Investigation of Laser-Induced Choroidal Neovascularization in the Rat

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PURPOSE. Choroidal neovascularization plays an important role in pathogenesis of age-related macular degeneration. Induction of neovascularization by laser photocoagulation in the rat fundus is an established animal model in which the effects of new therapeutic approaches are assessed. The purpose of this study was to compare different detection methods of laser-induced neovascularization in the rat.

METHODS. Laser spots were applied to the fundus of Long-Evans rats. Ten days after, four different methods were used to detect laser-induced neovascularization: (1) high-resolution angiography with fluorescein isothiocyanate-dextran, (2) immunohistochemical visualization of platelet endothelial cell adhesion molecule (PECAM-1), (3) visualization of intravascular lumens by peroxidase perfusion in the living rat with subsequent histologic analysis, and (4) histochemical representation of alkaline phosphatase in endothelial cells.

RESULTS. At the rim of the laser scars vessel-forming endothelial cells with intravascular dextran and peroxidase were present. Cross-sections demonstrated that these vessels originated from the retina. The center of the scars contained homogenous endothelial cells of choroidal origin, which was confirmed by immunohistochemistry and electron microscopy. In laser-treated eyes without FITC-dextran perfusion, scars showed unspecific fluorescence, making differentiation from specific FITC-dextran-associated fluorescence difficult.

CONCLUSIONS. In the rat model of laser-induced neovascularization, newly developed endothelial cells originate from the retina and the choroid. Whereas ring-like surrounding vessels come from the retina, flat endothelial cells in deeper layers are of choroidal origin or may originate from circulating endothelial precursor cells. Dextran angiography has to be regarded critically for visualizing the choriocapillaris and CNV in laser scars. PECAM-1 immunohistochemistry is best for detection and quantification of neovascularization in laser scars. (Invest Ophthalmol Vis Sci. 2003;44:5349–5354) DOI:10.1167/iovs.02-0732

Choroidal neovascularization (CNV) is a major cause of severe central vision loss in patients with exudative age-related macular degeneration (ARMD). In response to an increase of locally produced angiogenic factors, such as VEGF, IL-1α, prostaglandins, oxidized lipids, or FGF in a wounded area, vessels grow between Bruch’s membrane and the RPE as well as into the subretinal space.

However, therapeutic treatments with a reliable curative effect are not yet available, and therefore several studies have been performed to develop various strategies, including eradication of CNV by laser photocoagulation, obstruction of CNV by photodynamic therapy (PDT), prevention of angiogenesis by pigment epithelium-derived factor (PEDF), and treatment with steroids, anti-VEGF agents, or an analogue of fumagillin, TNP-470.

To demonstrate the success or failure of these therapeutic strategies, visualization and quantification of CNV are crucial. Therefore, our study was performed to test and to compare different methods for visualization of experimentally induced neovascularization in the rat by dextran angiography, immunohistochemistry, peroxidase perfusion, and histochemistry.

METHODS

Laser Photocoagulation

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-two-month-old Long Evans (LE) rats were anesthetized with 2 mg/kg ketamine (Ketanest; Parke-Davis, Berlin, Germany) or 2,2,2-tribromoethanol (Avertin; 50 mL/kg) and the pupils were dilated with tropicamide/phenylephrine. With a coverslip used as a contact lens, eight argon laser spots (150 mW, 100 ms, 100 μm; Coherent Novus 2000; Carl Zeiss Meditec, Oberkothen, Germany) were applied to each fundus in a circle around the optic disc. All methods described below were applied 10 days after photocoagulation.

FITC-Dextran Angiography

Angiography was performed on anesthetized rats generally by the method of D'Amato et al. and Edelman and Castro. Thirty minutes after intraperitoneal injection of 0.5 mL heparin, rats were perfused with 50 mL PBS and 5 mg/mL fluorescein isothiocyanate-labeled dextran (FITC-dextran; MW 2 × 10⁶, Sigma-Aldrich, Deisenhofen, Germany) through a low-perfusion cannula (20-gauge). The physiological blood pressure was maintained by the beating heart and by adjusting the height of the perfusate bottle. Thirty to 90 seconds later, the animals were killed by cervical dislocation. Eyes were enucleated and fixed in 4% paraformaldehyde overnight.

