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Friend or Foe? Resolving the Impact of Glial Responses in Glaucoma

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Abstract

Glaucomatous vision loss results from the progressive degeneration of optic nerve axons and the death of retinal ganglion cells. This process is accompanied by dramatic alterations in the functional properties and distribution of glial cells in both the retina and the optic nerve head in a reaction commonly referred to as glial activation. The recent availability of rodent and cell culture glaucoma models has substantially contributed to our knowledge of glial activation under glaucomatous conditions. Conclusions drawn from these studies have led to the refinement of existing hypotheses and the generation of new ones. Because these hypotheses encompass both protective and injurious roles for glia, the impact of specific aspects of glial activation are current topics of intensive research, speculation and debate in the field. With these unresolved issues in mind, this review will summarize recent progress in our understanding of the process of glial activation in the glaucomatous optic nerve head and retina.

Keywords

astrocytes; microglia; optic nerve head; retina; glaucoma; intraocular pressure; gliosis

Introduction

The optic neuropathy of glaucoma is characterized by a predictable pattern of visual field loss and optic disk cupping. Cupping reflects a loss of axons in the prelaminar optic nerve head (ONH) and a backward bowing of the lamina cribrosa accompanied by extensive remodeling of the associated extracellular matrix (ECM). In the retina, there is selective loss of retinal ganglion cells (RGC) and nerve fiber layer thinning. Elevated intraocular pressure (IOP) is the most recognized glaucoma risk factor and controlling IOP is the foundation of current glaucoma therapy. In addition, aging, diabetes, race, hypertension, myopia, steroid exposure and family history all contribute to the risk of developing this disease.[1]

Historically, it has been generally hypothesized that elevated IOP results in ischemic or mechanical injury to optic nerve axons at the level of the ONH leading to axon degeneration and RGC death. Currently, the specific cellular mechanisms by which elevated IOP, or any other risk factor, results in glaucomatous optic nerve degeneration and RGC loss are not fully understood. However, recent investigations suggest that glial cells may play strategic roles in this injury process.

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In glaucomatous neuropathology, glial cells of the retina and ONH are known to change their morphology and their protein expression in a process referred to as glial activation. The specifics of activation differ, depending on the cell type and localization. In the retina injured by experimental IOP elevation, glial fibrillary acidic protein (GFAP) appears upregulated in astrocytes and Müller cells. In corresponding ONH, the normal columnar organization of glial nuclei is disrupted and there is increased expression of ECM proteins. Eventually, axonal debris is phagocytosed, the neural area contracts and is replaced by a GFAP-positive astrocytic scar.[2] Under normal conditions, astrocytes and Müller cells are believed to maintain the homeostasis of extracellular ions, glucose and other metabolites, water, pH and neurotransmitters such as glutamate and gamma-aminobutyric acid. They are also thought to contribute to the blood-brain barrier.[3] In uninjured neural tissues, microglia, the immune cells of the brain, actively survey their microenvironment via extremely motile processes and protrusions.[4] Following injury, microglia hypertrophy, remove debris from the injured area and may proliferate, secrete cytokines and act as antigen presenting cells. In contrast to traumatic injuries that disrupt the blood-brain barrier, in glaucoma there is at present no evidence for invasion of inflammatory cells.[5]

In the glaucomatous activation of glia cells, it is critical to determine the impact of specific aspects of these processes. Hypothetically, some changes could be enhancements of the normal, supportive functions of glial cells that may protect axons and RGC from further injury, as illustrated classically by ischemic preconditioning.[6] Alternatively, glial activation may result in the triggering of processes that result in further injury, as illustrated by glutamate excitotoxicity with reperfusion following ischemia in brain.[7, 8] These dichotomous glial responses are undoubtedly accompanied by many epiphenomena that have no impact on neuronal or axonal survival and are merely secondary responses to axonal degeneration and a challenge to glial homeostasis.

While the alteration in glial cell function associated with glaucoma has drawn the attention of researchers for many years, at least 50% of all original research articles and 75% of all reviews on this topic have been published since 2000. In 2008, the ARVO Pfizer Research Institute sponsored a meeting focused on the role of glia in glaucoma and a report from that meeting should be in press soon (http://www.arvo.org/EWEB/DynamicPage.aspx?site=foundation&WebCode=4thannualconf). One reason for research progress in recent years is undoubtedly the development of rodent and cell culture models to examine various aspects of glial response in injury induced by elevated IOP exposure or other hypothesized injury factors.[9-13] This review summarizes recent progress in understanding the roles of astrocytes, Müller cells and microglia in the two neural regions most affected by glaucoma, the optic nerve head and ganglion cell layer of the retina. While undoubtedly closely interrelated, axonal degeneration and RGC loss appear to be separately regulated biological processes.[14] Therefore, progress in our understanding of glaucomatous glial reactions in the ONH and retina will be discussed separately. These discussions will be followed by a brief consideration of the impact of the important risk factor of age on glial reactions in glaucoma. Finally, in the conclusion, we will consider the implications of new developments and highlight areas warranting further investigation.

