

Overexpression of matrix metalloproteinase-2 mediates phenotypic transformation of lens epithelial cells

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Transforming growth factor- β (TGF- β) is known to be a causative factor in pathological fibrosis and the metastasis of cancer cells, through effects on molecules of the extracellular matrix (ECM). We evaluated the influence of TGF- β_1 on the gene expression of matrix metalloproteinase-2 (MMP-2) in lens epithelial cells (LECs). The results showed that TGF- β_1 induced the expression of mRNA for MMP-2 in LECs. Subsequently, in order to examine the role of MMP-2, we overexpressed MMP-2 in LECs by stable transfection. The MMP-2-overexpressing LECs showed typical indicators of a myofibroblast-like cell

phenotype, such as multiple layers of cells, elongated morphology, and expression of α -smooth muscle actin. We also showed that an MMP inhibitor blocked the TGF- β_1 -induced morphological change in LECs. These results demonstrate that MMP-2 plays a role in the transformation of LECs, which has implications for the pathological fibrosis of these cells.

Key words: cataract, epithelial-to-mesenchymal transformation, TGF- β_1 , transformation.

INTRODUCTION

Pathological fibrosis in various tissues involves modulation of the interaction between the cell and molecules of the extracellular matrix (ECM) [1,2]. Transforming growth factor- β (TGF- β) has been implicated as a strong inducer of fibrotic diseases in many cell types [3], and has been proposed as one of the most well characterized cytokines driving the transformation and pathological fibrosis of epithelial cells by modulating components of the ECM [4–6].

When a monolayer epithelium is transformed, the cells exhibit a bipolar and elongated shape, pseudopodia, microfilaments and enriched rough endoplasmic reticulum, which is similar to epithelial-to-mesenchymal transformation [7]. Loss of epithelial polarity and cell multi-layering are well known signs of pathogenesis, especially in oncogenesis, renal dysfunction and cataractogenesis, since alterations in cellular structure lead to cell and organ dysfunction [8–10].

The pathological remodelling of the ECM suggests that there may be alterations in the synthesis and/or degradation of the matrix proteins [4]. The matrix metalloproteinases (MMPs) form a family of structurally related metalloendopeptidases that are capable of degrading the different macromolecular components of the ECM. MMPs play a major role in normal tissue remodelling processes, such as embryonic development, bone growth and resorption, ovulation, uterine involution and wound healing [11]. On the other hand, abnormal expression of these proteinases is known to contribute to a variety of pathological processes, such as rheumatoid arthritis, atherosclerosis, pulmonary emphysema, and tumour invasion and metastasis [12]. At present, the MMP family can be classified into four different

groups: collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). The expression of MMPs is modulated by cytokines or growth factors in numerous cell types [13–17].

Anterior subcapsular cataract is a fibrosis-induced disease caused by the accumulation of ECM molecules and the transformation of lens epithelial cells (LECs) [18,19]. With regard to the roles of MMPs in ECM degradation, we hypothesized that MMPs play a crucial role in the TGF- β -mediated transformation of LECs by abnormal matrix degradation. Among the known MMPs, we focused on MMP-2 in our study, because the basal lamina of LECs is composed primarily of collagen type IV and laminin, which are known substrates for MMP-2 [20]. A few studies have been reported on the role of MMPs in lens pathology. Richiert and Ireland [21] and Tamiya et al. [22] showed that the secretion of MMP-2 and MMP-9 was stimulated by TGF- β or hydrogen peroxide in LECs. However, neither the gene expression of MMPs nor the effects of such expression in LECs has been examined. Thus the objectives of the present study were to examine the regulation of MMP-2 by TGF- β_1 in cultured LECs, and to determine specifically the role of MMP-2 in the transformation of LECs leading to fibrosis in these cells.

MATERIALS AND METHODS

Cell culture

Human LEC line HLE B-3 was kindly provided by Dr Usha Andley (University of Southern California Keck School of Medicine, Los Angeles, CA, U.S.A.) [23]. Cultures were maintained in minimum essential medium (Sigma, St. Louis, MO,

Abbreviations used: APMA, 4-aminophenyl mercuric acetate; ECM, extracellular matrix; LEC, lens epithelial cell; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; RT-PCR, reverse transcription-PCR; α -SMA, α -smooth muscle actin; TGF- β , transforming growth factor- β ; TIMP, tissue inhibitor of metalloproteinases; TNF- α , tumour necrosis factor- α .

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U.S.A.) containing 20% (v/v) fetal bovine serum (GIBCO, Gaithersburg, MD, U.S.A.) at 37 °C in a humidified 5% CO₂ atmosphere.

