Induction of endothelial cell proliferation in rat retinal venules by chemical and indirect physical trauma

Clark W. Deem, Sidney Futterman, and Robert E. Kalina

The capacity of retinal vessels to incorporate tritiated thymidine was studied in normal rats and in rats previously exposed to ocular trauma for the purpose of developing a model for the study of neovascularization in the retina. Needle puncture of the lens capsule caused intraocular inflammation and a 30- to 70-fold increase in the number of venular endothelial cells which incorporated tritiated thymidine. The time course of entry of venular endothelial cells into the S-phase of the cell cycle was studied following lens injury. An increase in the frequency of labeled cells was first detected six hours after lens injury and two hours after the appearance of leukocytes within the lumen of vessels. The number of endothelial cells in S-phase peaked sharply at eight hours and again at 40 hours following lens injury, indicating cell synchrony and a 32-hour duration for the complete cell cycle.

Key words: lens, lens injury, intraocular inflammation, cell division, retinal blood vessels, neovascularization, autoradiography.

Neovascularization occurs in the retina as a complication in diabetes mellitus,1 SS and SC hemoglobinopathies,2 and other less common disorders.3 The abnormal new vessels which arise are believed to originate from pre-existing retinal vessels, especially capillaries and venules.2 4 However, the cells of normal adult retinal vessels show virtually no mitotic activity.5 6 If an effective method for activating these non-cycling cells could be devised, it might provide an experimental model useful for study of the initial steps in neovascularization.

The mitotic activity of a cell population can be measured by autoradiography after exposure of the tissue to tritiated thymidine. Cells synthesizing DNA in preparation for mitosis will be labeled selectively. In this report, evidence is presented that certain physical or chemical insults to the rat eye are followed by the rapid onset of DNA synthesis in endothelial cells of the retinal vasculature.

Methods

The retinas of 350 to 400 gram male Sprague-Dawley rats were exposed to intravitreal tritiated
thymidine, specific activity 20 Ci per millimole (New England Nuclear Corporation), administrated while the animals were lightly anesthetized with ether and after surface anesthesia with topical 0.5 per cent proparacaine. Three micro-cytes of tritiated thymidine contained in a volume of 3 μl were injected intravitreally 2 mm. posterior to the limbus, using a 10 μl Hamilton syringe with a fixed 26-gauge needle, taking care to avoid lens injury. Alternatively, the tritiated thymidine was administrated systemically by intraperitoneal injection of 1 μCi per gram of body weight.

Animals were killed by ether overdose 30 minutes after exposure to tritiated thymidine, and their eyes promptly enucleated and fixed in 10 per cent neutral formalin for 24 hours. Retinas were removed and digested with 3 per cent trypsin, and the resulting vascular network was mounted on glass slides previously coated with alum-gelatin. The slides were treated with 0.5 per cent periodic acid, dipped in Kodak NTB-2 autoradiographic emulsion, and exposed for four days if the rats had received intravitreal thymidine or three weeks if they had been given systemic thymidine. Slides were developed with Kodak D-19, stained with Schiff's reagent, counterstained with hematoxylin, dehydrated in an ethanol series, coverslipped with xylene and permount, and examined by light microscopy. A cell was considered labeled if six or more silver grains were present over its nucleus. Labeled cells were categorized as either endothelial cells or intramural pericytes and according to location in a capillary (diameter less than 7 μm), postcapillary venule (diameter 7 to 15 μm), venule (diameter greater than 15 μm), or arteriole.

Eyes of additional groups of rats were physically or chemically insulted by one of several techniques: (1) the cornea was punctured centrally with a 30-gauge needle, allowing some loss of aqueous humor without touching the iris or lens. (2) The sclera was punctured 3 mm. posterior to the lateral corneoscleral limbus by passing a 26-gauge needle into the vitreous body. (3) A 3 μl volume of either 0.9 per cent sodium chloride, 5 per cent formic acid, 5 per cent depolymerized lactic acid, or 2 per cent trichloroacetic acid was injected into the vitreous by scleral puncture 3 mm. posterior to the lateral corneoscleral limbus using a 10 μl Hamilton syringe with a fixed 26-gauge needle, taking care to avoid touching the lens. (4) Retinal ischemia was produced with a suction opthalmodynamometer (fitted with a suction cup modified to fit the rat eye) so that the intraocular pressure was raised to and maintained at 500 mm. of mercury for periods of either five or ten minutes. Blanching of the red reflex occurred at a pressure of 100 to 110 mm. of mercury. (5) Capsular and cortical lens damage was produced by puncturing the lens with a 30-gauge needle either anteriorly through the cornea or posteriorly through the sclera 3 mm. posterior to the lateral corneoscleral limbus and puncturing until cataractous changes became apparent. Pupils had been dilated by the previously administrated topical anesthetic, allowing lens puncture without iris trauma. Four to 72 hours following each of these forms of ocular trauma, tritiated thymidine was administered and uptake was studied by autoradiography as described above.

