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MMP19 expression in the human optic nerve

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Purpose: The defining feature of glaucoma is excavation of the optic nerve head; however, the mechanism of this loss of tissue is not well understood. We recently discovered a copy number variation upstream of *matrix metalloproteinase 19* (*MMP19*) in a large, autosomal dominant pedigree with a congenital malformation of the optic disc called cavitary optic disc anomaly (CODA). Patients with CODA have abnormal optic discs that exhibit an excavated shape similar to cupping seen in glaucoma. The goal of this study is to characterize the localization of MMP19 within the human optic nerve.
Methods: The MMP19 protein in the optic nerve was evaluated with western blot analysis and with immunohistochemistry in sagittal and en face/cross sections of optic nerves obtained from healthy human donor eyes.
Results: The MMP19 protein was detected in the human optic nerve, retina, and RPE/choroid with western blot analysis, with highest expression in the retina and the optic nerve. Using immunohistochemistry, MMP19 was localized within the optic nerve to the extracellular space within the septa that separate bundles of optic nerve axons into fascicles. The presence of MMP19 within the optic nerve septa was further confirmed by the colocalization of MMP19 to this structure with type IV collagen. Strong labeling of MMP19 was also detected in the arachnoid layer of the optic nerve sheath. Finally, immunohistochemistry of the optic nerve cross sections demonstrated that MMP19 shows a peripheral to central gradient, with more abundant labeling along the edges of the optic nerve and in the arachnoid layer than in the center of the nerve.
Conclusions: Abundant MMP19 was detected in the optic nerve head, the primary site of pathology in patients with CODA. The localization of MMP19 to the optic nerve septa is consistent with its predicted secretion and accumulation within the extracellular spaces of this tissue. Moreover, the lateral localization of MMP19 observed in the optic nerve cross sections suggests that it might have a role in regulating adhesion to the optic nerve to the scleral canal and remodeling the extracellular matrix that provides the structural integrity of the optic disc. Dysregulation of MMP19 production might, therefore, undermine the connections between the optic nerve and the scleral canal and cause a collapse of the optic disc and the development of CODA. Similar processes might also be at work in the formation of optic disc cupping in glaucoma.

Progressive cupping of the optic disc is a key clinical sign of glaucoma. Although enlargement of the cup-to-disc ratio is most often associated with glaucoma, progressive cupping has also been documented in cases of patients with arteritic ischemic optic neuropathy and rarely in other ocular conditions [1-3]. Static excavation of the optic disc occurs with many congenital malformations, including optic pits, optic nerve coloboma, and morning glory disc anomaly. Autosomal dominant pedigrees that exhibit features of these congenital optic nerve abnormalities have been called cavitary optic disc anomaly (CODA) [4-6]. Longitudinal data were available in one large CODA pedigree, and several family members were shown to have progressive enlargement of optic nerve excavation, as is observed in glaucoma [6,7].

Several genes have been identified that are associated with optic nerve colobomas that are part of larger syndromes (*PAX6*-OMIM 607108 [8], *SHH*-OMIM 600725 [9], *MAF*-OMIM 177075 [10], and *CHX10*-OMIM 142993 [11]). However, until recently, no CODA genes had been identified. We identified a regulatory sequence mutation (triplication of an enhancer sequence) upstream of *matrix metalloproteinase 19* (*MMP19*-OMIM 601807) as the cause of CODA in a large pedigree [12]. This triplication appears to cause pathologic upregulation of *MMP19* gene expression. Matrix metalloproteinases (MMPs) are zinc-dependent extracellular proteases that are required for the breakdown of the extracellular matrix

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(ECM) in normal processes, such as embryonic development, cell surface receptor cleavage, apoptosis, and tissue remodeling. MMPs also have a role in disease states, such as arthritis, cardiovascular disease, and pathologic angiogenesis [13-16].