ONH ASTROCYTES AND RESPONSES TO GLAUCOMATOUS INJURY

Astrocytes are the most prevalent glial cell in the ONH and astrocyte studies in human and experimental glaucoma dominate the recent ONH literature. In the normal anterior portion of the ONH, astrocyte cell nuclei are arranged in columns separating the axon bundles, with their GFAP-labeled processes oriented perpendicular to the nerve fibers. In the lamina and at vascular and pial surfaces within the nerve head, astrocytes and their processes are interposed

between these tissue components and axons. Reactive astrocytes are generally characterized by hypertrophy, hyperplasia, as well as increased expression of GFAP and vimentin.[15] In glaucoma or with experimentally elevated IOP, ONH astrocytes in the prelaminar ONH round up and migrate, abandoning their columnar organization. In experimental glaucoma, GFAP immunolabeling intensity and mRNA expression vary during the course of the injury process and scar formation, suggesting variations in the organization and abundance of the cytoskeletal protein.[2, 16-18]

Astrocyte-Mediated Alterations in the ONH Extracellular Matrix (ECM)

Changes in ONH ECM composition and organization in glaucoma are well recognized, including the deposition of ECM materials in areas formerly occupied by axons. These changes are likely to contribute to the altered biomechanical properties of the glaucomatous ONH that may increase the vulnerability of remaining axons.[19, 20] Astrocytes are thought to play the major role in the process of ECM remodeling.[5] Fibrillar collagens I and III, basement membrane collagen IV, microfibrillar collagen VI, tenascin C and elastic ECM components increase and appear disorganized.[21-24] New evidence suggests that genetic differences in elastin exon splicing, synthesis rates and maturational crosslinking may contribute to the higher risk of glaucoma in individuals of African ancestry.[25] To model glaucoma, ONH astrocytes have been exposed to hydrostatic pressure or mechanical stretch *in vitro*, resulting in the upregulation of many of these same ECM components.[12, 13, 24, 26] Glaucoma modeling in the rat in our laboratory suggests that specific ONH ECM protein messages, such as fibulin 2 and tenascin c, are most increased early in the injury process while other ECM components, such as periostin, collagen VI and IV, show increases that parallel the extent of nerve degeneration. These data lead to the concept that ECM changes in glaucoma are not static. Instead, progressive and variable alterations in the composition of the ECM accompany and may play a direct role in the response to pressure-induced ONH injury as well as the recovery from axon loss.[18]

Increased tissue stress in the ONH from elevated IOP may be detected by glial cells via transmembrane integrin receptor signaling. Integrins α2, α3, α6, β1, and β4 are localized within the glial columns and laminar beams of the human ONH, providing receptors for fibrillar and basement membrane collagens, fibronectin, laminin, tenascin, periostin, complement 1q, matrix metalloproteinases, reelin, and astroctyic hemidesmosomes.[27-33] In human glaucoma, reduced α6 integrin in the prelamina may reflect astrocyte migration and reorganization, while increased α4 labeling suggests microglial activation.[28, 34]

While integrins act as mechanoreceptors, transducing the stresses induced by elevated IOP, the matrix metalloproteinases (MMP) are key elements in the regulation of ECM remodeling.[35] Several MMP and their inhibitors, the tissue inhibitors of MMP (TIMPs), are present in ONH astrocytes. MMP1, 2 and 3, as well as membrane type1-MMP appear to be increased in glaucomatous human and in monkey ONH following exposure to elevated IOP.[36-38] These responses may be specific to pressure-induced injury, as similar increases are not observed in the ONH following optic nerve transection.[36] In rat ONH following extensive axon degeneration, mRNA for several MMP are increased, while levels of TIMP1 showed the greatest increase in ONH with focal injury.[18] MMP-related remodeling of the ECM is likely to be a major contributor to the changes in the biomechanical properties of the glaucomatous ONH, but alterations in MMP levels may also contribute to an improved potential for axonal regeneration. Increased expression of MMP, coupled with decreased TIMP, have been linked to a more permissive environment for optic nerve axon outgrowth following crush injury.[39]

Altered Gap Junctional Communication

While integrins and other receptors allow astrocytes to sense extracellular changes, astrocytes are also metabolically and ionically linked by gap junctions, forming a syncytium. In our rat glaucoma model, we found a loss of immunohistochemical connexin-43 gap junctional labeling at three days following IOP elevation.[2] Similarly, ONH astrocytes exposed to hydrostatic pressure demonstrate decreased gap junctional coupling, apparently mediated by epidermal growth factor receptor and associated with an increase of intracellular and nuclear connexin-43 phosphorylation.[40] Loss of gap junctional communication may have mixed effects on glaucomatous injury since reduced communication restricts the spread of injurious ions or metabolites, but simultaneously intensifies local damage by reducing the buffering effect of their distribution. Inhibition of astrocytic gap junctional coupling is has also been associated with increased astrocyte proliferation and is supported by increased glucose utilization via the pentose-phosphate pathway to produce ribose for nucleic acid synthesis.[41]

Astrocyte Proliferation And Migration

When normal human ONH astrocyte cultures were exposed to hydrostatic pressure, Hernandez found a two-fold increase in the number of cells after one day and an approximately five-fold increase after three to five days.[12] At three days following IOP elevation in our rat glaucoma model, the labeling of anterior ONH glial nuclei with antibodies to proliferating cell nuclear antigen coincided with the first alterations in connexin-43 labeling and preceded obvious morphological alterations in the glial columns.[2] More recently, we used the same rat model and microarray analysis to compare gene expression in ONH with extensive axonal injury to that in the normal ONH. In this study, the most significantly regulated biological process was cell proliferation, an observation confirmed by an almost three-fold increase in ONH DNA content.[18] Also significantly affected were cell migration and adhesion processes. Pressure-induced cell proliferation was also indicated by a near doubling of optic nerve astrocytes in a mouse glaucoma model.[42] When astrocytes cultured from normal and glaucomatous human ONH were compared in functional studies, the glaucomatous astrocytes were less adhesive and migrated more rapidly, as reflected by gene expression profiling in the same study.[43] Considered together, these studies suggest that elevated pressure exposure results in ONH astrocyte proliferation, as well as altered adhesion and migration properties. Recently, ONH astrocyte migration *in vitro* has been shown to be regulated via multiple kinase signal transduction pathways.[44, 45]

