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# Modulation of HSV-induced angiogenesis in HSK pathogenesis

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To the Graduate Council:

I am submitting herewith a dissertation written by Sujin Lee entitled "Modulation of HSV-induced angiogenesis in HSK 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, Leon N. D. Potgieter, Albert T. Ichiki
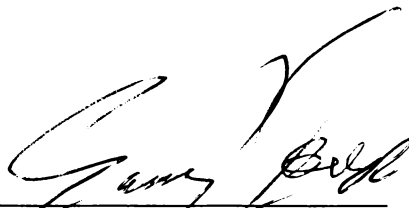
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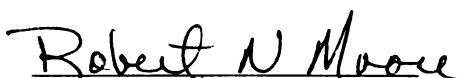(Original signatures are on file with official student records.)

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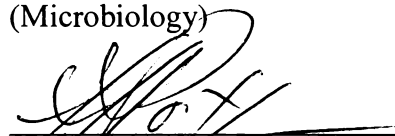
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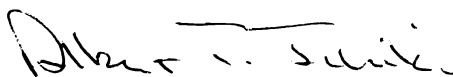
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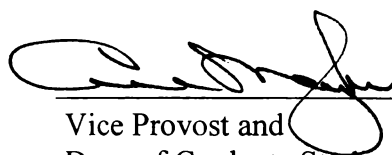
Leon N. D. Potgieter
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Albert T. Ichiki
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Accepted for the Council:

Vice Provost and
Dean of Graduate Studies

# MODULATION OF HSV-INDUCED ANGIOGENESIS IN HSK

# PATHOGENESIS

A

Dissertation Presented

For the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Sujin Lee

August 2002

# TABLE OF CONTENTS

# LIST OF FIGURES

# PART I


# GENERAL INTRODUCTION AND OVERVIEW

# CHAPTER 1

## MECHANISMS OF ANGIOGENESIS

The formation of a functional, integral vascular network is a fundamental process in the growth and maintenance of tissues. Vascularization occurs by three distinct processes: vasculogenesis, angiogenesis, and arteriogenesis (1).

Cell function and survival is dependent on oxygen and nutrient supply from blood vessels. During embryogenesis and organogenesis, the coordinated growth of endothelial cell ensures adequate vessel generation. This process called vasculogenesis, involves the de novo differentiation of endothelial cells (EC) from mesodermal precursors. The distinct process of blood vessels generation from pre-existing vessels, called angiogenesis, is required in adult life during the female reproductive cycle, tissue repair and wound healing (2). Angiogenesis is the mechanism of blood vessel formation after the first few days of embryogenesis, and is essential for all tissue growth (3). In adults, angiogenesis occurs during inflammatory reactions and cancer. The molecular mechanisms controlling angiogenesis are becoming clearer, and therapy targeting these processes is becoming closer to clinical trials. The understanding that the formation of blood vessels depends on the acquisition of a blood supply has led to the development of new therapies for cancer and other angiogenic diseases based on inhibition of neovascularization.

**Vasculogenesis**

Early in development, vessel formation occurs by a process referred to as vasculogenesis, in which endothelial cells differentiate and proliferate in situ within a previously avascular tissue, and then coalesce to form a primitive tubular network (4). This primary network includes some of the major vessels in the embryo such as the aorta and major veins. Blood vessels in the embryo form through vasculogenesis; through in situ differentiation of undifferentiated precursor cells (angioblast) to endothelial cell.

**Angiogenesis**

Angiogenesis is a complex phenomenon consisting of several distinct processes, which include endothelial migration and proliferation, extracellular proteolysis, endothelial differentiation and vascular wall remodeling (2). The term of angiogenesis was first used to describe the growth of endothelial sprouts from preexisting postcapillary venules (4). There are at least two different types: true sprouting of capillaries from pre-existing vessels, and non-sprouting angiogenesis or intussusception. Sprouting angiogenesis is the proteolytic degradation of the extracellular matrix is followed by chemotactic degradation and proliferation of endothelial cells. Almost all known angiogenesis-activating factors induce one or more of these activities in endothelial cells in vitro, but it is unclear which factors act in vivo. One may be VEGF, as it is an endothelial-specific growth and chemotactic factor. Non-sprouting angiogenesis is a process of splitting pre-existing vessels by transcapillary pillars or posts of extracellular matrix. Non-sprouting angiogenesis (intussusception) can occur by proliferation of

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endothelial cells inside a vessel, producing a wide lumen that can be split by transcapillary pillars, or fusion and splitting of capillaries.

**Regulation of angiogenesis**

*Basement membrane breakdown: proteolytic enzymes*

To initiate the formation of new capillaries, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the neighboring tissue (5). These processes of endothelial cell invasion and migration require the cooperative activity of the PA system and the MMPs. The PA is serine protease that converts plasminogen to plasmin. Plasmin has broad substrate specificity and degrades several ECM components, including fibrin, fibronectin, laminin and the protein core of proteoglycans (5). The MMP family consists of at least 16 members, which are expressed as latent enzymes with a similar domain structure (6). The activity of both PAs and MMPs is controlled at least three levels: 1) the expression of uPA, uPAR and MMPs is upregulated by angiogenic growth factors (7, 8) and cytokines (9). 2) pro-MMPs and pro-uPA need to be activated proteolytically (10), and 3) the activity of MMPs, plasmin, and uPA is regulated by TIMPs (11) and PAIs (12). PAs and MMPs are secreted together with their inhibitors, ensuring a stringent control of local proteolytic activity to preserve normal tissue structure.

*Endothelial cell migration and proliferation: angiogenic factors*

Endothelial cells start to migrate through the degraded matrix following proteolytic degradation of the ECM. They are followed by proliferating endothelial cells,

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which are stimulated by a variety of growth factors, some of which are released from degraded ECM (13). Angiogenesis can be divided into three classes (14). The first class consists of the VEGF family, the angiopoietins and ephrins, which specifically act on endothelial cells. These three different growth factor systems seem to have very different roles during vascular development. The first characterized vascular-specific growth factor, VEGF, maintains its position as the most critical driven of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenesis sprouting during development as well as in the adult (15). Angiopoietin 1 (16) and ephrin-B2 (17) are recruited for further remodeling and maturation of this initially immature vasculogenesis. Following vessel maturation, angiopoietin 1 seems to continue to be important in maintaining the quiescence and stability of the mature vasculature. Disruption of this stabilizing signal coincides with reinitiation of vascular remodeling in the adult. Such destabilization seems to involve the autocrine induction of a natural antagonist of angiopoietin 1, termed angiopoietin 2 (18). VEGFs, angiopoietins and ephrin B2 recapitulate their developmental roles during vascular remodeling in the adult, and administration of individual factors to the adult allows them to reprise these roles but not to trigger the entire process.

The second class contains most direct acting molecules, including several cytokines, chemokines (19) and angiogenic enzymes (20) that activate a broad range of target cells besides endothelial cells. The third group of angiogenic molecules includes the indirect acting factors, whose effect on angiogenesis results from the release of direct acting factors from macrophages, endothelial and tumor cells. The most extensively studied are TNF-$\alpha$ and TGF-$\beta$, which inhibit endothelial cell proliferation in vitro. In

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vivo, TGF-β induces angiogenesis and stimulates the expression of TNF-α, FGF-2, PDGF and VEGF by attracted inflammatory cells (21). TNF-α has been shown to increase the expression of VEGF and its receptors, IL-8 and FGF-2 by endothelial cells, thus explaining its angiogenic properties in vivo (22).

*Cell-cell and cell-matrix interaction: adhesion molecules*

The processes of cell invasion, migration and proliferation not only depend on angiogenic enzymes, growth factors and their receptors, but also are mediated by cell adhesion molecules (23). During invasion and migration, the interaction of the endothelial cells with ECM is mediated by integrins. Integrins are a group of cell adhesion receptors, consisting of non-covalently associated α and β subunits. Endothelial cells express several distinct integrins, allowing attachment to a wide variety of ECM proteins (24). Integrin αvβ3 was found to be particularly important during angiogenesis. In activated endothelium, αvβ3 suppresses the activity of both p53 and the p53 inducible cell cycle inhibitor, while increasing the bcl2, resulting in an anti-apoptotic effect (25).

# CHAPTER 2

## CHEMOKINES AND ANGIOGENESIS

Chemokines are small, cytokine-like, secreted proteins that regulate leukocyte transport by mediating the adhesion of leukocytes to endothelial cells, the initiation of transendothelial migration and tissue invasion (26, 27). Four families of chemokines have been described, based on the relative position of the conserved cystein residues: CC, CXC, C and CX3C, with the CC and CXC groups being by far the most common ones. The CXC chemokines are a unique family of cytokines that can regulate angiogenesis in a disparate manner. CXC chemokines are heparin binding proteins. On a structural level, they have four conserved cystein amino acid residues, with the first two cysteins separated by one nonconserved amino acid residues (28, 29). Although the CXC motif distinguishes this family from other chemokine families, a second domain dictates their angiogenic activity. The NH2 terminus of the majority of the CXC chemokines contain the ELR motif, a three amino acid (Glu-Leu-Arg), which procedes the first cystein amino acid of the primary structure of these cytokines (30). The family members that contain the ELR motif are potent promoters of angiogenesis (31). In contrast, members that are induced by interferons and lack the ELR motif are potent inhibitors of angiogenesis (32, 33). Thus members of the CXC chemokine family can either promote or inhibit angiogenesis, and the imbalance of the local expression of these chemokines may be important in the regulation of angiogenesis

CXC chemokine family members behave as angiogenic factors including interleukin-8, growth related genes (GRO-$\alpha$,$\beta$,$\gamma$), granulocyte chemotactic protein-2

(GCP-2) and neutrophil activating protein-2 (NAP-2) (31, 34). ELR+ CXC chemokines induce endothelial cell chemotactic and proliferative activity in vitro, and angiogenesis in vivo. Although a specific CXC chemokine receptor that mediates the angiogenic activity of these cytokines remains to be determined, the candidate CXC chemokine receptors for this effect are CXCR1 and CXCR2. Only IL-8 and GCP-2 specifically bind to CXCR1, whereas all ELR+ CXC chemokines bind to CXCR2 (35). The ability of ELR+ CXC chemokine ligands to bind to CXCR2 supports the notion that this represents the receptor for the mediation of angiogenic activity by ELR+ CXC chemokines. This is supported by the fact that CXCR2 has the greatest sequence homology with the recently described human Kaposi's sarcoma herpes virus G protein coupled receptor ( KSHV-GPCR) (36). IL-8 and GRO-$\alpha$ can act as agonists for KSHV-GPCR, and further augment the signaling of this receptor (37).

Also, the ELR+ CXC chemokines are important mediators of tumorigenesis related to their angiogenic properties, Studies in melanoma tumors support that all GROs play a significant role in mediating tumorigenesis related to both their mitogenic and angiogenic activities (38). The progression of ovarian carcinoma is dependent on successful angiogenesis, and IL-8 has been determined to play a significant role in mediating human ovarian carcinoma derived angiogenesis and tumorigenesis. The expression of IL-8, bFGF, and VEGF was examined in five different human ovarian carcinoma cell lines (39). The expression of IL-8 was directly correlated with neovascularization and inversely correlated with survival (39). Thus CXC chemokines have important implications in promoting angiogenesis.

The findings of ELR+ CXC chemokines in human tumors provides the unique opportunity to target a putative receptor for ELR+ CXC chemokine-mediated angiogenesis. The angiostatic members of the CXC chemokine family include PF4, monokine induced by IFN-$\gamma$ (MIG), and IFN-$\gamma$ inducible protein 10 (IP-10) (40,41). All three interferons stimulate the expression of IP-10. MIG is induced only by IFN-$\gamma$. Recently, a new ELR- member of the CXC chemokine family, IFN-inducible T cell alpha chemoattractant (I-TAC), is induced by IFN-$\gamma$ (42). All interferon inducible ELR- CXC chemokines are potent inhibitors of angiogenesis. This relationship of interferon and interferon-inducible ELR- CXC chemokines and their biological function are directly relevant to the function of IL-18 and IL-12. The ability of IL-18 and IL-12 to induce IFN-$\gamma$ and subsequent interferon-inducible ELR- CXC chemokines explains their ability to inhibit angiogenesis (43). Thus IL-12 and IL-18 will havea profound effect on the production of IP-10, MIG and I-TAC via the induction of IFN-$\gamma$.

