UVB-Mediated Induction of Interleukin-6 and -8 in Pterygia and Cultured Human Pterygium Epithelial Cells

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Objective. Pterygia are common ocular surface lesions that are thought to be induced by exposure to ultraviolet (UV) radiation. The hypothesis tested in the current study is that UV radiation modulates the expression of interleukin (IL)-6 and -8, which could promote the neovascularization and chronic inflammation regularly observed in pterygia. Methods. Immunohistochemical analysis was performed on 10 pterygia and 14 specimens of normal conjunctiva (4 of which contained limbus), to identify the cellular source of these cytokines. Pterygium epithelial cells were exposed to UVB (0–100 mJ/cm²) and the expression of cytokine mRNA and protein was determined by reverse transcription-polymerase chain reaction (RT-PCR), RNase protection assay (RPA), and enzyme immunoassay. Similarly, pterygium tissue in organ culture was UVB irradiated and the supernatants analyzed for cytokine production. Results IL-6 and -8 proteins were abundantly expressed, predominantly by the pterygium epithelium, with additional IL-8 immunoreactivity associated with the vascular endothelium. In contrast, significantly less staining for both cytokines was observed in normal conjunctiva, cornea, and limbus. Expression of both IL-6 and -8 mRNA and protein was induced in UVB-irradiated pterygium epithelial cells in a time- and dose-dependent manner. Similarly, IL-6 and -8 proteins were significantly elevated in UVB-treated compared with nonirradiated pterygia. Conclusions. This study provides the first direct experimental evidence that implicates UV in the pathogenesis of pterygia. The two proinflammatory cytokines that are induced by UV radiation may play a key role in the development of pterygia, by initiating blood vessel formation, cellular proliferation, tissue invasion, and inflammation. Strategies aimed at reducing ocular exposure to UV light may decrease the incidence and recurrence of pterygia. (Invest Ophthalmol Vis Sci. 2002;43:3430–3437)

Pterygia are wing-shaped inflammatory fibrovascular lesions that invade the cornea. They are characterized by cellular proliferation, tissue remodeling, and neovascularization. Immunohistologic features of pterygia suggest that these lesions may be derived from altered limbal epithelial stem cells.1 Injury or activation of these stem cells may initiate development of pterygium.

Although the pathogenesis of pterygia is still incompletely understood, there is considerable epidemiologic evidence implicating ultraviolet (UV) radiation as an initiating environmental factor.2-4 In addition, light-focusing experiments in the eye have provided an explanation for the location and shape of pterygia.3 Light and electron microscopic studies have identified elastic changes in the extracellular component of pterygia5 that resemble the actinic degenerative changes seen in chronic UV-exposed skin. Whether these degenerative changes are of primary importance or the development of pterygia is principally a consequence of altered cellular proliferation with associated tumorlike properties is unclear.6,7

Recently, we demonstrated expression of matrix metalloproteinases (MMPs) in resected pterygium specimens8,9 and localized these enzymes at the advancing edges of the lesions.10 In addition, we have presented in vitro data that suggest that proinflammatory cytokines (previously localized in pterygia11) can modify the expression of these extracellular matrix denaturing enzymes.9 These investigations imply that MMPs may play a significant role in tissue remodeling and invasion and in the dissolution of Bowman’s layer associated with pterygia.

Although MMPs may be important effector molecules in the pathogenesis of pterygia,8-10 the roles of cytokines and growth factors have yet to be established. Several studies have documented the expression of cytokines, such as tumor necrosis factor (TNF)-α, basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-β, and platelet-derived growth factor (PDGF) in pterygia and cultured pterygium cells.11,12 In addition, the localization of vascular endothelial growth factor (VEGF) in the pterygium epithelium and vascular endothelium (Di Girolamo N, Kumar RK, Coroneo MT, Wakefield D, unpublished observations, 2000), and the presence of intraepithelial capillaries in pterygia13 suggest a role for angiogenic cytokines in this disease.

