UVB-Elicited Induction of MMP-1 Expression in Human Ocular Surface Epithelial Cells Is Mediated through the ERK1/2 MAPK-Dependent Pathway

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PURPOSE. Pterygia are common, frequently recurring ocular surface lesions characterized by tissue remodeling, cellular proliferation, angiogenesis, and inflammation. The increased incidence of pterygia in persons exposed to excessive solar radiation suggests that ultraviolet (UV) light may play a critical role in the pathogenesis of this disease. These investigations were focused on the expression of collagenase-1 (matrix metalloproteinase [MMP]-1) in pterygia and cultured pterygium epithelial cells, to determine whether the expression of this protease could be modified after exposure to UVB.

METHODS. Pterygium, conjunctival, and limbal epithelial cells were subcultured and exposed to various amounts of UVB. The conditioned medium and RNA were harvested for analysis by gelatin zymography, Western blot analysis, ELISA, and RT-PCR. Furthermore, whole pterygium specimens were irradiated to determine secreted MMP-1 levels.

RESULTS. Immunohistochemical analysis revealed enhanced MMP-1 expression in pterygia that corresponded precisely with p63-positive epithelial cells. In contrast, significantly less MMP-1 reactivity was found in normal conjunctiva, limbus, and cornea. A dose- and time-dependent increase in MMP-1 was observed when pterygium epithelial cells were exposed to UVB with no significant modulation of inhibitor activity. MMP-1 was not affected in irradiated normal conjunctival epithelial cells or in pterygium fibroblasts but was induced in limbal epithelial cells. Although the induction of MMP-1 after UVB was not mediated by an intermediate soluble factor, the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) intracellular pathway was involved.

CONCLUSIONS. Collectively, these data support the hypothesis of the involvement of UV light and MMPs in the development of pterygia and may assist in devising new therapeutic approaches for the treatment and prevention of pterygia. (Invest Ophthalmol Vis Sci. 2003;44:4705–4714) DOI:10.1167/iovs.03-0356

Pterygia are highly vascularized, proliferative, and invasive ocular surface lesions that originate at the corneal limbus.1 Recent studies indicate that pterygia are a disregulation of wound healing, in which molecular events leading to apoptosis are modified.2–4 Pterygia may also be a degenerative disease,5 and several studies have identified members of the matrix metalloproteinase (MMP) class of enzymes in both pterygium cells6–9 and diseased tissue.7,10–12 One of the most widely accepted theories implicates ultraviolet (UV) radiation in the pathogenesis of pterygia.13–17 Although this evidence stems from epidemiologic data, studies in human skin have shown that UV can induce elastic connective tissue changes18 along with increased expression of inflammatory cytokines19,20 and MMPs.21,22 MMPs are a family of enzymes that include at least 20 members, most of which are active against extracellular matrix (ECM) components.23,24 Hence, they have been assigned essential roles in modeling and remodeling the ECM in normal and disease states.23–25 Although there is some overlap in substrate activity between the subgroups, which include the collagenases, gelatinases, stromelysins, and membrane-bound MMPs, the most specific are the collagenases (MMP-1, -8, and -13), which have the unique ability to denature interstitial collagen types I, II, and III. Of these enzymes collagenase-1/MMP-1 is the most ubiquitously expressed. Like other MMPs, MMP-1 is regulated at multiple levels, including transcription, proenzyme activation, and enzyme inhibition.26 The latter is a result of natural antagonists known as tissue inhibitors of metalloproteinase (TIMPs) which bind to the latent or active enzyme. An imbalance between MMP and TIMP activity is a likely cause of progressive panus invasion and irreversible tissue damage in rheumatoid arthritis27 and scleral matrix dissolution in scleritis.28 MMP-1 has also been implicated in cancer metastasis, in which human tumor cells transplanted with constructs containing antisense RNA for MMP-1 demonstrate a reduced capacity to invade and degrade collagen.29

Several reports have documented the induction of MMP-1 in UV-exposed human skin30 and cultured cells.31 This has been attributed to known UV-responsive elements in the promoter of MMP-1 and mutations in this sensitive region result in the loss of responsiveness.32 The UV-mediated induction of MMP-1 is associated with increased expression of several transcription factors32,33 and can be amplified by secondary mediators such as cytokines.34 The accumulation of MMP-1 RNA after UV or phorbol ester treatment is partially delayed and partly depends on protein synthesis.32,34

This investigation focused on MMP-1, one of the most abundant MMPs in pterygia.7–10,12 The goals of this study were to determine the expression of MMP-1 in pterygia, establish whether UVB modulates the expression of this enzyme, and determine the potential mechanism of induction.

