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

Bumseok Kim, Sujin Lee, Susmit Suvas and Barry T. Rouse

*J Immunol* 2005; 175:509-516; doi: 10.4049/jimmunol.175.1.509

http://www.jimmunol.org/content/175/1/509

**Why The JI?**

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

**References**  This article cites 32 articles, 15 of which you can access for free at: [http://www.jimmunol.org/content/175/1/509.full#ref-list-1](http://www.jimmunol.org/content/175/1/509.full#ref-list-1)

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**  Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Application of Plasmid DNA Encoding IL-18 Diminishes Development of Herpetic Stromal Keratitis by Antiangiogenic Effects

Bumseok Kim,2* Sujin Lee,2† Susmit Suvas,* and Barry T. Rouse3*

HSV-1 infection of the eye can cause a blinding immunoinflammatory stromal keratitis (SK) lesion. Using the mouse model, we have demonstrated that angiogenesis is an essential step in lesion pathogenesis because its inhibition results in diminished severity. The molecules involved in causing corneal angiogenesis are multiple and include the vascular endothelial growth factor (VEGF) family of proteins. In this report we show that application of plasmid DNA encoding IL-18 to the cornea of mice before HSV-1 ocular infection resulted in reduced angiogenesis and diminished SK immunoinflammatory lesions. The antiangiogenic effects of IL-18 treatment appeared to be mediated by inhibition of VEGF production in the cornea. We also showed that IL-18 controlled VEGF expression in vitro and also decreased CpG oligodeoxynucleotide-induced VEGF-dependent neovascularization. In addition the administration of IL-18-binding protein, an IL-18 antagonist, into the inflammatory eye resulted in elevated angiogenesis and increased VEGF expression. Our results indicate that IL-18 is an important endogenous negative regulator of HSV-induced angiogenesis resulting in reduced SK lesion severity. Our results could mean that IL-18 administration may represent a useful approach to manage unwanted angiogenesis. The Journal of Immunology, 2005, 175: 509–516.

Materials and Methods

Reagents

Phosphorothioate oligodeoxynucleotides (ODNs) were kindly provided by D. M. Klimman (Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Rockville, MD). The sequences of stimulatory ODNs used in this study were: ODN 1466, TCAACGTTGA, and ODN 1555, GCTAGACGTTAGCGT. Subsequent studies were performed using an equimolar mixture of ODN 1466 and ODN 1555.

Mice

Female 4- to 5-wk-old BALB/c mice were purchased from Harlan Sprague Dawley. To prevent bacterial infection, all mice received treatment with sulfamethoxazole/trimethoprim (Biocraft) at the rate of 5 ml/200 ml 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 (Knoxville, TN) are fully accredited by the American Association of Laboratory Animal Care.

Virus and corneal HSV infection

HSV-1 strain RE, kindly provided by Dr. R. Lausch (University of Alabama, Mobile, AL) was used in all procedures. Virus was grown in Vero cell monolayers (cat. no. CCL81; American Type Culture Collection), titrated, and stored in aliquots at −80°C until use. Corneal infections of all mouse groups were conducted under deep anesthesia induced by Avertin. The mice were scarified lightly on their corneas with a 30-gauge needle and infected with a 2-μl drop containing 10^6 or 3 x 10^7 PFU of HSV-1 strain RE that was applied to the eye and gently massaged with the eyelids.

Plasmid DNA preparation and administration

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

Cells

BALB/c mouse brain microvascular endothelial (MBE) cells were kindly provided by Dr. R. Auerbach (University of Wisconsin, Madison, WI). MBE cells were grown in DMEM supplemented with 10% FCS. They were maintained in culture at the University of Tennessee with no signs of cell senescence after they were received from the University of Wisconsin.

Cell proliferation assay (DNA synthesis assay)

Recombinant murine IL-18 (Medical & Biological Laboratories) was tested for the ability to stimulate proliferation of mouse endothelial cells (17). Accordingly, MBE cells (1000 cells/well) were seeded onto 96-well tissue culture plates and incubated in 10% DMEM for 12 h. To maximize activation MBE cells were then stimulated with recombinant basic fibroblast growth factor (bFGF) (10 ng/ml; R&D Systems) for 12 h in 0.4% DMEM. Activated MBE cells were treated with different recombinant proteins (IL-18 or OVA) in 0.4% DMEM at various doses (0.1, 1, 10, 100 ng/ml). After 24 h the cells were pulse-labeled with [3H]thymidine (1 μCi/ml) for 6 h. The level of thymidine incorporation was then measured using a beta scintillation counter (Inotech).

