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Retinal Ganglion Cell Apoptosis in Glaucoma Is Related to Intraocular Pressure and IOP-Induced Effects on Extracellular Matrix

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Abstract

Purpose—To investigate the effect of IOP on retinal ganglion cell (RGC) apoptosis and correlate the effects with IOP-induced changes in extracellular matrix (ECM) in the retina and optic nerve head (ONH) in glaucomatous rat eyes.

Methods—Thirty-seven Dark Agouti rats had elevated IOP induced in the left eye by hypertonic saline episcleral vein injections. Eyes were examined at 3 months histologically for RGC apoptosis and expression of specific ECM components.

Results—RGC apoptosis was significantly related to IOP exposure (integral Δ IOP $P < 0.001$; peak IOP $P < 0.01$). In the RGC layer, elevated IOP correlated positively to a significant increase in MMP-9 activity ($P < 0.001$), tissue inhibitor of matrix metalloproteinase (TIMP-1) ($P < 0.05$), and collagen I ($P < 0.01$), and negatively correlated to deposition of laminin ($P < 0.05$) and TGF- β 2 ($P < 0.05$). There was a significant correlation between MMP-9 activity and both RGC apoptosis ($P < 0.001$) and loss of laminin ($P < 0.01$). IOP exposure was also associated with increased deposition of TGF- β 2 and collagen I at the ONH ($P < 0.01$).

Conclusions—The results demonstrated that RGC apoptosis in glaucoma correlates strongly with elevated IOP and is significantly associated with IOP-induced changes in specific ECM components in the RGC layer. The study shows for the first time a link between MMP-9, laminin degradation, RGC apoptosis, and IOP exposure in glaucoma. The findings suggest that abnormal ECM remodeling in the glaucomatous retina may relate to RGC death and support the notion that the retina is a primary site of injury in glaucoma.

Glaucoma is a major cause of worldwide irreversible blindness. Vision loss in glaucoma is attributed to retinal ganglion cell (RGC) death, and intraocular pressure (IOP) is the major modifiable risk factor.¹ The underlying mechanisms that link elevated IOP to glaucomatous

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RGC death are not fully understood, though the process of RGC apoptosis is heavily implicated.²⁻⁴ Furthermore, the site of primary damage in glaucoma is controversial, although it is suspected to be either at the optic nerve head (ONH) or the retina and is complicated by the likelihood of the effects of secondary RGC degeneration.⁵

An established theory is that elevated IOP induces physical changes at the ONH, visualized clinically as optic disc cupping, which causes optic nerve axonal compression at the lamina cribrosa, blockage of axoplasmic flow, and interference in retrograde neurotrophin transport to RGCs, leading to cell death. The ONH has previously been investigated in both human and experimental animal models as a primary site of glaucomatous damage. These studies have shown extensive remodeling of the extracellular matrix (ECM), including collagen I and IV, TGF- β 2, and matrix metalloproteinase (MMP)-1.⁶⁻¹³ Hernandez⁸ has identified the astrocytes as the key cell type involved in this process at the ONH and has shown astrocytes to be activated by increased IOP. Yan et al.⁹ have shown that these active astrocytes are responsible for the production of the matrix-degrading enzymes (MMPs) that affect the pattern of matrix remodeling.¹⁰ It is believed that these effects are modulated by astrocyte production of TGF- β 2, which has been shown to be significantly increased in the glaucomatous ONH.¹²

Previous studies have shown a positive correlation of the loss of RGC axons to the level and the duration of IOP elevation in experimental rat glaucoma.^{14,15} It is estimated that approximately half of the total RGCs are lost after 2 to 3 months of IOP elevation.¹⁶⁻¹⁸ However, there has been little research on the direct effect of IOP on RGC apoptosis, although recent in vitro studies suggest RGC apoptosis may be induced directly by elevated IOP, supporting the theory that the retina is the primary injury site in glaucoma.¹⁹

The mechanisms by which RGC apoptosis occurs has recently been linked to specific ECM-related changes in MMP-9 and laminin expression in the retina.^{20,21} This is further corroborated by the fact that in the central nervous system (CNS), neuronal apoptosis is associated with increased MMP-9 activity.^{22,23}

We have investigated for the first time, as far as we are aware, the effect of IOP elevation on the level of RGC apoptosis in an in vivo model of glaucoma. As RGC apoptosis has been recently linked to specific ECM changes,^{20,21} we have evaluated the role of MMP-9, laminin, and TIMP-1 activity in the retina, and whether RGC apoptosis is at all related to IOP-induced effects on these targets. In addition, we have assessed the effects of IOP on changes in collagen I, IV, MMP-1, and TGF- β 2 at both the ONH and retina.

Methods

Animals

Adult male Dark Agouti (DA) rats, weighing 200 to 300 g, were treated with procedures approved by the UK Home Office and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Rat Model of Glaucoma

Thirty-seven DA rats, were anesthetized by intraperitoneal injections of ketamine (37.5%)/Domitor (25%; Pfizer Animal Health, Exton, PA) solution (0.75 mL ketamine, 0.5 mL Domitor, and 0.75 mL sterile water) at 0.1 mL/100 g.²⁴ The IOP was elevated in the left eye of each animal by injection of 50 μ L of hypertonic saline solution (1.80 M) into the episcleral veins,⁷ using a syringe pump (60 μ L/min; UMP2, World Precision Instruments, Sarasota, FL).¹⁴ Contralateral, nonsurgical eyes acted as the control.