Reverse transcription-PCR (RT-PCR)

Total RNA was isolated by using TRIZOL reagent (GIBCO). A sample of 1 µg of RNA was reverse-transcribed and subjected to PCR. The primer sequences specific for the genes examined and predicted product sizes were as follows: β -actin (350 bp), 5'-AGGCCAACCGCGAAGATTGACC-3' (sense) and 5'-GAAGTCCAGGGCGACGTAGCAC-3' (antisense); MMP-2 (969 bp), 5'-TATGGCTTCTGCCCTGAGAC-3' (sense) and 5'-TCCAAACTTCACGCTCTTCA-3' (antisense); tissue inhibitor of metalloproteinases-2 (TIMP-2) (353 bp), 5'-AGCACCACCAGAAGAAGAG-3' (sense) and 5'-CCAGGAAGGGATGTCAGAG-3' (antisense); MT1-MMP (642 bp), 5'-AACATTGGAGGACACCCA-3' (sense) and 5'-CCAGAAGAGAGCAGCATCAA-3' (antisense). All primer sequences (except those for β -actin) were designed by using Primer 3 primer picking software through the web site (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>) run by the Center for Genome Research at the Whitehead Institute for Biomedical Research (Cambridge, MA, U.S.A.).

Northern blot analysis

Total RNA (10 µg/lane) was electrophoresed on a 1% (w/v) agarose gel containing formaldehyde, transferred by capillary action to a nitrocellulose membrane (Optitrans BA-S; Schleicher & Schuell, Dassel, Germany) using a TurboBlotter transfer system (Schleicher & Schuell), and fixed by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA, U.S.A.). cDNA probes (25 ng) for MMP-2 and β -actin were labelled with 50 µCi of [α -³²P]dCTP (Amersham, Cleveland, OH, U.S.A.) by random priming (Random primed DNA labeling kit; Boehringer Mannheim). Blots were prehybridized at 42 °C for 3 h, and then hybridized in 50% formamide, 5 × SSPE [1 × SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 10 × Denhardt's solution (1 × Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA) and 0.5% SDS with [³²P]dCTP-labelled probes for 16 h. Blots were then washed with 0.2 × SSC/0.1% SDS (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) for 1 h at 65 °C and exposed to autoradiographic film. Equal loading was assessed by hybridization with a β -actin probe. A human MMP-2 cDNA clone was generously provided by Dr Gregory I. Goldberg (Washington University School of Medicine, St. Louis, MO, U.S.A.).

Western blot analysis

Culture supernatants of B-3 cells were centrifuged at 2000 g for 10 min to remove cell debris. The cells were extracted with cell lysis buffer (20 mM Tris/HCl, pH 7.5, containing 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 10% glycerol, 1 mM PMSF, 5 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin), and the same amount of protein was subjected to SDS/10% -PAGE and transferred to a nitrocellulose membrane (Hybond; Amersham). The membranes were blocked for 1 h in PBS/Tween containing 5% (w/v) non-fat milk powder, and incubated with primary antibody [1:1000 for α -smooth muscle actin (α -SMA); Sigma] in the blocking buffer for 45 min, and then washed in PBS/Tween. Blots were then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated anti-mouse antibody (Amersham) at room temperature for 45 min. The

proteins were visualized by using ECL detection reagents (Amersham).

Gelatin zymography

The MMP-2 activity of cell culture supernatants was determined as described previously [24]. Briefly, medium was centrifuged at 2000 g for 10 min to remove cell debris, and concentrated approx. 5-fold using a Centricon-10 instrument (Millipore, Bedford, MA, U.S.A.). Aliquots of 20 µl were applied to a non-reducing SDS/10% -polyacrylamide gel containing 0.1 mg/ml denatured gelatin. After electrophoresis, the gels were washed to remove SDS and incubated in an activating buffer (50 mM Tris/HCl, pH 7.5, 10 mM CaCl₂ and 1 µM ZnCl₂) for 18 h at 37 °C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% (v/v) ethanol/10% (v/v) acetic acid. 4-Aminophenyl mercuric acetate (APMA)-treated MMP-2 (Oncogene; cat. no. PF023) was used as a control for active MMP-2. The zone of enzymic activity was identified as a clear band on a blue background.

Lens organ culture and immunohistochemistry

Rat whole lenses were cultured as reported previously [25]. Briefly, lenses were dissected from 3-week-old Sprague-Dawley rats and incubated in Medium 199 (Sigma) containing 0.1% BSA and 1% penicillin/streptomycin. TGF- β ₁ was added to the medium at 10 ng/ml, and medium was changed every 2 days without further addition of TGF- β ₁. Control lenses were cultured in the absence of TGF- β ₁. After 5 days, the lenses were fixed in Carnoy's fixative (acetic acid/ethanol) and embedded in paraffin. The paraffin-embedded lenses were sectioned on a microtome at a thickness of 5 µm. The lens sections were incubated in 2% H₂O₂ for 5 min, in 20% (v/v) normal horse serum for 10 min, and in a 1:200 or 1:500 dilution of mouse anti-SMA (Sigma) for 2 h. The sections were then incubated in biotinylated anti-goat or anti-mouse IgG (Amersham) for 10 min, and visualized according to the manufacturer's protocol (UltraTek Streptavidin/Peroxidase Label; ScyTek Laboratories, Logan, UT, U.S.A.). The immunolabelled sections were counter-stained with haematoxylin.