Intracellular detection of VEGF

For intracellular detection of VEGF, MBE cells (5000 cells/well) were seeded onto 24-well tissue culture plates and incubated in 10% DMEM for 12 h. MBE cells were then stimulated with bFGF (10 ng/ml) for 12 h in 0.4% DMEM. Stimulated MBE cells were then treated with IL-18 or OVA as a control in 0.4% DMEM using various concentrations (0.01, 0.1, 1, 10, 100 ng/ml). After 24 h of incubation adherent cells were dispersed in trypsin-EDTA and resuspended in FACS solution. For intracellular staining for VEGF, cells were fixed in 4% paraformaldehyde and stained for VEGF by using biotinylated rat anti-mouse VEGF (1:40 dilution; R&D Systems) followed by streptavidin-PE (0.2 μg/ml; BD Pharmingen). Positive cells and mean fluorescence were measured by flow cytometry using a FACScan (BD Biosciences). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

Clinical observations

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

RNA extraction and RT-PCR

At 24 h following treatment the cells were harvested and RNA was extracted by using an RNeasy protect mini kit (Qiagen). For ocular infections, two corneas per time point were excised and transferred to RNAlater. Briefly, cells were lysed in RLT buffer (cat. no. 79216; Qiagen) and RNA was purified according to manufacturer’s instructions. An RNase-free DNase set (Qiagen) was used to remove any contaminating genomic DNA. Total cellular RNA (10 μg/ml) was reverse-transcribed using oligo(dT) primers and reverse transcriptase (Promega) according to protocols previously described (18). The cDNA was made by reverse transcription at 42°C for 90 min. The primer sequences for mouse VEGF RT-PCR were: 5'-GGCGGCTGCTCCAGCTC-3’ (sense) and 5'-TCACCGTCTGGCAGTGTCAC-3’ (antisense). RT-PCR products were 644 bp for mouse VEGF164 and 512 bp for mouse VEGF120. The primer sequences for murine IL-18 RT-PCR were: 5'-ATGCTGCCCATGTCAGAAG-3’ (sense) and 5'-TAACCTTGATGTAAGTGAGGAG-3’ (antisense). The amplification profile was 94°C for 1 min, 65°C for 1 min, and 72°C for 15 min for 30 cycles. The PCR products were separated by 1% agarose gel electrophoresis.

VEGF quantification of culture supernatants or corneal lysates by ELISA

The culture supernatants from recombinant protein (IL-18 or OVA) treated MBE cells, lysates from IL-18 DNA or IL-18-binding protein (2 μg/cornea; R&D Systems) treated CpG ODNs implanted, as well as HSV-1-infected cornea were used for the measurement of VEGF by a standard sandwich ELISA protocol. For preparation of corneal lysates, two corneas per time point (n = 4) were collected and minced with liquid nitrogen. Minced pieces were collected in 1 ml of DMEM without FCS and homogenized using an ultra sonicator (Heat Systems Ultrasonics). The lysates were then clarified by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was collected and stored at −80°C until further use. The ELISA plate was coated with anti-mouse VEGF capture Ab (100 μl/well of the capture Ab at 0.4 μg/ml; R&D Systems) and incubated at 4°C overnight. The plate was washed 5 times with 0.05% Tween 20PBS and blocked with 3% BSA for 2 h at 37°C. After washing, serially diluted corneal lysates were added to the plate and incubated at 4°C overnight. The plate was washed and then incubated with anti-VEGF biotinylated detection Ab (50 ng/ml, R&D Systems) for 2 h. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) was added. The color reaction was developed using ABTs (Sigma-Aldrich) and measured with an ELISA reader (Spectramax 340; Molecular Devices) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Intrastromal corneal injection and micropocket assay

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

In vivo angiogenic activity was assayed in the avascular cornea of mouse eyes as previously described (19). Briefly, mice were pretreated with plasmid DNA encoding IL-18 DNA and control GFP DNA intrastromally 1 day before implantation of pellets. Pellets for insertion into the cornea were made by combining known amounts of CpG ODNs, sulrafate (10 mg; Bulch Meditec), and hydron polymer in ethanol (120 mg/1 ml) as described previously (20). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 1 μg of CpG ODNs. Pellets containing CpG ODNs were implanted into an intracorneal pocket (1 mm from the limbus). The eyes were then evaluated for corneal neovascularization.
neovascularization. The extent of the neovessel ingrowth was recorded by direct measurement using calipers (Biomedical Research Instruments) under stereomicroscopy (Leica Microsystems). The length of the neovessels originating from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours were measured. Each clock hour is equal to 30° at the circumference. The angiogenic area was calculated according to the formula for an ellipse. \( A = [(\text{clock hours}) \times 0.4 \times (\text{vessel length in millimeters})] \times \pi/2 \).

Subconjunctival injection of IL-18-binding protein

Mice were treated with rIL-18-binding protein and control OVA protein (2 μg/10 μl of PBS per eye) subconjunctivally on days 0 and 2 after implantation of CpG ODNs pellets containing 500 ng or after infection with HSV-1 strain RE (3 × 10^5 PFU). The eyes were then evaluated for corneal neovascularization or herpetic SK lesion severity.