IOP Measurements

The IOP of both eyes in each rat was measured with the animal under inhalational anesthesia of a mixture of oxygen and isoflurane (Merial; Animal Health, Ltd., Essex, UK), with a handheld tonometer (Tonopen XL; Medtronic Solan, Jacksonville, FL) at the same time of day on all test days. IOP measurements were taken before surgery and at regular weekly intervals after surgery. After a drop of topical anesthetic (proxymetacaine 0.5%, Chauvin Pharmaceuticals Ltd, Essex, UK), 10 IOP readings were taken and averaged.²⁴ Peak IOP, defined as the maximum elevation in IOP attained at any time during the study period, was recorded in all animals after surgery. The IOP elevation in the surgical eye was calculated from the difference of the IOP between surgical operated and control eyes at each time point. For each animal, the Δ IOP integral, defined as the integral of IOP elevation over time, was calculated from the area under the curve as previously described.^{14,15} Animals were studied until 3 months after IOP elevation.

Identification of Retinal Ganglion Cells

So as to identify RGCs unequivocally, a subgroup of rats ($n = 12$) had RGCs retrogradely labeled by the application of DiAsp 4-(4-(didecylamino)styryl)-*N*-methylpyridinium iodide (4-Di-10-Asp; Molecular Probes-Cambridge Biosciences, Cambridge, UK) to both superior colliculi.^{18,24} These same rats underwent surgery to elevate IOP, as previously described, 10 days later, and were assessed for both surviving and apoptotic RGC counts.

Assessment of Retinal Ganglion Cell Apoptosis

To assess RGC apoptosis, fluorescent Alexa-488 –labeled annexin V (1.25 μ g in 5 μ L) was injected into the vitreous of both eyes of the rats ($n = 25$) 2 hours before they were killed. After death, the eyes were enucleated and fixed immediately in 4% fresh paraformaldehyde overnight, after which they were dissected at the equator, the lens and vitreous removed, and whole flat retinas obtained. In animals that had not undergone RGC retrolabeling, whole flat retinas were incubated either with 4,6-diamidino-2-phenylindole ($n = 12$; DAPI; Sigma-Aldrich, Poole, UK), diluted in 1:2500 with phosphate-buffered saline (PBS)²⁴ for 10 minutes to assess nuclei or with a mouse monoclonal 200-kDa anti-neurofilament ($n = 13$, NF-200; Sigma-Aldrich) to assess RGCs and their axons.²⁵ For immunostaining of NF-200, briefly, whole flat retinas were blocked in normal donkey serum 1:20 in PBTA solution (phosphate-buffered saline containing 10% bovine serum albumin, 0.1% Tween 20, and 1 g/L azide sodium) for 1 hour, followed by incubation with NF-200 antibody at 1:1000 in PBTA overnight. After they were washed in PBS, the retinas were incubated with Cy3-conjugated donkey anti-mouse IgG (AffiniPure; Jackson ImmunoResearch Laboratory, Inc., Cambridgeshire, UK) at 1:100 in PBTA for 2 hours. After a PBS wash, the retinas, stained with either DAPI or NF-200, were flattened by four radial cuts and mounted vitreous side up with glycerol/PBS solution (Citifluor, Ltd., London, UK).

The flat retinas labeled with annexin V and DiAsp were examined under confocal laser scanning microscopy with (LSM software; CLSM 510 META; Carl Zeiss Meditec, Oberkochen, Germany). Using $\times 16$ magnification, 81 adjacent microscopic fields (each measuring 0.329 mm²), radiating outward from the optic nerve head in the rat and accounting for 40% of the whole retina, were assessed, with a retinal montage being made for each whole retina.²⁴ The number of RGCs (DiAsp positive) and apoptotic RGCs (labeled with annexin V) were counted manually (MetaMorph software; Universal Imaging Corp., West Chester, PA) by observers masked to treatment protocols. The same strategy was used to assess apoptotic RGCs in eyes with no DiAsp.

For further assessment of the RGC layer, flat retinas stained with DAPI or NF-200 were also processed for frozen or paraffin-embedded sections. Briefly, for frozen sections, the whole

retina was demounted and cut into small pieces before being embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Sakura Finetec Corp., Torrance, CA) and frozen in liquid nitrogen. The frozen retinal blocks were then cut into sections $7\ \mu\text{m}$ in thickness and mounted in glycerol/PBS solution. Paraffin-embedded sections of $5\ \mu\text{m}$ thickness were also analyzed. Cross sections were examined and scanned by confocal microscopy.

Assessment of ECM Changes in Retina and ONH

To assess MMP-9 involvement in RGC apoptosis, whole retinas, and paraffin-embedded cross sections, stained with annexin V, were also immunostained with sheep anti-MMP-9 ($n = 12$, 1:250; The Binding Site, Ltd., Birmingham, UK), Cy5-conjugated donkey anti-sheep IgG, and using NF-200 or DiAsp immunofluorescence, to identify RGCs (AffiniPure; Jackson ImmunoResearch). The retinas were examined by confocal laser scanning microscopy and apoptotic RGCs, double-labeled by anti-MMP-9 antibody and annexin V, were counted.

To assess other ECM characteristics at the retina and the ONH, ECM components were studied in sequential $5\text{-}\mu\text{m}$ -thick paraffin-embedded hemiglobe cross sections through the ONH ($n = 13$) and retina ($n = 12$), from enucleated, fixed eyes. Primary antibodies used were rabbit anti-laminin (1:60; Chemicon International, Temecula, CA), rabbit anti-collagen type I (1:300; Chemicon International), mouse anti-collagen IV (1:150; Dako A/S, Glostrup, Denmark), sheep anti-MMP1 (1:750, The Binding Site, Ltd.), mouse anti-TIMP-1 (1:20; R&D Systems, Abingdon, UK), and rabbit anti-TGF- β 2 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA). A biotinylated anti-sheep and mouse secondary antibody (Dako, High Wycombe, UK), and a biotinylated swine anti-rabbit secondary antibody (Dako) were used accordingly, followed by a tertiary complex antibody (StreptABCComplex/HRP; Dako). Cross sections were stained with red dye (Vector Red; Vector Laboratories, Burlingame, CA) or diaminobenzidine (DAB) and counterstained with hematoxylin.

