MMP Inhibition Preserves Integrin Ligation and FAK Activation to Induce Survival and Regeneration in RGCs Following Optic Nerve Damage

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Purpose. Integrin adherence to the extracellular matrix (ECM) is essential for retinal ganglion cell (RGC) survival: damage causes production and release of ECM degrading matrix metalloproteinases (MMPs) that disrupt integrin ligation, leading to RGC death. The interplay of MMPs, integrins, and focal adhesion kinase (FAK) was studied in RGCs after optic nerve injury.

Methods. Optic nerve transection and optic nerve crush were used to study RGC survival and regeneration, respectively. Treatments were administered intravitreally or into the cut end of the optic nerve. RGC survival was assessed by fluorescence or confocal microscopy; cell counting, peptide levels, and localization were assessed by Western blot and immunohistochemistry.

Results. MMP-9 was most strongly increased and localized to RGCs after injury. Pan-MMP, MMP-2/9, and MMP-3 inhibition all significantly enhanced RGC survival and increased RGC axon regeneration. FAK activation was decreased at 4 days postaxotomy, when apoptosis begins. FAK inhibition reduced RGC survival and abrogated the neuroprotective effects of MMP inhibition, whereas FAK activation increased RGC survival despite MMP activation. Integrin ligation with CD29 antibody or glycine-arginine-glycine-aspartate-serine (GRGDS) peptide increased RGC survival after axotomy.

Conclusions. ECM-integrin ligation promotes RGC survival and axon regeneration via FAK activation.

Keywords: matrix metalloproteinase, integrin, focal adhesion kinase, retinal ganglion cell, neurotrophic factor, necroptosis

Injury to the mammalian central nervous system (CNS) often results in permanent neuronal and functional loss. Apoptosis is a primary death-inducing mechanism that contributes to this and has also been identified as an important mechanism in several neurodegenerative diseases.1–3 Suppression of apoptosis relies on the maintenance of survival signals, and one of the ways in which this proceeds is via focal adhesion kinase (FAK).4

The adherence of cells to the extracellular matrix (ECM) provides survival signals through the ligation and activation of ubiquitously expressed integrin receptors. A total of 18α and 8β integrin subunits combine to form at least 24 heterodimeric receptors (αβ), each binding to a limited subset of ECM components, such as collagen, laminin, arginine-glycine-aspartate (RGD)-containing proteins (fibronectin, fibrin), and immunoglobulin superfamiy proteins (VCAM, ICAM).5 Integrin receptors are a subset of dependence receptors,6 meaning that in the absence of their specific ligand they trigger apoptotic cell death.7 At least 7 alpha integrin subunits (α1, α2, α3, α4, α6, α8, αv) along with 3 β-subunits (β1, β2, β3) have been localized to the mammalian retina.8 Specifically, subunits α2, α3, α4, and β2 are localized to the ganglion cell layer (GCL), nerve fiber layer (NFL), and inner plexiform layer (IPL). Integrin β3 is also localized to the nerve fiber layer; β1 is found in the inner limiting membrane, and β1-3, β1-6, and αv are localized to the human optic nerve.9 Altogether, this demonstrates the localization of several α and β subunits to mammalian RGC somata and axons.

ECM degradation causes cultured neuron death, which can be prevented by integrin ligation.9,10 In the retina, integrin αββ1 antagonism leads to increased RGC apoptosis and a reduced number of RGCs during development.11 Additionally, integrin αββ1 mediates neurite outgrowth in embryonic retinal cells, and inhibition of β112 or αβ13 function in the developing visual system results in apoptosis. Furthermore, evidence indicates that ECM signaling collaborates with neurotrophic factor signal transduction to regulate synaptic development, neurite outgrowth, and cell survival.14 Thus, it is clear that ECM interaction plays a critical role in the survival of developing mammalian RGCs and other CNS neurons.

Matrix metalloproteinases (MMPs) are a group of enzymes that degrade the ECM. Their activity increases after retinal injury,15–17 inducing apoptosis due to loss of integrin ligation.
Downstream signal transduction from integrins relies on tyrosine kinase activity, which occurs via the recruitment and activation of FAK, a nonreceptor tyrosine kinase. FAK activation leads to phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK), and Src kinase activation, all of which are essential for the transduction of ECM-dependent survival signals via integrin receptors. These mechanisms also regulate the survival of injured CNS neurons and are required for neurotrropic factor rescue.

The present study examined the importance of FAK activation on RGC survival and regeneration after optic nerve injury. It also examined the factors influencing FAK activation: integrin binding to the ECM and its regulation by MMP activation. The evidence reported provides critical insight into the molecular mechanisms that govern neuron fate in the cellular environment of the CNS, and identifies FAK as an important target for activation in therapies designed to improve neuron survival after injury.

METHODS

Statistical Analysis

Data regarding cell survival, axon regeneration, and densitometric intensity was analyzed with Microsoft Excel (Microsoft, Redmond, WA, USA) and R software (R Statistical Computing, Vienna, Austria). All in vivo experiments consisted of 4 animals (8 eyes) unless otherwise indicated. Survival results were grouped by retinal eccentricity, and expressed as the mean number of RGCs/mm² ± SEM. Data were analyzed for significance with Student's t-test or ANOVA followed by Tukey's post-hoc test as appropriate. The threshold for significance was established as α = 0.05.