MATERIALS AND METHODS

Surgical Tissue Specimens

Resected pterygia (n = 10) and normal conjunctiva (n = 10) were obtained from the Prince of Wales Hospital, Sydney, Australia. Autologous tissue (n = 4) that remained after grafting was also used in this study and included segments of conjunctiva and limbus. Tissue was immediately fixed in formalin and embedded in paraffin.
resected pterygia were derived from six men and four women (mean age, 47.6 years). In addition, fresh pterygia (n = 7) was surgically removed and placed in organ culture, as previously described.10,11 Informed consent was obtained from each subject. All research protocols were approved by the University of New South Wales Ethics Committee and were performed in accordance with the tenets of the World Medical Association’s Declaration of Helsinki.

**Immunohistochemical Analysis**

Serial 4-μm sections of pterygia, conjunctiva, limbus, and cornea were processed for immunohistochemistry, as previously described.7 In brief, tissue sections were deparaffinized, hydrated, equilibrated in 0.05 M Tris-buffered saline (TBS; pH 7.6), and blocked with 20% goat serum in 2% BSA/TBS for 30 minutes at room temperature. Some tissue sections were incubated (10 μg/ml final) with an anti-human MMP-1 antibody (clone 41-1E5; ICN Biomedicals, Sydney, Australia) or with 7 μg/ml anti-human p63 antibody (clone A4A; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in 2% BSA/TBS. With each primary antibody that was used, a rigorous staining optimization protocol was applied. For p63, antibody concentration, time of exposure (30 minutes to 16 hours), and temperature of incubation (4°C–37°C) were performed in preliminary experiments. Antibody retrieval by either microwave treatment or enzymatic digestion was performed but not necessary to obtain optimal staining. Sections were extensively washed in TBS before the addition of a biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Sections were washed and incubated with horseradish peroxidase-conjugated streptavidin (Dako Corp, Carpinteria, CA) and the immunoreactivity developed by adding 3-amino-9-ethyl-carbazole (Sigma-Aldrich, St. Louis, MO). Control reactions included tissue sections with an antibody-antigen preabsorption mixture, omitting the primary antibody or replacing the primary antibody with a mouse isotype (IgG2a) negative control antibody (BD Biosciences, Lincoln Park, NJ). Some sections were counter-stained with hematoxylin. Differences in the staining intensity between pterygia, conjunctiva, limbus, and cornea were comparable, as diseased and normal tissue was analyzed under identical conditions in the same experimental run.

Conjunctival epithelial cells (CECs) and pterygium epithelial cells (PECs) were cultured in chamber slides, 36 36, and grown in the presence of 10% FBS/EMEM. Medium from semiconfluent cells was aspirated and the cells were washed three times with sterile PBS and left in serum-free medium for 16 hours, as previously described.7 This medium was replaced with 5 ml PBS, and the monolayer irradiated with UVB (0–100 mJ/cm²) using UVB bulbs (TL 20W/12 RS; Philips, Sydney, Australia) as previously reported.57–59

**Enzyme-Linked Immunosorbent Assay for MMP-1**

Supernatants derived from control, cytokine stimulated, and UVB-irradiated cells were analyzed by commercial ELISA (Biotrak; Amersham Pharmacia Biotech, Sydney, Australia) for MMP-1, precisely as described by the manufacturer. The optical density of each 96-well plate was read at 450 nm on a microplate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA).

