Laser Trabeculoplasty Induces Stromelysin Expression by Trabecular Juxtacanalicular Cells

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Purpose. The mechanism by which laser trabeculoplasty reduces elevated intraocular pressure in primary open-angle glaucoma has not been established. To test the hypothesis that trabecular extracellular matrix turnover is involved, stromelysin expression after laser treatment of anterior segment organ cultures was evaluated.

Methods. Argon laser trabeculoplasty, using typical clinical treatment parameters, was applied to anterior segment organ cultures. Stromelysin levels and activity were then evaluated at various times by immunoblots of Western transfers and by zymography. Stromelysin mRNA levels were evaluated by dot blot and by reverse transcription, followed by polymerase chain reaction amplification. Stromelysin protein was localized by immunohistochemistry, and image analysis was used for quantitation. Stromelysin mRNA was localized by in situ hybridization.

Results. Trabecular stromelysin protein, activity, and mRNA levels were detectably elevated by 8 hours and were several-fold higher by 24 hours after treatment. Stromelysin immunostaining was elevated dramatically in the juxtacanalicular and insert regions of the meshwork, but only modestly in other regions. Stromelysin mRNA increases also were localized primarily to these regions. The juxtacanalicular stromelysin immunostaining increase was sustained for at least 1 week, whereas the insert levels declined somewhat after day 2.

Conclusions. A stromelysin increase, localized primarily to the juxtacanalicular region of the meshwork, the putative site of the aqueous humor outflow resistance, should degrade trabecular proteoglycans, the putative outflow resistance source, and allow their uptake and further degradation by the juxtacanalicular cells. If diminished juxtacanalicular extracellular matrix turnover is responsible for the glaucomatous reduction in aqueous humor outflow, an increase in stromelysin in this specific area of the meshwork should ameliorate the problem. Thus, the observations support the working hypothesis and may explain the efficacy of this treatment for glaucoma. Invest Ophthalmol Vis Sci. 1996;37:795–804.

Elevated intraocular pressure, often associated with primary open-angle glaucoma, can be ameliorated by laser trabeculoplasty (LTP).1–5 Although LTP's effects are seldom permanent, its benefits can last for many years. It has been used extensively since its inception as a treatment modality.6–8 Although several hypotheses have been proposed to explain the efficacy of LTP,9–11 the actual mechanism(s) has not been established. We and others12–16 have observed an increase in trabecular cell division within the first 48 hours of treatment. In humans, most trabecular cell division is localized to the anterior portion of the meshwork, a triangular, nonfiltering region in which the meshwork "inserts" into the cornea beneath Schwalbe's line13 (see Fig. 1). After laser treatment, many of these cells divide; then, during the next week or two, the new cells migrate to the burn sites and repopulate the damaged tissue.14 In addition, we observe a shift in the glycosaminoglycan biosynthesis pattern for the first few days after laser treatment, which reverts to the normal pattern by days 7 to 10.11 We postulated that these trabecular insert cells serve as an induc-
Trabecular beams
Trabecular cells
Intratrabecular channels
Juxtacanalicular ECM & cells
Schlemm's Canal
Anterior Chamber
Cornea
Insert
Schlemm's Canal
Sclera

**FIGURE 1.** Diagrammatic radial cross-section through an idealized human aqueous humor outflow pathway. Cell nuclei are black, and the cell cytoplasm is clear; in the inset, stromal ECM has wide-spaced cross-hatching, and basement membranes have close-spaced cross-hatching. Aqueous humor must pass through the intratrabecular flow channels between the trabecular sheets and beams, through or between the deepest trabecular cells above the juxtacanalicular ECM, through the juxtacanalicular ECM, and through or between the cells lining Schlemm's canal. Approximate positions of the insert and juxtacanalicular regions of the meshwork are shown.

ible stem-like cell population and are responsible for the normal replacement of damaged trabecular cells in the remainder of the meshwork. However, the laser burns produced by standard LTP are small and are found only in the uveal or outer corneoscleral meshwork. Thus, these repopulating stem-like cells may serve a critical role in the normal trabecular meshwork, but they seem unlikely to be involved directly in the ameliorative effects of this treatment.

