Deficiency in Matrix Metalloproteinase Gelatinase B (MMP-9) Protects against Retinal Ganglion Cell Death after Optic Nerve Ligation

Shravan K. Chintala*, Xu Zhang*, Jeffrey S. Austin**, and M. Elizabeth Fini***

From the *Eye Research Institute, Oakland University, Rochester, MI 48309; **Bascom Palmer Eye Institute, University of Miami School of Medicine, PO Box 016880, Miami, FL 33101; ***New England Eye Center, Tufts University School of Medicine, and the Tufts Center for Vision Research, 750 Washington Street, Box 450, Boston, MA 02111

Running title: GelB deficiency protects against retinal cell death

*Corresponding Author: Shravan K. Chintala, Ph.D.  
Eye Research Institute  
409 Dodge Hall  
Oakland University, Rochester, MI, 48309  
Phone: (248) 370-2532; FAX: (248) 370-2006  
email: Chintala@oakland.edu

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Abbreviations: MMP: matrix metalloproteinase; GelB: gelatinase B; ECM: extracellular matrix; GCL: ganglion cell layer; RGC: retinal ganglion cells; ILM: inner limiting membrane; PA: plasminogen activator; PG: plasminogen; TUNEL: TdT mediated dUTP nick-end labeling.
Summary

Loss of retinal ganglion cells is the final endpoint in blinding diseases of the optic nerve such as glaucoma. To enable the use of mouse genetics to investigate mechanisms underlying ganglion cell loss, we adapted an experimental model of optic nerve ligation to the mouse, and further characterized post-surgical outcome. We made the novel finding that apoptosis of retinal ganglion cells correlates with specific degradation of laminin from the underlying inner limiting membrane, and an increase in gelatinolytic metalloproteinase activity. These changes co-localize with a specific increase in levels of the Matrix Metalloproteinase, gelatinase B (GelB; MMP-9). Using a transgenic mouse line harboring a reporter gene driven by the GelB promoter, we further show that increased GelB is controlled by activation of the GelB promoter. These findings led us to hypothesize that GelB activity plays a role in ganglion cell death and degradation of laminin. Applying the genetic approach, we demonstrate that GelB-deficient mice are protected against these pathological changes. This is the first report demonstrating a causal connection between GelB activity and pathological changes to the inner retina after optic nerve ligation.
Introduction

Retinal ganglion cells contribute to the innermost cellular layer of the neural retina, functioning to relay visual information to the processing centers in the brain. This transfer occurs through the ganglion cell axons, which bundle together as they exit from the eye to form the optic nerve.

Several eye diseases are characterized by damage to the optic nerve and specific loss of ganglion cells. Prominent among these is glaucoma, one of the three major causes of irreversible blindness in the United States today (1-3). An understanding of the mechanisms leading to ganglion cell loss in diseases of the optic nerve is essential for the design of much-needed therapeutic strategies to save sight.

Selective depletion of ganglion cells from the retina can be experimentally induced by application of several types of insults. These include models of ischemia/reperfusion injury due to short-term blood vessel occlusion (2,4), selective vascular ligation (5-7), photothrombosis (8), or occlusion of the carotoid artery (9). Eliminating the connection between ganglion cells and the brain by compressing or severing their axons also causes specific loss of ganglion cells. It is suggested that this may be due to blockade of retrograde neurotrophin transfer (10). Both mechanisms may contribute to ganglion cell loss following elevation of intraocular pressure above systolic blood pressure (12), which compresses structures at the optic nerve head, and is a major causal risk factor for glaucoma (13-19). Optic nerve ligation is thought to lead to ganglion cell loss by similar mechanisms (11). Most investigators in the field accept that optic nerve ligation is a useful experimental model for studying the pathophysiological mechanisms, which lead to optic nerve damage in glaucoma and related diseases (2).
The retina is part of the central nervous system (CNS), and though there are many specific
differences, there are also many commonalities. It is known that neuronal cell death in
pathologies of both retina and brain occurs by apoptotic mechanisms (13,6,15,17,20). An
increase in apoptotic cells is also found in diseases specific to the optic nerve such as glaucoma
(21,22). Current opinion holds that cell death is stimulated by an excess of the excitatory amino
acid, glutamate (23,24). According to this model, over-abundant glutamate is the end result of a
variety of insults including inappropriate electrical activity, ischemia/reperfusion injury, or
neurotrophin deprivation. These insults are thought to promote entry of calcium ions into the
cell, initiating downstream apoptotic events. Consistent with this idea, injection of glutamate
agonists into the hippocampus induces neuronal apoptosis (25). Similarly, injection of a
 glutamate agonist into the posterior chamber of the eye induces specific death of ganglion cells
(26,27).