**RNA Extraction and Reverse Transcription–Polymerase Chain Reaction**

Table 1. Primer Pairs Used for PCR Analysis

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>MMP-1</td>
<td>5’-GGT GAT GAA GCA GCC CAG G3’</td>
<td>5’-CAG TAG AAT GGG AGA GTC-3’</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>5’-TGG ACC TGT GTC CCA CCC CAC CCA CAG AGG-3’</td>
<td>5’-GGA CGG CAG GGA CTT CGA GGT-3’</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>5’-GCA GAT GTA GTC ATG AGG GC-3’</td>
<td>5’-TTG TCC TCT AGG AGG AG-3’</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>5’-CGA TCA AGG AGA AGA AGA AGG-3’</td>
<td>5’-GGT AGT AGG AGG ACT TGA TGT ACC-3’</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>5’-GCC AAA ATG TGC GAT GGG G-3’</td>
<td>5’-CTA GTA GGA GCG GAC AAG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-TGA TGA CAT CAA GAA GGT GGT GAA G-3’</td>
<td>5’-TGC TGG GAG GGC TGG GCC AT-3’</td>
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Previous studies (referenced) have successfully used these primer sets to amplify the respective gene products. GAPDH, glyceraldehyde 3 phosphate dehydrogenase; F, forward primer; R, reverse primer.
on the vascular endothelium (Fig. 1A, arrow) and stromal fibroblasts (data not shown). A bandlike pattern of intense MMP-1 staining was also evident in the basal epithelium of most pterygium specimens analyzed (Fig. 1A, arrowheads). MMP-1 also localized to connective tissue components in pterygia that resembled coiled or elastic matrix (Fig. 1M). Antibody preabsorption against recombinant MMP-1 confirmed the specificity of this result (Fig. 1N). In contrast, significantly less MMP-1 staining was observed in the normal conjunctival, limbal, and corneal epithelium (Figs. 1D, 1G, 1J, respectively). It was apparent that MMP-1-expressing epithelial cells were indented p65 positive (Figs. 1B, 1E, 1H). This was a relevant finding, as this transcription factor has been identified on proliferating epithelial cells and has been localized on limbal stem cells.40 It was also noted that the basal pterygium, conjunctival, and limbal epithelium stained more intensely than the superficial cells but the central cornea displayed little or no reactivity for this antigen (Fig. 1K). Tissue sections incubated with an isotype control antibody were completely void of any reactivity (Figs. 1C, 1F, 1I, 1L).

In Vitro Modulation of MMP-1 mRNA Expression by UVB

PECs were exposed to various amounts of UVB, and total RNA was isolated to determine MMP-1 and TIMP-1 mRNA expression by RT-PCR. Whereas MMP-1 mRNA was constitutively expressed in nonirradiated cells (Fig. 2A, lane 1), a dose-dependent increase in the expression of this enzyme was observed after UVB exposure. MMP-1 was enhanced by 2.3-, 3.7-, 4.6-, and 4.1-fold after 5, 20, 40, and 60 mJ/cm², respectively (Fig. 2A). In contrast, no significant modulation of TIMP-1 mRNA was noted after the same treatment (Fig. 2B).

In Vitro Modulation of MMP-1 Protein by UVB

PECs were irradiated with UVB ranging from 0 to 80 mJ/cm² and the conditioned medium collected to determine whether this treatment modifies MMP protein production. Gelatin zymography disclosed the absence of MMP-9 activity either under control conditions or after UVB exposure (Fig. 3A). Although MMP-2 was constitutively produced, this protease was not induced after UV exposure (Fig. 3A). The same conditioned medium was then analyzed by Western immunoblot for MMP-1 production. MMP-1 reached the maximum level when PECs were exposed to 20 mJ/cm² UVB and then gradually declined and was absent at higher exposures (Fig. 3C). MMP-1 and -9 (but not MMP-2) were significantly enhanced when cells were stimulated with PMA (Figs. 3A–D, lane 7). PMA was included as a positive control stimulus for both gelatinase species, because MMP-2 and -9 were not induced by the UVB treatment. The zymography and immunoblot results were confirmed and quantified by ELISA. MMP-1 protein was constitutively produced at relatively low levels but was significantly increased to 84.54 ± 0.99 ng/mL (P < 0.01) when PECs were exposed to 20 mJ/cm² UVB (Fig. 3E). Higher exposures (>50 mJ/cm²) resulted in a decline of MMP-1 to below detectable levels, perhaps due to cell death.57

