MMP and TIMP Expression in Quiescent, Dividing, and Differentiating Human Lens Cells

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PURPOSE. Matrix metalloproteinases (MMPs) and the tissue inhibitors of the MMPs (TIMPs) have been implicated in lens differentiation, growth, remodeling, and cataract. Hence, a gene expression analysis was undertaken in epithelial and fiber cells dissected from clear human donor lenses.

METHODS. The human lens was dissected into three regions: anterior epithelial, equatorial, and fiber cells. Primary lens cell cultures were also analyzed. cDNA was generated by reverse transcription of the mRNA portion of the total RNA isolated from each sample. Gene expression data were generated using quantitative real-time reverse transcription PCR. Data were analyzed in terms of cycle threshold number (Ct) and were normalized to endogenous 18S expression. Western blot analyses were carried out to confirm the presence of two critical MMPs.

RESULTS. Anterior and equatorial samples were uncontaminated by fiber cells because they showed high expression of α-crystallin genes but low expression of β- and γ-crystallins. The fibers had high expression of these genes and of MIP. MMP genes were expressed at uniformly low levels in the native tissues except for MMP-14 and -15 (MT1- and MT2-MMP, respectively). In fact, MT1-MMP declined in expression from the anterior epithelium to fibers, whereas MT2-MMP increased. The presence of MT1 and MT2-MMP proforms and faster migrating bands, indicating processed or activated forms, was confirmed at the protein level. TIMP genes were uniformly highly expressed in native tissues, with TIMP-3 having the highest expression in the epithelial tissues and TIMP-2 in the fibers. MMP expression was generally elevated in both sets of cultured cells, including MMP-2 and -9. TIMP genes were also relatively highly expressed in the cultured cells.

CONCLUSIONS. MMP expression is generally well regulated in native tissues, with relatively low expression of MMPs and high expression of TIMPs. Membrane-type MMPs (MT1 and 2-MMPs) were the most highly expressed; this is important in a tissue with relatively high membrane content but low extracellular space. The striking reciprocal patterns of expression of MT1-MMP and MT2-MMP indicate that these enzymes are of particular significance in lens function. (Invest Ophthalmol Vis Sci. 2007;48:4192–4199) DOI:10.1167/iovs.06-1371

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The lens is an ideal organ in which to study gene expression because it can be removed from the eye intact and without the complication of attendant nerve and blood supplies. In addition, constituent epithelia and fiber cells are arranged in a regular and accessible manner. The anterior epithelium, which faces the aqueous humor, represents a quiescent, nondividing population in the adult lens, and the cell division necessary to give rise to new fibers occurs strictly in the equatorial region. Newly formed fibers undergo no further cell division; rather, they elongate to form some of the longest cells in the body (for a review, see Forrest et al.3). Ultimately, in the fully differentiated form, they lose their cell organelles. In this study, we chose to investigate the gene expression of the matrix metalloproteinases (MMP) and the tissue inhibitors of the matrix metalloproteinases (TIMP) because not only are they thought to play a role in differentiation,2 they have roles to play in the development of cataract2,3 and posterior capsule opacification (PCO).8

Collectively, the MMPs form a family of zinc-dependent endopeptidases involved in the degradation of extracellular matrix (ECM) to participate in the remodeling of their cellular environment. These enzymes are classified into groups associated with their substrate specificity and structural biology. The collagenses, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others generally consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain and can be secreted from the cell or anchored at the cell surface. MMPs are specifically regulated at many levels (for reviews, see Woessner6 and Vosse and Nagase7).

Of particular interest in the lens are the gelatinases A and B (MMP-2 and -9), which are involved in corneal wound-healing processes8 and disorders.9 Specifically, they play different roles, MMP-9 in the early stages of wounding10 and MMP-2 in the ensuing ECM remodeling. MMP-2 and -9 are both present in aqueous and vitreous humors.11,12 In the normal lens, MMP-213 and -9 are expressed at low levels, and both are increased when the lens is stressed.3,4 Endogenously, MMPs are regulated by their inhibitors, the TIMPs, which control enzyme activity and proenzyme activation.11

It will be shown here that members of the membrane-type MMP (MT-MMP) subfamily became a particular focus for this study. Six have been identified to date.13,15–17 All are anchored to the cell surface, extending an extracellular catalytic domain through a transmembrane domain (MT1, MT2, MT3, and MT5-type MMPs also referred to as MMP-14, -15, -16, and -24, respectively) or a glycosphingolipidinositol link (MT4 and MT6-MMP, also referred to as MMP-17 and -25, respectively). Additionally, MT5-MMP species are shed from the cell surface.16

