In Vivo Significance of ICAM-1–Dependent Leukocyte Adhesion in Early Corneal Angiogenesis

Matthias D. Becker,1,4 Friedrich E. Kruse,1 Leila Azzam,1 Rainer Nobiling,2 Jürgen Reichling,3 and Hans E. Völcker1

PURPOSE. Numerous investigations have stressed the significance of leukocytes in early angiogenesis. Leukocytes invade the cornea, and the location of their extravasation corresponds to the site of vessel ingrowth. The interactions between leukocytes and vascular endothelium are mediated by various proteins, including adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). In this study, the role of ICAM-1 during early corneal angiogenesis was evaluated in vivo.

METHODS. Corneal neovascularization was induced in New Zealand White rabbits by use of intrastromal pellets containing 750 ng vascular endothelial growth factor (VEGF). The fluorescent dye rhodamine 6G was used to stain leukocytes in vivo. Leukocyte adhesion and vessel growth were quantified in vivo by high-resolution fluorescence angiography. To inhibit ICAM-1 interactions a microemulsion containing anti-ICAM-1 antibody was applied topically.

RESULTS. Limbal vessels showed increased leukocyte adhesion 24 hours after pellet implantation: The number of rolling and sticking leukocytes was significantly increased compared with the number in control animals (P < 0.01). Treatment with anti-ICAM-1 antibody resulted in reduced leukocyte sticking and increased leukocyte rolling. The area covered by new blood vessels was significantly diminished in eyes treated with anti-ICAM-1 (P < 0.05).

CONCLUSIONS. The results support the hypothesis that ICAM-1-mediated leukocyte adhesion is a key event in early angiogenesis. This model may serve for investigation of the significance of adhesion molecules by in vivo observation and quantification. (Invest Ophthalmol Vis Sci. 1999;40:612–618)

In numerous investigations the humoral and cellular aspects of angiogenesis have been examined. A variety of growth factors and antiangiogenic compounds have been studied to explore the mechanisms of ocular vascular growth (reviewed in Ref. 1). Earlier studies have provided evidence for a leukocytic infiltration in the corneal stroma during early stages of the angiogenic process, before the formation of new blood vessels. The location of this leukocytic extravasation corresponds to the site of vessel ingrowth. The pattern of leukocytic infiltration is well correlated with the amount, localization, depth of stromal involvement, and direction of the vascular invasion from the corneoscleral limbus.2–5 Leukocyte adhesion was proposed by Ferrara6 to be the “missing link” during early stages of angiogenesis. Infiltrating macrophages seem to promote all phases of the angiogenic process by the action of their secreted products. Each phase of the angiogenic cascade can be influenced by macrophage-derived factors, as has been reviewed by Poverini7 and Sunderkötter et al.8 Several angiogenic stimuli (e.g., interleukin-89) are also chemoattractants and activators of leukocytes, thus integrating the process of angiogenesis and leukocyte extravasation.

Of the many known factors that can induce ocular neovascularization in an animal model, vascular endothelial growth factor (VEGF) is a potentially key mediator of the changes in the microvasculature. In the past years extensive evidence has become available that VEGF is probably involved in all forms of ocular angiogenesis. It has been shown that VEGF is a multifunctional cytokine that plays a pivotal role in the regulation of angiogenesis. VEGF functions as a directly acting endothelial cell–specific mitogen, is an angiogenic factor in vivo, and is a stimulator of vascular permeability.10,11 In addition, antagonizing VEGF, one specific endothelial cell mitogen, may form a basis for an effective treatment of a variety of tumors and proliferative retinopathies, despite the many factors involved in angiogenesis.10

Most studies of the role of leukocytes during neovascularization are based on conventional histologic examination of angiogenic tissue specimens collected after death. We have recently developed a model for intravitral epifluorescence video microscopy of the ocular surface12 to investigate angiogenesis in living rabbits. This in vivo model can be used to visualize the dynamics of leukocyte adhesion and diapedesis to gain further insight into the pathologic course of inflammatory and neovascular diseases of the ocular surface. Therefore, the first purpose of this study was to examine the interaction between leukocytes and endothelium during the first days of the neovascular response during VEGF-induced corneal angiogenesis with this model, using the fluorescent dye rhodamine 6G to stain leukocytes in vivo.
Leukocytes bind to endothelial cells, to extracellular matrix, or to each other by use of cell adhesion molecules. Specific signals produced in response to wounding and infection control the expression and activation of adhesion molecules. The interactions and responses initiated by binding of cell adhesion molecules to their receptors and ligands play important roles in the inflammatory immune and angiogenic reactions. The process of leukocyte arrest is thought to be primarily mediated by $\beta_2$ integrins on the leukocyte surface and by intercellular adhesion molecule (ICAM)-1 (CD54) on the endothelium. Because ICAM-1 is one of the initial markers during angiogenesis, it was chosen as the subject of our investigation. ICAM-1 is an inducible surface glycoprotein that is widely distributed on endothelial cells and various parenchymal cells in a functionally active form. It has been shown to be a ligand for the following integrins on leukocytes: lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18), Mac-1 (CD11b/CD18), and p150.95 (CD11c/CD18). ICAM-1 and its interaction with the CD11/CD18 complex is one of the mediators for adhesion of leukocytes onto the blood vessel wall and their subsequent extravasation into the surrounding tissue. Experiments using monoclonal antibodies (mAbs) against either of these adhesion molecules have shown that they lead to the sticking of leukocytes observed by intravital microscopy.

