Up-Regulation of Glial Fibrillary Acidic Protein in Response to Retinal Injury: Its Potential Role in Glial Remodeling and a Comparison to Vimentin Expression

Geoffrey P. Lewis and Steven K. Fisher

Neuroscience Research Institute
Department of Molecular, Cellular, and Developmental Biology
University of California, Santa Barbara, Santa Barbara, California 93106

Intermediate filament proteins are a heterogeneous group of proteins that form 10-nm-diameter filaments, a highly stable cytoskeletal component occurring in various cell types. The up-regulation of one of these intermediate filament proteins, glial fibrillary acidic protein (GFAP), has historically been an indicator of "stress" in central nervous system (CNS) astrocytes. The retina also responds similarly to "stress," but the up-regulation of intermediate filaments occurs primarily in Müller cells, the retinal glia of the retina. This is a remarkable ubiquitous response in that a similar up-regulation can be observed in numerous forms of retinal degeneration. As a consequence of retinal detachment, a "mechanical" injury to the retina, GFAP, and another intermediate filament protein, vimentin, dramatically increase in Müller cells. Consistent with this up-regulation is the hypertrophy of these cells both within the retina and onto the photoreceptor and retinal surface of the retina. The function of this distinctive intermediate filament up-regulation in glial cells is unknown. In the retina the expression of intermediate filaments is differentially regulated in a polarized manner as the Müller cells hypertrophy, suggesting that there are some role in this process. Moreover, the response of intermediate filaments and the Müller cells differs depending on whether the retina has been detached or remodeled to the retinal pigment epithelium. The differential expression of these proteins may give insight into their role in the formation of glial scars in the retina and elsewhere in the CNS.

KEY WORDS: GFAP, Vimentin, Intermediate filaments, Müller cell, Glial, Retinal detachment, Retinal reattachment.
1. Introduction

The hallmark response to injury or stress in the central nervous system (CNS) is an up-regulation in astrocytes of intermediate filament proteins, bipolar, stable structural components of the cellular cytoskeleton. The cytoskeleton of animal cells is composed of 7-nm-diameter actin filaments, 25-nm microtubules, and 10-nm intermediate filaments. Unlike actin filaments and tubulin, ubiquitous cytoskeletal elements found in all cells and with specific known functions, intermediate filaments are found in a limited number of cell types and their expression varies greatly during development and under pathological conditions. Although this implies specialized roles for intermediate filaments, in fact, very little is known of their exact functions.

A. Intermediate Filament Types and Structure

Based largely on amino acid sequence homologies, intermediate filaments can be classified into six types: Type I (collagen and basic keratin), Type II (intermediate and basic keratin), Type III (desmin, glial filibrillary acidic protein (GFAP), vimentin, peripherin), Type IV (neurofilaments, z-protocollagen), Type V (lamins), and Type VI (neurilin). These classes of intermediate filaments are associated with specific cell types including epithelial cells (keratins), muscle cells (desmin, vimentin), neurons (neurofilaments, internerve, peripherin), CNS and muscle precursor cells (nestin), and astrocyte cells (GFAP, vimentin). The basic structure of an intermediate filament molecule consists of a central z-helical rod domain flanked on either side by a nonhelical amino-terminal and a carboxyl-terminal domain (Parry and Stec, 1992; Coulombe et al., 2000). Two molecules aggregate in parallel to form an intermediate filament dimer that takes on a coiled-coil conformation. Two dimers unite to form a tetramer of antiparallel protofilaments and multiple tetramers then assemble to form the typical 10-nm intermediate filament bundles. Whereas the central region consists of approximately 310 amino acids and is common to all intermediate filaments, the head and tail domains are variable in size, volume, and amino acid composition, providing immunogenic and functional specificity to the individual intermediate filament proteins. The filaments themselves can be composed of different intermediate filament proteins coexpressed to form heteropolymers. This depends on the cell type, developmental stage of the cell, and the most likely role of the cell in the tissue. In epithelial cells, for example, two types of keratins can copolymerize to form intermediate filaments. In astrocytes, three types of intermediate filament proteins can be found but their expression varies with age and apparent function. Although nestin and vimentin are the main intermediate filament proteins in immature
GFAP is the most studied of the intermediate filament proteins in glial cells. Although it was first isolated from patients with multiple sclerosis (Eng et al., 1971), it is now well established that it is the main intermediate filament protein in mature "normal" astrocytes in the CNS. A gradual increase in the expression of GFAP in astrocytes has been shown to occur throughout the life span of a number of species (Nichols et al., 1993; Eng and Lee, 1998). However, a rapid increase in the expression of the protein and its mRNA, and a concomitant increase in intermediate filaments, occurs within hours when astrocytes become reactive as a result of trauma, ischemia, disease, or chemical insult (Eng and Ghirnikar, 1994).