The MT-MMPs process proMMPs in the pericellular environment (proMMP-2) and degrade ECM components and cell surface molecules (for a review, see Noel et al.20). MT1-MMP is thought to be the main MT-MMP involved in the processing of proMMP-2 to activation when in complex with TIMP-2 and αvβ3 integrin,21–23 and indeed MT1-MMP has been detected in the normal human lens.15 MT2-MMP, however, activates proMMP-2 in a TIMP-2-independent manner.24

Lens fiber cells are continuously formed by differentiation of the equatorial epithelial cells. Differentiating cells withdraw
from the cell cycle and specialize by synthesizing lens crystallins. The activity of the MMPs and TIMPs has been implicated in normal and pathologic lens growth, development, and remodeling. MMP substrates include molecules involved in lens fiber migration and differentiation, such as cytokines, cell adhesion molecules, and growth factors.25–27 Cataract and PCO are major causes of visual acuity loss and blindness worldwide.28 Gene expression of the MMP and TIMP families was, therefore, investigated in regions of the normal adult human lens using quantitative real-time PCR as a prelude to a cataract study. Given these observations, we chose to investigate further some important MMPs by analyzing protein expression with the use of Western blotting techniques. To provide adequate quantities of starting material, previous gene expression studies used sample pools obtained from multiple donors by an experienced observer according to the Lens Opacification Classification System (LOCS).28–30 Because of the difficult nature of this assessment and the fact that many cataracts have, for example, nuclear and cortical involvement, it is important to study the properties of individual cataractous lenses.31 The purpose of this study was to analyze individual lenses. Hence, we developed the necessary techniques for gene expression analyses in small samples from normal, clear lenses to analyze expression patterns in different regions of the same lens.

**METHODS**

**Donor Lenses and Culture-Derived Cells**

Human donor eyes were obtained with full ethical permission (REC 94/Q0102/57) from the East Anglia Eye Bank or the Corneal Transplant Service (Eye Bank, Bristol, UK) after the cornea had been removed for transplantation purposes. Human donor lenses in this study were used in accordance with the tenets of the Declaration of Helsinki. All eyes were stored in individual sterile pots in an antibiotic wash medium before use. Dissection and primary culture methods have been described in detail elsewhere.32,33 For regional analyses, human donor eyes were obtained from the East Anglia Eye Bank (n = 6; age range, 55–77 years; mean, 67.5 years) less than 24-hours postmortem, and the lenses were dissected into three regions: anterior epithelium, equator, and fibers. The iris was removed, and a circular anterior epithelium was torn away. The lens nucleus was then released by hydrodissection, and residual fibers were sampled after careful removal using forceps. Finally, the lens capsule was dissected from the zonules. Each sample was immediately snap-frozen in liquid nitrogen.

Primary cultures generated from combined anterior and equatorial epithelial cells were investigated as a growth and remodeling system. For primary cell culture, isolated human donor lenses were obtained from the Corneal Transplant Service (Eye Bank, Bristol, UK). The lenses were transported in individual tubes containing sterile Eagle minimal essential medium (EMEM; Sigma, Dorset, UK). The center of the cell-free posterior capsule was punctured, and an incision was made across the diameter of the posterior capsule. Entomology pins (D1; Watkins and Doncaster Ltd., Kent, UK) were inserted at the edges of the capsule to secure the capsule at either end of the incision. Small cuts were then made in the capsule, near the pins, so that most of the posterior capsule could be removed with two curvilinear tears. The remaining capsule (anterior and equatorial regions) was then further secured with six additional pins, and the major fiber mass and residual fibers were carefully removed with forceps. Explants were cultured in EMEM for 24 hours and split into two halves, again pinned to the culture dish and cultured in the presence of 10% fetal calf serum (FCS; Gibco, Paisley, UK) for 1 to 2 weeks. Explants were then removed from culture dishes, and epithelial cells were left to grow to confluence in the presence of serum.