Sections were assessed for staining by two independent and masked observers who used a grading system and analysis method well known to our group,²⁶⁻²⁸ originally described by Shah et al.²⁹ (Scale: -4 to $+4$; where 0 is the same as the control eye, 1 is 1% to 25% of control, 2 is 26% to 50% of control, 3 is 51% to 75% of control, and 4 is $>75\%$ of control with the prefix + indicating more than and $-$ less than 0.)

Statistics

Mean values were calculated with 95% confidence intervals for IOP-related parameters. Statistical analysis was performed with Δ IOP integral and peak IOP for each eye for each parameter assessment. Since all data were parametric, Pearson's correlation coefficient was used to assess the strength of correlation.

Results

IOP Elevation in Surgical Eyes

Surgery produced an increase of IOP in all eyes with mean peak IOP elevation of 31.45 ± 3.81 mm Hg and mean Δ IOP integral of 537.65 ± 78.06 mm Hg days. The mean duration of IOP elevation was 57.40 ± 7.35 days.

Effect of IOP Exposure on RGC Apoptosis

Typical appearances of apoptotic RGCs in both control and IOP-elevated eyes are shown in Figure 1, with apoptotic cells labeled by annexin V (green), RGCs labeled by DiAsp (red), and retinal nuclei stained with DAPI (blue). Localization of apoptosis to the RGC layer was

confirmed by double staining of cells with annexin V and DiAsp (Figs. 1A, 1B) and with annexin V and DAPI in a retinal wholemount (Fig. 1C) and cross sections (Fig. 1D).

In normal control eyes, we found the mean number of RGCs to be $115,000 \pm 2,021$. We demonstrated RGC apoptosis in all eyes. The mean number of apoptotic RGCs in control eyes was 324.50 ± 49.93 , compared with 1516.22 ± 308.91 in surgical eyes. Compared with control eyes, the number of apoptotic RGCs was significantly higher in all glaucomatous eyes at 3 months after surgery (*t*-test, $P < 0.001$). We found a significant correlation of RGC apoptosis with integral Δ IOP (Pearson's $r = 0.766$, $P < 0.001$; Fig. 2A) and peak IOP (Pearson's $r = 0.533$, $P < 0.01$; Fig. 2B).

For the RGC counts at 3 months after elevation of IOP, the mean percentage loss of RGCs was $61.21\% \pm 7.54\%$. Again, we found a significant correlation between RGC loss and integral Δ IOP (Pearson's $r = 0.777$, $P < 0.005$; Fig. 2C) and peak IOP (Pearson's $r = 0.612$, $P < 0.05$; Fig. 2D).

IOP-Induced ECM Changes in Retina and ONH

Compared with control eyes, we found a decrease in laminin deposition in the RGC layer in all IOP-elevated eyes and showed a significant negative correlation with Δ IOP integral (Pearson's $r = -0.661$, $P < 0.05$; Figs. 3A, 3C, 3E). We also found an increase in TIMP-1 immunolabeling in IOP-elevated eyes and showed a significant correlation with IOP exposure (Pearson's $r = 0.605$, $P < 0.05$; Figs. 3B, 3D, 3F), but no change in MMP-1. We demonstrated a significant increase in collagen I deposition (Pearson's $r = 0.778$, $P < 0.01$) in the RGC layer with elevated IOP, but not in collagen IV. In addition, TGF- β 2 labeling showed a decrease in the RGC layer, and again, the change was significantly correlated with integral Δ IOP (Pearson's $r = -0.625$, $P < 0.05$).

We demonstrated intense MMP-9 immunofluorescence localized to the RGC layer compared with control eyes. The pattern of staining was discontinuous, with areas of skip lesions. Figure 4 illustrates MMP-9 (Fig. 4A, blue) colocalizing to apoptotic cells stained with Alexa-488 -labeled annexin V (Fig. 4B, green) and RGCs and axons labeled by NF-200 (Fig. 4C, red). There was a very strong correlation between the mean grade of MMP-9 immunostaining and integral Δ IOP (Pearson's $r = 0.898$, $P < 0.00$; Fig. 5A) and peak IOP (Pearson's $r = 0.721$, $P < 0.01$; Fig. 5B). Furthermore, MMP-9 immunoreactivity was significantly related to a decrease in laminin deposition (Pearson's $r = -0.726$, $P < 0.01$; Fig. 5C) and an increase in the number of apoptotic RGCs (Pearson's $r = 0.898$, $P < 0.001$; Fig. 5D).

For the ECM components in the ONH, we demonstrated an increase in TGF- β 2, collagen I, collagen IV, and MMP-1 in all glaucomatous compared with control eyes. TGF- β 2 was identified mainly in the transition region of the ONH (Figs. 6A, 6B) whereas collagen IV was deposited throughout the ONH. However, only TGF- β 2 and collagen I deposition appeared to correlate significantly with Δ IOP integral (Pearson's $r = 0.688$, 0.686 , respectively, $P < 0.01$; Figs. 6C, 6D). There were no significant difference between glaucomatous and control eyes, in collagen IV (Pearson's $r = 0.357$, $P = 0.345$) and MMP-1 (Pearson's $r = 0.446$, $P = 0.170$) staining. Histology demonstrated changes in the ONH contour in glaucomatous compared with control eyes, including scleral canal expansion or cupping with loss of nerve fibers (Figs. 6E, 6F).

Discussion

In this study RGC apoptosis correlated significantly with IOP exposure in a rat model of glaucoma. Elevated IOP was significantly positively related to expression of MMP-9,

TIMP-1, and collagen I in the RGC layer—the first time this has been shown. In contrast, it correlated negatively with laminin and TGF- β 2. The level of RGC apoptosis correlated significantly to MMP-9 activity, suggesting that this may be an important mechanism mediating glaucomatous RGC loss. The results also confirmed that TGF- β 2 and collagen I deposition correlates significantly with raised IOP at the ONH.