The gene for this 54-kD protein has been isolated and characterized in a number of species, including human, mouse, and rat (Lewis et al., 1984; Baleare and Cowan, 1985; Reeves et al., 1989; Bremer et al., 1993; Feinstein et al., 1992). Several studies examining astrocytes in culture have shown that transcription of the gene is highly complex, and that it is regulated by a combination of apparent ubiquitous positive, and tissue-specific negative elements (Miura et al., 1996; Sarkar and Cowan, 1991; Kameko and Sueoka, 1995; Bonnard et al., 1994; Maseo et al., 1993; Nakatani et al., 1996). Much less is known of GFAP gene regulation in other cell types that contain GFAP including Schwann cells, radial glia, Bergmann glia, and Müller cells in the retina although the induction of GFAP in Müller cells in mice with retinal dystrophy has been shown to be regulated at the level of transcription (Norby and Fu, 1989). Interestingly, it appears that the regulatory elements that stimulate GFAP transcription in Müller cells differ from those in astrocytes (Bremer, 1994; Verderber et al., 1995; Johnson, et al., 1995).

C. Functions of Intermediate Filaments in Glial Cells

Astrogial in the CNS perform a wide variety of functions including the formation of the blood-brain barrier, maintaining the ionic balance of the extracellular medium, uptake and metabolism of neurotransmitters, providing neurotrophic support to neurons, and playing a major role in the reaction to injury. Little is known of the exact function of intermediate filaments and any role they may play in these processes. However, recent studies using approaches such as antisense oligonucleotides to suppress GFAP expression...
in cultured astrocytes implicates GFAP in the formation of stable astrocytic processes and in growth regulation (Wennerstein et al., 1991; Kurita and Smith, 1993; Toda et al., 1994). Gene knockout studies have shown that mice lacking either GFAP or vimentin appear to develop normally (Gomi et al., 1995; Pelkey et al., 1995; Colucci-Guyon et al., 1994), however, subtle changes have been observed in other systems as a result of disrupting intermediate filament expression. GFAP knockout mice have shown signs of altered neuronal function including changes in motor control (Shibuki et al., 1996; McCall et al., 1998) as well as low-cortisol CNS demyelination (a function of oligodendrocytes) and locally impaired blood brain barrier function (Liedtke et al., 1996). Astrocytes cultured from GFAP knockout mice can still elaborate processes, but demonstrate an increased level of proliferation (Pelkey et al., 1998). Finally, astrocytes lacking both GFAP and vimentin have a reduced ability to maintain their volume in response to osmotic changes in the culture medium, whereas astrocytes lacking only one of the intermediate filament proteins can still respond (Zing et al., 1998).

One of the most studied aspects of intermediate filament expression is glial cells' response to CNS injury or disease. Observations in many systems over the past 2 decades suggest that they play a major structural role in the hypertrophy of astrocytes, the first step in the formation of glial scars (Ring and Glennner, 1994). As these cells expand in the CNS, often filling in space where neurons have died, they dramatically pack their cytoplasm with intermediate filaments. Interestingly, gene knockout studies have shown that the ability of astrocytes to form intermediate filaments appears to depend on whether the cell is in the reactive or nonreactive state. In addition, it was also shown that in general, copolymerization of different intermediate filament proteins in astrocytes appears necessary for the formation of functional intermediate filaments. In "normal" uninjured GFAP knockout mice, the astrocytes completely lack both GFAP and vimentin, indicating that in the nonreactive state, vimentin cannot self-polymerize, needing GFAP to polymerize into intermediate filaments (Pelkey et al., 1993; Elkasen et al., 1999). Following brain injury, which induces the astrocytes to become reactive, nestin becomes expressed and copolymerizes with vimentin to form intermediate filaments in the absence of GFAP. This allows glial scars to form in these animals. In vimentin knockout mice, reactive astrocytes are able to assemble some intermediate filaments made only of GFAP, but the filaments were in abnormal compact bundles rather than the characteristic loose bundles (Galou et al., 1996; Elkasen et al., 1999). Finally, it was demonstrated that glial scar formation was impaired in astrocytes deficient in both GFAP and vimentin, but not in mice that were missing one or the other. These data were interpreted to indicate that nestin, although present, cannot form intermediate filaments on its own (Pelkey et al., 1999). These studies suggest that although there is some degree of functional overlap between these two
II. Intermediate Filaments in the Retina