Microscopy

Samples were visualized using an Andor iXon 885+ EMCCD (Andor Technology Oxford Instruments, Belfast, Ulster, UK) camera mounted on a Leica DM LPSA microscope (Leica, Wetzlar, Hesse, Germany) with a Sutter Lambda XL light source (Sutter Instrument, Novato, CA, USA) for illumination and an Andor iXon 885+ camera and EM gain applied (property of Koeberle’s lab), or with an AxioObserverZ1 inverted motorized microscope (Carl Zeiss Microscopy, Jena, Thuringia, Germany) equipped with a Yokogawa CSU-XI SDC (Yokogawa Electric, Tokyo, Kantô, Honshu, Japan), an Axiocam 506 high-resolution camera, and a Photometrics Evolve 512 EMCCD high sensitivity camera spinning disk confocal, (Photometrics, Tuscon, AZ, USA, property of the University of Toronto Faculty of Medicine Microscopy Imaging Laboratory).

Optic Nerve Transection and Optic Nerve Crush

All animals were pathogen-free female Sprague Dawley rats treated in accordance with the University of Toronto Animal Handling and Surgical Guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Optic nerve transections were performed as previously described. Briefly, animals were anesthetized with isoflurane (2.5%, 0.8 L/min O2; Baxter, Deerfield, IL, USA) and placed in a stereotactic frame with a gas anesthesia mask to maintain sedation. The optic nerve was accessed via an incision in the tissue overlying the superior rim of the eye socket and the underlying extraocular muscles were exposed. Following retraction of the rectus muscles, the meningeal coverings of the nerve were dissected longitudinally to preserve the retinal blood supply. The optic nerve was gently lifted and cut approximately 2 mm from the back of the eye. Optic nerve injury specifically induces damage to RGCs, which follows a characteristic time course wherein the population of RGCs begins to decrease at 3 to 4 days postaxotomy and reaches its nadir by 12 to 13 days postaxotomy. This results in the death of approximately 90% of RGCs. Assessments of RGC survival in the current study were all based on this timeline. RGC density in a normal eye is approximately 2500 to 2000 cells/mm² (from the Central to Peripheral eccentricities), and all results are relative to this amount.

The optic nerve crush model is a well-established method to study axon regeneration in the nonpermissive environment of the adult CNS. The procedure is very similar to that for optic nerve transection; however, the meningeal sheath is not dissected. Instead, the nerve is crushed within the meningeal sheath using fine self-closing forceps for 5 seconds as previously described. RGC axon regeneration was assessed at 21 days postcrush to allow axons time to regenerate.

Following transection and crush procedures, the orbital contents were replaced in their original locations and the skin incision was sutured. Animals were removed from anesthesia and given injections of ketoprofen and sterile saline before being placed under a heat lamp until waking. Ophthalmic ointment was placed on the animals’ corneas to prevent desiccation, and postoperative discomfort was minimized with intraperitoneal injection injections of ketoprofen (5 mg/mL, dosage for rats: 0.1 mL/100 g body weight) 24 hours postsurgery.

RGC Transfection After Axotomy

FAK constructs were expressed in axotomized RGCs via transfection with FAK plasmid injected into the cut end of the optic nerve, as previously described. RGCs were transfected with cytomegalovirus (CMV) promoter based plasmids (kindly supplied by Kenneth M. Yamada; National Institutes of Health, Bethesda, MD, USA) encoding FAK constructs, constitutively active FAK (CA-FAK) or dominant-negative kinaseinactive FAK (DN-FAK). In vivo jetPEI (Qbio-Gen, Montréal, Quebec, Canada) complexed plasmids (1 μg/μL) were injected into the cut end of the optic nerve using a Hamilton syringe. Control plasmid injections consisted of green fluorescent protein (GFP) encoding plasmids.

Intracocular Injections

Intracocular injections were performed as previously described. A hydraulic injection system consisting of a pulled glass micropipette, coupled to a 10-μL Hamilton syringe through polyethyleneketone tubing was used to deliver solutions into the vitreous chamber. For intracocular injections, rats were anaesthetized with isoflurane in a stereotactic apparatus to prevent head movement and lens damage, which is known to release confounding soluble factors. Intracocular injections consisted of the broad-spectrum MMP inhibitor GM6001 (N-[(2R)-2-(hydroxyamidocarbonylmethyl)-4-methyl-pentanoyl]-L-tryptophan methylamide; 20 μM, 2 μL), GM6001 inactive negative control (GM-Neg; 20 μM, 2 μL), MMP-2/9 inhibitor (2R)-2-[(4-biphenylylsulfonyl)amino]-3-phenylproionic acid; 20 μM, 2 μL), MMP-3 inhibitor (N-isobutyl-(N-4-methoxyphenylsulfonyl)-glycylhydroxyamic acid; 20 μM, 2 μL), RGD peptide (arginyl-glycyl-aspartic acid; 10 mg/mL, 4 μL), GRGD ligating-antibody (1 mg/mL, 4 μL; catalogue # 556404; BD Pharmingen, Mississauga, Ontario, Canada), Cyclodextrin-RGD-fmk peptide (RGD negative control, 10 mg/mL, 4 μL), ANapec Inc., Freemont, CA, USA), YIGSR peptide (Tyrosine-Isoleucine-Glycine-Serine-Arginine; 10 mg/mL, 4 μL; Sigma-Aldrich), FAK inhibitor PF-573228 (25 mM, 4 μL; Sigma-Aldrich), FAK inhibitor FAK-I-14 (1,2,4,5-benzetetramine tetrahydrochloride; 25 mM, 4 μL; Tocris, Bristol, UK), platelet-derived growth factor (PDGF) receptor inhibitor AG1290 (25 mM, 2 μL; Sigma-
Western Blotting