Early studies demonstrated that *MMP19* is expressed in a broad range of tissues, including the intestine, ovary, spleen, lung, placenta, and thymus [17,18]. *MMP19* is also produced in several tumor cell lines [19]. MMP19 was later shown to be expressed in the optic nerve [12]. Several specific regulators of *MMP19* expression have been identified, including POU2F3 [20] and POU3F1 [20]. Moreover, *MMP19* expression is known to be altered during wound healing, and by several diseases, including psoriasis, eczema, and malignancies [21-24]. The activity of MMPs are also known to be modulated by tissue inhibitors of metalloproteinases (TIMPs). TIMP2 in particular has been shown to reduce MMP19 activity [18],

Northern blot analyses from multiple tissues suggest one predominant *MMP19* transcript [17,18]. However, sequence-based analyses of *MMP19* transcripts have detected alternative splicing and several distinct transcripts: variant 1 (NM_002429), variant 3 (NM_001272101), and variant 4 (NR_073606). Variant 1 encodes the canonical 57 kDa protein (Q99542–1) that is a propeptide with a signal sequence, which targets the enzyme for secretion. Other non-canonical isoforms of MMP19 are missing key sequences that encode functional domains, including the signal sequence, metal ion binding domains, and the active site of the enzyme [25].

MMP19 encodes a secreted protease [18] that is produced in the optic nerve [12], and previous studies have indicated that aggrecan, laminin, nidogen, fibronectin, and collagen are target molecules for MMP19's protease activity [26,27]. These observations suggest that MMP19 and its activity for digesting extracellular matrix structural proteins may be involved in important optic nerve diseases, including CODA and glaucoma. Currently, however, the role of *MMP19* in ocular health or disease is unknown. In this study, we investigate the localization of MMP19 in the human optic nerve to begin to understand the role of this protein in the development of disease in CODA and potentially in other optic nerve diseases, such as glaucoma.

METHODS

Ocular tissue preparations: Human donor eyes were obtained from the Iowa Lions Eye Bank after informed consent was received from the donors' families. All experiments were performed with full consent and in accordance with the Declaration of Helsinki. Within 5 h of death, the human donor eyes were fixed with 4% paraformaldehyde in PBS (1X; 1.06 mM KH_2PO_4, 155.17 mM NaCl, 2.97 mM Na_2HPO_4-$7H_2O$, pH 7.4). Three eyes from three donors were used for histological studies, and two eyes from two donors were used for biochemical experiments.

Western blot analysis: For western blot analysis, 4 mm punches of full thickness optic nerve head tissue were collected. The optic nerve head punch was prepared from eyes that had the optic nerve severed at the outer edge of the sclera. A disposable 4 mm trephine punch, centered on the optic nerve head, was pushed through the retina, Bruch's membrane, choroid, and sclera. This full thickness punch was used for the biochemical studies. Thus, these punches included a small rim of the peripapillary retina, as well as the prelaminar nerve, the lamina cribrosa, and a small portion of the retrolaminar nerve, terminating at the outer edge of the sclera. In addition, 6 mm punches of the neural retina and 6 mm punches of RPE/choroid tissue were collected from the eyes of two donors who were 72 and 80 years old. Tissue was homogenized in PBS with 1% Triton X-100 and a complete protease inhibitor cocktail tablet (Roche Diagnostic, Indianapolis, IN) for protein extraction. Total protein concentrations were quantified using the BioRad DC Assay following the manufacturer's instructions (BioRad, Hercules, CA). For each sample, 20 µg of total protein was combined with Laemmli buffer and β-mercaptoethanol, and the samples were loaded into a precast 4–20% Mini-PROTEAN TGX gel (BioRad) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) separation. After the proteins were blotted on a polyvinylidene difluoride (PVDF) membrane (BioRad), the membrane was subjected to brief methanol treatment and blocking with 3% bovine serum albumin (BSA, Research Products International, Mt Prospect, IL) in 10 mM PBS with 0.1% Tween-20 (v/v; PBS-T; Research Products International). The membrane was incubated with a polyclonal anti-MMP19 primary antibody (1:1,000, ab39002; Abcam, Cambridge, MA) and monoclonal anti-α-tubulin antibody (1:5,000, ab7291; Abcam) for 1.5 h. The membrane was washed in PBS-T three times for 5 min each, followed by incubation with the appropriate fluorescent secondary antibodies (Thermo Fisher Scientific, Waltham, MA). Three 5-min washes were performed in TBS with 0.1% Tween-20 (TBS-T; BioRad), and protein bands were detected with a VersaDoc Imager (BioRad).

