Use of the MIB-1 Antibody for Detecting Proliferating Cells in the Retina

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Purpose. To study intraretinal proliferation as a response to experimental retinal detachment using an antibody that recognizes the nuclear specific antigen Ki-67 in proliferating cells.

Methods. Experimental retinal detachments were produced in cats (1, 3, 7, and 28 days) and rabbits (1, 3, and 7 days). The animals were killed and the eyes were fixed and embedded in paraffin. Histologic sections were processed for immunohistochemistry using the MIB-1 antibody to detect the Ki-67 protein. Labeled cells were identified, and the proliferative response was quantified.

Results. In normal cat retina, approximately 0.05 cells per millimeter of retina are labeled. In cat retina detached for 1, 3, 7, or 28 days, the number of cells labeled by MIB-1 is 0.06, 5.03, 1.38, and 0.23 cells per millimeter of retina, respectively. MIB-1 labeling yields an approximate fivefold increase over the number of proliferating cells detected in retinal sections using ^{3}H-thymidine autoradiography. Detachment of the rabbit retina elicits a similar response as measured by MIB-1 immunohistochemistry.

Conclusions. In contrast to ^{3}H-thymidine, which labels cells in S-phase only, the MIB-1 antibody labels proliferating cells regardless of their location within the cell cycle. MIB-1 labeling, therefore, is a more accurate means of evaluating cellular proliferation in the retina and elsewhere in the central nervous system, and it is a relatively simple way of evaluating the effects of agents that may affect this response. Invest Ophthalmol Vis Sci. 1995;36:737–744.

Although cellular proliferation in the central nervous system (CNS) is an essential component of normal growth and development, abnormal cellular proliferation often occurs in response to injury, disease, or both. The cell types that proliferate after CNS injury include macrophages, microglia, astrocytes, oligodendrocytes, vascular, and blood-borne cells. Evidence suggests that glial proliferation after injury has an adverse effect on neuronal survival and axon regeneration in the CNS. Astrocytes, the most prevalent cell type in most glial scars, are thought to inhibit axonal regrowth.[1,2] More recently, however, glial proliferation has been proposed to be beneficial for neuronal recovery after injury by providing a substrate for axonal regeneration,[3] providing axonal remyelination,[4] and releasing growth factors that may facilitate neuronal survival.[5]

Detection of proliferating cells has traditionally relied on uptake and incorporation of radiolabeled nucleosides (e.g., tritiated thymidine) into the DNA of dividing cells, followed by tissue autoradiography. There are several drawbacks associated with tissue autoradiography, especially when using intact animals. It is time consuming, expensive, and requires potentially hazardous quantities of radioactivity to quantitate accurately the total number of dividing cells. Moreover, it is often difficult to identify specific cell types through the overlying photographic emulsion.

Gerdes and colleagues[6] identified an antibody that detects a nuclear-associated antigen present in all phases of the cell cycle except G_0.[7] Recently, Schlüter and colleagues[8] cloned and sequenced the cDNA coding for this protein. It was determined to be a large heterodimeric nuclear-associated protein (Ki-67) with no known homology to other nuclear-associated proteins. Subsequently, three monoclonal antibodies (MIB 1 to 3) that recognize this protein were raised by the same group.[9,10] These antibodies have been

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used frequently in cancer histopathology to determine the percentage of dividing cells in biopsied tissues and to gauge the aggressiveness of cellular proliferation in human neoplasms.[11]

To our knowledge, antibodies to Ki-67 have not been applied to studies of proliferation after CNS injury. Previously, ^{3}H-thymidine autoradiography was used to quantify the cellular proliferation induced by experimental retinal detachment.[12,13] In the current study, we take an immunohistochemical approach, using the MIB-1 antibody to detect proliferating cells in both cat and rabbit retinal sections after experimental retinal detachment. The results show that MIB-1 labeling follows the same time-course as that shown by ^{3}H-thymidine autoradiography but yields a more accurate measure of the overall magnitude of the proliferative response.