Western blots on whole retinas were performed as previously described.22 Briefly, total protein fractions were separated by SDS-PAGE (5%-20% acrylamide) and immunoblotted after semidry electrotransfer to nitrocellulose membranes (0.2-μm pore). Primary antisera for analyzing the activation and integrity of FAK consisted of rabbit-anti-p-FAK (Tyrs97/12; 1:1000; Cell Signaling Technology, Danvers, MA, USA) and rabbit-anti-FAK (1:1000; Cell Signaling Technology). Primary antisera for evaluating MMP levels in the retina consisted of rabbit-anti-MMP-3 (1:250; Abcam, Cambridge, Cambridgeshire, UK), rabbit-anti-MMP-9 (1:250; Abcam), rabbit-anti-MMP-12 (1:250; Abbcom), and rabbit-anti-MMP-2 (1:250; Cell Signaling Technology). Primary antisera were detected with a 1:1000 dilution of secondary antibody (horseradish peroxidase conjugated, cross reacted against rat serum antigens; Jackson Immunoresearch, West Grove, PA, USA). Chemiluminescent immunoreactive complexes were visualized using a Bio-Rad Fluor-S Max imager (Hercules, CA, USA). Loading was verified by visualizing protein bands with Ponceau S dye, and reprobing blots with a rabbit antisera directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000; 2118; Cell Signaling Technology).

Immunohistochemistry

Fluorescence immunohistochemistry was performed on transverse retinal sections as previously described.19,22,24,28 Primary antisera for characterizing MMP localization in the retinal were rabbit-anti-MMP-2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit-anti-MMP-3 (1:250; Abcam), rabbit-anti-MMP-9 (1:500; Chemicon, Fisher Scientific, Hampton, NH, USA), and rabbit-anti-MMP-12 (1:500; Abcam), guinea pig anti-RBPMS (RNA binding protein with multiple splicing, 1:1000; Phosphosolutions, Aurora, CO, USA), rabbit anti-GAP-43 (growth associated protein-43, ab16053, 1:1000; Abcam), and rabbit-anti-MMP-2 (1:250; Cell Signaling Technology). To ensure complete fixation, nerves were postfixed in 4% paraformaldehyde overnight at 4°C. Following removal of the cornea and lens, the eye cup was fixed in 4% paraformaldehyde for 1 hour followed by three washes of PBS containing 0.3% Triton X-100 and 3% normal serum. Incubation in secondary antisera consisted of fluorophore-labeled secondary antibody, PBS containing 0.3% Triton X-100, and 3% normal serum. Incubation in secondary antisera was done for 3 hours at room temperature. In between each incubation stage sections were rinsed 3 × 15 minutes in PBS. Final prepared samples were cover-slipped with 50/50 glycerol/PBS solution.

Regeneration was quantified by the number of growth cones as well as their distance, beyond the crush site. Distance bins were defined as 0 to 250, 250 to 500, and more than 500 μm. A total of four equally spaced transverse sections per nerve were examined.

Results

Localization of Matrix Metalloproteinases After Axotomy

We examined the localization of full-length and cleaved forms of MMP-3, -9, -12, and -2 throughout the retina and compared the results between control and axotomized retinas. These MMPs were specifically examined due to their reported roles in CNS pathology: both MMP-2 and -9 have been implicated in CNS damage, such as blood–brain barrier disruption, neuroinflammation, edema, and neuron death17; MMP-2 is expressed by RGCs,20 and active MMP-9 is expressed in the GCL in conjunction with RGC degeneration21; expression of both MMP-3 and -12 increases in the GCL following retinal damage, where they are thought to contribute to inflammation.32

Immunohistochemistry revealed very low levels of MMP-3, -9, and -12 in normal uninjured retinas (Figs. 1A, 1C, 1E) whereas MMP-2 immunoreactivity was more pronounced and localized to the outer plexiform layer (Fig. 1G). Immunoreactivity of MMP-3, -9, and -12 all increased following transection (Figs. 1B, 1D, 1F), whereas MMP-2 immunolocalization and expression did not appear to change after axotomy (Fig. 1H). Validation for antibodies detecting MMPs are demonstrated in Figures 11 through 1K. MMP-3 immunoreactivity appeared to be localized to the conical inner end-feet of Müller glia enveloping RGGs in the GCL (Fig. 1B). Further examination of 4- and 7-days post-axotomy retinas colocalized MMP-3 with GFAP, which is expressed by activated glial cells following retinal injury (Ockuly JC, et al. IOVS 2014;55:ARVO E-Abstract 1851), confirming our previous observation (Figs. 2C, 2D, 2G, 2H–J). Importantly, MMP-3 expression appeared to decrease from 4 to 7 days postaxotomy, suggesting more pronounced effects in the days immediately following optic nerve injury. MMP-2 immunoreactivity appeared at the GCL (Fig. 1B). Further examination of 4- and 7-days post-axotomy retinas colocalized MMP-3 with GFAP, which is expressed by activated glial cells following retinal injury (Ockuly JC, et al. IOVS 2014;55:ARVO E-Abstract 1851), confirming our previous observation (Figs. 2C, 2D, 2G, 2H–J). Importantly, MMP-3 expression appeared to decrease from 4 to 7 days postaxotomy, suggesting more pronounced effects in the days immediately following optic nerve injury. MMP-9 and -12 appeared to be strongly localized to the GCL (Figs. 1D, 1F), and was further examined in normal, 4-, and 7-day postaxotomy retinas via confocal microscopy (Fig. 3). MMP-9 appeared to be very weakly expressed in normal retinas, but increased postaxotomy at 4 days and again at 7
FIGURE 1. MMP-3, -9, and -12 increase in the GCL at 7d postaxotomy. Immunofluorescence micrographs of MMPs in transverse sections (14-μm thick) of normal or axotomized retinas. (A, C, E, G) MMP-3, -9, -12, -2 staining in normal nonaxotomized retinas. (B) MMP-3 immunoreactivity was observed in Müller cell radial processes and end-feet in the GCL. (D) MMP-9 immunoreactivity increased after axotomy and was localized to injured RGCs (arrows) and cells in the inner nuclear layer (INL). (F) MMP-12 immunoreactivity also increased after RGC axotomy and was localized to RGCs (arrows) and the INL. MMP-2 immunoreactivity was confined mainly to the border between the INL and the outer nuclear layer (arrows) in both
normal (G) and axotomized retinas (H). Scale bar: 50 μm. (I–L) Western blots demonstrating antibody specificity to their respective target MMPs. Molecular weight of the MMPs differs from that on later blots as these are recombinant forms only used to validate antibody specificity. Blots were run in quadruplicate, lanes 1 to 4 containing samples of recombinant MMP protein (MMP-2 and -9 from R&D Systems; MMP-3 and -12 from LS BioSciences). Each blot was probed with a specific antibody to show its specificity for its target MMP. Expected weight and identification for each MMP was accurate and is indicated. Cross-reactivity for the MMP-3 antibody was noticed very slightly with MMP-2; however, all other antibodies were exclusive.

