UVB-Mediated Induction of Cytokines and Growth Factors in Pterygium Epithelial Cells Involves Cell Surface Receptors and Intracellular Signaling

Nick Di Girolamo,1 Denis Wakefield,1 and Minas T. Coroneo2

PURPOSE. Pterygium is a proliferative, inflammatory, and invasive ocular surface disease associated with excessive ultraviolet (UV) exposure. This investigation was conducted to identify UV activated signaling pathways in pterygium epithelial cells (PECs) that mediate cytokine and growth factor production and to determine whether these pathways are sensitive to blockade by anti-inflammatory agents such as retinoic acid (RA) and interleukin (IL)-6, IL-8, and vascular endothelial growth factor (VEGF). Cytokine mRNA was assessed by reverse transcription-polymerase chain reaction (RT-PCR).

METHODS PECs were pretreated with or without inhibitors of the ERK1/2, JNK, and p38 (PD98059, SB202190, and SB203580, respectively) mitogen-activated protein kinases (MAPK) or with inhibitors of the tyrosine kinase activity of epidermal growth factor receptor (EGFR; PD153035) and platelet-derived growth factor (PDGF; AG1295); exposed to UVB (20 mJ/cm2) and then further treated with the same inhibitors. Media were harvested and analyzed by ELISA for IL-6, IL-8, and vascular endothelial growth factor (VEGF). Cytokine mRNA was assessed by reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS Inhibitors of ERK1/2, JNK, and p38 MAPKs significantly abolished the UVB-mediated increase in IL-6, IL-8, and VEGF. PD153035 reduced IL-8, AG1295 repressed IL-6, and both inhibitors partially downregulated VEGF production in UV-exposed PECs. RA and IFN-α dose dependently abrogated IL-6 and IL-8 but had no effect on VEGF expression after UV exposure.

CONCLUSIONS The results have identified a stress-induced intracellular pathway and potential cell-surface transmitters that may be relevant to pterygium development. Moreover, two anti-inflammatory/antiangiogenic agents were identified that reduced cytokine production in the study model. Topical application of these drugs may benefit patients with pterygia, potentially reducing the necessity for surgical intervention. (Invest Ophthalmol Vis Sci. 2006;47:2430–2437) DOI: 10.1167/iovs.05-1130

From the 1Inflammatory Diseases Research Unit, Department of Pathology, School of Medical Sciences, University of New South Wales, Sydney, Australia; and the 2Department of Ophthalmology, Prince of Wales Hospital, Sydney, Australia. Supported by Grant 350919 National Health and Medical Research Council of Australia. Submitted for publication August 24, 2005; revised December 5, 2005, and January 18, 2006; accepted March 13, 2006.

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Corresponding author: Nick Di Girolamo, Inflammatory Diseases Research Unit, Department of Pathology, School of Medical Sciences, The University of New South Wales, Sydney, Australia; n.digidrolamo@unsw.edu.au.

Pterygium is an invasive and proliferative disease of the human ocular surface particularly prevalent in sun-exposed individuals.1,2 Histologic examinations have identified foci of connective tissue elastosis3 and regions of severely damaged Bowman’s membrane,4 suggesting either direct damage due to solar radiation or indirect damage due to excessive proteolytic activity.5–9 Pterygia are characterized by an inflammatory infiltrate, composed of neutrophils,5 mast cells,6,10 and lymphocytes.5,11 They can present with a prominent vascular reaction1–12 that is likely to be exacerbated by excessive cytokine1,3 and growth factor13–18 production involving resident cells, as well as infiltrating leukocytes.

Recently, our laboratory has identified several key effector molecules that are likely to participate in the inflammatory, proliferative, and remodeling phase of this disease. These include interleukin (IL)-619 and IL-8,9 heparin-binding epidermal growth factor-like growth factor (HB-EGF),20 vascular endothelial growth factor (VEGF),4 and several matrix metalloproteinases (MMPs).5–7 Relevant to the abundant epidemiologic evidence implicating ultraviolet (UV) exposure as a probable trigger for this disease,1,2 these molecules are induced by physiological doses of UVB radiation in ex vivo pterygium specimens19 as well as in cultured pterygium epithelial cells (PECs).19,21 Our studies have also demonstrated that the induction of collagenase-1 (MMP-1) by UVB is specifically mediated by the ERK1/2 mitogen activated protein kinase (MAPK) pathway and did not involve other MAPK pathways—namely, p38 or JNK.21,22 Moreover, we noted that the UVB signal was partially transmitted by the epidermal growth factor receptor (EGFR).23 This too is highly relevant, as this receptor22,23 and other growth factor receptors, such as platelet-derived growth factor receptor (PDGFR),24 have been identified in pterygia.