METHODS

Cat Surgery

Retinal detachments were performed on cats as previously described.[14] Briefly, cats were anesthetized with 20 mg/kg Ketaset (ketamine HCL; Parke-Davis, Morris Plains, NJ) and 5 mg/kg Rompun (xylazine; Miles Labs, Shawne, KS). Subsequent injections of Rompun–Ketaset maintained deep anesthesia throughout the surgical procedure. In this procedure, the lens and vitreous were removed and a small glass pipette (external tip diameter 80 to 100 μm) was inserted between the neural retina and the retinal pigment epithelium (RPE) monolayer. Healon (0.25%; Pharmacia, Monrovia, CA) in balanced saline solution was then infused to separate the retina from the RPE. Eyes were removed and fixed at specified times (1, 3, 7, or 28 days) after the procedure. Some data included in this study originated from an earlier study of proliferation in which the animals were injected intraocularly with 200 μCi of ^{3}H-thymidine 2 hours before enucleation.[13] The autoradiographic cell counts were used for comparison with counts of MIB-1 labeled cells in the present study.

Rabbit Surgery

One-, 3-, and 7-day detachments were performed on New Zealand white rabbits. Retinal detachments were performed as in the cats, except that the lens and vitreous were not removed, and the infusion pipette was inserted through a small incision at pars plana.

Tissue Processing

After a specified number of days of detachment, animals received an overdose of sodium pentobarbital and the eyes were removed. The eyes were fixed briefly in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), the anterior chambers were removed, and the posterior portion of the globe including the retina was returned to fixative for at least 24 hours. The tissue was rinsed in 0.1 M phosphate-buffered saline (PBS) (pH 7.4), dehydrated in graded ethanol, and embedded in either wax (Paraplast X-tra; Fisher Scientific, Pittsburgh, PA) for immunohistochemistry[15] or LR White resin (Polysciences, Warrington, PA) for autoradiography.

Immunohistochemistry

For consistency, all immunohistochemical assays were performed using an automated histostainer (Techmate 1000, BioTek Solutions, Santa Barbara, CA). Four-micrometer thick wax sections were dried on microscope slides, dewaxed in xylene, and rehydrated in graded ethanol. The sections were microwaved two times for 5 minutes each (2 × 5 minutes) on high power in 200 ml of a 10 mM citrate buffer (pH 6.0) (to increase antigenicity) and allowed to cool to room temperature in the citrate buffer (BioTek Solutions), similar to the procedures of Cattoretti[16] and Shi.[17] Between microwaving incubations, 50 ml of room temperature dH_2O was added to each container of slides to compensate for evaporation during the microwaving procedure. Blocking with normal goat serum in PBS (pH 7.3) for 15 minutes immediately preceded overnight incubation with MIB-1 monoclonal antibody (Immunotech, Inc., Westbrook, ME) at 1:250 in PBS, followed by 5 × 5-minute rinses with PBS and incubation in biotin conjugated goat-anti-mouse secondary antibody (BioTek Solutions) for 1 hour. After rinsing 3 × 5 minutes in PBS, endogenous peroxidases in the tissue were quenched by incubation in 0.3% hydrogen peroxide in PBS for 15 minutes. The tissue was then incubated in avidin–horseradish peroxidase (BioTek Solutions) for 1 hour. After rinsing for 3 × 5 minutes in PBS, the tissue was incubated in PBS with diaminobenzidine (BioTek Solutions) and 0.02% hydrogen peroxide for 15 minutes to yield a brown precipitate. The tissue was then counterstained with hematoxylin (BioTek Solutions), and MIB-1-positive cells were counted.