days (Figs. 3G–I). There was weak colocalization between MMP-9 and RNA binding protein with multiple splicing (RBPMS), indicating that MMP-9 was predominantly located outside of RGCs (Figs. 3J–L). MMP-12 expression in normal retinas was also relatively weak and increased at 4 days postaxotomy; however, unlike MMP-9, it appeared to remain stable at 7 days (Figs. 3S–U). MMP-12 also appeared to overlap with RBPMS but not with 4',6'-diamidino-2-phenylindole (DAPI), indicating that it was predominantly localized to RGCs' cytoplasmic compartment. The increased expression of both MMP-9 and -12 after axotomy supports a role for both in RGC degeneration. However, their different localizations relative to injured RGCs suggests different mechanisms of action.

Full-length (inactive) and cleaved (active) MMP expression was examined in normal and 7-day postaxotomy retinal lysates via Western blot and optical density measurement. We found that full-length MMP-3 increased after injury without a concomitant increase in its cleaved form (Figs. 4A, 4B). Levels of both full-length and cleaved MMP-9 increased at 7 days postaxotomy (Figs 4C, 4D), implying importance in RGC death at this time. There was no significant increase in levels of either full-length or cleaved MMP-12 (Fig. 4E, 4F). There was no change in levels of full length MMP-2; however, cleaved MMP-2 decreased significantly at 7 days postaxotomy (Figs. 4G, 4H). Overall, these results indicate that MMP-9 is the most involved in retinal damage at 7 days postaxotomy.

**MMP Inhibitors Promote RGC Survival**

MMPs degrade the ECM, disrupting cell contact and resulting in death.7 We demonstrated the presence and activation of MMPs in the retina following optic nerve axotomy, and therefore examined the effect of MMP inhibition on RGC survival. Animals received intraocular injections of MMP inhibitors at 3 and 8 days postaxotomy and RGC survival was quantified at 14 days. Retinas treated with MMP inhibitors increased RGC survival (Figs. 5B–D) relative to those treated with vehicle or inactive MMP inhibitor analog (Figs. 5A, 5E). Injection of MMP-2/-9 inhibitor (Fig. 5B), MMP-3 inhibitor (Fig. 5C), or GM6001 (a broad-spectrum MMP inhibitor; Fig. 5D) all increased RGC survival at 14 days postaxotomy (Fig. 5E). This shows that MMP inhibition results in an approximately 2-fold increase in RGC survival at 14 days after injury.

Adherence of RGCs to laminin is known to induce signals essential to their survival.53 MMP-9 degrades laminin,54 and as such we examined the presence of laminin in the normal retina, at 4 and 7 days postaxotomy (Figs. 5, L–N). Our results show that laminin is slightly degraded at 4 days postaxotomy, but that it is very pronounced by 7 days postaxotomy. This suggests the loss of laminin contact as a factor inducing RGC death, and that this may be the predominant effect of MMP-9.

**Focal Adhesion Kinase Modulates RGC Survival**

ECM degradation induces the death of neurons in culture, which can be prevented by the ligation of cell-surface integrin receptors.10 FAK activation via phosphorylation at its Tyrrosine 397 (Y397) residue is a critical event in the transduction of integrin ligation signals,6 and therefore we investigated the degradation or activation of FAK in RGC degeneration after axotomy. At 4 days postaxotomy, the time when RGCs begin to die by apoptosis, FAK phosphorylated at Y397 (p-FAK) was reduced (Figs. 6A, 6B). Retinal cryosections of normal, 4-, and 7-day postaxotomy retinas were examined by confocal microscopy and revealed that p-FAK had decreased at 4 days postaxotomy and had decreased further at 7 days (Figs. 6I–N).

It has previously been shown that FAK is cleaved by caspases during apoptosis in mammalian cell lines55 and accordingly we observed increased FAK cleavage in the retina at 4 days postaxotomy (Figs. 7A, 7B) without observing a change in the total levels of FAK (Fig. 7A, optical density results
Combined with our results regarding FAK phosphorylation, this shows that FAK activity is reduced at 4 days postaxotomy, the time at which RGC loss begins. We further examined the role of FAK in RGC survival by inhibiting or augmenting its function. Intraocular injection of two different FAK inhibitors, FAK-I-14 (1,2,4,5-benzenetetraine tetrahydrochloride; Figs. 7D, 7F) or PF-573228 (Figs. 7E, 7F), at 3 days postaxotomy significantly reduced RGC survival at 7 days postaxotomy relative to control axotomized eyes (Fig. 7F), indicating that FAK inhibition exacerbates the effects of optic nerve transection. It should be noted that PF-573228 reduced RGC survival by a greater degree and in all retinal eccentricities whereas FAK-I-14 reduced RGC survival to a lesser degree, and only in the central and peripheral eccentricities. This suggests that PF-573228 was more effective at inhibiting FAK.