Re-expression of Developmental Proteins

Astrocytes from glaucomatous ONH may upregulate or re-express proteins normally associated with neural and glial differentiation during development.[5] Vimentin, characteristically found in immature astrocytes, appears increased in experimental glaucoma ONH in monkeys,[46] as well as in ONH astrocytes exposed to hydrostatic pressure.[47] Nestin, another developmental intermediate filament protein, is also upregulated in glaucomatous rat ONH.[18] The embryologically expressed, bidirectional signaling molecules Ephrin B1 and Eph1B are upregulated in experimental primate glaucoma ONH and in ONH astrocytes cultured from glaucomatous human ONH,[48] while in a genetic mouse glaucoma model, the upregulation of ephrin B2 and EphB2 in glia is associated with elevated axonal calcium and axon loss.[49] Ephrin signaling is involved in maintenance of shape, movement and attachment. Following neural injury, ephrins and Eph are generally upregulated and appear to have differential effects on axons and glia. Ephrins inhibit axon regeneration and induce growth cone collapse, while they stimulate glial activiation.[50]

Neurotrophins (NT) And Cytokines In Astrocytic Responses

NT and NT Receptors—Cultured human optic nerve astrocytes express and secrete NT of the nerve growth factor family and their associated Trk receptors.[51] Exposure of these cells to exogenous NT leads to receptor phosphorylation, astrocyte proliferation and an altered composition of secreted NT.[52] In addition, exposure of cultured ONH astrocytes to ischemic conditions resulted in altered NT and NT receptor expression.[53] In immunohistochemical studies of ONH responses in a rat glaucoma model, immunolabeling for brain-derived neurotrophic factor (BDNF) and NT4/5 in the ONH appeared increased by extensive injury, suggesting *in vivo* NT upregulation by ONH astrocytes.[2] These observations together suggest that altered autocrine or paracrine astrocytic NT responses may be evoked during ONH glaucoma pathogenesis.

Transforming Growth Factor Beta (TGFβ) and the TGFβ Superfamily—TGFβ contributes to glial proliferation, differentiation and the regulation of ECM composition.[54, 55] Although ONH immunolabeling for both TGFβ1 and TGFβ2 appeared increased in monkeys with experimental glaucoma,,[56] further studies using human glaucomatous ONH explants and bioassays have implicated astrocytic TGFβ2 as a key modulator of glaucomatous responses.[57] In addition, TGFβ2 stimulates cultured ONH astrocytes to increase production of basement membrane and other ECM components.[58] The role of TGFβ2 in the pathogenesis of glaucoma has been reviewed.[59] In our recent studies of ONH gene expression using our rat glaucoma model, we found evidence for differential regulation of TGFβ1 and TGFβ2 mRNA levels. The level of TGFβ1 increased linearly with increasing pressure-induced nerve injury, while that of TGFβ2 did not.[18]

In addition to TGFβ, other members of the TGFβ superfamily have been hypothesized to play roles in glaucomatous ONH injury. Microarray studies indicate an upregulation of betaglycan (TGFβ receptor III) in isolated glaucomatous ONH astrocytes.[43] In a rat glaucoma model ONH, mRNA for TGFβ receptor 1 and 2, plus a number of TGFβ pathway regulators were altered in expression.[18] Isolated human ONH astrocytes express mRNA and protein for TGFβ family members, including bone morphogenic proteins (BMP) 2, 4, 5 and 7, glial derived neurotrophic factor, their receptors and a number of BMP inhibitory and signaling proteins.[60, 61] BMP signaling pathway mediators are induced in ONH astrocytes by exogenous BMP4 application.[62] BMPs promote proliferation of glial precursors and astrocytic differentiation.[63] Together, these observations suggest that members of the TGFβ superfamily play diverse roles in the glaucomatous ONH responses that remain to be elucidated in greater detail.

Tumor Necrosis Factor Alpha (TNFα)—TNFα and TNFα receptor 1 are expressed in the glaucomatous human ONH[38, 64] Cultured ONH astrocytes express TNFα receptor and, in response to exogenous TNFα, produce inducible nitric oxide synthase and endothelin-1 (ET-1), two proteins proposed to play key roles in glaucomatous ONH responses.[64, 65]

Endothelin Dysregulation

Numerous reports of elevated concentrations of ET-1 in the aqueous humor and plasma of individuals with glaucoma support a role for the dysregulation of this potent vasoconstrictor in the pathogenesis of glaucoma.[66-70] Mechanical stress, ischemia and cytokines, such as TNFα and TGFβ, all induce the expression and secretion of ET-1 in ONH cells.[65, 71, 72] Administration of exogenous ET-1 to the ONH of monkeys or rats produces an optic neuropathy ascribed to ischemia that has demonstrated some clinical similarities to the optic neuropathy of glaucoma,[73, 74] including optic cup enlargement,[75] and regional axon loss.[76, 77]

Separately from its ability to induce ischemia, ET-1 is also an important glial mitogen.[78] Exposure of cultured ONH astrocytes to ET-1 results in a 30% increase in cells in a 96 hour period.[79] This proliferation appears mediated via several parallel signaling pathways.[80] In addition to the vascular and proliferative effects of ET-1, it also increases the expression of MMPs and their inhibitors, thus contributing to ECM remodeling.[81]

In rat eyes with elevated IOP, ET-1 levels in the aqueous humor increased and immunolabeling for ET-1 and ET receptor B appeared increased and co-localized with GFAP in the ONH.[82] ET receptor B mediates the vasodilative actions of ET-1 (in contrast to its vasoconstrictive functions mentioned above) and is associated with endosomal uptake and lysosomal degradation of extracellular ET-1, while ET receptor A mediates the vasoconstrictive effects of the signaling protein.[83] Recent studies of human glaucomatous and experimental monkey ONH have confirmed an increase in astrocytic ET receptor B immunostaining.[84] In general, levels of this receptor are down-regulated during astrocyte proliferation,[85] and, in fact, message levels for ET receptor B were found to be downregulated coincidental with evidence of ONH cell proliferation in our recent rat glaucoma microarray analysis.[18] Therefore, evidence from human and experimental glaucoma supports a role for increased ET-associated vasodilative responses as well as astrocytic proliferation.

