 6. Decreased level of VEGF mRNA in the cornea that was infected and treated with siVEGFmix with either local or systemic delivery.

Two corneas were collected at day 4 or 7 p.i. from mice that were infected with 1×10^5 PFU HSV-1 RE and were treated with siRNAs targeting VEGF-pathway genes at day 1 and 3 p.i. by either local (10 μ g, subconjunctival; S/C) or systemic (40 μ g, tail vein) administration and then VEGF mRNA level was measured by RT-PCR (A) or quantitative real-time PCR (B).

A



B



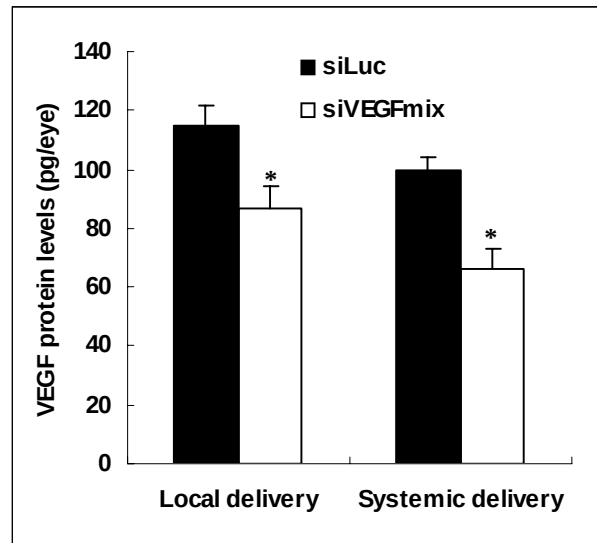


Figure 7. Reduced levels of VEGF protein in the cornea that was infected and treated with siVEGFmix with either local or systemic delivery.

At day 7 p.i. 2 corneas/mouse were processed to measure the VEGF protein levels. Levels of VEGF were estimated from supernatants of corneal lysates of mice infected with HSV-1 and treated with siRNAs targeting VEGF-pathway genes by an antibody capture ELISA as outlined in materials and methods. Results are expressed as mean $\pm$ SD of four separate mice (2 corneas per mouse). * Statistically significant differences in VEGF protein levels ($p < 0.05$) were observed between the groups.

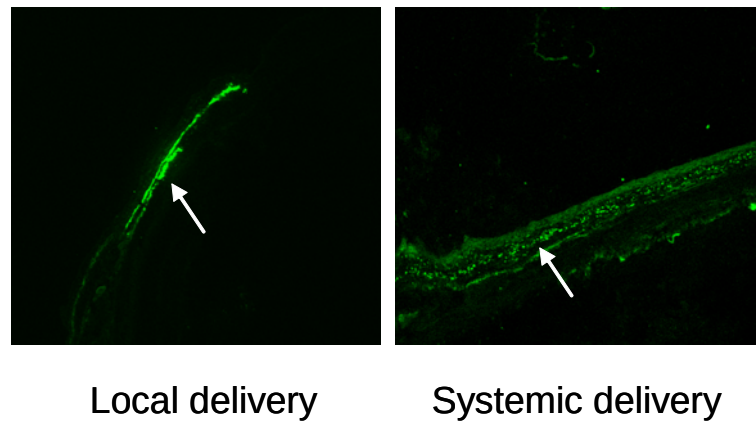


Figure 8. Ocular delivery of FITC-labeled siRNA.

siLuc-FITC (10 μ g for local injection and 40 μ g for systemic injection) with polymer or PBS control was delivered 24 h after the CpG ODN induction. 18 h after siLuc-FITC injection, eyes were frozen and six-micron-thick sections were cut. Local as well as systemic delivery of siRNAs with FITC fluorescences was demonstrated in the limbal area by microscopic observation. The white arrows indicate FITC fluorescences.

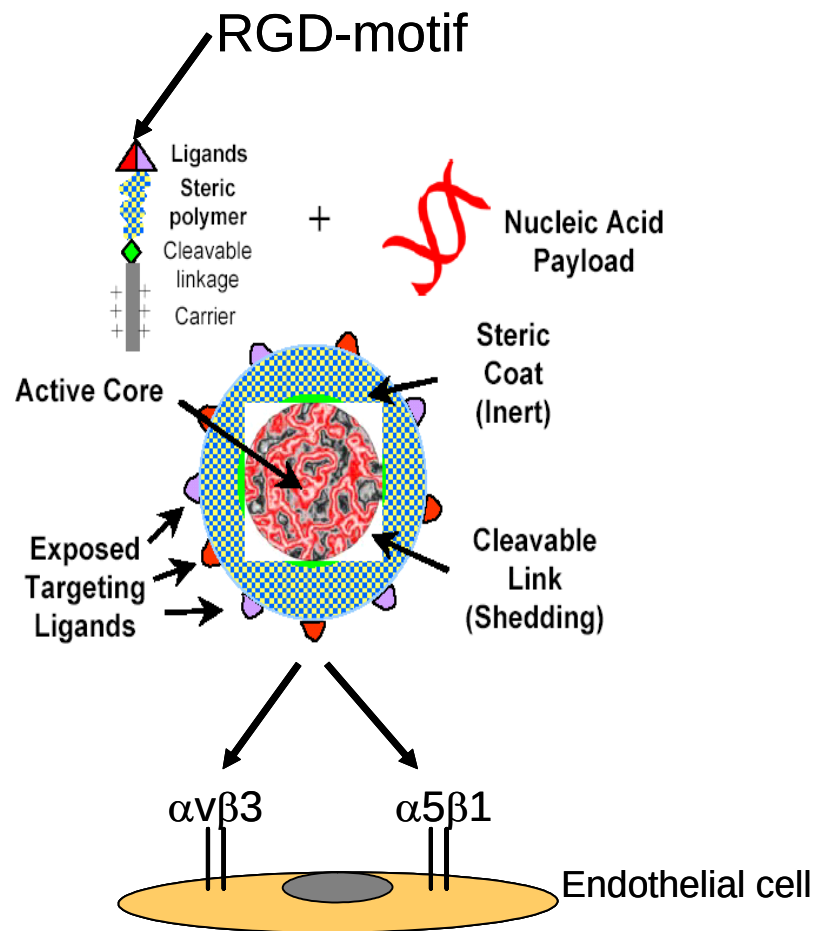


Figure 9. Schematic structure of TargeTran.

TargeTran contains three functional domains: a cationic core, a steric polymer, and a peptide ligand. The polymer electrically interacts with nucleic acids, and initiates self-assembly into nanoparticles that deliver the siRNAs selectively to cells with the receptor of the ligand.

Part III

Application of Plasmid DNA Encoding IL-18 Diminishes Development of Herpetic Stromal Keratitis by Antiangiogenic Effects

Research described in this chapter is a slightly modified version of an article published in 2005 in *The Journal of Immunology* by Bumseok Kim, Sujin Lee, Susmit Suvas and Barry T. Rouse

Kim, B., S. Lee, S. Suvas, and B. T. Rouse. 2005. Application of plasmid DNA encoding IL-18 diminishes development of Herpetic Stromal Keratitis by antiangiogenic effects. *J. Immunol.* 175(1): 509-516. Copyright 2005. The American Association of Immunologist, Inc.

In this chapter “we” and “our” refers to co-authors and me. My contributions in the paper include (1) selection of the topic (2) data analysis and interpretation (3) planning experiments (4) compiling and interpretation of the literature (5) understanding how results fit with the literature (7) compilation of contributions into one paper (8) providing structure to the paper (9) making graphs, figures and tables (10) writing and editing.

ABSTRACT

Herpes simplex virus-1 (HSV-1) infection of the eye can cause a blinding immunoinflammatory stromal keratitis (SK) lesion. Using the mouse model, we have demonstrated that angiogenesis is an essential step in lesion pathogenesis since its inhibition results in diminished severity. The molecules involved in causing corneal angiogenesis are multiple and include the vascular endothelial growth factor (VEGF) family of proteins. In this report we show that application of plasmid DNA encoding IL-18 to the cornea of mice prior to HSV-1 ocular infection resulted in reduced angiogenesis and diminished SK immuno-

inflammatory lesions. The antiangiogenic effects of IL-18 treatment appeared to be mediated by inhibition of VEGF production in the cornea. We also showed that IL-18 controlled VEGF expression *in vitro* and also decreased CpG oligodeoxynucleotide (ODN) induced VEGF dependent neovascularization. In addition the administration of IL-18 binding protein (IL-18 BP), an IL-18 antagonist, into the inflammatory eye resulted in elevated angiogenesis and increased VEGF expression. Our results indicate that IL-18 is an important endogenous negative regulator of HSV-induced angiogenesis resulting in reduced SK lesion severity. Our results could mean that IL-18 administration may represent a useful approach to manage unwanted angiogenesis.

INTRODUCTION

Herpes simplex virus (HSV) infection of the eye commonly results in a blinding T cell mediated immunoinflammatory lesion (1), (2). One prominent early event in the pathogenesis of stromal keratitis (SK) is neovascularization of the usually avascular cornea. Indeed, inhibiting the angiogenic process may result in diminished SK lesions (3). It has become apparent that angiogenesis is a complex multi-step process that includes proliferation and migration of endothelial cells from preexisting blood vessels in response to many angiogenic factors as well as degradation of the extracellular matrix which facilitates neovessel ingress (4). These multiple steps are likely subject to different control measures. For example, it is believed that the local changed balance between angiogenic and angiostatic molecules could switch an angiogenic phenotype (5). Some cytokines may participate in regulating this angiogenic switch. For instance, some cytokines such

as IL-8 (6), IL-6 (7), and IL-1 (8) stimulate angiogenesis, whereas others such as IL-12 (9), platelet factor 4 (PF4) (10) and IFN-inducible protein-10 (IP-10) (11) inhibit neovascularization. Recently, it became evident that IL-18, a cytokine that shares several properties of IL-12, may induce tumor regression that involves inhibition of angiogenesis by suppression of endothelial cell proliferation (12, 13) and promotion of IFN- γ release (12). Recent studies in a vascular ischemic disease model showed that *in vivo* application of plasmid DNA encoding murine IL-18 binding protein (IL-18BP, the endogenous inhibitor of IL-18) stimulated ischemia-induced angiogenesis indicating that IL-18 plays an antiangiogenic role in the ischemic model. Furthermore, the addition of IL-18 to human umbilical vein endothelial cells (HUVECs) completely abrogated VEGF-induced Akt phosphorylation in endothelial cells (14). In addition, it was reported that IL-18 knockout mice have abnormal retinal vascular development because of over-expression of several angiogenesis-associated factors in the retina (15). By contrast, IL-18 may induce endothelial cell migration and could act as an angiogenic factor (16), although this observation remains unconfirmed. Most reports indicate that IL-18 is on balance an antiangiogenic factor, but the precise mechanism by which IL-18 exerts this effect remains unresolved. In the present study, we have measured the effects of IL-18 on angiogenesis induced by HSV infection of the mouse eye. Our results demonstrated that its antiangiogenic effects occurred via inhibition of the expression of VEGF, a major factor in HSV induced ocular angiogenesis. To our knowledge this demonstration of a direct effect of IL-18 on VEGF expression *in vitro* has not previously been reported.

MATERIALS AND METHODS

Reagents

Phosphorothioate ODNs were kindly provided by Dennis M. Klinman (Biologics Evaluation and Research, FDA, U.S.A.). The sequences of stimulatory ODNs used in this study were: 1466, TCAACGTTGA, and 1555, GCTAGACGTTAGCGT. Subsequent studies were performed using an equimolar mixture of ODNs 1466 and 1555.

Mice

Female 4-to-5 week old BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). To prevent bacterial infection, all mice received treatment with sulfamethoxazole/trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5 ml/200 ml drinking water. All investigations followed guidelines of the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee (Knoxville) are fully accredited by the American Association of Laboratory Animal Care.

Virus and corneal HSV infection

HSV-1 strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile) was used in all procedures. Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA; Cat.no.CCL81), titrated, and stored in aliquots at -80°C until used. Corneal infections of all mouse groups were

conducted under deep anesthesia induced by Avertin. The mice were scarified lightly on their corneas with a 32 gauge needle and infected with a 2 μ l drop containing 10^6 or 3×10^5 plaque-forming units (PFU) of HSV-1 RE that was applied to the eye and gently massaged with the eyelids.

Plasmid DNA preparation and administration

Plasmid DNA encoding murine IL-18 was kindly provided by Dr. Kenji Okuda (Yokoyama City University School of Medicine, Japan) and pCDNA3.1-GFP was used as control. The plasmid DNA used in this work was inserted into the pCDNA3.1 expression vector (Invitrogen, San Diego, CA). The plasmid DNA was purified by a polyethylene glycol precipitation method. The quality of DNA was measured by electrophoresis on 1% agarose gel. Protein expression of the different plasmids was determined by dot blot after *in vitro* transfection of Chinese hamster ovary (CHO) cells.

Plasmid DNA (100 μ g) was suspended in 4 μ l sterile phosphate-buffered saline (PBS). Corneas were scarified using a 32 gauge needle in a criss-cross pattern, and the plasmid was administered intraocularly on 4 and 2 days before virus infection.

Cell culture

BALB/c mouse brain microvascular endothelial (MBE) cells were kindly provided by Dr. Auerbach (University of Wisconsin). MBE cells were grown in DMEM supplemented with 10% FCS. They were maintained in culture at the University of

Tennessee with no signs of cell senescence after they were received from the University of Wisconsin.

Cell proliferation assay (DNA synthesis assay)

Recombinant mouse IL-18 (Medical & Biological Laboratories Co., Woburn, MA) or OVA (Sigma Co., St. Louis, MO) was tested for the ability to stimulate proliferation of mouse endothelial cells (17). Accordingly, MBE cells (1,000 cells/well) were seeded onto 96 well tissue culture plates and incubated in 10% DMEM for 12 h. To maximize activation MBE cells were then stimulated with recombinant bFGF (10 ng/ml, R&D system) for 12 h in 0.4% DMEM. Activated MBE cells were treated with different recombinant proteins (IL-18 or OVA) in 0.4% DMEM at various doses (0.1, 1, 10, 100 ng/ml). After 16 hours cells were pulse labeled with ^3H -thymidine (1 $\mu\text{Ci/mL}$) for 6 h. The level of thymidine incorporation was then measured using a beta scintillation counter (Inotech, Lansing, Mich.).