The subsequent expression of interferon-inducible ELR- CXC chemokines may represent the final common pathway and explain the mechanism for the attenuation of angiogenesis related to interferons. Although all three IFN-inducible ELR- CXC chemokines specifically bind to the CXC chemokine receptor, CXCR3 (44). Potential mechanisms for the ELR- CXC chemokine, PF4, and its ability to inhibit angiogenesis that may be relevant to interferon-inducible ELR- CXC chemokines. The ability of PF4 to bind to glycosaminoglycans (GAG: heparin and heparan sulfate) with high affinity appears to be important to several of its biological functions. PF4 has been shown to inhibit bFGF and VEGF165 binding to their respective receptors (45, 46). One mechanism for this effect is related to the generation of PF4-bFGF or PF4-VEGF165

heterodimeric complexes, which impairs bFGF or VEGF 165 binding to their respective receptors. bFGF must undergo dimerization in the presence of endogenous heparin in order to bind to its receptors. VEGF 165 possesses heparin binding ability similar to bFGF. PF4 impairs VEGF 165 binding to its receptors on endothelium via a mechanism similar to what has been reported for its ability to inhibit bFGF. PF4 can inhibit a variety of endothelial cell mitogens at multiple levels.

These events may be relevant to interferon-inducible ELR- CXC chemokines because IP-10 has been shown to compete with PF4 for binding and inhibition of endothelial cell proliferation that may be related to inhibition of the cell cycle. This supports that interferon-inducible ELR- CXC chemokines may have similar mechanism for their inhibition of bFGF, VEGF, EGF, and ELR+ CXC chemokine-induced angiogenesis.

# CHAPTER 3

# HERPETIC STROMAL KERATITIS: ANGIOGENESIS AND HSK

Infections by herpes simplex virus (HSV) represent an expensive public health problem. Although only rarely a cause of mortality, HSV infections usually cause painful and often distressing lesions and are particularly troublesome since symptomatic recurrent disease is a common outcome once an individual has been infected. Recurrent lesions on the face and genitalia are the most common expression in some locations such as the eye and distressing results such as blindness can occur.

One unfortunate sequel to HSV infection is a chronic inflammatory lesion of the eye called herpetic stromal keratitis (HSK) (47). In humans, this is an important cause of vision impairment. The murine lesions resemble the human counterpart both clinically and histopathologically especially in susceptible mouse strains such as BALB/c and A/J mice infected with the RE strain of HSV-1. HSV-1 ocular infection of susceptible mice leads to transient mild epithelial lesions caused by virus replication and destruction of epithelial cells. These lesions quickly heal with little or no scarring and the cornea appears normal until around day 5-8 post infection. Subsequently, most infected animals develop progressive corneal opacity, edema, neovascularization, necrosis and ulceration (48), all of which become evident in a span of 2-3 weeks after infection. Although there is general agreement that CD4+ T cells are the principal cell types that organize the inflammatory lesions of HSK, usually viral antigens are not demonstrable during lesion progression (49). Following ocular infection, virus replication occurs primarily in epithelial cells and initiates an infiltration of polymorphonuclear (PMN) cells, the

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majority of which are neutrophils, into the underlying corneal stroma. This response peaks in intensity at 48 hr and then declines by 3-4 days p.i. This pattern of PMN influx correlates with the time when virus can be detected (50, 51). The neutrophils are involved in viral clearance and neutrophil response is trivial in response to mutant viruses such as ICP4-/- and ICP8-/- as well as UV inactivated virus. A second and more aggressive infiltration of immune cells including neutrophils occurs starting around day 7-8 p.i. which peaks between 15 and 20 days p.i. (50). This phase of HSK is orchestrated by CD4+ T cells of the Th1 phenotype (52). The notion that CD4+ T cells are the principal mediators of the lesion came primarily from studies showing that SCID or athymic mice failed to develop HSK unless reconstituted with CD4+ T cells (53, 54).

**Angiogenesis and the pathogenesis of HSK**

The pathogenesis of HSK involves the development of new blood vessels in the normally avascular cornea. Although most studies on HSK have focused on the participation of different cell types, the cytokines and chemokines induced, and the issue of which antigens act as targets for CD4+ T cell recognition, the event of new blood vessel ingrowth into the normally avascularized cornea has been virtually ignored. Although it remains unclear as to the likely multiple molecules responsible for HSV-induced angiogenesis, the event appears to be a necessary step in HSK pathogenesis.

Recently, Zheng et al. demonstrated that HSV ocular infection acts as a stimulus for the VEGF family of proteins, potent angiogenesis factors (55). This induction of VEGF appeared to occur as an indirect consequence of infection and appeared not to derive from virus infected cells. In fact, using GFP labeled virus and processing cells for

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VEGF expression by immunohistochemistry, no evidence for double producers was evident in the infected corneal epithelium. Similarly, the authors have infected peritoneal macrophage cell line in vitro with HSV, and been unable to detect cells that were both virus positive and VEGF positive. Thus it remains to be determined how HSV infection resulted in VEGF expression. Also, angiogenic sprouting during HSK development was evident at 24hr and increased in magnitude peaking between 15 and 20 days p.i. Inhibition of VEGF function with the fusion protein mFlt-IgG significantly inhibited both HSV driven angiogenesis and HSK. However, HSK angiogenesis and severity were not completely inhibited by anti VEGF likely because the process of HSV induced angiogenesis is complex with much more than VEGF involved. In addition, treatment of EMAPII (endothelial monocyte-activating polypeptide II) which is potent anti-angiogenesis cytokine resulted in diminished HSK severity (56). The effect of EMAPII was at least in part directed at the function of VEGF. EMAPII inhibited VEGF induced angiogenesis in micropocket assay. Also, EMAPII appears to function by causing apoptosis in corneal epithelial cells. Recently, mouse bioactive CpG containing oligodeoxynucleotides (ODN), but not control ODN, caused angiogenesis (57). This effect was significantly inhibitable by anti-VEGF antibody. Furthermore, HSV DNA itself could similarly cause angiogenesis with the effect also inhibitable by anti-VEGF antibody. Thus HSV DNA released from dying cells could be one mechanism of angiogenesis factor induction.

A novel observation was that angiogenesis represents a crucial event in HSK pathogenesis and that neovascularization provides a logical target for the therapeutic management of HSK. The process of new capillary formation from preexisting vessels,

angiogenesis, is a complex physiological event which is strictly controlled, occurring only very rarely under normal conditions. In contrast, there are number of serious disease, among them solid tumor growth, rheumatoid arthritis and several eye disease, which is characterized by unrestricted new capillary growth and which are described as angiogenic disease (58). As it currently stands, the research on angiogenesis should add to our knowledge about this topic and could lead to the ultimate development of new therapies for the control of HSK.

# CHAPTER 4

## SPECIFIC AIMS AND RATIONALE

Infection of the eye with HSV may result in blinding immunoinflammatory lesion in the cornea called herpetic stromal keratitis (HSK). Studies in mouse models have shown that HSK is a multistep process that primarily is the consequence of an immuno-inflammatory reaction orchestrated by CD4+ T cells. Although most studies on HSK have focused on the participation of different cell types, the cytokines and chemokines induced, and the issue of which antigens act as targets for CD4+ T cell recognition, the event of new blood vessel ingrowth into the normally avascularized cornea has been virtually ignored. Although it remains unclear as to the likely multiple molecules responsible for HSV-induced angiogenesis, the event appears to be a necessary step in HSK pathogenesis. As it currently stands, it remains unclear how HSV infection results in angiogenesis.

The specific aims are

1. To define how herpes simplex virus (HSV) infection of the cornea results in initial neovascularization of the cornea.

2. To determine the cellular and molecular mechanisms in HSV induced angiogenesis during herpetic stromal keratitis (HSK) pathogenesis.

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# PART II

# IL-12 SUPPRESSES THE EXPRESSION OF OCULAR IMMUNOINFLAMMATORY LESIONS BY EFFECTS ON ANGIOGENESIS

This part is lightly revised version of a paper by the same name published in the *Journal of Leukocyte Biology* in 2002 by Sujin Lee, Mei Zheng, Shilpa Deshpande, Seong Kug Eo, Thomas Hamilton, and Barry T. Rouse:

Lee, S., M. Zheng, S. Deshpande, S. Eo, H. Thomas, and B. T. Rouse. IL-12 suppresses the expression of ocular immunoinflammatory lesions by effects on angiogenesis. *J. Leuko.Biol.* 2002. 71: 469-476

# CHAPTER 1

## ABSTRACT

Topical application of plasmid DNA encoding IL-12 to the cornea of mice prior to ocular infection with herpes simplex virus type 1 (HSV) results in diminished corneal immunoinflammatory lesions. Such herpetic stromal keratitis (HSK) reactions in humans represent an important cause of blindness. The effect of IL-12 pretreatment acted via inhibitory effects on corneal neovascularization, rather than by inhibiting viral replication or the function of CD4$^+$ T cells that mediate HSK. The anti-angiogenesis induced by IL-12 DNA application was mediated indirectly via the cytokine IFN-$\gamma$ and one or both of two chemokine molecules, IP-10 and MIG. Thus IL-12 DNA administration lacked modulatory effects on HSK in GKO mice, indicating the necessary involvement of IFN-$\gamma$ induction for antiangiogenesis. In contrast, exposure of GKO mice to IP-10 DNA did suppress the severity of HSK. Furthermore, treatment with specific antisera to IP-10 and MIG in HSV infected mice abrogated the IL-12 induced an inhibitory effect on lesion severity. Taken together, these data indicate that the HSV induced ocular immuno-inflammatory lesions can be modulated by IL-12 and that this effect results from chemokine inhibition of angiogenesis. The use of antiangiogenesis therapy might represent a useful control measure against HSK.

## CHAPTER 2

## INTRODUCTION

Herpes simplex virus (HSV) infection of the eye can result in a blinding immunoinflammatory lesion in the corneal stroma (1). In humans this is an important cause of vision impairment. The lesion, as studied in animal model systems, occurs as a consequence of a CD4$^+$ T cell orchestrated immunopathological reaction, but the nature of target antigens which drive T cell activation remain ill-defined (1, 2). In humans, herpetic stromal keratitis (HSK) is controlled with anti-inflammatory drugs and severe cases may require corneal transplantation. It is anticipated that a full understanding of HSK pathogenesis may lead to novel therapies to control this distressing lesion.

Evidence to data implicates CD4$^+$ T cells of the type 1 phenotype as the mediator of HSK (3, 4). Moreover pretreatment with type 2 cytokines can suppress HSK severity (5, 6). In addition, treatment with the cytokine IL-10, before the full clinical phase develops, can result in lesion suppression or even resolution (5). HSV infection itself results in the production of a notable IL-12 cytokine response (7), which presumably helps set the stage for the type 1 T cell mediated CD4$^+$ inflammatory reaction. We anticipated that if animals were exposed to IL-12 prior to infection this would further potentiate type 1 CD4$^+$ T cell responses and result in more severe HSK lesions. Unexpectedly, however, ocular exposure to plasmid DNA encoding IL-12 resulted in diminished rather than exacerbated stromal lesions. Conceivably, the lesion modulating effects of IL-12 could be explained by the induction of antiviral cytokines such as IFN-γ, or the induction of cellular defenses such as NK cells known to exert protective effects against HSV infection (8). In addition, IL-12 has been reported to express

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immunosuppressive effects on CD4$^+$ T cell priming by inducing IFN-γ and iNOS activation that in turn causes T cell apoptosis (9). Furthermore IL-12 may inhibit tumor seemingly by mediated an antiangiogenesis effect (10).

As we have documented elsewhere, angiogenic sprouting into the normally avascular cornea appears to be an essential event in the pathogenesis of HSK (11, 12). This process appears as a necessary prelude to invasion by the CD4$^+$ T cells which drive the inflammatory reaction (11). The present report adds support to the notion that IL-12 is antiangiogenic and that the inhibitory effect of IL-12 on HSK lesion severity proceeds by an effect on angiogenesis rather than by antiviral or suppressive effects in T cell function. As observed with some tumor systems, the antiangiogenic effect of IL-12 appeared to act indirectly by inducing IFN-γ which in turn caused the expression of two antiangiogenic factors interferon inducible protein 10 (IP-10) and monokine induced by interferon gamma (MIG) (13). Accordingly, our results demonstrate that administration of IL-12 DNA to normal, but not IFN-γ -/- mice, caused upregulation of two cytokines IP-10 and MIG in the cornea. These inhibited HSV induced angiogenesis and consequently the severity of HSK. Our results are discussed in terms of novel approaches that merit testing to control HSK lesions.

# CHAPTER 3

## MATERIALS AND METHODS

**Mice**

Female 4- to 5- week old BALB/c and C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.). Female 4- to 5- week old iNOS KO mice and female 6- to 8- week old GKO mice purchased from The Jackson Laboratory (BarHarbor, ME). GKO mice contain a nonfunctional IFN-γ gene and are on a BALB/c background. BALB/c and C57BL/6 mice were housed conventionally, and KO mice were housed in sterile microisolator cages in the animal facility. All manipulations were performed in a laminar flow hood. To prevent bacterial infection, all mice received treatment with sulfamethoxazole/trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5ml/200ml of 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 are fully accredited by the American Association of Laboratory Animal Care.

