Cell proliferation in response to vitreous hemoglobin

Janice M. Burke, Eva Sipos, and Harold E. Cross

Rabbits were examined at intervals to 90 days after receiving two or three intravitreal injections, on consecutive days, of homologous hemoglobin or saline. Cell proliferation in the vitreous was assessed by scintillation counting and radioautography after intravitreal administration of \( ^3H \)-thymidine 4 hr prior to sacrifice. Two populations of vitreous cells phagocytize the vitreous hemoglobin and are stimulated to DNA synthesis. Cells that migrate into the vitreous in response to hemoglobin also contribute to total \( ^3H \)-thymidine uptake. Tritiated thymidine incorporation peaks between 5 to 10 days and again between 22 to 30 days after the first administration of hemoglobin. By 45 to 60 days after two injections and 90 days after three injections the vitreous cell proliferative activity has returned to normal. It is concluded that a bleeding event which leads to the release of hemoglobin in the vitreous stimulates a minor, transient vitreous cell proliferation and a more significant, but also transient, migration of cells into the vitreous. Aside from contributing by phagocytosis to vitreal clearing, no other functions have been ascribed to these cells.

Key words: vitreous, vitreous hemorrhage, phagocytosis, hyalocyte, cell proliferation

Both fibrinolytic and phagocytic responses are important in the removal of intravitreal blood. Both fibrinolytic and phagocytic responses are important in the removal of intravitreal blood. 3, 11, 12 The major population of vitreous cells, the hyalocytes, act as macrophages. 13-16 Intravitreal blood may stimulate this cell population to phagocytosis as well as promote the ingress of macrophages from extravitreal sources. 1, 4, 17 There is an increase in cells in the vitreous as a result of hemorrhage, but it is not known whether intravitreal proliferation contributes significantly to this change in cell number. Although vitreous hyalocytes can presumably divide, they do so only rarely under normal conditions. 18, 19

In this report we examine the magnitude and duration of the proliferative response to intravitreal hemoglobin.

Materials and methods

Seventy-two albino rabbits weighing 2 to 4 kg were used for these studies. The animals were anesthetized by intramuscular injection of fen-
tanyl/droperidol (Innovar-Vet; Pitman-Moore, Inc., Washington Crossing, N. J.) and by topical application of proparacaine hydrochloride (Alcaine; Alcon Laboratories, Inc., Fort Worth, Texas) prior to the ocular injections.

Hemoglobin, rather than whole blood, was used to mimic vitreous hemorrhage so as to avoid the introduction of blood cells. Homologous hemoglobin was prepared from four donor rabbits by the method of Regnault and sterilized through a 0.22 μm Millipore filter. To avoid intraocular pressure changes associated with the introduction of large volumes, the animals were given two or three intravitreal injections of 0.05 ml of hemoglobin (6.9 to 8.2 mg/0.05 ml) on consecutive days. The contralateral control eye received a similar volume of sterile saline. Injections were delivered with a 26-gauge needle inserted 3 to 4 mm posterior to the limbus, with the needle tip directed posteriorly to avoid damage to the lens. At intervals after the injections and just prior to sacrifice, the eyes were examined by indirect ophthalmoscopy after mydriasis with 1% atropine sulfate.

Four hours prior to termination, the rabbits received intravitreal injections of 12.5 μCi of 3H-thymidine (New England Nuclear; sp. act. 2 Ci/mmol) in 0.05 ml of sterile saline. The animals were sacrificed at intervals to 90 days by an intracardiac injection of secobarbitol (Repose; Diamond Laboratories, Des Moines, Iowa). In 52 animals the vitreous was removed by aspiration through an 18-gauge needle or by dissection via a circumferential incision at the ora serrata. Since the attachment of the uncompromised vitreous to the retina is firm in young rabbits, these procedures allow analysis predominantly of cells found in the central vitreous. The cells in each vitreous sample were pelleted by centrifugation and washed with phosphate-buffered saline to remove unincorporated label. Smears were made from a small fraction for radioautography with Kodak NTB2 liquid emulsion. The slides were exposed for 4 to 6 days and stained with hematoxylin and eosin. Smears that were not coated with emulsion were also prepared from both normal and injected vitreous for improved light microscopic observation of cell morphology. These smears were air-dried or fixed either in phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μm, and prepared for radioautography by the same procedure as for the vitreous smears. The number of labeled vitreous cells within approximately 100 μm of the vitreoretinal junction were counted at a magnification of 400× and expressed as number of labeled cells per microscope field diameter.

Results

Indirect ophthalmoscopy. After two or three hemoglobin injections on successive days, the eyes exhibited a dull red reflex which became brownish by day 4. A dense gray mass formed in the retrolental area on days 5 to 7. Strands and dark, flocculent material could be seen nasally and temporally. By days 7 to 10 the mass appeared as a dense gray to yellowish opacity that obscured other intraocular structures. The mass became smaller and less dense after day 30, with strands and flocculence at its periphery. The fundus was visible by day 45 in the eyes receiving two injections and by day 60 in those receiving three injections. By day 90 the fundus was clearly visible in all eyes, and the opacities were replaced by diffuse strands or membranes with some dark, flocculent material. There were variable signs of iris and/or perilimbal inflammation at early time periods.