Stable transfection

Human MMP-2 cDNA (obtained from Dr Gregory I. Goldberg) was double-digested with *EcoRV* and *NotI*, and then inserted into *EcoRV/NotI*-digested pIRES-neo vector (Clontech, Palo Alto, CA, U.S.A.). A sample of 3 µg of DNA was used to transfect 2 × 10⁶ HLE B-3 cells using the lipofectin transfection method according to the manufacturer's protocol (Gibco BRL). At 3 days after transfection, medium containing 500 µg/ml Geneticin (G418; Gibco BRL) was applied to select the transfectants. More than 60 Geneticin-resistant colonies were obtained from 2 × 10⁶ HLE B3 cells. Of these, 31 colonies were selected; the remaining cells were pooled after colony selection. The selected colonies were grown and expanded for further experiments, and maintained in medium containing 100 µg/ml Geneticin.

Electron microscopy

The transfected cells were grown on plastic tissue culture plates, and were fixed in 0.1 M sodium phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for 1 h. After washing with the same buffer, the samples were fixed in 1% osmium tetroxide for 10 min. The samples were washed and dehydrated using a series of ethanol, then subsequently cleared in propylene oxide and

soaked in 1:1 (v/v) mixture of ethyl alcohol and epon, followed by pure epon. The samples were embedded on pure epon, and incubated at 60 °C overnight. The semi-thin sectioned cells were observed with a light microscope after staining with Toluidine Blue. The cells for electron microscopy (Hitachi H-600 instrument) were sectioned at 90 nm by an ultramicrotome, and observed after staining with uranyl acetate for 30 min and lead citrate for 3 min.

RESULTS AND DISCUSSION

Expression of MMP mRNAs following treatment with TGF- β_1

Previous reports have shown that MMPs are regulated by various cytokines in different tissues [13–17]. The basal lamina of LECs is composed primarily of collagen type IV and laminin, which are the substrates for MMP-2 [20]. Thus we evaluated the expression of MMP-2 in response to various cytokines, namely interferon- γ , interleukin-1 α , TGF- β_1 , tumour necrosis factor- α (TNF- α) and fibroblast growth factor-2. We treated human LECs (HLE B-3) with the indicated cytokines (10 ng/ml each), and then examined the expression of MMP-2 mRNA using RT-PCR analysis. The results showed that the steady-state level of MMP-2 mRNA was increased by TGF- β_1 only (Figure 1A).

To examine the effects of TGF- β_1 on MMP-2 expression in more detail, we tested MMP-2 mRNA expression as a dose- and a time-dependent response to TGF- β_1 . Figure 1(B) indicates that the level of MMP-2 mRNA peaked at 10 ng/ml TGF- β_1 , and Northern blot analysis (Figure 1C) demonstrated that the expression of MMP-2 transcript increased in a time-dependent manner up to 12 h. The plateau of mRNA persisted till 36 h and had started to decrease at 48 h of treatment of TGF- β_1 . Subsequently we performed a zymography experiment in order to examine the pattern of expression of MMP-2 protein. HLE B-3 cells were incubated in the absence or presence of TGF- β_1 (10 ng/ml), and the accumulative culture media were collected at the indicated time points after treatment. Control samples were run in parallel with the TGF- β_1 -treated samples. Figure 1(D) shows that the secretion of pro-MMP-2 and active MMP-2 was enhanced by treatment with TGF- β_1 . These results are consistent with those of others [21], who reported that the secretion of pro-MMP-2 and active MMP-2 was stimulated by TGF- β . We did not observe any activity of 80 kDa or 92 kDa gelatinase, unlike Richiert and Ireland [21]. We used a human LEC line for the present study; in contrast, Richiert and Ireland used a primary culture of chicken annular pad cells [21]. Even though the differences in MMP subtypes exist between organisms, MMP-2 expression in response to TGF- β seems to be similar in human and chicken.

We also examined whether the regulatory factors believed to be involved in the activation of pro-MMP-2 are affected by TGF- β_1 , since the activation of pro-MMP-2 is tightly regulated by two other factors, TIMP-2 (an inhibitor of MMP-2) and MT1-MMP (an activator of MMP-2) [26,27]. Neither the level of mRNA for TIMP-2 nor that for MT1-MMP was affected by TGF- β_1 during the same period of treatment (Figure 1E). Thus the expression of TIMP-2 and MT1-MMP seemed to be constitutive in the presence or absence of TGF- β_1 in HLE B-3 cells. From the results shown in Figure 1, we conclude that the secretion of both pro-MMP-2 and active MMP-2 was increased by TGF- β , but that the level of expression of other regulatory factors for MMP-2 was not changed. If the constant levels of MT1-MMP and TIMP-2 mRNAs translate into constitutive protein expression, the mechanisms underlying the small degree of activation of MMP-2 in TGF- β_1 -treated cells remain to be explained. Nevertheless, the activation of MMP-2 evident in