Time-course experiments were performed in which PECs were irradiated with 20 mJ/cm² UVB and the supernatants were harvested at 24-hour intervals over 3 days. Again, MMP-9 was not detected and MMP-2, although present, was not induced over the 3-day time course when compared with control cells (Fig. 3B). Western blot analysis of the same conditioned medium disclosed an intense immunoreactive band corresponding to MMP-1 at 48 and 72 hours after UVB exposure (Fig. 3D). Although similar amounts of MMP-1 were produced...
after 24 hours in control versus UVB-irradiated cells, this enzyme was significantly elevated 20-fold after 48 hours and 80-fold after 72 hours (49.57 ± 1.82 ng/mL; P < 0.01; Fig. 3F).

**TIMP Expression in UVB-Irradiated PECs**

Although expression of TIMP-1 mRNA was examined and shown not to be influenced by UVB (Fig. 2B), the expression of the remaining members of this family of inhibitors was assessed by RT-PCR. The results demonstrated relatively unaltered TIMP-1, -2, -3, and -4 (Fig. 4A–D) mRNA levels in control (Fig. 4E) compared with cells irradiated with 5 and 20 mJ/cm² UVB.

TIMP protein production was assessed by reverse zymography in which two prominent inhibitor bands were identified. The 28-kDa TIMP-1 and the 22-kDa TIMP-2 proteins were constitutively produced and accumulated in control cells over the 3-day time course (Fig. 4F, lanes 2–4). This production did not differ significantly from cells exposed to UVB (Fig. 4F, lanes 5–7). However, cytokine stimulation (Fig. 4F, lane 10) moderately elevated TIMP-1 by 1.85-fold and increased TIMP-2 by 1.3-fold when compared with untreated cells (Fig. 4F, lane 4).
Culture and Characterization of Human Epithelial Cells

Primary CECs (Fig. 5A), PECs (Fig. 5B), and LECs (not shown) were morphologically indistinguishable but differed significantly from pterygium fibroblasts (Fig. 5C). Both CECs and PECs displayed similar filamentous cytokeratin expression when stained with the AE5 (Figs. 5D, 5E, respectively) and AE3 antibody (data not shown). Likewise, LEC stained positively for both markers, and each of the three cell types displayed greater than 97% purity as determined by flow cytometry and (data not shown).

Effect of UV Light and Cytokines on Epithelial Cells

CECs, PECs, and LECs were irradiated with 20 mJ/cm² UVB, and the conditioned media harvested at 24-hour intervals over 3 days and examined by zymography. The results indicate that CECs were not responsive to UV-light with respect to MMP-1 production, as lytic bands corresponding to MMP-1 were not evident (Fig. 6A, arrowhead). In contrast, UVB enhanced MMP-1 production in both PECs (Fig. 6B, arrow) and LECs (Fig. 6C, arrow). These results were further confirmed and quantified by ELISA, where a significantly greater amount of MMP-1 was detected in PECs and LECs compared with CECs after UVB exposure (see Table 2). Of interest was that MMP-9 was not detected in the cell lines analyzed, but they all produced similar amounts of latent and active MMP-2 (Fig. 6).

Next, the CECs were stimulated with proinflammatory cytokines to determine whether MMP-1 production could be induced in these cells with an alternative stimulus. Here CECs, PECs, and LECs were treated over 72 hours with the combination of TNF-α/H9251 and IL-1. These cytokines have been documented to increase the expression of MMPs in pterygium-derived cells. In contrast to the UV treatment, cytokine stimulation enhanced both latent and active MMP-2 in CECs (Fig. 6D, lane 2), PECs (Fig. 6D, lane 4), and LECs (Fig. 6D, lane 6) compared with cells cultured under control conditions. Although no lytic