**MMP and TIMP Expression in Human Lens Cells**

**Total RNA Extraction and cDNA Generation**

Total RNA was extracted from tissue and primary cultured cells (RNeasy micro kits; Qiagen, West Sussex, UK) in accordance with the manufacturer’s instructions. In the initial step, RLT buffer (containing β-mercaptoethanol) was added to Eppendorf tubes containing snap-frozen tissues, or they were added directly to PBS-washed culture monolayers. Cultured cell lysates were removed with a cell scraper. Frozen tissue was homogenized with an Eppendorf homogenizer, and both sample sets were then passed through a needle and syringe. The remainder of the protocol was as described by the manufacturer and included a DNase step. Quality control was maintained with an RNA analyzer (Bioanalyser 2100; Agilent, West Lothian, UK) and an RNA lab chip (6000 Nano lab chip; Agilent) to ensure that 28S and 18S rRNA bands were clearly evident in total RNA samples. RNA was quantified with a spectrophotometer (ND-1000; NanoDrop, Wilmington, DE). For the 18 samples analyzed, the 260/280 ratio ranged from 1.8 to 2.2 (mean, 2.0). Where possible, total RNA was immediately used for cDNA generation or was briefly stored at −80°C. Generation of cDNA was performed with reverse transcriptase (Superscript II; Invitrogen, Paisley, UK) and random primers (Promega, Southampton, UK), according to standard protocols.

**Real-Time PCR**

Quantitative real-time PCR was used to analyze mRNA expression for all genes in native tissue and culture-derived cells (for a comprehensive description of MMPs and TIMPs primer/probe sets used, see Nuttall et al.34). Predesigned gene expression assays were purchased (TaqMan; Applied Biosystems, Foster City, CA) for the following genes: **MIP** (NCBI reference sequence [RefSeq] at the time of publication; NM_012064.1), γ-A-crystallin (NM_014617.2), β1-crystallin (NM_005208.3), α-crystallin (NM_000594.2), and αB-crystallin (NM_001885.1). Assuming 100% efficiency in the reverse transcription reaction, either 1 ng or 5 ng cDNA was used in real-time PCR reactions performed using a real-time PCR machine (ABI7700; Applied Biosystems). Reagent-based assays (TaqMan Universal PCR Master Mix; No AmpErase UNG; Applied Biosystems) with all PCR reagents were used according to the manufacturer’s instructions. The amount of amplification associated with priming from genomic DNA contamination was evaluated with control reverse transcription reactions containing all reagents without reverse transcriptase. Conditions for the PCR reaction were 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles, each consisting of 15 seconds at 95°C and 1 minute at 60°C. The cycle number at which amplification entered the exponential phase (cycle threshold [Ct]) was determined, and this number was used as an indicator for the amount of target RNA in each tissue analyzed. To determine the relative RNA levels in the samples, standard curves for each primer/probe set were prepared by taking cDNA from one sample and making twofold serial dilutions covering the range equivalent to 20 ng to 0.625 ng RNA (for 18S analysis, the range was 1 ng to 0.03125 ng). Differences in the total amount of RNA present in each sample were normalized to endogenous 18S ribosomal RNA gene expression, as previously described.34

**Protein Extraction, Quantification, and Western Blotting**

Each experiment was performed at least three times. Frozen tissues were incubated at 4°C for 10 minutes in buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/mL aprotinin.35 Samples were homogenized with an Eppendorf homogenizer, followed by passage through needle and syringe, and then were further incubated at 4°C for 10 minutes. Lysates were precleared by centrifugation at 13,000 rpm at 4°C for 10 minutes, and the protein content of the soluble fraction was assayed by a bicinchoninic acid protein assay (Pierce, Rockford, IL). Detergent-insoluble fractions were produced by solubilizing pelleted material in reducing sample loading.
buffer containing 10% (wt/vol) SDS, 20% glycerol, 0.1% (wt/vol) bromophenol blue, 125 mM Tris-HCl, pH 6.8, and 5% β-mercaptoethanol. Equal amounts of reduced protein (100 μg) per sample were loaded onto 10% SDS-PAGE gels for electrophoresis and transfer to polyvinylidene difluoride (PVDF) membrane (NEN Life Science Products, Boston, MA) with a semidry transfer cell (Trans-Blot; Bio-Rad, Herts, UK). Proteins were detected with a chemiluminescent blot analysis system (ECL; Amersham Biosciences, Amersham, UK) with primary antibodies against MT1-MMP (AB8102; Chemicon, Temecula, CA) and MT2-MMP (M4442 [Sigma] and MAB3320 [Chemicon]).

Statistical Analysis
When statistical analysis was performed, one-way ANOVA with Tukey post hoc analysis was used to confirm a significant difference in the data between lens regions.