In the mammalian retina, neurofilaments, based on current immunocytochemical data, are limited to expression in horizontal and ganglion cells. Surprisingly, the expression of neurofilaments in these neurons shows an almost "giant-like" response following injury to the retina, dramatically swelling the cell bodies and newly sprouted neurites as they grow widely throughout the retina (Coble et al., 2005; Fisher and Lewis, 2003). GFAP and vimentin are found in the two glial cell types, astrocytes and Müller cells, the radial glia of the retina. As discussed previously, up-regulation of intermediate filaments is in the brain and spinal cord in response to injury and disease. In astroglia, in the retina of most species, however, it is the Müller cells rather than the astrocytes that show the dramatic increase in intermediate filaments. Indeed, the astrocyte response to retinal injury is relatively undefined by comparison to that of the Müller cell.

A. Müller Cells and Astrocytes

The two retinal glial types have very different morphologies and locations in the retina. Retinal astrocytes, with their typical stellate appearance, have their cell bodies located primarily in the ganglion cell layer where their processes form rows along the vitreal surface as they follow ganglion cell axons toward the optic nerve (Figs. 1D and 2E, green). The Müller cells are radial glia that span the entire width of the retina (Fig. 1A), from Dreher et al., 1992. They are large cells with a complex morphology, even by comparison to other radial glia in the CNS. Here we will focus on the Müller cells of the domestic cat retina. Although Müller cells in all vertebrates share general morphological characteristics, there are many specializations peculiar to a species or broader group of vertebrates (Dreher et al., 1992; Sarthy and Rupp, 2001). For example, in the feline retina, the innermost region of the cell possesses a club-shaped "end foot" that lies adjacent to the vitreous cavity, essentially forming the cellular border between the retina and vitreous. In many species, however, this end foot is highly branched. The central region of the cell contains lateral processes. A cell body with its nucleus resides in the inner nuclear layer, and a honeycomb of branches weaves between photoreceptor cell bodies in the outer nuclear layer. Numerous
FIG. 1 Image showing the potential morphology of the Müller cell, as well as the potential distributions of processes within the cell. (A) A drawing of a Müller cell (from Orklo et al., 1980) from the ferret retina showing the club-shaped end foot in the GCL, numerous lateral processes that branch from the main trunk in the IPL, a honeycomb of processes that weave around. Flavocerotic cells in the ONL, and an apical process that interdigitate with
The highly polarized morphology of the Müller cell is reflected in the distribution of several specific proteins. For example, the transmembrane glycoprotein, C6M4, is restricted to the apical portion, whereas the intermediate inner segment. 

B. Distribution of GFAP and Vimentin in Normal Retina

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filament proteins GFAP and vimentin are restricted almost exclusively to the end foot domain with only occasional filaments extending deeper than the border between the ganglion cell layer and the inner plexiform layer (Fig. 1B and C). It is not known what limits these filaments to this particular cytoplasmic compartment (Fig. 1B). In contrast, GFAP appears uniformly distributed throughout the cytoplasm of astrocytes. In addition, in tissue sections, the relative intensity of immunolabeling for GFAP is much greater in astrocytes than in Müller cells. Feline astrocytes, unlike Müller cells, do not express detectable amounts of vimentin. Indeed in Müller cells from a “normal” feline retina, vimentin is much more abundant than GFAP, but, as noted above, its expression is limited to the end foot (Fig. 1C). The relative abundance of the two intermediate filament proteins becomes apparent in double-labeled experiments with antibodies to both (Fig. 1D). Anti-GFAP, tagged with a green-fluorescing secondary antibody, is obvious in the astrocytes where there is no signal from the antibody to vimentin, tagged with a red-fluorescing secondary antibody. In a “normal” retina the Müller cell labeling appears generally skewed toward the red end of the spectrum although some “yellow” fluorescence can be seen where for “green” anti-GFAP labeling overlaps with the “red” from the anti-vimentin. When the double-labeled image is separated into its two individual channels, it becomes clear: but the Müller cells contain both GFAP and vimentin, whereas the astrocytes contain almost exclusively GFAP (Fig. 1E and F). At the electron microscopic level, the antibody to both of these proteins localize most heavily to clusters of 10-nm filaments in the end foot region of the Müller cells (Fig. 1G). Labeling also occurs away from the filaments, presumably indicating the presence of GFAP monomers that have not polymerized into filaments. In the Müller cells, the relative abundance of the intermediate filament proteins varies from cell to cell and even regionally within a cell (Fig. 1D and I). In some cases there appears to be an almost independent expression of GFAP and vimentin whereas in other cases they appear to overlap completely. It is their apparent colocalization to the same filament by immunoelectron microscopy that provides evidence for their ability to copolymerize within cells (Fig. 1H).