Statistical analysis

Significant differences between groups were evaluated by using the Student \( t \) test. A value \( p < 0.05 \) was regarded as a significant difference between two groups.

Results

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

An initial experiment was performed to determine whether the expression of IL-18 in corneas before HSV infection had any effect on the severity of SK lesions. To express IL-18 in the cornea, mice were treated with 100 μg of plasmid DNA encoding IL-18 on their scarified corneas 4 and 2 days before virus infection, with control animals receiving the plasmid DNA encoding GFP. Subsequently, the corneas of mice were infected with 10^6 PFU of HSV-1 strain RE. As shown in Fig. 1, A and B, IL-18 pre-exposure resulted in decreased average severity and percentage of animals with clinical lesions compared with control DNA-treated mice. A comparison of average severity scores on days 12, 15, and 21 postinfection (p.i.) was all significantly less in the IL-18-treated than the control group (p < 0.05). Recently IL-18 was claimed to exert antiviral effects (20) against HSV-1 infection (21) due to augmentation of IFN-γ. This mechanism, however, seemed not to explain our findings because comparisons of viral yields and duration of detectable virus in IL-18 pretreated and control cornea showed no statistically significant differences (data not shown).

Suppression of HSV-induced angiogenesis

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

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

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

Inhibition of CpG-induced corneal neovascularization by IL-18

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

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

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

Additional experiments regarding the antiangiogenic effects of IL-18 were performed in vitro using MBE cell line that produces VEGF endogenously. To investigate whether IL-18 inhibits VEGF expression and production in vitro, different concentrations of rIL-18 were incubated with MBE cells prestimulated by 10 ng/ml bFGF for 12 h as described in Materials and Methods. At 24 h after IL-18 treatment cell supernatants or cells were collected and VEGF expression and production were measured by RT-PCR, ELISA, or intracellular staining, respectively. As shown in Fig. 4A, expression of both the 120 and 164 isoforms of VEGF was reduced by rIL-18 in a dose-dependent manner. Furthermore, VEGF production in cell lines was significantly reduced in IL-18-treated cell lines compared with control OVA protein-treated cell lines (p <
The intensity of intracellular VEGF fluorescence was markedly decreased by the addition of IL-18 (Fig. 4C and D). This down-regulation of VEGF production and expression occurred in a dose-dependent manner and was clearly evident when compared with cells incubated with OVA. In addition, VEGF expression was not decreased by incubation with different concentrations of rIL-12, a cytokine that shares several properties of IL-18 (data not shown). To exclude the possibility of nonspecific gene suppression by IL-18, we also measured the expression of bFGF, but no suppression of this gene was detected (data not shown). Furthermore, no apoptosis of cells following IL-18 treatment was observed by the annexin V staining method (data not shown).

Inhibitory effects of IL-18 on endothelial cell proliferation

Angiogenesis involves growth of endothelial cells (proliferation and migration) and other vascular components from existing blood vessels (24). The effect of IL-18 on endothelial cell proliferation was tested using the MBE cell line. In these experiments cells recently stimulated by 10 ng/ml bFGF were washed and exposed to different concentrations of IL-18. After 24 h incubation the effect on proliferation was measured by a short-term pulse with [3H]thymidine. At the concentration of 100 and 10 ng/ml, IL-18 inhibited significantly endothelial cell proliferation by 53 and 30%, respectively (p < 0.05) (Fig. 5). IL-18 was shown to inhibit endothelial cell proliferation in a dose-dependent way. These data also suggest that inhibition of angiogenesis by IL-18 is mediated by the suppression of endothelial cell proliferation.

Up-regulation of VEGF by IL-18-binding protein in the inflammatory cornea

A previous study showed that several angiogenesis-associated factors including VEGF were overexpressed in the retinas of IL-18 knockout mice and indicated that the endogenous IL-18 regulates VEGF expression in the retina (15). Therefore, we tested whether endogenous IL-18 may also regulate VEGF expression in the cornea. First we measured endogenous IL-18 mRNA expression in naive and virus infected cornea. As shown in Fig. 6A, IL-18 was present in normal corneal extracts, and the IL-18 mRNA expression was slightly increased in the SK cornea. It is known that

---

**FIGURE 4.** Suppression of VEGF expression and production by IL-18 incubation in vitro. MBE cells that normally produce VEGF endogenously were prestimulated by 10 ng/ml bFGF for 12 h and then incubated with different concentrations of rIL-18 for 24 h. Cells were collected and VEGF mRNA levels were measured by RT-PCR (A). Cell supernatants were used to measure VEGF protein levels by ELISA as described in Materials and Methods. Statistically significant differences in VEGF protein levels were observed between the groups (*, p < 0.05; **, p < 0.01) (B). MBE cells incubated with IL-18 for 24 h were collected and resuspended in FACS solution (PBS/3% FCS containing 0.1% sodium azide). The cells were then fixed in paraformaldehyde and stained for VEGF by using biotinylated rat anti-mouse VEGF164 Ab followed by streptavidin-PE. Positive cells and mean fluorescence were measured by flow cytometry (C and D). VEGF expression was not decreased by incubation with different concentrations of rIL-12, a cytokine that shares several properties of IL-18 (data not shown).

