Limiting the Proliferation and Reactivity of Retinal Müller Cells During Experimental Retinal Detachment: The Value of Oxygen Supplementation

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• PURPOSE: To assess the role of hypoxia in inducing the proliferation, hypertrophy, and dysfunction of Müller cells in detached retina and the effectiveness of supplemental oxygen in limiting these reactions.

See also pp. 155–164 and 231.

• METHODS: Retinal detachments were produced in the right eye of each of 13 cats; the cats survived surgery for 3 days, during which six were kept in normoxia (room air, 21%) and seven in hyperoxia (70% oxygen). Retinas were labeled for proliferation with an antibody (MIB-1) to a cell cycle protein (Ki-67), for evidence of hypertrophy employing antibodies to the intermediate filament protein glial fibrillary acidic protein (GFAP) and to β-tubulin and for disturbance of glutamate neurochemistry employing antibodies to glutamate to a glutamate receptor (GluR-2) and to glutamine synthetase.

• RESULTS: Results from the two animals kept in normoxia after retinal detachment confirmed previous reports that detachment caused the proliferation of Müller cells, the hypertrophy of Müller cell processes, and the disruption of glutamate recycling by Müller cells. Oxygen supplementation during detachment reduced Müller cell proliferation and hypertrophy and reduced the abnormalities in the distributions of glutamate, GluR-2, and glutamine synthetase.

• CONCLUSIONS: Oxygen supplementation reduced the reaction of retinal Müller cells to retinal detachment, limiting their proliferation and helping to maintain their normal structure and function. In the clinical setting, oxygen supplementation between diagnosis and reattachment surgery may reduce the incidence and severity of glial-based complications, such as proliferative vitreoretinopathy. (Am J Ophthalmol 1999;128:165–172.

A MAJOR COMPLICATION OF RETINAL DETACHMENT is the development of proliferative vitreoretinopathy even after seemingly successful reattachment. The reattached portion of retina becomes a focus for glial scarring, which spreads into the surrounding retina, including regions not previously detached.[1,2] One factor in proliferative vitreoretinopathy may be the response of retinal glial cells, particularly Müller cells, to detachment.[3]

The Müller cell response resembles the "reactive" response of astrocytes to injury of other parts of the central nervous system and involves three principal components. The Müller cells proliferate, with the rate of their proliferation rising sharply to a maximum within 3 to 4 days of retinal detachment but continuing for weeks or months.[4,5] The processes of Müller cells hypertrophy and grow abnormally into the vitreous humor and along the outer surface of the retina, between the photoreceptors and the retinal pigment epithelium.[2,6–9] In addition, detachment changes the distributions of amino acid transmitters within the retina.[10,11] It was hypothesized that the glial changes induced by detachment are caused by hypoxia and hypo-

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glycemia that result when the outer layers of retina are separated from the choriocapillaris, their source of nutrients. Evidence has been found that supplementing the oxygen available to the retina during retinal detachment limits the degeneration of photoreceptors.[12] In these experiments, we tested whether supplementing the oxygen available to the outer retina, by enriching the air inspired to 70% oxygen, would mitigate the proliferative response of retinal glia.

METHODS

THE STUDY WAS PERFORMED USING ADULT CATS (*FELIS domesticus*), and protocols were approved by the Animal Ethics Committee of the University of Sydney and the Animal Care Council of the University of California Santa Barbara. All procedures conformed to the standards set forth by the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgery to produce experimental detachment of the retina, animal management, and the conditions of oxygen management, have been described.[12] The detachments were all of 3 days' duration. Observations were made in a total of 13 animals, of which six remained in room air (that is, 21% oxygen) after the detachment had been made, and the other seven were kept in air enriched with oxygen to 70%.