As a corollary to this experiment, axotomized RGCs were transfected with plasmids encoding a constitutively-active (CA-FAK) or a DN-FAK. Injection of GFP encoding plasmids showed that nerve application produced a maximal RGC transfection rate of approximately 35% (Figs. 7G–I). CA-FAK plasmids significantly increased RGC survival at 7 days postaxotomy, whereas DN-FAK significantly reduced it (Fig. 7J). Furthermore, DN-FAK transfection abolished the previously observed neuroprotective effect of GM6001 (Fig. 7K), demonstrating that FAK activity is required for neuroprotection via broad-spectrum MMP inhibition. These results indicate that FAK activation is reliant on ECM contact, presumably due to integrin ligation.

Figure 3. MMP-9 and -12 increase at 4 and 7 days postaxotomy. Confocal micrographs of transverse retinal sections showing MMP-9 and -12 expression and localization in normal, 4-, and 7-day postaxotomy retinas. (A–C, M–O) DAPI stained images of cell nuclei in the GCL. (D–F, P–R) RBPMS-labeled images, indicating RGC nuclei, in the GCL. (G–I) Localization of MMP-9 in retinas from each timepoint. Expression of MMP-9 is clearly increased over time and appears to be localized predominantly to the extracellular space. (S–U) Localization of MMP-12 in retinas from each timepoint. Expression of MMP-12 appears to increase by 4 days postaxotomy and remain constant at 7 days. (J–L) Merged images of MMP-9 expression at all timepoints. Expression of MMP-9 appears to increase over time and is localized to the extracellular space around RGCs. (V–X) Merged images of MMP-12 expression at all timepoints. Expression of MMP-12 appears to be maximal at 4 days postaxotomy and be localized to the soma of RGCs.
FIGURE 4. Western blots showing levels of latent (full-length proenzyme), active (cleaved), and optical density measurement of active/GAPDH whole-retina preparations. (A, B) MMP-3, (C, D) MMP-9, (E, F) MMP-12, (G, H) and MMP-2 in lysates of normal (n = 4) or 7-day axotomy (n = 4) retinas. GAPDH loading controls are shown below each blot. Optical density results represent mean ± SEM (**P < 0.01, *P < 0.05).
Integrin Ligation Promotes Retinal Ganglion Cell Survival After Injury

After demonstrating that MMP and FAK activity are each able to modulate RGC survival, we examined whether integrin ligation was the junction point between them. The RGD motif is predominantly associated with the fibronectin component of the ECM and is recognized by over 20 known integrin receptors; however, it preferentially binds $\alpha v \beta 3$ (IC$_{50} = 28 \pm 3$) and $\alpha v \beta 5$ (IC$_{50} = 260 \pm 42$) and $\alpha v \beta 6$ (IC$_{50} = 1480 \pm 62$). Its effects on neuron survival have been reported to be concentration dependent. A soluble mutated version of the RGD peptide, Cyclo-RAD-fluoromethylketone (RAD) was used as a control treatment. The CD29 (HUTS-21) antibody specifically ligates $\beta 1$ integrin, and it was injected intraocularly to investigate its effects on RGC survival. To verify this, YIGSR peptide, which also binds to $\beta 1$ integrin without inducing cell survival, was also injected intraocularly.

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Administration of RAD peptide did not improve RGC survival after axotomy relative to untreated controls (Figs. 8A, 8E). In contrast, RGD peptide yielded a 3-fold increase in RGC survival (Figs. 8B, 8E), indicating that the latter was protective, presumably via integrin ligation. Likewise, administration of the CD-29 antibody resulted in a 4-fold increase in RGC survival (Figs. 8D, 8E), whereas YIGSR peptide did not improve it (Figs. 8C, 8E). Taken together, these results demonstrate that integrin ligation is able to effectively induce RGC survival; however, it should also be noted that CD-29 significantly increased RGC survival relative to RGD-treated retinas (Figs. 8B, 8D, 8E), suggesting that ligation of β1 integrin is more effective at inducing neuron survival. To examine the mechanism of RGC protection via integrin ligation, we surveyed the relative levels of p-FAK present in the retina at 4 days postaxotomy following the administration of RAD, RGD, YIGSR, and CD-29 via Western blot (Figs. 8E, 8G). As expected, our results indicated a significant increase in p-FAK following treatment with RGD relative to RAD peptide. However, we did not observe a significant increase in p-FAK in CD-29 relative to YIGSR, or in CD-29 relative to RGD, treated retinas.