Cell Counts

In both cats and rabbits, cell nuclei filled with the dark brown peroxidase precipitate were counted per millimeter of retinal length using an ocular micrometer attached to a Zeiss Universal light microscope (Carl Zeiss, Oberkochen, Germany). Cell types were classified according to their retinal stratification as well as their morphology and, thus, were categorized as residing in one of eight compartments: vitreous, ganglion cell, inner plexiform, inner nuclear, outer plexiform, outer nuclear, inner–outer segments, or RPE. Furthermore, because retinal stratification and

morphology are reasonable indicators of cell type, this analysis yielded quantitative data on the proliferation of individual cell types. The number of cells counted by autoradiography and immunohistochemistry were corrected by the method of Abercrombie[18] to approximate the population of labeled nuclei in microtome sections. In addition, when comparing data from 1-μm (autoradiography) and 4-μm (immunohistochemistry) thick sections, the latter were divided by 4 to yield data quantitatively comparable to that collected from 1-μm sections. All animals were treated in accordance with the guidelines established in the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

RESULTS

MIB-1 Labeling in the Cat

Sections of both normal and detached cat retina were processed for immunohistochemistry with the MIB-1 antibody (Figs. 1A to 1E). Few labeled cells were detected in normal retinas (Fig. 1A) or at 1 day after retinal detachment (Fig. 1B). At 3 days after retinal detachment, however, the MIB-1 antibody labeled numerous cells throughout the retina (Fig. 1C), and labeled examples of all nonneuronal retinal cell types were identified. At 7 days, the number of labeled cells decreased markedly, and at 28 days, there were few MIB-1-positive cells (Figs. 1D, 1E). Tissue processed with the primary antibody omitted showed no labeling (data not shown).

Proliferating RPE cells are more difficult to detect using ^{3}H-thymidine autoradiography, presumably because of the difficulty in sampling infrequently dividing cells in a monolayer.[12,13] Using the MIB-1 antibody, however, we found that the labeling pattern of RPE cells followed the same time-course as the other nonneuronal cells within the neural retina (data not shown).

MIB-1 Labeling in the Rabbit

Sections from normal and detached rabbit retina were processed for immunohistochemistry with the MIB-1 antibody (Figs. 1F to 1I). Virtually no MIB-1 labeled cells were found in normal retinas or in those retinas detached for 1 day (Figs. 1F, 1G). By 3 days, however, there was a significant increase in MIB-1-labeled cells (Fig. 1H), and labeled examples of all nonneuronal retinal cell types were identified. At 7 days after retinal detachment (Fig. 1I), there was a decrease in the number of MIB-1-labeled cells compared to the 3-day detachment, a decline similar to that found in the cat retina at that time.

Correlation Between Immunohistochemistry and Autoradiography

There is a strong correlation between data derived from MIB-1 immunohistochemistry in the present study and data from radiolabeling with ^{3}H-thymidine obtained in our earlier study[13] (Fig. 2). In the autoradiographic study, 0.01, 0.04, 1.1, 0.22, and 0.07 radiolabeled cells per millimeter of retina were found in normal, 1-, 3-, 7-, and 28-day retinal detachments, respectively. Using MIB-1 immunohistochemistry, we detected 0.05, 0.06, 5.03, 1.38, and 0.23 labeled cells per millimeter of retina in normal, 1-, 3-, 7-, and 28-day retinal detachments. Although the overall time-course is the same, with a large increase noted at 3 days and a decline by 7 days, the MIB-1 procedure yields approximately a fivefold increase in the number of labeled cells at most of the five timepoints.

The two methods also compare favorably when analyzing the distributions of labeled cell types at the peak (3 days) of the proliferative response (Fig. 3). In general, it does not appear that one method preferentially labels any single cell type or subpopulation of cells. Both methods of detection indicate that Müller glia and astrocytes together comprise more than half the total number of labeled cells. There is a difference in the percentage of proliferating vascular cells, although this observation is complicated by the fact that the retinal regions surveyed for autoradiography were more vascularized than those sampled by immunohistochemistry.