RGC death in experimental glaucoma has been shown to occur by the process of apoptosis.^{2,3} Our data demonstrated that RGC apoptosis correlated positively with both Δ IOP integral and peak IOP in the rat model, supporting the theory that the level of IOP elevation determines the extent of RGC loss.³ Although our incidence of apoptosis was similar to that reported in previous studies (0.5%–3.0% of the total population of RGCs; normal RGC count in our model, 115,000 cells or 2,213 cells/mm²),^{2,3,16} it was higher than recorded in studies in which TUNEL was used as the method of detection. This discrepancy may be due first, to the increased identification of positive cells with annexin V, and also because of its administration *before* the animal was killed. Compared with TUNEL, which detects mainly the DNA fragmentation phase of the pathway (representing only a small fraction of cells undergoing apoptosis at any single time point), annexin V detects translocated membrane phosphatidylserine (PS), which occurs from an early stage and during a major part of the process of apoptosis. Even so, the number of dying RGCs labeled by annexin V was still lower than that previously defined by light microscopy by Quigley et al.² (10% of ganglion cells).

Several studies have investigated RGCs or their axonal loss after experimental IOP elevation in the rat.^{3,16-18} There is a suggestion that RGC death after IOP elevation occurs in two phases: the fast phase, occurring in the initial 3 weeks with a rate of 12% RGC loss per week, followed months later by a second, slow-phase rate of 2%.¹⁷ The mechanism in the early phase may be pressure related, with recent evidence of neuronal cells undergoing apoptosis within 2 to 20 hours after being subjected to elevated IOP.¹⁹ The second phase, occurring a month or so after elevated IOP, is also believed to involve apoptosis but through the process of secondary degeneration—a process that causes the death of RGCs that survive the primary insult but are injured by toxic effects of the primary degenerating neurons. This may explain the lag period between RGC apoptosis and the initial event of IOP elevation.¹⁵

RGC and axonal loss has been estimated to be >50% at 2 months after IOP elevation, similar to our own findings.¹⁴⁻¹⁶ Compared with previous work which concentrated on the absolute reduction of RGC counts,¹⁴⁻¹⁶ in this study, we labeled and counted apoptotic cells throughout the whole retina, and then analyzed RGC attrition as a function of IOP exposure. As far as we know, this has not been investigated before.

The persistence of RGC apoptosis above baseline levels at 3 months after surgery supports a cumulative effect of IOP over time. This effect has been observed by other investigators who have shown a significant positive correlation of Δ IOP integral with the percentage of axonal loss occurring in glaucomatous rats.^{7,14,15} The mechanisms of this effect are still not established. One possible explanation is that ONH remodeling is still occurring at 3 months in this model, as confirmed by an increase in TGF- β 2 and collagen I immunoreactivity in this study, and this causes RGC axonal compression.^{12,30} In addition, the surviving axons may still be susceptible to the cumulative effect of IOP elevation.

A possible new mechanism by which RGC apoptosis actually occurs in relation to IOP exposure at the site of the retina highlighted in this study, is the association of ECM changes in the retina, in particular, the strong correlation of MMP-9 expression in RGCs with IOP exposure and RGC apoptosis. It is possible that IOP-induced changes in specific ECM components or cytokines in the retina contribute to an increase in MMP-9 activity. There is

accumulating evidence that changes in the status and composition of the ECM affect the function of a variety of different cells and modulate the synthesis and release of MMPs and vice versa.^{31,32} An in vitro study has recently shown that specific matrix components, including fibronectin, collagen I and IV, induce an increase in MMP-2 and -9 from human glomerular mesangial cells.³² In addition, ECM provides adherence signals that control cell function and survival.³¹ Changes in specific ECM components can interrupt cell–cell and cell–ECM interactions, leading to cell death.

Our finding that enhanced MMP-9 activity in apoptotic RGCs paralleled the decreased deposition of laminin in the RGC layer suggests increased degradation of the ECM at the retinal site in response to IOP exposure. Laminin is an ECM component believed to facilitate cell attachment and survival.³³ If cells begin to secrete large amounts of proteases (such as MMP-9) it is only logical that ECM components such as laminin may disappear. As laminin may provide survival signals through interactions with cellular integrins, the reduction or lack of laminin may lead to the interruption of cell–ECM communication making cells more susceptible to apoptosis, or anoikis (the form of apoptosis caused by lack of substrate-derived survival signals).³³ In support of our finding, MMP-9 activity has been linked to neuronal cell death in brain injury or ischemia.^{22,34,35} Moreover, brain neuronal death is associated with loss of laminin³⁶ and MMP-9 expression. Recently, MMP-9 – deficient mice have been demonstrated to have reduced RGC loss as a result of limited laminin degradation in a model of optic nerve ligation and RGC death.²⁰ This same group has subsequently demonstrated a correlation between MMP-9 upregulation and a decrease in laminin immunostaining in the RGC layer.^{21,37} Our study demonstrates for the first time a link between MMP-9, laminin degradation, RGC apoptosis, and IOP exposure in glaucoma.

One interpretation of our observations is that retinal cells, possibly RGCs, increase secretion of MMP-9 as a direct response to raised IOP, and that this in turn leads to MMP-9-induced loss of laminin and consequently, RGC apoptosis. This notion supported by previous findings from Chintala et al.²⁰ and Zhang et al.²¹ However, our data do not exclude the possibility that the mechanical effect of elevated IOP could cause RGC axonal damage in the region of the ONH, leading to retrograde damage of the RGC cell body, which in turn may induce an increase in MMP-9 activity, resulting in the changes in the ECM. An alternative theory for IOP inducing an increase in MMP-9 expression could be that it is an indirect effect mediated by glutamate—a major excitatory neurotransmitter. Glutamate appears to be increased by different types of stimuli, including injury, ischemia, and elevated IOP.³⁸⁻⁴⁰ Glutamate is known to be involved in the activation of MMP-9, with the demonstration of MMP-9 mRNA and protein being up-regulated by the glutamate receptor agonist, kainite, in rat hippocampus.⁴¹ Furthermore, a recent study has demonstrated that activation of retinal glutamate receptors upregulates MMP-9. This upregulation has been attributed to the development of laminin loss and retinal degeneration in this model,²¹ and suggests that any insult leading to glutamate-mediated activation of MMP-9 could lead to RGC apoptosis.