Cryostat, Vibratome (Technical Products International, Polysciences, Warrington, Pennsylvania), and wax sections were employed to analyze the retinal tissue. The techniques used to prepare and label cryostat and Vibratome sections have been described.[13]

The primary antibodies involved in this study were anti-glial fibrillary acidic protein (GFAP; Dako, Carpinteria, California), 1:500; anti-Ki67 (MIB-1) (Immunotech, Inc, Westbrook, Maine), 1:100; anti-β tubulin (gift of Dr M. Klymkowsky, University of Colorado, Boulder, Colorado), 1:1,000; antiglutamate (gift of Dr R. Marc, University of Utah, Salt Lake City, Utah) 1:100; anti-GluR-2 (Chemicon, Temecula, California), 5 μg per ml; and antiglutamine synthetase (gift of Dr P. Linser, Whitney Laboratories, St Augustine, Florida), 1:600. Secondary antibodies conjugated to Cy2 or Cy3 were employed at 1:200 (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania).

Proliferating cells in detached retina were fixed in wax to obtain thin sections for quantitation. Sections were cut at 4 μm, dewaxed in xylenes, rehydrated, and steamed for 20 minutes in a 10-mM citrate buffer (pH 6) to increase antigenicity.[5] Sections were blocked in 2% bovine serum albumin in phosphate-buffered saline (PBS) for 30 minutes and incubated in the primary antibody (MIB-1, or anti-Ki67) for 2 hours and in the biotin-conjugated secondary antibody for 1 hour. Endogenous peroxidases were quenched by incubation with 0.3% hydrogen peroxide in PBS for 15 minutes followed by incubation in avidin-horseradish peroxidase for 1 hour. Finally, after being rinsed in PBS, the tissue was incubated in diaminobenzidene and 0.02% hydrogen peroxide in PBS for 15 minutes to yield a brown precipitate.

MIB-1 recognizes a large nuclear-associated protein (Ki-67) that is present in all stages of the cell cycle except G_0, thereby labeling all proliferating cells.[5]

The observations regarding MIB-1 and antibodies for glutamine synthetase, glutamate, and β-tubulin were made on four animals kept in room air and four kept in hyperoxia; the observation of GFAP labeling was made in four animals kept in room air and seven kept in hyperoxia.

Labeling was compared for each of the markers between areas of retina in the same section, with one region from detached retina and another from retina still attached. The detachments were made in the midperipheral retina, well away from the area centralis and the peripheral margin of the retina. To quantify proliferation, the number of MIB-1^+ profiles were counted based on millimeter of retinal length, employing a calibrated ocular micrometer. Counts were made in three cases of normoxic detachment and four cases of hyperoxic detachment, with counts for attached and detached regions recorded separately. In each case, counts were averaged from a total of six sections, three from each of two separate areas along the available series of sections.

RESULTS

PROLIFERATING CELLS WERE RECOGNIZED BY THEIR LABELing with the MIB-1 antibody. Labeled nuclei were extremely rare in attached retina (Figure 1A), but they were numerous in retina detached in normoxia (Figure 1B). As previously reported, most proliferating cells were confined to the inner nuclear layer,[4,5] and their numbers and distribution within the layer suggest that most if not all are the nuclei of Müller cells. MIB-1^+ cells were also seen in hyperoxic detached retina, again concentrating in the inner nuclear layer (Figure 1C). However, their numbers were much lower than in normoxic detached retina (compare Figure 1, B and C). These trends are shown quantitatively in Figure 1D, which summarizes counts over several sections in three normoxic and four hyperoxic detachments. The mean frequency of MIB-1^+ cells in hyperoxic detached retina was 12% of that in normoxic detached retina. On a two-tailed *t* test, the probability that the normoxic and hyperoxic samples could have been drawn from the same sample was low ($P < 0.002$).