Tissue damaging stimuli activate specific repair and remodeling mechanisms. These processes
are precisely regulated through reciprocal interaction between the resident tissue cells and their
extracellular matrix supporting structures. Integral to this are two enzyme systems: the
plasminogen activator (PA)-plasminogen (PG) system, and a family of enzymes known as the
Matrix Metalloproteinases (MMPs). The PA-PG system effects removal of fibrin matrices and
also plays a part in converting inactive pro-MMPs to their active forms. MMPs perform these
same functions, but within a more comprehensive context as both effectors and regulators of
tissue remodeling (28,29). MMP substrates include essentially all extracellular matrix
components, as well as a wide array of molecules involved in intracellular adhesion, cell:matrix
interaction, and cell signaling (29,30).
Both the PA-PG system and the MMP family have been implicated in the cascade of events leading to neuronal apoptosis in the CNS (31-36). More specifically, mice deficient for tissue plasminogen activator (tPA) or PG were found to be resistant to neuronal destruction induced by excitotoxins (37,38). Similarly, mice deficient for the Matrix Metalloproteinase gelatinase B (GelB; MMP-9) were found to be resistant to both ischemic and impact injury to the cerebrum (39,40). In contrast, deficiency of a related MMP, gelatinase A (gelA; MMP-2) had no effect on the course of the injury response (41). Laminin loss occurs in response to injection of glutamate into the hippocampus, and this is also mediated by the PA-PG proteolytic system (37,38). In this system, laminin loss in response to excitotoxicity was found to contribute to neural cell death.

The starting point for the current study was the observation that GelB is expressed at low constitutive levels specifically in the ganglion cell layer of the retina (42). This led us to hypothesize that this particular MMP might promote pathological changes that occur in diseases of the optic nerve. To investigate this hypothesis, we adapted an experimental model of optic nerve ligation to the mouse, allowing us to take a genetic approach to further define pathological mechanisms.
Materials and Methods

Mice and optic nerve ligation. All experiments with mice were performed according to protocols approved by the Institutional Animal Care and Use Committee. Mice were used for experiments at 6-8 weeks of age. For experiments characterizing retinal injury and MMP expression, normal adult CD-1 mice were used (Charles River Breeding Labs). For experiments investigating the role of GelB (MMP-9) in retinal injury, the GelB-deficient strain (GelB^{−/−}) on the 129SvEv/CD-1 mixed background was used. A corresponding matched littermate control line (GelB^{+/+}) on the same background was used as the control for GelB-deficient mice (43). GelB-deficient mice show delayed growth of long bones due to impaired vascularization of the growth plate, but ultimately attain normal adulthood (43). Eyes of GelB-deficient mice are of normal size and corneas and lenses are clear (44). Routine histological examination of adult eyes revealed no obvious anatomical changes in the retina between gel-B deficient homozygous mice and their normal counterparts.

Prior to surgery, mice were anesthetized by an IP injection of 2% Avertin (0.017 ml/g body weight). The surgical procedure was an adaptation of a method for optic nerve ligation previously described for rats (11). Briefly, a 6-0 nylon ligature was placed around the optic nerve and tightened until blood flow in all the retinal vessels was stopped, as viewed under an operating microscope. After 30 to 60 min, re-perfusion of tissue was allowed by removal of the suture. Contralateral eyes served as controls. Animals were euthanized at various time points after performing the optic nerve ligation procedure and eyes were enucleated for analysis.
Tissue sectioning, histology, apoptosis assay, and fluorescence microscopy. Eyes were embedded in Optimal Temperature Cutting (OCT) compound (Miles, Elkhart, IN). Transverse, 8-µm thick cryostat sections were cut and placed onto Super-frost plus slides (Fisher Scientific, Pittsburgh, PA). Histological staining was performed on sections following fixation in 4% paraformaldehyde for 30 minutes at room temperature. To detect apoptotic cells, the TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed using a kit (In Situ Cell Death Detection Kit with Fluorescein; Boehringer-Mannheim, Germany) according to the protocol provided by the manufacturer. Counterstaining was performed for 2 min with propidium iodide (1 mg/ml) to define the nuclei of cells. Tissue sections were examined using a Nikon E400 light microscope equipped for epifluorescence and the image was captured with a digital camera. Images acquired individually were either merged (for Fig. 5A) or showed separately (for Fig. 1B).

Retrograde labeling and quantification of labeled cells. Retrograde labeling of ganglion cells was performed as described previously (18). Briefly, 1.5 ul of a 5% solution of Fluorogold in 0.9% sodium chloride (Fluorochrome Inc., Englewood, CO, USA) was injected into the superior colliculi of anesthetized mice immobilized in a stereotaxic apparatus. One week after Fluorogold application, the optic nerve was ligated as described above. At various time points thereafter, the animals were euthanized and their eyes were enucleated and fixed in 4% paraformaldehyde for 1 h. Eyes were bisected at the equator, the lenses were removed, and the posterior segments were fixed for an additional 30 min. To prepare flat mounts, retinas were dissected from the underlying sclera/choroid, flattened by six radial cuts, and mounted vitreal side up on superfrost slides. Retinal ganglion cells labeled with Fluorogold were counted in six microscopic fields of
retina under a fluorescence microscope at 40X magnification. Labeled cells from four to six selected fields of identical size were counted. The selected fields were located at approximately the same distance from the optic disk to account for the variation in RGC density as a function of distance from the optic disk. Quantification of ganglion cell loss was performed using Scion image analysis software (Scion Corporation, Frederick, MD).