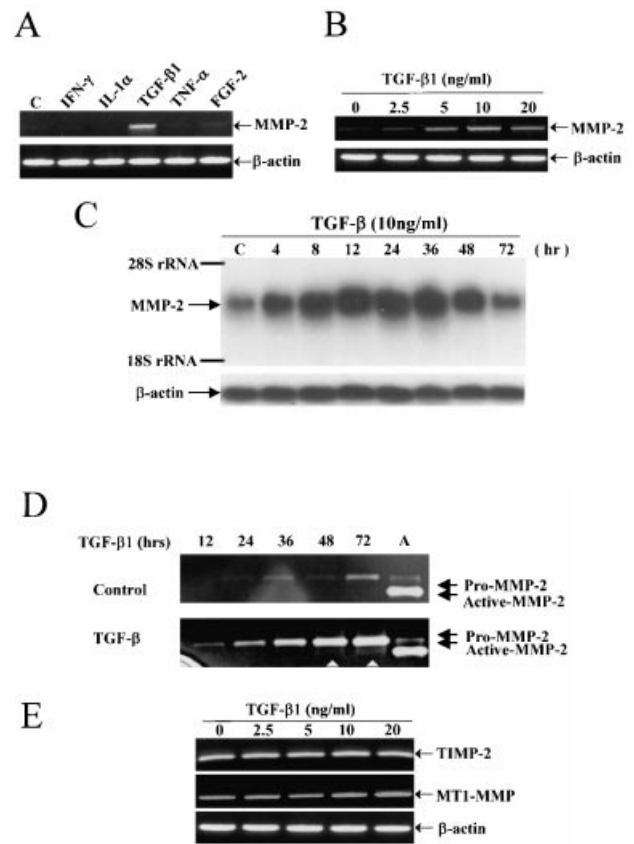


Figure 1 TGF- β_1 induces the expression of MMP-2 in LECs

(A) B-3 human LECs were seeded at 3×10^5 cells/well in collagen IV-coated 6-well plates. Following incubation for 16 h in serum-free minimum essential medium, the cultures were treated with or without cytokines [interferon- γ (IFN- γ), interleukin-1 α (IL-1 α), TNF- α , TGF- β_1 or fibroblast growth factor-2 (FGF-2)] at a concentration of 10 ng/ml for each cytokine. Total cellular RNA was isolated 24 h after cytokine treatment and RT-PCR was performed. (B) RT-PCR analysis of MMP-2 mRNA levels in the presence of TGF- β_1 : dose-response study. LECs were treated with various concentrations of TGF- β_1 for 24 h, and then total cellular RNA was isolated and subjected to RT-PCR analysis. (C) Effect of time on the expression of MMP-2 in LECs induced by 10 ng/ml TGF- β_1 . Total cellular RNA was isolated after the indicated times and subjected to Northern blot analysis. (D) Zymography experiment for MMP-2 activity. B-3 cells were incubated in the absence or presence of TGF- β_1 . The accumulative culture media were collected at the indicated time points after treatment with TGF- β_1 , and concentrated 5-fold using a Centricon-10 concentrator. The samples were subjected to 10%-PAGE in the presence of 0.1 mg/ml denatured gelatin. Following electrophoresis, the gels were washed to remove SDS and incubated in activation buffer for 18 h at 37 °C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% ethanol/10% acetic acid. Lane A contains APMA-treated MMP-2 as a control for active MMP-2 (25 ng). The triangle indicates active MMP-2. (E) RT-PCR analysis of TIMP-2 and MT1-MMP.

Figure 1(D), if not counteracted by increased levels of TIMP-1, -3 or -4, could contribute to TGF- β_1 -induced lens pathogenesis.

A basal level of MMP-2 expression exists in the cell line that we used in the present study. It is likely that a basal level of MMP-2 activity is required in an *in vitro* cell culture system because the cells have to migrate to maintain a monolayer as cell numbers increase, in contrast with the situation *in vivo*, where cell migration does not take place unless wound healing occurs. Furthermore, the activity of MMP-2 is tightly regulated by TIMP-2 (cellular MMP-2 inhibitor), so that the basal level of MMP-2 will be inhibited by endogenous TIMP-2. However, overexpressed MMP-2 is likely to overwhelm the endogenous level of TIMP-2, which will cause lens pathogenesis.