IL-8 is a multifunctional cytokine with angiogenic,14 neutrophil chemotactic,15 and keratinocyte proliferative activity.16 This cytokine has also been shown to induce the production of MMPs.17 IL-8 is a product of activated monocytes and fibroblasts and of endothelial and epithelial cells. Similarly, IL-6 is a pleiotropic proinflammatory cytokine synthesized by various cells, such as fibroblasts, endothelial cells, and keratinocytes, in response to numerous cytokines including TNF-α and IL-1. Similar to IL-8, IL-6 can also induce the expression of MMPs.18,19

Consistent with the potential involvement of cytokines and angiogenic factors and the possible role of UV radiation in the development of pterygia, previous studies have shown that some of these mediators can be induced by UV radiation. Kennedy et al.20 exposed human corneal fibroblasts to physiological doses of UVB and demonstrated significant expression of IL-1, -6, -8, and TNF-α. Ansel et al.21 demonstrated an upregulation of the same cytokines in corneal epithelium after exposure to UV radiation. Their data suggest that this induction is mediated by nuclear factor (NF)-κB. In similar experiments, expression of IL-6 mRNA was maximally enhanced 2 to 6 hours after UVB irradiation in human keratinocytes.22 and in UVA-exposed skin fibroblasts.23

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The purposes of this study were to determine the expression and cellular source of IL-6 and -8 in pterygium tissue, compared with normal conjunctiva, cornea, and limbus, and to determine whether UVB irradiation modulates the expression of these cytokines in pterygium tissue ex vivo and in cultured pterygium epithelial cells (PECs). Our results establish a correlation between exposure to UV light and expression of cytokines that may offer some insight into the pathogenesis of pterygia.

**MATERIALS AND METHODS**

**Ocular Tissue**

For immunohistochemical analysis, excised primary pterygia (n = 10) and normal conjunctival tissue (n = 14) were obtained at surgery from the Prince of Wales Hospital, Sydney, Australia. Four of the conjunctival specimens, containing normal limbus, were excess (1–2 mm³) tissue after free conjunctival autografting. Tissue specimens were fixed in formalin and paraffin embedded according to routine procedures. Fresh primary pterygia (n = 8) were obtained for ex vivo organ culture experiments. Informed consent was obtained from each subject and the experimental protocol was approved by the University of New South Wales Ethics Committee and performed in accordance with the tenets of the World Medical Association’s Declaration of Helsinki.

**Immunohistochemical Analysis**

Tissue blocks were serially sectioned (2–4 μm), placed on slides treated with 3-aminopropyltrietoxysilane (Sigma, Sydney, Australia), and processed for immunohistochemistry, as previously described. Briefly, sections were deparaffinized in xylene, rehydrated, quenched for endogenous peroxidase with methanol/H₂O₂, and incubated with a 1:5 dilution of goat serum for 30 minutes. To facilitate antigen retrieval, sections were incubated in 0.01 M citrate buffer (pH 6.0). Sections were then incubated with a 1:50 dilution of anti-IL-6 (R&D Systems, Minneapolis, MN) and processed for immunohistochemistry, as previously described. Immunohistochemistry was performed with an avidin-biotinylated peroxidase complex technique (lsbio, Denmark), covered with 250 μL of PBS and incubated for 40 min/cm² UVB (as described earlier). The other half of each pterygium was exposed to ambient light (for the same length of time) in a laminar flow cabinet (Westinghouse, Sydney, Australia). All tissue specimens were subsequently washed in PBS and incubated for 72 hours in serum-free medium. Supernatants were harvested, stored in small aliquots at −70°C, standardized for total protein (BSA Protein Assay Kit; Pierce, Rockford, IL), and appropriate dilutions of 0.5 μg total protein analyzed by ELISA for IL-6 and -8 production (see below).