**In situ proteinase activity assay.** This technique identifies tissue sites where proteinases are actively available to catalyze cleavage of their substrates. It employs FITC-labeled DQ-gelatin, (Molecular Probes, Eugene, OR), a substrate for gelatinolytic MMPs, which emits a fluorescent signal when cleaved (45). Unfixed sections of tissue prepared by cryostat were incubated overnight at 37 degrees C with reaction buffer (0.05M Tris-HCl, 0.15M NaCl, 5 mM CaCl₂, and 0.2 mM NaN₃, pH 7.6) containing 40 µg/ml DQ-gelatin. The resulting enzymatic activity pattern was observed by fluorescence microscopy.

**Retinal tissue extraction.** Eyes were cut in half at the equator and lens and vitreous were removed. The retina was then gently peeled off with fine forceps and two retinas were placed in each 1.5 ml tube. The tissues were homogenized on ice in 40 ul of RIPA buffer (1% nonidet-P40, 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃VO₄, 5 mM EDTA, 0.1 mg/ml aprotinin, 1 mM PMSF [phenylmethionylsulfonyl fluoride], pH 7.4). Homogenates were centrifuged at 10,000 rpm for 5 min at 4°C and the supernatants were collected. The total protein concentration in supernatants was determined using the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA).
Gel Zymography. The presence of specific MMP protein species in retinal homogenates was determined by zymography (74) according to our standard lab protocol (46). This technique detects both latent proenzymes and active enzyme forms based on the capacity for activation of proenzymes by SDS. In brief, retinal extracts containing equal amounts of protein (20 µg) were mixed with SDS gel-loading buffer, and then loaded without reduction or heating onto 10-12% SDS polyacrylamide gels containing 0.1% gelatin (Sigma, St. Louis, MO). Following electrophoresis, the gels were washed to remove SDS, developed under conditions optimal for MMPs (pH 7.4, 10 mM CaCl₂), and then stained with Coomasie Brilliant Blue-R (Sigma). The location of proteinase species in the gels could be easily visualized as clear bands in a blue background of stained substrate. A sample containing GelA (MMP-2) and GelB (MMP-9) (which has been confirmed by zymography)- the conditioned culture medium from rabbit corneal fibroblasts treated with phorbol myristate acetate (46) was co-electrophoresed for comparison. In addition, a reduced molecular weight size standard was included on all gels (Life Technologies, Gaithersberg, MD).

Western blot analysis. Samples of retinal tissue extract containing equal protein (20 µg) were mixed with sample buffer, and separated on 10-12% SDS-polyacrylamide gels. After electrophoresis was complete, the proteins were transferred out of the gels to nylon membranes. The membranes were blocked with 10% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween-20, and then probed with either a rabbit polyclonal antibody raised against mouse GelB (a kind gift from Dr. Robert M. Senior, Washington University School of Medicine, St Louis, MO) or a polyclonal anti-laminin antibody developed in rabbit using laminin purified from the basement membrane of Englebreth Holm-swarm (EHS) mouse sarcoma
(Sigma, St. Louis, MO). After washing again, the membrane was incubated with peroxidase-conjugated secondary antibody at room temperature for 1 hr. Finally, the proteins were detected using a chemiluminescence kit (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Purified mouse GelB (MMP-9) and GelA (MMP-2) (Chemicon, Tamecula, CA) were used as standards.

**Immunohistochemistry.** For detection of MMPs, sections of tissue prepared by cryostat were fixed by dipping slides in 4% paraformaldehyde at room temperature. For detection of laminin, tissue sections were immersed briefly in acetone at –20 degrees C according to the method of Libby et al., (47). Sections were subsequently processed for indirect immunofluorescent localization. The primary antibody probes used were a rabbit polyclonal antibody raised against mouse GelB (described above), a mouse monoclonal antibody raised against mouse GelB (Neomarkers, CA), a rabbit polyclonal antibody raised against laminin derived from Engelbreth sarcoma (Sigma St. Louis, MO) and a monoclonal antibody against murine nidogen (Chemicon International, Temecula, CA). The secondary antibody was either FITC- or rhodamine-labeled. Antibody binding was visualized by fluorescence microscopy.