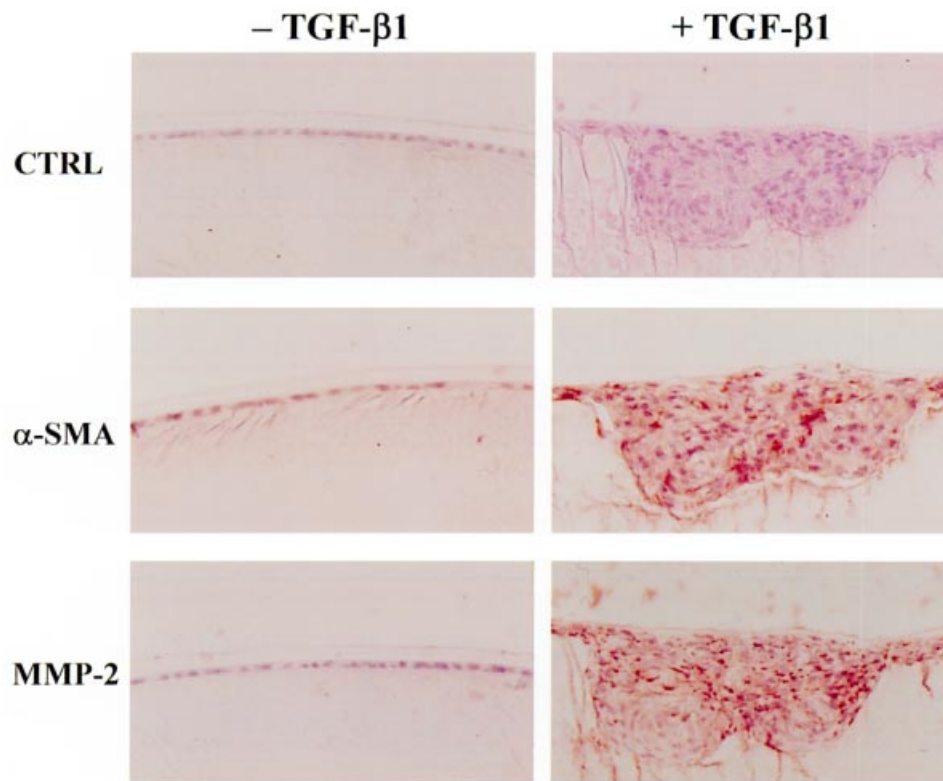


Figure 2 Immunohistochemical analysis of MMP-2 expression induced by TGF- β_1 in rat lenses

Whole lenses were dissected from 3-week-old Sprague–Dawley rats and incubated in the presence of 10 ng/ml TGF- β_1 . Control lenses were cultured without the addition of TGF- β_1 . After 5 days, the lenses were fixed, embedded in paraffin and sectioned for histological analysis using anti-MMP-2 and anti- α -SMA antibodies. The primary antibody was omitted from the control (CTRL).

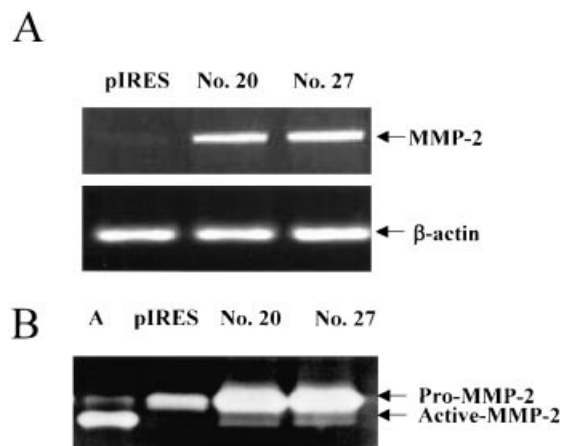


Figure 3 LECs stably transfected with MMP-2 cDNA overexpress MMP-2

(A) RT-PCR analysis of the overexpression of MMP-2 in stably transfected clone nos. 20 and 27, following transfection with pIRES containing cDNA encoding MMP-2. 'pIRES' indicates vector only. Total RNA was collected from 3×10^5 cells from stably transfected cell lines, and 1 μ g of RNA was subjected to RT-PCR. (B) Zymography for the overexpression of MMP-2 in stably transfected clones. The culture media were collected from 3×10^5 cells from stably transfected cell lines. The same amount of protein was applied to SDS/10%-PAGE containing 1 mg/ml gelatin. The clear zone was visualized for MMP-2 activity. Lane A contains APMA-treated MMP-2 (25 ng) as a control for active MMP-2.

We examined the expression of MMP-2, MMP-3 and MMP-9 in response to the cytokines tested above; however, neither MMP-3 nor MMP-9 was induced by TGF- β_1 , although interleukin-1 α and TNF- α induced MMP-3 and MMP-9 respectively (results not shown). Thus MMP-2 was of special interest, because numerous reports have shown that TGF- β is a critical inducing mediator of anterior subcapsular cataract [25,28]; among the various cytokines tested, MMP-2 was induced only by TGF- β_1 . We and others have shown that TGF- β_1 induces genes that are the markers for fibrotic diseases, such as connective tissue growth factor, fibronectin, α -SMA and collagen type I, in various tissues as well as in LECs [28–30]. Therefore induction of MMP-2 by TGF- β_1 would seem to be one of the factors that play a role in the fibrosis of LECs.