Along with an increase in MMP-9 activity, we also showed an increase in TIMP-1 in the RGC layer, correlated with the degree of IOP exposure. In a previous study, both MMP-9 and TIMP-1 were shown to be induced in ischemic rat brain, regulated in a cell- and time-dependent manner in association with neuronal death.²² TIMP-1 is usually recognized as an inhibitor for MMPs, specifically MMP-9, to maintain ECM homeostasis.⁴² Therefore, the increase in TIMP-1 may serve to counterbalance the simultaneous increase in MMP-9.⁴³ However, recent studies have shown that TIMP-1 has neuroprotective effects that are independent of its ability to inhibit MMPs.⁴⁴ In normal conditions, RGCs express significant levels of TIMP-1, but low constitutive levels of MMP-9.^{10,37,45} The increase in retinal TIMP-1 demonstrated in this study is likely to facilitate its neuroprotective effect on

RGCs through both inhibition of MMP-9 and antiapoptotic actions. This would be in keeping with the theory that ECM remodeling continuously occurs in the retina after exposure to raised IOP.

Our analysis of changes in ECM at the ONH site is consistent with previous studies.^{8,11,46} We demonstrate a clear relationship between TGF- β 2 deposition and IOP exposure, supporting the previous observations implicating the association of TGF- β with ONH changes in ECM in glaucoma.^{12,13} The increased expression in TGF- β in the ONH may be as a stress response to raised IOP, in that TGF- β has been highlighted as a key molecule stimulated by mechanical stress.⁴⁷ In contrast to the ONH, we found a significant reduction in TGF- β 2 deposition in the retina with increased IOP exposure. TGF- β is one of the most potent modulators of wound healing throughout the body.¹³ Given that TGF- β 2 is a major stimulant of ECM and an inhibitor of MMPs, it may not be surprising that the low level of TGF- β 2 in the retina is associated with increased activity in MMP-9 and decreased deposition in laminin. One study has shown that a decrease in TGF- β and an increase in MMPs are associated with vascular cell apoptosis.⁴⁸ It is well established that the effects of TGF- β are cell specific and concentration dependent, with different functions being maximally stimulated at different concentrations.⁴⁹ The biphasic behavior of TGF- β may offer an alternative explanation to why we found low levels of TGF- β 2 in the RGC layer and high levels in the ONH and why both may be associated with increased levels of RGC apoptosis.

Taken together, our observations have demonstrated that RGC apoptosis in glaucoma correlates strongly with IOP exposure and that RGC apoptosis is significantly related to specific IOP-induced changes in RGC layer ECM components. Our data suggest that the retina may be the primary site of injury in glaucomatous neuropathy and implicate MMP-9 activity as being involved in the development of RGC apoptosis in glaucoma. To the best of our knowledge, this is the first report of RGC apoptosis in relation to RGC layer ECM remodeling.

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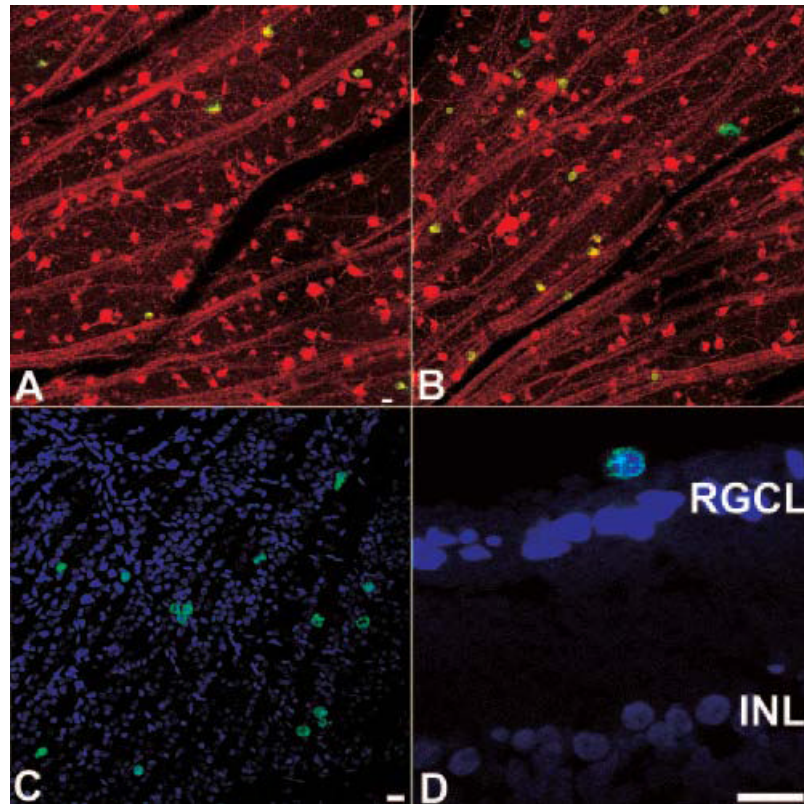


Figure 1.

RGC apoptosis was detected in all rat eyes, although it was considerably greater in glaucomatous retinas. Immunohistochemistry confirmed that apoptotic cells were localized to the RGC layer (RGCL). Typical fluorescence micrographs from the control eye (**A**) and the contralateral, IOP-elevated eye (**B–D**) show apoptotic cells stained by Alexa-488 – labeled annexin V (*green*) and RGCs labeled by DiAsp (**A, B, red**), with DAPI as a nuclear stain (**C, D, blue**), both in retinal wholemounts (**A–C**) and cross sections (**D**). INL, inner nuclear layer. Scale bars, 15 μm .