RESULTS

Distribution of Lens-Specific Gene Expression in Lens Regions
It was important to demonstrate that the dissected regions of the lens contained relatively pure populations of cells and, in particular, that the anterior and equatorial cells were not contaminated with fiber remnants. The β- and γ-crystallins are the major soluble components of the fibers. Indeed, PCR analysis of the different regions corresponded with the expected distributions (Fig. 1). In particular, γA-crystallin gene expression was absent from the epithelial cell populations, in concurrence with Wang et al.,36 because this protein is produced relatively late in fiber cell differentiation.37 Similarly, the expression of the major intrinsic protein (MIP), which was largely absent from the epithelial populations, was characteristically associated with fiber cells. Human lens epithelial cells contain little or no αA crystallin but a significant amount of αB-crystallin.36 Again, this is borne out by the present PCR data shown in Figure 1. Next, MMP and TIMP expression were investigated in detail.

Distribution of MMP and TIMP Gene Expression in Lens Regions Determined by CT Analysis
Figure 2 demonstrates MMP and TIMP gene expression data prepared from six donors and primary epithelial cultures derived from four donors. The CT value (see Methods) was used to classify gene expression as very high (CT ≤ 20), high (21–25), moderate (26–30), low (31–38), or absent.
Absent or low levels of MMP gene expression were determined in the three lens regions, except for the membrane-type MMPs—MT1-MMP (MMP-14) and MT2-MMP (MMP-15). There was low expression of gelatinase A (MMP-2) and negligible gelatinase B (MMP-9). Generally, regional MMP expression levels occurred in the order of anterior cells > equator cells > fiber cells. However, though MT1-MMP expression declined from anterior to fiber cells, MT2-MMP expression increased. Relatively high levels of the four inhibitory TIMPs were maintained throughout; the lowest in abundance was TIMP-4.

\( C_T \) Analysis of MMP and TIMP Gene Expression in Cultured Primary Lens Cells

A different picture emerges after primary cell culture. Upregulation of several MMPs was apparent, including the gelatinases (MMP-2, -9) and the stromelysins (MMP-3, -10, -11). Of the collagenases, MMP-1 was high but -8 and -13 were low. MMP-19 and MT5-MMP (MMP-24) were also elevated. Upregulation of TIMP-1 gene expression occurred in primary culture.

Distribution of MMP and TIMP Gene Expression in Lens Regions and Cultured Cells Using the Relative Standard Curve Method

A more quantitative analysis of these data was obtained by expressing the data normalized with respect to the level of 18S message (Fig. 3). Of the MMPs, MMP-14 and -15 showed the greatest levels of expression, with MMP-23 and -24 at a much lower level. In primary cultured cells, MMP-2, -9, -10, -23, and -24 were expressed at higher levels than those observed in native cells, whereas MMP-14 and -15 were expressed at levels

\( C_T \) was used to classify gene expression as either very high (\( C_T \leq 20 \)), high (\( 21 < C_T < 25 \)), moderate (\( 26 < C_T < 30 \)), low (\( 31 < C_T < 38 \)), or absent (\( 39 < C_T < 40 \)).
similar to those observed in the native cells. Regarding inhibitors, TIMP-3 and -2 were the most highly expressed. In native anterior and equatorial cells, the relative expression of TIMPs followed the sequence $3 > 2 > 4 > 1$; this was similar to the sequence determined in primary cultured cells. In fibers, the sequence was $2 > 3 > 4 = 1$.

**Protein Expression of MT1-MMP in the Normal Human Lens**

Two bands were detected on Western blot analysis of extracts from anterior epithelial (AE), equatorial (E), and fiber (F) protein fractions using an antibody that recognizes the catalytic domain of human MT1-MMP (Fig. 4). These bands likely corresponded to the previously reported pro- and active forms at 63 kDa and 60 kDa, as judged from the migration of a recombinant MT1-MMP standard representing the human prodomain, catalytic domain, and hemopexin domain (CC1043; Chemicon).

**FIGURE 3.** MMP and TIMP gene expression in each of the three regions of the lens. The $x$-axis represents the gene of interest/18S expression expressed as mean $\pm$ SEM. Data were analyzed by one-way ANOVA and Tukey post hoc analysis. Significance was determined at $P \leq 0.05$ (*) in that region compared with the other two regions and with the region indicated.