Evidence of Oxidative Stress

Oxygen-dependent metabolism inevitably produces reactive oxygen species, such as superoxide, peroxide and hydroxyl radicals. These are normally detoxified by endogenous antioxidant processes. During periods of hypoxia, metabolic or other cellular stress, reactive oxygen species may accumulate to a point that critical cellular functions are damaged. In various neurodegenerative diseases, localization of tissue markers of oxidative stress to lesion areas has led to suggestions that oxidative injury contributes to the disease process. Exposure to oxidant insult may also upregulate antioxidant levels to enhance endogenous neuroprotection. For example, exposure to a lipid peroxidation product, 4-hydroxynonenal, increases levels of ONH astrocytic glutathione and antioxidant enzymes.[86] Therefore, oxidative stress may play a role as a primary injurious event, contribute to the propagation of injury, or provide a stimulus to increase endogenous neuroprotective mechanisms or more downstream by-products of the injury process. The relative importance of each of these roles in neurodegeneration remains unclear.[87]

Oxidative stress can promote the production of advanced glycosylation end-products (AGEs) that are commonly associated with aging and diabetes, and AGEs exacerbate oxidative stress.[88-90] Recently, increased immunolabeling for AGEs and the AGE receptor, RAGE, have been found in the ONH of glaucomatous human eyes.[91] While labeling for AGEs was primarily associated with the lamina cribrosa, RAGE was co-localized with GFAP, suggesting that astrocytic responses to extracellular AGEs may play a role in glaucomatous glial activation and migration.

Nitric Oxide Dysregulation

The isoforms of nitric oxide synthase (NOS), neuronal, inducible (iNOS) and endothelial, play important roles in neural transmission, defense responses and vascular dilation, respectively. It has been hypothesized that, in reperfusion injury following ischemia, excessive nitric oxide production by NOS may react with superoxide to produce the damaging free radical peroxynitrite. While human glaucomatous and rat glaucoma model ONH showed positive immunolabeling for all three isoforms, the apparent increase of iNOS in ONH glial cells in glaucomatous human ONH led to the hypothesis that overproduction of iNOS leads to axon loss.[92-95] In support of this hypothesis, cultured astrocytes were shown to increase iNOS mRNA and protein in response to exposure to hydrostatic pressure,[96] and two iNOS inhibitors

were found to be neuroprotective in experimental studies using a rat glaucoma model.[97, 98] In the same rat glaucoma model, IOP control with timolol was found to decrease both iNOS and nNOS ONH immunolabeling.[99] However, other studies of human glaucoma and experimental rodent glaucoma models found no evidence of iNOS upregulation, nor was pharmacologic iNOS inhibition found to be neuroprotective.[100-102] Therefore, the role iNOS in the pathogenesis of glaucomatous ONH injury remains controversial.

Epidermal Growth Factor Receptor (EGFR) Activation

EGFR is widely involved in the regulation of growth and differentiation of cells, including neuroglia. The linking of EGFR activation to the induction of iNOS in cultured human astrocytes led to observations that EGFR is upregulated and phosphorylated in human glaucomatous ONH. Further *in vitro* studies demonstrated that exposure of human ONH astrocytes to increased hydrostatic pressure resulted in receptor phosphorylation and nuclear translocation, suggesting that EGFR activation plays a role in glaucoma pathogenesis.[103] Activation of EGFR led to the induction of cyclooxygenase-2 and prostaglandin e_2 in ONH astrocytes,[104, 105] affected the three-dimensional organization of astrocytes and their interaction with neurites,[106] and appeared associated with the process of astrocyte activation and migration.[45, 107] These observations led to the hypothesis that the activation of ONH astrocytic EGFR plays an injurious role in glaucoma.[108] However, EGFR activation in astrocytes can also promote cell survival, glutamate transport and functional support of neurons, suggesting the need for a more complete understanding of the potentially varied roles of this receptor in the ONH.[109, 110]

Dysregulation of Steroid Metabolism

Corticosteroid exposure is a well known risk factor for glaucoma through its potential effects on aqueous humor outflow via the trabecular meshwork.[111] However, the effects of steroids on ONH glial cells are only beginning to be explored. When cultured porcine ONH astrocytes were exposed to dexamethasone, the expression levels of two genes implicated in glaucoma pathogenesis, optineurin and myocillin, were altered, suggesting that corticosteroid exposure may have *in vivo* effects on ONH astrocyte function.[112]

