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Cellular Events Associated with Inflammatory Angiogenesis in the Mouse Cornea

Cord Sunderkötter, Waltraud Beil, Johannes Roth, and Clemens Sorg

From the Institute of Experimental Dermatology, University of Münster, Münster, Federal Republic of Germany

The aim of this study was to establish an angiogenesis model in the mouse and to define immunohistochemically the cellular events that precede angiogenesis. After chemical cauterization of the murine cornea, neovascularization was observed within 36 hours. The cellular infiltrate was analyzed by using antibodies on cryostat and paraffin sections and by histochemical staining for mast cells. It was found that neither T lymphocytes nor mast cells nor macrophages in a more mature stage of development were part of the infiltrate that preceded the ingrowth of new blood vessels. Instead, the infiltrating cells appearing from 3 hours on were granulocytes and inflammatory monocytes, as detected by an antibody against the calcium-binding protein MRP14. The authors conclude that the induction of angiogenesis during nonspecific inflammation is associated with the early influx of myelomonocytic cells, but not with the infiltration of mature macrophages, T lymphocytes, or mast cells. This study shows that immunohistochemical analysis of cauterized murine corneas presents a useful tool for further studies on cells and cell products involved in the angiogenic process. (Am J Pathol 1991, 138:931–939)

Formation of new capillaries is involved in many physiologic and pathophysiologic processes. The most common conditions for pathophysiologic neovascularization are inflammation and wound healing, although much insight into the angiogenic events has originally been gained from studies on tumor angiogenesis.[1] Tumor angiogenesis, however, is a very complex angiogenic process because it not only involves the activity of infiltrating cells but also of tumor cells. In inflammatory angiogenesis, no additional neoplastic growth potential is involved. There still remain, however, many cells and factors that have been given a major role in the induction of neovascularization.[2–4] With regard to the different cell types, macrophages,[5,6] lymphocytes,[7] and mast cells[8,9] have been portrayed as most prominent.

The angiogenic potential of most cells and substances has been determined by bioassays, for example, the corneal implantation assay or the chorioallantois membrane assay in the hatched chicken egg. As angiogenesis has been suggested to present a cascadelike process similar to the clotting system,[10] it would be helpful to know the temporal sequence in which certain cells and cytokines appear *in situ* during neovascularization. This would also clarify whether those cells that showed angiogenic potential in bioassays are also involved *in situ* during pathophysiologic angiogenic processes. If they do not appear in conjunction with the formation of new blood vessels, their necessity for the induction of neovascularization would appear questionable.

A comprehensive analysis of angiogenic processes could be obtained by immunohistochemical studies. By this method, the spatial and temporal appearance of various cells and their subtypes in conjunction with angiogenesis could be revealed. It would also allow one to detect the presence of various cytokines and to locate the cells by which they are expressed. As there are several genetically defined strains of mice, and because many immunohistochemical reagents have been developed for use in mice, we re-evaluated the mouse cornea as a model for angiogenesis. In modification of a technique described earlier,[11,12] the center of the avascular murine cornea was cauterized to evoke a nonspecific inflammation with subsequent neovascularization. This model subsequently allowed the immunohistochemical analysis of the cellular events that precede angiogenesis. It was found that neither T lymphocytes nor mast cells appear to be mandatory for the induction of neovascularization. Neither are more mature phenotypes of macrophages part of the pre-angiogenic infiltrate. Most cells in the infiltrate, however, contained MRP14,[13] a calcium-

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Address reprint requests to C. Sunderkötter, Institute of Experimental Dermatology, University of Münster, Von-Esmarch-Str. 56, 4400 Münster, FRG.

binding protein that is expressed during differentiation of myelomonocytic cells.[14] It is present in granulocytes and in infiltrating monocytes, but absent in mature macrophages.[13,15]

Materials and Methods

Animals

BALB/c mice were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, FRG).