**Virus**

HSV-l strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile, AL) was used in all procedures. Virus was grown in Vero cell monolayers (ATCC cat.no.CCL81 ), titrated, and stored in aliquots at -80°C until used.

**Plasmid DNA preparation**

Plasmid DNA encoding murine IL-12 was kindly provided by Dr. Kenji Okuda (Yokohama City University School of Medicine, Yokohama, Japan). Plasmid DNA encoding murine IP-10 was constructed by PCR amplification of the full coding region. All plasmid DNAs used in this work were inserted into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, Calif). The plasmid DNAs were purified by polyethylene glycol precipitation by the method of Sambrook et al. (14) with some modifications. The quality of DNA was measured by electrophoresis on 1 % agarose gel. The protein expression of the different plasmids was determined by RT-PCR and dot blot after in vitro transfection into Chinese Hamster Ovary (CHO) cells.

**Corneal HSV infection**

Corneal infections of all mouse groups were conducted under deep anesthesia induced by the inhalant anesthetic methoxyfurane (Methofane; Pittman Moore, Mondelein). The mice were lightly scarified on their corneas with a 27 gauge needle, and a 2.5μl drop containing $1\times10^6$ PFU of HSV-1 RE for BALB/c, $1\times10^7$ PFU of HSV-1 RE for C57BL/6 and iNOS KO mice, and $1\times10^4$ PFU of HSV-1 RE for GKO mice 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

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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 previously described (12). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5mm toward the corneal center. The score of the 4 quadrant of the eye were then summed to derive the NV index (range 0-16) for each eye at a given time point (12).

**Virus recovery and titration**

Swabs of the corneal surface were collected at various time points post infection. The swabs were put into sterile tubes containing 500μl of DMEM with 10 IU of penicillin/ml and 100μg of streptomycin (Life Technologies, Grand Island, NY)/ml and stored at - 80°C. For detection and quantification of virus in the swabs, the samples were thawed and vortexed. Individual subsamples (200μ of each sample) were further diluted, and viral titers were determined by a plaque assay performed on Vero cells as described elsewhere (15).

**Plasmid DNA administration**

100μg of plasmid DNA was suspended in 4μl of sterile PBS. Corneas were scarified using a 27 gauge needle in a criss-cross pattern and the plasmid was intraocularly administered on 6 and 3 days before virus infection.

**HSV-specific lymphoproliferation**

This assay has been described in detail elsewhere (16). Briefly, at day 15 following HSV ocular infection, the splenocytes of vector, IL-10 or IL-12 DNA treated mice were enriched for T cells by a nylon wool column and used as responder populations. These T cells were restimulated in vitro with irradiated syngeneic enriched naïve dendritic cell (DC) or DC infected with UV-inactivated HSV (MOI of 1.5 before UV inactivation) and incubated for 5 days at 37°C. Con A (5μg/ml) was used as a polyclonal positive control and incubated for 3 days. Eighteen hours before harvesting, [$^3$H] thymidine was added to the cultures.

**Cytokine assay**

For cytokine (IFN-γ) assay, splenocytes from mice were suspended in 10% RPMI 1640, and $10^6$ cells in 1 ml were stimulated in vitro with irradiated syngeneic enriched DC pulsed with UV-inactivated HSV (MOI, 5.0. before UV inactivation). Similar number of cells were Con A stimulated (5μg/$10^6$ cells/ml) in 96 well plates. Plates were incubated at 37°C for 72h. The supernatant fluid was collected and stored at -80°C until use. These supernatants were screened for the presence of IFN-γ by ELISA as described previously (16).

**Isolation of RNA**

At day 3 post infection, corneas were carefully dissected, freed of scleral tissues, minced and homogenized in TRI Reagent (Molecular Research Center, Cincinnati, OH).

Total RNA was isolated by manufacturer's protocol. All the procedures including RT-PCR were performed in a laminar flow hood.

**RT-PCR**

Total cellular RNA (10μg/ml) was reversed transcribed using oligo (dT) primers and reverse transcriptase (Promega) according to protocols described previously (17). The cDNA was made by the reverse transcription reaction incubated at 42°C for 90 min. The cDNA (2μl) was subjected to 35 cycles of amplification as described (5) using primers. The primers used follows: β-actin-1, 5'-GTGGGGCGCCCCAGGCACCA-3'; β-actin-2, 5'-CTCCTTAATGTCACGCACGAT-3'; IFN-γ-1, 5'-ATGAACGCTACACACTGCAT C-3'; IFN-γ-2, 5'-GCAGCGACTCCTTTTCCGCTT-3'; IP-10-1, 5'-ACCATGAACCCA AGTGCTGCCGTC-3'; IP-10-2, 5'-GCTTCACTCCAGTTAAGGAGCCCT-3'; MIG-1, 5'-ACTCAGCTCTGCCATGAACTCCGC-3'; MIG-2, 5'-AAAGGCTGCTCTGCCAG GGAAGGC-3'; IL-10-1, 5'-ATGAAATATACAAGTTATATC-3'; IL-10-2, 5'-TTAGC TTTTCATTTTGATCAT-3'. The PCR products were separated by agarose gel electrophoresis.

**Preparation and administration of Abs**

Rabbit polyclonal Abs to IP-10 and MIG were produced by Biosynthesis (Lewisville, TX) using synthetic peptides selected from the IP-10 and MIG protein sequences (CIHIDDGPVRMRAIGK and CISTSRGTIHYKSLKDLKQFAPS) coupled to

carrier protein KLH. Mice were given intraperitoneally 250µl of antibodies to IP-10 and/or MIG 1 day before infection and 3 day after infection.

**Corneal micropocket assay**

In vivo angiogenic activity was assayed in the avascular cornea of BALB/c mouse eyes, as previously described (18). Briefly, mice were pretreated with plasmid DNA encoding IP-10 twice intraocularly before implantation. Pellets for insertion into the cornea were made by combining rhVEGF-165 (40µg, R&D system), sulcralfate (10mg, Bulch Meditec) and hydron polymer in ethanol (120mg/1ml ethanol, Interferon Sciences), and applying the mixture to a $15\times15mm^2$ piece of synthetic mesh (Tetko). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 90ng of VEGF. Pellets containing rhVEGF were implanted into an intracorneal pocket (1mm from the limbus) afterwhich the eyes were 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. The number of vessels originating from the limbus was counted over the entire orbit, and the area of angiogenesis was calculated according to the formula for an ellipse.

A=[(clock hours)×0.4×(vessel length in mm)×π]/2. Each clock hours is equal to 30° at the circumstance.

**Statistical analysis**

Significant differences between groups were evaluated using the Student's $t$ test.

CHAPTER 4

RESULTS

**IL-12 DNA decreases the severity and incidence of HSK**

Since murine HSK appears to be an inflammatory lesion orchestrated mainly by

type 1 cytokine producing CD4^{+} T cells (1, 2), expression of IL-12 on the cornea prior to

infection was expected to enhance the severity of HSK. In fact, however, as shown in Fig

1, the opposite outcome was observed. In such experiments, susceptible BALB/c mice

were exposed to 100μg of IL-12 DNA on the ocular surface 6 and 3 days before virus

infection. In each experiment, animals received either IL-12 DNA or vector DNA and

then $10^6$ PFU of HSV-1 RE on their scarified corneas. As is clearly evident (Fig. 1), the

majority of animals given IL-12 DNA showed lesions of diminished severity and

incidence in comparison to vector DNA treated individuals. By day 10, the IL-12 DNA

treated mice had significantly reduced clinical lesions and corneal opacity ($p<0.05$)

compared to vector treated controls. These differences continued at days 12 ($P<0.05$), 15

($P<0.05$) and 20 ($P<0.01$). In the IL-12 DNA treated eyes, approximately 80-90% of eyes

showed controlled or resolved lesions during a 21 day observation period. In vector DNA

treated eyes, resolution was evident in a maximum of 10% of eyes. These results indicate

that IL-12 DNA possesses an inhibitory effect on the expression of HSK lesions.

However, IL-12 given after HSV infection had no significant effect on lesion severity

(data not shown).

Three possibilities were considered to explain the inhibitory effect of IL-12.

Firstly, IL-12 exposure would likely result in IFN-γ expression in the cornea which in

turn could be antiviral. As documented in a later section, exposure of the cornea to IL-12

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DNA did result in IFN-$\gamma$ mRNA expression. However, measurement of the duration and concentration of virus in ocular washing at various times after infection in IL-12 and vector-treated mice revealed no significant differences (Fig. 2). A second explanation for the inhibitory effect of IL-12 DNA was that IL-12 could exert immunosuppressive effects on $CD4^+$ T cell priming as reported in some other systems (9). As shown in Fig. 3, evidence for any such immunosuppression was not obtained. Accordingly, measurement of HSV specific proliferative (mainly a function of $CD4^+$ T cell function) (P=0.3) and cytokine production of splenocytes at 15 day post infection (P=0.4) revealed no evidence of immunosuppression. In contrast pretreatment with IL-10 DNA did result in diminished HSV specific proliferative responses.

Antiangiogenic effects were the third possibilities to explain by IL-12 DNA inhibition of HSK. The data recorded in Fig. 4, support this mechanism. In these experiments, the extent of angiogenic sprouting from the limbus into the normally avascular cornea was recorded at various times following HSV infection in IL- 12 DNA and vector DNA treated mice. As is evident, the extent of angiogenesis was significantly reduced on day 5 and at subsequent examination periods in animals that received IL-12 DNA pre-exposure. As is also shown, expression of IP-10 DNA also caused reduced angiogenesis (described later).

**IL-12 inhibition of HSK acts via IFN-$\gamma$ induction**

In several systems where IL-12 exerts functional effects, it does so by inducing intermediary cytokines such as IFN-$\gamma$ (19,20). Other activities such an immunosuppression may be the consequence of iNOS induction (9, 21, 22). To test the

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role of both IFN-γ and iNOS activity during the IL-12 induced inhibition of HSK, the effects of IL-12 pretreatment was compared in ocularly infected normal and knockout mice. Fig. 5C records the inhibitory effects of IL-12 DNA pretreatment in BALB/c and IFN-γ -/- mice. Whereas IL-12 pretreatment inhibited the severity of HSK in BALB/c mice, the effect was not evident in GKO mice (P=0.7). In the latter mice, animals were infected with $10^2$ less virus than given to BALB/c mice, since GKO mice die of encephalitis if infected with the virus doses used to produce HSK in BALB/c mice [23]. To measure the influence of iNOS expression, C57BL/6 (B6) mice and B6 background iNOS -/- mice were used. Both groups of mice were IL-12 or vector DNA treated prior to HSV infection and the severity of HSK lesions compared. Fig. 5A and 5B show IL-12 DNA pretreatment resulted in diminished HSK lesion severity in both B6 and iNOS -/- mice, with the IL-12 inhibitory effect of IL-12 even more apparent in the iNOS -/- mice. These data indicate that the antiinflammatory effect of IL-12 likely dose not proceed via nitric oxide production as has been reported to occur in some other systems (9, 22).


**Possible mechanism by which IL-12 mediates antiangiogenesis**

In some tumor systems IL-12 was observed to mediate antiangiogenesis, acting indirectly on intermediary steps such as IFN-γ production that in turn induced IP-10 and MIG antiangiogenic factors (13, 24). The modulating effect of IL-12 DNA pretreatment on HSK and corneal angiogenesis would also seem to involve a similar mechanism. Thus as shown in Fig 6, topical administration of IL-12 DNA led to increased expression of mRNA for IFN-γ as well as for both IP-10 and MIG. There was no upregulation of IL-10 mRNA, another possible mediator of HSK suppression as shown in previous work (5, 6).

The upregulation of IP-10 and MIG mRNA appeared to be the consequence of IFN-γ expression since measurement of IP-10 and MIG mRNA in IL-12 DNA treated GKO corneas revealed no detectable IP-10 and MIG mRNA expression.

In another experiment, the effects on HSK lesion severity was measured in mice pretreated with either IP-10, IL-12 or vector DNA prior to infection with HSV. As is readily apparent (Fig. 7A), BALB/c mice pretreated with IP-10 DNA had reduced HSK lesion severity and incidence almost to the same degree, as did those animals pretreated with IL-12 DNA. Furthermore, IP-10 DNA pretreated animals also showed reduced angiogenesis scores in comparison to vector DNA exposed animals (Fig. 4). Interestingly, IL-12 DNA pretreatment had no effect on HSK expression in GKO mice, preexposure of such mice to IP-10 DNA did result in significantly reduced HSK lesion severity and incidence (Fig. 7B). Taken together the above data support the idea that IL-12 reduces HSK expression via effects on angiogenesis mediated indirectly by IFN-γ induced IP-10 and MIG expression.