**FIGURE 5.** Inhibition of endothelial cell proliferation by IL-18. MBE cells prestimulated with bFGF (10 ng/ml) were incubated with various concentrations (100, 10, 1, 0.1 ng/ml) of rIL-18 or OVA as described in Materials and Methods. After 18 h cells were pulse labeled with [3H]thymidine (1 μCi/ml) for 6 h. The amount of radioactivity incorporated into the TCA-precipitable material was measured. * Statistically significant differences in radioactive levels (p < 0.05) were observed between the IL-18 and OVA-treated groups.
IL-18-binding protein functions as an IL-18 antagonist by binding to IL-18 and blocking its biological activity (14). To investigate the effect of endogenous IL-18 on corneal angiogenesis, IL-18-binding protein was used to block endogenous IL-18 in both virus-infected and CpG-implanted eyes. The corneas of mice were infected with 3 × 10^5 PFU of HSV-1 strain RE followed by treatment with 2 μg of rIL-18-binding protein in the subconjunctival area at 0 and 2 days after virus infection (3 × 10^5 PFU) with rIL-18-binding protein or control OVA (2 μg/10 μl) and subsequently scored for lesion severity by slit-lamp biomicroscopy. Statistically significant differences in SK and angiogenic score were observed between the groups (*, p < 0.05; **, p < 0.01). Days 0 and 2 after implantation with CpG ODN (500 ng) into the cornea, mice were given 2 μg per eye of IL-18-binding protein or OVA protein as a control group by subconjunctival injection. The angiogenic area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). At days 4 and 7 after CpG pellets implantation IL-18-binding protein or OVA-treated corneas were processed to measure the VEGF mRNA expression. VEGF mRNA levels were measured by RT-PCR (E). At day 4 after CpG pellet implantation IL-18-binding protein or OVA-treated corneas (four corneas per group) were processed to measure the VEGF protein level (F). Levels of VEGF were estimated from supernatants of corneal lysates of mice treated with IL-18-binding protein by an Ab capture ELISA as described in Materials and Methods.

**Discussion**

In the present study, we show that the administration of plasmid DNA encoding IL-18 to the cornea of mice before HSV ocular infection results in diminished immunoinflammatory lesions. The diminished SK lesion severity by IL-18 treatment was caused by the inhibitory effect on corneal neovascularization, an essential step in the pathogenesis of herpetic SK. One major factor induced by HSV ocular infection that is angiogenic is VEGF (19). By both in vitro and in vivo approaches we show that IL-18 resulted in a diminished VEGF response, although the actual mechanism by which this was mediated was not defined. Application of IL-18 up-regulated in the IL-18-binding protein-treated corneas compared with those of the OVA-treated mice (Fig. 6, E and F). We could exclude the possibility that IL-18-binding protein itself is an angiogenic factor because injection of IL-18-binding protein into the normal cornea failed to cause significant levels of angiogenesis (data not shown). Taken together our results indicate that endogenous IL-18 may control the angiogenesis induced by inflammation.
controlled VEGF expression in endothelial cells and also decreased HSV- or CpG-induced VEGF-dependent neovascularization. In addition the administration of IL-18-binding protein, an IL-18 antagonist, into the inflammatory eye resulted in elevated angiogenesis and increased VEGF expression. We interpret these observations to indicate that IL-18 is an important endogenous negative regulator of HSV-induced angiogenesis resulting in reduced SK lesion severity.

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

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

The overexpression of several potent angiogenic factors including VEGF, bFGF, and platelet-derived growth factor A genes in the retina (15), or the up-regulation of VEGF mRNA in the hind limb after ischemic injury, has been previously reported in IL-18 knockout mice (14). Our data again support the idea that IL-18 has angiostatic function and shows its function in the SK model. Although it was reported that IL-18 induces endothelial cell migration and tube formation (16), the issue could be that endothelial cell proliferation induced by potent angiogenic factors may be more important than endothelial cell migration for the process of angiogenesis. This issue is under further investigation.

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

Acknowledgments
We thank Dr. Dennis M. Kliman for kindly supplying CpG ODN and Dr. Kenji Okuda for supply of plasmid DNA encoding murine IL-18.

Disclosures
The authors have no financial conflict of interest.

References