Species Comparison Using MIB-1

A comparison of the labeling pattern in cats and rabbits is shown in Figure 4. The pattern is essentially the same in both species, although the rabbits have approximately 30% fewer labeled cells at the presumptive peak (3 days after detachment) of the response. It should be noted that the numbers of proliferating astrocytes and vascular cells in the rabbit retinas can be increased significantly by sampling in the region of the "medullary rays," where the populations of these cell types are higher than elsewhere in the retina. To avoid an undue bias and to allow for a more accurate comparison to data collected from cat retina, these regions were not included in our sampling procedures.

DISCUSSION

Quantification of the Proliferative Response

Accurate quantification of the proliferative response in heterogeneous tissues, such as retina, where cells are dividing asynchronously, is difficult. Usually, it is not feasible to deliver ^{3}H-nucleosides continuously to an intact animal for an extended period of time. In the

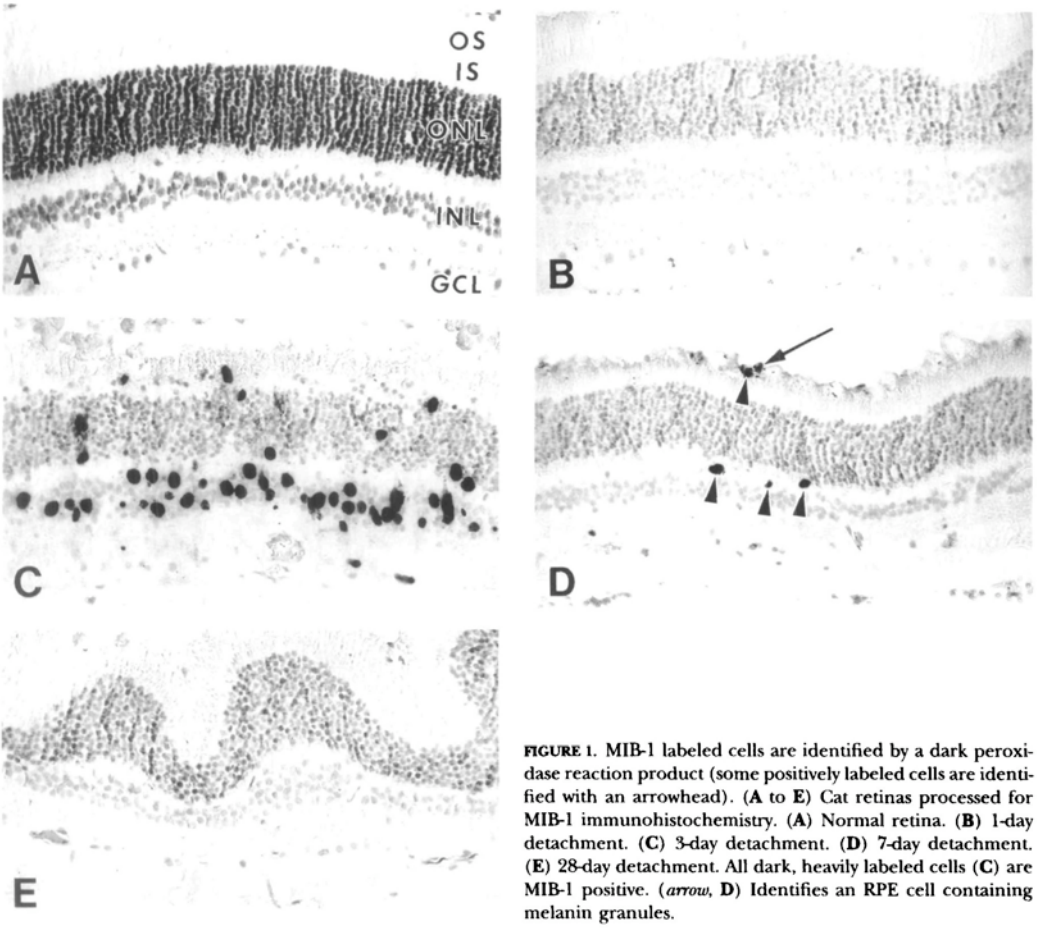

FIGURE 1. MIB-1 labeled cells are identified by a dark peroxidase reaction product (some positively labeled cells are identified with an arrowhead). (**A** to **E**) Cat retinas processed for MIB-1 immunohistochemistry. (**A**) Normal retina. (**B**) 1-day detachment. (**C**) 3-day detachment. (**D**) 7-day detachment. (**E**) 28-day detachment. All dark, heavily labeled cells (**C**) are MIB-1 positive. (*arrow*, **D**) Identifies an RPE cell containing melanin granules.