Flatmount Preparation and Digital Images

All flatmounts were prepared as described by McMenamin. The eyes were sectioned at the equator, and the anterior half and the vitreous removed. The retinas were isolated and investigated by light microscopy. The posterior eye segment containing the sclera and choroid was dissected into quarters by four radial cuts and mounted on a slide. All flatmounts described here and later were examined by a fluorescence microscope (Axioplan 2 Imaging; Carl Zeiss Meditec) using FITC, Cy3, and lipofuscin filters (Analysetechnik Feuerbach, Tübingen, Germany). For FITC: excitation 471 nm, emission 503 nm; for Cy3: excitation 550 nm, emission 570 nm; and for lipofuscin: excita-
tion 405 nm; emission 475 nm. All digital images are taken under the same conditions. The images of the laser scars were captured with an analog video camera (Axioplan 2 Imaging, Carl Zeiss Meditec) coupled to a computer with image-analysis software (Openlab, ImproVision, Inc., Lexington, MA).

**Immunohistochemical Visualization of Endothelial Cells**

Eyes were enucleated and fixed in 4% paraformaldehyde. Flatmounts were prepared as described. Several of these animals had been perfused by FITC-dextran as described earlier. In the first set of experiments the following immunohistochemical protocol was used. The tissue was labeled with platelet endothelial cell adhesion molecule (PECAM-1; purified rat-anti-mouse CD 31 monoclonal antibody; Pharmingen, Heidelberg, Germany) as the primary antibody overnight (dilution 1:100). The secondary antibody was a biotinylated goat anti-rat IgG (Amersham Pharmacia Biotech, Freiburg, Germany), incubated overnight in a dilution of 1:400. The third agent was Cy3-labeled streptavidin (Fluorolink; Amersham Pharmacia Biotech), diluted 1:1000 and incubated for 1 hour at room temperature. In control immunohistochemistry, the primary antibody was omitted. In the next set of experiments, we used purified mouse anti-rat PECAM-1 monoclonal antibody (PharMingen) for specific binding of PECAM-1 (1:200 dilution) and goat anti-mouse IgG conjugated with Cy5 (1:500 dilution; Dianova, Hamburg, Germany) as a secondary detection antibody. As a control, immunostaining was performed without the primary antibody.

**Control**

Laser scars without dextran perfusion and without immunohistochemical labeling were examined by fluorescence microscopy (FITC-filter).

**Histochemical Visualization of Intravasal Peroxidase**

Rats were anesthetized and 50 mg horseradish peroxidase (type II, MW 40,000; Sigma-Aldrich) in 200 μL PBS were injected into the jugular vein. Thirty to 90 seconds later, animals were killed by cervical dislocation. The eyes were enucleated, fixed in 1.8% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight. Peroxidase was visualized by the method of Karnovsky. The anterior part of the eye, vitreous, and retina were removed, and the posterior eye cup was kept in 5 mg 3,3-diaminobenzidine-tetrahydrochloride (DAB; Sigma-Aldrich) in 0.1% EDTA in Ca²⁺/Mg²⁺-free PBS after 2 hours at room temperature. The retina and RPE were removed, and the tissue was washed in 0.1 M cacodylate buffer (pH 7.4) at 4°C. After fixation with 2% paraformaldehyde for 1 hour, the tissue was washed in 0.1 M cacodylate at 4°C overnight. Tissues were incubated with a fresh, filtered solution consisting of 40 mL 0.1 M Tris buffer (pH 9.2) with 20 mg fast blue RR salt (Sigma-Aldrich) and 4 mg naphtol AS-MX phosphate (Sigma-Aldrich) dissolved in 0.2 mL dimethyl sulfoxide (DMSO: Sigma-Aldrich) at 37°C for 30 minutes. After the medium was changed once, the tissue was washed and postfixed at 4°C for 24 hours. The tissues were washed and bleached in 30% H₂O₂ at 4°C for 3 days. The tissues were then repeatedly washed, treated with 1% catalase in 0.1 M cacodylate (pH 7.4; 4°C, 2 hours), and washed again. Radial cuts were made, and the tissues were flatmounted on slides for light microscopy.

**RESULTS**

By all methods laser scars were visualized 10 days after photocoagulation. Ninety percent of the applied laser burns led to scar formation. Two forms of scars were detected: 30% were similar to the scar shown in Figures 1a-c, without overlying retina; 70% of the scars were similar to the one in Figure 2a, revealing patches of retinal tissue overlying parts of the scar. Figures 1a–c presents images of one laser scar, detected by light microscopy (Fig. 1a, bright field) and by fluorescence microscopy for FITC-dextran (Fig. 1b) and Cy3-PECAM-1 (Fig. 1c).

**FITC-Dextran Angiography**

Dextran-perfused vessels were seen regularly at the rim of the laser scars (Fig. 1b) whereas the center of the scars showed diffuse background fluorescence. Even when we focused through the flatmount, the choriocapillaris vasculature could not be demonstrated, either near to or at a distance from the scars. In contrast, flatmounts with overlying retina and separately prepared retinas showed well-perfused retinal vessels.