Intracellular detection of VEGF

For intracellular detection of VEGF, MBE cells (5,000 cells/well) were seeded onto 24 well tissue culture plates and incubated in 10% DMEM for 12 h. MBE cells were then stimulated with bFGF (10 ng/ml) for 12 h in 0.4% DMEM. Stimulated MBE cells were then treated with recombinant IL-18 or OVA as a control in 0.4% DMEM using various concentrations (0.01, 0.1, 1, 10, 100 ng/ml). After 24 h of incubation adherent cells were dispersed in trypsin-EDTA and resuspended in FACS solution. For intracellular staining for VEGF the cells were

then fixed in paraformaldehyde and stained for VEGF by using biotinylated rat-anti-mVEGF164 antibody (0.2 µg/well, R&D system) followed by streptavidin-PE (0.2 µg/well, BD Pharmingen). Positive cells and mean fluorescence were measured by flow cytometry using a FACScan (BD Biosciences, Mountain View, CA). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

Clinical observations

The eyes were examined on different days after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Co, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing stromal keratitis. The severity of angiogenesis was recorded as described previously (3). In reference to the angiogenic scoring system, the method relied on quantifying the degree of neovessel formation based on three primary parameters: 1) the circumferential extent of neovessels (as the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant of the circle; and 3) the longest neovessel in each quadrant was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ~0.4 mm (radius of the cornea is ~1.5 mm). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovessel index (range, 0 to 16) for each eye at a given time point.

RNA extraction and RT PCR

At 24 h following IL-18 treatment the cells were harvested and RNA was extracted by using an RNeasy protect mini kit (Qiagen, Valencia, CA). For ocular infections, 2 corneas/time point were excised and transferred to RNAlaterTM. Briefly, cells were lysed in RLT buffer (Qiagen, catalog no. 79216) and RNA was purified according to manufacturer's instructions. An RNase-free Dnase set (Qiagen) was used to remove any contaminating genomic DNA. Total cellular RNA (10 µg/ml) was reversed-transcribed using oligo (dT) primers and reverse transcriptase (Promega, Madison, WI) according to protocols described previously (18). The cDNA was made by reverse-transcription at 42°C for 90 min. The primer sequences for mVEGF RT-PCR were: 5'-GCGGGCTGCCTCGCAGTC-3' (sense) and 5'-TCACCGCCTTGGCTTGTCAC-3' (antisense). RT-PCR products were 644 bp for mVEGF164 and 512 bp for mVEGF120. The primer sequences for mIL-18 RT-PCR were: 5'-ATGGCTGCCATGTCAGAAG-3' (sense) and 5'-TAACTTTGATGTAAGTTAGTGAGAG-3' (antisense). The amplification profile was 94°C for 1 min, 65°C for 1 min, and 72°C for 15 min for 30 cycles. The PCR products were separated by 1% agarose gel electrophoresis.

VEGF quantification of culture supernatants or corneal lysates by ELISA

The culture supernatants from recombinant protein (IL-18 or OVA) treated MBE cells, lysates from IL-18 DNA or IL-18 BP (2 µg/cornea, R&D system) treated CpG ODNs implanted, as well as HSV-1 infected cornea were used for the measurement of VEGF by a standard sandwich enzyme-linked immunosorbent

assay (ELISA) protocol. For preparation of corneal lysates, two corneas/time point (n = 4) were collected and minced with liquid nitrogen. Minced pieces were collected in 1 ml of DMEM without FCS and homogenized using an ultra sonicator (Heat systems-Ultrasonics, NY, U.S.A.). The lysates were then clarified by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was collected and stored at –80°C till further use. The ELISA plate was coated with anti-mouse VEGF capture antibody (100 µl/well of the capture antibody at 0.4 µg/ml, R&D system) and incubated at 4°C overnight. The plate was washed with 0.05% tween20/PBS and blocked with 3% BSA for 2 h at 37°C. After washing, serially diluted corneal lysates were added to the plate and incubated at 4°C overnight. The plate was washed and then incubated with anti-VEGF biotinylated detection Ab (50 ng/ml, R&D system) for 2 h. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory, PA, U.S.A.) was added. The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Intrastromal corneal injection and micropocket assay

A nick in the epithelium and anterior stroma of a BABL/c mouse cornea was made in the mid periphery with a 32 gauge needle (Becton Dickinson, Franklin Lakes, NJ) under direct microscopic observation. A blunt 32 gauge needle with a 30° bevel was introduced into the corneal stroma and advanced to the corneal center. 2 µl (1 µg of IL-18 or GFP DNA) of plasmid solution was injected under pressure into the stroma to separate corneal lamellae and disperse the plasmid.

In vivo angiogenic activity was assayed in the avascular cornea of mouse eyes as previously described (19). Briefly, mice were pretreated with plasmid DNA encoding IL-18 DNA and control GFP DNA intrastromally 1 day before implantation of pellets. Pellets for insertion into the cornea were made by combining known amounts of CpG ODNs, sucralfate (10 mg, Bulch Meditec) and hydron polymer in ethanol (120 mg/1 ml ethanol, Interferon Sciences), and applying the mixture to a 15×15 mm² piece of synthetic mesh (Tekto). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 1 µg of CpG ODNs. Pellets containing CpG ODNs were implanted into an intracorneal pocket (1 mm from the limbus). The eyes were then evaluated for corneal neovascularization. The extent of the neovessel ingrowth was recorded by direct measurement using calipers (Symbol of Quality, biomedical research instruments, Rockville, Maryland) under stereomicroscopy (Leica Microsystems, Wetzlar, Germany). The length of the neovessels originating from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours were measured. Each clock hour is equal to 30° at the circumference. The angiogenic area was calculated according to the formula for an ellipse. $A = [(clock\ hours) \times 0.4 \times (vessel\ length\ in\ mm) \times \pi] / 2$.

Subconjunctival injection of IL-18 BP

Mice were treated with recombinant IL-18 BP and control OVA protein (2 µg/10µl PBS per eye) subconjunctivally on day 0 and 2 after implantation of CpG ODNs pellets containing 500 ng or after infection with HSV-1 RE (3×10^5 PFU).

The eyes were then evaluated for corneal neovascularization or herpetic SK lesion severity.

Statistical analysis

Significant differences between groups were evaluated by using the Student's t test. $P < 0.05$ was regarded as a significant difference between two groups.

RESULTS

Application of Plasmid DNA encoding IL-18 decreases the severity and incidence of SK

An initial experiment was performed to determine if the expression of IL-18 in corneas prior to HSV infection had any effect on the severity of SK lesions. To express IL-18 in the cornea, mice were treated with 100 µg of plasmid DNA encoding IL-18 on their scarified corneas four and two days before virus infection, with control animals receiving the plasmid DNA encoding GFP. Subsequently, the corneas of mice were infected with 10^6 plaque-forming units (PFU) of HSV-1 RE. As shown in Fig. 1 (A and B), IL-18 pre-exposure resulted in decreased average severity and percentage of animals with clinical lesions compared to control DNA treated mice. A comparison of average severity scores on days 12, 15, and 21 p.i. was all significantly less in the IL-18 treated than the control group ($p < 0.05$). Recently IL-18 was claimed to exert antiviral effects (20) against HSV-1 infection (21) due to augmentation of IFN- γ . This mechanism, however, seemed not to explain our findings since comparisons of viral yields and duration of detectable

virus in IL-18 pretreated and control cornea showed no statistically significant differences (Data not shown).

Suppression of HSV-induced angiogenesis

One essential step in the pathogenesis of SK is angiogenesis mediated by several angiogenesis factors. Thus, one explanation considered to explain the inhibitory effects of IL-18 on SK was anti-angiogenesis. To record such an effect, levels of neovascularization were measured in the corneas by biomicroscopy in groups of mice either pretreated with IL-18 DNA or control plasmid prior to HSV infection. The results shown in Fig. 1C indicate that levels of angiogenesis were significantly less at most time points tested in the IL-18 DNA compared to control plasmid treated animals.

Suppressed expression of VEGF in the HSV-1 infected cornea by IL-18

Previous reports on HSV-1 induced angiogenesis have identified VEGF as a molecule that is highly expressed in the infected eye and clearly involved in angiogenesis (19). To evaluate whether treatment of IL-18 DNA inhibits HSV-1 induced angiogenesis by decreased expression of VEGF gene in the cornea we measured VEGF mRNA and protein levels in IL-18 pretreated and HSV-1 infected corneas at day 2 and 4 after infection. As shown in Fig. 2A and B, VEGF mRNA and protein levels were lower in those that received IL-18 DNA compared to controls given control DNA at day 4 ($p < 0.05$). This result suggests that the target gene of IL-18 with angiostatic function is the VEGF molecule in the SK cornea.

Inhibition of CpG-induced corneal neovascularization by IL-18

A previous study demonstrated that CpG containing ODNs encapsulated in hydropolymer pellets induce VEGF mediated angiogenesis when inserted into corneal micropockets (22, 23). Hence, attempts were made to determine if VEGF expression and function were affected by IL-18. In the first approach, the effects of IL-18 plasmid expression in the eye were tested for the ability to affect the extent of angiogenesis caused by CpG ODNs using a corneal micropocket assay. In this experiment pretreatment of IL-18 DNA markedly inhibited (approximately 60% inhibition) the extent of CpG-induced angiogenesis compared to control DNA treated animals (Fig. 3A and B).

To evaluate whether treatment of IL-18 diminished the production of VEGF protein in the CpG stimulated cornea, VEGF protein levels were compared in IL-18 and control DNA treated corneas at day 4 and 7 after CpG implantation. As shown in Fig. 3C, VEGF protein levels were significantly lower ($p < 0.05$) in the IL-18 DNA treated group, but significant differences from controls were only evident in the day 7 samples. These results show that administration of IL-18 inhibited neovascularization by suppression of VEGF production in the CpG induced inflammatory cornea.

Suppression of VEGF expression and production *in vitro* by IL-18

Additional experiments regarding the anti-angiogenic effects of IL-18 were performed *in vitro* using an endothelial cell line derived from mouse brain endothelial (MBE) cell that produces VEGF endogenously. To investigate whether IL-18 inhibits VEGF expression and production *in vitro*, different concentrations

of recombinant IL-18 were incubated with MBE cells pre-stimulated by 10 ng/ml FGF-2 for 12 h as described in materials and methods. At 24 h after IL-18 treatment cell supernatants or cells were collected and VEGF expression and production were measured by RT-PCR, ELISA, or intracellular staining, respectively. As shown in Fig. 4A, expression of both the 120 and 164 isoforms of VEGF was reduced by recombinant IL-18 in a dose dependent manner. Furthermore, VEGF production in cell lines was significantly reduced in IL-18 treated cell lines compared to control OVA protein treated cell lines (Fig. 4B, $P < 0.05$ or $P < 0.01$). Also, the intensity of intracellular VEGF fluorescence was markedly decreased by the addition of IL-18 (Fig. 4C and D). This downregulation of VEGF production and expression occurred in a dose-dependent manner and was clearly evident when compared with cells incubated with OVA. In addition, VEGF expression was not decreased by incubation with different concentrations of recombinant IL-12, a cytokine that shares several properties of IL-18 (data not shown). To exclude the possibility of non-specific gene suppression by IL-18, we also measured the expression of bFGF, but no suppression of this gene was detected (data not shown). Furthermore, no apoptosis of cells following IL-18 treatment was observed by the Annexin-V staining method (data not shown).

Inhibitory effects of IL-18 on endothelial cell proliferation

Angiogenesis involves growth of endothelial cells (proliferation and migration) and other vascular components from existing blood vessels (24). The effect of IL-18 on endothelial cell proliferation was tested using the endothelial cell line MBE. In these experiments cells recently stimulated by 10 ng/ml FGF-2 were washed

and exposed to different concentrations of IL-18. After 24 h incubation the effect on proliferation was measured by a short term pulse with ^3H -thymidine. At the concentration of 100 and 10 ng/ml, IL-18 inhibited significantly endothelial cell proliferation by 53 and 30% respectively ($P < 0.05$) (Fig. 5). IL-18 was shown to inhibit endothelial cell proliferation in a dose-dependent way. These data also suggest that inhibition of angiogenesis by IL-18 is mediated by the suppression of endothelial cell proliferation.

Upregulation of VEGF by IL-18 BP in the inflammatory cornea

A previous study showed that several angiogenesis-associated factors including VEGF were overexpressed in the retinas of IL-18 KO mice and indicated that the endogenous IL-18 regulates VEGF expression in the retina (15). Therefore, we tested whether endogenous IL-18 may also regulate VEGF expression in the cornea. First we measured endogenous IL-18 mRNA expression in naïve and virus infected cornea. As shown in Fig. 6A, IL-18 was present in normal corneal extracts, and the IL-18 mRNA expression was slightly increased in the SK cornea. It is known that IL-18 BP functions as an IL-18 antagonist by binding to IL-18 and blocking its biological activity (14). To investigate the effect of endogenous IL-18 on corneal angiogenesis, IL-18 BP was used to block endogenous IL-18 in both virus infected and CpG implanted eye. The corneas of mice were infected with 3×10^5 PFU of HSV-1 RE followed by treatment with 2 μg of recombinant IL-18 BP in the subconjunctival area at day 0 and 2 after virus infection. As shown in Fig. 6 (B and C), IL-18 BP application resulted in more severe SK lesions and increased angiogenesis compared to control OVA treated mice. A comparison of average

severity scores after day 4 p.i. or angiogenesis scores spanning all time points were all significantly higher in the IL-18 BP treated group than the control group (* $p < 0.05$, ** $p < 0.01$). In addition, corneas were implanted with 500 ng of CpG ODNs pellets and then treated with 2 μ g of recombinant IL-18 BP by subconjunctival injection at day 0 and 2 after pellet implantation. The IL-18 BP treatment was shown to significantly increase the CpG-induced angiogenesis (Fig. 6D). We further investigated the expression levels of VEGF mRNA and protein in the CpG stimulated and IL-18 BP treated cornea. VEGF mRNA and protein were upregulated in the IL-18 BP treated corneas compared to those of the OVA treated mice (Fig. 6E and F). We could exclude the possibility that IL-18 BP itself is an angiogenic factor, since injection of IL-18 BP into the normal cornea failed to cause significant levels of angiogenesis (data not shown). Taken together our results indicate that endogenous IL-18 may control the angiogenesis induced by inflammation.