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein normally prominent only in astrocytes. In the attached retina, GFAP was prominent in astrocytes at the inner surface and could be detected in the radial processes of Müller cells near the inner surface of the retina

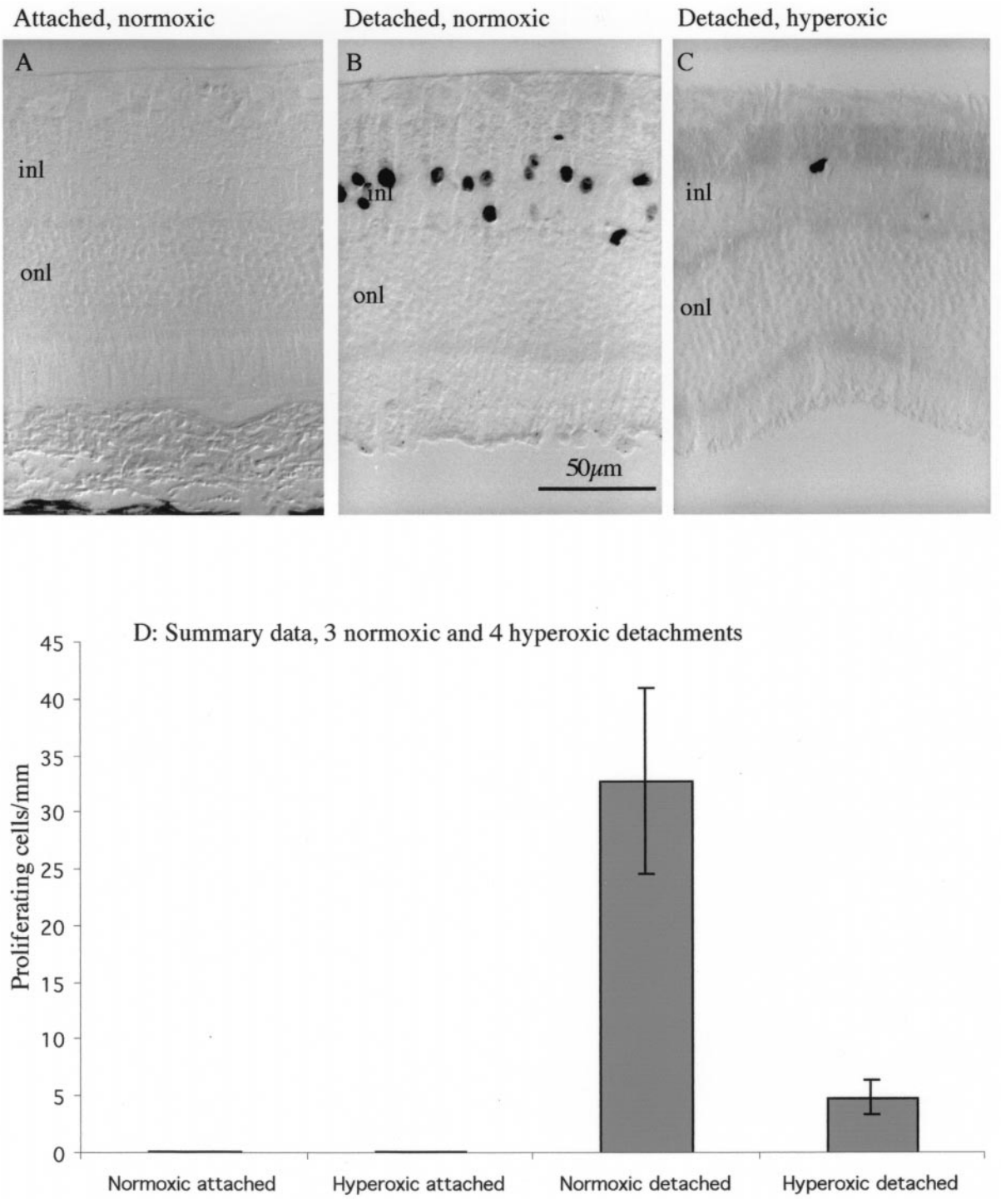

FIGURE 1. Effect of oxygen supplementation on proliferation in detached retina. MIB-1–labeled cells (proliferating cells) appear dark. (A) MIB-1 labeled cells were rare in attached retina. (B) MIB-1 labeled cells were numerous in normoxic detached retina. They concentrated in the middle regions of the inner nuclear layer, suggesting that they are Müller cells. (C) MIB-1 labeled cells were present in hyperoxic detached retina but in low numbers. inl = inner nuclear layer, onl = outer nuclear layer. (D) Means and SDs for the frequency of MIB-1 labeled cells in attached and detached retina. For normoxic detached retina, the mean number of labeled cells per mm was 32.8 (SD 8.2) and for hyperoxic detached retina, 4.9 (SD 1.5). On a *t* test the probability that normoxic detached and hyperoxic detached counts were drawn from the same population was low ($P < .002$.)