**GelB promoter activity.** Construction of CD-1 transgenic mouse line 3445 was previously described (48). The transgene consists of bases –522 to +19 of the GelB promoter linked to a β-galactosidase reporter gene. Promoter activity in these mice closely parallels the expression of the endogenous GelB gene (48). To visualize this activity, retinal cryostat sections (8 um) were fixed for 25 min in buffered 4% paraformaldehyde, washed with phosphate buffered saline, then incubated with 2% X-gal solution (a substrate for β-galactosidase) at 30 degrees C for 5 to 12h. Tissue localization of the blue reaction product was observed by light microscopy.
In vitro analysis of laminin degradation. For analysis of endogenous laminin-degrading proteinase activity, retinal extracts were prepared from different mouse treatment groups as described above. Equal amounts (20 ug) of specific extracts were then mixed as appropriate to the experiment and incubated for 2 h in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl. To determine the capacity of GelB to initiate laminin degradation in retinal extracts, 50 ng of purified murine GelB (from Chemicon International, Temecula, CA) was added to equal amounts of retinal extract (20 ug) and the mixture was incubated at 37 degrees C for 2 h. The GelB had been previously treated with the organomercurial, amino-phenyl-mercuric-acetate (APMA), to convert the proenzyme to its active form (49). For analysis of laminin content after incubation, all extracts were loaded onto 4-20% gradient SDS-polyacrylamide gels and electrophoresed under reducing conditions. After electrophoresis the proteins were transferred to nylon membranes and probed with anti-laminin antibodies as described above.

Results

Ganglion cell death in the mouse retina by apoptosis after optic nerve ligation. As previously demonstrated in rats, optic nerve ligation in mice resulted in a striking decrease in retinal thickness. This was specifically due to a decrease in the total number of cells present in the ganglion cell layer (GCL), and a reduction in the thickness of the inner plexiform layer (IPL) containing cytoplasmic extensions from these cells (Fig. 1A). In addition, there was a smaller reduction in the thickness of the inner nuclear layer (INL). TUNEL assay was performed over a time course of one to four days after surgery to learn whether cell loss was due to apoptosis. TUNEL-positive cells were observed in the retinal ganglion cell layer at all time points, with a
peak at two days after optic nerve ligation (Fig. 1B). Essentially no TUNEL-positive cells were found in control retinas.

In addition to ganglion cells, the retinal ganglion cell layer also contains displaced amacrine cells, the end feet of glial and Muller cells, and any leukocytes which infiltrate in response to injury. To determine whether cells lost after optic nerve ligation were ganglion cells, and to quantify their loss, we specifically marked these cells by injecting the site where their axons terminate in the brain (the superior colliculus) with the fluorescent tracer, fluorogold. One week after injection, ganglion cells were fluorogold positive as a result of retrograde transport of the tracer (Fig. 1C). Loss of fluorogold positive cells was observed as early as two days after optic nerve ligation and cell loss was even greater after 7 days (Fig. 1C & D). In contrast, the number of labeled cells remained unchanged in control retinas over the time course examined (Fig. 1C & D).

Optic nerve ligation leads to an increase in gelatinolytic metalloproteinase activity in the retinal ganglion cell layer and loss of laminin from the inner limiting membrane. An in situ gelatinase activity assay was employed to compare the level of gelatinolytic activity in the normal and injured retina of CD-1 mice. Two days after optic nerve ligation, gelatinolytic activity was localized on tissue sections to the inner aspect of injured retinas, but this activity was not found in control retinas (Fig 2A). No activity was observed when the procedure was performed on sections from injured retinas in the presence of 50 uM 1,10-phenanthroline. This is a metal ion chelator which inhibits MMPs due to its affinity for Zn2+, which is required for enzymatic activity (data not shown). These experiments demonstrate that a gelatinolytic metalloproteinase
activity appears in the inner retina following optic nerve ligation, co-localizing with ganglion cell apoptosis.

Laminins are major ECM components of the inner limiting membrane of the ganglion cell layer (47,50-53). Laminins associated with neurons and blood vessels in the brain disappear from the site of excitotoxin injection, and their loss is temporally and spatially coincident with neuronal cell loss (54). Immunofluorescent localization indicated positive staining for laminin in a bright sheet-like pattern in the inner limiting membrane of uninjured control retinas from CD-1 mice examined in this study (Fig. 2B). In contrast, a fragmented pattern of staining was observed following optic nerve ligation. The pattern of nidogen staining was unchanged after optic nerve ligation (Fig. 2C), indicating specificity of laminin loss. These data associate loss of the laminin of the inner limiting membrane with ganglion cell death after optic nerve ligation.

**GelB expression is induced in the retinal ganglion cell layer after optic nerve ligation.**

Gelatin/casein gel zymography was used to assess changes in neutral proteinases in CD-1 mice retina following optic nerve ligation. The levels of a gelatinase with an apparent molecular weight (105 kDa) appropriate for the proenzyme form of mouse GelB (44) was dramatically increased in extracts derived from injured retinas as compared to uninjured controls (Fig. 3A). The levels of this proteinase increased from one day to two days after injury, but had returned to control levels by four days. Inclusion of 10 mM EDTA (ethylene diaminotetraacetic acid) in the zymograms during development inhibited the appearance of this proteinase band demonstrating metal dependence for enzyme activity, consistent with identity as a matrix metalloproteinase (data not shown). In contrast, there was essentially no change in the level of a 65 kDa gelatinase
of the appropriate size to be the proenzyme form of the related MMP, Gelatinase A (GelA; MMP-2). Western blot analysis confirmed the identity of the 105 kDa gelatinase as pro-GelB and the time course of proenzyme increase after optic nerve ligation was consistent with the zymography data (Fig. 3B). Immunofluorescent localization for GelB on retinas, two days after optic nerve ligation showed that the increased levels of immunoreactive GelB protein were localized to the ganglion cell layer (GCL) (Fig. 3C), in correlation with the location of TUNEL-positive cells. Activation of GelB proenzyme is associated with cleavage from the N-terminus, and a reduction in molecular size. However, most observations indicate that the amount of active GelB at any given time in vivo is only a small percentage of the total (39;44;55). Therefore, the increase in the amount of GelB proenzyme is consistent with an increase in overall gelatinase activity in the retinal ganglion cell layer. Taken together, these data correlate GelB expression in the retinal ganglion cell layer with increased gelatinolytic metalloproteinase activity and ganglion cell apoptosis following optic nerve ligation.