Elevated levels of 3-hydroxysteroid dehydrogenases (HSD) are found in astrocytes from glaucomatous human and experimental monkey ONH. These enzymes are induced by oxidative stress and metabolize many endogenous steroid isoforms. 3α-HSD immunostaining appears intensified in reactive astrocytes in human and experimental monkey glaucomatous ONH and, when normal astrocytes are exposed to hydrostatic pressure, 3α-HSD mRNA is increased[113] In the same study, an enhanced conversion of androgen 5α-dihydrotestosterone to neuroactive 5 α-androstane-3 α,17β-diol (3α-diol) by astrocytes from glaucomatous eyes was observed. Cultured ONH astrocytes exposed to 3α-diol demonstrate increased levels of androgen receptor (AR) protein and evidence of the activation of kinase signaling pathways, demonstrating that astrocytes are potential targets of neuroactive androgens.[114] In ONH from monkeys with experimental glaucoma, demonstration of increased astrocytic immunostaining for AR and for nuclear factor kappa b, a transcription factor implicated in the upregulation of AR, led to the suggestion that altered androgen metabolism may play a significant, potentially neuroprotective, role in glaucoma pathogenesis.[115]

Astrocytic Immune Responses

Glaucomatous ONH, as well as cultured ONH astrocytes subjected to ischemic conditions, interleukin 10 or interferon gamma, demonstrate increased levels of major histocompatibility complex antigens. Immune responses may also be enhanced by oxidative stress. When ONH astrocytes were treated with reactive oxygen species, they expressed major histocompatibility complex class II proteins and became more potent inducers of T-cell activation.[116] This

suggests that ONH astrocytes can function as antigen presenting cells and may evoke immune responses that contribute to the initiation and progression of glaucomatous injury.[117]

Roles for ONH Microglia In Glaucoma

ONH microglia are much less numerous than astrocytes and their responses in glaucomatous ONH injury have not been extensively studied. Given their important roles in phagocytosis, surveillance and immune responses,[4, 118-120] a better understanding of their functions in the normal and glaucomatous ONH is warranted. For instance, it is intriguing that nitric oxide production by isolated microglia is at least an order of magnitude greater than that for astrocytes.[121] Microglia are distributed throughout the ONH, particularly in association with the elements of the vasculature. In glaucoma, they hypertrophy, reorganize, and increase in density near the peripapillary choroid.[17, 122] These activated microglia demonstrate increased immunolabeling for TNFα, TNF receptor-1, TGFβ2, proliferating cell nuclear antigen, cyclooxygenase 2, MMP and TIMP proteins.[64, 95] In our rat glaucoma model, the expression level of a microglial marker, ionized calcium binding adapter molecule 1, was positively correlated with the extent of axonal degeneration.[18] TNFα-associated microglial activation has been proposed to lead to loss of optic nerve oligodendroglia in a mouse glaucoma model.[123] In addition, the increased immunolabeling for integrin α4 that we observed in human glaucomatous ONH and the upregulation of many microglial associated messages in glaucomatous rat ONH may also reflect microglial activation.[18, 28] Reactive microglia are also implicated as sources of reactive oxygen species, as well as initiators of both protective and destructive immune responses.[124]

GLAUCOMATOUS RESPONSES IN RETINAL GLIA

Three glial cell types are recognized in the inner retina: astrocytes, Müller cells (together termed macroglia), and microglia. Located within the nerve fiber layer, astrocytes can appear either elongated, with processes oriented parallel to the nerve fiber bundles and without apparent vascular contact, or stellate, having an oval appearance with processes crossing nerve fiber bundles and contacting the vasculature. Stellate astrocytes are primarily located at the level of RGC soma.[125, 126] Müller cells, whose nuclei lie within the inner nuclear layer, have vertically oriented processes that span the entire thickness of the retina, their inner foot processes forming the internal limiting membrane. Ultrastructural studies indicate that interactions among these glia exist, since desmosomes are present between astrocytes and Müller cells, although gap junctions exist only between astrocytic foot processes.[126] Also, both appear to contribute to the internal limiting membrane, the vascular glia and glial sheaths of the retinal ganglion cells. Microglia appear ovoid or amoeboid in the normal retina, usually within the ganglion cell and inner plexiform layers.

Macroglia appear to supply metabolic support for RGC cell bodies and axons, modulate the extracellular ionic balance, regulate local concentrations of neurotoxic compounds and produce neurotrophic factors. [126, 127] [127-129] They are thought to function as intraretinal phagocytic cells during development, in various retinal diseases and following optic nerve transection.[130]

Retinal Glial Cell Activation in Glaucoma

Glial activation in the glaucomatous retina has been suggested to have both injurious and protective effects on RGC.[130-132] Evaluations of retinal sections from glaucomatous eyes have demonstrated an increase in label for the catalytic forms of mitogen activated protein kinases in glial cells, leading to the suggestion that these signaling pathways may be responsible for glial activation. Further, activation of these pathways is also associated with cell survival and glial cells are relatively protected in the glaucomatous retina as compared to RGC, which lack similar kinase activation.[133] However, processes that enhance glial survival in injured

retinas could simultaneously sustain their supportive functions. This alternative was suggested by the observation that exposure to elevated IOP increases Müller cell expression of the anti-apoptotic protein, BCL-2, which may enhance Müller cell survival and effective function, thus indirectly protecting RGC.[134] Another example of the potential of glial cells to offer protection to RGC is the demonstration that Müller cells can be a source of neurotrophic support.[135] This possibility is discussed further below.

Experimental elevation of IOP also results in retinal microglial activation, identified by an increase in the size and number of microglial cells throughout the retina.[130, 136] Proliferation of microglia in the inner retina has also been demonstrated in a genetic mouse glaucoma model.[137] These responses, as well as those of macroglia, were temporally and spatially correlated with downregulation of neuronal markers, suggesting that glial responses are closely associated with glaucomatous neuronal degeneration. However, whether glial responses play a pivotal role in RGC death, or simply represent a secondary reaction to neuronal damage, such as the removal of cellular debris, is currently unknown.