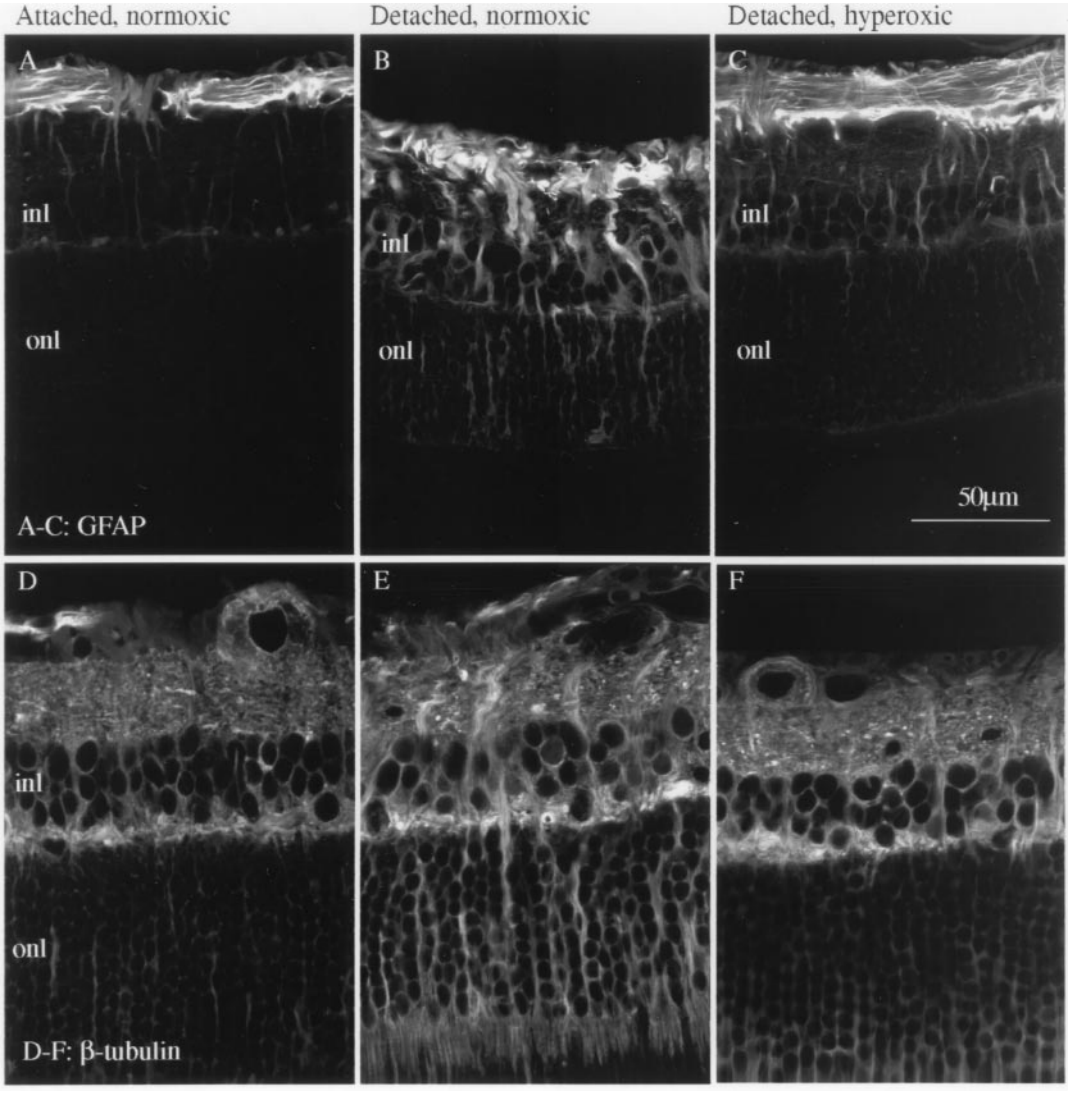

FIGURE 2. Effects of oxygen supplementation on intermediate filaments and microtubules of Müller cells in detached retina. Immunolabeling for GFAP in (A) normoxic attached retina, (B) normoxic detached retina, and (C) hyperoxic detached retina. GFAP-labeled structures appear white. Immunolabeling for β-tubulin in (D) normoxic attached retina, (E) normoxic detached retina, and (F) hyperoxic detached retina. Beta-tubulin–labeled structures appear white. inl = inner nuclear layer; onl = outer nuclear layer.

(Figure 2A). After detachment in normoxia, GFAP labeling of Müller cells was considerably more prominent, confirming previous studies[8,13] and enabling their radial processes to be traced as far as the outer limiting membrane (Figure 2B). Hyperoxia during detachment reliably reduced this upregulation of GFAP in Müller cells, but it did not eliminate it (Figure 2C).

Beta-tubulin is a major component of microtubules. In attached retina (Figure 2D) β-tubulin was present in Müller cells, allowing some of their processes to be identified. After detachment in normoxia (Figure 2E) the β-tubulin labeling of Müller cell processes was increased. The processes also appeared swollen and hypertrophic, which confirmed previous findings.[14] Hyperoxia during detachment reduced but did not eliminate the upregulation of β-tubulin in Müller cells and the hypertrophy of their processes (Figure 2F).

In normal attached retina, glutamate was prominent in neurones, such as the large ganglion cell at the top of Figure 3A, and did not label strongly in the radial processes of Müller cells, confirming previous work.[10] In normoxic detached retina (Figure 3B), glutamate became prominent in Müller cell processes.[11] Oxygen supplementation during detachment (Figure 3C) prevented the accumulation of glutamate in Müller cells and preserved its presence in neurones.

The glutamate receptor GluR-2 was prominent in attached retina only in the inner end feet of Müller cells