C. Giel Cell Intermediate Filaments: Responses to Retinal Stress

As is the case with astroglia, GFAP expression in retinal Müller cells can change dramatically in response to a variety of retinal degenerative conditions or injuries, including inherited diseases (retinitis pigmentosa, muscular dystrophies), mechanical injuries (penetrating wounds, retinal detachment), or stress (ischemia) (Table 1). Interestingly, the baseline level of GFAP in Müller cells varies among species. There can be little to none in the cell, as in the
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(continued)
mouse, rat, or ground squirrel retina, or there can be a moderate amount, as in the cat or human. In most species studied, however, "stress" induces a progressive increase in both GFAP and vimentin in the cell over time until the entire cell label with antibodies to the proteins and is filled with intermediate filaments. The ground squirrel is a dramatic exception. Its Müller cells appear remarkably intact with respect to change in intermediate filament protein expression (Leiberg et al., 2002; Sakata et al., 2003). Virtually all other species studied respond very similarly to "stress" by up-regulating intermediate filament expression. Indeed, the number of conditions in which GFAP is up-regulated in Müller cells is extensive (Table 1) and in all cases the response is remarkably uniform. When the increase is caused by trauma there is a rapid accumulation of intermediate filaments that occurs within a few days of the insult (Esser et al., 1994; Gutrin et al., 1996; Lewis et al., 1994; Wen et al., 1998; Cao et al., 2002; Chen and Weber, 2002). In the case of inherited degenerations, the build-up can occur gradually over months or years (as is also the case during normal aging) (Madigan et al., 1994). In addition, the GFAP response to injury appears to increase in magnitude with age, showing minimal up-regulation in early postnatal ages (Cao et al., 2002). Although often associated with conditions that cause photoreceptor cell death, the accumulation of GFAP in Müller cells is probably not a local direct effect of cell death; it can either precede photoreceptor cell death, as in the case of the Mysel's cat or "rd" mouse, or follow, as observed in the "rd" mouse (Ekstroem et al., 1988). Although the mRNA for GFAP rapidly increases in Müller cells after injury to the retina and returns to basal levels a few days later (Sakata and Egad, 1985; Humphrey et al., 1987), the intermediate filaments, once formed, can remain elevated for months (Lewis et al., 1989; Humphrey et al., 1987; Secor et al., 1999).
1. Retinal Detachment: a Specific Form of Retinal Stress

During development of the eye, the neural retina and retinal pigment epithelium (RPE) become closely apposed and remain so throughout the life span of the animal. As the animal matures, the photoreceptor outer segments of the rods and cones grow into the extracellular space and interdigitate with the apical microvillar processes of the RPE. Although cellular mechanisms connecting the photoreceptors to the RPE are poorly understood, they are normally kept such that the retina “attached” to the back of the eye. These retinal layers can be separated, however, resulting in a “retinal detachment” and blindness to that retinal region. The causes of retinal detachment may occur in many different circumstances, as a result of trauma, disease, or high myopia, but recently it has become a part of a surgical procedure for treating macular degeneration by macular translocation (de Juan et al., 1998; Eckardt et al., 1999). In addition, it occurs as part of proposed therapies for the injection of substances into the subretinal space to prevent photoreceptor degeneration (Lewis et al., 1998) or in RPE or retinal transplantation in eyes with retinal degeneration (Bok, 1993; Del Cerro et al., 1997). Regardless of the initiating event, intermediate filaments dramatically increase in the Müller cells.

We create retinal detachments experimentally by inserting a glass micropipette between the neural retina and the underlying RPE and infusing a solution of sodium hyaluronate (Healon; 0.25%) in a balanced salt solution to elevate the retina and prevent it from realigning to the RPE (Lewis et al., 1996). Although this produces a relatively small hole through the retina, the detachment produces the same result as a human rhegmatogenous detachment where there is a large tear in the retina (Sethi et al., 2001). As a result of the detachment, a cascade of events involving Müller cells can be observed. As soon as 15 min after detachment various transcription factors become activated (Geller et al., 2001). Within a day the cells begin to change their morphology, especially in the foot region where lateral branches appear to sprout. Some also proliferate and in these cells the nuclei migrates out of its normal position in the inner nuclear layer toward the outer retina. These “activated” Müller cells will hypertrophy extensively, growing thicker and more heavily branched within the retina and often extending out onto the retinal surfaces (see below). The proliferation of Müller cells peaks between 3 and 4 days after a detachment is created (Fischer et al., 1991; Geller et al., 1995), but the growth of their processes, especially their extensions into the newly created space between the neural retina and the RPE or “subretinal space” continues for as long as the retina is detached (Lewis et al., 1989).