To determine whether the elevated level of GelB in injured retina is due to increased transcription, we used transgenic mouse reporter line 3445. This line carries a transgene construct consisting of a beta-galactosidase reporter gene linked to DNA sequences between -522 and +19 of the GelB gene which contain transcriptional promoter activity. The GelB gene sequences drive reporter gene expression in these mice in a manner identical to that of the endogenous GelB gene (48). Therefore, these mice serve to indicate the location of GelB transcriptional promoter activity under homeostatic conditions and following induction in response to specific stimuli. Constitutive GelB promoter activity was observed as a dot-like staining pattern in the ganglion cell layer (GCL) of control retinas from line 3445 mice (Fig. 3D,
control), as previously shown (56). However, this staining pattern covered considerably more area and was much more intense in retinas after optic nerve ligation (Fig. 3D, ligated). These results suggest that optic nerve ligation induces GelB promoter activity in cells of the retinal ganglion cell layer.

**GelB deficiency protects against pathological changes in the retina after optic nerve ligation.** Based on the spatial and temporal association of GelB induction with ganglion cell death and loss of laminin from the inner limiting membrane after optic nerve ligation, we reasoned that GelB might contribute to these changes and that GelB deficiency might, therefore, have a protective effect. To address this hypothesis, we applied the optic nerve ligation model to a line of GelB deficient mice (GelB−/− mice), and used a matched littermate line for comparison (GelB+/+ mice). Zymography and Western blot analysis (Fig. 4) indicated that the level of GelB was dramatically increased in injured retinas from normal littermate control mice (GelB+/+ mice), but was absent from the retinas of GelB-deficient mice (GelB−/− mice). In contrast, the amount of a proteinase species, which co-electrophoresed on zymograms with the GelA proenzyme was essentially the same in extracts from normal and GelB-deficient mice.

Retinas from GelB-deficient mice (GelB−/− mice) remained relatively intact, with little reduction in the ganglion cell layer following optic nerve ligation. No TUNEL-positive cells were observed in injured retinas from GelB-deficient mice (Fig. 5A). Moreover, laminin immunoreactivity was relatively unaffected in GelB deficient mice (GelB−/− mice) after optic nerve ligation (Fig. 5B).
Western blotting was used as a second method to assess laminin loss after optic nerve ligation (Fig. 5C). The antibody employed for this experiment was raised against the alpha (440 kDa) and beta/gamma (220 kDa) chains of purified mouse laminin, but the alpha1 chain is not bound by this antibody on western blots (57,58,59). In our study, an immunoreactive protein band of around 200-220 kDa – the size of the beta/gamma chains - was observed with similar intensity in retinal extracts from both GelB-deficient mice (GelB−/−) and normal CD-1 mice. The intensity of this band was reduced in extracts from retinas of normal mice after optic nerve ligation. In contrast, the band intensity did not change in retinal extracts from GelB-deficient mice after optic nerve ligation.

**Rescue of GelB deficiency restores laminin loss from the inner limiting membrane after optic nerve ligation.**

To provide evidence for increased proteolytic activity against laminin in retinas after optic nerve ligation, equal amounts of retinal extracts from surgically-treated mice were incubated with extracts from normal mice, and laminin degradation was analyzed by western blotting. The intensity of the laminin band was reduced in the presence of retinal extract from surgically-treated mice (Fig. 6A, lane 4), and this reduction was inhibited in the presence of the MMP inhibitor EDTA (Fig. 6A, lane 5). When retinal extract from surgically-treated mice was added to extract from GelB-deficient mice, the higher molecular weight laminin band was again reduced (Fig. 6B, lane 4). These results provide evidence that loss of laminin in surgically-treated mice is due to an EDTA-sensitive proteinase activity.
To determine whether GelB could cause degradation of laminin present in the inner limiting membrane of the retina, purified GelB proenzyme was activated by treatment with an organo-mercurial (amino-phenyl-mercuric-acetate) and added to retinal extracts prepared from normal mice that had not undergone optic nerve ligation surgery. Western blotting demonstrated a reduction in the intensity of the immunoreactive laminin band after addition of active GelB to extracts (Fig. 6A, lane 2). Addition of EDTA, which inhibits GelB activity due to its capacity to chelate zinc and calcium ions, blocked this reduction (Fig. 6A, lane 3).