It has been hypothesized frequently that the normal resistance to aqueous humor outflow resides within the trabecular juxtacanalicular extracellular matrix (ECM), particularly within the glycosaminoglycan side-chains of the trabecular proteoglycans. Numerous lines of evidence support this idea, although it has not been proven. However, it is also likely, but even less clearly established, that the glaucomatous obstruction to aqueous humor outflow resides within this region and may be caused by some problem with these same proteoglycans. We have hypothesized that the regulation of trabecular ECM turnover is the key modulator of aqueous humor outflow in normal and in glaucomatous eyes.

Trabecular ECM turnover appears to be achieved, as in other tissues, by members of the matrix metalloproteinase family. Trabecular cells make and secrete interstitial collagenase, gelatinase A, gelatinase B, and stromelysin, as well as their tissue inhibitors, TIMPs. Their production by trabecular cells is tightly controlled. These proteinases cleave selective ECM components, disrupting the intricate supramolecular organization of the ECM and allowing their endocytosis and intracellular degradation. Biosynthetic replacements are then secreted and assembled extracellularly, forming new ECM. Extracellular matrix component half-lives vary considerably depending on tissue function. The trabecular proteoglycans have average half-lives of approximately 1.5 days, compared to 7 to 10 or more days for the cornea and sclera.

Stromelysin has the broadest substrate specificity of the metalloproteinase family members, degrading the globular domains of proteoglycan core proteins, laminin, fibronectin, type IV collagen, and a variety of other proteins. Thus, if the hypotheses mentioned above are all correct, stromelysin is a primary candidate for regulating trabecular aqueous outflow resistance.

To evaluate the possibility that trabecular stromelysin levels are altered by LTP and, thus, are responsible for the trabecular remodeling reported by oth-
ers27–31 after this treatment, we measured trabecular stromelysin protein, activity, and mRNA levels at various times after laser treatment. To determine where, within the meshwork, these changes might occur, we used in situ hybridization and immunohistochemistry coupled with image analysis to localize stromelysin mRNA and protein levels after laser treatment. The most reasonable compromise between conducting these experiments in cell culture and in vivo was to use the anterior segment explant organ culture system that we and others5,10,12,13,20–22,32–38 have characterized in considerable detail. This organ culture system retains the trabecular ultrastructure and morphology, mimicking many aspects of the in vivo meshwork’s behavior for several weeks.

MATERIALS AND METHODS

Culture and Treatments

Paired human anterior segment explants, comprised of the intact cornea and a 5-mm rim of sclera with the undisturbed trabecular meshwork, were cultured serum free for 1 week before treatments, as previously described.12,15,20–22 The tenets of the Declaration of Helsinki were observed, and the institutional human subjects committee approved all protocols. Laser treatments involved application of 50 uniformly spaced burns 50 μm in diameter over 180° of the anterior–central meshwork using an argon dye laser in blue–green mode (peaks at 488 and 514 nm) with a duration of 0.1 second at 0.75 W.11-13 Sham-treated explants were handled in parallel without actually applying burns. Explants were then returned to culture for the times designated.

Stromelysin Western Immunoblots and Zymograms

Conditioned culture medium was concentrated up to 20-fold and stored at -20°C until used. Western transfers of proteins from sodium dodecyl sulfate–polyacrylamide gel electrophoresis were probed with a rabbit anti-peptide antibody made to a unique sequence from human stromelysin and were peptide-affinity purified.20,21,39 Zymograms to assess stromelysin activity used 0.1% β-casein as a substrate and were conducted as described previously with undiluted culture medium and without sample reduction.20,21,39 Western immunoblots and zymograms used sample concentrations that were within the linear ranges of sensitivity.21

RNA Analysis

For dot blots, total cellular RNA was isolated40 from four dissected meshworks, pooled, and dot blotted to nitrocellulose.41 Blots were probed with stromelysin cDNA, which was 32P-radiolabeled using random primer extension, washed to high stringency, and autoradiographed.41 DNase and/or RNase treatments of aliquots of samples and parallel applications of several concentrations of stromelysin cDNA were used to establish specificity of the responses. Different dilutions of each sample were applied and probed as adjacent dots. For reverse transcription–polymerase chain reaction (RT–PCR) amplification, the total RNA (isolated as above) from single explants was reverse transcribed to cDNA for 40 minutes at 42°C with Superscript (1 U in 20 μl; Gibco/BRL, Gaithersburg, MD) and 5-μM random hexamers in 10 mM Tris (pH 8.4), 90 mM KCl, 0.2 mM each dNTP and 1 mM MnCl2. Aliquots (2 μl) of cDNA were amplified by PCR using T7I DNA polymerase (2 U in 80 μl; Epicentre, Madison, WI), 125 nM each (sense and antisense) PCR primer, 0.2 mM each dNTP, 10 mM Tris (pH 7.5), 100 mM KCl, 1.75 mM MgCl2, and 0.75 mM EGTA. Twenty-five PCR cycles (95°C, 15 seconds; 55°C, 1 minute, 72°C, 2 minutes) were used with a final 10-minute extension at 72°C. In some experiments, PCR products were radiolabeled by adding 32P-dCTP to the PCR reaction. All mRNA analysis was conducted within the linear portions of parallel dilution curves with conditions optimized for duplex amplification of the cDNAs for stromelysin and glyceraldehyde-3-phosphate dehydrogenase (which we had shown previously to be unchanged by this treatment in these cells). Polymerase chain reaction products were digested with several restriction enzymes and fragments migrated at the sizes predicted from their sequences.