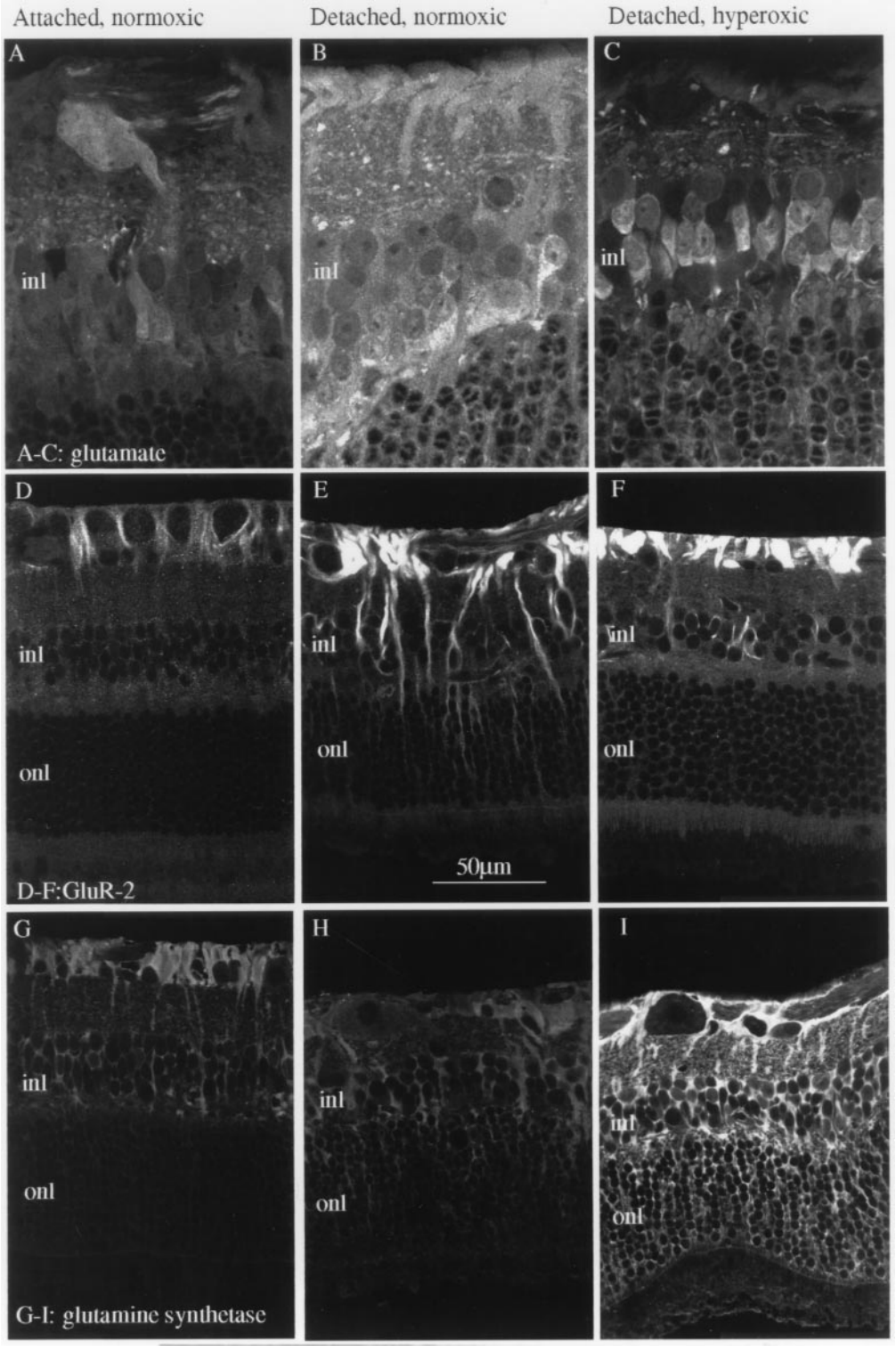

FIGURE 3. Effects of oxygen supplementation on glutamate neurochemistry in detached retina. Immunolabeling for glutamate in (A) normoxic attached retina, (B) normoxic detached retina, and (C) hyperoxic detached retina. Immunolabeling for the glutamate receptor GluR-2 in (D) normoxic attached retina, (E) normoxic detached retina, and (F) hyperoxic detached retina. Immunolabeling for glutamine synthetase in (G) normoxic attached retina, (H) normoxic detached retina, and (I) hyperoxic detached retina. inl = inner nuclear layer; onl = outer nuclear layer.