We then attempted to learn whether addition of exogenous GelB could rescue the GelB-deficient (GelB−/−) phenotype. Western blotting demonstrated a reduction in the intensity of the immunoreactive laminin band after addition of active GelB to extracts from GelB-deficient mice (Fig. 6B, lane 2). Addition of EDTA inhibited this reduction (Fig. 6B, lane 3).

These results provide a causal connection between GelB activity and both ganglion cell death, and specific loss of laminin from the retinal inner limiting membrane following optic nerve ligation.

Discussion

Apoptosis of retinal ganglion cells represents the final endpoint in optic nerve diseases such as glaucoma and results in irreversible loss of sight. Adapting a model for optic nerve ligation to the mouse, we found that apoptosis of retinal ganglion cells correlates with specific degradation of laminin from the underlying inner limiting membrane and with a ganglion cell-associated increase in gelatinolytic metallo-proteinase activity. These changes co-localize with a specific
increase in levels of a specific Matrix Metalloproteinase (MMP), gelatinase B (GelB; MMP-9), controlled (at least in part) by activation of the GelB promoter. Our findings led us to hypothesize that induction of GelB expression plays a direct role in ganglion cell death and loss of laminin from the inner limiting membrane of the retina in our model. In strong support of this hypothesis, we show that mice made genetically deficient for GelB are protected against these pathological changes.

The retina can be considered as an extension of the brain, and activity of GelB has been previously associated with cell death in experimental models of brain injury (34,39,60). Moreover, loss of laminin from affected areas of the brain in response to injury has been shown to contribute to neuronal cell death (37,38,61,62). It is interesting to speculate about mechanisms. One likely possibility is that cell attachment to matrix activates intracellular signaling pathways for cell survival (67). This would be similar to the mechanisms proposed for “anoikis” the process whereby matrix attachment facilitates epithelial cell survival (68,69). Another idea worth considering is that matrix attachment facilitates retrograde transport of cell survival factors such as brain-derived neurotrophic factor (BDNF). The delayed time course of cell death after ligation of the optic nerve in our study is consistent with such a mechanism.

There is evidence for MMP involvement in neural cell death in a number of CNS disorders (63,64). However, this is the first report demonstrating a causal connection between a specific MMP - GelB - and degradation of laminin. It is possible that this connection is direct; MMPs, including GelB (65), can catalyze degradation of laminins to some extent. However, the connection may also be indirect. Thus, other laminin-degrading proteinases are activated by
MMPs (66) and MMPs can alter the activity of cytokines that control expression of laminin-degrading proteinases (29).

The fact that the PA-PG system is necessary for laminin degradation in response to neural injury in the brain (37,38) is not inconsistent with a similar role for GelB. *In vitro*, plasmin directly activates proMMPs to active MMPs and plasmin is known to act on proGelB (70,71). Recent studies using mice deficient in plasmin(ogen) indicate that *in vivo* activation of pro-GelB may occur via plasmin-dependent or plasmin-independent mechanisms (72). Thus combining our data with those of the previous studies suggests a cooperative interaction between the PA-PG system and GelB in degradation of laminin following neural injury.

Apoptosis is a mechanism integral to organ formation during development. Ganglion cell death occurs postnatally in the retina, as a normal part of developmental patterning. A prominent degeneration of cells in the ganglion cell layer occurs in mice in the immediate postnatal period, peaking at 2-4 days, and ceasing by day 11 (73); comparable observations have been made in the chicken, rat, and hamster. A low level of GelB is expressed by ganglion cells in developing and adult mice (42,56). However, we have observed no significant difference in the number of cells in the retinal ganglion cell layer of GelB deficient mice as compared to their normal littermates. Thus, while GelB may still play a part in programmed cell death during retinal development, other mechanisms may compensate for its loss. This difference between requirement for a specific MMP in developmental and pathological remodeling processes has been a common observation for the majority of the MMP-deficient mouse models studied.
In conclusion, the current study provides the first evidence that induction of GelB activity following optic nerve ligation causes pathological changes to the retina, including ganglion cell death and specific loss of laminin from the inner limiting membrane of the retina. Other studies have shown that laminin loss contributes to cell death. Our findings suggest that GelB inhibitors might serve as retinal protective agents in the treatment of optic nerve diseases, including glaucoma.

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References


Figure Legends

Figure 1. Ganglion cell death in the mouse retina by apoptosis after optic nerve ligation.

Tissue analysis was performed at the indicated time after optic nerve ligation in CD-1 mice, and compared to untreated controls.