DISCUSSION

In the present study, we show that the administration of plasmid DNA encoding IL-18 to the cornea of mice prior to HSV ocular infection results in diminished immunoinflammatory lesions. The diminished SK lesion severity by IL-18 treatment was caused by the inhibitory effect on corneal neovascularization, an essential step in the pathogenesis of herpetic SK. One major factor induced by HSV ocular infection that is angiogenic is VEGF (19). By both *in vitro* and *in vivo* approaches we show that IL-18 resulted in a diminished VEGF response, although the actual mechanism by which this was mediated was not defined. Application of

IL-18 controlled VEGF expression in endothelial cells and also decreased HSV or CpG induced VEGF dependent neovascularization. In addition the administration of IL-18 binding protein (IL-18 BP), an IL-18 antagonist, into the inflammatory eye resulted in elevated angiogenesis and increased VEGF expression. We interpret these observations to indicate that IL-18 is an important endogenous negative regulator of HSV-induced angiogenesis resulting in reduced SK lesion severity.

The activity of endogenous IL-18 can be regulated by IL-18 BP, a secreted protein possessing a high-affinity binding and ability to neutralize IL-18 (25). Recent several studies associated with IL-18/IL-18 BP signaling have shown that IL-18 BP efficiently inhibits the IL-18 activity in many inflammatory disease models (14, 26). Since endogenous IL-18 was present in normal as well as infected corneas, IL-18 BP was applied to the infected or CpG implanted eye to demonstrate the antiangiogenic role of endogenous IL-18 in our SK model. Blocking of endogenous IL-18 by administration of IL-18 BP increased VEGF expression and angiogenesis in the virus infected as well as CpG implanted cornea, further demonstrating that IL-18 is antiangiogenic. Since VEGF expression in the inflammatory cornea was localized to inflammatory cells including neutrophils and macrophages, it seems that endogenous IL-18 may control VEGF expression in inflammatory cells as well as proliferating endothelial cells. In addition, the fact that there is no angiogenesis in the normal cornea treated with IL-18 BP supports the idea that endogenous IL-18 regulates VEGF expression in the inflammatory environment.

IL-18 was first identified as an IFN- γ inducing factor and proinflammatory cytokine (27), but it may mediate several additional biological functions. For example, IL-18 can induce TNF- α or Fas-ligand related cell cytotoxicity (28) and mediate inflammatory tissue damage (29). In addition, IL-18 can skew naïve T cells towards a Th-1 type phenotype by decreased synthesis of IL-10 and increased production of IFN- γ (30). IL-18 is a member of the IL-1 family, with structural similarity and some common biological activities (31). However, IL-18 most closely resembles IL-12, as both are potent inducers of IFN- γ (27). Although IL-12 has also been reported to be a potent anti-angiogenic factor through effects on IFN- γ signaling (32), the function of IL-18 on angiogenesis has not been clearly defined. Previously we showed that the anti-angiogenic effects of IL-12 on HSV induced angiogenesis was mediated by IFN- γ induced antiangiogenic chemokines such as IP-10 and MIG (9). However the antiangiogenic effects of IL-18 appeared to differ from IL-12. Thus IL-18, unlike IL-12, could inhibit endothelial cell proliferation and had direct inhibitory effects on VEGF production *in vitro* and *in vivo*. Furthermore, IL-18 treatment had no effect on IFN- γ induction in endothelial cells (data not shown), indicating that the antiangiogenic effects of IL-18 does not appear to involve signaling through IFN- γ as occur with IL-12.

The over-expression of several potent angiogenic factors including VEGF, bFGF, and PDGF-A genes in the retina (15), or the upregulation of VEGF mRNA in the hindlimb after ischemic injury, has been previously reported in IL-18 KO mice (14). Our data again support the idea that IL-18 has angiostatic function and shows its function in the SK model. Although it was reported that IL-18 induces

endothelial cell migration and tube formation (16), it could be that endothelial cell proliferation induced by potent angiogenic factors may be more important than endothelial cell migration for the process of angiogenesis. This issue is under further investigation.

Taken together our results indicate that IL-18 may act as an endogenous negative regulator of angiogenesis caused by inflammatory stimuli. In fact the eye has low levels of IL-18 which if inhibited by administration of IL-18 BP, subsequent levels of HSV induced angiogenesis and SK become significantly increased. Manipulating the function of IL-18 could represent a useful approach to control unwanted angiogenesis.

LIST OF REFERENCES

1. Deshpande, S. P., M. Zheng, S. Lee, and B. T. Rouse. 2002. Mechanisms of pathogenesis in herpetic immunoinflammatory ocular lesions. *Vet Microbiol* 86:17.
2. Gangappa, S., S. P. Deshpande, and B. T. Rouse. 1999. Bystander activation of CD4(+) T cells can represent an exclusive means of immunopathology in a virus infection. *Eur J Immunol* 29:3674.
3. Zheng, M., M. A. Schwarz, S. Lee, U. Kumaraguru, and B. T. Rouse. 2001. Control of stromal keratitis by inhibition of neovascularization. *Am J Pathol* 159:1021.
4. Lee, S., M. Zheng, B. Kim, and B. T. Rouse. 2002. Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus. *J Clin Invest* 110:1105.
5. Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353.
6. Koch, A. E., P. J. Polverini, S. L. Kunkel, L. A. Harlow, L. A. DiPietro, V. M. Elner, S. G. Elner, and R. M. Strieter. 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 258:1798.
7. Banerjee, K., P. S. Biswas, B. Kim, S. Lee, and B. T. Rouse. 2004. CXCR2^{-/-} mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6-induced neovascularization. *J Immunol* 172:1237.
8. Biswas, P. S., K. Banerjee, B. Kim, and B. T. Rouse. 2004. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. *J Immunol* 172:3736.

9. Lee, S., M. Zheng, S. Deshpande, S. K. Eo, T. A. Hamilton, and B. T. Rouse. 2002. IL-12 suppresses the expression of ocular immunoinflammatory lesions by effects on angiogenesis. *J Leukoc Biol* 71:469.
10. Maione, T. E., G. S. Gray, J. Petro, A. J. Hunt, A. L. Donner, S. I. Bauer, H. F. Carson, and R. J. Sharpe. 1990. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* 247:77.
11. Arenberg, D. A., S. L. Kunkel, P. J. Polverini, S. B. Morris, M. D. Burdick, M. C. Glass, D. T. Taub, M. D. Iannettoni, R. I. Whyte, and R. M. Strieter. 1996. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J Exp Med* 184:981.
12. Coughlin, C. M., K. E. Salhany, M. Wysocka, E. Aruga, H. Kurzawa, A. E. Chang, C. A. Hunter, J. C. Fox, G. Trinchieri, and W. M. Lee. 1998. Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. *J Clin Invest* 101:1441.
13. Cao, R., J. Farnebo, M. Kurimoto, and Y. Cao. 1999. Interleukin-18 acts as an angiogenesis and tumor suppressor. *Faseb J* 13:2195.
14. Mallat, Z., J. S. Silvestre, S. Le Ricousse-Roussanne, L. Lecomte-Raclet, A. Corbaz, M. Clergue, M. Duriez, V. Barateau, S. Akira, A. Tedgui, G. Tobelem, Y. Chvatchko, and B. I. Levy. 2002. Interleukin-18/interleukin-18 binding protein signaling modulates ischemia-induced neovascularization in mice hindlimb. *Circ Res* 91:441.

15. Qiao, H., K. H. Sonoda, Y. Sassa, T. Hisatomi, H. Yoshikawa, Y. Ikeda, T. Murata, S. Akira, and T. Ishibashi. 2004. Abnormal retinal vascular development in IL-18 knockout mice. *Lab Invest* 84:973.
16. Park, C. C., J. C. Morel, M. A. Amin, M. A. Connors, L. A. Harlow, and A. E. Koch. 2001. Evidence of IL-18 as a novel angiogenic mediator. *J Immunol* 167:1644.
17. Bastaki, M., E. E. Nelli, P. Dell'Era, M. Rusnati, M. P. Molinari-Tosatti, S. Parolini, R. Auerbach, L. P. Ruco, L. Possati, and M. Presta. 1997. Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. *Arterioscler Thromb Vasc Biol* 17:454.
18. Babu, J. S., S. Kanangat, and B. T. Rouse. 1993. Limitations and modifications of quantitative polymerase chain reaction. Application to measurement of multiple mRNAs present in small amounts of sample RNA. *J Immunol Methods* 165:207.
19. Zheng, M., S. Deshpande, S. Lee, N. Ferrara, and B. T. Rouse. 2001. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. *J Virol* 75:9828.
20. Kimura, K., K. Kakimi, S. Wieland, L. G. Guidotti, and F. V. Chisari. 2002. Interleukin-18 inhibits hepatitis B virus replication in the livers of transgenic mice. *J Virol* 76:10702.

21. Fujioka, N., R. Akazawa, K. Ohashi, M. Fujii, M. Ikeda, and M. Kurimoto. 1999. Interleukin-18 protects mice against acute herpes simplex virus type 1 infection. *J Virol* 73:2401.
22. Zheng, M., D. M. Klinman, M. Gierynska, and B. T. Rouse. 2002. DNA containing CpG motifs induces angiogenesis. *Proc Natl Acad Sci U S A* 99:8944.
23. Klinman, D. M., M. Zheng, M. Gierynska, and B. T. Rouse. 2002. DNA Containing Bioactive CpG Motifs Promote Angiogenesis. *Drug News Perspect* 15:358.
24. Folkman, J. 2003. Fundamental concepts of the angiogenic process. *Curr Mol Med* 3:643.
25. Dinarello, C. A., D. Novick, M. Rubinstein, and G. Lonnemann. 2003. Interleukin 18 and interleukin 18 binding protein: possible role in immunosuppression of chronic renal failure. *Blood Purif* 21:258.
26. Dinarello, C. A. 2004. Interleukin-18 and the treatment of rheumatoid arthritis. *Rheum Dis Clin North Am* 30:417.
27. Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, and et al. 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378:88.
28. Dao, T., K. Ohashi, T. Kayano, M. Kurimoto, and H. Okamura. 1996. Interferon-gamma-inducing factor, a novel cytokine, enhances Fas ligand-mediated cytotoxicity of murine T helper 1 cells. *Cell Immunol* 173:230.

29. Ushio, S., M. Namba, T. Okura, K. Hattori, Y. Nukada, K. Akita, F. Tanabe, K. Konishi, M. Micallef, M. Fujii, K. Torigoe, T. Tanimoto, S. Fukuda, M. Ikeda, H. Okamura, and M. Kurimoto. 1996. Cloning of the cDNA for human IFN-gamma-inducing factor, expression in *Escherichia coli*, and studies on the biologic activities of the protein. *J Immunol* 156:4274.
30. Wereszczynska-Siemiatkowska, U., J. Kosel, and A. Siemiatkowski. 2004. [Biological properties of interleukin 18]. *Pol Merkuriusz Lek* 16:279.
31. Joosten, L. A., R. L. Smeets, M. I. Koenders, L. A. van den Bersselaar, M. M. Helsen, B. Oppers-Walgreen, E. Lubberts, Y. Iwakura, F. A. van de Loo, and W. B. van den Berg. 2004. Interleukin-18 promotes joint inflammation and induces interleukin-1-driven cartilage destruction. *Am J Pathol* 165:959.
32. Voest, E. E., B. M. Kenyon, M. S. O'Reilly, G. Truitt, R. J. D'Amato, and J. Folkman. 1995. Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst* 87:581.

APPENDIX

Figure 1. Inhibition of SK lesion severity (A), incidence (B), and HSV-induced angiogenesis (C) by IL-18 DNA administration.

Groups of animals ($n=6$) were treated intraocularly at 2 and 4 days before virus infection with 100 μ g IL-18 DNA (o) or control GFP DNA (●). Two days after the second treatment, animals were infected with 10^6 PFU of HSV-1 RE on their scarified corneas and subsequently scored for lesion severity by slit-lamp biomicroscopy. The data are compiled from three independent experiments. Statistically significant differences in SK (A) and angiogenic (B) score were observed between the groups (* $p < 0.05$, ** $p < 0.01$).

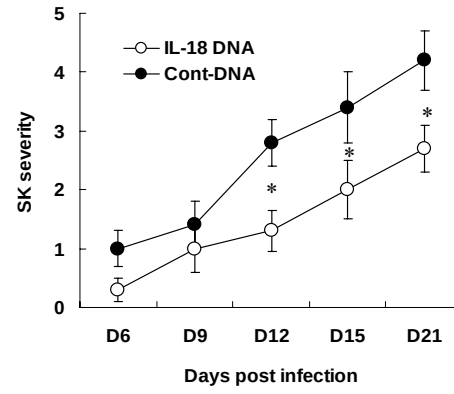
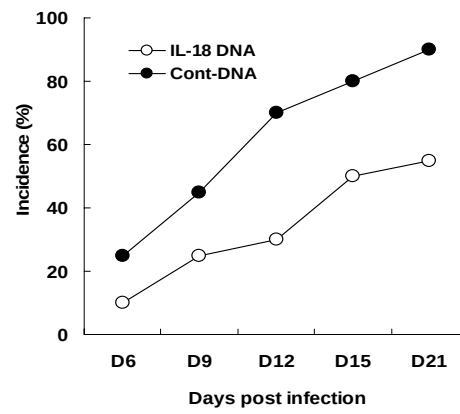
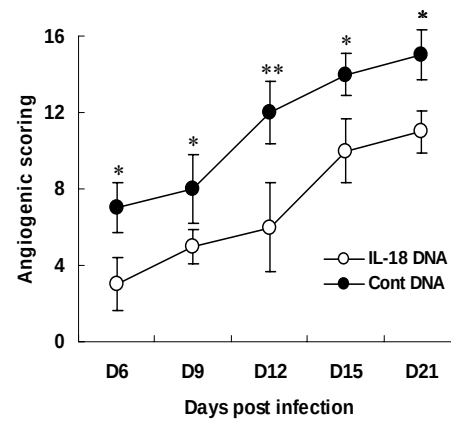
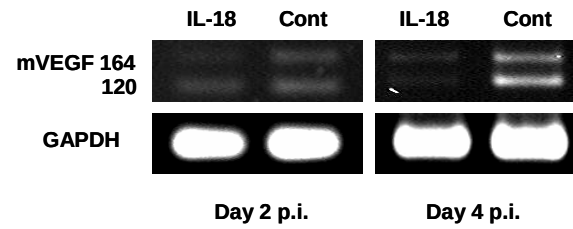
A**B****C**

Figure 2. Reduced levels of VEGF mRNA and protein in the HSV-1 infected cornea by IL-18 treatment.