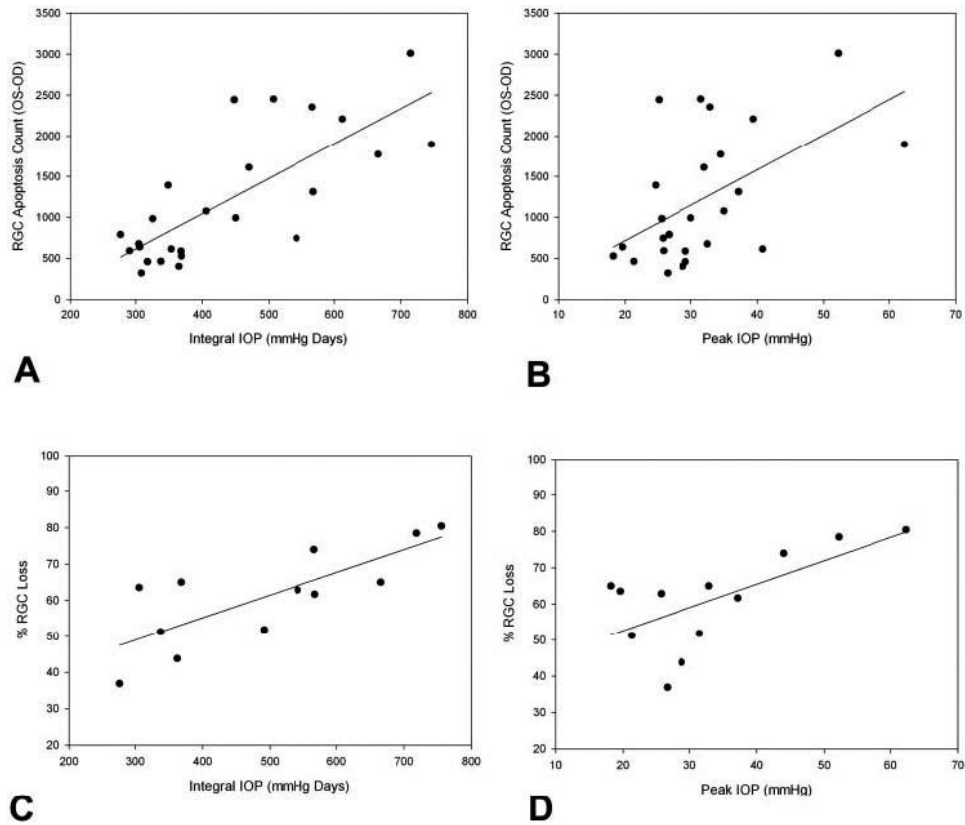


Figure 2. RGC apoptosis correlated positively with both Δ IOP integral (**A**) and peak IOP (**B**) (Pearson's $r = 0.766$ and 0.533 , $P < 0.001$ and < 0.01 , respectively). Similarly, there was a significant correlation between the percentage loss of RGCs and Δ IOP integral (**C**) and peak IOP (**D**) (Pearson's $r = 0.777$ and 0.612 , $P < 0.005$ and < 0.05 , respectively).