(A) Frozen cross sections (8 um) of eyes prepared two days after optic nerve ligation were stained with H&E and the retinas were observed by light microscopy (n=3). GCL=ganglion cell layer, IPL=inner plexiform layer, INL=inner nuclear layer, ONL=outer nuclear layer. Note the loss of ganglion cells in the inner retina. (40x magnification)

(B) TUNEL assay was performed on frozen cross sections (8 um) of eyes two days after optic nerve ligation; retinas were examined by fluorescence microscopy to identify apoptotic cells (n=3). The lower panel shows representative sections of the TUNEL assay. The bright green spots over the light green background are the TUNEL-positive cells. Arrows indicate such cells in the GCL. The photographs in the upper panel are similar sections stained with propidium iodide to identify all nuclei. Some TUNEL-positive staining is also present in the inner nuclear layer. GCL=ganglion cell layer, INL=inner nuclear layer, IPL=inner plexiform layer. (40x magnification)

(C) Retrograde labeling of ganglion cells with fluorogold was performed one week before optic nerve ligation. Two days, four days and one week later, flat retinal mounts were prepared as described in methods and ganglion cell density was determined using a fluorescence microscope (n=3 for each experimental group). The selected fields were located at approximately the same distance from the optic disk to account for the
variation in RGC density as a function of distance from the optic disk. (40x magnification)

(D) Quantitative estimation of ganglion cell loss after optic nerve ligation. Retinal ganglion cell density in representative images obtained from fluorogold labeled flat retinal mounts in the experiment described above (in figure C) were counted using Scion Image analysis. The data was represented as the percentage of fluorogold labeled cells remaining at different time intervals after optic nerve ligation. All comparisons are significant. Statistical significance was determined using the Student t-test. n=3, p<0.005.

Figure 2. Optic nerve ligation leads to an increase in gelatinolytic metalloproteinase activity in the retinal ganglion cell layer and selective loss of laminin from the inner limiting membrane.

(A) Tissue analysis was performed two days after optic nerve ligation in normal CD-1 mice, and compared to untreated controls. Unfixed frozen sections were incubated with FITC-labeled DQ-gelatin for in situ zymography and observed by fluorescence microscopy (n=3). Arrows indicate the localization of gelatinolytic activity in the inner retina (green). GCL=ganglion cell layer, INL=inner nuclear layer, IPL=inner plexiform layer. (40x magnification)

(B) Indirect immunofluorescent staining for laminin. Frozen retinal cross sections were incubated with a polyclonal antibody against mouse laminin (green). Propidium iodide was used to stain the nucleus. The photographs shown are the overlapping images of laminin (green) and propidium iodide (red) staining (n=3). Arrows indicate laminin
staining in the inner limiting membrane. GCL=ganglion cell layer, INL=inner nuclear layer, IPL=inner plexiform layer (40x magnification)

(C) Indirect immunofluorescent analyses of laminin and nidogen. Frozen retinal cross sections were incubated with a polyclonal antibody against mouse laminin (red, upper panel), and a monoclonal antibody against nidogen (red, lower panel). Hoechst dye was used to stain the nucleus. The photographs shown in the upper panel are the overlapping images of laminin (red) and Hoechst dye (blue) staining. The photographs in the lower panel are the overlapping images of nidogen (red) and Hoechst dye (blue) staining. Arrow indicates the band of laminin staining, and arrowhead indicates the fragmented appearance of laminin. GCL=ganglion cell layer, ILM=inner limiting membrane, IPM=inner plexiform layer, INL=inner nuclear layer, ONL=outer nuclear layer (40x magnification)

Figure 3. GelB expression is induced in the retinal ganglion cell layer after optic nerve ligation.

(A) Retinal extracts were prepared from optic nerve ligated (Lig.) or uninjured control (Cont.) CD-1 mouse eyes over a time course from one to seven days after optic nerve ligation (n=3 for each experimental group). Equal aliquots of protein (20 ug) were analyzed by gelatin zymography. For comparison, the samples were co-electrophoresed with a sample of purified mouse GelB (MMP-9, GelB Std.) and with reduced molecular weight size standards (not shown). The migration position of the clear bands representing GelB proenzyme (105kDa), and GelA (MMP-2) proenzyme (65 kDa) are indicated.
(B) Retinal extracts were prepared from injured (Ligated) or uninjured control (Control) CD-1 mice eyes over a time course from six hours to seven days after optic nerve ligation (n=3 for each experimental group). Equal aliquots of protein (20 ug) from retinal extracts derived from uninjured control (Control) or injured (Ligated) eyes were analyzed by Western blotting. Figure indicates that GelB expression increases over a time course up to 2 days after optic nerve ligation. For comparison, the samples were co-electrophoresed with a sample of purified mouse GelB (GelB Std.) and with reduced molecular weight size standards (not shown).

(C) Immunofluorescent localization of GelB protein (bright red) occurs specifically in the ganglion cell layer (GCL) of injured retinas. Figure shows a bright GelB staining in injured retinas two days after optic nerve ligation (n=3) in CD-1 mice. (40x magnification)

(D) Two days after optic nerve ligation in GelB/lacZ transgenic reporter mouse line 3445, eyes were enucleated and frozen sections were prepared (n=3). Sections were incubated with 2% X-gal solution to determine β-galactosidase reporter gene activity. Retinas were observed by light microscopy. The areas of GelB promoter activity are stained blue. Figure shows increased GelB promoter activity (arrows) in ganglion cell layer two days after optic nerve ligation. GCL=ganglion cell layer. (40x magnification)

Figure 4. Demonstration that GelB is not produced in the retinas of mice made genetically deficient for GelB.