Immunohistochemistry: Wedges of the retina and the optic nerve, or the retrolaminar optic nerve alone, were cryopreserved in sucrose solution and embedded in optimal cutting temperature solution [28]. A polyclonal anti-MMP19 primary antibody (1:200, ab39002; Abcam) was used in the

colorimetric and fluorescent immunohistochemical studies. Seven micron thick tissue sections were obtained using a Microm H505E cryostat (Waldorf, Germany) and were mounted on Superfrost Plus slides (Ted Pella, Redding, CA) as previously described [29,30].

For the immunofluorescence microscopy, sections were blocked in 1X PBS with 0.1% BSA for 15 min followed by incubation with anti-MMP19 for 1 h at room temperature. Following the primary incubations, the sections were washed for 5 min in 1X PBS three times and then incubated with 1:200 donkey anti-rabbit Alexa Fluor® 488-conjugated secondary antibody (A-21206; Thermo Fisher Scientific) and 4'6-diamidino-2-phenylindole (DAPI) for 30 min in the dark protected from light. Sections were washed 3 × 5 min, mounted in Aqua-Mount (Thermo Scientific), observed under fluorescence microscopy with an Olympus BX41 microscope (Melville, NY), and images were collected with a SPOT RT digital camera (Diagnostic Instruments; Sterling Heights, MI). For some experiments, sections were dual labeled with *Ulex europaeus* agglutinin-1 (UEA-I, Vector) and monoclonal antibody directed against collagen type IV (Developmental Studies Hybridoma Bank, Iowa City, IA).

For the colorimetric immunohistological analyses, the sections were blocked for 15 min in 1% horse serum in PBS and were then incubated for 1 h with anti-MMP19 primary antibody, followed by 3 × 5 min washes in PBS. Sections were then overlaid with biotinylated secondary antibody for 30 min (1:100 dilution from Vector Laboratories, Burlingame, CA). Following 3 × 5 min washes, the sections were treated with avidin biotin horseradish peroxidase complex, washed, and developed in peroxidase substrate (Vector Laboratories). Images were captured with an Olympus BX41 microscope using the SPOT RT camera.

To confirm the specificity of the MMP19 antibody, full-length recombinant MMP19 protein (ab132174, Abcam) was preincubated with 1:10 diluted MMP19 antibody at a 5X excess. After 1 h of preincubation, antibody alone or antibody plus recombinant protein was added to the tissue sections, and labeling was performed as described.

RESULTS

Western blot analysis: As part of studies to evaluate the expression of MMP19 within the human eye, we assessed the binding of polyclonal anti-MMP19 antibody with western blot analysis of protein isolated from the human optic nerve, retina, and RPE/choroid samples (Figure 1). The same western blot was also labeled with anti-α-tubulin as a loading control. Anti-MMP19 antibody strongly labeled a band of approximately 40 kDa in optic nerve and retina lysates that is around the predicted size of the activated MMP19 protein [12,31]. Weaker labeling of secreted/cleaved MMP19 protein was also observed in one of the RPE/choroid samples. Some full-length MMP19 (about 57 kDa) was detected in the optic nerve and RPE/choroid samples. These experiments confirmed the expression of MMP19 in the human optic nerve tissue and the specificity of the MMP19 antibody, and indicate that the majority of MMP19 in the optic nerve head is in the activated form.

We further investigated the tissue localization of MMP19 with immunohistochemical analysis of the human optic nerve