**IP -10 and MIG mediate antiangiogenesis in the HSK model**

To further evaluate the role of IP-10 and MIG in antiangiogenesis mediated by IL-12 DNA two further sets of experiments were performed. In the first, mice were pretreated with IL-12 DNA, infected with HSV and then either given control Ig or polyclonal rabbit anti IP-10 or/and anti MIG antisera. These sera were shown to mediate appropriate specific effects in previous studies (25, 26, 27). The results show that treatment with either anti IP-10 ($P<0.05$) or anti MIG ($P<0.02$) partially reversed the IL-12 inhibitory effect on HSK. When both antisera were combined, the IL-12 DNA

inhibitory effect was abrogated (Fig. 8). In separate experiments, the effect of IP-10 DNA was tested for antiangiogenesis in a corneal micropocket assay using the potent angiogenesis factor VEGF to induce angiogenesis. As is recorded in Fig. 9, IP-10 DNA resulted in 58 % inhibition of VEGF induced angiogenesis.

# CHAPTER 5

## DISCUSSION

This report demonstrates that the application of plasmid DNA encoding IL-12 to the cornea of mice prior to ocular infection with HSV results in diminished immunoinflammatory lesions. Such herpetic stromal keratitis (HSK) reactions represent an important cause of human blindness (1). The effect of IL-12 pretreatment acted via inhibitory effects on corneal neovascularization, rather than by inhibiting viral replication or the function of CD4$^+$ T cells that mediate HSK. The antiangiogenesis induced by IL-12 DNA application was mediated indirectly via the cytokine IFN-$\gamma$ and one or both of two chemokine molecules IP-10 and MIG. Thus IL-12 DNA administration had no modulatory effect on HSK in GKO mice, indicating the necessary involvement of IFN-$\gamma$ induction. In contrast, exposure of GKO mice to IP-10 DNA did suppress the severity of HSK. Furthermore, suppression with specific antisera of IP-10 and MIG expression in HSV infected mice abrogated the IL-12 induced inhibitory effect on HSK. Taken together, our results indicate that the ocular immunoinflammatory lesion that results from HSV infection can be modulated by IL-12 and that this effect results from chemokine inhibition of angiogenesis.

As first shown by our group (5), and confirmed by others (28), the surface application of plasmids encoding various proteins is a convenient means of expressing molecules in the cornea. By such an approach, we showed that pre-exposure to Th2 chemokines such as IL-4 and IL-10 reduced HSK lesions caused by HSV infection. Such

lesions are immunoinflammatory with CD4$^+$ T cells producing type I cytokines such as IFN-$\gamma$ and IL-2, the principal mediators of the inflammation (4). During lesion resolution Th2 cytokines may predominate (5). Initial experiments with IL-12 DNA administered to the eye were done in an attempt to exacerbate lesions and to facilitate disease induced by nonvirulent mutant viruses. Surprisingly, however, lesions were diminished rather than exaggerated. Since IL-12 induces IFN-$\gamma$, as shown to occur in the cornea in the present report, a logical explanation for the modulatory effect on HSK lesion severity was the antiviral activity of IFN-$\gamma$. Indeed, clearance of HSV infection from peripheral sites often appears to be a correlate of IFN-$\gamma$ production (29, 30). In line with this observation, animals unable to produce IFN-$\gamma$ (GKO mice), are markedly more susceptible to HSV infection (23). Nevertheless, such mice can still express HSK, as long as they are infected with lower, non-lethal doses of virus (23). In our model, although IL- 12 DNA resulted in IFN-$\gamma$ expression, this appeared inadequate to curtail viral replication. Thus, in IL-12 DNA treated mice, levels and duration of viral expression were almost identical to those in control vector DNA treated animals.

The HSK lesions are mediated principally by CD4$^+$ T cells although the identity of antigens which drive these cells have yet to be identified. Conceivably the inhibitory effects of IL-12 administration might result from immunosuppressive effects of IL-12 on CD4$^+$ T cell priming. In fact, such a mechanism was advocated to explain the inhibitory effects of IL-12 on CD4$^+$ T cell mediated uveitis (9). In this instance, suppression was mediated by upregulation of iNOS which led to NO production. The latter interfered with Bcl 2 regulated apoptosis in developing CD4$^+$ effector cells (9). Such a mechanism

appeared not to be the explanation for our observations. Thus the HSV specific CD4$^+$ T cell responses appeared normal in IL-12 treated mice. In addition, the inhibitory effect of IL- 12 on HSK expression was unimpaired in mice unable to express iNOS because of gene knockout. Indeed, in such mice the inhibitory effects of IL-12 were even more marked but we have no explanation for this observation.

The hypothesis favored to explain the inhibitory effect of IL-12 on HSK lesions was an effect on corneal neovascularization. Accordingly, ocular HSV infection results in angiogenic sprouting into the normally avascular cornea (12). A molecular explanation for such events is lacking but the VEGF family of potent angiogenesis factor appears as involved (12). Angiogenesis appears necessary during HSV pathogenesis to permit appropriate access of CD4$^+$ T cells and some other inflammatory components to the corneal stroma (11). In support of such ideas, we have shown elsewhere that inhibition of angiogenesis with a cytokine that causes vascular endothelial cell apoptosis results in diminished HSK lesions (12). The present report further demonstrates the relationship of angiogenesis and HSK lesion expression. Thus we show that IL-12 pretreatment results in diminished angiogenesis which correlated with reduced HSK lesions. The effect of IL-12 appeared as indirect with IL-12 serving to upregulate IFN-γ which in turn caused expression of two CXC chemokines IP-10 and MIG. These latter molecules are the actual angiogenesis inhibitors. At least for IP-10, we demonstrated, using a corneal micropocket assay, that it could inhibit the angiogenesis effect of VEGF, a factor involved in HSV angiogenesis (12). Others have also shown the inhibitory effect of IP-10 against angiogenesis caused by fibroblast growth factor (31). Moreover, it was apparent that

inhibition with specific antisera of either IP-10 or MIG (but preferably both simultaneously) reversed an IL-12 induced effect on angiogenesis and HSK expression. That IFN-γ was an essential component of the antiangiogenesis, was supported by observations that the IL-12 induced effect did not occur in GKO mice. Taken together our data support the mechanism advocated to explain the antitumor effect of IL-12 observed in some systems. Namely that IL-12 induces IFN-γ which then upregulates antiangiogenic chemokines (13, 25, 32).

The molecular mechanism by which IP-10 and MIG inhibit angiogenesis has yet to be established. To this end, studies in the cornea may represent a more convenient and accessible model than those in solid tumors or in vitro systems. Evidence supports the fact that at least two types of receptors can be involved in responses to IP-10 and MIG (33, 34, 35). These are heparan sulfate proteoglycans (HSPG) as well as CXCR3. Curiously, CXCR3 can be expressed on effector T lymphocytes and engagement of the receptor by IP-10 or MIG can result in chemotaxis. Such an event could recruit inflammatory T cells to the cornea, and conceivably serve to increase the severity of HSK. However, since neovascularization may be necessary to permit invasion by CD4^{+} T cells, the effect on angiogenesis, likely mediated by HSPG receptor engagement (35) will be dominant. Future therapy of HSK could benefit from targeting angiogenesis receptors such as HSPG. Such issues merit further investigation.

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**APPENDIX**

**Figure. 1. BALB/c mice are protected from HSK by IL-12 DNA administration.**

Groups of animals (n=7) were treated intraocularly at 3 and 6 days before virus infection with 100μg of either IL-12 DNA (_) or vector DNA (_). 3 Day after 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 complied from seven independent experiments.
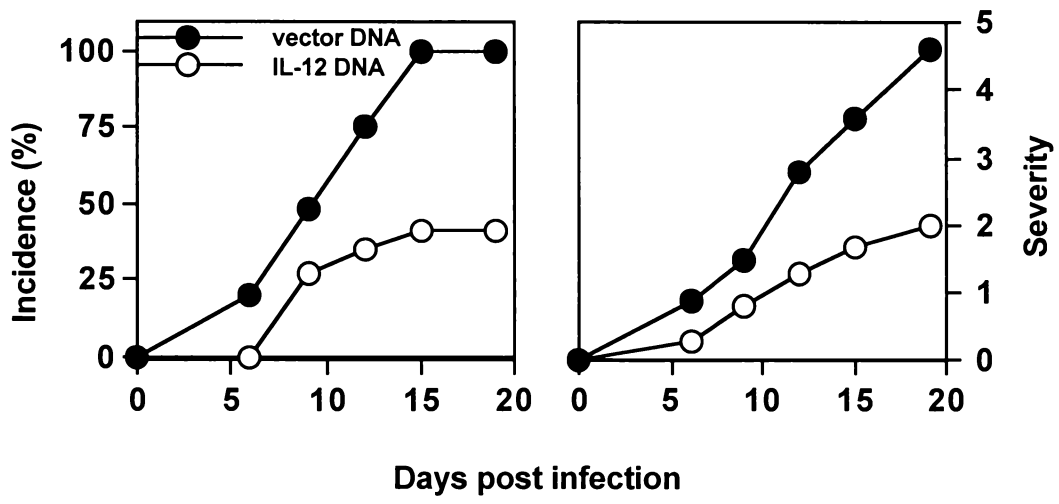
Days post infection

49

**Figure. 2. Persistence of virus following HSV-1 RE infection.**

BALB/c mice were treated with IL-12 DNA (_) or vector DNA (_) and then infected with $10^6$ PFU (n=8) of HSV-1 RE on their scarified corneas. Eye swabs were collected every day post infection, and the virus titer was determined by the agarose overlay method. The virus titer was calculated as log PFU per milliliter. Data represent an average of 8 numbers of mice per group. *P= 0.5 at day 4 post infection. (P value represents that there is no significant difference between vector and IL-12 DNA treated group.
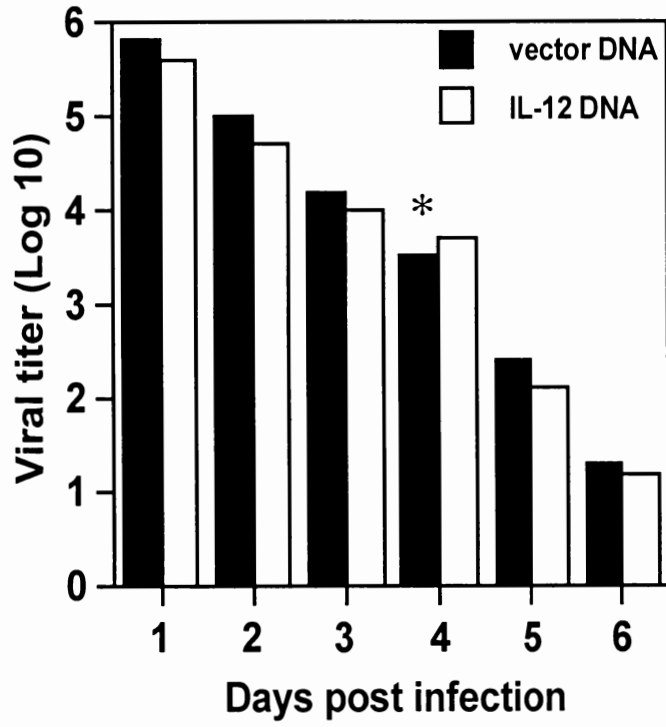
**Figure. 3. HSV-specific Th-cell proliferative responses (A) and IFN-γ production (B) of HSV stimulated splenocytes.**

Groups of animals (n=7) were treated intraocularly at 3 and 6 days before virus infection with 100μg of either IL-12 DNA (_) or vector DNA (_). 3 Day after second treatment, animals were infected with $10^6$ PFU of HSV-1 RE on their scarified corneas. Mice were sacrificed 15 days following HSV-1 ocular infection. Stimulation indices were calculated by cpm of HSV infected cells/cpm of uninfected cells. For the measurement of IFN-γ, the T cells were restimulated in vitro with irradiated DC infected with UV-inactivated HSV-1 KOS. Seventy-two hours later, culture supernatants were collected and analyzed for IFN-γ by ELISA. Significant differences (P<0.01) between IL-10 DNA and vector DNA treated mice are indicated by an asterisk (Stimulation Index), and a double asterisk (IFN-γ production).
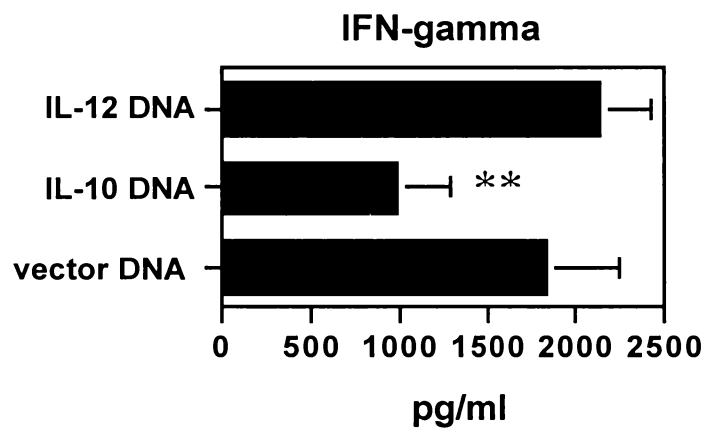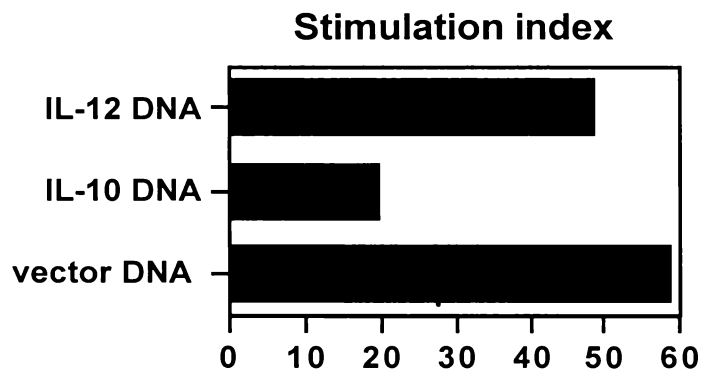
## Stimulation index