a. Up-regulation of Intermediate Filament Proteins

Because the changes in Müller cell architecture closely mirror the dramatic changes in intermediate filament expression in the feline retina and because the Müller cells of the
ground squirrels do not change either. It is attractive to assume a functional relationship between the two. The view has often been expressed that changes in Müller cell architecture are either induced or stabilized by the increase in intermediate filament expression. Limited experimental evidence for either of these possibilities exists. Circumstantial evidence from our model suggests a potential link between the expression of GFAP and vimentin and specific directional growth of Müller cells, and thus possibly different functional roles for each. In general, GFAP and vimentin mirror each other in their responses to detachment. There are, however, some subtle differences in their pattern of expression that become apparent only with double-labeled experiments. Vimentin is the predominant intermediate filament in normal Müller cells (shown in red in Fig. 1; Fig. 2A). By 1 day after detachment, both intermediate filament proteins have increased in amount and now extend about midway across the retina with a shift in the balance toward GFAP expression (green, Fig. 2B). This trend becomes even more apparent by 7 days with anti-GFAP labeling predominating (green, Fig. 2C). Occasionally, an isolated Müller cell will appear predominantly immunolabeled for vimentin (red, Fig. 2C, arrow). It is in these cells that we believe grow beyond the outer limiting membrane and form glial scars on the photoreceptor surface, at least in the feline retina (Fig. 2F and inset, arrow).

**b. Origin of Intermediate Filament Up-regulation**

It might be expected that intermediate filaments would grow outwardly from the cell body and into the opposite poles of the Müller cells. This is not the result observed. However, instead, the increase in both GFAP and vimentin originates from the end foot, the site of accumulated intermediate filaments in the normal retina. Because the antibodies recognize free subunits as well as polymerized filaments, this observation suggests the proteins are being synthesized locally in the end foot and not in the cell body. In support of this is the evidence that mRNA for GFAP can be localized to the end foot by in situ hybridization (Sarthy and Fu, 1989; Erickson et al., 1992). Taken together this suggests that the mRNAs for intermediate filament proteins is exported from the nucleus to the end foot where protein synthesis occurs and then the filaments expand from an initial "growth region" that exists there. This sorting of mRNAs to distal parts of the cell may not be unique to the retina because GFAP mRNA also has been observed in the processes of reactive astrocytes and radial glial in human spinal cord and brain (Landry et al., 1994).

**c. Changes in Morphology Parallel Intermediate Filament Changes**

The architecture of the end foot is the first to change as a result of retinal detachment. This change can readily be observed by visualizing the reorganization of the intermediate filament cytoskeleton and begins at the same time
that there is a shift in the balance of vimentin and GFAP expression within that compartment. In section through the retina, the morphological changes are relatively subtle with the end feet appearing more elongated and branching (Fig. 2A-C). When viewed in a retina that mount, however, the changes are unmistakable (Fig. 2D and E). Initially, the double-labeled Müller cell end feet appear predominantly as vimentin-labeled, club-shaped structures situated between the rows of astrocytic processes brightly labeled with anti-GFAP, and unlabeled “holes” containing the ganglion cell somata (Fig. 2D). Following just 1 week of detachment, the club-shaped end feet appear to have transformed into a “sea” of processes extending laterally across the retina (Fig. 2F). In addition, there has been a dramatic shift toward anti-GFAP labeling. However, the remodeled end feet processes do not extend beyond the confines of the neuron retina into the vitreous cavity, strikingly different than what occurs at the opposite pole of the cells where they readily grow into the subretinal space. Although the inner limiting membrane is not visualized by this technique, conventional histology shows that the Müller cell end feet are indeed “confined” by this layer of basement membrane. Just as detachment permits the Müller cells to grow into the subretinal space, it is retinal reattachment that appears to permit the end feet to extend into the vitreous cavity and form the equivalent of “glial scars” on this surface of the retina. Thus, reattachment halts their growth into the subretinal space and stabilizes their growth into the vitreous. Surprisingly, the ratio of the two types of intermediate filaments differs in Müller cell processes that form these so-called extraretinal “membranes.” In the early stages of growth, processes extending into the subretinal space show dominant labeling for anti-vimentin, whereas cells extending into the vitreous cavity appear primarily anti-GFAP labeled. These differences continue to hold true even for relatively complex, late-stage cellular “membranes” found on these two retinal surfaces, although in later stages it is the edges of the membranes that show the major differences in intermediate filament expression (Fig. 2F and G). Thus, a sequence of changes in the expression of intermediate filaments and architecture of Müller cells can be proposed.