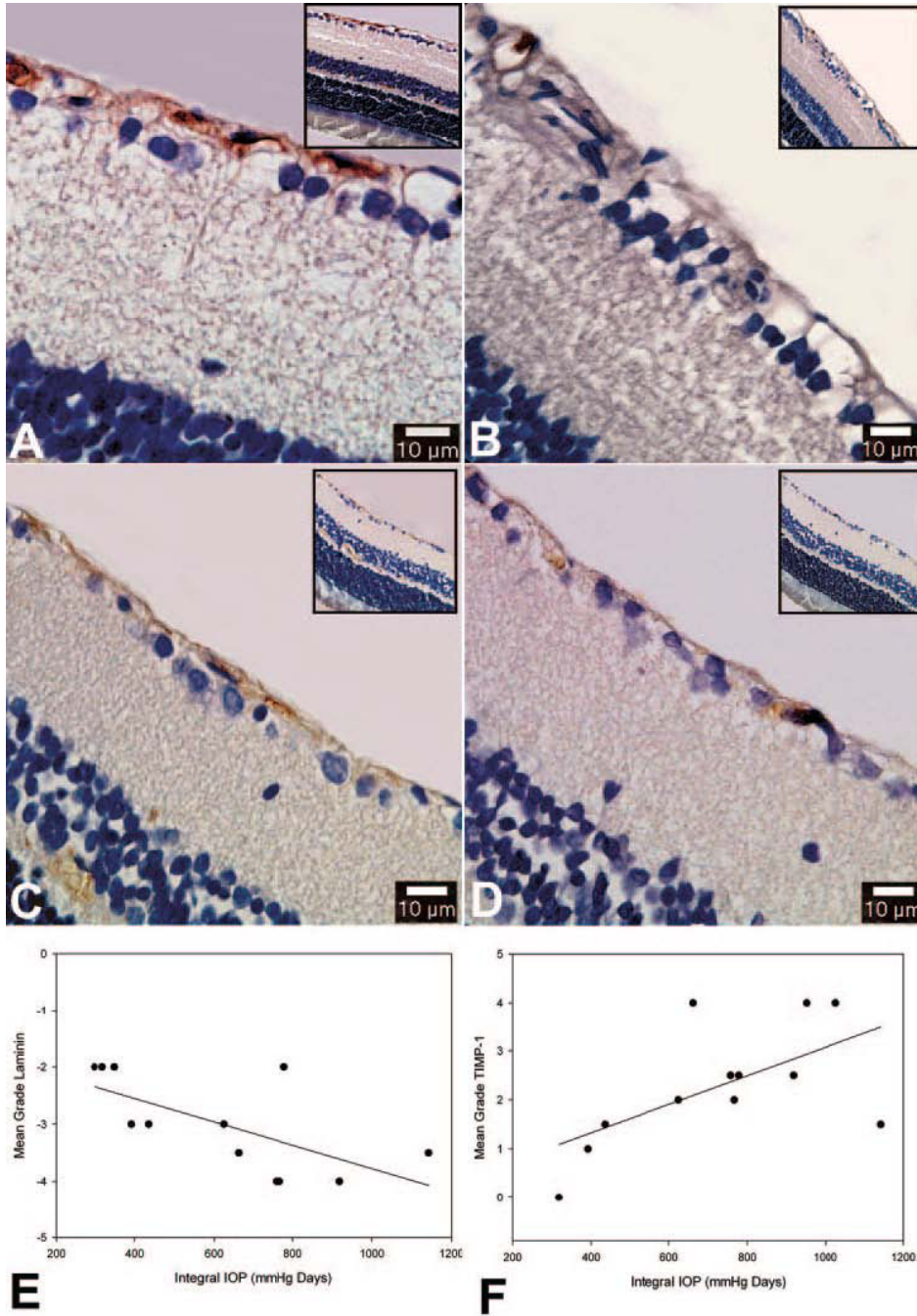


Figure 3. Immunohistochemistry on paraffin-embedded cross sections shows typical staining in control (A, B) and glaucomatous (C, D) eyes of laminin (A, C) and TIMP-1 (B, D). *Insets:* Same cross section of retina at low magnification. Immunostaining demonstrated a decrease in laminin (C) and an increase in TIMP-1 (D) in all glaucomatous eyes compared with the control. On analysis, loss of laminin (E) and increased TIMP-1 expression (F) correlated significantly with Δ IOP integral (Pearson's $r = -0.661$ and $+0.605$, respectively, $P < 0.05$). Scale bars, 10 μ m.

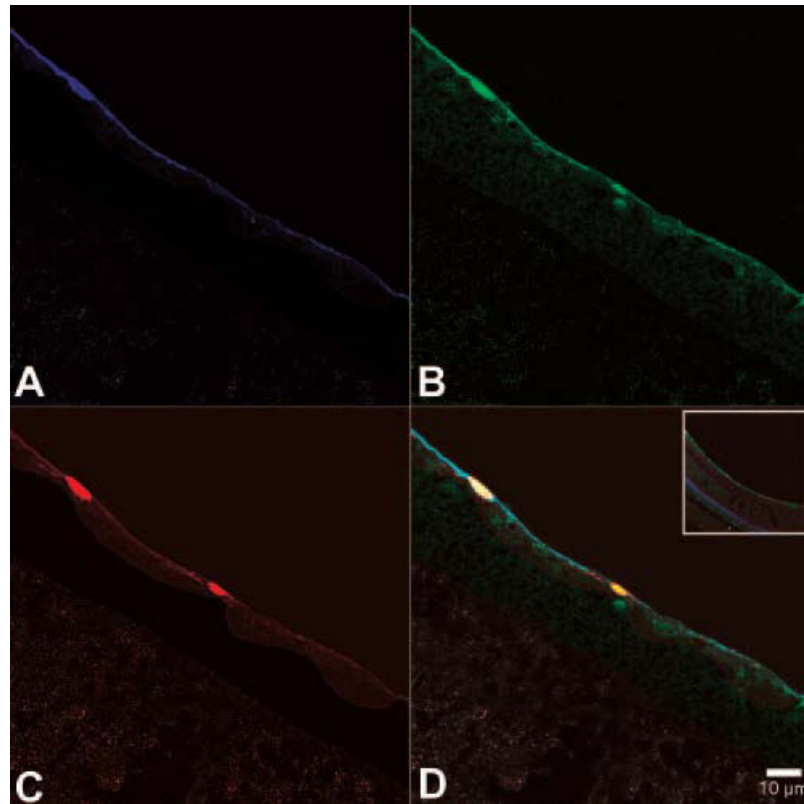


Figure 4. Immunohistochemistry of frozen cross sections illustrated MMP-9 (**A**, *blue*) colocalizing to apoptotic cells in the RGC layer stained with Alexa-488 –labeled annexin V (**B**, *green*) and RGCs labeled with NF-200 (**C**, *red*). (**D**) Composite image and low-magnification (*inset*) demonstrated discontinuity in MMP-9 immunostaining with areas of skip lesions. Bar, 10 μm .