**PDGF and EGF Induced Retinal Ganglion Cell Survival Relies on Extracellular Matrix Adhesion**

PDGF and EGF receptor ligation can induce FAK activation. To determine whether this is responsible for RGC rescue after MMP inhibition, we delivered intraocular injections of PDGF...
FIGURE 7. FAK deactivation reduces RGC survival, FAK activation increases it. (A, B) Western blot of total and cleaved FAK from whole retinal lysates and optical density measurement of cleaved FAK/GAPDH. GAPDH loading controls are shown below. FAK phosphorylation (Y397), which is required for activation, was reduced at 4 days postaxotomy. There was no discernible change in the levels of full-length FAK; however, axotomized retinas showed increased levels of cleaved, inactivated FAK (B). (C–E) Epifluorescence micrographs of Fluorogold retrogradely labeled RGCs in flatmounted retinas at 7 days postaxotomy. Intraocular injection of two FAK inhibitors, PF-573228 or FAK-I-14 (1,2,4,5-benzenetetramine...
tetrahydrochloride; reduced RGC survival compared with 7-day controls. (F) Graph representing mean RGC densities ± SEM at 7 days postaxotomy in retinas treated with FAK inhibitors. The FAK inhibitor PF-573228 produced a substantial reduction in RGC survival after intraocular injection. FAK-I-14 (1.2 ± 5-Benzamidine tetrahydrochloride) was less effective, reducing survival in the central and peripheral retina only. ∗∗P < 0.01 relative to control. (**P < 0.01 relative to control. (G–I) Fluorescence micrographs showing the expression of GFP in axotomized RGCs, following injection of plasmids into the transected optic nerve stump. Images were taken at different retinal eccentricities as follows: (G) central, (H) midperipheral, and (I) peripheral. (J) Graph showing the effects of CA-, or DN-FAK plasmid treatments on RGC survival at 7 days after axotomy. RGCs were transfected at the time of axotomy, by the application of plasmids to the transected optic nerve stump. RGC densities in retinas that received CA-FAK were significantly higher than retinas that received control GFP encoding plasmids (Ctrl). DN-FAK treatment significantly reduced RGC survival at 7 days postaxotomy, compared with control treatment. ∗<P < 0.05 relative to control; ∗∗<P < 0.01 relative to control). (K) Graph showing the showing the effect of DN-FAK plasmid treatment on GM6001-mediated neuroprotection. RGC densities in retinas that received DN-FAK in combination with the broad-spectrum MMP inhibitor GM6001 were significantly lower than retinas that received GM6001 alone. Control GFP encoding plasmids did not reduce RGC survival in a similar manner (**P < 0.01 in relation to GM6001 negative control [GM6001 Neg Ctrl] and GM6001 + DN-FAK).

and EGF: neither significantly improved overall RGC survival relative to controls (Fig. 8H). This indicates that PDGF or EGF receptor binding does not compensate for the loss of ECM contact and is insufficient to induce FAK activation. However, it should be noted that following PDGF treatment, survival of “central” retinal eccentricity RGCs was increased significantly, suggesting that PDGF has a stronger effect than EGF, and may have an impact on FAK activation.

In addition, we investigated whether PDGF or EGF receptor ligation is responsible for the prosurvival effect of MMP inhibition via combined intraocular injection of GM6001 and either a PDGF receptor inhibitor (AG1296) or an EGF receptor inhibitor (PD153035). Neither treatment improved RGC survival relative to the administration of GM6001 alone (Fig. 8I). As the effects of ECM ligation and EGF or PDGF receptor ligation are not cumulative, we suspect that they are proceeding via the same mechanism to activate FAK and preserve RGC survival after injury.

MMP Inhibition Enhances Axon Regeneration by Injured Retinal Ganglion Cells

FAK activity promotes axon extension in cultured neurons, and we investigated its effects on RGC axon regeneration following optic nerve crush. Crucially, we first documented the presence of activated FAK (p-FAK) in regenerating axons. To overcome the nonpermissive CNS environment, we administered intraocular injections of the ROCK inhibitor (Y27632), which has been shown to prevent cell death by another mechanism. Under these conditions, p-FAK was co-localized with regenerating axons both proximally and distally to the crush site 21 days postcrush via retrograde Cholera Toxin-FITC tracing (Figs. 9A–C).

We next examined if MMP inhibition with GM6001 would increase the length and number of regenerating axons. In control eyes, few axons extended beyond the crush site (Fig. 9D; arrowheads). Intraocular injection of GM6001 at 3 and 10 days postcrush resulted in pronounced axonal sprouting and regeneration within the proximal nerve stump and enhanced axon outgrowth across the injury site (Figs. 9E, 9F). GM6001 treatment resulted in significantly greater numbers of regenerating axons at all sampling distances (0–250, 250–500, >500) in the distal optic nerve (Fig. 9G), showing that MMP inhibition promotes the RGC axons regeneration.

DISCUSSION

Our results demonstrate an important relationship between the ECM, integrin receptors, and FAK in RGCs. We show that MMP-3, -9, and -12 are all active following optic nerve axotomy but that they are differently localized and therefore likely have different mechanisms of action. We show that FAK is degraded and its activation is reduced in the days following optic nerve axotomy, but that its activation, and RGC survival, can be rescued by administration of soluble ECM components. The same mechanisms involved in survival may also be important in neurite regeneration as we also show that inhibition of MMPs increases RGC axon regeneration.

MMPs induce neuron death by breaking down the ECM and disrupting integrin ligation – a mechanism thought to play a role in glaucomatous RGC death. MMP-9 upregulation is known to induce RGC death, while its deficiency is protective; accordingly, we found an increase in the expression of MMP-9 proenzyme across the entire retina and in the GCL at 7 days postaxotomy. In addition, we found that that laminin, a target of MMP-9 digestion, is degraded in the GCL by 7 days postaxotomy. Taken together, this indicates that MMP-9 may be activating RGC survival by breaking down the ECM and disrupting their adherence to it.