Chemical Cautery

One hundred twenty female BALB/c mice (10 weeks old; weight, 25 to 30 g) were anesthetized by intraperitoneal injection of 3 mg ketamine (Ketanest, Parke Davis, Berlin, FRG) and 0.01 mg xylazine (Rompun, Bayer, Leverkusen, FRG). Corneas on the left-hand side were cauterized with an applicator stick of silver nitrate (Lunar Caustic Pencil, B. Braun, Melsungen, FRG). The tip of the applicator was applied to the center of the cornea for 1 second so that a grayish-white patch (1-mm diameter) of cauterized tissue marked the point of contact. Animals were treated with 2 mg ketamine subcutaneously every 12 hours for analgesia. Sixteen animals were exposed to anesthetic agents alone to see if side effects from the narcotics (eg, intermittent depression of corneal reflex) would also lead to inflammation with subsequent angiogenesis. Eyes of five untreated animals were used for evaluation of normal values. After cauterization, all animals were kept under a sterile hood (Flow, Meckenheim, FRG) to avoid microbial superinfection.

At regular intervals corneas were examined under a stereomicroscope (Zeiss, Köln, FRG) for signs of neovascularization.

Tissue

Fifteen cauterized eyes and two controls were taken at 1 hour, 3, 6, 12, 24, 36, 48, and 72 hours after application of silver nitrate.

After administration of an overdose of ketamine and xylazine, eyes were removed during the final stage of anesthesia. Five eyes each were 1) immediately snap frozen in liquid nitrogen and stored at −80°C; 2) fixed and kept in 4% buffered formalin before being embedded in paraffin; 3) processed as described below for histochemical staining of mast cells.

Histologic Preparation

Frozen and paraffin-embedded eyes were cut sagitally in the plane of the corneal lesion. Cryostat (5-μ) sections were cut on a Minotome cryostat (SLEE, Mainz, FRG), fixed in cold acetone at 4°C for 10 minutes, and stored at −80°C. Paraffin sections of 4 μ were cut on a rotation microtome (Autocut, Reichert Jung, Nuβloch, FRG) and mounted on slides coated with 0.1% poly-L-lysine.

Immunohistochemical Staining

The following antibodies were used.

Polyclonal rabbit antiserum against recombinant MRP14,[13] provided by Dr. J. Brüggen (Ciba-Geigy AG, Basel, Switzerland).

MAb F4/80, Rat IgG2b, against murine macrophages[16] in a more mature stage of development,[17] provided by Dr. S. Gordon (Oxford, UK).

MAb BM8, rat IgG2a, against murine tissue macrophages.[18]

MAb against Ia antigen, rat IgG2b (Hybritech, Liege, Belgium).

Anti-L3T4, rat IgG2b, against murine helper T cells (Becton Dickinson, Heidelberg, FRG).

Anti-Lyt2, rat IgG2a, against murine suppressor T cells (Becton Dickinson).

Goat $F(ab')_2$ anti-rat IgG and goat $F(ab')_2$ anti-rabbit IgG, both conjugated with peroxidase (Dianova, Hamburg, FRG).

Antibodies were diluted in 1% bovine serum albumin (BSA) in PBS with concentrations to a maximum of 0.5 μg IgG/ml. For negative controls, specific antibodies were replaced by nonspecific rabbit IgG and rat IgG in the corresponding IgG concentrations. All these controls were negative, indicating specificity of our antibodies.

Staining with all antibodies except the MRP14 antiserum had to be done on frozen sections. They were thawed and dried for 40 minutes, fixed in acetone (10 minutes), and placed into phosphate-buffered saline (PBS)-buffer. Endogenous peroxidase was blocked by 0.12 mol/l NaN_3 (Merck, Darmstadt, FRG) and 0.01 mol/l H_2O_2 (Merck) in PBS for 10 minutes at room temperature. To prevent nonspecific protein binding of the antibodies, sections were placed in 1% BSA (in PBS) for 1 hour. The specific antibody was applied for 1 hour. After thorough washing in 0.01% BSA (in PBS), sections were incubated for 1 hour with the conjugated second antibody. Substrate reactions were performed with 0.01 mol/l H_2O_2 using amino-9-ethyl-carbazol (Sigma, Deisenhofen, FRG) as chromogen. Sections were counterstained with Mayer's hemalun (Merck).