Immunohistochemistry and Image Analysis

Nonserial 100-μm vibratome sections of paraformaldehyde-fixed, agarose-embedded explants were taken randomly from each quadrant of the explants. Sections were heparitinase-treated,22 washed in 1% Triton X-100 and 0.5% hydrogen peroxide, blocked, incubated with affinity-purified primary anti-stromelysin peptide antibody,20,21,39 and processed with biotinylated protein A and horseradish peroxidase-conjugated ABC Vectastain kit (Vector, Burlingame, CA). Sections were paraformaldehyde fixed again, dehydrated, flat embedded, and sectioned to 3 μm for analysis. Some were lightly counterstained with toluidine blue to provide structural orientation and cell information. Microscopic images of sections were digitized with a solid state, high-resolution color camera, which was red-green-blue-interfaced to a Vidas Image Processing System (Kontron Bindanalyse, Munich, Germany). Stromelysin immunostaining and toluidine blue cell staining were determined individually. Threshold values were established for the primary colors to discriminate areas exhibiting counterstaining from areas exhibiting immunostaining. Restrictive ranges were used for stromelysin immunostaining...
areas, essentially counting pixels above the threshold discriminator intensity level. Results are presented as immunostaining per total cellular and ECM staining to provide normalization on the basis of trabecular cells. Similar patterns were obtained when analyzed on the basis of direct immunostaining levels or when conducted on sections not counterstained. Results were verified independently by subjective intensity scoring conducted by two or more uninformed observers.

In Situ Hybridization
Vibratome sections (200 μm and otherwise as above) were delipidated and permeabilized with graded ethanol washes, rehydrated, digested with proteinase K, acetylated, prehybridized in buffer with 33% deionized formamide, and hybridized with probe overnight at 42°C. The oligonucleotide probes (antisense and sense control) were digoxigenin end-labeled with terminal transferase and identified with horseradish peroxidase conjugated to an anti-digoxigenin antibody. The nickel-diaminobenzidine reaction product was copper-H2O2 oxidized and silver intensified. Slides were postfixed and processed into JB-4 plastic, resectioned at 4 μm onto glass slides, and photographed with differential interference contrast microscopy.

RESULTS

Figure 1 shows a diagrammatic cross-section through the trabecular meshwork, allowing identification of the insert and the juxtacanalicular, corneoscleral, and uveal regions of the trabecular meshwork. The boxed inset (enlarged) shows the juxtacanalicular ECM and cells with large cross-hatched areas representing stromal ECM and small cross-hatched areas representing basement membranes adjacent to the cells. Cells are clear and have black nuclei.

Modulation of Stromelysin Enzymatic Activity and Protein Levels by Laser Wounding

Zymogram analysis of stromelysin enzymatic activity in the medium from two sets of paired eyes (Fig. 2A) showed an increase of several-fold in response to laser treatment at 24 hours. Four bands of activity were seen (exposure to sodium dodecyl sulfate activates pro-stromelysin, although minimal autolysis occurs during migration). The 62- and 60-kDa bands were, respectively, glycosylated and nonglycosylated pro-stromelysin, and the 50- and 45-kDa bands were the respective activated forms.