## IFN-gamma



pg/ml

53

**Figure. 4**. **Administration of IL-12 DNA or IP-10 DNA inhibits angiogenesis.**

Groups of animals (n=7) were treated twice with 100μg of either IL-12 (_), IP-10 (_) or vector DNA (_) intraocularly 6 and 3 days before virus infection and were infected with $10^6$ PFU of HSV-1 RE on their scarified corneas 3 day after second treatment of DNAs. The animals were then examined for the extent of angiogenesis as described in Materials and Methods. The data are complied from three independent experiments. * $P<0.05$ between IL-12 DNA or IP-10 DNA and vector DNA at days 5, 7, 10, 14 and 18 post infection.
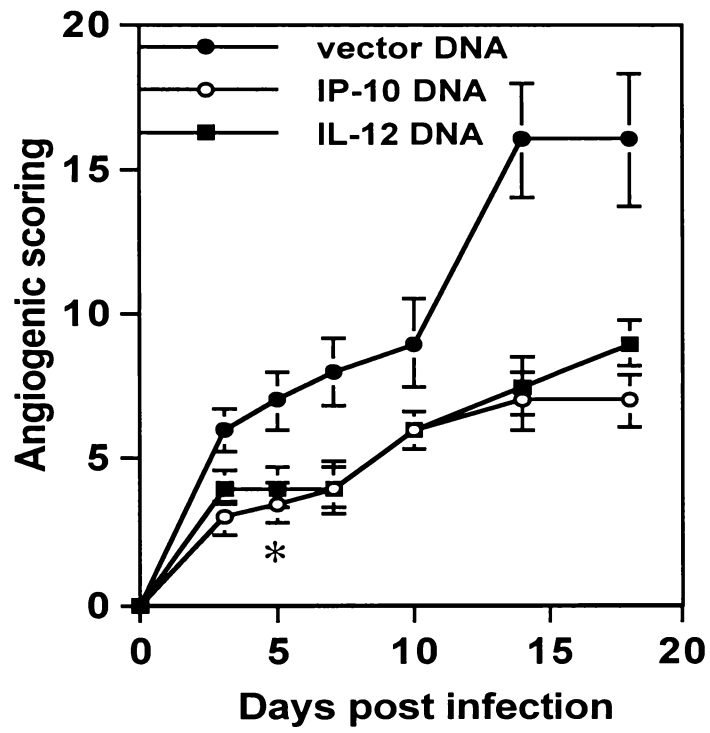
**Figure. 5. IL-12 DNA treatment fails to protect GKO mice (B) from HSK, but not iNOS KO mice (A).**

Groups of BALB/c, C57BL/6, and iNOS KO mice (n=7) were infected with $10^6$ PFU and GKO mice (n=7) were infected with $10^4$ PFU of HSV-1 RE on their scarified corneas. These mice were given 100μg of each of IL-12 DNA (_) and vector DNA (_) intraocularly 3 and 6 day before virus infection. The mice were examined clinically by slit lamp biomicroscopy, and the severity of lesions was scored on a 0-to-5 scale as described in Materials and Methods. Data are complied from three independent experiments. Significant reductions of HSK severity and incidence in IL-12 DNA treated C57BL/6 and iNOS KO mice are indicated by an asterisk (P<0.05).
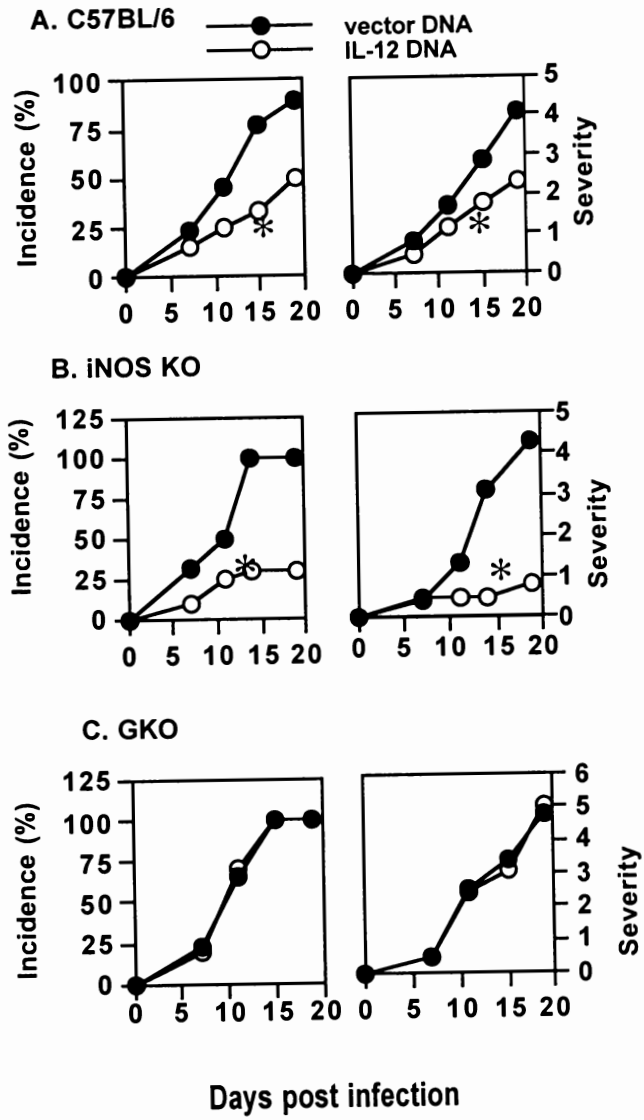
A. C57BL/6 — ● vector DNA
— ○ IL-12 DNA

B. iNOS KO

C. GKO

Days post infection

57

**Figure. 6. Expression of transcripts for IFN-γ, IP-10 and MIG in corneas of BALB/c or GKO mice.**

Groups of BALB/c mice (n=7) or GKO mice(n=7) received 100μg of IL-12 DNA or vector DNA and infected with $10^6$ PFU ($10^4$ PFU for GKO mice) of HSV-1 RE on their scarified corneas. At 3 day post infection, corneas were isolated from mice and treated with Trizol reagent. Total RNAs were extracted from the corneas as described in Materials and Methods. The RNA samples were subjected to RT-PCR analysis. Similar results were obtained in two separate experiments. Lane 1: cornea from naïve BALB/c mice, Lane 2: cornea from vector DNA treated BALB/c mice, Lane 3: cornea from IL-12 DNA treated BALB/c mice, Lane 4: cornea from vector DNA treated GKO mice, Lane 5: cornea from IL-12 DNA treated GKO mice.
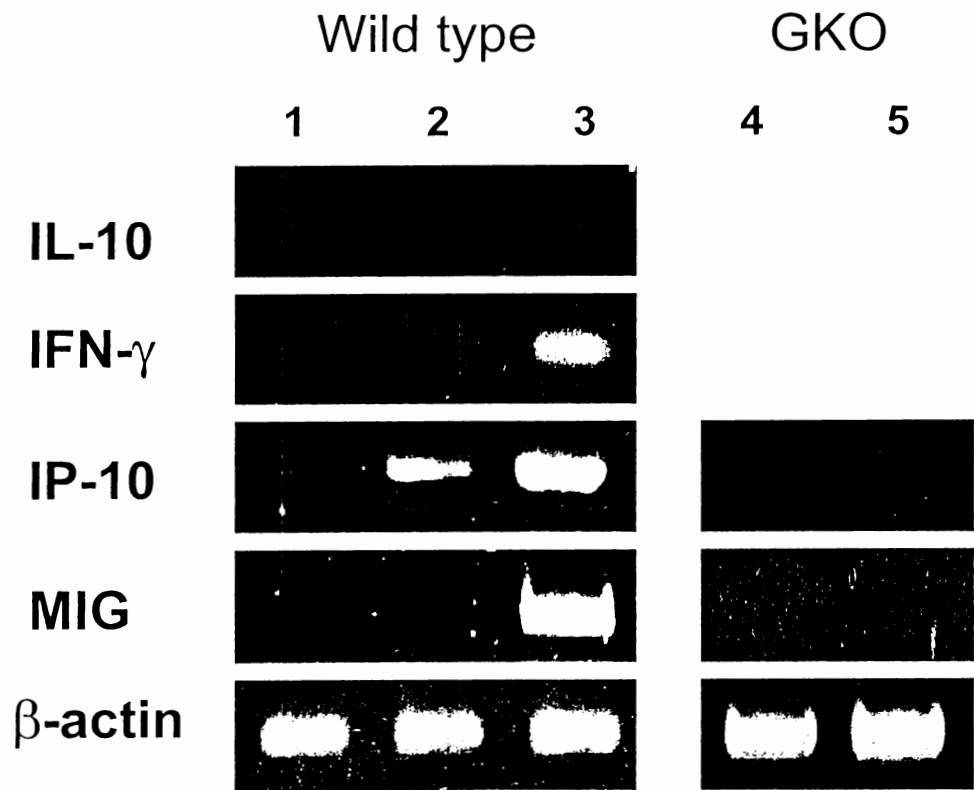
**Figure. 7. Inhibitory effect of IP-10 DNA on lesion severity of HSK in BALB/c (A) and GKO (B) mice. (IP-10 DNA decreased the incidence as well as lesion severity in both BALB/c and GKO mice-incidence data not shown.)**

Groups of mice (n=7) were treated intraocularly with IP-10 DNA (_) or vector DNA (_) 3 and 6 day before virus infection and infected with $10^6$ PFU ($10^4$ PFU for GKO mice) of HSV-1 RE on their scarified corneas. The mice were examined clinically by slit lamp microscopy, and the severity of lesions was scored on a 0-to-5 scale as described in Materials and Methods. Data are complied from three independent experiments. Significant reductions of HSK severity in IP-10 DNA treated BALB/c and GKO mice are indicated by an asterisk (P<0.05).
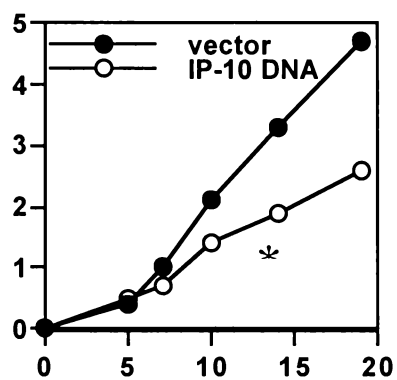
A. BALB/c    B. GKO
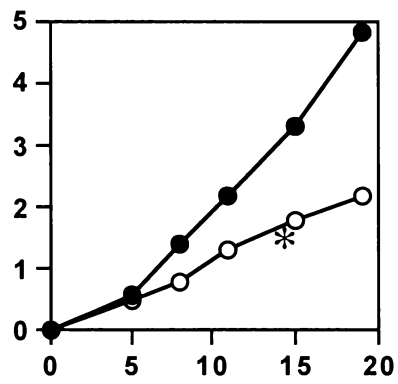
Days post infection

**Figure. 8. Antibodies to IP-10 and MIG inhibit IL-12 DNA mediated protective effect on HSK.**

Groups of mice (n=7) were treated intraocularly with IL-12 DNA or vector DNA 3 and 6 day before virus infection. These mice were injected intraperitoneally with control Ig (_), anti-IP-10 (_), anti-MIG (Δ) or anti-IP-10 plus anti-MIG (◊) 1 day before virus infection and 5 day after virus infection. Groups of mice received $10^6$ PFU of HSV-1 RE on their scarified corneas. The mice were examined clinically by slit lamp biomicroscopy, and the severity of lesions was scored on a 0-to-5 scale. Data are complied from two independent experiments. A significant reduction of the effect of IL-12 DNA in anti IP-10 plus anti-MIG treated mice is indicated by an asterisk (P<0.01).
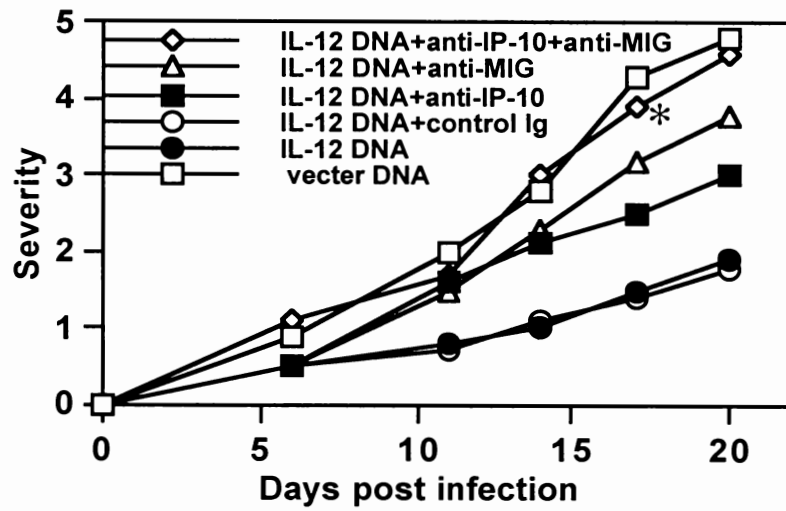
**Figure. 9. Pretreatment of IP-10 DNA inhibits recombinant VEGF induced angiogenesis.**

Groups of mice (n=7) were treated intraocularly with IP-10 DNA or vector DNA, and hydrogen pellets containing rhVEGF (90ng) were implanted into the corneal pockets. The total number of neovessels originating in the limbus and the area of neovascularization were calculated. A significant reduction of angiogenesis in IP-10 DNA treated mice is indicated by an asterisk (P<0.01).