Paraffin sections could be used for staining with the antiserum against MRP14. They were deparaffinized in Rothihistol (Roth, Karlsruhe, FRG) for 5 minutes and rehydrated in a graded series of alcohol (96%, 70%, 50%, distilled water) before being placed into PBS buffer.

Deparaffinized sections were also used for routine staining with hematoxylin and eosin (H&E).

Histochemistry

Mast Cells

For detection of connective tissue mast cells, three eyes per time interval were fixed in 4% buffered formalin for 6 hours and embedded in paraffin. Embedding procedure was shortened from 24 to 8 hours to avoid washing out of soluble proteoglycans. Deparaffinized (6 μ) and also cryostat sections were stained for 1 hour in 0.5% Toluidine blue (Merck) dissolved in 0.5 N HCl, rinsed in running tap water, dried, and examined by oil immersion. As the presence of mucosal mast cells could be masked by formalin fixation, two eyes were fixed in an isotonic formaldhyde-acetic acid mixture[19] and in Carnoy's fixative[20] before being processed as described above.

Eosinophils

For the detection of eosinophils, deparaffinized sections fixed in buffered formalin were stained for 5 minutes in 0.2% Eosin Y (Sigma) dissolved in 1 N NaOH. Additionally autofluorescence of eosinophils[21] was examined by a Zeiss Standard microscope equipped with an HBO 200-W super pressure mercury lamp at 520 to 560 nm.

Microscopic Evaluation

Sections were analyzed with a Zeiss photomicroscope III. For cell counting, sections were examined at 425-fold magnification using an ocular endowed with an eyepiece graticule (Zeiss). Within the graticule field, the number of positive cells and the total number of cells were recorded. Two areas of each cornea were evaluated separately (Figure 1): the corneoscleral limbus (L), ie, the vascularized end of the sclera, and the originally avascular corneal stroma (C).

At the corneoscleral junction, two graticule fields were evaluated, starting at the anterior margin of the retina (os serrata). In the adjoining part of the originally avascular cornea, a stretch of three graticule fields was evaluated. Cell counting was controlled by a second observer.

Results for each antibody at the investigated intervals were expressed as arithmetic means (n = 5) of the absolute number of positive cells and also as arithmetic means (n = 5) of the percentage of positive cells related to the total number of cells.

The U-test according to Mann and Whitney (for values without normal distribution) was performed to determine significant differences in the number of positive cells between the different time periods. Values of $P > 0.05$ were considered not to be significant (n = 5).

Results

Stereomicroscopic Observations

The cornea of murine eyes comprises most of the external hemisphere of the bulbus. Stereomicroscopic observation of untreated eyes disclosed a ring of two to three small vessels surrounding the cornea at the corneoscleral junction (limbus). From these corneoscleral vessels, only few capillary arcades and branches were emerging toward the transparent corneal stroma.

Within 3 hours after cauterization, the vessels at the corneoscleral limbus began to dilate. Between 6 and 12 hours, the corneas became increasingly cloudy. After 1