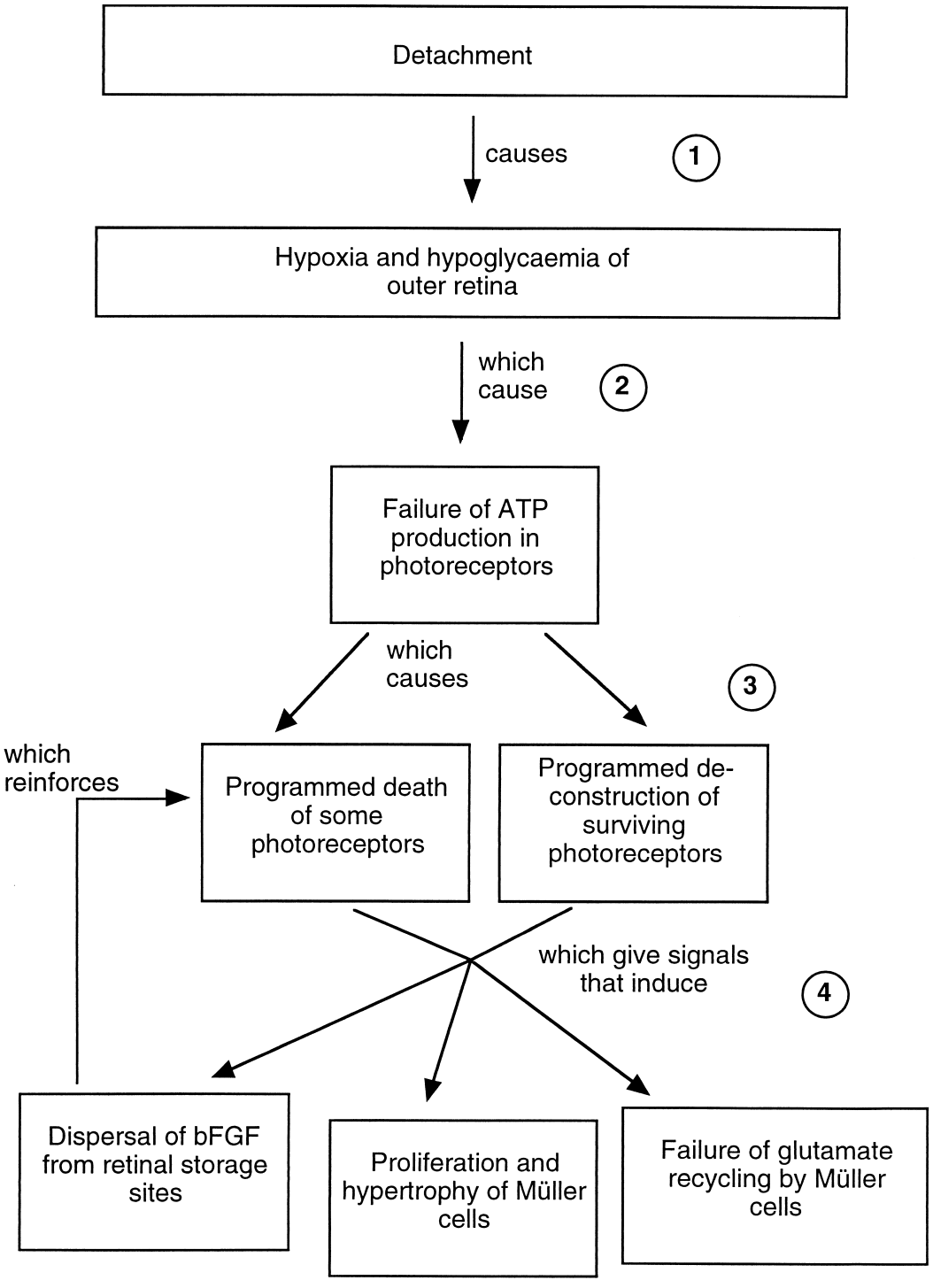

FIGURE 4. Hypothesis of the genesis of retinopathy of detachment. The first three stages of genesis have a relatively strong empirical base.[12] The sequence of events at stage 4 is more speculative. We have suggested that the glial responses to detachment are mediated by signals from surrounding neurones as they undergo death or deconstruction. Although speculative, this suggestion can be tested, which should clarify the interactions of glia and neurones in detachment retinopathy.

(Figure 3D), but in detached retina that were kept in normoxia it was prominent as far outward as the outer nuclear layer (Figure 3E). Hyperoxia during detachment reduced but did not eliminate this upregulation of GluR-2 (Figure 3F).

Detachment caused a reduction of the glutamine synthetase labeling in the inner processes of Müller cells (Figure 3, G and H), consistent with a previous report.[13] This loss was prevented by hyperoxia during detachment (Figure 3I). In an unexpected response, glutamine syn-

thetase was upregulated in hyperoxic detached retina to a level above those found in attached retina, either normoxic (Figure 3G) or hyperoxic (not shown).

DISCUSSION

THESE RESULTS PROVIDE EVIDENCE THAT OXYGEN SUPPLEmentation during retinal detachment reduces the proliferation of Müller cells caused by detachment, reduces the hypertrophy of their processes, and stabilizes glutamate neurochemistry. These effects of oxygen supplementation confirm that the glial reaction to detachment results partly from hypoxia. They suggest that in humans the glial proliferation that sometimes follows retinal reattachment may be reduced in occurrence and severity if the patient receives oxygen supplementation between diagnosis and surgery.