Figure 3. Dose- and time-dependent induction of MMP-1. PECs were irradiated with 0 to 80 mJ/cm² of UVB (A, C, E) or stimulated with PMA (A-D, lane 7) and the conditioned media collected after 72 hours (A, C, E). Other cells were cultured under control conditions (no UV) or irradiated with 20 mJ/cm² of UVB (B, D, F), the supernatants harvested after 24 (B, D, lanes 1 and 2), 48 (B, D, lanes 3 and 4), and 72 (B, D, lanes 5 and 7) hours and analyzed by zymography (A, B), Western blot (C, D), and ELISA (E, F). Arrows: active forms of MMP-2 and -9. A low molecular weight, unstained (A, B) and prestained (C, D) protein ladder was used to estimate the size of the gelatinolytic and immunoreactive bands. Data points in (E) and (F) represent the mean ± the SD of triplicate samples. *Significant difference; P < 0.01.

Figure 4. TIMP mRNA and protein expression in UVB-treated PECs. PECs were exposed to 0, 5, 20 mJ/cm² of UVB. Total RNA was purified, and equal amounts analyzed by RT-PCR to determine the steady state mRNA expression for TIMP-1 (A), TIMP-2 (B), TIMP-3 (C), TIMP-4 (D), and GAPDH (E). To determine TIMP protein activity, conditioned medium from PECs cultured under control conditions, irradiated with 20 mJ/cm² UVB, and stimulated with TNF-α and IL-1 was analyzed by reverse zymography (F). Supernatants were harvested after 24, 48, and 72 hours. The identity of the protected bands was disclosed by comparison with rhTIMP-1 (T1), rhTIMP-2 (T2), and the previously characterized TIMPs from cytokine (Ctk) stimulated PECs. Molecular weights were estimated by comparison with a low molecular weight protein ladder (MW). Similar results were obtained in two other experiments.
bands corresponding to MMP-1 were visible on the zymograms, similar levels of MMP-1 were found in CECs, PECs, and LECs by ELISA (Table 2).

Modulation of MMP-1 by Exogenous Cytokines and PEC-Derived Soluble Factors

In a previous study, we established the absence of both TNF-α and IL-1 in the supernatants from UVB-irradiated PECs, whereas both IL-6 and -8 were localized in pterygia and significantly enhanced by UVB.37 The late induction of MMP-1 protein after UV exposure (Figs. 3, 6) suggests that this increased expression may be through a secondary messenger. This arm of the study was designed to determine whether UV-inducible cytokines act in an autocrine manner to amplify the expression of MMP-1. PECs stimulated with various doses of IL-8 (Fig. 7A), IL-6 (data not shown), or the combination of IL-6 and -8 (data not shown) were not responsive with respect to MMP-1 production. These data suggest that MMP-1 expression could be upregulated by other soluble mediators. To test this hypothesis, conditioned media from control or UVB-irradiated PECs was collected and incubated with quiescent CECs, PECs, and LECs to determine whether a soluble factor(s) could stimulate MMP-1. Only conditioned media from 24 and 48 hours after UV irradiation was used, because these supernatants contained low to undetectable levels of this enzyme. Soluble proteins produced by PECs in response to UV exposure did not modulate MMP-1 production by PECs (Fig. 7B), CECs (Fig. 7C), or LECs (data not shown). No significant difference in MMP-1 production was noted when the same supernatants were analyzed by ELISA (data not shown).

Involvement of Extracellular Signal-Regulated Kinase (ERK)1/2 in the Induction of MMP-1 after UVB Exposure

PECs were treated with specific chemical inhibitors to identify the potential MAPK pathway(s) involved in the induction of MMP-1 after UV radiation. After UVB exposure, PECs were immediately incubated with various concentrations of PD98059 or SB202190. Although no significant modulation of MMP-1 protein expression was observed after treatment with SB202190 (Fig. 8B), MMP-1 was significantly decreased in a dose-dependent manner after exposure to PD98059 (Figs. 8A, 8C) where a 50% reduction in MMP-1 protein was noted with 25 μM PD98059. Furthermore, MMP-1 mRNA expression was moderately decreased (~40%) when cells were incubated with PD98059 (Fig. 8D, lane 3), but was not modulated by SB202190 (Fig. 8D, lane 4) when compared with UVB-exposed PECs (Fig. 8D, lane 2).