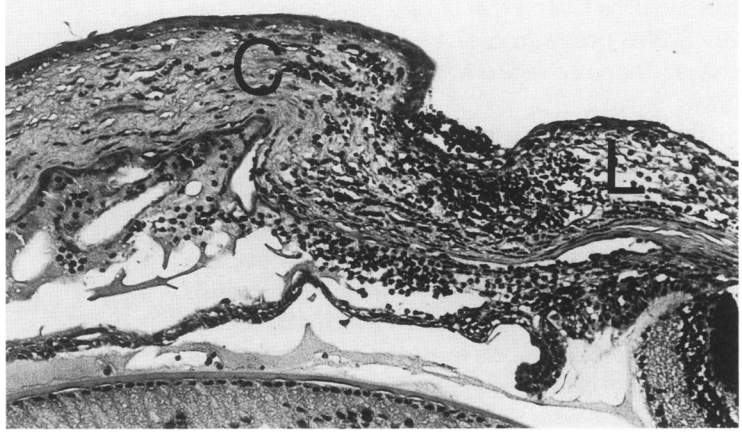

Figure 1. *Paraffin section of cornea, 24 hours after cauterization. Cellular infiltrate at the corneoscleral limbus (L) is extending into the corneal stroma (C). (H&E, ×144).*

day, short capillary extensions were reaching the cornea in 80% of the treated eyes.

Thirty-six hours after cauterization, all eyes showed several new capillaries that were extending radially into the corneal stroma (Figure 4a). They formed arcades from which other microvessels continued to invade the cornea. After 3 days, about two thirds of the distance between the corneoscleral limbus and the central lesion were traversed by new vessels. After 4 to 5 days, the lesion itself was vascularized.

Control eyes (contralateral eyes of treated animals and eyes of untreated animals that were exposed to the anesthetic agents) did not present new blood vessels or other signs of inflammation such as cloudiness of the cornea or dilation of vessels.

Histologic Findings

The vascularized limbus (corneoscleral junction) and the adjoining part of the originally avascular corneal stroma (Figure 1) were recognized to be important for the evaluation of the inflammatory infiltrate in angiogenesis. The limbus with the overlying conjunctiva presents the source of blood vessels and of all infiltrating cells. Investigating this area allows a qualitative and quantitative analysis of the infiltrate and of early endothelial reactions. The adjoining corneal stroma is the site where new vessels can first be detected and where all cells preceding vascular ingrowth can be identified. Both areas were evaluated separately.

Normal Cornea

Immunohistochemical sections of normal corneas disclosed the presence of several mononuclear phagocytic cells within the vascularized corneoscleral limbus. On average 17 cells (per two graticule fields) were BM8 positive and 9.6 cells were F4/80 positive (Figure 2). Compared with the total number of cells, this corresponds to 29.3% BM8 positive and 17.4% F4/80 positive macrophages. The percentage of MRP14-positive cells was low (5%).

The corneal stroma presented a low cell content, composed primarily of fibrocytes and mononuclear cells. The cell density gradually declined from the limbus toward the cornea. At the beginning of the avascular zone the percentage of resident macrophages was comparatively high (23% BM8-positive cells within three graticule fields). The overall prevalence of BM8- and F4/80-positive macrophages in the whole avascular cornea was about 10%. Only 1% of all corneal cells stained MRP14 positive. Ia-positive cells were found in the corneal stroma (9,2% Ia-positive cells) and also in the corneal epithelium (Langerhans cells).

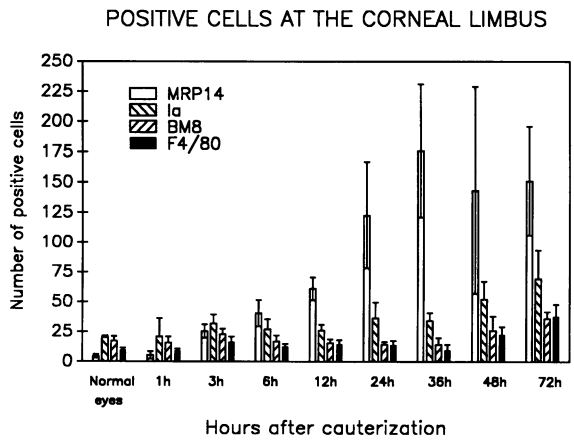

Figure 2. *Absolute number of MRP14-, Ia, BM8-, and F4/80-positive cells at the corneoscleral limbus.*

Neither at the limbus nor inside the avascular stroma did we see more than two L3T4- or Lyt2-positive cells. Mast cells were found at the corneoscleral limbus but not in the avascular stroma of the cornea. They belonged to the connective tissue type, as their number (three to six cells per section) did not vary by use of different fixation methods.