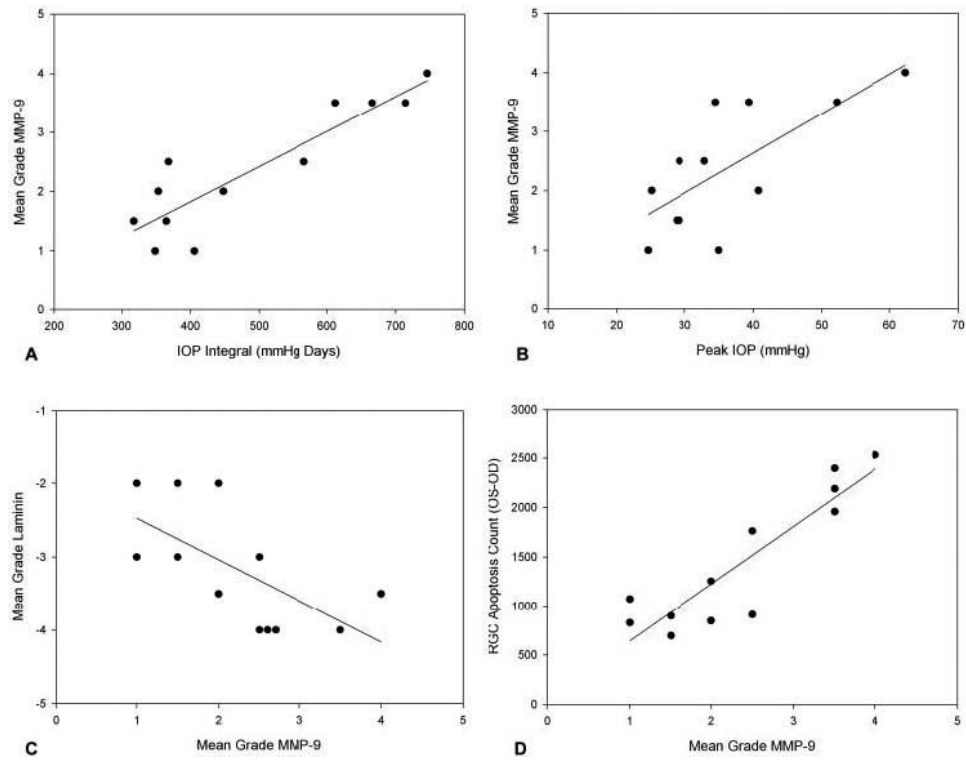


Figure 5. Increase in MMP-9 immunoreactivity in glaucomatous eyes was strongly positively correlated with Δ IOP integral (**A**, Pearson's $r = 0.898$, $P < 0.001$) and peak IOP (**B**, Pearson's $r = 0.721$, $P < 0.01$). A correlation between MMP-9 upregulation and a decrease in laminin immunostaining in the RGC layer was also demonstrated (**C**, Pearson's $r = -0.726$, $P < 0.01$), as also a direct relationship between MMP-9 and the number of apoptotic RGCs (**D**, Pearson's $r = 0.898$, $P < 0.001$).

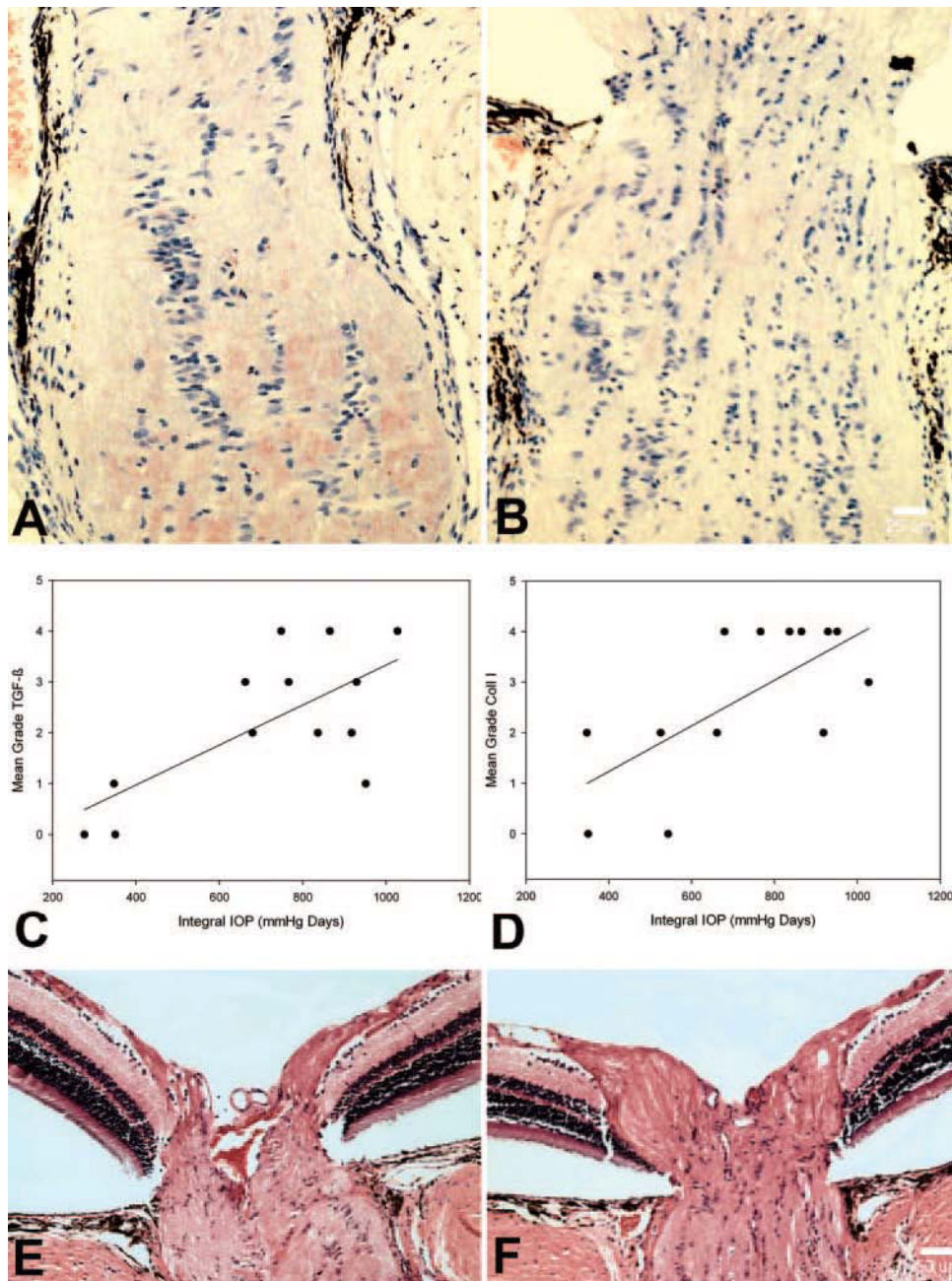


Figure 6. Immunohistochemistry confirmed changes in ECM components in the rat ONH in glaucomatous eyes (A), including increased expression of TGF- β 2 (red) compared with control (B) in the transition region. TGF- β 2 (C) and collagen I (D) deposition were positively associated with an increase in Δ IOP integral (Pearson's $r=0.688$ and 0.686 , respectively, $P < 0.01$). Histochemistry (hematoxylin and eosin) confirmed scleral canal expansion and cupping with loss of nerve fibers in a typical glaucomatous eye (E) compared with a control eye (F).