Previous study of MMP-3 has shown that, in the rat retina, its activation is maximal at 3-days posthigh IOP injury. Our results agree with this as we found its expression to be maximal in the GCL at 4 days postaxotomy, after which time it begins to decline. Western blots did not demonstrate an increase in MMP-3; however, we suspect that its expression may be camouflaged by inclusion of the whole retina. Confocal examination of MMP-3 colocalized it to activated glia in the GCL, as has been suggested previously (D’Onofrio PM, et al. IOVS 2012;53:ARVO E-Abstract 3479) supporting the notion that it is involved in RGC death, but that it is secreted by glial cells as opposed to RGCs in response to damage. Analysis of MMP-12 expression did not reveal a significant increase after injury; however, microscopy appeared to indicate an increase in the GCL, specifically in the cytoplasm of RGCs. Similarly to MMP-3, we suspect that the increase in RGCs is camouflaged by low levels of MMP-12 expression in the rest of the retina. Apparent MMP-12 localization to the RGC soma suggests that it is functioning intracellularly to induce RGC death. MMPs, specifically MMP-12, has been localized intracellularly following injury, where it can play a variety of roles related to apoptosis initiation. As such, we suspect that the role of MMP-12 after axotomy may go beyond ECM degradation and affect the intracellular processes of RGCs.

Contrary to our findings regarding other MMPs, we found a significant decrease in MMP-2 across the entire retina but an apparent increase in its presence in the outer plexiform layer (OPL). Although MMP-2 and MMP-9 are expressed by Mueller glia, only MMP-9 appears to play a role in breaking down the ECM. In conjunction with out findings, this indicates that MMP-2 likely does not play a direct role in RGC death; however, it may propagate damage to the rest of the retina, particularly to the OPL.

Overall, our data indicate that MMP-9 is secreted by RGCs and that it plays an important role in RGC death after injury. However, it is important to note that MMP-3 and -12, although not increased throughout the retina, still appear to be secreted in very high levels in the GCL, although their mechanisms of action on RGC death appear to vary from that of MMP-9.
We show that administration of MMP-3, -2/-9, or the broad-spectrum MMP inhibitor GM6001 improves RGC survival after axotomy. Combined with our results regarding MMP localization, we therefore conclude that MMP-3 and -9 contribute to RGC death. A role for MMP-12 is also possible; however, it was not specifically inhibited and will require further research. The literature indicates that MMP-9 specifically affects neuron survival as it is linked to the destruction of laminin, an integral component of the ECM found in the GCL. The importance of laminin is exemplified by the fact that cultured RGCs survive significantly better by being raised on laminin media rather than collagen type I or IV, fibronectin, or poly-L-lysine. Indeed, laminin contact with RGCs also effectively induces axon growth and elongation. Fibronectin binds with integrin α5β1, while laminin targets α3β1; in both cases the presence of the β1 subunit appears to be the source of their involvement in RGC survival. This interaction is essential as it leads to the activation of FAK and Akt, as well as the increased...
expression of Bcl-XL. Loss of laminin contact is sufficient to arrest these survival signals and induce apoptosis, strongly suggesting that laminin degradation by MMP-9 is a key event contributing to RGC death after axotomy. Our results support this view as we found a clear decrease in laminin near RGCs in the days following axotomy and concurrent with an increase in MMP-9 expression.

We hypothesized that RGC death relies on the abolition of ECM-integrin ligation and subsequent FAK deactivation. Our results support this hypothesis as we found that following optic nerve transection, CA-FAK increased the number of surviving RGCs whereas DN-FAK and pharmacologic FAK inhibitors reduced it. This shows that FAK activity effectively opposes RGC death signals and improves survival, a result that is further supported by the observed reduction in surviving RGCs following FAK activity. Furthermore, we found decreased levels of phosphorylated FAK and increased levels of cleaved FAK at 4 days postaxotomy, the time point at which RGCs begin to rapidly die after injury. Taken together, this evidence strongly suggests that the deterioration of FAK activity is an important event in the onset of RGC death.

In order to link the deterioration of the ECM to FAK activity levels, we administered the broad-spectrum MMP inhibitor GM6001 along with DN-FAK following axotomy. We found that co-delivery of DN-FAK abolished the survival-inducing effect of GM6001, demonstrating that FAK activity is required for GM6001-mediated neuroprotection. Thus, the beneficial effects of MMP inhibition appear to rely on downstream activation of FAK, suggesting that they occur via enhanced ECM binding and activation of integrin receptors on RGCs.

Integrin subunits are prevalent on RGCs, and we hypothesized that they function as the interface between ECM contact and activation of FAK and other survival-promoting mechanisms. To examine this, peptides encoding the soluble integrin ligand-motif were administered. Immunostaining with anti-RGD antibodies showed that these peptides promote integrin-ligation and cell survival by mimicking fibronectin and binding to \( \alpha 5\beta 1 \) and \( \alpha v\beta 1 \) integrins, whereas soluble RGD peptides most common.