**Immunohistochemical Visualization of Endothelial Cells**

Endothelial cells labeled by PECAM-1 were present in the center (Fig. 1c) and at the rim (Fig. 2a) of the laser scars. Circular endothelial cells formed vascular tubes (Fig. 2a), corresponding to those visualized by dextran angiography (Fig. 1b). The flatmount in Figure 2a originated from an animal that was perfused by FITC-dextran before labeling with PECAM-1. Therefore, dextran-perfused vessels are also visible at the rim of the scar.

When Figure 1b is compared with Figures 2a and 2b, it becomes clear that only immunohistochemistry provided a clear image of the choriocapillaris vasculature, whereas in dextran angiography the choriocapillaris was not visible.

Control preparations of immunohistochemistry without the first antibody showed no fluorescence (Fig. 1e). However, the fluorescence signal was made more intense by use of a lipofuscin filter set. In this case, nonspecific fluorescence may be due to the formation of lipofuscin-like material.

**Control**

In the control, laser scars without any treatment by fluorescent markers (no dextran perfusion, no immunohistochemistry), were examined by fluorescence microscopy with FITC (Fig. 3) and lipofuscin filter sets. Even in these scars, a background fluorescence was present, especially in the center of the scars (Fig. 3, star). This fluorescence must not be confused with fluorescence expressed in the newly formed vessels. We suggest that this nonspecific fluorescence is due to formation of lipofuscin-like material and/or cell debris derived from damage to the tissue during laser photocoagulation.

**Histochemical Visualization of Intravasal Peroxidase**

Histologic sections provide a detailed picture of the laser scars (Fig. 4). The black reaction product of peroxidase enzyme activity was visible within vessels located in the retina, but not in the choriocapillaris (Figs. 4a, 4b). The large peroxidase-perfused vessel in Figure 4b is a higher magnification of the edge of a laser scar, corresponding to the circular vessels in Figures 1b, 2a, and 6a. In contrast to the peroxidase-positive intraretinal vessels, the choriocapillaris was not perfused by
peroxidase, either near to or at a distance from the laser spots (Fig. 4a). Only constricted arterioles almost without a lumen were filled with peroxidase (Fig. 4a). By electron microscopy, no extravasation of reaction product was noted.

Cells marked by white arrows in Figure 4a were classified as endothelial cells by electron microscopy (Fig. 4c). They are flat cells in the choroid, surrounded by their basement membrane.

Figure 5 is a schematic drawing of a histologic section, showing a laser scar. Retinal neovascularization is distinguished from newly formed endothelial cells in the choroid. Whereas retinal vessels are perfused by peroxidase (Fig. 4a) and dextran (Fig. 4b), the choriocapillaris vessels are not (Fig. 4a). Endothelial cells in the choroid, drawn at the bottom of the scar, are visualized by PECAM-1 (Fig. 1c) and electron microscopy (Fig. 4c). The dotted line indicates the supposed line of separation, when the retina was removed during flat-mount preparation.

**Histochemical Visualization of Alkaline Phosphatase in Endothelial Cells**

The reaction product of alkaline phosphatase was present in the vessel-forming endothelial cells at the rim of the laser scars (Fig. 6a). Endothelial cells of the choriocapillaris vasculature were represented as well (Fig. 6b). The quality of the staining was high, even though cacodylate-buffered solutions and EDTA may reduce the enzyme activity. However, the omission of...
both inhibitors of alkaline phosphatase activity can further improve the quality of the staining.

DISCUSSION

Four different methods were chosen in our experiments to assess the formation of laser-induced neovascularization in the rat fundus and four main conclusions were drawn: (1) PECAM-1 immunohistochemistry is the best method for detection and quantification of neovascularization in laser scars and of the choriocapillaris. (2) Dextran-filled vessels surrounding the laser scars do not originate from the choroid, but from the retina. (3) Flat endothelial cells in the center of the scars originate from the choriocapillaris or perhaps from circulating endothelial progenitor cells. (4) Histologic techniques are important in obtaining detailed information.