1. In normal retina intermediate filaments are confined to the end foot compartment; vimentin predominates.

2. Detachment induces the up-regulation of both vimentin and GFAP with GFAP predominating slightly.

- During 1 to 3 days of detachment, up-regulation appears first in the end foot.

- There are concurrent architectural changes in the end foot.

- Three days and beyond, intermediate filaments grow outward from the end foot into cell body, lateral branches, and outer nuclear layer (ONL). GFAP dominates.
FIG. 2 Laser scanning confocal images of sections double-labeled with anti-GFAP (green) and anti-vimentin (red) showing the up-regulation of intermediate filaments in Müller cells and consequent hypertrophy of the cells that occurs following retinal detachment (RD) in the feline retina. (A) In the normal retina these proteins are present in the end feet portion of the Müller cell with anti-vimentin predominating. The image appears yellow where there is overlap of the two proteins. (B) One day after detachment both intermediate filament proteins increase within
i. There is hypertrophy of the main process and lateral branches.

ii. Seven days and beyond, the balance shifts to vimentin expression in a few “random” processes in the ONL.

iii. These vimentin-rich processes extend beyond the outer limiting membrane (OLM) and form subretinal scars.

iv. Reattachment keys the spread of both GFAP and vimentin into the outer part of the Müller cell.

b. Subretinal growth is stopped.

c. There is continued up-regulation of intermediate filaments in the end foot region; vimentin predominates.

d. GFAP predominates in apparent “random” end feet that grow through the outer limiting membrane to form epiretinal glial scars.

d. Retinal Astrocytes Retinal astrocytes also undergo cellular remodeling following retinal detachment (as shown in the flat mounts in Fig. 2D and E) and are a component of epiretinal membranes, as are Müller cells (Gissem et al., 1999b), presumably migrating out of the retina in response to retinal reattachment. Elsewhere in the CNS, astrocytes up-regulate their expression of GFAP in response to injury or disease (Eng and Chunnak, 1994). In the feline retina, however, it is difficult to see if modest changes in GFAP expression occur in the astrocytes because they have a high baseline level of expression. It is also true that we have received little experimental attention because their response tends to be overshadowed by the much more dramatic response of Müller cells. Astrocyte reactivity, however, can be illustrated in a

as Müller cells but anti-GFAP labeling begins to be more prominent as seen by the presence of more yellow color in the end feet and more green color extending into the INL. (C) At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates. At 7 days after detachment, anti-GFAP labeling predominates.

(1) Normal flat-mounted retina integrated at the level of the Müller cell and flat-mounted view from the vitreal side of the retina. The club-shaped end feet are predominantly labeled with anti-vimentin (red) and the astrocytes are exclusively labeled with anti-GFAP (green). The circles with red labeling represent cell bodies of the GCL. (E) Seven days detached (detached from 5). Viewed at the level of the Müller cell on flat showing the change in morphology of the end foot and the astrocyte processes. Anti-GFAP labeling is also more prominent compared to normal. (F) Seven-day detached flat-mounted retina viewed from the vitreal side showing a complex subretinal glial scar that is labeled most intensely with anti-vimentin. (G) Twenty-eight day detached (detached from 5) retina viewed from the vitreal side showing a complex epiretinal “membrane” (glial scar). Although many intermediate filament proteins are present, anti-GFAP labeling (green) dominates the “leading edges” of the membrane whereas anti-vimentin labeling (red) occurs predominantly in the central region. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OLN, outer nuclear layer; IS, photoreceptor inner segments; BM, Bruch’s membrane.)
III. Regulation of Intermediate Filament Protein Expression