**UVB Irradiation of Pterygia**

To determine the effect of UV light on the production of cytokines, fresh pterygia (n = 8) were surgically excised and cut symmetrically in half by the surgeon (MTC). Tissue was placed in sterile saline and transported immediately (<2 hours) to the laboratory on ice. One half of each pterygium was placed in a 24-well plate (Nunc, Roskilde, Denmark), covered with 250 μL of sterile PBS, and irradiated with 40 mJ/cm² UVB (as described earlier). The other half of each pterygium was exposed to ambient light (for the same length of time) in a laminar flow cabinet (Westinghouse, Sydney, Australia). All tissue specimens were subsequently washed in PBS and incubated for 72 hours in serum-free medium. Supernatants were harvested, stored in small aliquots at −70°C, standardized for total protein (BSA Protein Assay Kit; Pierce, Rockford, IL), and appropriate dilutions of 0.5 μg total protein analyzed by ELISA for IL-6 and -8 production (see below).

**Enzyme Immunoassays**

Human IL-1β, -6, and -8 (Immunotech, Marseilles, France) and human TNF-α (Duoset; Genzyme Diagnostics, Cambridge, MA) were quantified with sandwich immunooassays. Cytokines in supernatants from control, PMA-treated, or UVB-irradiated PECs or UVB-exposed pterygia were captured on antibody-coated 96-well plates and detection performed precisely as directed by the manufacturer. The optical density of the reaction product was read at the appropriate wavelength with a microplate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA).

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

Total RNA was extracted (RNagents Total RNA Extraction Kit; Promega, Sydney, Australia) from control and UVB-exposed PECs, as previously outlined. Reverse transcription was performed according to the manufacturer’s instructions (Preamplification System for First-Strand cDNA Synthesis kit; Gibco BRL, Gaithersburg, MD), as previously described. RT-PCR products were analyzed by PCR, in which 100 μL of each of the forward and reverse gene-specific primers for IL-6, IL-8, and GAPDH were used (Table 1). Initially, a 2-minute hot start at 95°C was performed to denature the double-stranded cDNA, followed by 26 to 32 cycles of PCR (each cycle: 95°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds), and the reactions terminated with a 2-minute extension at 72°C. The cycle number was predetermined so that the products formed fell within the linear portion of the amplification curve. Products were visualized on a 1.2% agarose gel, precast with ethidium bromide. Semi-quantitative assessment was performed.
formed on computer (Gel Doc 2000 and Quantity One software programs; Bio-Rad, Sydney, Australia).

RNase Protection Assay

An RNase protection assay (RPA) was used to determine the mRNA expression of multiple cytokines in UV-stimulated PECs. The template for this assay included IL-6 but not IL-8. This assay was performed as previously described26 with minor modifications as specified by the manufacturer. Briefly, the RNA samples (10 μg) were hybridized with [α-32P]labeled RNA probes (RiboQuant Human Template Set; BD Biosciences, Mountain View, CA) at 56°C for 12 to 16 hours. Unpaired RNA was degraded by treatment with RNase A and RNase T1 at 37°C for 30 minutes. The purified and protected fragments were denatured and electrophoresed in a standard 6% polyacrylamide, 7 M urea, 0.5%
Localization of IL-6 in Pterygia

The expression and distribution of IL-6 in diseased pterygium and control conjunctival, corneal, and limbal tissue was assessed. IL-6 protein was demonstrable in all pterygium specimens, predominantly associated with the superficial epithelium (Figs. 1A–C, 1J), but was absent in the basal pterygium epithelium (Figs. 1A–C, 1J, arrows). Very faint staining for this cytokine was occasionally detected in some vascular endothelial cells (Fig. 1J, arrowheads). IL-6 reactivity in normal conjunctiva (Fig. 1D), cornea (Fig. 1I), and limbus (Fig. 1H) was minimal. The limbal epithelium was identified by the palisades of Vogt (Fig. 1G). No goblet cells were present in the limbal epithelium. Cells in the basal limbal epithelium were similar to those of the cornea but were generally smaller and more closely packed (Fig. 1G). No immunoreactivity developed when tissue specimens were incubated with an appropriate isotype control antibody (Fig. 1E) or when the primary antibody was omitted (data not shown). As a positive control, inflamed tonsillar tissue was assessed for IL-6 protein expression. Similar to the pattern observed in pterygia, there was moderate staining of the superficial mucosal epithelium for this cytokine (Fig. 1F) as well as reactivity in some vascular endothelial cells (micrographs not shown) and no staining was found in the basal epithelium. IL-6 protein was differentially expressed at the advancing edge of the pterygium. Although the basal pterygium epithelium displayed minimal-to-absent immunoreactivity and the superficial pterygium epithelium demonstrated moderate IL-6 staining, a group of differentiated, proliferating, and invading squamous epithelial cells displayed intense cellular staining for this cytokine at the invading edge (Fig. 1J).