Cauterized Corneas

MRP14-positive Cells

One hour after cauterization we observed a slight increase of MRP14-positive cells at the vascularized limbus. This increase (Figure 2) became significant after 3 hours (U test, alpha = 0.05). At this time, MRP14-positive cells were also invading the avascular stroma, which resulted in a significant increase of MRP14-positive cells inside the cornea after 6 hours (Figure 3). Most cells were

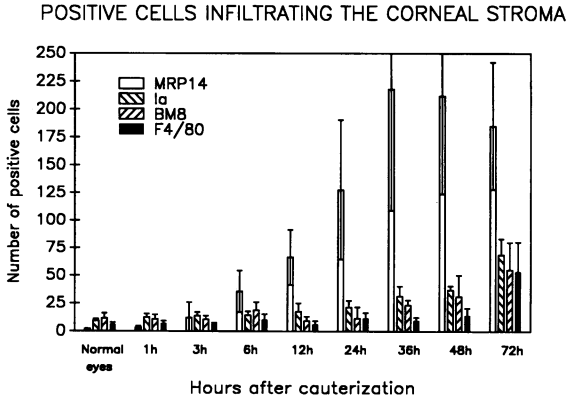

Figure 3. *Absolute number of MRP14-, Ia-, BM8-, and F4/80-positive cells in the originally avascular corneal stroma.*

infiltrating the connective tissue layers close to the corneal epithelium (Figure 4b) and had reached the lesion after 12 hours. Thus complete infiltration of the cornea corresponded with the cloudy appearance of the cornea at that time. Figure 4c shows MRP14-positive cells around dilated blood vessels at the limbus. After 24 and 36 hours, when neovascularization was in full progress, the growing infiltrate inside the cornea contained between 86% and 88% MRP14-positive cells. This percentage was slightly reduced to 77% after 3 days when macrophages in a more mature stage of development gained in number. During the first 3 days, cross-sections of new vessels were seen primarily in the superficial part of the corneal stroma, following the streak of infiltrating cells.

The majority of MRP14-positive cells were granulocytes. After 12 hours they also began to comprise a num-