It has been shown that inhibition of ICAM-1 by mAbs can significantly reduce leukocyte adhesion in various models, for example phorbol-ester-induced rabbit lung inflammation, myocardial infarction, and endotoxin-induced uveitis in rabbits. Argenbright et al. reported that intravenous administration of mAbs against ICAM-1 can block CD5a-induced leukocyte adhesion in the microcirculation of the rabbit mesentery, visualized by use of intravital microscopy. We were therefore interested to see whether the administration of antibodies against ICAM-1 could alter the course of corneal neovascularization. The second purpose of this investigation was to explore whether a monoclonal antibody against ICAM-1 can affect leukocyte adhesion in vivo during VEGF-induced corneal angiogenesis and reduce vascular growth consecutively.

**METHODS**

The model for microcirculation studies of the anterior segment has been described earlier, and we therefore limit ourselves to a brief description. All experiments were performed on New Zealand White rabbits in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All surgical procedures including the intravitreal microscopic examinations were performed under intramuscular general anesthesia with 10 mg/kg body weight (BW) ketamine-hydrochloride and 0.125 mg/kg BW xylazine. The intravenous application of 0.2 mg/kg BW diazepam (Valium; Roche, Gränzach-Wyhlen, Germany) avoided nystagmus and reduced artifacts caused by movements of the eye.

To stimulate a predictable, persistent, and reproducible neovascular response we used a modified rabbit corneal microsutured assay to induce corneal blood vessels in response to intrastromally administered VEGF. To achieve a continuous stimulus, 750 ng VEGF (R&D, Minneapolis, MN) was incorporated in methylcellulose pellets and surgically implanted in a stromal pocket within the cornea approximately 2 mm from the limbus in slight modification of the description of Crum et al. Rhodamine 6G (0.15 mg/kg body weight; Sigma, Deisenhofen, Germany) was applied intravenously as a fluorescent marker to stain leukocytes.

In this study we used the clone RR1/1; (Bender MedSystems, Vienna, Austria) of a mAb anti-human ICAM-1, IgG1 subclass, which inhibits rabbit neutrophil function through cross reactivity to rabbit ICAM-1 (personal communication, Robert Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). A murine IgG1 mAb served as an isotype control (Southern Biotechnology, Birmingham, AL).

The premicroemulsion (oleo-tensol) consisted of 33% polyethylene glycol (PEG)-20-glycerol-oletinol; 66% vegetable monoglycerides, diglycerides, and triglycerides; 0.5% phosphatidylcholine; and 0.5% vitamin E acetate and was kindly supplied by René A. Duss, Klosters, Switzerland. The liquid components (PEG-20-glycerol, glycerides, and vitamin E acetate) were mixed and stirred at room temperature. Phosphatidylcholine was then added with stirring until a clear premicroemulsion resulted. The premicroemulsion was stored in the dark at room temperature.

The premicroemulsion was mixed 5:1 with anti-ICAM-1 or isotype-matched control mAb to obtain an antibody concentration of 12.5 $\mu$g/ml in the final microemulsion. The milky mAb-containing microemulsion was topically applied (50 $\mu$l) three times daily on the day before surgery and on days 1 through 3.

By use of an intravitreal microscope (Orthoplan; Leica, Wetzlar, Germany) and a long-distance objective (L20/0.32P, UT 32/0.5, $\times 20$ magnification; Leica) the experimental setup allowed an 8-mm working distance. The entire eye was not touched during the investigations. The microscopic images were taken by a video camera (CF 8/1; Kappa, Gleichen, Germany) and recorded on a videotape for further off-line quantification. The off-line quantification of the video tapes was performed with a special analysis software developed for microcirculation research (CapImage; Biomedical Engineering, Heidelberg, Germany).