There were no vitreous changes in the saline-injected control eyes nor any evidence of induction of intraocular hemorrhage. Both control and experimental eyes demonstrated a variable focal inflammation at the site of repeated injection.
Histology and radioautography. Although few cells were recovered from the central vitreous of uninjected eyes, two types could be seen in smear preparations of this tissue (Fig. 1). The nucleus of one cell type was usually dense and lymphocyte-like but might also be lobulated to resemble the cells described below that are common after vitreal injury. Granules could sometimes be seen in the narrow rim of cytoplasm, suggesting that these cells represent the vitreal hyalocytes found in tissue section. The other cell type had a large oval pale-staining nucleus and an expansive, poorly defined cytoplasm. No additional morphologic features or distinguishing tinctorial properties of these cells were revealed by the Papanicolaou staining method (Fig. 2).

Effort to avoid contamination by extravitreal tissues leads to dissection of a variable number of cells in each vitreous sample after hemoglobin injection. For this reason it was difficult to make quantitative estimates of increases in cell number. Examination of smears indicated that most cells of the injured vitreous (Fig. 2) were indistinguishable from the hyalocyte type, with lobulated nuclei seen in untreated vitreous. Large numbers of cells were found in tissue section by day 4 in the anterior vitreous near the ciliary body.22

Both the hyalocyte-like and the larger cells of the injured vitreous could contain hemoglobin-filled phagocytic granules (Fig. 3). The granules were larger and more frequently seen in the hyalocyte-like cell. Both types of vitreous cells also labeled with \(^{3}\)H-thymidine (Figs. 4 and 5). Cell counts of vitreous smears indicated that at a maximum (day 7 after three injections), 1% to 2% of the vitreous cells incorporated the DNA precursor during a 4 hr labeling period. No labeled nuclei were seen in any binucleate or multinucleate cells.

Intravitreal hemoglobin also stimulated DNA synthesis in other intraocular tissues, as evidenced by the presence of \(^{3}\)H-thymidine-labeled nuclei in the ciliary epithelium, its subjacent connective tissue, throughout the retina, and in the retinal pigmented epithelium.22

\(^{3}\)H-thymidine incorporation. There is some individual variation in vitreous \(^{3}\)H-thymidine incorporation levels between rabbits, but measurements of both untreated eyes of an individual yield similar results (Table I). Intravitreal saline promotes no significant change in \(^{3}\)H-thymidine incorporation as compared to untreated vitreous (\(^{3}\)H for untreated vitreous: 3.7 ± 0.8 cpm/cell × 10^5; for saline-injected: 4.2 ± 0.6).

Samples in which examination of vitreous smears revealed no contamination by extravitreal tissues were used for quantitative evaluation of \(^{3}\)H-thymidine incorporation. Changes in isotope uptake after intravitreal hemoglobin injection are given in Fig. 9. There are two apparent waves of proliferation after two or three hemoglobin doses. The first peak of DNA synthesis after three injections is slightly earlier and of a greater magnitude than after two injections. The second

<p>| Table I. (^{3})H-thymidine incorporation, untreated vitreous |
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<table>
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<tr>
<th>Rabbit vitreous</th>
<th>(^{3})H(cpm/cell × 10^5)</th>
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<tbody>
<tr>
<td>1. Right eye</td>
<td>5.6</td>
</tr>
<tr>
<td>Left eye</td>
<td>5.6</td>
</tr>
<tr>
<td>2. Right eye</td>
<td>1.2</td>
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<td>Left eye</td>
<td>1.1</td>
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<tr>
<td>3. Right eye</td>
<td>4.5</td>
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<td>Left eye</td>
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saline-treated vitreous. Except for one vitreous sample from day 22, PMNs were absent after day 10.

Binucleate cells with voluminous, pale, eosinophilic cytoplasm were seen in random saline- or hemoglobin-injected eyes (Fig. 7). Large multinucleate giant cells were found in vitreous smears at later stages after hemoglobin administration (Fig. 8). No labeled nuclei were seen in any binucleate or multinucleate cells.

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Fig. 1. Unusually dense cluster of cells in a smear of normal vitreous. Single arrows indicate the large, pale-staining cells, and double arrows indicate the smaller, hyalocyte-like cells. (×400.)

Fig. 2. Smear of vitreous on day 4 after two hemoglobin injections stained by the Papanicolaou method. Most cells after injury contain a lobulated nucleus, and intermediates can be seen relating them to the smaller hyalocyte-like cells (double arrows). The larger vitreous cells are indicated (single arrows). (×360.)

Fig. 3. Radioautograph of a vitreous smear on day 5 after three hemoglobin injections. Both the larger (arrow) and the hyalocyte-like cells (double arrows) contain hemoglobin-filled phagocytic granules. None of these cells is labeled with \(^{3}H\)-thymidine. (×680.)

Fig. 4. Radioautograph of a vitreous smear on day 5 after three hemoglobin injections, showing a labeled nucleus in a large vitreous cell (arrow). An unlabeled hyalocyte-like cell is also shown. (×775.)