At day 2 and 4 p.i. 2 corneas/mouse that were pretreated with IL-18 and infected with 1×10^6 pfu HSV-1 RE were processed to measure the VEGF mRNA or protein levels. VEGF mRNA levels were measured by RT-PCR (A). Levels of VEGF protein were estimated from supernatants of corneal lysates of mice by an antibody capture ELISA as outlined in materials and methods. Results are expressed as mean $\pm$ SD of four separate mice (2 corneas per mouse). * Statistically significant differences in VEGF protein levels ($p < 0.05$) were observed between the groups at day 4 after viral ocular infection (B).

A



B

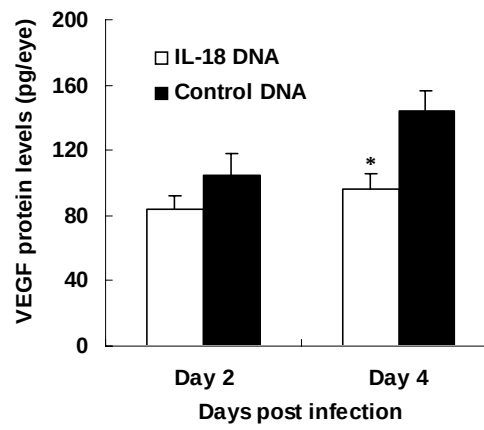
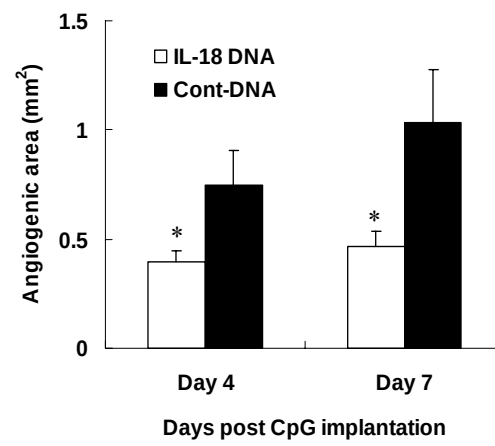


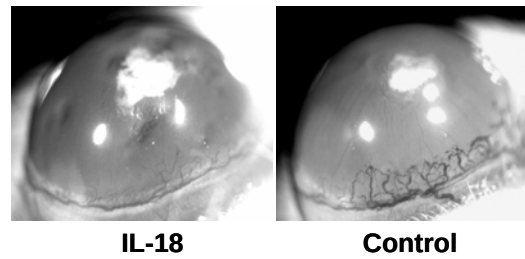
Figure 3. Inhibition of the CpG ODN-induced angiogenesis via diminished VEGF production by IL-18 pretreatment.

24 h before implantation with CpG ODN (1 µg) into the micropocket of mouse corneas, mice were given 1 µg per eye of IL-18 or GFP control plasmid by intrastromal injection. The angiogenesis area (A) was measured on day 4 and 7 after the CpG pellets implantation (four mice per group). Images were taken by stereomicroscopic imaging system at day 4 after CpG pellets implantation (original magnification, x40) (B). At day 4 and 7 after CpG pellet implantation 2 corneas/mouse were processed to measure the VEGF protein levels (C). Levels of VEGF were estimated from supernatants of corneal lysates of mice pretreated with IL-18 and implanted with CpG ODN (1 µg) by an antibody capture ELISA as described in materials and methods. Results are expressed as the mean ± SD of four separate mice (2 corneas per mouse). Statistically significant differences in angiogenic areas (A) and VEGF protein levels (C) (* $p < 0.05$) were observed between the groups.

A



B



C

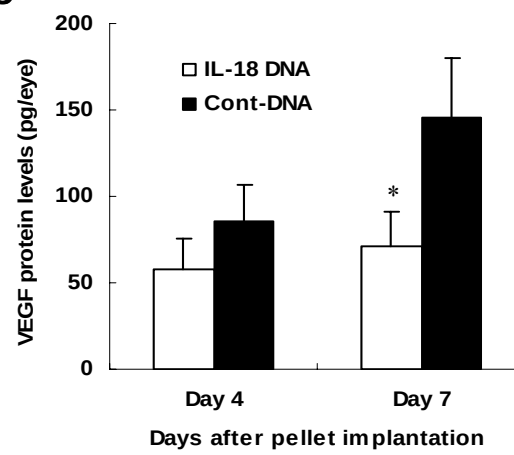
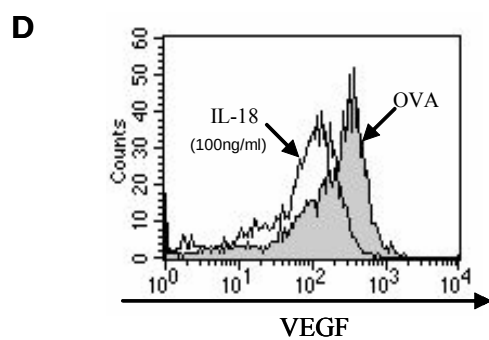
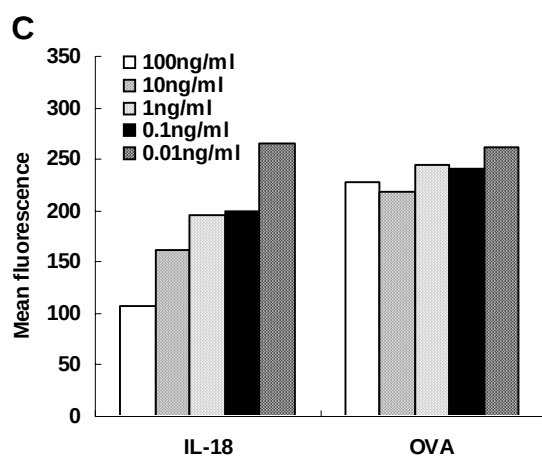
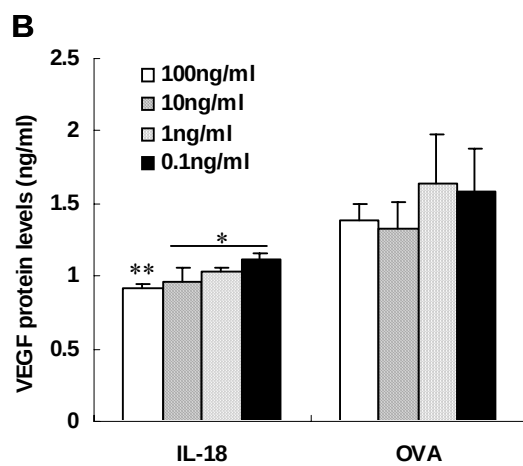
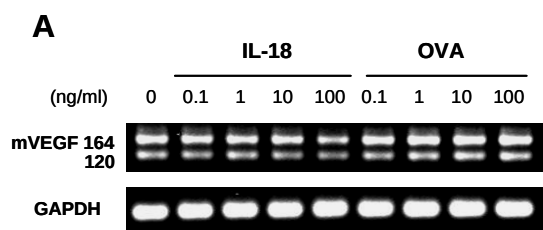


Figure 4. Suppression of VEGF expression and production by IL-18 incubation in vitro.

MBE cells that normally produce VEGF endogenously were prestimulated by 10 ng/ml FGF-2 for 12 h and then incubated with different concentrations of recombinant IL-18 for 24 h. Cells were collected and VEGF mRNA levels were measured by RT-PCR (A). Cell supernatants were used to measure VEGF protein levels by ELISA as described in materials and methods. Statistically significant differences in VEGF protein levels were observed between the groups (* $p < 0.05$, ** $p < 0.01$) (B). MBE cells incubated with IL-18 for 24 h were collected and resuspended in FACS solution (PBS-3% FCS containing 0.1% Sodium Azide). The cells were then fixed in paraformaldehyde and stained for VEGF by using biotinylated rat-anti-mVEGF164 antibody followed by streptavidin-PE. Positive cells and mean fluorescence were measured by flow cytometry (C and D). VEGF expression was not decreased by incubation with different concentrations of recombinant IL-12, a cytokine that shares several properties of IL-18 (data not shown).



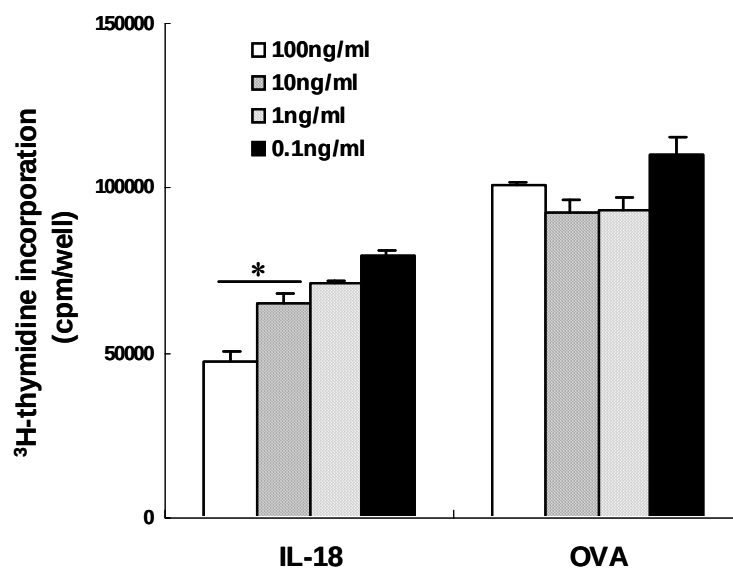


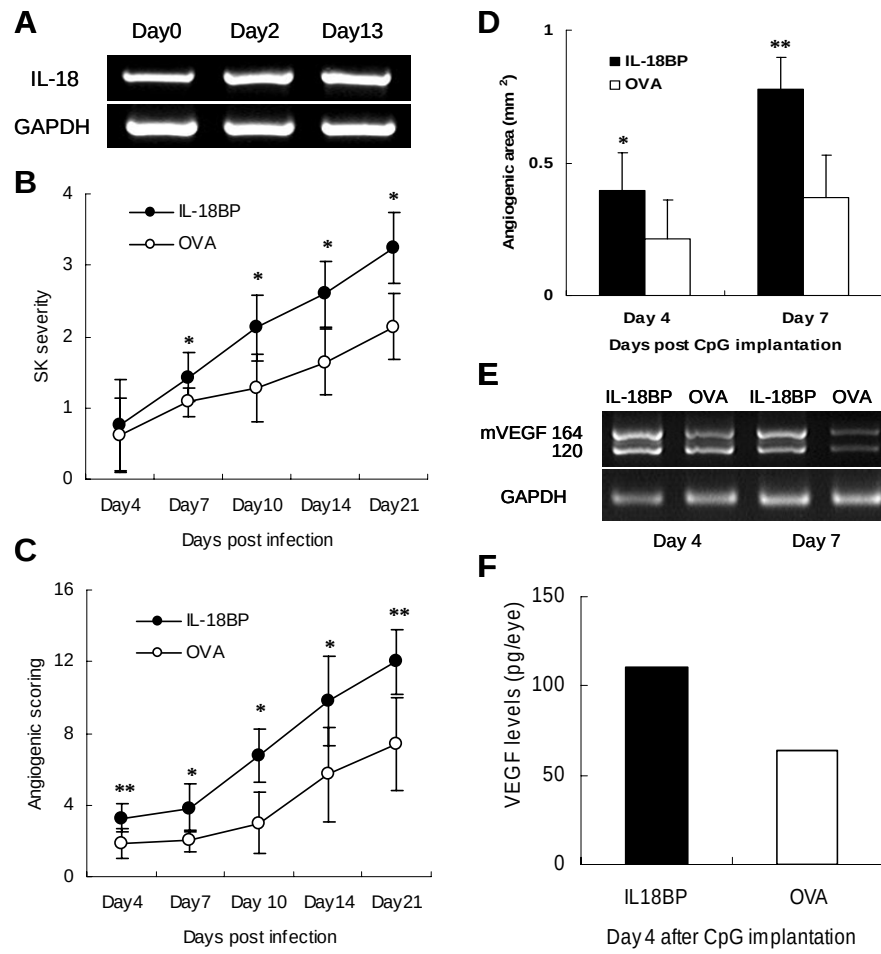
Figure 5. Inhibition of endothelial cell proliferation by IL-18.

MBE cells pre-stimulated with FGF-2 (10 ng/ml) were incubated with various concentrations (100, 10, 1, 0.1 ng/ml) of recombinant IL-18 or OVA as described in Material and Methods. After 18 hours cells were pulse labeled with ^3H -thymidine (1 $\mu\text{Ci/mL}$) for 6 h. The amount of radioactivity incorporated into the trichloroacetic acid-precipitable material was measured. * Statistically significant differences in radioactive levels ($p < 0.05$) were observed between the IL-18 and OVA treated groups.

Figure 6. Increased angiogenesis and upregulation of VEGF by IL-18 BP.