Leukocytes were stained in vivo and classified by fluorescence microscopy, according to their interaction with the endothelial lining for at least 30 seconds, as either firmly adherent leukocytes or as moving leukocytes, which are represented by rolling and free-flowing cells. Adherent leukocytes were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 seconds. The numbers represent the number of cells per square millimeter of vessel surface, calculated from the diameter assessed by digital planimetry and the length (200 $\mu$m) of the particulate vessel segment, with the following formula:

$$\text{Number of cells per mm}^2 \text{ of endothelial surface} = \frac{\text{Number of sticking cells in 200-}$\mu$m vessel segment} {\text{Diameter of 200-}$\mu$m vessel segment} \times 10^6 \times \pi \times 200$$

Rolling leukocytes are defined as cells that are intermittently in contact with the vascular endothelial lining and are considerably slower than free-flowing leukocytes in the center stream. The number of rolling and free-flowing leukocytes passing through the observed vessel segment within 30 seconds was counted and quantified. The leukocyte flux repre-
sent the percentage of free-flowing and rolling leukocytes in all moving leukocytes and is independent from the total leukocyte count.

Ten venules in the limbus of each animal were randomly chosen and recorded once at each observation time point. Intravitreal microscopic examination was performed before surgery to exclude inflammation in baseline values. Further intravitreal microscopic observations were completed on the right eye of each animal on days 1, 2, 3, 4, 7, and 11 after pellet implantation. The animals were grouped as follows: Group 1 included eyes with VEGF-containing pellets ($n = 6$). Eyes with empty pellets ($n = 2$) and untouched eyes ($n = 1$) served as control specimens to exclude influences of phototoxic effects and operative trauma. Group 2 contained VEGF-implanted eyes, which were topically treated with anti-ICAM-1 microemulsion ($n = 5$) controlled with nonspecific Ig-mAb ($n = 6$), and untreated eyes with implantation of empty pellets. The length of neovascular vessels and the corneal surface covered by new blood vessels was quantified on days 7 and 11 with a caliper under a stereomicroscope by two observers in a double-blind manner.

All measured and calculated values are given as means ± SE. Probability values were calculated using a $t$-test. $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered significant. Each parameter was quantified off-line at least three times, and mean values of each single measurement were recorded as a result of the parameter in the individual vessel. The experiments were terminated after 5 minutes of observation to avoid phototoxic damage to the vessel under investigation.

**RESULTS**

**Leukocyte Adhesion**

The number of firmly adherent (sticking) leukocytes in VEGF-stimulated limbal venules significantly increased in group 1 from $36 ± 32$ adherent cells/mm$^2$ of endothelial lining inbaseline values to a maximum of $158 ± 89$ cells/mm$^2$ on day 4 ($P < 0.001$), whereas the number of sticking leukocytes in control groups (empty pellet, no pellet) remained in a normal range of 20 to 60 cells/mm$^2$, with no significant difference from baseline values (Fig. 1).

The topical treatment with anti-ICAM-1 antibody in VEGF-stimulated venules of group 2 (VEGF/anti-ICAM) resulted in a significant reduction of leukocyte adhesion (maximum of $84 ± 41$ sticking leukocytes/mm$^2$ on day 4; $P < 0.01$) compared with eyes treated with nonspecific isotype-control mAb microemulsion (VEGF-IgG) (Fig. 2). However anti-ICAM-treated eyes had significantly more firmly adherent leukocytes than eyes with empty pellets on days 1 through 4 ($P < 0.01$; Fig. 2). In contrast, statistical analysis of isotype-control application in VEGF-stimulated eyes (Fig. 2) versus VEGF alone (Fig. 1) revealed no significant difference. All values of sticking leukocytes were independent of the diameter. Diameter measurements showed a progressive dilatation of vessels in VEGF-stimulated eyes from approximately $40 \mu m$ to $80 \mu m$, which was unaffected by microemulsion anti-ICAM-1 or control IgG treatment.

The increase in leukocyte flux (percentage of rolling cells in all moving leukocytes) in group 1 was statistically significant in VEGF-stimulated venules compared with control vessels on days 1, 2, 4, and 7 (Fig. 3, $P < 0.01$). The fraction of rolling leukocytes was greater in VEGF eyes treated with anti-ICAM-1 mAb than in VEGF eyes treated with control mAb (Fig. 4). Although this increase in rollers was not statistically significant, it was consistent with recently published data. Statistically, the amount of rolling leukocyte flux in anti-ICAM-1-treated eyes with empty control pellets was significant on days 1 through 11, except for day 4.

**Blood Vessel Formation**

In all animals with VEGF-containing pellets small vascular sprouts originating from limbal vessels could be observed in the peripheral cornea on days 2 and 3 from central limbal
FIGURE 2. Group 2: the number of firmly adherent leukocytes in VEGF-stimulated eyes could significantly be diminished by topical treatment with anti-ICAM-1 containing microemulsion. The results are compared with IgG-isotype control treatment and empty pellets (mean ± SE).

vessels. Newly formed, perfused vessels were first observed on day 5. On day 7 the area covered by new blood vessels was 7 ± 3 mm² in animals treated with VEGF pellets and increased to 26 ± 9 mm² on day 11 (Fig. 5). The application of anti-ICAM-1 resulted in a decreased neovascular growth and consequently in a reduction of the area covered with blood vessels. The area was 4 ± 3 mm² in mAb-treated eyes on day 7 versus 16 ± 3 mm² on day 11. Statistical analysis revealed a significant difference on day 11 (P < 0.05).