Induction of MMP-1 in UVB-Irradiated, Organ-Cultured Pterygia

After localizing MMP-1 in pterygia and determining an optimal dose of radiation that augmented this enzyme in cultured PECs, pterygium tissue was surgically removed, cut symmetrically into halves, and exposed to 20 mJ/cm² of UVB. After the conditioned media were standardized for total protein content, MMP-1 was measured by ELISA. High levels of MMP-1 were detected in nonirradiated pterygia (Fig. 9, unfilled histograms and inset lanes 1 and 3), whereas significantly higher amounts (2.7-fold) were observed after UVB exposure (Fig. 9, black histograms and inset lanes 2 and 4).

DISCUSSION

Over recent years, several studies have focused on the likely involvement of proteolytic enzymes of the metalloproteinase class in the pathogenesis of pterygia7–12,14 and the potential role of UV light in the development of this lesion15,16,37,38 Previously, we reported the abundant expression of MMP-1 in pterygium tissue,7 at the invading pterygium edge,10 and in cultured PECs.7 The current investigation extends these observations and suggests that MMP-1 is a likely candidate enzyme in pterygium formation, because UV light regulates this enzyme at the level of transcription and translation. Of interest, although...
MMP-1 was increased in a time- and dose-dependent manner in cultured PECs, the same could not be said for its counterregulatory molecules, the TIMPs. Likewise, MMP-1 protein was significantly elevated in UVB-exposed organ-cultured pterygia. These data imply a possible imbalance in favor of enzymatic over inhibitory activity that may facilitate pterygium invasion through the normal cornea.

MMP-1 was the first described and is to date the best characterized member of the MMP family. This enzyme is one of three interstitial collagenses capable of selectively cleaving fibrillar collagen at a single locus. For this reason, it is one of the most important ECM rate-limiting enzymes in humans. Knowledge of the activation and expression status of MMP-1 in pterygia is therefore relevant, as interstitial fibrillar collagen makes up a large component of this lesion. This was a major reason that we focused our attention on MMP-1, although other enzymes with broader substrate specificity, such as MMP-3, may also be relevant. Other reasons for studying MMP-1 include the enzyme’s potential to promote directed cell migration, and hence it may be regarded as a potent chemoattractant. Similarly, type I collagen peptides generated by MMP-1 have chemotactic activity for human leukocytes. In the present investigation, ECM-bound MMP-1 was observed in pterygia (Fig. 1M), and its affinity for the collagen molecule is well established. It is likely that matrix-bound MMP-1 forms a reservoir of latent enzyme. Once activated, MMP-1 may release chemotactic collagen peptides and promote leukocyte infiltration and inflammation. MMP-1 can also mediate tumor cell invasion and keratinocyte migration through type I collagen in vitro. Likewise, migrating human keratinocytes actively involved in reepithelialization (wound healing) acquire a collagenolytic phenotype in vivo. This is relevant to pterygia.

### Table 2. MMP-1 Levels in UVB-Exposed Ocular Surface Epithelial Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MMP-1 (ng/mL)</th>
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<tbody>
<tr>
<td>PEC</td>
<td>49.6 ± 11.5</td>
</tr>
<tr>
<td>LEC</td>
<td>45.8 ± 10.5</td>
</tr>
<tr>
<td>CEC</td>
<td>1.7 ± 0.6</td>
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</table>

Ocular surface epithelial cells derived from pterygia (PEC), limbus (LEC), and conjunctiva (CEC) were exposed to 20 ml/cm² UVB or stimulated with a combination of cytokines, and the supernatants were analyzed by commercial ELISA. Values for MMP-1 are from triplicate readings with standard deviations.

### Figure 6. MMP production in UVB-irradiated epithelial cells. Human CECs (A, D, lanes 1 and 2), PECs (B, D, lanes 3 and 4), and LECs (C, D, lanes 5 and 6) were irradiated with 20 ml/cm² UVB (A–C, lanes 2 to 4), stimulated with TNF-α and IL-1 (D, lanes 2, 4, and 6), or cultured under control conditions (A–C, lane 1; D, lanes 1, 3, and 5). The supernatants were harvested after 24, 48, and 72 hours of treatment (A–C) or 72 hours (D) after treatment then analyzed by gelatin zymography (A–D). Arrows: faint doublet corresponding to MMP-1 (B, C); arrowheads: little or no detectable gelatinolytic activity (A, D).