Mice were infected as described in the material and methods. At different time points p.i. the corneas ($n = 4$) were isolated and stored at -80°C . Total RNA was extracted from the samples and reverse transcribed into cDNA. PCR was performed to detect the IL-18 in both uninfected and HSV-infected corneas (A). Groups of animals ($n = 4$) were treated subconjunctivally at 0 and 2 days after virus infection (3×10^5 PFU) with recombinant IL-18 BP(●) or control OVA (○) ($2 \mu\text{g}/10 \mu\text{l}$) and subsequently scored for lesion severity by slit-lamp biomicroscopy. Statistically significant differences in SK (B) and angiogenic (C) score were observed between the groups (* $p < 0.05$, ** $p < 0.01$). Day 0 and 2 after implantation with CpG ODN (500 ng) into the cornea, mice were given $2 \mu\text{g}$ per eye of IL-18 BP or OVA protein as a control group by subconjunctival injection. The angiogenic area (D) was measured on day 4 and 7 after the CpG pellet implantation (four mice per group).

At days 4 and 7 after CpG pellets implantation IL-18 BP or OVA treated corneas were processed to measure the VEGF mRNA expression. VEGF mRNA levels were measured by RT-PCR (E). At day 4 after CpG pellet implantation IL-18 BP or OVA treated corneas (4 corneas per group) were processed to measure the VEGF protein level (F). Levels of VEGF were estimated from supernatants of corneal lysates of mice treated with IL-18 BP by an antibody capture ELISA as described in materials and methods.



Part IV

VEGF Receptor 2 Based DNA Immunization

Prevents Herpetic Stromal Keratitis by

Antiangiogenic Effects

In this chapter “we” and “our” refers to co-authors and me. My contributions in the paper include (1) selection of the topic (2) data analysis and interpretation (3) planning experiments (4) compiling and interpretation of the literature (5) understanding how results fit with the literature (7) compilation of contributions into one paper (8) providing structure to the paper (9) making graphs, figures and tables (10) writing and editing.

ABSTRACT

Stromal keratitis (SK) is an immunoinflammatory eye lesion caused by herpes simplex virus-1 (HSV-1) infection. It has been previously shown that abnormal angiogenesis in normally avascular cornea contributes the pathogenesis of SK lesions and vascular endothelial growth factor (VEGF) family of proteins is responsible for pathological corneal neovascularization. In this report, we targeted the proliferating vascular endothelial cells expressing VEGF receptor 2 (VEGFR-2, FLK-1) in the SK cornea by immunization with attenuated *Salmonella typhimurium* harboring murine FLK-1. FLK-1 based DNA vaccination resulted in diminished angiogenesis caused by HSV-1 infection or by recombinant VEGF corneal implantation. Strong CTL against endothelial cells expressing FLK-1 was evident in the FLK-1 immunized group, indicating that FLK-1 specific CD8⁺ T cells are responsible for the antiangiogenic effects. In addition *in vivo* CD8⁺ T cell depletion resulted in the abrogation of the antiangiogenic immune response. Our results indicate that FLK-1 based DNA vaccination efficiently induces a T cell mediated immune response against activated endothelial cells overexpressing

FLK-1 and this approach may represent a useful therapeutic strategy for SK angiogenesis.

INTRODUCTION

Virus infections contribute to the onset of several inflammatory autoimmune diseases (1-5). One autoimmune disease involved in this category is herpetic stromal keratitis (SK), a corneal inflammatory lesion following herpes simplex virus-1 (HSV-1) infection (6-8). In the mouse model, lesions of SK appear to be mediated by autoreactive CD4⁺ T cells (9, 10). In addition, several inflammatory cells including neutrophils (11) and macrophages (12-14) are infiltrated to the immune privileged cornea. Since one of the early events in the pathogenesis of SK is abnormal angiogenesis of the usually avascular cornea, it has been demonstrated that infiltrated inflammatory cells producing various angiogenic factors are the primary contributors to the uncontrolled process of angiogenesis after HSV infection (15). These leaky new blood vessels in the stromal layer of cornea may become a route for supply of inflammatory cells and CD4⁺ T cells, and subsequently cause blindness. Indeed, inhibition of neovascularization by antiangiogenic approaches could result in diminished SK lesions (16). Therefore, several strategies to inhibit unwanted angiogenesis have been proposed for SK lesion therapy (16-18).

It has recently been shown that multiple angiogenic molecules were responsible for the HSV-induced angiogenesis (15, 19-21). Extensive studies in SK, as well as tumor models, have demonstrated that vascular endothelial growth factor (VEGF), one of the potent angiogenic factors, significantly contributes the

pathological process of angiogenesis (15, 22, 23). Previous SK studies have shown that HSV infection resulted in the induction of VEGF expression by CpG-containing motifs of HSV DNA (24) as well as infiltrated inflammatory cells (15). Additionally, virus infection stimulates release of various cytokines including IL-1 (21, 25) and IL-6 (20), and subsequently they induce VEGF production. Accordingly, neutralization of VEGF with antibody (16), knock-down of VEGF family genes with siRNAs targeting VEGF, VEGF receptor 1, and 2 (VEGFR-1 and -2) (17), or application of DNA encoding antiangiogenic genes (26) diminished HSV-induced angiogenesis and SK lesions.

The role of VEGF in the process and regulation of angiogenesis has been intensely investigated (27). It has been known that two receptor tyrosine kinases, VEGFR-1 and 2 are related to the biological effects of VEGF (27). Although both receptors are involved in angiogenesis, the VEGFR-2 signaling pathway is used more for the studying of pathological angiogenesis since VEGFR-2 is highly overexpressed in the activated endothelial cells and its signaling induces proliferation and migration of endothelial cells (28). Therefore, several antiangiogenic or immunotherapeutic approaches targeting endothelial cells expressing VEGFR-2 have been used to inhibit pathological angiogenesis and tumor growth, including immunization with VEGFR-2 protein (29) or mRNA-loaded dendritic cells (30), with paraformaldehyde-fixed endothelial cells (31), or disruption of VEGFR genes by various methods (32). These approaches indicate that VEGFR-2 is highly implicated as a therapeutic target for inhibition of unwanted angiogenesis. In the present study, we have immunized the mice with an attenuated *Salmonella typhimurium* harboring FLK-1 cDNA to induce FLK-1

specific immune response. Here we show that our immunization stimulates a potent antiangiogenic response and efficiently reduces SK lesions through the inhibition of HSV-induced ocular angiogenesis.

MATERIALS AND METHODS

Bacterial strains

Attenuated *S. typhimurium* Aro/A (strain SL7207) transformed with pcDNA3.1-FLK-1 or -GFP was kindly provided by R.A. Reisfeld (Scripps Research Institute, La Jolla, California, USA). The construction of expression vector encoding murine FLK-1 was described previously (28). Protein expression of the different plasmids was determined by immunohistochemical staining after *in vitro* transfection of Cos-7 cells.

Mice

Female 5-to-6 week old BALB/c and C57BL/6J mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). To prevent bacterial infection, all mice received treatment with sulfamethoxazole/trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5 ml/200 ml drinking water after virus infection. All investigations followed the guidelines of the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee (Knoxville) are fully accredited by the American Association of Laboratory Animal Care.

Oral immunization and corneal HSV infection

Mice were immunized by oral gavage 3 times at 2-wk intervals with 100 μ l sodium bicarbonate (NaHCO_3) buffer containing 1×10^8 *S. typhimurium* transformed with pcDNA3.1-FLK-1 or -GFP as a control. Corneas of mice were infected 1 wk after final immunization. HSV-1 strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile) was used in all procedures. Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA; Cat.no.CCL81), titrated, and stored in aliquots at -80°C until used. Corneal infections of all mouse groups were conducted under deep anesthesia induced by Avertin. The mice were scarified lightly on their corneas with a 32 gauge needle and infected with a 2 μ l drop containing 5×10^5 plaque-forming units (PFU) of HSV-1 that was applied to the eye and gently massaged with the eyelids.

Clinical observations

The eyes were examined on different days after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Co, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing stromal keratitis. The severity of angiogenesis was recorded as described previously (16). In reference to the angiogenic scoring system, the method relied on quantifying the degree of neovessel formation based on three primary parameters: 1) the circumferential

extent of neovessels (as the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant of the circle; and 3) the longest neovessel in each quadrant was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ~ 0.4 mm (radius of the cornea is ~ 1.5 mm). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovessel index (range, 0 to 16) for each eye at a given time point.

Corneal micropocket assay

In vivo angiogenic activity was assayed in the avascular cornea of mouse eyes as previously described (16). Briefly, corneas of mice immunized with *S. typhimurium* transformed with pcDNA3.1-FLK-1 or -GFP as a control were implanted pellete containing 200 ng recombinant VEGF protein (R&D system). Pellets for insertion into the cornea were made by combining known amounts of VEGF protein, sucralfate (10 mg, Bulch Meditec) and hydron polymer in ethanol (120 mg/ml ethanol, Interferon Sciences), and applying the mixture to a 15×15 mm² piece of synthetic mesh (Tekto). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 200 ng of VEGF. Pellets containing VEGF were implanted into an intracorneal pocket (1 mm from the limbus). The eyes were then evaluated for corneal neovascularization. The extent of the neovessel ingrowth was recorded by direct measurement using calipers (Symbol of Quality, biomedical research instruments, Rockville, Maryland) under stereomicroscopy (Leica Microsystems, Wetzlar, Germany). The

length of the neovessels originating from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours were measured. Each clock hour is equal to 30° at the circumference. The angiogenic area was calculated according to the formula for an ellipse. $A = [(\text{clock hours}) \times 0.4 \times (\text{vessel length in mm}) \times \pi] / 2$.

CTL assay

CTL activity was assessed by a standard 4-h ^{51}Cr release assay against labeled target cells as previously described (28). One week after final immunization, splenocytes were collected from mice immunized with FLK-1 or GFP DNA. Splenocytes were cocultured with irradiated (3000 rad) and mitomycin incubated HEV (endothelial cell lines expressing VEGFR-2), CT-26 (murine colon carcinoma cells), YAC cell lines. Five days later, the activated T cells were harvested and tested for cytotoxicity.

Depletion of CD8⁺ T cells

We depleted CD8⁺ T cells of animals vaccinated with pcDNA3.1-FLK-1 or GFP by i.p. injection of 1 mg rat anti-mouse monoclonal antibody to CD8 (ATCC, CRL-1971) one day before VEGF pellet implantation.

Confocal microscopy

For immunofluorescence staining, eyes were frozen in optimum cutting temperature (OCT) compound (Miles, Elkart, IN). Seven-micron-thick sections were cut, air dried, and fixed in cold acetone for 10 min at -20°C. Nonspecific

binding was blocked with 3% BSA-PBS-0.05% Tween 20 for 2 h at 37°C. For detection of endothelial cells expressing VEGFR-2, the sections were incubated with mixture of FITC-coupled monoclonal anti-mouse CD31 (0.5 µg/ml, BD Pharmingen, San Diego, CA) and biotin-labeled anti-VEGFR-2 antibody (1 µg/ml, R&D systems) in 1% BSA-PBS-0.05% Tween 20. For detection of both endothelial and CD8⁺ T cells in the stroma, the sections were incubated with a mixture of FITC-coupled monoclonal anti-mouse CD8 and biotin-labeled anti-CD31 antibody (0.5 µg/ml, BD Pharmingen, San Diego, CA) in 1% BSA-PBS-0.05% Tween 20. For detection of both CD8⁺ and CD4⁺ T cells, the sections were incubated with a mixture of FITC-coupled monoclonal anti-mouse CD8 and biotin-labeled anti-CD4 antibody (0.5 µg/ml, BD Pharmingen, San Diego, CA) in 1% BSA-PBS-0.05% Tween 20. After incubating overnight at 4°C, slides were washed thoroughly in PBS, and streptavidin-conjugated Alexa Fluor 546 (1 µg/ml) (Molecular Probes) in 1% BSA-PBS was added for 1 h at room temperature. Slides were mounted with Vectashield without propidium iodide (Vector Laboratories, Burlingame, CA). Images were captured with a Leica SP2 laser scanning confocal microscope (Leica, Deerfield, IL).

Adoptive transfer of Thy1.1 CD8⁺ T cells

CD8⁺ T cells were isolated from FLK-1 based DNA immunized Thy 1.1⁺ C57BL/6.PL (H-2^b) mice. Single-cell suspensions of pooled spleens were prepared from immunized mice at day 7 after final immunization, and CD8⁺ T cells were purified by incubating the splenocytes with biotin-conjugated anti-CD8 (15 µg/10⁸ cells) mAb in PBS-2% FCS for 15 min at 4°C. Positive magnetic separation was

then performed with LS⁺ columns (Miltenyi Biotec, Auburn, CA) according to the suggested protocol. The purity of cells ranged from 90-95%. The purified Thy 1.1 CD8⁺ T cells (5×10^6 per mouse) were adoptively transferred into Thy 1.2 mice 24 h before virus ocular infection or VEGF pellet implantation.

Flow cytometric analysis

Single-cell suspensions were prepared from the SK cornea. Briefly, four corneas per group were dissected, pooled together, and digested with 60 U/ml Liberase (Roche Diagnostics) for 60 min at 37°C in a humidified atmosphere of 5% CO₂. After incubation, the corneas were disrupted by grinding with a syringe plunger on a strainer (70 µm) and a single-cell suspension was made in complete RPMI 1640 medium. Next, the single-cell suspension obtained from corneal samples was stained for FACS. Briefly, single cells were first blocked with an unconjugated anti-CD32/CD16 mAb (0.5 µg/ml, BD Pharmingen, San Diego, CA) for 30 min in FACS buffer and stained by anti-Thy 1.1-PerCP antibody (BD Pharmingen, San Diego, CA) for 30 min. Finally, the cells were washed three times and samples were acquired on a FACScan (BD Biosciences). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

Statistical analysis

Significant differences between groups were evaluated by using the Student's t test. $P < 0.05$ was regarded as a significant difference between the two groups.