% Inhibition of Neovascularization
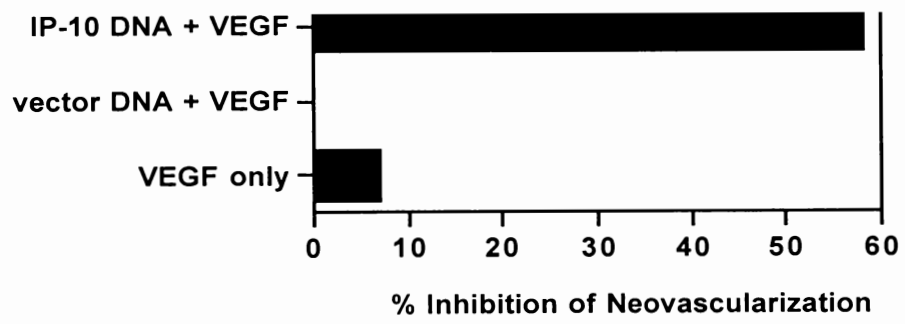
# PART III

# MATRIX METALLOPROTEINASE-9 PLAYS A MAJOR ROLE IN ANGIOGENESIS CAUSED BY OCULAR INFECTION WITH HERPES SIMPLEX VIRUS

This part is a lightly revised version of a paper by the same name published in the *Journal of Clinical Investigation* in 2002 by Sujin Lee, Mei Zheng, Bumseok Kim, and Barry T. Rouse:

Lee, S., M. Zheng, B. Kim, and B. T. Rouse. Matrix metalloproteinase-9 plays a major role in angiogenesis caused by ocular infection with herpes simplex virus. *Journal of Clinical Investigation*. 2002. In press.

# CHAPTER 1

## ABSTRACT

In this report, we demonstrate that HSV infection of the cornea results in the up-regulation of the matrix degrading metalloproteinase enzyme MMP-9. This enzyme was shown to contribute to the neovascularization process that occurs in the corneal stroma in response to HSV infection. The likely source of MMP-9, at least initially after infection, was neutrophils that were signaled to invade the cornea soon after infection. Corneal infiltrating neutrophils were shown to express MMP-9 and preventing the neutrophil response with specific mAb diminished MMP-9 expression as well as the extent of angiogenesis. Further supporting a role for MMP-9 in HSV induced corneal angiogenesis was the observation that inhibition of MMP-9 with the specific inhibitor TIMP-1 resulted in reduced angiogenesis. In addition, angiogenesis was diminished in ocularly infected MMP-9 -/- mice. Our results demonstrate that MMP-9 is involved in angiogenesis caused by HSV. Since angiogenesis appears to represent a vital step in the pathogenesis of herpetic stromal keratitis these results indicate that targeting MMP-9 for inhibition should prove useful for the therapy of HSK.

## CHAPTER 2

## INTRODUCTION

One of the more distressing lesions caused by herpes simplex virus (HSV) infection is vision impairment and blindness resulting from a chronic immunoinflammatory reaction in the corneal stromal. Studies on experimental herpetic stromal keratitis (HSK) in animal models have revealed that the pathogenesis involves numerous cellular and molecular participants. One early essential event appears to be neovascularization of the normally avascular corneal stroma. This event is assumed to be necessary to assist corneal access of some of the cellular orchestrators of HSK (1). In line with this notion, inhibition of new blood vessel development serves to moderate the severity of HSK lesions(2, 17). Currently, the mechanisms by which HSV infection results in corneal angiogenesis remain ill-defined, and more than likely involves multiple participants could be involved. These include the VEGF family of proteins, potent angiogenesis factors under both physiological and pathological circumstances and known to be produced following ocular infection with HSV (2). The VEGF proteins, as well as certain chemokines, induce angiogenesis by binding to receptors on vascular endothelial cells causing them to undergo growth and movement. Other molecules influence angiogenesis by breaking down the extracellular matrix so facilitating neovessel growth. Matrix degrading proteases include the collagenases (eg, matrix metalloproteinase 2 and 9) and heparanases (3). Several MMP proteins appear involved in tumor angiogenesis, but the role of such molecules in viral induced angiogenesis has not been described.

In the present report, we have evaluated if MMP-9 is involved in angiogenesis following infection of the mouse eye with HSV-1. Our results demonstrate that MMP-9,

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whilst undetectable in normal eyes, is produced in the cornea in response to HSV infection. A prominent cell type that produces MMP early after infection was invading neutrophils. When MMP-9 levels were suppressed, as could be achieved by neutrophil depletion, by inhibition with the specific inhibitor TIMP-1, or by using mice strains unable because of gene knockout to produce MMP-9, HSV induced angiogenesis was inhibited. In addition, the results further demonstrate that inhibition of angiogenesis is reflected in the reduced severity of subsequent HSK lesions. Our observations are discussed in terms of the value of controlling angiogenesis in the therapy of herpetic eye disease.

# CHAPTER 3

## MATERIALS AND METHODS

### Mice

Female 4- to 5- week old BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, Ind). Female 4 week old 129 Sv/Ev mice were purchased from Taconic Farms (Germantown, NY). MMP-9 K/O mice were kindly provided by Drs Robert M. Senior and J.Michael Shipley (Washington University School of Medicine, St.Louis, MO). BALB/c and 129 Sv/Ev mice were housed conventionally, and K/O mice were housed in sterile microisolator cages in the animal facility. To prevent bacterial infection, all mice received treatement with sulfamethoxazole/trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5ml/200ml of 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 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, AL), HSV-1 KOS 1.1 (kind gift of Dr.David Knipe, Harvard Medical School, Boston, MA) and the mutant virus n12 (ICP4-/-) (gift from Dr.David Knipe) were used. Viruses were grown in Vero cell monolayers (ATCC cat.no. CCL81), titrated, and stored in aliquots at $-80^{\circ}C$ until used. UV inactivation of the wild type virus was carried out for 5 minutes.

## Corneal HSV infection

Corneal infections of all mouse groups were conducted under deep anesthesia induced by the inhalant anesthetic methoxyfurane (Methofane; Pittman Moore, Mondelein). The mice were lightly scarified on their corneas with a 27 gauge needle, and a 2.5µl drop containing $1 \leftrightarrow 10^6$ PFU of HSV-1 RE for mice was applied to the eye and gently massaged with the eyelids.

## Plasmid DNA preparation

Plasmid DNA encoding murine TIMP-1 was kindly provided by Dr.Dylan Edwards (University of East Anglia School of Biological Sciences, Norwich, England). TIMP-1 DNA was inserted into the pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA). The pCDNA3 was used as a negative control against TIMP-1 DNA. The plasmid DNAs were purified by polyethylene glycol precipitation by the method of Sambrook et al. (4) with some modifications. The quality of DNA was measured by electrophoresis on 1% agarose gel. The protein expression of TIMP-1 DNA was determined by RT-PCR and dot blot after in vitro transfection into Chinese Hamster Ovary (CHO) cells.

## Plasmid DNA administration

100µg of plasmid DNA was suspended in 4µl of sterile PBS. Corneas were scarified using a 27 gauge needle in a criss-cross pattern and the plasmid was administered on 3 and 6 days before virus infection.

## 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 nectotizing stromal keratitis. The severity of angiogenesis was recorded as previously described (5). 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 4 quadrant of the eye were then summed to derive the NV index (range 0-16) for each eye at a given time point (5).

## Gelatin Zymography

Zymographic assays for gelatinase were performed as described previously (6). In brief, protein extracts (20μg) of corneas from mice were subjected to SDS-PAGE on gelatin-containing acrylamide gels (8% acrylamide and 1% gelatin) under nonreducing conditions. After electrophoresis, gels were washed three times with 2.5% Triton X-100 for 3 hrs to remove SDS. Gels were then rinsed briefly with water followed by incubation overnight at 37ΥC in reaction buffer containing 50mM Tris, pH7.5, 0.15M NaCl, 10mM CaCl$_2$ and 0.05% (w/v) Sodium azide. The gels were stained with 0.5% (w/v) Commassie Brilliant Blue R-250. Gelatinolytic activity was detected as a transparant band against a dark blue background.

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**Depletion of neutrophils with mAb**

Clone RB6-BC5 was kindly provided by Dr. E.Balish (University of Wisconsin Medical School, Madison, WI) with permission of Dr. R.L. Coffman (PharMingen, San Diego, CA). The cells were grown in RPMI 1640 with 10% FBS. Hybridoma cells were injected i.p. (5↔10$^6$ cells/mouse) into BALB/c nude mouse. Ascitic fluid was collected, centrifuged at 400↔g for 15min, pooled, and stored at –20ΥC until ready for use. Delipidized ascitic fluid containing rat IgG2b Abs against HLA-DR5 (clone SFR3-DR5; ATCC, Rockville, MD) was used as isotype control. The ascitic fluids were titrated for the Ab content using an indirect ELISA as described previously (7). BALB/c mice were administered 500µg of anti Gr-1 Ab i.p. on day –3 and +1 with HSV-1 RE on the cornea. Control mice were treated similarly with rat anti-HLA DR5 Ab.

**Immunohistochemistry**

Eyes were removed and snap frozen in OCT compound (Miles, Elkhart, IN). Six-micron-thick (6µm) sections were cut, air dried and fixed in cold acetone for 10min. The sections were then blocked with 3% BSA and stained with anti-mMMP-9 (Santa Cruz Biotech) for 3hrs, which was followed with biotinylated anti-goat Ig (Santa Cruz, Biotech) for 1hr. Frozen sections were also stained with biotinylated anti-Gr-1 (Pharmingen) for 3 hrs. The sections were then treated with horseradish peroxidase-conjugated streptavidin (1:1,000, Jackson Immunoresearch Laboratories, Inc) and 3,3'-diaminobenzidine substrate (Biogenex, San Ramon, CA) and then counterstained with hematoxylin.

## Corneal micropocket assay

In vivo angiogenic activity was assayed in the avascular cornea of BALB/c mouse eyes, as previously described (8). Briefly, mice were pretreated with plasmid DNA encoding TIMP-1 twice intraocularly before implantation. Pellets for insertion into the cornea were made by combining rhVEGF (40µg, R&D system), sulcralfate (10mg, Bulch Meditec) and hydron polymer in ethanol (120mg/1ml ethanol, Interferon Sciences), and applying the mixture to a 15↔15mm$^2$ piece of synthetic mesh (Tekto). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 90ng of VEGF. Pellets containing rhVEGF were implanted into an intracorneal pocket (1mm from the limbus). Then the eyes were 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. The number of vessels originating from the limbus was counted over the entire orbit, and the area of angiogenesis was calculated according to the formula for an ellipse.

A=[(clock hours)↔ 0.4↔ (vessel length in mm)↔ π]/2

Each clock hours is equal to 30 at the circumstance.


## Statistical analysis

Significant differences between groups were evaluated using the Student's *t* test.