### Figure 7. Effect of cytokines and soluble factors on MMP-1 production in PECs. PECs were stimulated with several concentrations of IL-8, the supernatants were harvested after 72 hours and analyzed by Western blot (A). Conditioned medium from PMA-treated PECs (lane 6) was run in parallel. Quiescent PECs (B) and CECs (C) were exposed to conditioned media derived from PECs cultured under control conditions (B, C, lanes 3 and 5) or conditioned media derived from PECs irradiated with 20 ml/cm² UVB (B, C, lanes 4 and 6) for 24 hours (B, C, lanes 3 and 4) or 48 hours (B, C, lanes 5 and 6). Some cells were incubated in fresh basal medium for the same time (B, C, lane 2). After the addition of either conditioned or basal medium, each cell line was incubated a further 48 hours. The supernatants were removed and analyzed by zymography (B, C). A low molecular weight protein standard was run in parallel (B, C, lane 1). Arrowheads: absence of lytic activity corresponding to MMP-1.
that are present in other MMP promoters and are responsive to absence of critical elements in the promoter region of this gene observed in our study. A possible explanation may be the through a direct or indirect pathway. Petersen et al.30 demonstrated a dose-dependent and Herrmann et al. 47 a time course-

FIGURE 8. Inhibition of MMP-1 production in PECs by PD98059. PECs were irradiated with 20 ml/cm2 UVB and treated with various concentrations of PD98059 (A, C) or SB202190 (B), and the supernatants were harvested after 72 hours and analyzed by zymography (A, B) or ELISA (C). Some cells were incubated with the diluent control DMSO (V) after irradiation, whereas other cells were cultured under control conditions (−C) or stimulated with PMA (+C). The data in (C) are the mean ± SD of triplicate experiments. *P < 0.05 or **P < 0.01. (D) RNA was also extracted from cells that were mock irradiated (lane 1), UVB irradiated (lane 2), UVB irradiated and treated with either 10 μM PD98059 (lane 3) or 10 μM SB202190 (lane 4) after 24 hours in culture and analyzed by RT-PCR. Assay controls were identical with those used in Figure 2.

because this lesion resembles a disregulated model of wound healing.

The notion that UV radiation may be a causative factor in the development of pterygia is derived from extensive epidemiologic studies.13–17 In our recent investigations, UV exposure amplified the production of inflammatory cytokines and growth factors in cultured PECs and organ-cultured pterygia.37,38 The present study extends these observations to determine whether the UV-mediated induction of MMP-1 is through a direct or indirect pathway. Petersen et al.30 demonstrated a dose-dependent and Herrmann et al. 47 a time course-
dependent induction of MMP-1 in UVA-irradiated human fibroblasts. As in the current investigation, these studies noted a minimal increase in TIMP-1, suggesting that both genes were dysregulated in response to UV. Corroborating in vivo data were presented by Scharffetter et al.,18 who demonstrated intense localized MMP-1 mRNA in dermal fibroblasts in UVA-exposed human skin. These data also support the hypothesis of UV-mediated tissue damage in pterygia and other sunlight-related diseases, as proteolysis may be in excess relative to inhibitory activity. In dermal fibroblasts, the UVA-mediated induction of MMP-1 is through an autocrine pathway97 that is initiated by the early production of IL-1α/β (within hours), which in turn induces IL-6 and subsequently MMP-1.34

Minimal erythematous doses of UVB have been shown to increase the transcription, translation, and activation status of MMP-1, -3, and -9 but not of MMP-2 in human skin.22 Likewise, no modulation of MMP-2 expression after UVB exposure was observed in our study. A possible explanation may be the absence of critical elements in the promoter region of this gene that are present in other MMP promoters and are responsive to UVB.22 Intense mRNA and protein staining for MMP-1 has been reported in keratinocytes throughout the epidermis of UVB-treated compared with nonirradiated human skin.31 Furthermore, immunoreactivity for this enzyme was noted throughout the collagenous matrix in UVB-exposed skin,23 a result that corroborates our findings (Fig. 1M). MMP-1 was not induced when either human CECs (Fig. 6A) or pterygium fibroblasts (data not shown) were irradiated with UVB, and the fact that keratinocytes rarely respond to UVA suggests that different UV spectra may trigger alternate intracellular signal transduction pathways in certain cells. It has also been noted that cells of differing lineage acquire diverse UV-responsive phenotypes.34