RESULTS

VEGFR-2 is highly expressed in the neovessels of SK cornea

Previous studies on HSV-1 induced angiogenesis have identified VEGF as a protein that is highly expressed in the infected cornea and clearly involved in HSV specific angiogenesis (15). It has been known that VEGFR-2, one of the VEGF receptors, is highly overexpressed in proliferating endothelial cells under pathological conditions. To test whether this receptor is overexpressed in the HSV-1 induced abnormal blood vessels, we observed VEGFR-2 expression in the cornea after HSV-1 infection. Infection with 5×10^5 PFU of HSV-1 RE into the scarified eye induced corneal inflammation and strong angiogenesis sprouted from the limbal vessels, starting at day 3 after virus infection. To determine whether VEGFR-2 is specifically expressed in the proliferating endothelial cells of SK cornea, we stained the endothelial cells with fluorescein isothiocyanate (FITC) antibody against CD31 (PECAM) and biotinylated antibody with streptavidin-alexa fluor 546 against VEGFR-2. Microscopic observation revealed that endothelial cells making up the newly grown blood vessels in the HSV infected corneal stroma expressed CD31 as well as VEGFR-2 (Fig. 1). Thus, we could confirm the idea that VEGFR-2 is highly expressed in the proliferating endothelial cells of HSV infected cornea.

A FLK-1 encoded DNA immunization inhibits the severity of SK and HSV-1 induced angiogenesis

One essential step in the pathogenesis of SK is angiogenesis mediated by several angiogenesis factors including the VEGF family of proteins. Thus, one explanation

considered to explain the inhibitory effects on SK is antiangiogenesis. To evaluate such an effect, we tested our hypothesis by demonstrating that an effective antiangiogenic immune response was induced against HSV-1 RE corneal infection by an orally administered DNA vaccine encoding murine FLK-1 or GFP as a control carried by attenuated *S. typhimurium*. Protein expression of FLK-1 was demonstrated by immunohistochemical staining by transfected Cos-7 cells (Fig. 2A). Gene transfer from attenuated *S. typhimurium* into Peyer's patches by GFP expression at 16 h after oral administration was demonstrated by confocal microscopic observation (Fig. 2B).

To evaluate whether immunization with attenuated *S. typhimurium* targeting FLK-1 inhibits the development of herpetic SK, mice were orally administered with 10^8 CFU of attenuated *S. typhimurium* 3 times over a 4 week period, at 2 week intervals. One week after final immunization, the corneas of mice were scarified and infected with 5×10^5 HSV-1 RE. The levels of SK severity and neovascularization were measured in the corneas by biomicroscopy in groups of mice either pre-vaccinated with pcDNA3.1-FLK-1 or control GFP plasmid prior to HSV infection. As shown in Fig. 3, significantly decreased average SK severity and percentage of animals with clinical lesions were observed in mice following immunization with the FLK-1-encoded DNA. A comparison of average severity scores after day 4 p.i. were all significantly less in the FLK-1 DNA immunized than the control group ($p < 0.05$, 0.01). Furthermore, the results shown in Fig. 3 indicate that levels of angiogenesis were significantly less at most time points tested in the FLK-1 DNA compared to control plasmid immunized animals ($p < 0.01$).

FLK-1 specific CD8⁺ T cells are responsible for the antiangiogenic effects in SK cornea

Previous studies on SK angiogenesis have shown that inhibited signaling of VEGFRs highly expressed in angiogenic endothelial cells could reduce the severity of SK (17). To evaluate whether immunization with FLK-1 encoded DNA inhibits HSV-1 induced angiogenesis by FLK-1 specific immune cells, we observed the SK cornea samples to reveal the localization of endothelial cells targeting immune cells by confocal microscopy. Since it has been reported that immunization with attenuated *Salmonella* encoding either non-self or self antigens induces strong cytotoxic CD8⁺ T cell response (28, 33-35), we stained CD8⁺ T cells with FITC and endothelial cells with biotinylated antibody against CD31 in the SK cornea. Microscopic examination showed an association of infiltrated CD8⁺ T cells with neovessels in the corneal stroma (SK day 21) of mice immunized with FLK-1 DNA and infected with HSV-1 (Fig. 4). Almost no CD8⁺ T cells were observed in the cornea of control mice. To exclude the possibility of non-specific staining of CD8 antibody to CD4⁺ T cells since many CD4⁺ T cells infiltrate into the corneal stroma around 10 days after HSV infection, we stained CD8⁺ T cells with FITC and CD4⁺ T cells with biotinylated antibody in the SK cornea. As shown in Fig. 4, both CD8⁺ and CD4⁺ T cells were observed in the cornea of animals immunized with FLK-1 DNA, but only CD4⁺ T cells were found in the SK cornea of control group. These results suggest that FLK-1 specific CD8⁺ T cells are responsible for the antiangiogenic effects in HSV-1 induced angiogenesis.

Inhibition of VEGF-induced corneal neovascularization by FLK-1 immunization

To test the antiangiogenic effects of FLK-1 DNA immunization in the eye, the cornea of immunized mice were given 200 ng recombinant VEGF pellets using a corneal micropocket assay method. In this experiment, immunization with FLK-1 DNA markedly inhibited (approximately 50% inhibition) the extent of VEGF-induced angiogenesis compared to control DNA treated animals at days 4 and 7 after pellet implantation (Fig. 5A and B).

To demonstrate the involvement of FLK-1 specific CD8⁺ T cells with antiangiogenic response in the eye, CD8⁺ T cells were depleted before VEGF pellet implantation. As shown in Fig. 5A and B, *in vivo* depletion of CD8⁺ T cells resulted in the abrogation of the antiangiogenic response induced by FLK-1 DNA immunization. This result demonstrates the antiangiogenic effects of CD8⁺ T cells by FLK-1 DNA immunization.

Induction of T cell mediated lysis by FLK-1 DNA immunization

To test FLK-1 targeting CTL immune response, we measured FLK-1 specific cytotoxicity against endothelial cell lines, which constitutively express VEGFR-2, with 4 hours ⁵¹Cr-release assay using splenocytes from mice immunized with FLK-1 DNA. One week after final immunization, splenocytes were collected from mice immunized with FLK-1 or GFP DNA and cocubated with irradiated HEV cells. After 5 days cocubation, CTL assay was performed with endothelial cell lines. FLK-1 DNA immunization resulted in a significantly higher level of CTL

responses against the endothelial cells expressing VEGFR-2, compared with control immunization (Fig. 6A). However, neither immunization was effective in evoking cytotoxicity against CT-26 cells not expressing FLK-1, indicating VEGFR-2 specific lysis by FLK-1 immunization (Fig. 6B). In addition, neither vaccination induce cytotoxicity against YAC cells, which are specific cell lines for NK cell cytotoxicity, excluding direct lysis by NK cells (Fig. 6C).

Adoptive transfer of FLK-1 specific CD8⁺ T cells efficiently inhibits angiogenesis

To further establish the role of FLK-1 specific CD8⁺ T cells in suppressing corneal angiogenesis, an adoptive transfer system was used. CD8⁺ T cells were isolated from FLK-1 based DNA immunized Thy 1.1⁺ C57BL/6.PL (H-2^b) mice and transferred into Thy 1.2 recipients. Mice were then infected ocularly with HSV-1 or implanted with VEGF pellets into the cornea to induce angiogenesis. The results expressed in Fig. 7A and B indicate that the severity of lesions and extent of angiogenesis induced by HSV infection or VEGF pellet implantation were reduced if animals received CD8⁺ T cells of FLK-1 based DNA immunized mice. To reveal the localization of adoptive transferred Thy 1.1⁺ CD8⁺ T cells in the SK cornea, we stained Thy 1.1⁺ cells with biotinylated antibody against Thy 1.1 and endothelial cells with FITC against CD31. Microscopic observation showed infiltration of few Thy 1.1⁺ cells near neovessels in the cornea stroma of mice adoptively transferred with Thy 1.1⁺ CD8⁺ T cells of FLK-1 based DNA immunized mice. Almost no Thy 1.1⁺ cells were observed in the cornea of mice adoptively transferred with Thy 1.1⁺ CD8⁺ T cells of control DNA immunized

mice (Fig. 7C). In addition, at day 14 p.i. flow cytometry analysis revealed a 3 fold increase in the number of Thy 1.1⁺ cells per cornea of mice adoptively transferred with Thy 1.1⁺ CD8⁺ T cells of FLK-1 based DNA immunized mice than the control eye (Fig. 7D).

DISCUSSION

Our study shows that oral immunization with attenuated *S. typhimurium* carrying FLK-1 gene results in reduced SK severity by inhibition of ocular angiogenesis. Antiangiogenic effects of FLK-1 DNA immunization were caused by induction of cytotoxic CD8⁺ T cells against FLK-1. Previously, our studies have demonstrated that VEGF, one of the major angiogenic factors induced by HSV-1 ocular infection, plays an essential role in the formation of neovessels induced by inflammation in the normally avascular cornea (15, 16). Additionally, various proinflammatory cytokines and chemokines are overexpressed by HSV infection and these released molecules in inflammatory cornea subsequently induce the expression of VEGF directly or indirectly. For example, it was reported that IL-1 and IL-6 are upregulated in the cornea by HSV infection and then these cytokines induce VEGF expression *in vitro* and *in vivo* (15, 16, 20, 21, 25). CXC chemokines such as MIP-2 or KC (CXCL3) were also produced by viral stimulation and these chemokines induce recruitment of polymorphonuclear leukocytes (PMN), one of major inflammatory cells producing VEGF, into the SK cornea (36, 37). Furthermore, CpG motifs of HSV DNA directly induce VEGF expression from inflammatory cells (24). Accordingly, various antiangiogenic

strategies targeting VEGF family genes including VEGF and its receptors could successfully diminish immunoinflammatory SK lesions (16, 17).

Although it seems that VEGF is the major molecule as a final trigger of HSV-1 induced angiogenesis, knock down of VEGF may not completely inhibit pathological angiogenesis. Since angiogenesis is a complicated process in the cornea during HSV infection, we assume that VEGF is not the sole mediator responsible for the HSV induced angiogenesis in the cornea. Possibly, other angiogenic molecules such as fibroblast growth factor-2 (FGF-2) may be involved in this process without relation to VEGF molecules. Therefore approaches that target pathological blood vessels might be an attractive strategy for antiangiogenic therapy. In response to angiogenic molecules, newly formed blood vessels express various angiogenic receptors and integrins (38, 39). Among these surface molecules, VEGFR-2 is highly overexpressed on the cell surface of activated and proliferating endothelial cells under pathological conditions (27, 28). This finding suggests that control of VEGFR-2 expressing endothelial cell growth may be an efficient approach for the treatment of neovascular disease causing unwanted angiogenesis. Accordingly, various immunotherapeutic strategies targeting VEGFR-2 of uncontrolled endothelial cells have been suggested by investigators mainly for the purpose of inhibition of tumor progression and angiogenesis (28-31, 33, 34). Interestingly, although VEGFR-2 is autologous, our vaccination strategy, as well as those that have been previously introduced, could efficiently break peripheral T-cell tolerance against self-antigen, VEGFR-2 (28, 30, 33, 34). The precise mechanism by which oral DNA vaccination targeting self-antigen exerts immune response remains unresolved and this issue is under further investigation.

Induction of CTL responses against FLK-1 by oral immunization demonstrates that FLK-1 specific CD8⁺ T cells might kill or control the proliferating endothelial cells, leading to reduced angiogenesis and subsequently diminished SK lesions. We could further show that angiogenesis induced by VEGF pellet implantation was inhibited in the immunized mice. To demonstrate involvement of CD8⁺ T cells in antiangiogenic immune response, neutralizing antibody to deplete CD8⁺ T cells was used. As was expected, depletion of CD8⁺ T cells abrogated the antiangiogenic activity in the immunized and VEGF pellet implanted mice. However, this method could not be used in herpetic SK model since HSV-1 infected mice without CD8⁺ T cells might induce severe encephalitis caused by virus spread into the central nervous system. Thus we used the adoptive transfer system in the SK model to demonstrate the antiangiogenic immune activity of CD8⁺ T cells. Finally, the adoptive transfer of CD8⁺ T cells collected from immunized mice into non-immunized and HSV infected mice could efficiently reduce clinical SK lesions by antiangiogenic immune response. Our data again support the idea that FLK-1 specific CD8⁺ T cells demonstrate an angiostatic function and show this activity in the SK model.

In summary, we demonstrated that oral immunization with attenuated *S. typhimurium* harboring murine FLK-1 could break peripheral T cell tolerance and induce CTL against self-antigen VEGFR-2. This FLK-1 specific immune response resulted in reduction of herpetic SK lesion severity by its antiangiogenic effects. This novel approach may represent a novel therapeutic strategy for herpetic SK as well as neovascular diseases.

LIST OF REFERENCES

1. Fujinami, R. S. 2001. Viruses and autoimmune disease--two sides of the same coin? *Trends Microbiol* 9:377.
2. Oldstone, M. B. 1998. Molecular mimicry and immune-mediated diseases. *Faseb J* 12:1255.
3. Olson, J. K., J. L. Croxford, and S. D. Miller. 2001. Virus-induced autoimmunity: potential role of viruses in initiation, perpetuation, and progression of T-cell-mediated autoimmune disease. *Viral Immunol* 14:227.
4. Whitton, J. L., and R. S. Fujinami. 1999. Viruses as triggers of autoimmunity: facts and fantasies. *Curr Opin Microbiol* 2:392.
5. Wucherpfennig, K. W. 2001. Mechanisms for the induction of autoimmunity by infectious agents. *J Clin Invest* 108:1097.
6. Garweg, J. G., and M. Halberstadt. 2002. [The pathogenesis of herpetic keratitis]. *Klin Monatsbl Augenheilkd* 219:477.
7. Deshpande, S. P., S. Lee, M. Zheng, B. Song, D. Knipe, J. A. Kapp, and B. T. Rouse. 2001. Herpes simplex virus-induced keratitis: evaluation of the role of molecular mimicry in lesion pathogenesis. *J Virol* 75:3077.
8. Thomas, J., and B. T. Rouse. 1997. Immunopathogenesis of herpetic ocular disease. *Immunol Res* 16:375.
9. Gangappa, S., S. P. Deshpande, and B. T. Rouse. 2000. Bystander activation of CD4⁺ T cells accounts for herpetic ocular lesions. *Invest Ophthalmol Vis Sci* 41:453.
10. Gangappa, S., S. P. Deshpande, and B. T. Rouse. 1999. Bystander activation of CD4(+) T cells can represent an exclusive means of immunopathology in a virus infection. *Eur J Immunol* 29:3674.