Given the results of our previous investigations, the present study was designed to explore whether a similar intracellular pathway and mode of signal transmission is responsible for cytokine production in a well-defined in vitro system. To assist us in elucidating the potential mechanism, we developed a working model (Fig. 1). In brief, we hypothesize that UVB radiation triggers pterygium growth by activating (1) extracellular molecules (such as growth factor receptors), (2) intracelular signaling pathways (such as the MAPKs), and (3) nuclear transcription factors (such as AP-1) that ultimately result in enhanced production of effector proteins including cytokines, growth factors, and MMPs. In this study, experiments were designed to address several key questions specifically: What intracellular signaling pathways are responsible for the UVB-mediated induction of IL-6, IL-8, and VEGF? Are cell-surface receptors such as the EGFR and PDGFR capable of transmitting this signal? Are antiproliferative/antiangiogenic agents capable of suppressing cytokine expression in UVB-exposed epithelial cells?

Surgical intervention25 accompanied by adjunct therapy with β-irradiation26 or mitomycin-C (MMC)27 have been used to treat pterygia. However, follow-up studies have identified unacceptable recurrence rates with the use of these latter agents. Significantly better results have been observed with...
Inhibition of Cytokine Production by RA and IFN-α

Conjunctival autografts and amniotic membrane transplantation. In addition to understanding the molecular mechanisms that regulate pterygium development after UV stimulation, our research was also intended to determine whether surface receptors were involved in transmitting the UVB signal, some cells were preincubated for 1 hour in 0 to 1.0 μM PD153035 (Calbiochem, CA), an inhibitor of the tyrosine kinase activity of the PDGFR. Whereas cells were pretreated in other experiments with RA (0–0.1 μM; Sigma-Aldrich), or IFN-α (0 to 15,000 U; PBL Biomedical Laboratories, Piscataway, NJ). After the preincubation period, the medium containing the respective agents was replaced with PBS and the monolayers irradiated with 20 ml/cm² of UVB (ITL 20W/12 RS bulbs; Philips, Sydney, Australia), as previously reported. This amount of radiation equated to a 3-minute exposure and was an amount that did not affect cell morphology or viability. UVB light intensity was monitored and calibrated before each experiment with the aid of a radiometer (ILL400A, International Light, Newburyport, MA). After each exposure, cells were rinsed once with PBS and placed in fresh serum-free medium with the respective agents and incubated for a further 48 hours. Supernatants were collected, cleared of any cell debris by centrifugation, and stored frozen in small aliquots at −70°C. Compounds tested in these experiments were nontoxic at the doses used.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted (RNeasy Total RNA Extraction Kit; Promega, Sydney, Australia) after 24 hours as previously outlined.
from control (mock-irradiated), and UVB-exposed PEC that were pre- and posttreated with the agents described earlier. This time point was selected as it corresponded to the peak in cytokine and growth factor transcriptional activity. Reverse transcription was performed according to the manufacturer’s instructions (Preamplification system for first strand cDNA synthesis kit; Invitrogen-Gibco, Gaithersburg, MD), as previously described. An aliquot (1 μL) of cDNA was amplified by PCR with 100 nM each of the forward and reverse gene specific primers for IL-6, IL-8, VEGF (reverse 5’-CTG TAT CTT TGC CGT TCT-3‘; forward 5’-CCA TGA ACT TTC TGC TGT CTT-3‘), and GAPDH. The VEGF primers used in the study were designed to amplify at least three of the four spliced variants including VEGF121, VEGF165, and VEGF206. 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 number of cycles was predetermined for each PCR product. PCR products were visualized on either 1.2% or 2% agarose gels that contained ethidium bromide. Semiquantitative assessment was performed after normalization to GAPDH (Gel Doc 2000; Bio-Rad, Sydney, Australia; and the Quantity One software program; Bio-Rad).

**Enzyme Immunoassays**

Human IL-6, IL-8, and VEGF (Quantikine; R&D Systems, Minneapolis, MN) were quantified using sandwich immunoassays. The VEGF ELISA used in this study specifically detected VEGF165, one of the soluble variants of this growth factor. Cytokines in supernatants from control (mock irradiated), or UVB-exposed PECs that were incubated with specific agents, were captured on antibody-coated 96-well plates and detected precisely as directed by the manufacturer. The optical density of the reaction product was read at the appropriate wavelength using a microplate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA).