FIGURE 3. Group 1: percentage of rolling leukocytes in VEGF-stimulated limbus venules versus empty control pellets and untreated eyes. VEGF elicits significantly increased leukocyte rolling (mean ± SE).

DISCUSSION

The recruitment and migration of macrophages and monocytes by VEGF in vivo can be mediated by VEGF receptors. Two high-affinity receptors for VEGF, flt-1/VEGF-R1 and VEGF-R2, have been characterized. The VEGF receptors, specifically flt-1/VEGF-R1, have been shown, not only in corneal endothelial cells and cultured pericytes but in many other cell types including monocytes and mast cells. These receptors may
therefore contribute to chemotaxis of these cell types induced by VEGF.

Firm binding and subsequent emigration of leukocytes through vascular endothelium is mainly mediated by the ICAM-1-LFA-1 complex. Lu et al.\(^2\) showed recently that VEGF increases retinal vascular ICAM-1 expression in postcapillary venules significantly compared with expression of vascular cellular adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin.

Figure 4. Group 2: rolling leukocyte fraction in anti-ICAM-1 treated eyes versus IgG-isotype and empty-pellet control eyes. There is a trend on day 1 and day 2 in anti-ICAM-1 treated eyes that as a compensation of inhibited leukocyte sticking, initially more leukocytes roll along the endothelial lining. This finding was not statistically significant (mean ± SE).

Figure 5. Vessel growth shown as area of corneal surface in square millimeters affected by neovascularization. The dashed lines (n = 5 eyes) represent eyes treated with anti-ICAM1-containing microemulsion, whereas the solid lines (n = 6 eyes) show the neovascular growth in eyes treated with control mAb. On day 7 the area covered by new blood vessels was 7 ± 3 mm\(^2\) (mean ± SD) in animals with VEGF pellets versus 4 ± 3 mm\(^2\) in mAb-treated eyes and increased to 26 ± 9 mm\(^2\) versus 16 ± 3 mm\(^2\) on day 11 (p < 0.05).
They showed that VEGF-treated endothelial cell monolayers bound 1.9 times more neutrophils than untreated monolayers in a flow chamber. Our findings document for the first time in vivo, ICAM-1-dependent leukocyte adhesion in blood vessels participating in active neovascularization (Fig. 6). Recently, Melder et al.²⁹ showed that VEGF promotes adhesion of activated natural killer cells to tumor endothelium, whereas basic fibroblast growth factor (bFGF) inhibits adhesion through the regulation of ICAM-1 and VCAM-1 on tumor vasculature. This was shown in vitro by significant VEGF-induced activated natural killer cell adhesion to endothelial cell monolayers and in vivo by showing that local administration of bFGF prevents adhesion of activated natural killer cells to the tumor vasculature in the severe-combined-immunodeficiency disease mouse cranial window model. We have supported these data by other findings of our group that show that anti-ICAM-1 treatment of eyes with bFGF-containing pellets had no effect on microvascular parameters including leukocyte adhesion (Becker M, unpublished data, 1997).

Numerous substances have been reported to induce angiogenesis, and many of these mediators are produced by macrophages.³⁰ These include polypeptide growth factors, cytokines, prostaglandins, and proteolytic enzymes.³⁰ Macrophages can mediate new capillary growth, either by secreting factors that act directly on angiogenesis or by modifying the extracellular matrix.³⁰ In our study, treatment with anti-ICAM-1 reduced not only leukocyte adhesion, but also neovascular growth (although the onset of corneal neovascularization reduced not only leukocyte adhesion, but also neovascular growth (although the onset of corneal neovascularization occurred in a microemulsion preparation. The corneal epithelial defect after pellet implantation could be used by the mAb to diffuse to limb vessels, because antibodies can diffuse through the corneal stroma.³⁲ Transconjunctival penetration with secondary limbal and stromal invasion of anti-ICAM-1 should also be considered.

In summary, our results show for the first time in vivo evidence that VEGF-induced angiogenesis is at least in part mediated by ICAM-1 expression. The ICAM-1 pathway of leukocyte adhesion seems to be an important event in early angiogenesis, even before the first endothelial sprouts occur.

References


Figure 6. Adherent leukocytes in limbus vessels 2 days after VEGF stimulation recorded by rhodamine angiography. White spots are leukocytes (arrows). Bar, 100 μm.