Argon laser photocoagulation is an established method for generating choroidal neovascularization (CNV) in animal models.\(^1\) High laser energy causes rupture of Bruch’s membrane, and, under the influence of various angiogenic factors, an ingrowth of choroidal vessels under the RPE and into the subretinal space takes place. Although in this model, pathogenesis of the neovascularization is different from ARMD, formation of CNV is believed to follow the same pattern, and identical angiogenic factors are expressed by the RPE and endothelial cells: FGF,\(^13\) VEGF (Ogata N, et al. IOVS 1996;37: ARVO Abstract 124), and TGF-\(\beta\). The energy of the laser affects the appearance of the subsequent scars, however, and has to be taken into account when comparing neovascularization in this model. The laser energy we used was similar to that used by other investigators (e.g., Mori et al.\(^3\)) Mature vessels are present from 10 days after photocoagulation,\(^5,6\) and therefore this time was chosen for our experiments.

For the angiography, a high molecular weight dextran (MW 2 × 10⁶) that was bound to a fluorescein marker and did not leak out of the CNV was chosen.\(^7,14\) Advantages of dextran angiography examined in flatmounts are the representation of the whole retina and a higher resolution of certain regions,\(^7\) as well as the possibility of quantification by measuring the fluorescence. Angiography is faster and easier to perform than histologic methods.

Whereas dextran-perfused vessels were regularly present at the rim of the scars and in the retina 10 days after laser

![Figure 3](image-url)  
**Figure 3.** Flatmount of a laser scar without treatment, examined by a fluorescence microscope (FITC-filter). Even without immunohistochemical staining or perfusion by fluorescent markers, a certain fluorescence is present in the scars. This makes differentiation from specific FITC-dextran-related fluorescence difficult. This nonspecific fluorescence (**star**) is probably due to formation of lipofuscin-like material and/or cell debris derived from damaged tissue and must not be confused with the fluorescence of newly formed vessels.

![Figure 4](image-url)  
**Figure 4.** Peroxidase, light microscopy. (a) At the laser scar the retina sinks deeply into the choroid (**left**). Next to the scar (**right**), retinal tissues, including photoreceptors (ROS), are well preserved. Vessels inside the retina are perfused by the black reaction product of peroxidase (**black arrow**). Choriocapillaris vessels are not perfused by peroxidase (**black arrow**). Only the deeper choroid shows a constricted arteriole blocked by peroxidase (**arrowhead**). Peroxidase perfusion does not pass from there into the choriocapillaris. Flat endothelial cells in the choroid (**thin white arrows**) without a visible lumen seem to correspond to those in Figure 1c. One of them is represented in detail by electron microscopy in (e). **Dotted line:** the plane of separation when the retina was removed from the flatmounts. (b) Higher magnification, taken from the rim of a scar. A large peroxidase-perfused vessel (**arrows**) is visible within the retina (R). This vessel corresponds to the circular ones in Figures 1b, 2a, and 6a. (c) Electron microscopic demonstration of the cells, marked by **thin white arrows** in (a). This is a flat, single endothelial cell in the choroid at the bottom of a laser scar, surrounded by its basement membrane. R, retina; Ch, choroid; e, erythrocytes; M, melanocytes.

![Figure 5](image-url)  
**Figure 5.** Schematic drawing of a histologic section through a laser scar. There are large neovascularizations within the retina, corresponding to those in Figures 1b, 2a, 4a, 4b, and 6a. They are perfused by dextran and peroxidase. At the bottom of the scar there are flat, single endothelial cells lying within the choroid, but apparently not part of vessels (compare Figs. 1c, 4c). Choriocapillaris vessels are perfused neither by dextran nor by peroxidase. **Dotted line:** the plane of separation when the retina is removed from the flatmount.
treatment, dextran perfusion of the choriocapillaris was usually not detectable (Fig. 1b). If the choriocapillaris were perfused, the typical pattern would be the same as that in Figure 2. Thus, the use of dextran for demonstration of CNV in laser scars in the rat should be regarded cautiously.

In contrast to the choriocapillaris, retinal vessels were always well perfused by dextran, demonstrating that the intravenous injection technique succeeded. Separately prepared retinas demonstrated the same regular perfusion as flatmount preparations with overlying retina. Within the retina, dense networks of vessels were found when focusing through the different planes.

Because the choriocapillaris vessels are not perfused but the retinal vessels are, it is likely that the circular vessels visualized in laser scars by dextran angiography come from the retina. This finding is supported by the results of peroxidase perfusion in the living rat (discussed later).

The background fluorescence in the center of the scars is most likely explained by an autofluorescence due to formation of lipofuscin-like material after laser photocoagulation-induced damage. This is indicated by laser scars showing autofluorescence without dextran perfusion. Under the FITC filter it can be seen as green-orange fluorescence.

For the demonstration of vessels within laser scars as well as the choriocapillaris vasculature, immunohistochemical staining with a PECAM-1-antibody, a classic marker for endothelial cells, is more helpful. PECAM-1 is an integral membrane protein on endothelial cells that mediates cell-to-cell adhesion.