Cell-cell signaling mechanisms that may regulate intermediate filament protein expression are poorly understood. Although current evidence suggests that diffusible factors are likely involved. Indeed, putative transmembrane response elements (RREs) and transcription factor binding sites have been identified in the rat GFAP gene promoter region (Gomes et al., 1993). These include thyroid hormone RE, estrogen RE, glucocorticoid RE, nuclear factor I, nitric oxide, transforming growth factor-B (TGF-B) inhibitory element, cAMP, PEP, and nuclear factor III. Factors that have been shown to modulate GFAP expression include basic fibroblast growth factor (bFGF), TGF-B, interleukin-1, interleukin-6, leukemia inhibitory factor, tumor necrosis factor, interferon gamma, and colony-stimulating factor (CNTF). In the retina, the intravitreal injection of either bFGF or CNTF increases the expression of both GFAP and vimentin in Müller cells (Lewis et al., 1992).
Sattly et al., 1997; Y. Wang et al., 2002. Furthermore, it has been shown that CNTF can induce GFAP expression in Müller cells through the JAK-STAT signaling pathway (Y. Wang et al., 2002). The increase in GFAP
FIG. 4 (A-C) Light microscopic images showing that basic fibroblast growth factor (bFGF) can upregulate GFAP and stimulate proliferation of Müller cells. (A) Normal retina showing anti-GFAP labeling (black, immunogold particles) in the outer two of Müller cells. (B) Four weeks after an intravitreal injection of bFGF into a normal eye, anti-GFAP labeling increases in the Müller cells. A glial scar (top of the photomicrograph) is present in the vitreous and has caused a large retinal fold. (C) Four days after intravitreal injection of bFGF into a normal eye, neuronal cells, including Müller cells and astrocytes, are labeled with anti-GFAP, similar to black, immunogold grains in the astrocytes. (D-G) A series of lower-magnification images illustrating that detachment induces an increase in GFAP in Müller cells and reattachment halts the build-up. (D) Normal retina labeled with anti-GFAP. (E) A day after detachment, the retina is reattached for 1 day after reattachment. (F) Three days after the retina is reattached for 1 day, anti-GFAP labeling increases in the Müller cells and extends into the ONL. (G) Retina detached for 3 days and reattached for 28 days, the increase in GFAP is "rectified" as observed after 3 days of detachment and does not continue to increase.
IV. Consequences of Gliarial Scar Formation in the Retina

Gliarial scarring formation in the CNS following injury may impede the regeneration of neuronal processes, especially axons, and the retina is no exception. A variant of this scenario, Subretinal scarring on the surface of photoreceptors, is seen in the retina left detached for 28 days. In this case, GFAP levels continue to increase in Müller cells as they hypertrophy throughout the retina and form glial scars on the surface of photoreceptors. (A) Laser scanning confocal images showing that glial scars appear to retract Müller cells. (B) Confocal images of Müller cells in normal retina show typical neuroglial GFAP (green) and hypereosinophilia throughout the retina. (C) Subretinal scar tissue in a normal retina shows Müller cells with increased GFAP levels.

Intraocular injection of bFGF into normal eyes can also induce gliarial scarring in the vitreous (Fig. 4C, arrows) and proliferation of both astrocytes and Müller cells (Fig. 4C, black silver grains). This response is consistent with a role of retinal detachment or reattachment. After retinal detachment, a penetrating wound to the retina, laser photocoagulation, or exposure to damaging light, there is a build-up of intermediate filaments in Müller cells distant from the site of injury with the response decreasing with distance from the site. Again suggesting that diffusible factors are involved (G. P. Lewis and S. K. Fisher, unpublished observations; Bignami and Dahl, 1973; Humphrey et al., 1993; Verderber et al., 1995). Because Müller cells have been shown to have a rich source of bFGF (Glaser et al., 1990; Buerd et al., 1985; Hagenmaier et al., 1991), it is conceivable that bFGF is released or activated following detachment and acts as the stimuli for increased expression of GFAP and vimentin both within and peripheral to the detachment. Indeed, the expression of bFGF and CNF, as well as GFAP, increases in response to continuous bright light exposure to the retina (Wein et al., 1998).

Finally, cell death does not appear to be essential to stimulating an increase in intermediate filament expression. In cases in which animals were raised in elevated oxygen, or in models of retinal ischemia, where no detectable neuronal loss occurs, GFAP levels still become elevated in Müller cells (Penn et al., 1988; Osborne et al., 1991; Fitzgerald et al., 1990).
inhibit the regeneration of the photoreceptor outer segments following retinal detachment (Fisher and Anderson, 2001). Thus, inhibition of subretinal scar formation is of practical interest. Antisense oligonucleotides have been used with some success to inhibit intermediate filament assembly in the CNS (Yu et al., 1991). Both intravenously injected brain-derived neurotrophic factor (BDNF) and dexamethasone environmental oxygen have been shown to be relatively effective in preventing the increase in intermediate filaments and the formation of Müller cell scars following retinal detachment (Lewis et al., 1998a,b). Whether these effects are direct or indirect is not known. In addition, retinal reattachment, following a period of detachment long enough to start the increase in intermediate filament expression, can essentially "freeze" the up-regulation of Müller cells so that there is no further increase (Lewis et al., 2001, 2002) (Fig. 4G). Reattachment does not cause a reversal of the increase within 28 days in the feline retina (Lewis et al., 2001) but may cause a slight decrease in expression after 150 days in the primate retina (Oehrlein et al., 1990). The highly stable intermediate filaments, once formed, may therefore remain for some time, even though their mRNA has been shown to return to normal levels within a few days after damage to the retina by bright light (Santhy and Egal, 1995).