The expression or the activity of MMP-2 in response to TGF- β_1 seems to be regulated in a tissue- or cell-type-specific manner, in a complex fashion. For example, MMP-2 activity was inhibited by TGF- β in mesangial cells [31], but was increased in prostate cancer due to the increased stability of MMP-2 mRNA [32]. Although there have been numerous reports concerning the stimulation of MMP-2 activity, only a few cell types show induced MMP-2 transcription in response to TGF- β_1 , such as gingival fibroblasts, renal cell carcinoma and human saliva gland [33–35]. Here we show that the steady-state level of MMP-2 mRNA is increased by TGF- β_1 in LECs. Therefore the stimulation of secretion by TGF- β is due mainly to transcriptional activation or the increased stability of MMP-2 mRNA, although

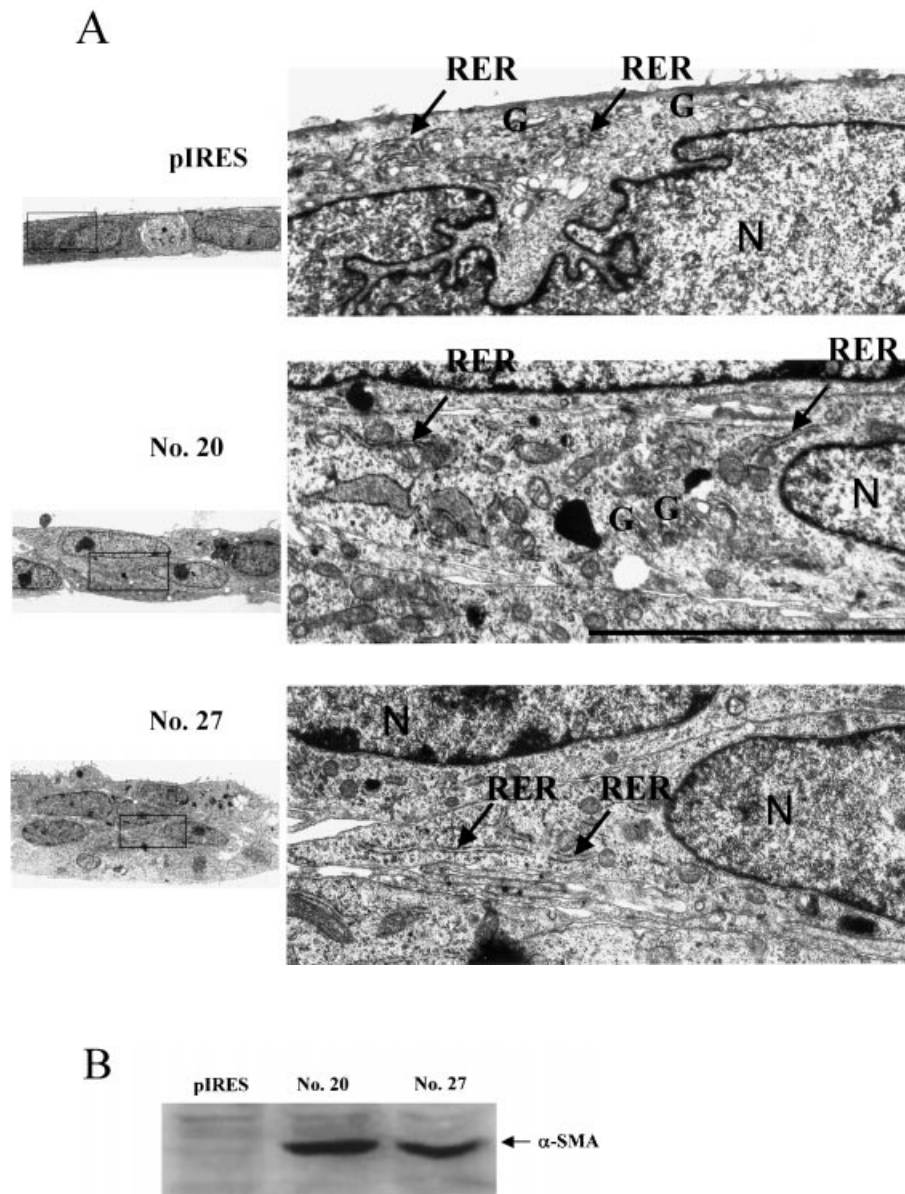


Figure 4 Overexpression of MMP-2 mediates the phenotypic transformation of LECs

(A) Electron microscopic observations showing altered cell morphology and arrangement in the MMP-2-overexpressing cells. Cells were plated in 60 mm plates and grown until they reached over 90% confluency. The cells were fixed with glutaraldehyde, sectioned at 90 nm thickness and subjected to transmission electron microscopy. The arrow indicates rough endoplasmic reticulum; N, nucleus; G, Golgi apparatus. Scale bar = 5 μ m. (B) Western blot analysis for α -SMA protein in the MMP-2-overexpressing cell line. Nos. 20 and 27 indicate the independent clones that were transfected with pIRES containing cDNA encoding MMP-2. 'pIRES' indicates vector only.

we still cannot rule out the other possibilities, such as stimulated protein synthesis and protein export.

Presence of MMP-2 within the anterior subcapsular plaques of rat lenses cultured with TGF- β_1

Enhancement of MMP-2 expression by TGF- β_1 in cultured LECs suggested that the enzyme might play a role in the development of anterior subcapsular cataracts. Therefore we employed a well characterized rat lens organ culture model of anterior subcapsular cataract [25] to test whether MMP-2 is correlated with anterior subcapsular cataract induction by TGF- β_1 . Lenses incubated with TGF- β_1 for 5 days developed anterior

opacities showing cellular plaques (Figure 2), as reported previously [25], while rat lenses cultured without TGF- β_1 displayed a normal monolayer of LECs.