**Figure 9.** MMP inhibition enhances axon regeneration following optic nerve crush. (A–C) Epifluorescence micrographs showing the colocalization of Cholera Toxin B-FITC anterograde labeling (CTB-FITC [A]) and phospo-FAK (pFAK-Y397 [B]) in regenerating RGC axons within transverse sections of optic nerve (21 days after crush). These animals were treated with the ROCK inhibitor Y27632 in order to promote regeneration. pFAK immunoreactivity was present in the majority of anterogradely labeled RGC growth cones and axons in the vicinity of the crush site (yellow arrow), including axons that bypassed by crush site as shown in the merge (C). (D–F) Composite images of epifluorescence micrographs showing GAP-43 immunostaining in longitudinal sections of optic nerve at 21 days after optic nerve crush and intraocular delivery of GM6001 (days 3 and 10). The crush site in each nerve is marked by a vertical arrow, and the retina (not visible) is located beyond the left-hand side of each image. MMP inhibition via intraocular injection of GM6001 induced robust axon regeneration within the proximal segment of the optic nerve and enhanced axon regeneration across the lesion site (arrowheads). The majority of regenerating RGC axons were located in the first 250 µm beyond the crush site. (G) Quantification of the average number of regenerating axons per section (mean ± SEM) at different distances from the crush site. The number of axons was quantified in three different bins (<250, 250–500, or >500 µm; **P < 0.01, relative to control).
ly disrupt integrin activity by competitive binding; however, the effect of soluble RGD peptides appears to be dependent on concentration, with lower concentrations of free RGD promoting cell survival and higher concentrations inducing cell death. Despite this, our results showed increased RGC survival following RGD peptide treatment; we suggest that the acute application of a single bolus of RGD peptide, followed by rapid clearance from the vitreous, resulted in lower effective concentrations and a survival promoting effect on RGCs. Further support for the role of integrin ligation in promoting RGC survival comes from our finding that intraocular delivery of an antibody directed against CD29 (β1 integrin) successfully increased RGC survival. Presumably, the effects of RGD and CD29 function through the activation of FAK, as this method of integrin activation has previously been used to stimulate integrin signal transduction and FAK activation in immune cells. Our data show that CD29 ligation has potential therapeutic value in the injured adult CNS, and implicates integrin ligation as an important factor in the survival of injured RGCs. To test the effect of β1 integrin ligation on RGC survival, we also administered a YIGSR peptide via intraocular injection. The YIGSR sequence specifically binds to β1 integrin; however, it did not increase RGC survival. This result agrees with previous studies that found 50% less p-FAK in cells cultured on YIGSR relative to those cultured on laminin even though β1 integrin expression was constant, and that found that the YIGSR peptide effectively halts cancer growth.

We conducted Western blots to examine levels of p-FAK in retinas at 4 days postaxotomy following treatment with RAD, RGD, YIGSR, or CD29 at 3 days postaxotomy. Our results indicated that only RGD treatment significantly increased p-FAK levels. However, there was an obvious difference in p-FAK levels in YIGSR- and CD29-treated retinas, and although it was not statistically significant, we suggest that the time point of 4 days postaxotomy was too early to assess levels of p-FAK after injury. In addition, we suggest that a single timepoint may not be enough to capture the effect of CD29 on p-FAK levels, and that sampling at later times may reveal a sustained increase that could explain its improvement of RGC survival. As such, the lack of RGC survival in response to YIGSR peptide treatment serves as further evidence of the importance of β1 integrin ligation as well as FAK signaling in the induction of RGC survival after axotomy.

PDGF or EGF ligation has been reported to increase FAK activation; however, this effect may ultimately be dependent on C-terminal FAK association with integrins located at focal adhesions. Our data demonstrate that neither PDGF nor EGF inhibition affected RGC survival in the presence of the broad-spectrum MMP inhibitor GM6001, indicating that integrin ligation induces survival signals independently of PDGF or EGF. We also demonstrated that PDGF or EGF administration alone did not significantly increase RGC survival after axotomy, except in the case of the central eccentricity of the PDGF-treated retinas. This suggests that PDGF and EGF alone do not induce RGC survival, but that they require concurrent integrin ligation. Another possible explanation is that PDGF and EGF treatments delay RGC death slightly so that by 14 days postaxotomy levels of surviving RGCs have not yet reached their nadir. Given our evidence, we conclude that PDGF and EGF activate FAK via the same mechanism as integrin activation and that they may in fact require FAK activation to have a positive effect on RGC survival. Previous research demonstrating that FAK and PDGF are required for extracellular receptor kinase (ERK)-stimulated vascular cell growth supports this interpretation. Consequently, evidence indicates that FAK, and PI3K or ERK, are required for the transmission of PDGF generated survival signals via FAK phosphorylation at Y397 or S910, respectively. Together, these results all support our conclusion that survival signaling as a result of PDGF signaling requires FAK.

To keep damaged RGCs alive, survival pathways must overcome death-inducing signals. FAK contributes to this function by activating PI3K as well as nuclear factor-kB (NF-kB) via the ERK1/2 and p38/MAPK pathways. NF-kB activation serves as a junction point where the FAK and TRADD pathways converge and the balance of these factors determines if cell fate will progress toward survival or death. We have previously shown that inhibition of caspase-6 increases axon regeneration following optic nerve crush, however the mechanisms involved remain unclear. Evidence indicates that caspase-6 cleaves FAK during apoptosis, which may account for limited axon regrowth. As we have shown, FAK activity is critical for RGC survival, and previous work has found it to be important in axon extension in cultured hippocampal and dorsal root ganglion neurons. Our evidence agrees with this and demonstrates a substantial regenerative response in the proximal optic nerve together with avoidance of the central lesion site by most regenerating axons. This suggests that MMP inhibition enhances the intrinsic regenerative capacity of RGCs. In addition, FAK promotes PI3K activation in cultured neurons, which is necessary for axon outgrowth; similar mechanisms likely contribute to the axon regeneration that we observed after optic nerve crush. It should also be noted that axon regeneration was induced following administration of MMP inhibitors near the RGC soma, which is located far from the crush site on the optic nerve: the regeneration signals induced by MMP activation can therefore act at a distance and are not solely due to the preservation of focal adhesions.

CONCLUSIONS

The results presented in this study demonstrate a major role for FAK in the survival and regeneration of injured RGCs, which may also translate to other CNS neurons. FAK activity appears to rely on integrin activation through contact with the ECM. There are several classes of growth-inhibitory cues, including ligands of the Nogo receptor and myelin-associated glycoprotein, that inhibit RGC axon regeneration after injury, likely limiting the extent of regeneration after MMP inhibition. As such, it is clear that the effects of the FAK pathway are invaluable in the pursuit of axon regeneration.

Pursuing strategies to preserve the RGC population and stimulate their regenerative capacity continue to be important topics in the field of neuroscience, and based on our evidence, FAK may be central to these pursuits.

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