Stromelysin levels, assessed in the culture medium by immunoblot of Western transfers, were elevated above sham-treatment levels by 8 hours and remained high at 24 hours after laser treatment (Fig. 2B). Analysis of densitometric scans of these bands from several gels showed that the elevation averaged approximately 4-fold and 8-fold, respectively, at these two times when all four bands were included (not shown). The same four bands of stromelysin immunostaining were typically apparent (Fig. 2B); because the Western samples were reduced and the zymogram samples were not, the four bands run at slightly different molecular weights. The exact ratio of bands was different between the two types of identification because the specific activity of each was not necessarily identical to the immunostaining intensity. A significant portion of the enzyme was in the activated, mature proteinase state as assessed by both methods. Verification of the
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Modulation of Stromelysin mRNA Levels by Laser Trabeculoplasty

Stromelysin mRNA levels, assessed by probing dot blots, were significantly elevated by 8 hours and were elevated dramatically at 24 hours after LTP treatment (Fig. 3A). In pools from four paired-eye explants, the sham-treated trabecular meshwork mRNA levels were nearly undetectable at both time points. Stromelysin mRNA levels in trabecular cells from laser-treated, paired-eye explants were many-fold higher at two dilutions of the total RNA applied (Fig. 3A). DNase pretreatment had minimal effect on pooled dot density, whereas RNase pretreatment eliminated probe binding (data not shown). Within the range of these analyses, the autoradiograph was approximately linear when subjected to densitometric analysis (data not shown).

Analysis of stromelysin mRNA levels by semiquantitative RT-PCR (Fig. 3B) reinforced the dot blot observations. Analysis of individual meshworks at 8 hours or at 24 hours (Fig. 3B) showed that transcripts for the "housekeeping gene," glyceraldehyde-3-phosphate dehydrogenase, remained relatively constant, whereas stromelysin transcripts increased dramatically. Careful analysis of the conditions for duplex amplification of these two transcripts was necessary to achieve linear responses, and all bands were lighter than photographically optimum to remain within the linear ranges. Densitometric analysis showed similar results to those seen with the dot blots (not shown), although the sham-treated controls were detectable because of the degree of amplification of signal with RT-PCR.

In Situ Hybridization of Stromelysin mRNA After Laser Treatment

When laser-treated explants were probed by in situ hybridization with a stromelysin antisense oligonucleotide probe, similar stromelysin transcript level in...
FIGURE 4. Photomicrographs of stromelysin in situ hybridization at 24 hours after sham or laser treatments. (A) Sham-treated explant probed with antisense oligonucleotide to stromelysin mRNA. (B) Laser-treated explant probed with antisense strand oligonucleotide to stromelysin mRNA. S shows the position of Schlemm's canal; arrowheads show typical positively stained cells. Orientation is approximately the same as in the drawing in Figure 1; Nomarski optics were used.

creases were observed (Figs. 4A, 4B). The probe staining was cytoplasmic, and the difference between sham- and laser-treated explants was dramatic at 24 hours. In addition, the localization of stromelysin mRNA was not uniform throughout the meshwork but was constrained primarily to the juxtacanalicular and deepest corneoscleral regions (Fig. 4B) and, to some degree, to the insert regions (not shown). Sense strand controls (not shown) were similar to the sham controls (Fig 4A). In both controls, an occasional trabecular cell with positive staining could be found.

Stromelysin Immunohistochemical and Image Analysis of Localization

Immunohistochemical staining demonstrated a similar dramatic increase in juxtacanalicular and insert stromelysin immunostaining levels at several times after laser treatment (Fig. 5). The immunostaining in sham controls was low; only an occasional cell was surrounded by stromelysin in its ECM. In the LTP-treated samples, strong staining was observed in the ECM around many cells within both the insert and juxtacanalicular regions (Fig. 5), with only modest staining elsewhere within the meshwork. In the absence of counterstaining, the brown staining was more apparent (Fig. 5F), although cellular details were difficult to ascertain.

Image analysis of stromelysin immunostaining in the total meshwork showed an approximate 4-fold increase by 8 hours, an 8-fold increase by 16 hours, and sustained elevation for as long as 1 week (Fig. 6A). When this immunostaining was regionalized, insert staining (Fig. 6B) was considerably elevated for as long as 48 hours, but it declined by 1 week. Juxtacanalicular staining was still elevated more than 8-fold at 1 week (Fig. 6C). Comparison of the juxtacanalicular stromelysin immunostaining with that of the remainder of the central meshwork (uveal and corneoscleral) shows that the localization was nearly 4-fold greater at 16 hours and more than 6-fold greater at 1 week (Fig. 6D).