Intermediate Filament Protein Expression in Activated Retinal Macroglia

The intermediate filament protein, GFAP, is exclusively expressed in macroglia and is generally used as a marker for astrocytes. In normal eyes, retinal astrocytes label with antibodies to GFAP, while Müller cells demonstrate little labeling.[130, 138] Increased GFAP expression occurs in a wide variety of retinal pathologies including ischemia and macular degeneration and is commonly used to identify glial activation. Multiple investigations using various experimental and genetic glaucoma models indicate that GFAP expression is increased, probably in both retinal Müller cells and astrocytes,[17, 130, 132-134, 137-142143] although there is some evidence of decreased expression in retinal astrocytes.[144] A study of GFAP-labeled astrocytes in human glaucomatous retinas found evidence of increased cell density in the peripapillary region and in association with the vasculature, while nerve fiber layer astrocytes did not appear altered.[145] In a murine glaucoma model, the upregulation of GFAP in macroglia following exposure to elevated IOP appeared to occur without evidence of proliferation.[137]

There are conflicting reports as to whether expression of vimentin, another intermediate filament protein present in retinal glia, is altered by elevated IOP.[17, 138, 143] Nestin, a third intermediate filament, considered by some a marker of neural progenitor cells, has been identified in Müller cells and some astrocytes of the ganglion cell layer. Increased expression of nestin in experimental rat glaucoma may represent an early, neuroprotective response to neural injury.[142]

Heat shock proteins (HSP) Upregulation

HSP function as cellular chaperones by assisting in protein folding. They have been found to increase in expression following injury and are thought to enhance neuronal survival. HSP immunolabeling was increased in glaucomatous human retinas, where it was primarily localized to RGC, although there was some increase in labeling of glial portions of the internal limiting membrane.[146] Increased retinal HSP expression has been found in several models of chronically elevated IOP as well.[138, 143, 147, 148] In a rat experimental glaucoma model, pharmacologic HSP induction offered neuroprotection.[149] In another study, elevated IOP exposure increased immunoreactivity to HSP in the nerve fiber layer, and colocalized with cells labeled by GFAP and vimentin, suggesting that glial cell HSP may play a neuroprotective role.[143]

Altered Iron Regulation

Studies in several animal models with elevated IOP and in human glaucoma eyes suggest a potential role in glaucoma for proteins involved in iron regulation. All of these studies have found upregulation of ceruloplasmin message in the retina,[138, 139, 150, 151] with some detecting commensurate elevation in protein levels.[150, 151] Significantly, immunohistochemistry localized this increase to the Müller cells and inner limiting membrane, which is composed primarily of Müller cell end feet.[151] Ceruloplasmin, a glycoprotein whose role in copper transport in the blood is well known, is synthesized in CNS glial cells, and by retinal macroglia.[152] Here it is believed to play a role in converting ferrous iron, which is toxic, to ferric iron, which binds to transferrin, a protein responsible for intracellular delivery of iron. Interestingly, retinal expression of transferrin, as well as ferritin, a major, non-toxic storage form of iron, has also been found to increase following experimental elevation of IOP in monkeys and in human glaucoma eyes.[150] Since all three proteins are involved in iron regulation, and may function as antioxidants, it has been proposed that upregulation of these proteins is an endogenous protective retinal response, mediated most likely by glial cells, to excess iron or oxidative stress.[150, 151] Whether this response is a direct effect of elevated IOP or a response to RGC injury is unknown, and the possibility that these responses might actually contribute to the primary mechanisms of RGC loss has not been totally ruled out. Resolving these possibilities could be critical to eventually developing strategies that manipulate these or other iron-regulating genes to produce effective neuroprotective treatments for glaucoma

NT and NT Receptor Responses in Macroglia

The survival of RGC may depend upon the availability of NT, and it has been proposed that RGC death in glaucoma results from loss of neurotrophic support due to the obstruction of retrograde transport of brain-derived neurotrophic factor (BDNF) and its TrkB receptor at the ONH.[153] Gene transfer to increase RGC BDNF expression or activate TrkB-mediated survival signaling pathways has been shown to enhance both somal and axon survival in rat glaucoma models.[154, 155] Interestingly, adenoviral transfection of Müller cells with a transgene expressing BDNF delayed RGC death following optic nerve transection.[135] A similar experiment in a glaucoma model has not yet been performed, but a comparable response would suggest another potentially exciting, neuroprotective role for glial cells in this condition.

Both BDNF and TrkB can be synthesized locally within the retina, and could affect RGC survival in either autocrine or paracrine fashion.[156-158] In a model of experimental IOP elevation, retinal levels of NT and receptor mRNA were altered, including the upregulation of TrkC receptor, apparently localized to the Müller cells, and a late increase in p75, a pro-apoptotic receptor that also has the ability to enhance cell survival pathways under specific circumstances.[159] In addition, exposure to glutamate has been shown to increase expression for several NT in cultured Müller cells.[160] These observations suggest that the reactive response of retinal glial cells in glaucoma may include events that directly affect the survival or loss of RGC.