Tissue analysis was performed two days after optic nerve ligation on GelB deficient mice (GelB<sup><-/-></sup>) and on mice of the matched normal littermate control line (GelB<sup><+/-></sup>) of the same
background (n=3 for each experimental group). Extracts from each strain were analyzed by
gelatin zymography and western blotting. The migration position of the GelB proenzyme (105
kDa) and the GelA (MMP-2) proenzyme (65 kDa) is indicated.

**Figure 5. GelB deficiency prevents death of retinal ganglion cells and degradation of
laminin from the inner limiting membrane after optic nerve ligation.**

(A) TUNEL assay was performed on frozen retinal cross sections (8 um) from GelB-
deficient control mice and mice, two days after they had undergone optic nerve ligation
(GelB⁻⁻; n=3). Retinal sections were counterstained with propidium to stain the nucleus
of the cells. The images shown are overlapping images of TUNEL (green) and
propidium iodide counter staining. GCL=ganglion cell layer. Note the absence of
TUNEL positive cells both in control and optic nerve ligated retinas in GelB-deficient
mice. (40x magnification)

(B) Indirect immunofluorescent analysis of laminin in frozen retinal sections from optic
nerve ligated and control eyes in GelB-deficient mice (GelB⁻⁻), (n=3). Frozen cross-
sections of eyes were incubated with a polyclonal antibody against laminin. Propidium
iodide was used to stain the nucleus. The photographs shown are the overlapping images
of laminin (green) and propidium iodide (red) staining. Arrow indicates the band of
laminin staining. GCL=ganglion cell layer. All the photographs were taken at 40x
magnification.

(C) Equal protein (20 ug) from retinal extracts prepared from control (C) and optic nerve
ligated mice (L) from both wild type CD-1 mice and GelB-deficient mice (GelB⁻⁻) were
electrophoresed on 4-20% polyacrylamide-SDS gels under reducing conditions. The
proteins were transferred onto nitrocellulose membrane and probed with a polyclonal antibody against mouse laminin. The laminin band is indicated with an arrow.

Figure 6. Exogenous GelB addition rescues the GelB-deficient phenotype.

Equal amounts of protein (20 ug) from retina extracts derived from normal CD-1 mice (panel A) or GelB deficient mice (GelB−/−)(panel B) were incubated with APMA-activated GelB, or with 20 ug of retinal extract from optic nerve ligated CD-1 mice, in the presence or absence of 1 mM EDTA for 2 h at 37 C. Extracts were electrophoresed on 4-20% polyacrylamide-SDS gels under reducing conditions, blotted onto nitrocellulose membrane, and probed with anti-laminin antibody. The laminin band is indicated with an arrow.
Chintala et al., Figure 1

(A) H&E Staining

Control

Ligated

GCL
IPL
INL
ONL

(B) Propidium Iodide

Control

Ligated

GCL
IPL
INL

(TUNEL)

Control

Ligated

GCL
IPL
INL

(C) Day 2

Day 4

Day 7

Control

Ligated

(D) Number of fluorescent labeled cells/40x field

Day 2

Day 4

Day 7

Control

Ligated
Chintala et al., Figure 4

GelB\(^{+/+}\) vs GelB\(^{-/-}\)

<table>
<thead>
<tr>
<th></th>
<th>Cont.</th>
<th>Lig.</th>
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<tbody>
<tr>
<td>GelB-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gelA-</td>
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<td></td>
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Gelatin Zymography

Western blot
Chintala et al., Figure 5

(A) Control vs. Ligated

- TUNEL Assay
  - GCL
  - INL
  - ONL

(B) Laminin staining
  - GCL
  - INL
  - ONL

(C) CD-1 mice vs. GelB (-/-)

<table>
<thead>
<tr>
<th>CD-1 mice</th>
<th>GelB (-/-)</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>Day 1</td>
</tr>
<tr>
<td>Cont. Lig.</td>
<td>Cont. Lig.</td>
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### Table 1: Protein Expression in CD-1 Mice and GelB(-/-) Mice

<table>
<thead>
<tr>
<th>Normal extract</th>
<th>CD-1 mice</th>
<th>GelB(-/-)</th>
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<tbody>
<tr>
<td>gelB</td>
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<td>+</td>
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<tr>
<td>Ligated CD-1 extract</td>
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<td>+</td>
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<tr>
<td>EDTA</td>
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### Image 1: Western Blot Analysis

- **Lanes:**
  - Lane 1: 198-, 176-, 113-, 63-, 49-
  - Lane 2: 198-, 176-, 113-, 63-, 49-

- **Notes:**
  - Arrow indicates a specific band.
Deficiency in matrix metalloproteinase gelatinase B (MMP-9) protects against retinal ganglion cell death after optic nerve ligation
Shravan K. Chintala, Xu Zhang, Jeffrey S. Austin and M. Elizabeth Fini

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