α -SMA is used as a marker for transformed fibrotic cell types in various tissues [30,36–39], and is also observed in LECs in pathological conditions [8,40]. Therefore we examined immunoreactivity for α -SMA and MMP-2 in rat lenses incubated with TGF- β_1 . Strong immunoreactivity for α -SMA and MMP-2 was observed within the subcapsular plaques, while sections incubated with normal rabbit IgG instead of the primary antibody showed no reactivity. These results suggest that transformation of LECs by TGF- β_1 is well correlated with the increased expression of α -SMA and MMP-2. This result led us to examine whether the

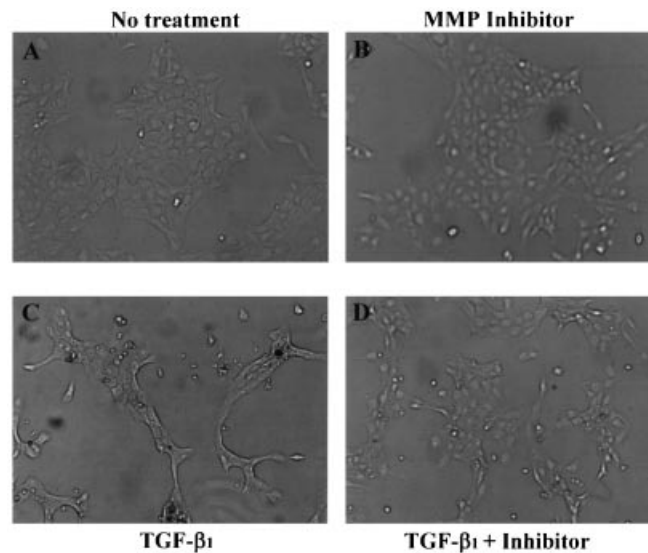


Figure 5 An MMP inhibitor blocks the morphological changes induced by TGF- β_1

B-3 cells were plated on to a four-well chamber slide with medium containing 1% serum. After 5–6 h of adaptation to low serum, the cells were treated with nothing (**A**), 1 μ M MMP inhibitor (Calbiochem; cat. no. 444241) alone (**B**), 10 ng/ml TGF- β_1 (**C**), or TGF- β_1 plus MMP-2 inhibitor (**D**). After 40 h, the cells were observed with a phase contrast microscope. TGF- β_1 -treated cells showed an elongated and scattered morphology; in contrast, cells treated with TGF- β_1 plus MMP inhibitor did not show any morphological changes.

increased amount of MMP-2 may participate in the transformation of LECs.

Overexpression of MMP-2 induces transformation of LECs

Since TGF- β is a multi-potent cytokine that affects various aspects of cellular physiology, we stably transfected LECs with MMP-2 in order to examine the influence of the overexpressed metalloproteinase in the absence of other possible effects of TGF- β . The cDNA for MMP-2 was cloned into a mammalian expression vector (pIRES-neo) and stably transfected into the HLE B-3 cell line. After colony selection, we used two highly MMP-2-expressing clones out of 31 stably transfected clones for further experiments. Most of the stable transfectants showed lower growth rates or impaired attachment (results not shown). In order to confirm the overexpression of MMP-2, we used RT-PCR to show that MMP-2 was overexpressed in clones 20 and 27 when compared with the control, which was transfected with vector only (Figure 3A). We also examined the overexpression of MMP-2 protein by a zymography experiment (Figure 3B). The results showed that the MMP-2-overexpressing clones 20 and 27 had higher expression of pro-MMP-2 and active MMP-2 compared with the control.

In order to demonstrate phenotypic differences in MMP-2-overexpressing LECs, we observed the morphology of the transfectants by electron microscopy. Figure 4(A) shows that overexpression of MMP-2 induced multilayered and bipolar-shaped cells containing enriched rough endoplasmic reticulum (arrow), which are characteristics of transformed epithelial cells. Furthermore, the Golgi apparatus (indicated as G in Figure 4A), which is typically placed at the apical side of epithelial cell, was pushed to the side of the cell in the MMP-2-overexpressing clones.

The most common fatal malignant adenocarcinomas arise from polarized epithelial cells of the lung, mammary gland, prostate, colon, pancreas and urinary tract. The early signs of adenocarcinoma are cell multi-layering and loss of apical polarity,

which leads to cellular dysfunction [9,10,19]. Even though little is known about the mechanisms involved, these morphological changes are well known indicators of the pathological status of these tissues. The LECs of patients with anterior polar cataract have a similar transformed cell morphology, accompanied by enhanced migration [41]. Our results imply that the overexpression of MMP-2 induced morphological changes in LECs, and suggest that MMP-2 may play a crucial role in the pathogenesis of fibrotic diseases.