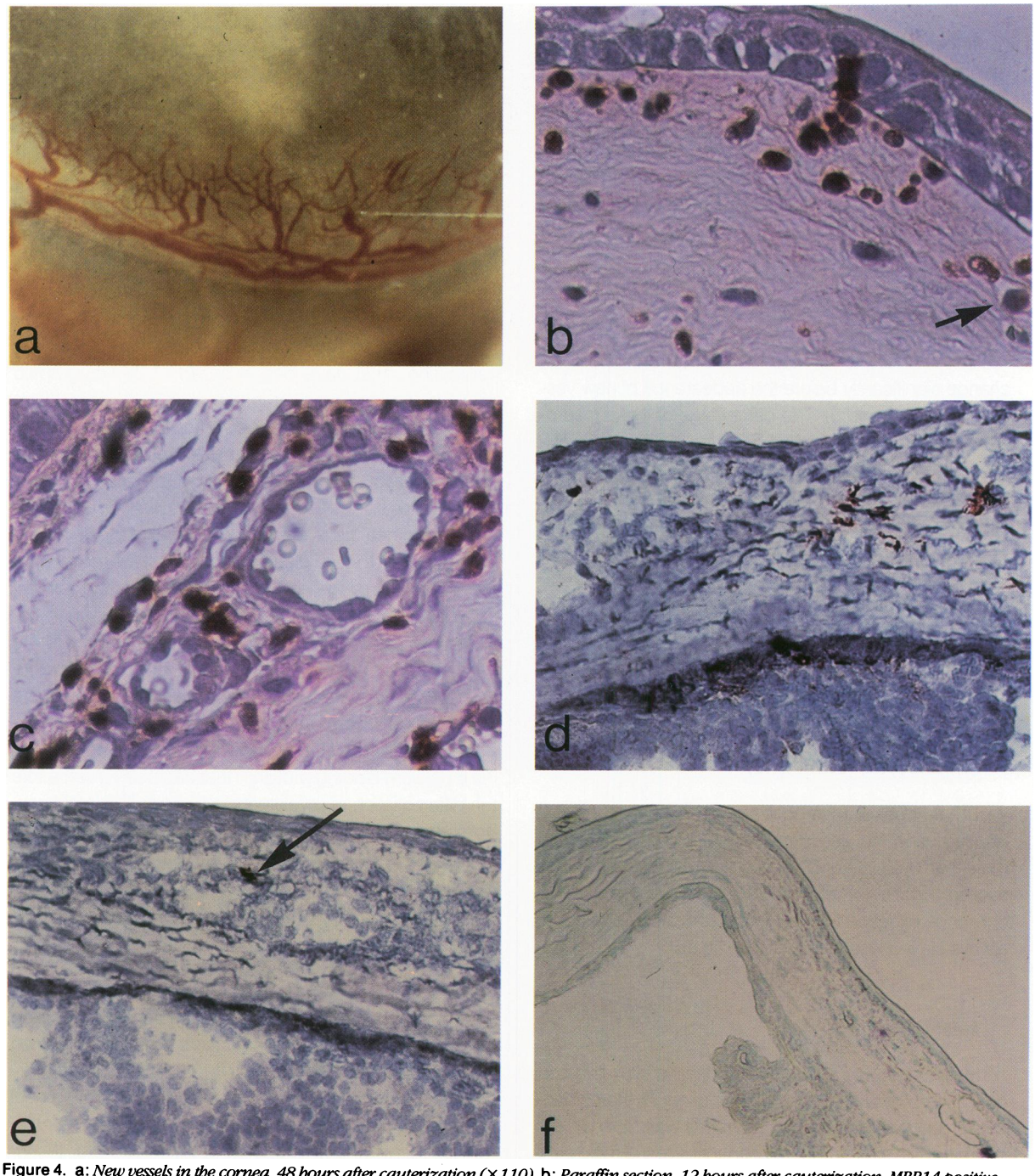

Figure 4. **a:** *New vessels in the cornea, 48 hours after cauterization (×110).* **b:** *Paraffin section, 12 hours after cauterization. MRP14-positive granulocytes and monocytes (arrow) are infiltrating the corneal stroma close to the epithelium (×576).* **c:** *Paraffin section of corneal limbus, 12 hours after cauterization. Infiltrate of MRP14-positive cells around dilated blood vessel (×576).* **d:** *Cryostat section of infiltrated limbus (left) and corneal stroma (right). F4/80-positive macrophages, 24 hours after cauterization (×225).* **e:** *Cryostat section of infiltrated limbus. Only one L3T4-positive lymphocyte (arrow) present 24 hours after cauterization (×225).* **f:** *Paraffin section (6 μ), 12 hours after cauterization. Two connective tissue mast cells present at the limbus, but none in the corneal stroma. Toluidine blue (×144).*

ber of mononuclear cells (Figure 4b). Exact quantitative differentiation of MRP14-positive granulocytes and monocytes by evaluating nuclear shape, however, was difficult because of intense staining of the cytoplasm.

No eosinophils could be detected, either by staining with Eosin Y or by autofluorescence.

Ia Antigen

At the limbus, the number of cells carrying the Ia antigen was not rising markedly in the first 24 hours (Figure 2). Inside the avascular cornea, they showed a slight increase (Figure 3), which became significant (compared with untreated eyes) 24 hours after injury. Their percentage in the infiltrate, however, was far below that in normal corneal tissue, which shows that the majority of infiltrating cells were MRP14-, but not Ia-, positive.