Fig. 5. Radioautograph of a vitreous smear on day 5 after three hemoglobin injections, showing a labeled hyalocyte-cell. (×775.)

Discussion

In smears of the central portion of untreated rabbit vitreous, two populations of cells are identified. One of these cell types is the macrophage-like hyalocyte that has been described in the vitreous of several species. The other larger cell type has not been previously identified. Vitreous cells other than hyalocytes have been found (fibrocytes, plasma cells, "hypertrophic glial cells"), but the appearance of the larger cell type described here is not consistent with that of any of these. The lack of morphologic intermediates relating the larger cells to the hyalocyte-like cells suggests they are differ-
ent cell types. However, we cannot rule out the possibility that the two morphologies are indicative of different physiologic states of the same cell type, especially in view of the observation that some populations of both cells respond by phagocytosis and DNA synthesis to the presence of intravitreal hemoglobin. It is not surprising that the larger cells have not been previously characterized, since electron microscopic analyses have concentrated on the more cellular cortex rather than the central vitreous. No cells resembling the larger cells of the vitreal smears are visible in tissue section when the eye is fixed whole for light microscopy, but the central vitreous is poorly preserved by these techniques. We are presently undertaking an electron microscopic examination of the cells of the normal central rabbit vitreous.

The ophthalmoscopic appearance of the eye after intravitreal injection of hemoglobin is similar to that observed by others following hemoglobin or whole blood injection in the rabbit. The resolution process after the intravitreal administration of hemoglobin differs from that after whole blood injection in that after hemoglobin injection the steps of hemolysis and fibrinolysis are bypassed. Early stages of clearing of the hemoglobin (or breakdown products) presumably involve diffusion, either anteriorly via the ciliary processes and iris stroma or posteriorly through the inner limiting lamina into the retina. Despite early differences the vitreous cellular response to hemoglobin, as indicated by light microscopy of smears and sections of whole eyes, is similar to the response to whole blood, both in terms of cell type and time frame. As after whole blood administration, an early PMN influx is not a consistent or dominant vitreous response to hemoglobin. Most of the immigrating cells are of the monocytic type indistinguishable in smear preparations from the resident vitreous macrophages (hyalocytes). As after whole blood injection, multinucleate macrophages can be found in the vitreous treated with hemoglobin. The infrequent binucleate cells described here may result from modulation of vitreous macrophages. Although they were not seen in untreated vitreous, they can be found in saline-injected vitreous.

Smears of vitreous demonstrate that the membranes and masses seen ophthalmoscopically after hemoglobin injection consist of a coalescence of cells and hemoglobin. Al-
Fig. 9. Changes in \(^{3}H\)-thymidine incorporation in cells of the central vitreous as determined by scintillation counting of samples after two (circles and solid line) or three (triangles and broken line) intravitreal injections. The data are related to cell number in the sample, and counts for each hemoglobin-injected eye are individually corrected for the counts from the contralateral saline-injected control eye. There are two waves of incorporation, the magnitude and duration of which are affected by the number of doses of hemoglobin received.

Fig. 10. Number of \(^{3}H\)-thymidine-labeled nuclei found in the vitreous cortex after three intravitreal injections of hemoglobin. The number of nuclei was determined by microscopic examination of the vitreoretinal juncture as described in Methods. Each point represents the mean of counts from two to five eyes.

Though the masses break down in time, some fine strands containing cells with hemoglobin-laden phagocytic vacuoles may remain for extended periods. As has been previously suggested,\(^4\) \(^ {23}\) the vitreous response that leads to clearing can become quiescent before the process is complete.

The data concerning vitreous \(^{3}H\)-thymidine incorporation indicate the proliferative capacity of both resident and invasive cells. Isotope incorporation into the larger vitreous cells provides evidence that resident cells can undergo DNA synthesis in response to hemoglobin. Although the hyalocytes cannot be distinguished from invasive monocytic cells, the labeling of cells lying very near the vitreoretinal junction at early time periods after hemoglobin administration suggests that the resident cells of the hyalocyte-type are also stimulated to DNA synthesis.

The major peaks of vitreous \(^{3}H\)-thymidine incorporation correlate in time with the major waves of monocytic immigration.\(^4\) This observation implies that some of the population of immigrating cells are actively proliferating. However, proliferation makes a minor contribution to the increase in vitreous cell number after hemoglobin administration, since only a small fraction of the cells incorporate \(^{3}H\)-thymidine. A similar proliferative response of immigrating vitreous cells was observed by Gloor\(^19\) after photocoagulation of the rabbit retina.

In the absence of other pathological conditions, the cellular response in the vitreous to either hemoglobin or whole blood is a transitory one. Aside from their contribution to
vitreal clearing, other effects of vitreous cells after hemorrhage are unknown. The presence of intravitreal blood components is associated with changes in the physical structure of the vitreous and with proliferative changes in cells of adjacent tissues such as the retina. The vitreous cells are presently being investigated to determine if they contribute to matrix degradation and/or mediate extravitreal proliferation.

REFERENCES