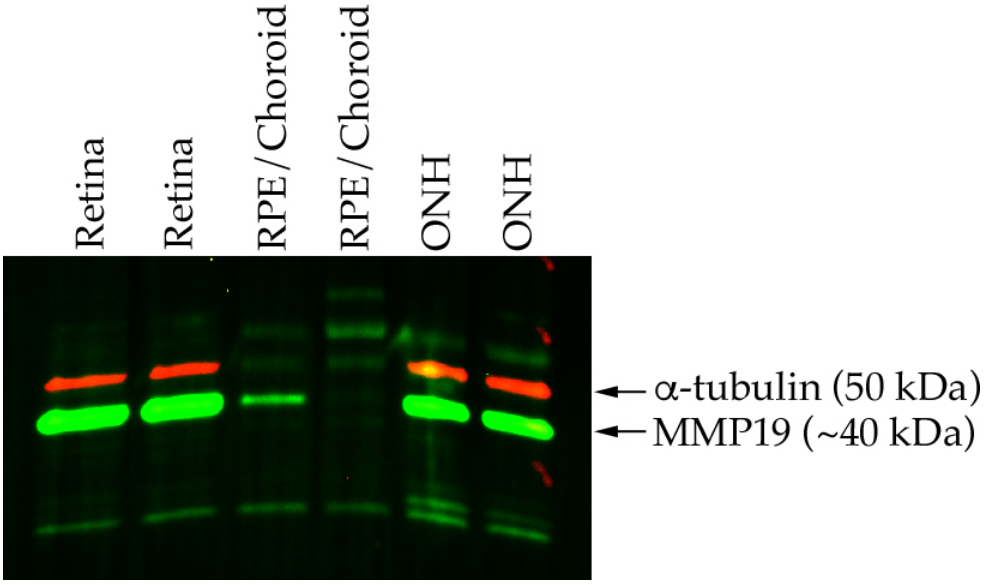

Figure 1. Western blot of the human retina, RPE/choroid, and optic nerve head. The membrane was probed with an anti-MMP19 antibody (about 40 kDa, green) and an anti-α-tubulin antibody (50 kDa, red). Duplicate lanes represent proteins from two donors.

just posterior to the scleral canal. MMP19 was detected throughout cross sections of the optic nerve. Polyclonal anti-MMP19 antibody strongly labeled the septa surrounding fascicles of optic nerve axons (Figure 2A). The intensity of the MMP19 labeling appeared higher at the periphery of the optic nerve and in the arachnoid with decreased abundance in the center of the nerve (Figure 2B).

The specificity of the anti-MMP19 antibody for immunohistochemical analyses was tested with blocking experiments. Polyclonal anti-MMP19 antibody was incubated with full-length, active MMP19 before the antibody was used as a primary antibody for immunohistochemical labeling of the human optic nerve. Incubation of the anti-MMP19 antibody with the MMP19 protein showed remarkable inhibition of labeling of the extracellular structures of the optic nerve (Figure 2C,D), confirming the specificity for MMP19 protein.

Lower magnification images of the optic nerve show that MMP19 labeling is strongest at the lateral edges or periphery of the optic nerve and diminished gradually toward the center of the nerve near the central retinal artery and vein (Figure 2E). The intensity of MMP19 labeling along the circumference of the optic nerve places MMP19 near the interface between the optic nerve and the scleral canal.