**Statistical Analysis**

Cytokine levels in the culture supernatants were expressed as mean ± SD. Difference in cytokine level between control and UV-treated cells was assessed by one-way analysis of variance, followed by the Newman-Keuls test for multiple comparisons of treatment groups with the control group. A commercial software package (Prism; GraphPad Software, San Diego, CA) was used for analysis.

**RESULTS**

**Mediation of Induction of IL-6, IL-8, and VEGF**

To determine whether cytokine and growth factor induction after UVB exposure is mediated by a MAPK pathway, PECs were pretreated, irradiated, and posttreated with specific intracellular pathway inhibitors. Initially, we noted a significant upregulation of IL-8, IL-6, and VEGF protein production (Fig. 2) after UVB exposure. The addition of specific MAPK inhibitors (SB202190, SB203580, and PD98059) abrogated IL-8, IL-6, and VEGF production, respectively, after irradiation (Fig. 2). These agents inhibited IL-8 (Fig. 2A) and IL-6 (Fig. 2B), whereas VEGF production was only partially, but still significantly, reduced. Hydrocortisone suppressed the production of all three proteins to near control levels, whereas the diluent control (DMSO) had no significant effect on cytokine production (Fig. 2).

Similar experiments were conducted to determine whether selective MAPK inhibitors were effective at the level of gene transcription. Indeed, when PECs were exposed to the same level of UVB radiation, we observed a 5.5-, 1.8-, and 1.5-fold increase in IL-8, IL-6, and VEGF gene expression, respectively (Figs. 3A, 3B, 3C). Furthermore, the addition of the MAPK inhibitors (SB202190, SB203580, and PD98059) significantly suppressed IL-8 (Fig. 3A) and IL-6 (Fig. 3B), whereas they only moderately inhibited VEGF (Fig. 3C) mRNA expression. The results of this analysis closely resembled the cytokine protein production data (Fig. 2).

**Cell-Surface Receptors and Transmission of the UVB Signal**

Next, we investigated whether cell-surface receptors (previously localized in pterygia) might function as transmitters of the UVB signal. In a recent investigation, the EGFR was found to be partially responsible for transmitting the signal that resulted in the induction of collagenase-1 (MMP-1). We demonstrated, using the same experimental model, that EGFR, but not PDGFR, was responsible for relaying the UVB signal. In a recent investigation, the EGFR was found to be partially responsible for transmitting the signal that resulted in the induction of collagenase-1 (MMP-1). We demonstrated, using the same experimental model, that EGFR, but not PDGFR, was responsible for relaying the UVB signal. In a recent investigation, the EGFR was found to be partially responsible for transmitting the signal that resulted in the induction of collagenase-1 (MMP-1). We demonstrated, using the same experimental model, that EGFR, but not PDGFR, was responsible for relaying the UVB signal.
Inhibition of Cytokine Production by RA and IFN-α

We simulated environmental UV exposure in a controlled laboratory setting and demonstrated that a single physiological dose of this agent increased the production of several inflammatory and angiogenic mediators (Figs. 2–6).19,20 Enhanced production of IL-6,45 IL-8,46 and VEGF47 has been documented in UVB-irradiated human skin keratinocytes. Given that the skin is the largest and most exposed organ of the body, this comes as no surprise and is thought to be a tissue response to injury. The ocular surface is subjected to similar environmental stress and cytokines such as IL-1, -6, and -8 and TNF-α have been shown to be induced in UV exposed corneal epithelial cells48 and corneas in vivo.49

Multiple UV exposure to the skin triggers downstream events resulting in cell differentiation, proliferation, invasion, inflammation, and angiogenesis.46,50 Our model is not sufficiently robust to accommodate several exposures. Furthermore, a monolayer of epithelial cells cultured under serum-minimized conditions does not accurately represent the complex cell-to-cell and cell–matrix interaction of an intact tissue. Therefore, we cannot comment on the potential epithelial influence on the stromal component, but we postulate that cell-to-cell cross-talk is an integral part of pterygium development.4

Recently, our research has focused on mapping the likely molecules and pathways that support our working hypothesis.
In addition to demonstrating the UVB-mediated enhanced expression of key proinflammatory cytokines (IL-6 and IL-8) and growth factors (VEGF and HB-EGF), we have identified several MAPK pathways that are activated in response to this stress signal (Fig. 1). Furthermore, we have identified two stress sensitive cell-surface receptors (EGFR and PDGFR) as potential signal transmitters, as specific tyrosine kinase inhibitors of these receptors suppressed cytokine production after UVB exposure. Although the mechanism of action is not entirely clear (Fig. 1), RA and IFN-α significantly abrogated both IL-6 and IL-8 mRNA expression and protein production in our model (Figs. 5, 6).