11. Thomas, J., S. Gangappa, S. Kanangat, and B. T. Rouse. 1997. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. *J Immunol* 158:1383.
12. Bauer, D., A. Schmitz, N. Van Rooijen, K. P. Steuhl, and A. Heiligenhaus. 2002. Conjunctival macrophage-mediated influence of the local and systemic immune response after corneal herpes simplex virus-1 infection. *Immunology* 107:118.
13. Bauer, D., S. Mrzyk, N. van Rooijen, K. P. Steuhl, and A. Heiligenhaus. 2000. Macrophage-depletion influences the course of murine HSV-1 keratitis. *Curr Eye Res* 20:45.
14. Bauer, D., S. Mrzyk, N. Van Rooijen, K. P. Steuhl, and A. Heiligenhaus. 2001. Incidence and severity of herpetic stromal keratitis: impaired by the depletion of lymph node macrophages. *Exp Eye Res* 72:261.
15. Zheng, M., S. Deshpande, S. Lee, N. Ferrara, and B. T. Rouse. 2001. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. *J Virol* 75:9828.
16. Zheng, M., M. A. Schwarz, S. Lee, U. Kumaraguru, and B. T. Rouse. 2001. Control of stromal keratitis by inhibition of neovascularization. *Am J Pathol* 159:1021.
17. Kim, B., Q. Tang, P. S. Biswas, J. Xu, R. M. Schiffelers, F. Y. Xie, A. M. Ansari, P. V. Scaria, M. C. Woodle, P. Lu, and B. T. Rouse. 2004. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial

- growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. *Am J Pathol* 165:2177.
18. Deshpande, S., K. Banerjee, P. S. Biswas, and B. T. Rouse. 2004. Herpetic eye disease: immunopathogenesis and therapeutic measures. *Expert Rev Mol Med* 2004:1.
 19. Lee, S., M. Zheng, B. Kim, and B. T. Rouse. 2002. Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus. *J Clin Invest* 110:1105.
 20. Banerjee, K., P. S. Biswas, B. Kim, S. Lee, and B. T. Rouse. 2004. CXCR2^{-/-} mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6-induced neovascularization. *J Immunol* 172:1237.
 21. Biswas, P. S., K. Banerjee, B. Kim, and B. T. Rouse. 2004. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. *J Immunol* 172:3736.
 22. Ferrara, N. 2004. Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist* 9 Suppl 1:2.
 23. Stewart, R. J., D. Panigrahy, E. Flynn, and J. Folkman. 2001. Vascular endothelial growth factor expression and tumor angiogenesis are regulated by androgens in hormone responsive human prostate carcinoma: evidence for androgen dependent destabilization of vascular endothelial growth factor transcripts. *J Urol* 165:688.

24. Zheng, M., D. M. Klinman, M. Gierynska, and B. T. Rouse. 2002. DNA containing CpG motifs induces angiogenesis. *Proc Natl Acad Sci U S A* 99:8944.
25. Biswas, P. S., K. Banerjee, M. Zheng, and B. T. Rouse. 2004. Counteracting corneal immunoinflammatory lesion with interleukin-1 receptor antagonist protein. *J Leukoc Biol* 76:868.
26. Lee, S., M. Zheng, S. Deshpande, S. K. Eo, T. A. Hamilton, and B. T. Rouse. 2002. IL-12 suppresses the expression of ocular immunoinflammatory lesions by effects on angiogenesis. *J Leukoc Biol* 71:469.
27. Ferrara, N., H. P. Gerber, and J. LeCouter. 2003. The biology of VEGF and its receptors. *Nat Med* 9:669.
28. Niethammer, A. G., R. Xiang, J. C. Becker, H. Wodrich, U. Pertl, G. Karsten, B. P. Eliceiri, and R. A. Reisfeld. 2002. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med* 8:1369.
29. Li, Y., M. N. Wang, H. Li, K. D. King, R. Bassi, H. Sun, A. Santiago, A. T. Hooper, P. Bohlen, and D. J. Hicklin. 2002. Active immunization against the vascular endothelial growth factor receptor flk1 inhibits tumor angiogenesis and metastasis. *J Exp Med* 195:1575.
30. Nair, S., D. Boczkowski, B. Moeller, M. Dewhirst, J. Vieweg, and E. Gilboa. 2003. Synergy between tumor immunotherapy and antiangiogenic therapy. *Blood* 102:964.

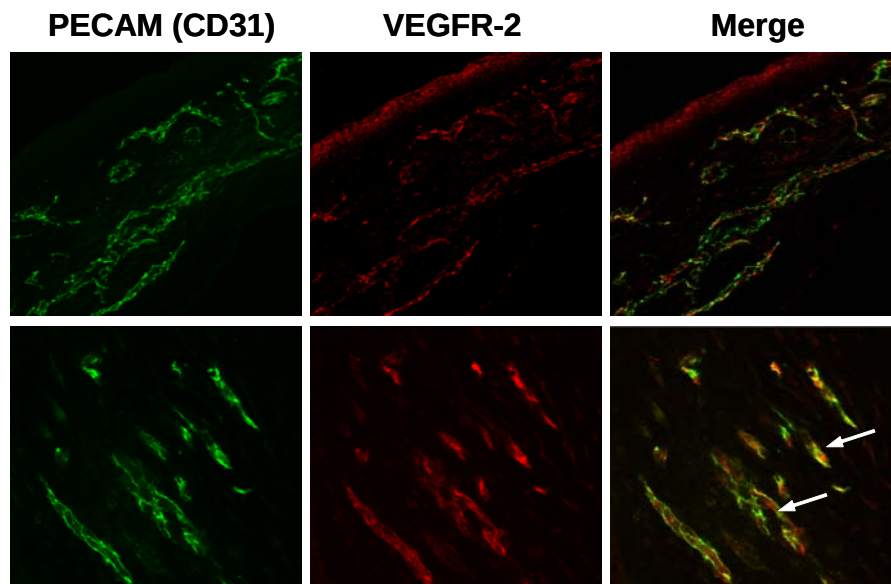
31. Wei, Y. Q., Q. R. Wang, X. Zhao, L. Yang, L. Tian, Y. Lu, B. Kang, C. J. Lu, M. J. Huang, Y. Y. Lou, F. Xiao, Q. M. He, J. M. Shu, X. J. Xie, Y. Q. Mao, S. Lei, F. Luo, L. Q. Zhou, C. E. Liu, H. Zhou, Y. Jiang, F. Peng, L. P. Yuan, Q. Li, Y. Wu, and J. Y. Liu. 2000. Immunotherapy of tumors with xenogeneic endothelial cells as a vaccine. *Nat Med* 6:1160.
32. Song, W., Q. Sun, Z. Dong, D. M. Spencer, G. Nunez, and J. E. Nor. 2005. Antiangiogenic gene therapy: disruption of neovascular networks mediated by inducible caspase-9 delivered with a transcriptionally targeted adenoviral vector. *Gene Ther* 12:320.
33. Feng, K. K., H. Y. Zhao, H. Qiu, J. X. Liu, and J. Chen. 2005. Combined therapy with flk1-based DNA vaccine and interleukin-12 results in enhanced antiangiogenic and antitumor effects. *Cancer Lett* 221:41.
34. Feng, K., H. Zhao, J. Chen, D. Yao, X. Jiang, and W. Zhou. 2004. Anti-angiogenesis effect on glioma of attenuated *Salmonella typhimurium* vaccine strain with flk-1 gene. *J Huazhong Univ Sci Technolog Med Sci* 24:389.
35. Niethammer, A. G., F. J. Primus, R. Xiang, C. S. Dolman, J. M. Ruehlmann, Y. Ba, S. D. Gillies, and R. A. Reisfeld. 2001. An oral DNA vaccine against human carcinoembryonic antigen (CEA) prevents growth and dissemination of Lewis lung carcinoma in CEA transgenic mice. *Vaccine* 20:421.
36. Su, Y. H., X. T. Yan, J. E. Oakes, and R. N. Lausch. 1996. Protective antibody therapy is associated with reduced chemokine transcripts in herpes simplex virus type 1 corneal infection. *J Virol* 70:1277.

37. Thomas, J., S. Kanangat, and B. T. Rouse. 1998. Herpes simplex virus replication-induced expression of chemokines and proinflammatory cytokines in the eye: implications in herpetic stromal keratitis. *J Interferon Cytokine Res* 18:681.
38. Hynes, R. O. 2002. A reevaluation of integrins as regulators of angiogenesis. *Nat Med* 8:918.
39. O'Reilly, M. S. 2002. Vessel maneuvers: vaccine targets tumor vasculature. *Nat Med* 8:1352.

APPENDIX

Figure 1. Expression of VEGFR-2 on the proliferating endothelial cells of neovessels of SK cornea.

The mice were scarified lightly on their corneas with a 32 gauge needle and infected with a 2 µl drop containing 5×10^5 PFU of HSV-1 RE that was applied to the eye. At day 21 post infection, eyes were frozen and seven-micron-thick sections were cut, air dried, and processed for immunofluorescences staining as described in materials and methods. Briefly, for detection of endothelial cells expressing VEGFR-2, the sections were incubated with a mixture of FITC-coupled monoclonal anti-mouse CD31 (0.5 µg/ml) and a biotin-labeled anti-VEGFR-2 antibody (1 µg/ml). On the next day, slides were incubated with streptavidin-conjugated Alexa Fluor 546 (1 µg/ml). Slides were mounted with Vectashield without propidium iodide. Images were captured with a Leica SP2 laser scanning confocal microscope. The white arrows indicate the cross section of endothelial cells expressing CD31 as well as VEGFR-2, lining the lumen of a blood vessel in the corneal stroma.



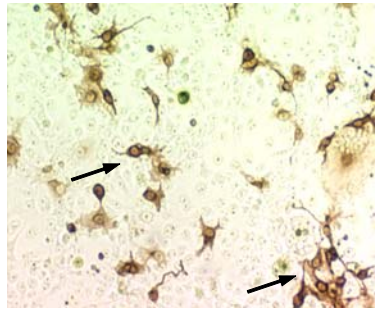
SK Cornea Day 21

Figure 2. Functionality of FLK-1 expression and gene transfer from attenuated salmonella typhimurium to Peyer's patches.

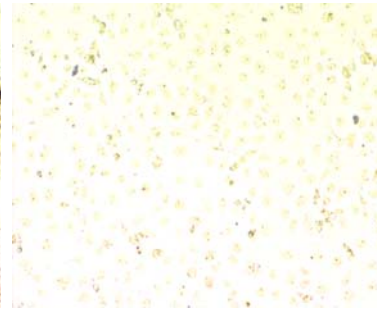
FLK-1 expression was verified by immunohistochemical staining by transfected Cos-7 cells. The black arrows indicate the cells expressing FLK-1 protein (A). Gene transfer from attenuated *S. typhimurium* into Peyer's patches by GFP expression at 16 h after oral administration was demonstrated by confocal microscopic observation. The white arrows indicate the cells expressing GFP transferred from attenuated *S. typhimurium* based GFP DNA (B).

A. Cos-7 cell transfection

VEGFR-2

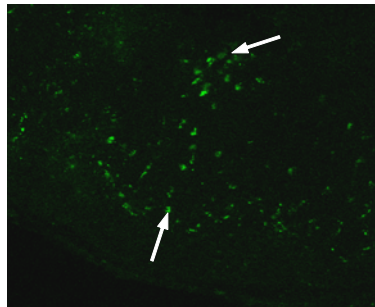


Control



B. Gene delivery into Peyer's patch

***Salmonella*-EGFP**

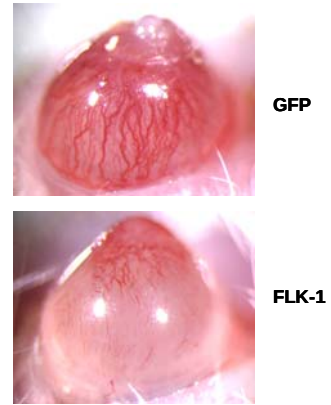
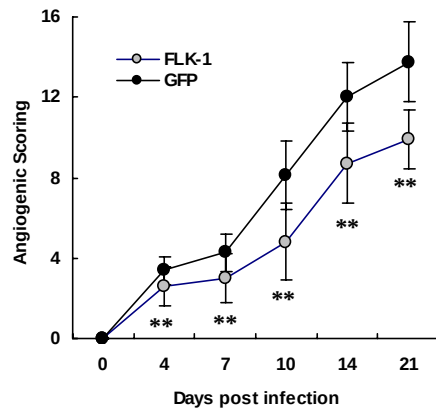
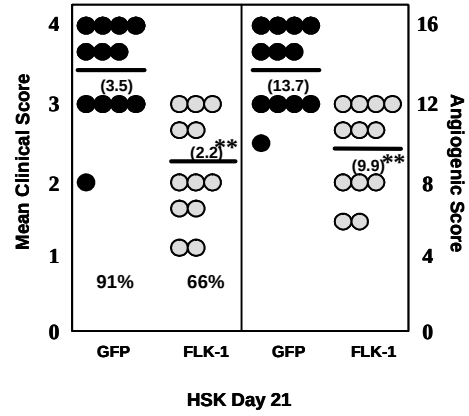
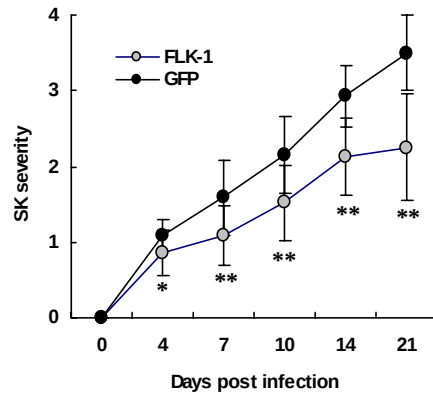


Control



Figure 3. Inhibition of SK lesion severity, incidence, and HSV-induced angiogenesis by FLK-1 DNA immunization.