α -SMA is a well known marker of myofibroblast-like cells, and accumulates in tissues characterized by fibrosis or anterior subcapsular cataract, but not in normal tissue [8,28,39,42]. Therefore, in order to examine the role of MMP-2 in the expression of α -SMA, we performed a Western blot analysis. This showed (Figure 4B) that clones 20 and 27 had increased levels of α -SMA. The expression of α -SMA may be required for alterations in cell morphology and migration, since a change in the interaction between the cell and the ECM due to MMP-2 may be a stimulatory signal for the cells to transform and to have a migratory character. Thus α -SMA may be a necessary factor in order for the cells to migrate and to change morphology. In fact, it is well documented that α -SMA is necessary for filopodia formation and cell locomotion [39,43,44].

In order to confirm that MMP-2 is involved in the transformation of LECs induced by TGF- β_1 , we treated cells with 1 μ M (2R)-2-[(4-biphenylsulphonyl)amino]-3-phenylpropionic acid (Calbiochem; cat. no. 444241), an MMP-2/-9 inhibitor, in the presence or absence of TGF- β_1 . TGF- β_1 -treated LECs changed to an elongated and scattered morphology, while cells treated with nothing or with the MMP inhibitor had a round shape and intact cell-cell interactions (Figure 5). In contrast, cells treated with MMP inhibitor plus TGF- β_1 showed an epithelial-like morphology. These results suggest that MMP-2 plays a role in TGF- β_1 -induced phenotypic transformation. We used an MMP inhibitor that inhibits both MMP-2 and MMP-9, so we cannot exclude the possibility that MMP-9 may also be involved in TGF- β_1 -induced transformation; however, we did

not observe MMP-9 activity in the zymogram in the presence or absence of TGF- β_1 in LECs. Thus MMP-9 did not seem to play a role in the TGF- β_1 -induced transformation.

Although treatment with the MMP-2 inhibitor had an inhibitory effect on TGF- β_1 -induced transformation, it was not able to reverse the morphological changes observed in MMP-2-overexpressing clones. Since it took several months to establish the MMP-2-overexpressing cell lines for stable transfection and colony selection, the cells were exposed to MMP-2 for several months. It is likely that long-term exposure of MMP-2 could induce irreversible transformation. We have not tested whether transformation could be blocked if the inhibitor were added from the beginning of the stable transfection. Thus the MMP inhibitor may block or slow down the process of cellular transformation, but cannot reverse it.

It has been shown that transformed epithelial cells exhibit not only morphological changes, but also alterations in gene expression [6,40]. The transformation of epithelial cells into myofibroblast-like cells has been implicated as the mechanism underlying diseases accompanying fibrosis in various tissues, such as the lung, kidney, liver and lens of the eye [6,7,28,45,46]. Of particular interest were two key findings: first, TGF- β_1 induces MMP-2 mRNA expression in LECs; secondly, MMP-2 promotes the phenotypic transformation of LECs into myofibroblast-like cells. Thus the present work presents novel observations with respect to the transforming ability of MMP-2.

Although it has been hypothesized that modulation of the ECM leads to cell transformation, a direct influence of MMP-2 in cell transformation has not been shown until now. Our results show for the first time that overexpression of MMP-2 induced α -SMA expression and morphological changes. Furthermore, an MMP inhibitor blocked the TGF- β -induced morphological change in LECs. These results suggest that the degradation of the ECM by MMP-2 alters cell-ECM interactions, which may be a critical signal for changes in cell-type-specific gene expression.

Only a limited amount of MMP-2 seemed to be converted into active MMP-2 in the TGF- β_1 -treated samples (Figure 1D), because pro-MMP-2 is predominant over active MMP-2. It is conceivable that the factors required for MMP-2 activation were not greatly enhanced by TGF- β_1 , particularly under our experimental conditions, which included serum-free media; it is possible that the MMP-2 activation might be markedly enhanced in the presence of serum. It has been reported that several factors (such as plasminogen activator and bivalent ions) present in serum are involved in the activation of MMPs, and are also activated by TGF- β_1 [47,48]. It is also possible that other regulatory factors are involved in the activity of MMP-2. Thus TGF- β_1 responsiveness with regard to the activity of MMP-2 might differ *in vivo* and *in vitro*. Our results clearly demonstrate that TGF- β_1 was a strong inducer of MMP-2 in LECs. These data suggest that TGF- β_1 modulates the interaction between the cell and the ECM by regulating MMP-2 gene expression in LECs.

We also observed that the MMP-2-overexpressing clones seemed to grow more slowly than control cells (results not shown). At present, it is not clear whether the growth rate is low, or whether they cannot adhere and thus die because of 'anoikis'. Nonetheless, the overexpression of MMP-2 resulted in disrupted cell-cell interactions, overexpression of α -SMA and altered cell morphology.

Our findings emphasize the role of MMPs in various fibrotic diseases. The present study of metalloproteinase expression further expands our understanding of possible physiological modulators of ECM that may be important for epithelial cell character.

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