Receptor signaling is a complex process and can be mediated through direct binding of ligand (ligand-dependent) or indirectly by physical stress such as osmosis and UV radiation (ligand-independent; Fig. 1). Although several EGFR, PDGFR ligands have been identified in pterygium, we previously proposed that these receptors are activated through a ligand-independent pathway. Despite this circumstantial evidence, it is tempting to speculate that the UVB-mediated cytokine–growth factor induction observed in the present study may have been caused by a secondary mediator such as another cytokine (e.g., TNF-α or IL-1). We have excluded this possibility, as several proinflammatory cytokines including TNF-α and IL-1 were not induced by UVB in our model (data not shown). Furthermore, in a previous investigation, we attempted to measure both IL-1 and TNF-α in supernatants derived from UVB irradiated PECs using commercial ELISAs. However, neither cytokine was detectable, nor were mRNA transcripts identified by an RNase protection assay, further confirming the irrelevance of these cytokines in our assay system. These results closely resemble those of other investigators who demonstrated that the UV-mediated induction of VEGF in epithelial cells is totally independent of TNF-α. Likewise, Blaudschun et al. demonstrated that the UVB-mediated induction of VEGF in skin keratinocytes is dependent on ATRA and IFN-α. PECs were pre- and then posttreated with either RA (0.1 μM) or IFN-α (15,000 U) after UVB (20 mJ/cm²) irradiation. Total RNA was isolated 24 hours later and analyzed by RT-PCR. The values shown above each band indicate the x-fold increase compared with nonirradiated cells after standardizing to GAPDH expression. Similar results were obtained with PECs from another donor.

Figure 5. Downregulation of IL-6 and IL-8 protein by RA and IFN-α. PECs were pre- and then posttreated with RA (A, C, E) or IFN-α (B, D, F) after UVB (20 mJ/cm²) irradiation. Supernatants were harvested 48 hours later and analyzed by ELISA. Some cells were mock irradiated (Control) or incubated with the diluent control (DMSO) without exposure. A similar pattern of inhibition was observed with at least one other diseased epithelial cell line. Data represent the mean ± SD of results in triplicate experiments. *P < 0.01.

Figure 6. Suppression of IL-6 and IL-8 mRNA by RA and IFN-α. PECs were pre- and then posttreated with either RA (0.1 μM) or IFN-α (15,000 U) after UVB (20 mJ/cm²) irradiation. Total RNA was isolated 24 hours later and analyzed by RT-PCR. The values shown above each band indicate the x-fold increase compared with nonirradiated cells after standardizing to GAPDH expression. Similar results were obtained with PECs from another donor.

(Fig. 1).
on phosphorylation of the EGFR and not due to ligand binding. Irrespective of the mode of activation, EGFR signaling has been reported to facilitate cell proliferation, apoptosis, angiogenesis, and metastasis. For this reason, tyrosine kinase inhibitors of such receptors have been used to treat patients with advanced malignancies and may be a nonsurgical strategy worth considering for pterygia.

Once initiated, receptor phosphorylation proceeds within minutes, with subsequent activation of intracellular signaling pathways. In the current investigation, we focused our attention on the MAPK pathway for several reasons. First, we have documented the exclusive involvement of the ERK1/2 in the UVB-mediated induction of MMP-1 in PECs. Second, JNK, p38, and ERK1/2 are all activated, although to varying degrees, in UVB exposed skin keratinocytes. Third, activation of one or more of these pathways amplifies nuclear transcription factor expression (c-jun and c-fos) resulting in cytokine production in skin and ocular surface epithelial cells. Finally, MAPKs have overlapping as well as contrasting effects relating to apoptosis and survival and these processes have been a topic of debate in pterygia.

In the current investigation we noted that RA and IFN-α inhibited IL-6 and IL-8 (but not VEGF) in UV-irradiated fibroblasts. This finding is highly relevant, as these cytokines have been identified in pterygium specimens, and, given their mitogenic, angiogenic, and proinflammatory activity, selective repression of these effector molecules may have therapeutic application. Several investigators have successfully used topical IFN-α and RA to treat primary as well as recurrent corneal/conjunctival neoplasia without side effects. Features of corneal neoplasia resemble pterygia. These include a strong mitogenic, angiogenic, and proinflammatory activity, se-

References

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