It is striking that the reactions of retinal neurones and glia to detachment are diametrically opposed; neurones (photoreceptors) degenerate,[12] whereas glia proliferate. Both responses are driven at least in part by hypoxia, suggesting that they are closely connected. Two further elements of the response of the retina to detachment, both driven by hypoxia, are suggested. One is the dispersal of bFGF from its normal storage sites in both glia (astrocytes and Müller cells) and neurones, which has been described.[12] Given the protective effect of bFGF on photoreceptors,[15,16] this dispersal may be an important factor in photoreceptor death. The second element is a disturbance of the neurochemistry of glutamate, which involves the accumulation of glutamate and a loss of glutamine synthetase activity in Müller cells.[11] Recognition that these various components of the retina's reaction to detachment have a common cause in nutrient starvation leads to the suggestion that the detached retina is undergoing a linked series of reactions that could be called the retinopathy of detachment.[11]

An important function of glial cells in the central nervous system is the recycling of transmitters. Müller cells are known to express glutamate receptors in the normally functioning retina and to contain high levels of the enzyme glutamine synthetase, whose action is to transform glutamate to glutamine (which has no transmitter activity) and to make the glutamine available to neurones for the resynthesis of glutamate.[17,18] Presumably, because their glutamine synthetase activity is high, Müller cells do not normally accumulate glutamate.[10] In the detached retina, glutamine synthetase levels in Müller cells fall (Figure 3B), and GluR-2 labeling indicates that glutamate receptor levels in Müller cells increase. Presumably as a result, glutamate levels rise in Müller cells. It is unclear whether these changes play a role in inducing photoreceptor degeneration in detached retina.

These results, in combination with a long series of results from earlier studies,[2,12,19] permit the proposal of a tentative theory as to the genesis of the retinopathy of detachment (Figure 4).

- Detachment separates the photoreceptors from the choriocapillaris, the source of their energy nutrients, inducing hypoxia and hypoglycemia in the outer layers of neural retina.
- Hypoxia and hypoglycemia induce the programmed death of some photoreceptors and the programmed deconstruction of the survivors. Dying and damaged photoreceptors release still unknown signals, which cause the dispersal of bFGF from its normal storage sites, the proliferation and hypertrophy of Müller cells to repair damage, and the downregulation of glutamine synthetase activity in Müller cells, causing a redistribution of retinal glutamate and glutamate receptors.
- The dispersal of bFGF increases the vulnerability of photoreceptors to damage.
- The proliferation and hypertrophy of Müller cells cause gliosis of the outer limiting membrane, which prevents re-establishment of normal relations between photoreceptors and retinal pigment epithelial (RPE) cells when reattached.

The model in Figure 4 is specific to the neural retina. A fuller model of the retinopathy of detachment may need to include structures adjacent to the neural retina, especially the RPE.[20]

These results come from an experimental model that deserves further exploration to include longer periods of detachment and the effect of delay in supplementation. Clinical trials of oxygen supplementation in the treatment of retinal detachment also deserve consideration.

There would appear to be few obstacles to such trials, other than those of case selection and the resources required for any such trial. Oxygen is toxic, and current practice in respiratory medicine would need to be incorporated in any trial, including the limiting of oxygen exposure and the use of antioxidants. Current practice in respiratory medicine is to limit the use of high oxygen levels to a few days, whereas current ophthalmic practice is to repair attachments within 1 or a few days of diagnosis; these two constraints seem compatible.

The reaction of Müller cells to retinal detachment was greatly reduced by oxygen supplementation, as was the proliferation of Müller cells. Their neurochemical signatures were stabilized, suggesting that the Müller cells maintained their normal role in transmitter recycling and that their hypertrophy was limited. In the treatment of retinal detachment, oxygen supplementation between diagnosis and reattachment surgery may reduce the incidence and severity of glial-based complications, such as proliferative vitreoretinopathy. Further experimental studies and careful clinical trials will be necessary to test this possibility.

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