**FIGURE 4.** Detection of MT1-MMP in anterior epithelial (AE), equatorial (E), and fiber protein fractions using anti-MMP14 catalytic domain. Fiber fractions were electrophoresed along side control human MMP-14 prodomain, catalytic domain, and hemopexin domain with a molecular weight of 58 kDa. Bands representing proprotein and activated enzyme were running at approximately 63 kDa and 60 kDa, respectively.
high throughout. When C regulation of several of the MMPs. On the other hand, with the MMPs in native cells, whereas primary cultures predicted up-expression relative to the other TIMPS. expression, in which curve analysis resulted in lowering of threshold number, used here, the data could be expressed in terms of cycle gent-soluble fiber fractions, however, little proenzyme or ac- and prominent activation/breakdown products at 48 kDa seen when the enzyme was fully activated, as described by the antibody manufac- turer. Marker lane (M) sizing is indicated (kDa).

DISCUSSION

Several methods have been used for illustrating gene expres- sion in a wide range of tissues. In the fluorometric method used here, the data could be expressed in terms of cycle threshold number, \( C_T \), and as relative RNA expression in terms of a standard housekeeping gene such as \( GAPDH \) or \( 18S \). \( C_T \) values indicated low or very low expression for most of the MMPs in native cells, whereas primary cultures predicted up-regulation of several of the MMPs. On the other hand, with the \( C_T \) method, TIMP expression (of at least 1–3) was uniformly high throughout. When \( C_T \) values were analyzed by the standard curve method and normalized to 18S, it was possible to conduct a more quantitative analysis of the relative expression across the different tissues. The resultant pattern was, however, similar to that obtained for \( C_T \) analysis, except for TIMP-1 expression, in which curve analysis resulted in lowering of expression relative to the other TIMPS.

Regardless of which method is used, it is important to have well-characterized tissues from which to generate data. The present study demonstrated that it was possible to obtain reproducible data from single lenses when they are dissected into three regions (Figs. 1–5). Furthermore, several studies from this laboratory and others (for a review, see Duncan et al.\(^{39} \)) have emphasized the multifactorial nature of cataract and have demonstrated the importance of analyzing individual, MMP and TIMP Expression in Human Lens Cells

Protein Expression of MT2-MMP in the Normal Human Lens

Detection of MT2-MMP is shown in Figure 5. In detergent-soluble protein fractions prepared from all lens regions, the proenzyme was shown at 72 kDa, activated enzyme at 55 kDa, and activation/breakdown products at 48 kDa. These were the most predominant, as described by the manufacturer. In deter- gent-soluble fiber fractions, however, little proenzyme or ac- active enzyme banding was evident. Smaller bands were seen when MT2-MMP was detected in soluble fractions. These lower molecular weight bands were likely to have been MT2-MMP breakdown products, which could have included the hemopexin domain, the transmembrane region, the cytoplasmic domain, or various combinations of the three. Activated MT2-MMP at 55 kDa was determined in detergent-insoluble protein fractions.
that altered fibroblasts in areas of degeneration behind pterygia constitute a type of noninvasive tumor (stimulated cell growth).47

A unique aspect of this study has been the ability to assess the relative intensity of MMP/TIMP expression in different regions of the lens. The most significant finding has been the relative downregulation of expression in MT1-MMP and the concomitant upregulation of MT2-MMP in the transition from quiescent epithelia to differentiated fiber cells. Although the upregulation of MT2-MMP is reportedly associated with an increase in growth drive,47 no reports have been published of its association with differentiation. One report states that MT2-MMP acts as an antiapoptotic factor in cancer cells.48 It is interesting that fiber cells ultimately lose contact with the basement membrane capsule; normally, epithelia-derived cells undergo apoptosis when this occurs. Perhaps one role of MT2-MMP is to inhibit cell death in elongating nucleated fiber cells. One potential substrate for MT2-MMP in the migratory equatorial epithelia and fiber cells of the lens could be laminin49 because these cells express α5β1 integrin, which is the putative laminin receptor for lens fiber cells.50–52 The Western blot analysis carried out to support the PCR data indicated that not only is the MT2-MMP protein expressed in fiber cells, it is also processed and activated.

There is a long history of drugs initially developed to treat one or more ocular conditions that are later found to carry an increased risk for cataract formation.53 It is, therefore, worth mentioning that latanoprost, which has proved useful in reducing intraocular pressure, also increases MMP-1 expression while it downregulates MT-MMPs in trabecular meshwork and in ciliary body cells.54,55 No study of the effect of latanoprost on MT-MMP expression in lens cells has yet been carried out.

In conclusion, this is the first study to report a global analysis of MMP and TIMP family members in the lens. Interestingly, relatively high expression of the membrane-type MMPs was observed throughout the human lens and showed regional differences. The striking reciprocal patterns of expression of MT1-MMP and MT2-MMP reported here indicate that these enzymes are of particular significance in lens function.

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