previous autoradiographic study,[13] rough estimates of the magnitude of the retina's proliferative response were calculated for a 24-hour period using several assumptions. First, an average nuclear diameter of 6 μm was assumed when correcting raw cell counts in a given section thickness.[18] Second, it was assumed that a single intravitreal injection was equivalent to a 2-hour "pulse" of ^{3}H-thymidine. Third, it was assumed that the number of radiolabeled cells during the 2-hour "pulse" would be representative of each 2-hour interval throughout an entire 24-hour period. Accordingly, the corrected counts were multiplied by 12 to approximate the number of proliferating cells in a given 24-hour period. This corrected cell count per millimeter of retina was multiplied by 10 to yield an estimate of the number of radiolabeled cells per centimeter of retinal length and then was multiplied by 10,000 to obtain a final estimate of the number of labeled cells for a 1-cm^2 piece of retina. Using this method, the number of proliferating cells at the peak of the proliferative response (day 3) in the cat was estimated to be 1.3×10^6 cells/cm^2 of retina.

Estimates of the magnitude of the response obtained using MIB-1 labeling as the proliferation index avoids many of the problems associated with ^{3}H-thymidine autoradiography. The Ki-67 antigen is present and available for detection throughout the cell cycle, which averages about 24 hours for most cell types.[19] Therefore, no assumptions regarding the "pulse" labeling interval or magnitude of labeling after the "pulse" need to be made to arrive at an estimate for one 24-hour period. To allow direct comparison between estimates obtained from 4-μm thick sections for immunohistochemistry in this study and 1-μm thick sections for autoradiography in the previous study, raw counts of MIB-1 labeled cells per millimeter of