Localization of IL-8 in Pterygia

Formalin-fixed, paraffin-embedded pterygia were serially sectioned and stained to determine the distribution of IL-8. Intense immunoreactivity for this cytokine was observed in pterygia, particularly in superficial epithelium (Figs. 2A, 2G) and the vascular endothelium (Fig. 2B), although not all the vascular endothelial cells were reactive (Figs. 2B, 2G, arrowhead). A similar pattern of IL-8 staining was observed in a specimen of inflamed ocular tissue (derived from a donor conjunctival site, post pterygium surgery) that served as a positive control (Fig. 2F). Occasionally, IL-8 was detected in resident stromal pterygium fibroblasts (micrographs not shown). IL-8 protein was also detected in the majority of normal human conjunctival (Fig. 2C), limbal (Fig. 2D), and corneal (Fig. 2G, inset) tissue specimens analyzed but the intensity and extent of staining for this cytokine were significantly less than in pterygia. Consistent with the potent neutrophil chemotactic activity exhibited by IL-8, recruitment of neutrophils was demonstrated in most pterygium specimens. These cells were found either adherent to blood vessel walls (Fig. 2E), extravasating from blood vessels (micrographs not shown), or among pterygium epithelial cells (Fig. 2E). Epithelial expression of IL-8 protein was most striking at the advancing edge of the pterygium, where a cluster of differentiated, proliferating, and invading squamous epithelial cells exhibited intense cell-associated staining for this cytokine (Fig. 2G). In contrast, the basal columnar epithelium demonstrated minimal IL-8 reactivity (Fig. 2G).

Dose Effect of UV Light on Cytokine Production in PECs

Monolayers of semiconfluent PECs were irradiated with various doses of UVB, and IL-6 and -8 levels in the supernatants quantified by enzyme immunoassay. Exposure of PECs to UVB resulted in a dose-dependent induction of both IL-6 and -8 (Fig. 3). IL-6 production was significantly increased at doses of 20 mJ/cm² (2.2-fold induction) and 40 mJ/cm² (4.6-fold induction), relative to control values. The production of IL-8 paralleled that of IL-6. This cytokine was significantly increased (7.5-fold) when exposed to 20 mJ/cm² of UVB and peaked (71-fold) when PECs were exposed to 40 mJ/cm² of UVB (Fig. 3). IL-1β and TNF-α were not detected in the same conditioned media. Doses of UV ranging from 0 to 40 mJ/cm² did not cause morphologic changes, nor was there a loss in cell viability as assessed by trypan blue exclusion. However, PECs exposed to greater than 40 mJ/cm² UVB changed from a flattened, cuboidal appearance to spindly and irregular cells, some of which detached from their substratum, and there was a substantial reduction in viability. Treatment with the positive control stimulus PMA potently increased production of IL-6 (8.6-fold) to a level of 15,460 ± 181 pg/mL (P < 0.01) and significantly enhanced IL-8 levels to 12,894 ± 590 pg/mL (P < 0.01), which represented a 170-fold induction.
Time-Course–Dependent Induction of Cytokines after UV Exposure

A dose of 20 mJ/cm² of UVB irradiation that caused no change in cell morphology or viability was used to assess the time course of secretion of IL-6 and -8. Exposure of PECs to this dose resulted in a significant induction (5.9-fold) of IL-6 above constitutive levels at 24 hours after irradiation, with maximal production (twofold increase over baseline) achieved at 72 hours after exposure to UV light (Fig. 4A). Similarly, IL-8 was rapidly and significantly induced (4.9-fold) at 24 hours, with a peak at 48 hours after exposure to UV irradiation, which represented a 4.7-fold increase above baseline levels (Fig. 4B).