Groups of animals ($n = 6$) were orally administered with 10^8 CFU of attenuated *S. typhimurium* 3 times over a 4 week period, at 2 week intervals. One week after final immunization, the corneas were scarified and infected with 5×10^5 HSV-1 RE. The levels of SK severity and neovascularization were measured in the corneas by biomicroscopy in groups of mice either pre-vaccinated with pcDNA3.1-FLK-1 (o) or control GFP (●) plasmid prior to HSV infection. The data are compiled from three independent experiments. Statistically significant differences in SK and angiogenic score were observed between the groups (* $p < 0.05$, ** $p < 0.01$). Images were taken by stereomicroscopic imaging system at day 21 after virus infection (original magnification, $\times 40$)



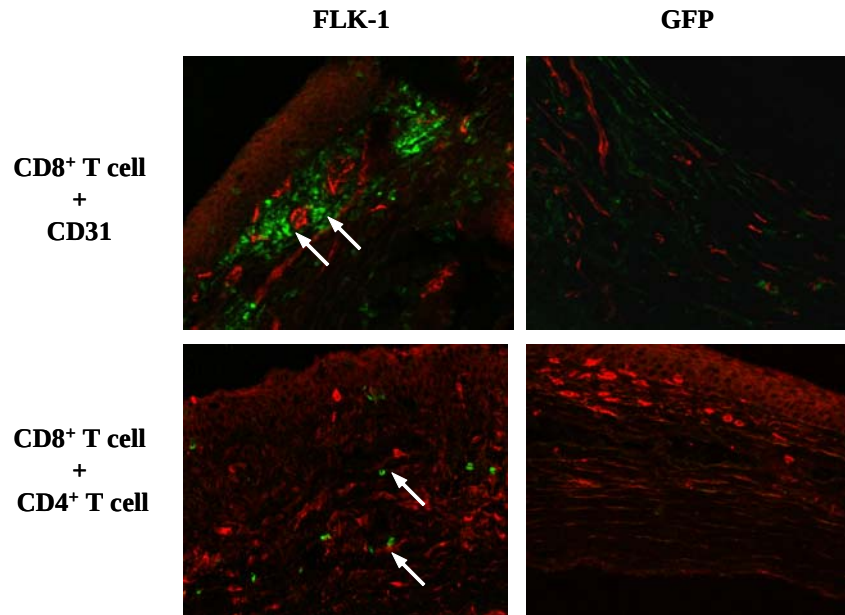


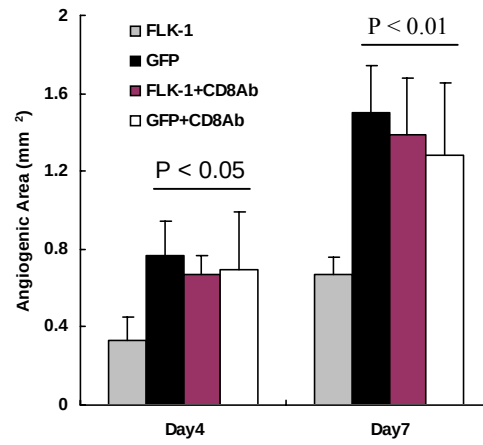
Figure 4. Involvement of FLK-1 specific CD8⁺ T cells for the antiangiogenic effects in SK cornea.

Upper panels depict the localization of CD8⁺ T cells (FITC, Green) and endothelial cells (Biotin + Alexa, Red) in the corneal stroma (SK day 21) of mice immunized and infected with HSV-1. Lower panels depict the infiltration of CD8⁺ (FITC, Green) and CD4⁺ (Biotin + Alexa, Red) T cells in the SK (day 10) cornea of mice immunized and infected with HSV-1. The white arrows indicate the infiltrated CD8⁺ T cells in the corneal stroma.

Figure 5. Inhibition of VEGF-induced corneal angiogenesis by FLK-1 immunization.

The cornea of mice (n = 6) immunized with either attenuated *S. typhimurium* harboring pcDNA3.1-FLK-1 or -GFP was given 200 ng recombinant VEGF pellets (*) using a corneal micropocket assay method. Furthermore, to demonstrate the involvement of FLK-1 specific CD8⁺ T cells with antiangiogenic response in the eye, CD8⁺ T cells of immunized mice were depleted before VEGF pellet implantation. The angiogenesis area (A) was measured on days 4 and 7 after the VEGF pellet implantation. Images were taken by stereomicroscopic imaging system at day 4 after VEGF pellet implantation (original magnification, × 40) (B).

A



B

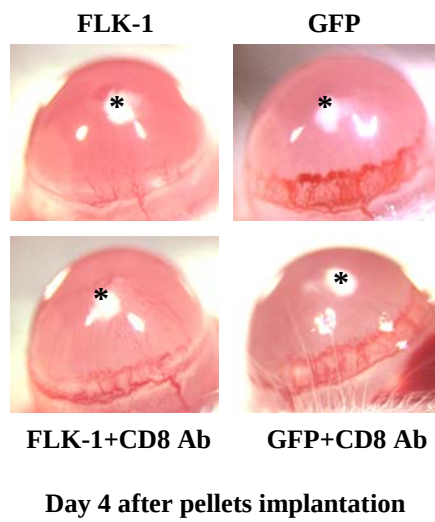
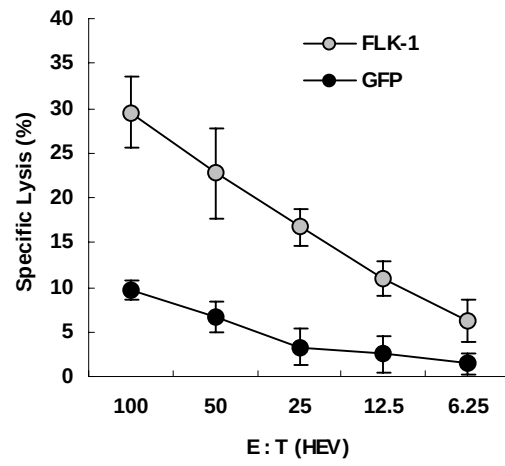


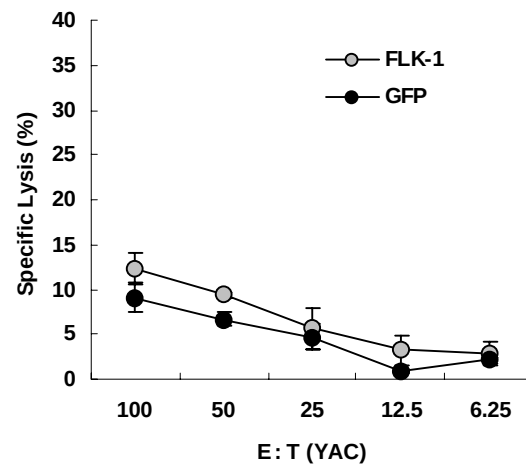
Figure 6. Specific lysis of endothelial cells by FLK-1 specific CD8⁺ T cells.

Splenocytes from FLK-1 or GFP DNA immunized mice were collected and coincubated with irradiated HEV, CT-26, or YAC cells. After 5 days coincubation, the activated T cells were harvested and tested for cytotoxicity.

A



B



C

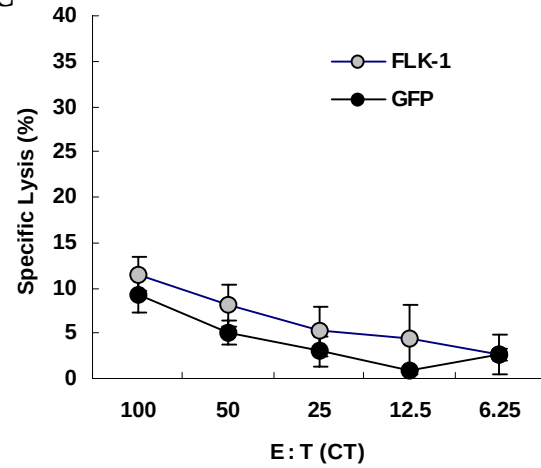
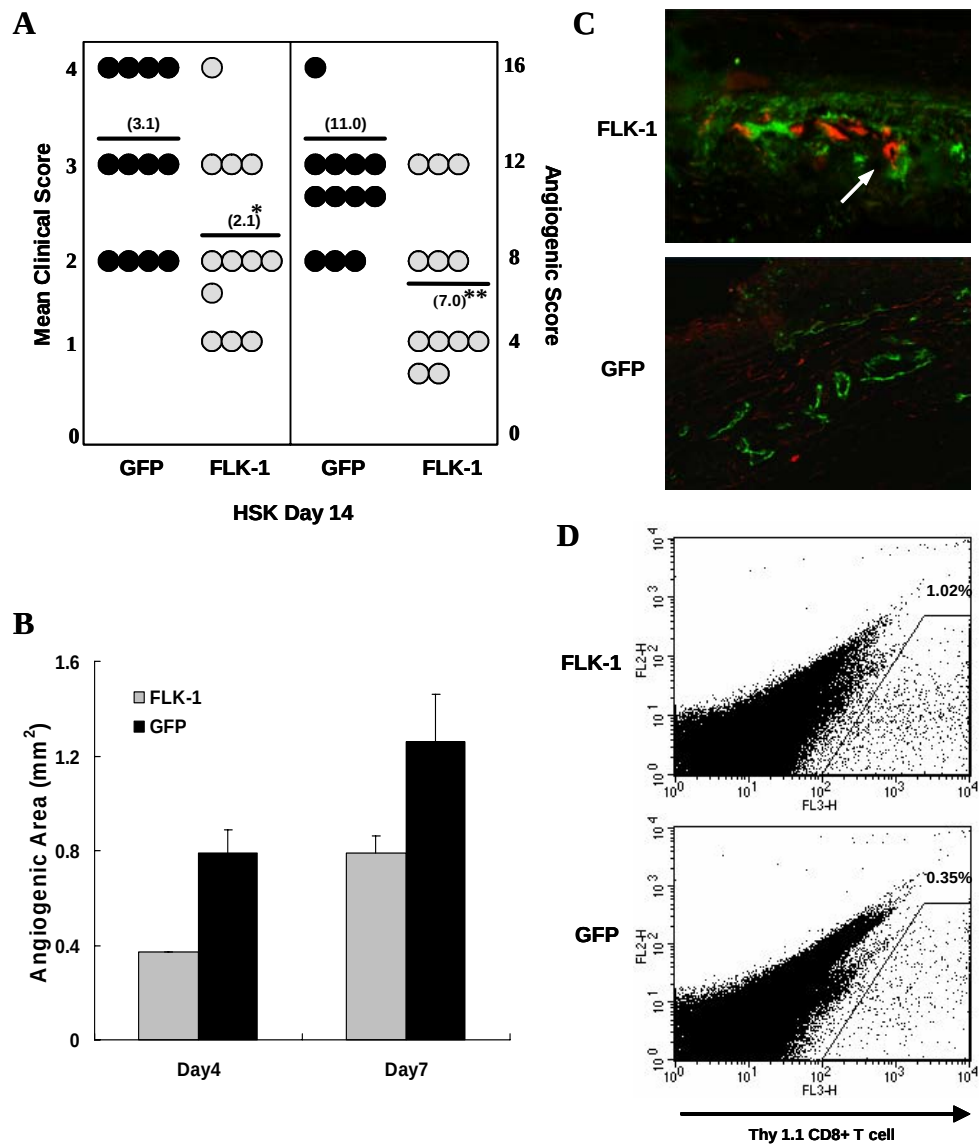


Figure 7. Inhibition of angiogenesis by adoptive transfer of FLK-1 specific CD8⁺ T cells.

CD8⁺ T cells were isolated from FLK-1 based DNA immunized Thy 1.1⁺ C57BL/6.PL (H-2^b) mice (n = 4) and transferred into Thy 1.2 recipients. Mice (n = 6) were then infected ocularly with HSV-1 RE or implanted with VEGF (200 ng) pellets into the cornea to induce angiogenesis. The levels of SK severity and neovascularization (A) or angiogenic area (B) were measured in the corneas by biomicroscopy in groups of mice that received CD8⁺ T cells of FLK-1 (o) or EGFP (●) based DNA immunized mice. Statistically significant differences in SK and angiogenic score were observed between the groups (* $p < 0.05$, ** $p < 0.01$). The localization of adoptive transferred Thy 1.1⁺ CD8⁺ T cells (Biotin+Alexa, Red) and endothelial cells (FITC, Green) in the SK cornea is depicted. The white arrow indicates the infiltrated Thy 1.1⁺ CD8⁺ T cells around endothelial cells in the corneal stroma (C). Four corneas of mice of each group were pooled together and the number of Thy 1.1⁺ cells was enumerated by FACS analysis as described in materials and methods. The number in the right corner represents the percentage of Thy 1.1⁺ cells (D).



Vita

Bum Seok Kim was born on January 12, 1975, in Chonju, South Korea. He grew up in Chonju and attended Youngsaeng High School, graduating in 1993. He was then admitted to the College of Veterinary Medicine, Chonbuk National University, Chonju for his Veterinary education in March of 1993. He developed a keen interest in Veterinary Pathology towards the end of his undergraduate tenure in the lab of Dr. Byung Moo Rim. After finishing his second year, he served in the military from February to August of 1995 and visited the lab of Dr. Robert M. Jacobs, Department of Pathology, Ontario Veterinary College, Guelph University, Canada from August of 1995 to February of 1996. He then returned to Korea and received his Doctor of Veterinary Medicine (D.V.M.) degree in 1998. At that time he continued his work in the lab of Dr. Rim and completed a Masters of Science (M.S.) degree in February of 2000. He worked in the department of diagnostic pathology, National Veterinary Research and Quarantine Service, Anyang, South Korea in 2001. He joined the University of Tennessee, Knoxville in August 2001 and received a Doctor of Philosophy (Ph.D) degree from the graduate program of Comparative and Experimental Medicine, Department of Pathobiology, College of Veterinary Medicine in August 2005. He plans on pursuing further post doctoral training.