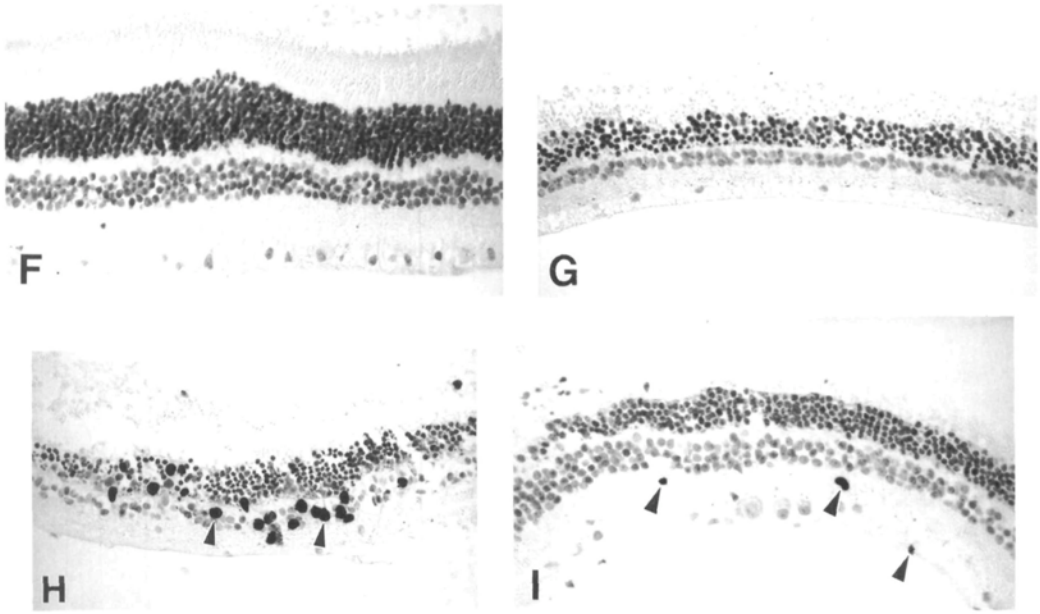

FIGURE 1 (*continued*). (**F** to **I**) Rabbit retinas processed for MIB-1 immunohistochemistry. (**F**) Normal retina. (**G**) 1-day detachment. (**H**) 3-day detachment. (**I**) 7-day detachment. (**C, H**) Approximate peak of the proliferative response for cats and rabbits, respectively. Note the same time-dependent proliferative response between species. Twenty-eight day retinal detachments were not performed on rabbits.

retina were divided by 4, corrected for section thickness by the method of Abercrombie,[18] and then multiplied by 100,000 to yield labeled cells per cm^2 of retina. Using this method, the number of proliferating cells on detachment day 3 is estimated to be approximately 5×10^5 cells/cm^2 of retina.

Although this immunohistochemical estimate represents a large population of proliferating cells, it is substantially smaller than the original autoradiographic estimate. Thus, it is likely that some of the assumptions required for arriving at the estimate based on autoradiographic data were flawed. The overestimate was probably due to difficulties in estimating the amount of time the ^{3}H-thymidine is present for uptake into dividing cells, estimating the length of S-phase, estimating the length of exposure time necessary to detect cells with little ^{3}H-thymidine incorporation, and detecting cells just entering or

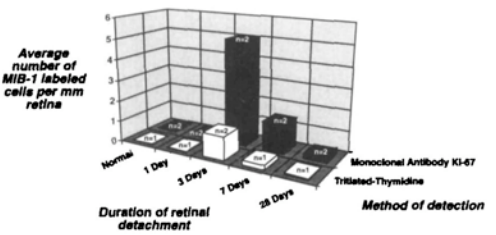

FIGURE 2. A comparison of the mean number of ^{3}H-thymidine labeled (*white bars*) and MIB-1 antibody labeled (*black bars*) cells per millimeter of cat retina in normal, 1-, 3-, 7-, and 28-day retinal detachments. All data are corrected by the method of Abercrombie,[18] and the MIB-1 data are corrected for the difference in section thicknesses used in the two studies.

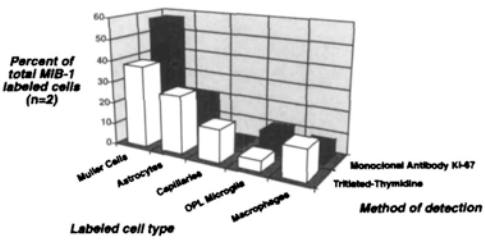

FIGURE 3. The percentage of different cell types labeled with ^{3}H-thymidine or MIB-1 in response to 3-day retinal detachments in cat. The cell types compared here were selected based on data in Fisher.[13] Cells are classified by cell type based on retinal location and morphology.

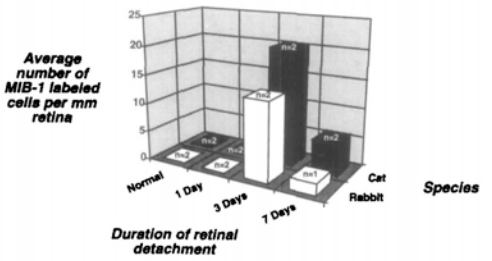

FIGURE 4. A comparison of the MIB-1 labeling pattern in cats and rabbits in normal, 1-, 3-, and 7-day retinal detachments. Data were corrected by the method of Abercrombie.[18]

exiting S-phase. If one cell cycle is assumed to be 24-hours with a 6-hour S-phase,[19] one would expect a fourfold increase in the number of proliferating cells detected by immunohistochemistry. We find a fivefold increase in the present study.