# CHAPTER 4

## RESULTS

### HSV Infection Results in MMP-9 Expression

Corneas were taken at various time points after ocular infection with HSV-1 and analyzed by zymography for the presence of the collagen degrading enzymes MMP-2 and MMP-9. Two bands of activity were evident. A band identified as MMP-2 was present in normal corneal extracts. Moreover its levels appeared not to vary at different times after virus infection (Fig 1). A second band, identified as MMP-9 was not present in normal tissue but was present at varying amounts in extracts from virus infected animals. A peak of activity appeared at 48 hr p.i. followed by a decline then subsequently a second rise of activity starting around day 7. The secondary peak corresponded with the developing clinically evident HSK (Fig. 1).

The primary peak of MMP-9 activity was suspected to derive from neutrophils which were previously to be prominent stromal invaders at around 2 days post infection (9,10). Evidence that such invading neutrophils were a source of MMP-9 was obtained by different lines of experimentation. Firstly, histological sections of corneas at 2 days post infection revealed prominent inflammatory cell infiltrates (Fig. 2A). The majority of invading cells were neutrophils most of which could be shown by immunocytochemistry to possess intracellular MMP-9 (Fig.2B). Cells positive for MMP-9 were not found in the virus infected epithelium nor was it evident in any cells in sections from uninfected animals. A second approach incriminating neutrophils as the source of MMP-9 demonstrated that diminished MMP-9 levels were present in corneal extracts of animals

Naive

Day 2 p.i.

A

B

BALB/c

MMP-9 KO

C
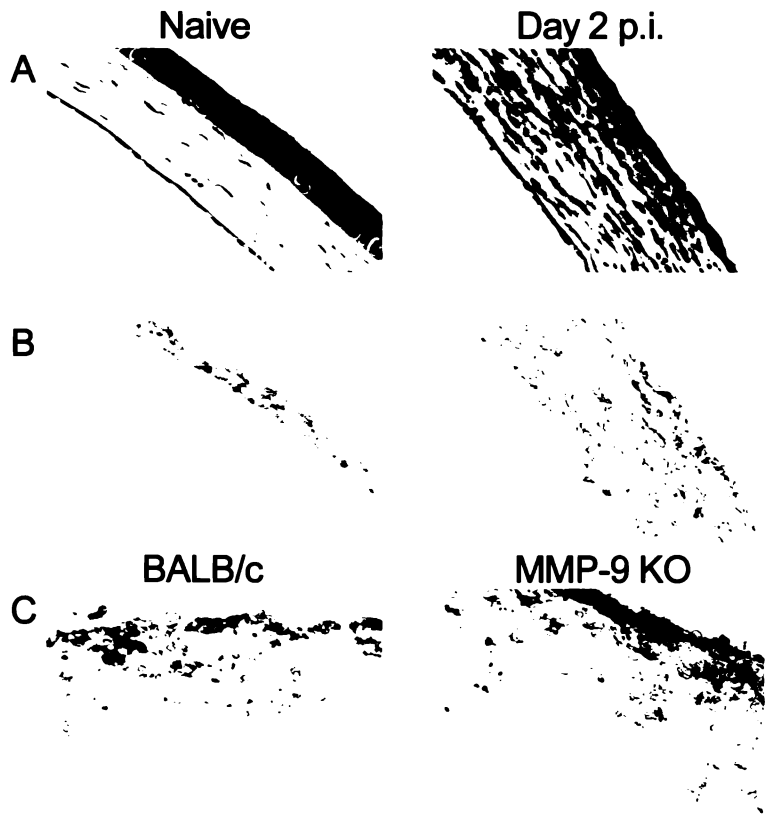
that were virus infected but depleted of neutrophils by treatment with specific anti-neutrophil mAb (Fig. 3).

Finally, infection of eyes with UV inactivated virus or replication defective HSV mutants, which induce negligible neutrophil responses, also failed to cause detectable levels of MMP-9 production (Fig 4). Taken together, these experiments support a role for corneal stroma invading neutrophils as a principal source of MMP-9 production early after ocular HSV infection.

## MMP-9 Knockout Mice Show Diminished Angiogenesis and Reduced Severity of HSK

Mice lacking MMP-9 expression because of gene knockout have reduced angiogenesis in several tumor systems (11,12). As shown in Fig. 5, a similar outcome was evident in mice ocularly infected with HSV-1. Thus in a comparison of the extent of ocular angiogenesis in MMP-9 -/- mice compared to 125 Sv/Ev control animals, levels were around 50% less in the MMP-9 -/- animals when measured over a 20 day test period. These differences were shown to be statistically significant (P<0.01). Furthermore, when animals were followed to measure the severity HSK on days 12 and 21 p.i., the severity was significantly less in the MMP-9 -/- animals.

In an additional experiment, ocularly infected MMP-9 -/- animals were analyzed to determine if a stromal neutrophil response was evident in such animals. As shown in Fig. 2C, a prominent response was indeed present at 48 hr p.i. Once again these data

support the notion that invading neutrophils are in normal animals a principal source of MMP-9.

**Effect of MMP-9 inhibitor on HSV induced angiogenesis**

The metalloproteinase MMP-9 is known to be inhibited by TIMP-1, with this molecule serving to minimize the duration of MMP-9 activity in some tumor angiogenesis systems (13). To measure the effect of TIMP-1 on HSV-1 induced MMP-9 induction, animals were given either plasmid DNA encoding TIMP-1 or control plasmid DNA vector on the cornea. Animals were treated at 3 and 6 days prior to HSV-1 infection, after which the extent of angiogenesis was compared in TIMP-1 and vector DNA treated animals. Preliminary experiments in uninfected mice revealed that TIMP-1 DNA treatment led to TIMP-1 mRNA expression in corneal extracts (data not shown). The results in Fig. 6 indicate that the extent of angiogenesis in TIMP-1 plasmid DNA treated animals was significantly decreased compared to vector treated animals during the 3-5 day observation period. As a further measure of the effect of TIMP-1 inhibition on MMP-9, corneal extracts were taken at 48 hr from TIMP-1 and vector DNA treated animals for measurement of MMP-9 levels by zymography. The results shown in Figure 7 demonstrate significantly diminished MMP-9 signals in samples from TIMP-1 treated animals. These experiments provide further evidence for a role of MMP-9 in HSV induced angiogenesis.

**TIMP-1 DNA Inhibits VEGF Induced Angiogenesis by its Effect on MMP9 Activity**

In a previous report, we demonstrated that ocular infection with HSV induces the

potent angiogenesis factor VEGF family of proteins with these playing an important role in corneal angiogenesis (2). Since TIMP-1 is not expected to inhibit VEGF, and these molecules are present following HSV infection, observing the inhibitory effects of TIMP-DNA on HSV angiogenesis was perhaps surprising. Hence experiments were done using a corneal micropocket assay to measure the effect of pretreatment of eyes with TIMP-1 DNA on the angiogenic response to VEGF. The results shown in Figure 8, indicate that such pretreatment diminished by about 50% neovascularization caused by VEGF. To determine if levels of MMP-9 might account for such differences corneal extracts from mice with VEGF containing and control micropockets were tested by zymography for MMP-9. Unlike normal corneas VEGF implanted eyes do produce MMP-9. Moreover in TIMP-1 DNA pretreated VEGF implanted eyes MMP-9 levels were markedly diminished compared to VEGF implanted vector treated eyes (Fig 9). These results demonstrate that MMP-9 represents a significant component of HSV induced angiogenesis being also involved indirectly in angiogenesis caused by other known angiogenesis factors.

# CHAPTER 5

## DISCUSSION

In this report, we demonstrate that HSV infection of the cornea results in the up-regulation of the matrix degrading metalloproteinase enzyme MMP-9. This enzyme was shown to contribute to the neovascularization process that occurs in the corneal stroma in response to HSV infection. The likely source of MMP-9, at least initially after infection, was neutrophils that are signaled to invade the cornea soon after infection. Corneal infiltrating neutrophils were shown to express MMP-9 and preventing the neutrophil response with specific mAb diminished MMP-9 expression as well as the extent of angiogenesis. Further supporting a role for MMP-9 in HSV induced corneal angiogenesis was the observation that inhibition of MMP-9 with the specific inhibitor TIMP-1 resulted in reduced angiogenesis. In addition, angiogenesis was diminished in ocularly infected MMP-9 -/- mice. Our results demonstrate that MMP-9 is involved in angiogenesis caused by HSV. Since angiogenesis appears to represent a vital step in the pathogenesis of herpetic stromal keratitis (14) these results indicate that targeting MMP-9 for inhibition should prove useful for the therapy of HSK.

Neovascularization of the cornea is a prominent event following HSV infection of the mouse eye. Growth of new blood vessels from the limbus are evident as early as 24 hr p.i. with vessels in some instances growing to the central cornea by 14 days p.i. (15). By this time, lesions of HSK have become evident. Characteristically, the virus replicates for only a few days in corneal epithelial cells, but angiogenesis and stromal immunoinflammatory reaction progress in severity for 2-3 weeks (16,17). Since

inhibiting angiogenesis, at least if begun early, diminished the severity of HSK, we have suggested that neovascularization of the cornea is a necessary step in the pathogenesis of HSK (17). It likely serves to provide access to the cornea of T lymphocytes that orchestrate the immunoinflammatory lesions of HSK. A crucial unsolved problem is to explain how a relatively brief period of HSV replication in the corneal epithelium leads to the rapid angiogenic sprouting from vessels at the corneal limbus which ultimately may involve almost the whole cornea. We anticipate that the infection signals the production of many molecules involved both in angiogenesis and inflammation. This report is the first to document a potential role of MMP-9 in HSV induced angiogenesis. However, MMP-9 is well known as involved in several tumor angiogenesis systems (18,19,20) and has also been shown to participate in ocular wound healing (21,22).

The role of MMP-9 is assumed to play in angiogenesis is to facilitate the growth of new blood vessels by breaking down the extracellular matrix (23). Numerous groups of molecules participate in angiogenesis by inducing growth of vascular endothelial cells (24,25). In the HSV model, previous results have shown that the VEGF family of proteins are involved (2). In addition, certain chemokines such as MIP-2, are known to be produced during HSV-1 ocular infection (26), are also expected to contribute to angiogenesis. Consequently, abrogating angiogenesis where so many different types of molecules are seemingly involved, could prove to be problematic.

MMP-9, however, represents a logical target for therapy, since this component acts as the principal degrader of the ECM, at least in certain tumor angiogenesis systems

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(27,28). Moreover ECM degradation appears to represent an essential step in angiogenesis (29), facilitating the growth of angiokine stimulated vascular components. Consequently the function of MMP-9 is expected to amplify the effect of other angiogenesis factors. Indeed, in our studies, we demonstrated that inhibiting MMP-9, as could be done with the specific inhibitor TIMP-1, also served to inhibit angiogenesis mediated by VEGF. Such results could mean that the combined use of reagents that inhibit MMP-9 along with specific inhibitors of certain angiogenesis factors may achieve more compete control of ocular angiogenesis. We are currently testing such notions in our HSV angiogenesis model.

Early after HSV infection, the cellular course of MMP-9 was shown to be the neutrophils that promptly invade the corneal stroma following HSV infection of the overlying epithelium. Preventing this, neutrophil response both diminished detectable levels of MMP-9 and reduced the extent of angiogenesis. However, it is not clear if neutrophils remain as the only source of MMP-9 nor in fact if the function of MMP-9 is necessary after the initial stages of HSV induced angiogenesis. It could well be that only a certain degree of corneal neovascularization is actually required to permit invasion by inflammatory T cells and other cell types involved in lesions of HSK. In line with this, our preliminary investigations have indicated that there is no benefit in terms of the progress of HSK, of inhibiting neovascularization after the initial stage. Ultimately to fully control HSK lesions could require approaches that reverse established angiogenesis. This issue is under investigation in our laboratory.

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**APPENDIX**

**Figure 1. MMP-9 is present in cornea during HSK, and MMP-9 activities are highly correlated with HSK legion severity.**

Groups of animals were infected with $10^6$ PFU of HSV-1 RE on their scarified corneas. The mice were examined clinically by slit lamp microscope, and the severity of lesion was scored on a 0 to 5 scale (A). At day 2 post infection, animals were sacrificed, corneas excised and pooled from 6 eyes, and homogenized. The concentrations of protein were measured by Bradford method. Corneal extract samples (20μg/lane) were analyzed by gelatin zymography. Numbers above gel represent days post infection. High MMP-9 activities were measured at day 2, 15, 18 and 20 post infection. The data are complied from three independent experiments.