The Potential of Glutamate Excitotoxicity

Initial reports of elevated levels of glutamate, a major central nervous system excitatory neurotransmitter, in the vitreous of glaucomatous human eyes[161] and in glaucoma models[161] introduced the possibility that RGC death in glaucoma could be mediated by excitotoxicity from prolonged stimulation of glutamate receptors, including NMDA receptors, and elevated intracellular calcium. It was hypothesized that elevation of glutamate resulted from a reduction in glutamate transporters, normally responsible for internalization of glutamate, and the reduced activity of glutamate synthetase, which is responsible for metabolizing glutamate. Glutamate transporters and glutamine synthetase have been located in retinal astrocytes and Müller cells,[162] suggesting an important role for glial cells in this

process.[163] In fact, glutamine, a precursor in the formation of glutamate, and glutathione, a metabolite of glutamate, have both been found increased in Müller cells in monkeys with elevated IOP.[164, 165] A recent study of mice deficient in glutamate transporters has demonstrated reduced levels of glutathione in Müller cells coupled with spontaneous RGC and optic nerve degeneration without elevated IOP. RGC loss in these mutants was prevented by administration of glutamate receptor blockers.[166] However, in rat glaucoma models, there are conflicting reports regarding alterations in retinal glutamine synthetase and glutamate transporter levels.[134, 167, 168]

The possibility that excess glutamate contributes to RGC death in glaucoma is controversial. Studies in monkeys[169, 170] and rats[171] with experimental glaucoma, as well as human glaucoma eyes,[172] have failed to confirm initial reports of elevated vitreous glutamate levels. Recent work using imaging of retinal ganglion cell calcium failed to show functional evidence of altered glutamate clearance in retinas from rats with chronically elevated intraocular pressure and optic nerve and retinal damage .[173] In other studies, glutamate was found to stimulate upregulation of NT expression in Müller cells, potentially augmenting the supply of NT.[160] Finally, cell culture and in vivo analyses have demonstrated that the RGC is relatively invulnerable to high concentrations of glutamate and NMDA, despite substantial injury to amacrine cells.[174] While the glutamate hypothesis presents an intriguing view of how altered function of retinal glial cells might contribute to RGC death, the initial release of findings of multicenter clinical trial of memantine, an NMDA inhibitor, in patients with chronic glaucoma reported no significant benefit of drug treatment compared to placebo. (http://agn360.client.shareholder.com/releasedetail.cfm?ReleaseID=290764)

Evidence of immune System Dysfunction

The observation that glaucomatous visual loss can proceed in the apparent absence of elevated IOP suggests that a number of other factors may influence this process. Among others, the potential that the immune system may contribute to glaucomatous nerve damage through an autoimmune mechanism has been suggested and thoroughly reviewed by Tezel and coworkers.[131, 175, 176] Both normal tension and open angle glaucoma patients have been found to have increased serum autoantibodies to small HSP.[177-179] In addition, the direct application of antibodies to small HSP appears to induce apoptosis in cultured retinal ganglion cells.[179] These observations, coupled with the evidence that glial cells have the capacity to be antigen-presenting cells, suggests that retinal glia may contribute to immune responses that ultimately are destructive to RGC.[146116, 175]

Involvement of the immune system in glaucomatous processes is suggested by the dramatic upregulation of components of the complement cascade in monkey and rat retinas following experimental IOP elevation and a mouse genetic glaucoma model with a demonstrated immune component.[132, 138, 139, 180] Complement expression may play a role in the process of glial activation. Increased expression and immunolocalization of complement cascade components and the membrane attack complex were found in RGC soma and along the vitreal surface in both human glaucoma and a rat model of elevated IOP, but did not co-localize with GFAP.[141] The upregulation of complement cascade components has been confirmed in a genetic mouse glaucoma model in which immunolabeling of immature glial or Müller cells with complement antibodies was associated with elimination of inner plexiform layer synapses.[181, 182] This linkage offers an explanation for the shrinkage and remodeling of the dendritic tree in specific RGC subtypes that has been demonstrated in experimental primate glaucoma[183] and suggests a role for glial cells in this degenerative process.

TNFα, The TNF Receptor Superfamily and RGC Apoptosis

Specific increases in the number of cells labeling for TNFα and its receptor have been described in the inner retina of glaucomatous human eyes compared to normals.[184] These increases were apparently localized to glial cells and RGC, respectively, suggesting that this aspect of glial cell reaction in glaucoma may also contribute to RGC death. When isolated retinal glial cells were exposed to increased hydrostatic pressure, they secreted TNFα and induced apoptosis in co-cultured RGC.[185] Increased levels of immunoreactivity for Fas and FasL, a member of the TNF receptor superfamily, werefound to correlate with increases in labeling for both microglia and astrocytes.[140] While activation of the Fas/FasL receptor can be protective under certain circumstances, it often results in apoptosis via caspases. The exclusive co-localization of caspases with GFAP in the retina in a rodent glaucoma model led to the suggestion that glial cells are actively involved in the initiation of RGC apoptosis.[186] More recent evidence of increased Müller cell reactivity for Fas-associated death domain (FADD), an important component for Fas-caspase-mediated apoptosis, further strengthens the possibility that glial cells contribute to RGC apoptosis from elevated IOP.[140]

Retinal Microglial Responses

As is the case in the ONH, the specific roles of retinal microglia in glaucomatous injury have not been as extensively studied as those of other retinal glia. There are numerous reports of microglial proliferation and hypertrophy in retinas in experimentally induced glaucoma.[130, 131, 136, 137, 140] They, too, express Fas/FasLimmunoreactivity, which increases in experimental glaucoma.[140] In a genetic mouse glaucoma model, microglia were the only glial cells to proliferate in the retina during the course of the disease.[137] In this same model, suppression of microglial activation with minocycline appeared to be protective of RGC integrity.[187] Clearly, much more work remains to be done to understand better how retinal microglia influence RGC death in glaucoma.