In accordance to the results of dextran angiography, vessel-forming endothelial cells in laser scars were present, predominantly at the margin (Fig. 2a), but there were also flat endothelial cells in the middle of the scars. Measurement of the fluorescence signal makes it possible to obtain a semiquantitative evaluation of the vasculization. However, it should be performed under the same conditions and the same physiological state. In contrast to the angiography, the endothelial cell marker gives a detailed picture of the choriocapillaris vasculature (Figs. 2a, 2b). This is also true of staining with alkaline phosphatase, a membrane-bound enzyme of endothelial cells, with which a good representation of neovascularization surrounding the laser scars (Fig. 6a) as well as the choriocapillaris vasculature (Fig. 6b), is possible. We did not observe the variation in enzyme expression and intense background staining that have been cited as objections to this technique.

To get a clearer picture of the regions of interest, histologic techniques were used (Fig. 4). The tight connection between the retina and RPE and therefore the choroid after laser treatment, in addition to the sinking of retinal structures toward the choroid, makes it comprehensible that within the area of the laser scar parts of the retina may adhere to the flatmount (compare Fig. 2a) when the retina is stripped off. The dotted lines in Figures 4a and 5 indicate the line of separation. This explains also the small patches of retinal tissue beneath the laser scar in Figure 2a. The enzyme product of intravenous injected peroxidase is visible as a black reaction product in the retinal vessels, whereas the choriocapillaris is not perfused in the same section (Figs. 4a, 4b). Peroxidase perfusion does not go beyond the large choroidal arterioles which appear to be constricted (Fig. 4a, arrowhead). Thus, the perfused vessels at the rim of the laser scars more likely originate from the retina rather than the choroid.

Generally peroxidase penetrates out of capillaries through transcytosis and intercellular clefts between endothelial cells. In our study, however, animals were killed before extravasation could take place, which was confirmed by electron microscopy.

In the center of the scars, flat endothelial cells were seen by light and electron microscopy which seemed not to form vascular tubes (Figs. 4a, 4c, 5). These cells were located within the choriocapillaris and correspond most likely to the endothelial cells in Figure 1c, shown in the center of the scars by immunohistochemistry. These cells are not visualized by dextran angiography. They are probably not arranged as vascular tubes with lumens, or they may be part of the choriocapillaris, which was not perfused by FITC-dextran. It is also possible that these endothelial cells are within the original walls of the choriocapillaris, representing a repair process. Hypertrophied, hyperosmotic endothelial cells with reduced lumina are reported as a feature of capillaries in injured tissue. These cells are thought either to come from the choroid or to derive from circulating precursors. Thus, experimentally induced neovascularization in laser scars may originate from choroidal and retinal vasculature, as outlined in the schematic drawing in Figure 5.

The explanation for nonperfusion of the choriocapillaris, even distant from the laser spots is not clear. Because the eyes were enucleated only 30 to 90 seconds after injection, the markers could not have already left the choriocapillaris. Because the retina was perfused, it can be excluded that the eyes were enucleated before choroidal filling. Laser photocoagulation influences the perfusion of the surrounding the choriocapillaris by capillary occlusion, and, as a result, perfusion of noncoagulated fundus areas should improve. This was not seen in our experiments, which showed no peroxidase-perfused vessels in the choriocapillaris, even at a distance from the laser spots. It has been reported that high molecular weight labels such as horseradish peroxidase and FITC can influence vascular transport; however, the retinal vessels were well perfused and showed no signs of obstruction.

Leakage of peroxidase out of the choriocapillaris was excluded by electron microscopic examination; leakage of dextran can be ruled out because of its high molecular weight. Another explanation could be that anesthesia influenced the choriocapillaris perfusion. Differences in blood flow regulation between retinal and choroidal microvasculature are known—for example, the role of NO in vascular autoregulation or the influence of varying intraocular pressures.

Whatever the reason for this nonperfusion of the choriocapillaris, the following conclusion can be drawn independently: When creating laser-induced neovascularization in an animal model of CNV, the observed vessels not only originate from the choroid, but also from the retina. When dextran angiography represents retinal vessels and not choroidal vessels, the relevance of this model as a model of ARMD has to be reconsidered.

Although different conclusions may be reached by other protocols, of the four tested methods used in these studies, the most useful and time saving was the immunohistochemical staining of endothelial cells by PECAM-1. The use of more than one method (for example FITC-dextran angiography and PECAM-1 immunohistochemistry) can contribute to better...
characterization and evaluation of vascularization after laser photocoagulation.

References