BM8 and F4/80

The number of BM8- and F4/80-positive cells did not change significantly before the appearance of new vessels (Figures 2 and 3). The percentage of these macrophages even declined because of the predominant infiltration of MRP14-positive cells. One day after cauterization, when the first capillaries were reaching the cornea, only 5.5% of the infiltrating cells in the cornea were BM8 positive and only 7.8% were F4/80 positive (Figure 4d). Only after 48 hours did the number of BM8- and F4/80-positive cells increase significantly in both the limbus (Figure 2) and the cornea (Figure 3). Their percentage remained lower than observed in tissues of untreated eyes.

Lymphocytes (L3T4, Lyt2)

The number of T lymphocytes did not change significantly during the period investigated. Before day 1 no more than two Lyt2- or L3T4-positive cells were found per section (Figure 4e) and after that their average number only once exceeded three per section; their percentage always stayed less than 2% in all areas.

Mast Cells

Mast cells could not be detected in the infiltrated corneal stroma by any of the methods used. At the corneoscleral junction, no mucosal mast cell was found in the infiltrate while the number of connective tissue mast cells (Figure 4f) remained unchanged during the course of neovascularization.

Discussion

The aim of this study was to establish a model for angiogenesis in the mouse that would allow the immunohistochemical detection of cells and substances involved in angiogenic processes. We then used this model to perform an analysis of the early cellular events associated with the induction of angiogenesis.

The cornea can easily be surveyed macroscopically and microscopically, and it allows the detection of newly formed blood vessels earlier than other models of angiogenesis. In the mouse, availability of a great variety of inbred strains permits analysis of genetic influences and assures reproducibility. Many antibodies against inflammatory cells, but also against angiogenic cytokines, are available for use in mice. Their number exceeds that of reagents applicable in other species for which the cornea model has already been established.

After chemical cauterization of the corneal apex, new capillaries became visible from 24 hours onward. As new capillaries can only be detected macroscopically when they are perfused with blood, the actual onset of vascular sprouting must have begun earlier. Autoradiographic studies demonstrated an increase of DNA synthesis in capillary endothelium 17 hours after thermal cautery of the rat cornea.[22] Migration of endothelial cells during corneal neovascularization starts before the onset of endothelial mitosis.[23] Consequently we suggest that also in the mouse cornea the events inducing angiogenesis should be expected to take place within the first 24 hours.

We analyzed the early cellular events during corneal neovascularization by histochemical and immunohistochemical methods. Inflammatory cells began to infiltrate the avascular corneal stroma 3 hours after cauterization. Before the appearance of new capillaries (24 to 36 hours after cauterization), infiltrating cells were almost exclusively identified by the antibody against MRP14. MRP14 is a calcium-binding protein[13] that has been given a role in differentiation of myelomonocytic cells.[14] It is only expressed by early infiltrating monocytes and by granulocytes, but not by mature macrophages.[13,15] Mature macrophages, T lymphocytes, eosinophils, or mast cells were not part of the infiltrate before the ingrowth of new capillary sprouts.

Minor damage to the corneal epithelium that did not elicit a strong corneal infiltrate subsequently did not lead to neovascularization.[24] Whenever cauterization resulted in a marked infiltrate of inflammatory cells, newly formed vessels followed the route of the early infiltrate, ie, they were first detected in the outer layers of the corneal stroma. In our experimental setting, the inflammatory infiltrate therefore appears to be a prerequisite for the induction of angiogenesis.

Previous studies on cauterized rat corneas have also

shown that the early infiltrate contained many granulocytes.[25–27] We showed that the early infiltrate also contained some mononuclear cells that were MRP14 positive, but not L3T4 or Lyt2 positive.