The significance of glial scars in the retina remains as much an enigma as in the brain and spinal cord. Do reactive glial cells help or hinder the regenerative process? Do they provide molecular factors that save neurons or do they contribute to some instances to cell death? Although there may be some type of balance between the effects, it is quite clear that Müller cells contribute to two known causes of blindness: subretinal fibrosis, where their growth in the subretinal space inhibits outer segment regeneration, and proliferative vitreoretinopathy, where their growth in the vitreous produces mechanical traction and reattachment. In addition, in our model of detachment and reattachment, the glial scars provide apparent substrates for the aberrant growth of neovessels from horizontal cells and ganglion cells (Fig. 4H) (Lewis et al., 1998;Bloblutz et al., 2003). Although many of the glial and neuronal changes reported in feline retina have also been identified in complex human detachments (Sethi et al., 2003; Fisher and Lewis, 2005), to our knowledge there is no information on the effects of the more subtle changes on visual outcome. It is difficult, however, to envision the changes being "positive."

The great hypertrophy of Müller cell processes within the retina can greatly alter the architecture of the retinal tissue but its effects on vision are completely unknown. The light-scattering effect of filamentous proteins is well known and it seems likely that the immense increase in intermediate filaments, especially those at right angles to the light path, in some way alter image formation by the photoreceptor array. The inhibition of their expression (or functionality) may prevent the formation of sub-epiretinal membranes in the retina, both of which put patients at risk for impaired vision or
V. Summary

Our studies of detachment and reattachment illustrate the general properties and apparent significance of intermediate filaments to the overall responsiveness of Müller cells, especially in glial scar formation. They also uncover new, potentially significant questions about the role of intermediate filaments in these cells. In particular, the differential expression of intermediate filament proteins in Müller cell processes as they form glial scars on the opposing retinal surfaces raises the issue of different functions for GFAP and vimentin. It also raises the issue of mechanisms for the differential compartmentalization of these two proteins as well as for maintaining the balance of expression. Continued research in the control of intermediate filament expression seems imperative if we are to fully understand these processes.

The retina, an approachable part of the brain (Dowling, 1987), provides an excellent model system in which to understand the role of intermediate filaments in the CNS.

References


Fig. 2. laser-scanning confocal images of sections double-labeled with anti-GAP-43 (green) and anti-anti-GAP-43 (red) antibodies showing the reorganization of intermediate filaments in the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer.

Fig. 3. Immunofluorescence micrographs of sections double-labeled with anti-GAP-43 (green) and anti-anti-GAP-43 (red) antibodies showing the reorganization of intermediate filaments in the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer. Anti-GAP-43-labeled axons were evident in the center of the outer nuclear layer.
LEONG AND FISHER. FIG. 3. Laser scanning confocal images showing the intracellular (green) labeling pattern in the gondad epithelium after detachment (RD) and culture with RD. (A) Normal retina labeled with anti-GFAP (green). Labeling occurred primarily intracellularly with little uptake by Muller cell foot processes. Labeling with anti-axon-sheath (green) and anti-glial fibrillary acidic protein (GFAP) antibodies showed little change in the pattern of staining observed in normal retina. (B) Thirty-five days after detachment, retina showing few GFAP-positive cells. Labeling with anti-axon-sheath (green) and anti-glial fibrillary acidic protein (GFAP) antibodies showed little change in the pattern of staining observed in normal retina. (C) Seven days after detachment, retina showing increased GFAP labeling in the inner nuclear layer. Labeling with anti-axon-sheath (green) and anti-glial fibrillary acidic protein (GFAP) antibodies showed little change in the pattern of staining observed in normal retina.

ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; OCL: outer nuclear layer; GС: ganglion cell layer; IN: inner nuclear layer; ONL: outer nuclear layer; RD: retinal detachment; GFAP: glial fibrillary acidic protein; A: anterograde; P: peroxidase.