Paraffin sections (5 μm) of selected eyes 24 or 72 hours after lens puncture were stained with hematoxylin and cosin and examined by light microscopy.

Results

Thymidine uptake in normal eyes. Essentially no labeling of capillary or arteriolar cells could be detected in normal rats following either intravitreal or systemic tritiated thymidine. However, a small number of venular (>15 μm in diameter) and postcapillary venular (<15 μm in diameter) endothelial cells did incorporate the

Table I. Effect of ocular trauma on the subsequent incorporation of intravitreal tritiated thymidine into endothelial cell nuclei of rat retinal venules. (Number of labeled cells per 1,000 cells counted)*

<table>
<thead>
<tr>
<th>Insult</th>
<th>Venule size</th>
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<tbody>
<tr>
<td></td>
<td>&gt;15 μm</td>
</tr>
<tr>
<td>None</td>
<td>0.66 ± 0.27</td>
</tr>
<tr>
<td>Puncture of cornea</td>
<td>0.62 ± 0.23</td>
</tr>
<tr>
<td>Puncture of sclera</td>
<td>1.04 ± 0.65</td>
</tr>
<tr>
<td>Injection, 0.9%</td>
<td>2.04 ± 1.81</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.36 ± 0.31</td>
</tr>
<tr>
<td>Ischemia for 5 min</td>
<td>0.10 ± 0.57</td>
</tr>
<tr>
<td>Ischemia for 10 min</td>
<td>8.7 ± 3.6</td>
</tr>
<tr>
<td>Injection, 2% trichloroacetic acid</td>
<td>30.3 ± 11.8</td>
</tr>
<tr>
<td>Injection, 5% lactic acid</td>
<td>27.8 ± 14.4</td>
</tr>
<tr>
<td>Injection, 5% formic acid</td>
<td>22.0 ± 4.1</td>
</tr>
<tr>
<td>Lens puncture, anterior</td>
<td>19.6 ± 4.2</td>
</tr>
<tr>
<td>Lens puncture, posterior</td>
<td>19.6 ± 4.2</td>
</tr>
</tbody>
</table>

*Each value represents pooled results from at least three experimental animals. In each retinal preparation from 4,000 to 6,000 venular cells were counted.

Tritiated thymidine was injected 42 hours following trauma.
label into DNA during a brief exposure (Table I). In six retinas of the control group, eleven labeled endothelial cells were found among 19,410 counted in large venules, and two labeled cells out of 13,590 counted in small venules. In the capillaries of each retina no labeled cells were found among the more than 50,000 endothelial cells or 35,000 intramural pericytes counted, giving label frequencies, respectively, of <0.02 and <0.03 cells per thousand. The distribution of labeled cells was identical for systemic or intravitreal administration of tritiated thymidine. Following systemic administration, grain counts over labeled nuclei numbered 10 to 30, compared with 25 to 100 observed over labeled nuclei after intravitreal injection. Because shorter exposure times could be used and smaller quantities of isotope were required, tritiated thymidine was administered intravitreally in subsequent experiments.

**Thymidine uptake after ocular trauma.**

After puncture of the globe through the cornea or sclera, injection of 0.9 per cent sodium chloride, or production of ocular ischemia, there was no significant increase in the number of venular endothelial cells that became labeled after exposure to tritiated thymidine (Table I). In each of these experiments, the retinal vessels appeared normal and no infiltration of leukocytes or other sign of intraocular inflammation was apparent. However, intravitreal injection of 5 per cent lactic acid, 5 per cent formic acid, or 2 per cent trichloroacetic acid, or puncture of the lens either anteriorly through the cornea or posteriorly through the sclera caused a marked increase in the labeling of venular endothelial cells by thymidine (Table I), and aggregations of mononuclear and polymorphonuclear leukocytes within the lumina of venules.

Damage to the lens was followed by a greater than 30-fold increase in the number of endothelial cells of retinal venules that became labeled by exposure to tritiated thymidine 42 hours following the trauma (Table I). This effect could be reproduced consistently regardless of direction of lens puncture. The increase in labeling of endothelial cells after any form of trauma occurred only in venules and postcapillary venules, with cells of the capillary net and arterioles remaining conspicuously inactive (Figs. 1 and 2). Labeled endothelial cells were encountered generally at random along the length of retinal venules from the optic disc to the periphery, with higher labeling frequencies in venules of larger caliber (Table I). A decrease in the frequency of labeled venular endothelial cells within 1 mm. of the ora serrata is probably explained by the relative absence of large caliber venules (>15 μ) in this area. An exception to the random pattern of venular labeling is the presence of scattered closely adjacent pairs of labeled venular endothelial cell nuclei in a venule and postcapillary venular tributary of a rat retina isolated 30 minutes following an intravitreal injection of tritiated thymidine given 42 hours after lens puncture. Cells of the capillary network and arteriole remain unlabeled. (×100.)
Endothelial cell proliferation in retinal venules

Fig. 2. Tritium-labeled endothelial cell nuclei in a retinal venule from a rat retina isolated 30 minutes following an intravitreal injection of tritiated thymidine given 42 hours after lens puncture. (×400.)