Multiple UV exposures were not performed in the current investigation, and this is perhaps relevant to pterygia, as this lesion often develops over decades. Exposure of human skin to multiple doses of UVB resulted in sustained maximum MMP-1 levels over 7 days.23 Accumulation of matrilysin (MMP-7) and metalloelastase (MMP-12) has been reported in sun-damaged and photosensitized human skin in a bandlike pattern below basal keratinocytes, similar to the MMP-1 staining observed in the present study (Fig. 1A, arrowheads). The same study demonstrated increased MMP-12 after multiple exposures to UVB.50

From our tissue localization studies (Fig. 1) and our ex vivo (Fig. 9) model, it could be argued that levels of MMP-1 reflect the hyperplasia and inflammation that characterize the disease. The high and variable level of this enzyme in nonirradiated specimens is supporting evidence for this hypothesis. However, the significant induction of MMP-1 after UVB exposure suggests a probable role for this agent in the pathophysiology of the disease. Furthermore, no reliable animal model for the disease is available for accurate assessment of the pathophysi-

FIGURE 9. Induction of MMP-1 in UVB-exposed pterygia. Fresh surgical specimens of pterygia were cut symmetrically into halves. One half was irradiated (■ and inset, lanes 2 and 4) and the other half was treated under control conditions (□ and inset, lanes 1 and 3). Supernatants were harvested after 72 hours and MMP-1 levels determined by ELISA or Western blot (inset). Conditioned media in lanes 1 and 2 and in lanes 3 and 4 were derived from patients 2 (P2) and 3 (P3), respectively.
stem cells features that include a distinct cytokeratin profile, p63 expression (data not shown), and an extensive proliferative capacity. Both cell types are also sensitive to the effects of UVB (Fig. 6). In contrast, the CECs have a significantly reduced lifespan and do not respond to UVB with respect to MMP-1 production. It is then tempting to speculate that limbal stem cells in vivo may be more susceptible to and altered by environmental agents. Their activation in situ may result in enhanced invasive and proliferative capacity as they express a wider range of effector molecules.

Although the expression of p63 was not the main focus of the current investigation, our immunohistochemical data resemble findings in a previous study in which little or no staining for p63 was detected in the peripheral and central corneal epithelium (Fig. 1K) but was abundant in the limbus, predominantly in the basal limbal epithelium. This increased staining may be an indicator of enhanced proliferative capacity associated with the least differentiated cells that reside in the basal regions of the limbus. One notable difference between the two studies was the absence of any p63 staining in the superficial limbus, attributable to slight protocol modifications.

Currently, three distinct MAPK pathways have been characterized in detail: ERK1/2, JNK/SAPK, and p38 MAPK. All three pathways can be activated by UV light to varying degrees. This rationale was used to identify the intracellular pathway involved in our model. SB202190 (an inhibitor of p38) had no marked effect on MMP-1 levels, whereas PD98059 (an inhibitor of ERK1/2) potently inhibited the UVB-mediated induction of this protease by at least 50% (Fig. 8). Future studies will focus on components of AP-1 (namely c-Jun and c-Fos), for several reasons: (1) They are both immediate early genes that can be induced by UV. To our knowledge, this is the first study to establish a link between UVB exposure and the induction of matrix-denaturing enzymes in pterygia. The sum of our previous and current data indicate potentially harmful secondary effects of UVB on the induction of proinflammatory cytokines and growth factors, an imbalance in the expression of MMP-1 compared with TIMP-1, and the overexpression of MMP-1 by PECs compared with CECs. If UV light is an environmental factor involved in the pathogenesis of pterygia, then the best form of disease prevention may be ocular protection.

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