## Days post infection



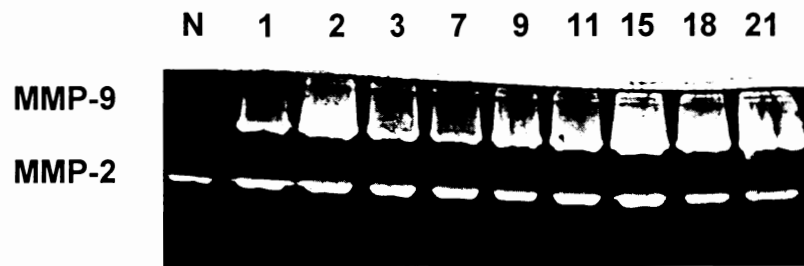MMP-9

MMP-2

N   1   2   3   7   9   11   15   18   21

**Figure 2. MMP-9 and neutrophil infiltration into corneas of BALB/c and MMP-9 KO mice.**

BALB/c and MMP-9 KO mice were infected with $10^6$ PFU HSV-1 RE on their scarified corneas. At day 2 p.i. mice were sacrificed, and the eyes were snap-frozen in OCT compound. (A) Histopathology of infiltrating cells in the cornea of BALB/c. (left; naïve, right; day 2 p.i). (B) Immunohistochemistry for MMP-9. (left; naïve, right; day 2 p.i). (C) Immunohistochemistry for Gr-1 (left; BALB/c, right; MMP-9 KO) (magnification, X200)

**Figure 3. Neutrophil depeletion markedly diminishes MMP-9 production.**

Groups of mice received intraperitoneally with anti-Gr-1Ab twice as described in Materials and Methods, and groups of mice received an irrelevant control isotype matched rat Ab. Groups of mice were infected on the scarified cornea with $10^6$ PFU of HSV-1 RE. At day 2 post infection, animals were sacrified, corneas excised and pooled from 6 eyes, and homogenized. Corneal extract samples (20µg/lane) were analyzed by gelatin zymography (A). Lane 1 : HSV-1 infected corneas after PMN depletion, Lane 2 : HSV-1 infected corneas after injection of isotype control Ig.
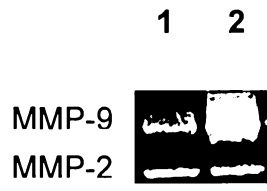
MMP-9
MMP-2

1    2

**Figure 4. Replication defective HSV mutants fail to produce MMP-9.**

BALB/c mice were infected on the cornea with $10^6$ PFU of the specified virus after scarifying the cornea with a 27 gauge needle. At day 2 post infection, animals were sacrificed, corneas excised and pooled from 6 eyes, and homogenized. The concentrations of protein were measured by Bradford method. Corneal extract samples (20μg/lane) were analyzed by gelatin zymography. Similar results were obtained in three independent experiments. Lane 1 : $10^6$ PFU of UV inactivated HSV-1 RE, Lane 2 : $10^6$ PFU of ICP4 -/-, Lane 3 : $10^6$ PFU of HSV-1 KOS, Lane 4 : $10^6$ PFU of HSV-1 RE, Lane 5 : trauma control, Lane 6 : naïve.
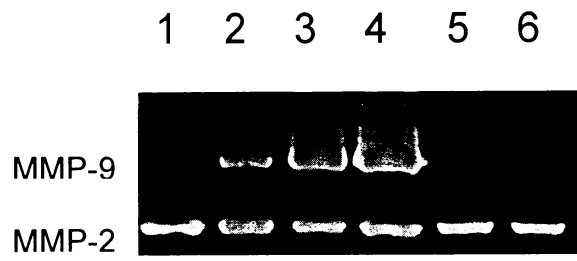
MMP-9

MMP-2

1  2  3  4  5  6

**Figure 5. MMP-9 knock out mice are protected from HSK pathogenesis.**

Groups of 129 Sv/Ev wild type (4 wk old) and MMP-9 K/O mice (4 wk old) were infected with $10^6$ PFU of HSV-1 RE on their scarified corneas. The mice were examined clinically by slit lamp microscope, and the severity of lesions was scored on a 0 to 5 scale. The mean clinical scores at day 5, 10, 15 and 19 are ploted for all groups. Also, the animals were examined for the extent of angiogenesis as described in Materials and Methods. * $P<0.05$ between WT and K/O mice at day 5 (angiogenic scoring) and at day 10 (HSK lesion severity).
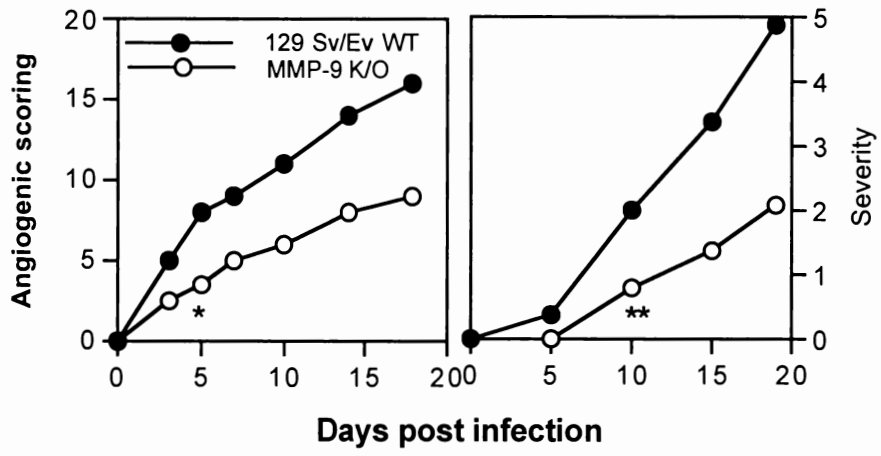
**Figure 6. TIMP-1 DNA inhibits angiogenic scoring and HSK lesion severity.**

Groups of animals were treated twice with 100μg of TIMP-1 DNA and vector DNA intraocularly 6 and 3 day before virus infection and were infected with $10^6$ PFU of HSV-1 RE on their scarified corneas 3 day after second treatment of DNAs. The mice were examined clinically by slit lamp microscope, and the severity of lesions and the extent of angiogenesis were scored as described in Materials and Methods. Data are complied from three independent experiments. Significant differences (P<0.05) between TIMP-1 DNA and vector DNA treated mice indicated by an asterisk (angiogenic scoring), and a double asterisk (severity).
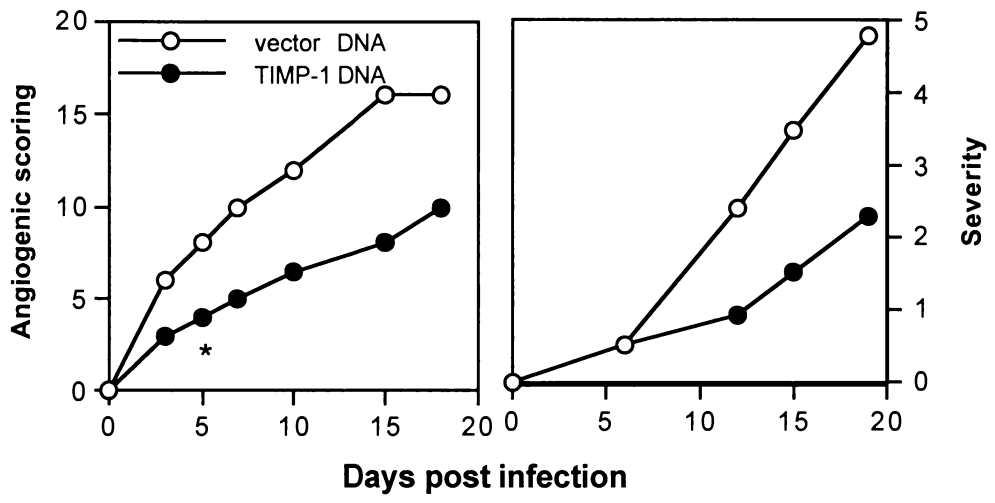
**Figure 7. TIMP-1 DNA inhibits MMP-9 activity at day 2 post infection.**

Groups of animals were treated twice with 100μg of TIMP-1, vector and β-gal DNA intraoculary 6 and 3 day before virus infection and were infected with $10^6$ PFU of HSV-1 RE on their scarified corneas 3 day after second treatment of DNAs. At day 2 post infection, animals were sacrificed, corneas excised and pooled from 6 eyes, and homogenized. Corneal extract samples (20μg/lane) were analyzed by gelatin zymography. Lane 1 : human zymography standard. Lane 2 : corneas treated with vector DNA. Lane 3 : corneas treated with β-gal DNA. Lane 4 : corneas treated with TIMP-1 DNA.
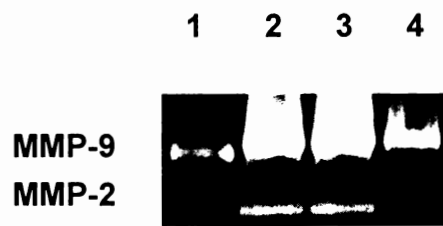
100

**Figure 8. Pretreatment of TIMP-1 DNA inhibits recombinant VEGF induced angiogenesis.**

Groups of mice were treated intraocularly with TIMP-1 DNA or vector DNA, and hydrogen pellets containing rhVEGF (90ng) were implanted into the corneal pockets. The total number of neovessels originating in the limbus and the area of neovascularization were calculated.
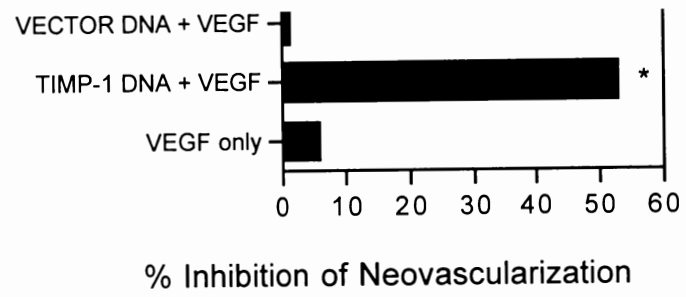
% Inhibition of Neovascularization

**Figure 9. TIMP-1 DNA reduces VEGF induced angiogenesis by inhibition of MMP-9 activity.**

Groups of mice were treated intraocularly with TIMP-1 DNA or vector DNA, and hydrogen pellets containing rh VEGF (90ng) were implanted into the corneal pellets. At day 4 post treatment, animals were sacrificed corneas were excised and pooled from 8 eyes, and homogenized. The concentrations of protein were measured by Bradford method. Corneal extract samples (20 μg/lane) were analyzed by gelatin zymography. Similar results were obtained in three independent experiments. Lane 1 : corneal sample from TIMP-1 DNA treated group. Lane 2 : corneal sample from vector DNA treated group.

# PART IV

# CONCLUSION

This study was aimed to determine the role of angiogenesis and to modulate HSV induced angiogenesis in HSK pathogenesis. The results from this study indicate that angiogenesis is an essential step in HSK pathogenesis.

In the first part of this study, the efficacy of plasmid DNA encoding IL-12 administered prophylactically was investigated for their modulating effects on lesion and angiogenesis caused by HSV-1 ocular infection. Thus, plasmid DNA encoding IL-12 was applied to the ocular surface, since it was supposed this might help push the HSV-specific immune response to subsequent ocular infection more towards the pathogenic Th1 pattern. Unexpectedly, however, the results demonstrated that ocular treatment by IL-12 DNA led to suppression of HSV-1 induced angiogenesis and lesion severity in HSK pathogenesis. Lesions were diminished in IL-12 DNA pretreated animals, and 80-90% of animal controlled or even resolved lesions compared to 10% in controls. The inhibitory effect of IL-12 on angiogenesis is mediated by intermediate proteins IFN-γ and two CXC chemokines, such as IP-10 and MIG.

In the second part of this dissertation, the role of MMP-9, a stromal matrix degrading enzyme, was investigated. This study shows that the MMP-9 was upregulated following virus infection of the cornea. Two peaks were evident. One, at 48 hr post infection, correlated with the initial neutrophil response. In fact, such cells were shown to produce MMP-9, with neutrophil suppression inhibiting MMP-9 levels in corneal extracts. The MMP-9 appeared to be associated with angiogenesis since its inhibition with the specific inhibitor TIMP-1 led to significant inhibition of angiogenesis. Similarly MMP-9 KO mice had diminished angiogenesis and HSK in comparison to control mice.

Therefore, based on the studies presented here, it can be concluded that

angiogenesis is necessary in HSK pathogenesis. Although it remains unclear as to the likely multiple molecules responsible for HSV-induced angiogenesis, from the above experiments we found one major molecule, MMP-9. Furthermore, antiangiogenic molecules such as IL-12, IP-10 and TIMP-1 ameliorated HSK lesion severity and incidence.

In conclusion, we hope that these studies should add to our knowledge about angiogenesis in HSV-1 ocular infection and could lead to the development of new therapies for the control of lesion severity in HSK pathogenesis.

# VITA

Sujin Lee was born in Seoul, South Korea, on August 3, 1969. She entered Hallym University in 1988 and obtained her Bachelor of Science degree in Genetic Engineering in February 1992. She continued her study in the same university and obtained a Master of Science degree in Biochemistry in February 1994. She worked until 1996 as an teaching assistant in the Department of Biochemistry, College of Medicine, Yonsei University.

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