Fig. 3. Two closely adjacent pairs of labeled endothelial cell nuclei in a venule from a rat retina isolated 30 minutes following an intravitreal injection of tritiated thymidine given 42 hours after lens puncture. (×250.)

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Fig. 3. Two closely adjacent pairs of labeled endothelial cell nuclei in a venule from a rat retina isolated 30 minutes following an intravitreal injection of tritiated thymidine given 42 hours after lens puncture. (×250.)

Endothelial cells (Fig. 3). Mononuclear and polymorphonuclear leukocytes were present within the lumina of retinal vessels 42 hours after lens injury.

Occasional small intraocular hemorrhages were unavoidable but were not associated with alterations in the amount of labeling. Lens puncture resulted in the rapid swelling of the lens followed by mature cataract formation as early as eight hours and invariably by 42 hours. Histologic sections of eyes fixed 24 hours after lens trauma revealed rupture of the lens capsule with marked swelling of the lens and partial herniation of lens cortex through the capsular rupture. An inflammatory process characterized by a mixed polymorphonuclear and mononuclear cellular response and a fibrinous exudate involved lens, iris, ciliary body, anterior chamber, and vitreous body. Eyes fixed 72 hours after lens injury revealed similar but considerably decreased evidence of inflammation. Some eyes contained moderate numbers of red blood cells in the vitreous.

Time course of entry of venular endothelial cells into S-phase of cell cycle. The first effect observed following anterior lens puncture was the appearance after four hours of mononuclear and polymorphonuclear leukocytes within the lumina of retinal vessels, especially larger veins. Increased thymidine uptake was first noted within endothelial cells of venules at six hours, peaking at eight hours, falling back to near control levels by 32 hours, and peaking again at approximately 40 hours (Fig. 4). The number of venular endothelial cells which incorporated tritiated thymidine decreased slowly thereafter. In-
intraluminal aggregation of leukocytes was maximal at 16 to 24 hours following trauma, decreased gradually thereafter, and was nearly absent by 72 hours. There was no increase in capillary or arteriolar labeling during this time, and labeled capillary intramural pericytes were not observed.

Discussion

Virtually all cells of adult rat retinal blood vessels, like those of the mouse, appear to be of the noncycling type. As a consequence of some forms of ocular trauma, a few of these cells become "un-blocked," incorporating tritiated thymidine into DNA and, thereafter, presumably enter mitosis. The susceptible cell population appears to be limited to endothelial cells of the large and small venules.

The biphasic course of DNA synthesis observed after ocular trauma suggests that cell proliferation is sustained through at least two generations. Endothelial cells synthesizing DNA at eight hours presumably undergo subsequent mitosis at 24 to 32 hours during the period in which the drop in labeling is observed. Some daughter cells would appear to remain in the active cell cycle, accounting for the sharp increase in tritiated thymidine uptake at 40 hours. The time interval between these peaks of DNA synthesis indicates a duration for the complete cell cycle of about 32 hours. The appearance of closely adjacent pairs of labeled cells at 40 hours suggests that they are daughter cells resulting from a parent endothelial cell which had previously undergone mitosis at that site.

Since intravitreal injections of acids stimulate venular endothelial cell DNA synthesis in the absence of lens damage, it is unlikely that lens injury releases an agent with specific mitogenic effect. However, puncture of the lens during intravitreal injection will interfere with analysis of mitogenic effects of injected agents. In a study in which intravitreal lactic acid injections provoked retinal vasoproliferation, it was not stated that evidence of lens damage was sought and excluded.

Thymidine incorporation which follows lens damage and is associated with intravitreal acid injection is preceded by intravascular aggregation of leukocytes. It is not yet clear whether the activation of DNA synthesis in venular endothelial cells is related to an agent originating in the damaged ocular tissues which has been carried to the retina by meridional flow or to a substance released by the abundant leukocytes or to a combination of these and perhaps other factors. If activated cells continue to divide, neovascularization may result. It remains to be determined whether or not cells once activated will
demonstrate continuing increased mitotic activity after the leukocyte response has subsided.

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REFERENCES