If care is taken to account for the presence of medullary rays in the rabbit retina and the differences in intraretinal circulation that occurs between rabbits and cats (or primates), the rabbit retina appears to be a reasonable model in which to test agents that modify the proliferative response. Rabbit and cat retinas probed with the MIB-1 antibody showed essentially the same time-course of proliferation after detachment (Fig. 4). All nonneuronal cell types (Müller cells, astrocytes, macrophages, RPE, microglia, endothelial cells, pericytes, and hyalocytes) were identified. Differences between labeling in the two species can be accounted for by the fact that the retinal vasculature in the rabbit resides in the medullary rays and not within the neural retina proper. Similarly, because the optic axons collectively form these rays, it is also the region of the retina with the highest concentration of astrocytes. Therefore, data collected from the rabbit retina will depend on whether sampling was taken from a region containing the medullary rays or from more peripheral retina. In rabbits, the Müller cells have a mean density of approximately 7,300 cells/mm^2 of retina.[20] Based on this density and on the number of MIB-1 labeled Müller cells in rabbit retinas detached for 3 days (1744 cells/mm^2), we estimate that a significant number of these cells, up to 25%, may be participating in the proliferation response.

Retinal Injury Leads to Proliferation

Although it is still unclear what causes proliferation in the CNS after injury, wound healing in general is a dynamic process influenced by many factors.[21–26] Invasive injury to the CNS leads to rapid proliferation of astrocytes, oligodendrocytes, microglia, and hematopoietic cells at or near the site of injury.[27–32] The number of proliferating cells typically peaks within a 2- to 5-day "window" after injury, and then declines progressively with time.[33,34]

As part of the CNS, the retina responds to injury in a similar way. Retinal detachment elicits a variety of cellular responses, including the proliferation of all nonneuronal cell types therein.[12,13,35] The proliferative response peaks by 3 to 4 days after detachment and returns to baseline levels by 14 days.[13] Detachment also involves the invasion and proliferation of blood-borne cells that could mediate both intraretinal proliferative and inflammatory responses, as well as tissue repair and regeneration.[22] In detachment, as well as in other retinal insults, the infiltration of blood-borne cells, along with the migration and proliferation of RPE and other nonneuronal retinal cell types (such as Müller cells), can lead to subretinal, intraretinal, and/or epiretinal "membranes" (or "scars") that manifest themselves in a variety of distinct pathologic conditions, including proliferative vitreoretinopathy, proliferative diabetic retinopathy, subretinal fibrosis, diabetes, and macular pucker.[35–37] Indeed, RPE cells, astrocytes, Müller cells, microglia, retinal vascular cells, hyalocytes, lymphocytes, and tissue macrophages have all been proposed to contribute to the formation of these cellular assemblages.[36,38,39] Currently, treatment of these fibrotic "membranes" in and around the retina involves their surgical removal, with no pharmacologic therapy available for preventing their recurrence.

CONCLUSION

It has been shown that proliferation associated with retinal detachment can lead to visual impairment, blindness, or both.[36] Inhibition of the proliferative response may enhance the ability of the retina to reattach and remain attached to the RPE after corrective surgery.[35,40] Based on the evidence from this study, the use of MIB-1 antibody labeling provides an accurate, reliable, and relatively simple technique for studying cellular proliferation in the retina and probably offers a more accurate means of quantification in whole animal experiments than does ^{3}H-thymidine autoradiography. Identification of the proliferating cell types, as well as the magnitude and time-course of the response, may provide critical insight into the eventual treatment of any retinal condition in which cellular proliferation is suspected to play a role. A variety of anti-mitogenic factors, drugs, growth modulators, or regulators could inhibit proliferation in the retina. The ideal therapeutic agent, however, would be one that exerts its antiproliferative effect in the absence of significant neurotoxicity. Use of the MIB-1 detection method, in conjunction with appropriate animal models of retinal injury or disease, provides a powerful tool for identifying and evaluating such an agent.

Key Words

Ki-67, autoradiography, retinal detachment, and gliosis

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