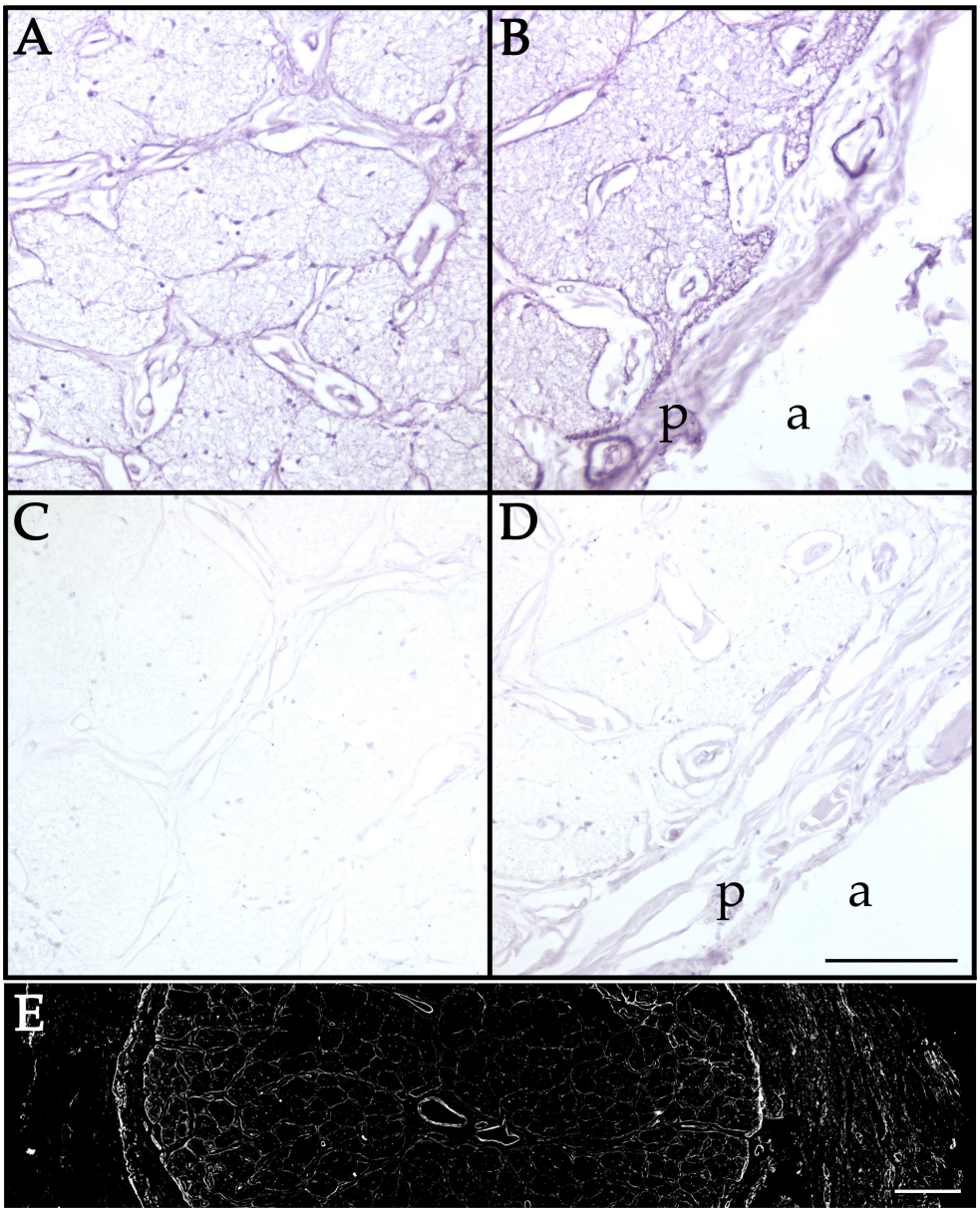

Figure 2. Immunohistochemistry of the human retrolaminar optic nerve. Anti-MMP19 antibody was used to label a central region (**A**) and a peripheral region (**B**) of the retrolaminar optic nerve (purple labeling indicates the reaction product). **C**, **D**: The anti-MMP19 antibody was preincubated with full-length MMP19 protein before immunolabeling. **E**: A grayscale, negative image of the MMP19 immunolabeling for the entire width of the optic nerve is presented. Scale bar (**D**) = 100 µm; scale bar (**E**) =500 µm. a, arachnoid mater; p, pia mater.

MMP19 was further evaluated within the optic nerve with colabeling studies using immunofluorescence microscopy. MMP19 (green) colocalized in the extracellular space with collagen (red) in the septa surrounding fascicles of the optic nerve (Figure 3G–I). No colocalization with UEA-1 (vascular endothelium) was detected (Figure 3J–L) within the capillaries in the optic nerve, although MMP19 was detected in large vessel walls. A similar pattern of MMP19 detection was identified along the periphery of the optic nerve cross sections. MMP19 and collagen colocalized in the extracellular spaces with collagen surrounding fascicles of the optic nerve axons (Figure 3A–C). Colocalization of MMP19 with collagen was detected within the exterior of the vessel walls in the outer edge of the optic nerve (Figure 3A–C). However, there was no colocalization with UEA-1 within the interior, vascular endothelium, of these vessels (Figure 3D–F). Finally, strong labeling of MMP19 was observed within the arachnoid layer of the optic nerve sheath (Figure 3A,C).