DISCUSSION

Using several methods to analyze trabecular stromelysin expression after standard clinical-parameter laser treatment, we found a dramatic increase in laser-treated compared to sham-treated paired-eye control explants. This increase was observed initially in both the insert and the juxtacanalicular regions of the meshwork, with modest changes in other regions. The juxtacanalicular stromelysin elevation was sustained even after the insert levels began to decline.

Using several methods of analysis provided strong evidence for the validity of these observations. Zymogram and Western immunoblot analyses demonstrated specificity by the distinctive gel migration pattern. Although it can be argued that some of this stromelysin was produced by other cells in the explant, the immunohistochemical data argue strongly against this. Dot blot and RT-PCR data also argue that the source of the stromelysin was the meshwork because this tissue was dissected before extraction of the RNA. Immunohistochemistry and in situ hybridization defined the localization. Restriction analysis of the PCR product and the placement of the primers in separate exons argued against genomic contamination or false-band amplification. RNase, but not DNase, pretreatment eliminated the dot blot binding and the RT-PCR product. The relatively constant amount of the housekeeping gene PCR product argued that similar amounts RNA were used and that PCR amplification was uniform. The various methods required careful use to maintain linearity in their quantitation, but the increases were so large that this was not a problem;
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FIGURE 5. Stromelysin immunohistochemical staining of trabecular sections from sham- and laser-treated explants. At 24 hours after sham or laser treatment of paired-eye explants, immunohistochemical analysis was conducted and final sections were photographed. The toluidine blue counterstain turned the brown stromelysin immunostaining greenish; nuclei stained dark blue, and cells and extracellular matrices stained light blue. A and B are photomicrographs of the insert region, respectively, of sham- and laser-treated paired-eye explants. C and D are higher magnification views of the same region. E and F are, respectively, sham- and laser-treated explants in which the view was through the deep corneoscleral and juxtacanalicular regions, including Schlemm’s canal. Schlemm’s canal is marked by the letters E and F, and a branch of Schlemm’s canal shows above the F. (G) Another laser-treated explant showing immunostaining in the juxtacanalicular region with Schlemm’s canal below.

in addition, dilution curves were used in parallel to define the linear ranges.

The increase in stromelysin in the insert region probably was associated with trabecular cell division, which was localized primarily to this region as we previously reported. The juxtacanalicular stromelysin increase was in accord with the structural remodeling of this region that others have reported after laser treatment. Although stromelysin was originally named proteoglycanase, it does exhibit activity against several other ECM proteins. Proteoglycan turnover was probably the most important because these ECM components are thought to provide much of the normal trabecular resistance to outflow. However, some other
ECM components may be involved in this process. The trabecular ECM biosynthetic response was likely to be important because outflow could be modulated by changing the amount of proteoglycans present in the ECM or by changing the composition or nature of these macromolecules. We presume that trabecular ECM turnover preceded the biosynthesis of replacement ECM components, but this has not been absolutely established.9,10

Two scenarios could explain why reduced ECM turnover produces reduced aqueous humor outflow facility in glaucoma9,10: reduced ability of trabecular cells to respond to the signals or feedback information by adjusting the outflow resistance; misregulation or incorrect signaling so that the trabecular cells could, but do not, maintain the appropriate outflow resistance. The existence of some type of signaling pathway to maintain this complex system is essential, although the molecular components of the actual signaling system are unknown.

We find that stromelysin remains elevated for at least 1 week after LTP. Previous microscopic analysis of trabecular ECM remodeling after LTP27–31 suggests that these changes are sustained for years. This is compatible with the clinical picture, wherein LTP often reduces intraocular pressure for from 5 to 15 years.1–6 It seems unlikely that stromelysin levels remain elevated for more than a few weeks, although we have not studied beyond 1 week. Thus, consequent ECM remodeling is probably extensive if it can produce these sustained effects.

In related studies, we recently reported46 increased levels of gelatinase B and TIMP1 expression, but not of gelatinase A or TIMP2 expression, by trabecular explants after similar laser treatments; in that study, no regional localization was determined. These observations, and the enhanced stromelysin currently reported, support the hypothesis that matrix metalloproteinases are critically involved in regulating normal aqueous humor outflow facility. Although the juxtacanalicular stromelysin increase after LTP does not prove that this is the central mechanism responsible
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for the efficacy of LTP in reducing elevated intraocular pressure, it does provide strong support for this interpretation.

Key Words

- glaucoma
- laser trabeculoplasty
- matrix metalloproteinase
- stromelysin
- trabecular extracellular matrix

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