Induction of IL-6 and -8 mRNAs in UVB-Irradiated PECs

Total RNA was extracted from PECs and analyzed by RT-PCR to examine the effect of 20 mJ/cm² UVB irradiation on expression of cytokine mRNA 24 hours after irradiation. IL-6 mRNA was constitutively expressed in untreated PECs, but was induced 2.2-fold after exposure to UVB (Fig. 5A). Similarly, IL-8 mRNA was increased by 18-fold after UVB irradiation (Fig. 5B). The expression of the house-keeping gene GAPDH remained relatively unaltered after UV treatment (Fig. 5C).

Multi-cytokine Gene mRNA Analysis

Having localized both IL-6 and -8 in pterygia and demonstrated the UVB-mediated induction of both cytokines in cultured PECs, a multi-cytokine RPA was performed to determine whether UVB radiation could influence the expression of other relevant cytokines. Of the eight detectable cytokines, IL-6 was the only mRNA species observed, a result that corroborated the RT-PCR (Fig. 5A). After standardizing the RNA loading and signal intensity to GAPDH, IL-6 was constitutively expressed in nonstimulated cells (Fig. 6, lane 1), but was enhanced approximately 1.8-fold in UVB-exposed PECs (Fig. 6, lane 2).

Induction of IL-6 and -8 in UVB-Irradiated Pterygia

IL-6 (Fig. 7A) and -8 (Fig. 7B) proteins were significantly ($P < 0.05$) enhanced (1.5-fold and 1.7-fold, respectively) in UVB-exposed pterygia compared with the corresponding nonirradiated tissue specimens. High levels of both cytokines were noted in nonirradiated pterygia, and significant variations in cytokine concentration were observed between pterygium samples, which may reflect differences in pterygium development or disease activity at the time of surgery.

DISCUSSION

In the present study, we examined the expression of two potent proinflammatory cytokines in pterygium specimens, as
well as the effect of UVB irradiation on cytokine expression by both pterygium tissue and by epithelial cells in culture. We demonstrated abundant immunoreactivity of IL-8 in pterygium epithelium and in the vascular endothelium. Similarly, abundant expression of IL-6 was identified in the pterygium epithelium. A striking feature of the pattern of expression of these two cytokines was that they appeared to be markedly upregulated at the advancing or invading edge of the pterygium, with moderate expression elsewhere in the superficial epithelium and little expression in the basal epithelium (Figs. 1, 2). The specific roles of IL-6 and -8 in pterygia are uncertain, but it is tempting to speculate that IL-8 may be responsible for the accumulation of leukocytes, formation of new blood vessels, and proliferation of PECs, because previous studies have documented its potent angiogenic, neutrophil chemotactic, and keratinocyte proliferative activities. Similar to IL-8, the pluripotent cytokine IL-6 has been assigned various roles including angiogenesis, in that it has been shown to induce VEGF; mitogenic and tumor cell growth factor activity; metastatic activity, in that its overexpression has been shown to correlate with cancer; and antiapoptotic activity. The data presented in the current investigation suggest that IL-6 and -8 may act in concert to promote inflammation, cellular proliferation, and angiogenesis in pterygia, because minimal expression of both cytokines was observed in normal conjunctiva and cornea. Although pterygia are thought to arise at the limbus, significantly less staining was revealed for both IL-6 (Fig. 1H) and -8 (Fig. 2D) in the superior limbus, suggesting that limbal tissue in this region is relatively quiescent.