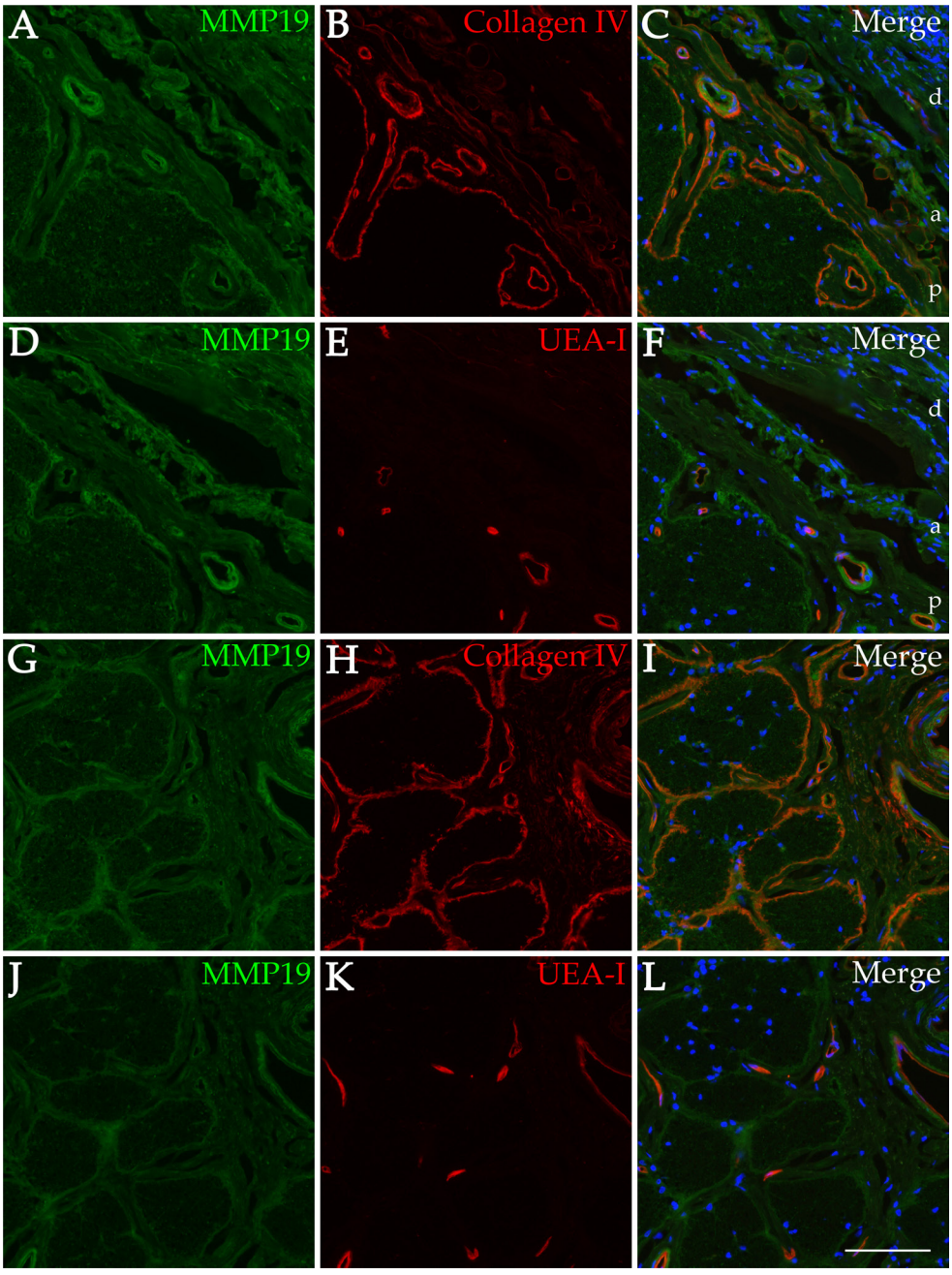

Figure 3. Immunofluorescence analysis of the human retrolaminar optic nerve. MMP19 immunolabeling is present in the peripheral (**A**, **D**, green) and central regions (**G**, **J**, green) of the optic nerve, as well as collagen IV (**B**, **H**, red) and UEA-I lectin labeling (**E**, **K**, red). The merged images show colocalization between MMP19 and collagen IV (**C**, **I**), while MMP19 and UEA-I do not overlap except in large vessel walls (**F**, **L**). Sections were counterstained with 4'6-diamidino-2-phenylindole (DAPI; **C**–**L**). Scale bar = 100 µm. d, dura mater, a, arachnoid mater; p, pia mater.