T lymphocytes have been shown to be angiogenic, especially in transplant experiments.[7,28–30] This so-called lymphocyte-induced angiogenesis[7] may thus be a phenomenon associated with immunologically specific inflammatory reactions (eg, graft-versus-host reactions).

Similar to lymphocytes, connective tissue mast cells did not appear in the corneal infiltrate before, or in conjunction with, the emergence of new blood vessels. Neither did their number increase at the limbus. The presence of mucosal mast cells could be excluded by using different fixation techniques.[19,20] Thus we propose that during angiogenesis mast cells are not required to precede new capillary sprouts, as was previously suggested from observations in the CAM-assay.[31] In another study, mast cells could be detected in vascularized corneal stroma during rejection of corneal xenografts, but this was at a later stage of the host-*versus*-graft reaction.[32] As in our model, mast cells were confined to the corneal limbus during the first 3 days. Mast cells however, have been shown to have supporting effects on angiogenic processes,[9,33] especially during tumor growth.[34] The mechanisms so far remain unclear. Release of histamine, for example, could enhance capillary permeability with subsequent exudation of fibrin or fibrinogen. This exudate can provide a migratory matrix for endothelial cells,[35] as it does during tumor growth.[36] It does not explain, however, the directed growth of capillary sprouts as observed in the cornea. The role of mast cells in providing heparin for protection of basic fibroblast growth factor (FGF) in the murine cornea was discussed in a previous paper.[24]

Macrophages in a more mature stage of development were present at the limbus and in the corneal stroma of untreated eyes. In cauterized eyes, however, their number did not increase before 36 hours postcauterization. This was past the onset of angiogenesis. The number and percentage of Ia-positive cells in normal corneas were similar to those of BM8- and F4/80-positive cells. The slight increase of Ia-positive cells inside the corneal stroma during the first 36 hours may be attributed to Ia expression on some of the early infiltrating monocytes.

In previous studies, only activated but neither unstimulated resident macrophages nor granulocytes, have been shown to induce angiogenesis after implantation into the corneas of several species.[5,37–39] Attempts to define an angiogenic subtype of macrophage more closely[38] have been limited by the lack of suitable reagents.

In our study, the preangiogenic infiltrate was composed of several granulocytes and a small fraction of inflammatory monocytes, defined by their expression of MRP14. Because granulocytes, in contrast to macrophages, have not shown angiogenic potential in corneal bioassays,[39] the angiogenic activity of the early infiltrate could be attributed to MRP14-positive cells of the monocyte-macrophage lineage. For further clarification it would be helpful to investigate if these cells do release angiogenic substances or if they can activate resident macrophages to do so. In a recent study,[24] we showed that basic FGF and tumor necrosis factor-α, two well-described angiogenic cytokines,[3,6] could not be detected in infiltrating or resident macrophages before the onset of angiogenesis. Other angiogenic cytokines such as IL-1,[40] IL-6[41] or TGF-β[42] have yet to be examined immunohistochemically for their presence during corneal neovascularization. Soluble growth factors released by activated macrophages, however, may not present the sole signal for endothelial cells to begin the complex process of neovascularization. Changes in the extracellular matrix have also been shown to be potent inducers of angiogenic processes.[4,43] Alterations in the composition of the extracellular matrix can be conveyed to endothelial cells by integrinlike receptors,[44] and possible molecular mechanims for the response by endothelial cells have recently been reviewed.[45] Some of the components of the extracellular milieu with the capacity to promote capillary growth are fibrin and its degradation products[35,46] and fragments of hyaluronic acid.[47] In the cauterized cornea, inflammatory monocytes and granulocytes were the only cells present before the formation of new blood vessels. They showed a directed migration toward the lesion, probably along a chemotactic gradient, and new vessels followed their path. Their cytokine repertoire may be limited, but they are known to be a rich source of degrading enzymes. Thus appropriate degradation of proteins in the extracellular matrix by these early infiltrating cells may present yet another important step in the induction of the angiogenic cascade.

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