Although the pathogenesis of pterygia is still poorly understood, epidemiologic evidence suggests that environmental stress may have a role. Of the potential agents, UV irradiation has received the greatest attention. In the present study, we observed the induction of IL-6 and -8 mRNA and protein in UVB-irradiated PECs (Figs. 3, 4, 5, 6) and in UVB-exposed pterygia (Fig. 7). It is well established that ocular surface epithelial cells produce these cytokines either constitutively or in response to a stimulus. Corroborating data have also been presented by other investigators, who have shown that UVB-exposed human skin keratinocytes produce TNF-α and IL-8, which correlates with increased expression of E-selectin and accumulation of neutrophils. Other studies in UVB-irradiated and implanted human cutaneous melanomas have demonstrated increased expression of IL-8 that correlates with angiogenesis, tumorigenicity, and metastatic ability, possibly because of enhanced expression of MMPs. In addition, abundant epidermal expression of bFGF and VEGF has been noted in UVB-exposed mouse skin. Similar in vitro investigations...
have shown increased production of IL-1, -6, and -8 and TNF-α in cultured human corneal fibroblasts,20 and corneal epithelium21 after UVB irradiation. de Vos et al.22 irradiated human keratinocytes with exposures similar in duration and dose to those used in the present study and found that IL-6 mRNA was enhanced in a time- and dose-dependent manner. Other investigators have linked UVB irradiation to the production of the immunosuppressive cytokine IL-10 from human keratinocytes,18,19 making it likely that they would also induce TIMPs,18,19 and their tissue inhibitory proteins such as MMPs. Both classes of proteins may be responsible (directly or indirectly) for initiating a series of concurrent events, such as increased cellular proliferation and migration, angiogenesis, inflammation, apoptosis, and tissue invasion and degradation, that may lead to formation of pterygium.

Despite detecting immunoreactivity for both IL-6 and -8 in the superficial cells of resected pterygia, significantly elevated amounts were detected in a group of proliferating and migrating epithelial cells at the advancing edge. This pattern of staining was identified when tissue specimens were oriented so that Bowman’s layer and the advancing edge could be distinguished (Figs. 1J, 2G). Although this was a surprising result, cytokines in the superficial-to-intermediary layers of the pterygium epithelium may diffuse to the more basal epithelium and signal those cells for the induction of other gene products such as the MMPs.

The results of the present study, together with those presented by other investigators has led us to develop a hypothetical model of how pterygia form (Fig. 8). We propose that UV light could be the initial trigger that activates epithelial cells at or near the limbus to produce cytokines such as IL-6 and -8. These multifunctional proteins set up a cascade of events that include inflammation,15 proliferation,16,26 angiogenesis,14,27 and apoptosis.30 In other models, cytokines may be able to induce the expression of MMPs17–19 and their tissue inhibitors (TIMPs),18,19 making it likely that they would also indirectly affect the rate of tissue remodeling, such as destruction of Bowman’s membrane and the invasion of pterygium. The abundant expression of IL-8 and the obvious increased leukocyte infiltration is consistent with its chemotactic activity and suggests that the accumulation of neutrophils in pterygium may be due in part to the expression of this cytokine. It is also clear from other studies that apoptosis may be directly related to UV exposure. It is well established that UV is mutagenic to the p53 tumor-suppressor gene,39 and abnormal p53 expression has been reported in pterygia.40–41 Although p53 has recently received considerable attention in pterygia, an alternative mechanism of apoptosis could be through the induction of IL-630 or the TIMPs42,43 (Fig. 8).

In a recent investigation, Wang et al.44 demonstrated the potential role of UV in the abnormal elastin accumulation in pterygia. They demonstrated several point mutations in the 3’ untranslated region of the elastin gene in UV-irradiated normal conjunctival fibroblasts that were absent from untreated conjunctival fibroblasts, but were identified in nonexposed pterygium fibroblasts. Thus, it is likely that elastosis in pterygium may be due to UV-mediated damage,5 because a similar histologic pattern has pterygium demonstrated in solar-exposed skin.45 Induction of proteolytic enzymes by proinflammatory cytokines may also result in pterygium elastosis, as has been observed in human skin.25,46

We conclude that the two multifunctional cytokines IL-6 and -8 are expressed in pterygia and that their production may be significantly enhanced by UVB radiation. Minimizing UV light exposure may be the best approach to preventing development of these lesions.

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References