DISCUSSION

The most striking feature of CODA is congenital excavation of the optic disc, which is similar to cupping of the optic disc, the key diagnostic sign of primary open-angle glaucoma. In CODA and primary open-angle glaucoma, there is a loss or absence of retinal ganglion cell axons at the optic disc that results in a thinner rim of neural tissue, as well as deeper and larger optic cups. The specific molecular pathways that lead to this central optic disc feature of CODA and glaucoma are mostly unknown. We have used genetic studies of large CODA pedigrees to identify the genes that cause optic disc excavation and to investigate the biologic mechanisms of these types of optic nerve damage.

Our previous studies suggested that an enhancer mutation causes a pathological increase in *MMP19* gene expression, which, in turn, causes CODA [12]. The experiments in this report provide evidence to support this hypothesis. We show that the MMP19 protein is most abundant in the arachnoid and at the lateral edges of the optic nerve, where the lamina cribrosa is inserted into the scleral canal supports the optic disc. Here, extracellular MMP19 would have access to the structures that attach the lamina cribrosa to the scleral canal as well as the opportunity to digest and undermine this attachment. Based on in vitro luciferase expression data [12], we hypothesize that the enhancer mutation we have discovered leads to excess MMP19 production. We propose that pathologically elevated MMP19 results in an imbalance between matrix synthesis and turnover, compromising the insertion of the lamina cribrosa into the scleral canal and permitting its collapse and the deep excavated optic disc morphology that is characteristic of CODA.

More recently, several studies have suggested a possible role for the translaminar pressure gradient as a potential mechanism in cupping of the optic disc [32,33]. Translaminar pressure has been defined as intraocular pressure (IOP), prelaminar pressure, minus cerebrospinal pressure (as a surrogate for retrolaminar pressure). Cerebrospinal fluid (CSF) is produced by the choroid plexus and circulates throughout the central nervous system in the subarachnoid space [34]. CSF pressure may be transmitted to the retrolaminar space by the CSF within the subarachnoid space that surrounds the optic nerve. Consequently, the retrolaminar pressure may be modulated by the activity of protease enzymes, such as MMP19 that are produced in these retrolaminar optic nerve structures and may alter access of CSF to subarachnoid optic nerve spaces. It is possible that MMP19 may influence translaminar pressure and promote cupping.

The current study has several limitations. Although we predict that MMP19-mediated remodeling of the optic nerve head in glaucoma and CODA may undermine the insertion of the lamina cribrosa, we have not demonstrated this, and additional studies on glaucomatous and unaffected optic nerve heads are necessary. Determining specific molecular targets of MMP19 in the human optic nerve will also be important in understanding the role of MMP19 in optic nerve disease. Moreover, although MMP19 is present at the lamina cribrosa, we collected less evidence for an expression gradient at the laminar portion of the optic nerve. Finally, although MMP19 upregulation appears to be a feature of CODA, evaluating the abundance and distribution of this protein in glaucoma will be important in future studies.

In summary, primate and human studies of optic disc cupping in experimental glaucoma have demonstrated that high intraocular pressure stimulates remodeling of the lamina cribrosa and its insertion into the scleral canal that involves MMPs [35-38]. Ultimately, remodeling results in the back-bowing of the lamina cribrosa and a posterior shift of the insertion of the lamina cribrosa into the sclera. Several aspects of our studies of MMP19 suggest that MMP19 might have a key role in this process. We have demonstrated that MMP19 is localized in the extracellular spaces of the optic nerve and is most abundant at these lateral margins. Here, MMP19 is ideally positioned to undermine the lamina cribrosa insertion, permit its posterior movement, and ultimately lead to formation of a deeper optic cup. It is also possible that MMP19 activity in the retrolaminar spaces alters CSF fluid dynamics, which may increase translaminar pressure and promote cupping. This hypothesis suggests that MMP19 might also have an important role in cupping of the optic nerve in glaucoma, as well as in the pathophysiology of CODA. Additional studies of MMP19 and optic nerve morphology using animal models are needed to further test this hypothesis.

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