AGING AND GLIAL RESPONSES IN GLAUCOMA

Aging is an important risk factor for glaucoma and also a consideration when modeling glaucoma in animals. We routinely use mature adult (8 month old) rats, but the use of very elderly animals does impact experimental responses. For example, in comparison to adult rats, we find that the optic nerve of aged (28 month old rats) is significantly more susceptible to injury when exposed to minimally elevated IOP (40 to 80 mm Hg-days).[188]

This increased susceptibility may reflect alterations in the responses of glia in the aged ONH to pressure-induced injury. To begin to explore this, we have examined gene expression patterns in adult and aged ONH using our rat glaucoma model.[189] In general, although we found that gene expression responses to elevated IOP exposure were similar in both studies, the aged study identified only about 24% of the number genes identified in the adult study. In addition, the dynamics of the regulated responses were often altered. For example, in aged ONH, the pressure-induced increase in the mRNA for matricellular protein periostin was 47% of that in adult ONH, while the increase in that for microglial IBA1 was 220%.[190]

We found that aging also impacts the retinal responses to exposure to elevated IOP. In the aged retina, there is a significant augmentation the pressure-induced reduction of axonal cytoskeletal neurofilament H mRNA coupled with an attenuation of neuroprotectiveTIMP1 upregulation, as compared to the pressure-injured adult retina. [188] The relationship between aging responses and the function of specific glial cells in the retina is largely unexplored. However, in comparing gene expression in adult and aged retinas, we found that aging alone significantly upregulated the retinal mRNA for GFAP, the commonly used marker for astrocyte activation.[191]

For both the retina and ONH, a clear understanding the effect of aging on the specific responses of each glial cell type in these complex tissues remains a current challenge in deciphering the mechanisms of glaucomatous neuropathy.

DISCUSSION

While recent years have dramatically increased our knowledge of the responses of glial cells in glaucomatous conditions, obviously more investigation is needed to clarify the impact of these responses on RGC and axonal survival. The upregulation of HSP in glaucomatous retinas is a case in point.[138, 143, 147, 148] While the chaperone functions of HSP are generally accepted to be protective, the production of autoantibodies to HSP in human glaucoma patients raises the potential that these "neuroprotective" proteins may trigger autoimmune responses that contribute to RGC loss.[177-179] It also illustrates the potential complexity of organismal, as opposed to cellular, responses.

Future investigations are needed to localize and discriminate between responses that occur simultaneously in RGC and glia. In many cases, even when a glaucomatous response is known to be glial, it has not been localized to a specific glial cell type. Because ONH astrocytes and retinal Müller cells are the most abundant glial cell types in each location, they have been more extensively studied, yet it is important to discriminate between their responses those of other glial cells. In particular, the responses of microglial cells have generally been underemphasized, despite the fact that their immune surveillance and phagocytic functions leave them beautifully equipped to participate in the glaucomatous process.

A corollary to this is the observation that endogenous glial cell responses may or may not affect adjacent RGC or axons. For example, the direct neuroprotective effects of HSP upregulation in glia are likely endogenous. In contrast, glial upregulation of neurotrophins offers the potential to enhance RGC survival in a paracrine fashion .

In addition, as pointed out in a recent review,[192] glial cell contributions to the glaucomatous process may change during the course of the disease, from ones that are initially defensive and protective to those that are directed to recovery and scar formation. Our own examination of ONH in rat glaucoma illustrates the complexity of ECM gene expression changes during the course of increasing axonal involvement in the degenerative process.[18]

Additionally, early glial responses may not necessarily be protective. Injured glial cells may respond to glaucomatous insults in a way that enhances their own survival while simultaneously diminishing their functional support of axons or RGC. For example, early glial, presumably astrocyte, proliferation within the ONH occurs in response to elevated IOP in our rat glaucoma model, as evidenced by upregulation of cell cycle genes and dramatic increase in total ONH DNA.[18] Similarly, human glaucoma is associated with an increase in immunostaining for ONH synuclein gamma, a protein associated with mitotic checkpoint regulation.[193, 194] ONH astrocyte proliferation almost certainly has a negative impact on the integrity of remaining axons, because of the anatomical association of each astrocyte with multiple individual axons. Astrocyte proliferation, coupled with altered adhesion and cellular migration, implies a significant disruption of their relationships to axons. It likely diminishes the functional role of astrocytes as metabolic mediators between the axons and their vascular supply of nutrients. This may be especially important in the anterior ONH, where the metabolic demands of unmyelinated axons are likely greater.[195, 196] To compound this situation, glucose utilization is increased in proliferating glia to produce ribose for nucleic acid synthesis.[41] It is possible, therefore, that pressure-induced glial proliferation may result in a state of local axonal glucose deprivation in the nerve head, even in the presence of normal vascular perfusion.

In discussing glaucomatous glial responses, the relative effects of proliferation and hypertrophy have been generally ignored. In contrast to the above-discussed likelihood that astrocyte proliferation may result in passive axonal injury, glial cells undergoing hypertrophy may maintain, or even augment their specialized functions that support RGC and axons. Recently, using the DBA2j mouse glaucoma model, retinal glial responses have been characterized as non-proliferative.[137] Clearly, more study is needed to clearly differentiate between glial proliferative and hypertrophic responses and their significance. In this regard, sources of proliferating cells need to be identified. In the adult retina, Müller cells have the capacity to become progenitor cells.[197] In the myelinated optic nerve, oligodendroglial progenitor cells, and their role in multiple sclerosis, have also been extensively studied.[198] However, it is not known if the normal ONH has a pool of progenitor cells, although a pool of presumably immature astrocytes, characterized by immunopositivity to GFAP, vimentin and nestin, has been described.[199]

In conclusion, the use of recently developed animal and cell culture models for glaucoma has led to the rapid expansion of our knowledge of the responses of glial cells in the ONH and retina. In many cases, these models have been verified by replicating observations originally derived from studies of human glaucoma specimens. Further refinement and application of these models offer the promise of a better understanding of glial responses to glaucomatous conditions and the potential that this knowledge will lead to enhanced therapeutic intervention in this disease.

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