Proliferative Effects of Heparin-Binding Epidermal Growth Factor–like Growth Factor on Pterygium Epithelial Cells and Fibroblasts

Timothy M. Nolan, Nick Di Girolamo, Minas T. Coroneo, and Denis Wakefield

PURPOSE. To investigate the growth promoting and chemotactic effects of heparin binding epidermal growth factor-like growth factor (HB-EGF), recently shown to be upregulated by ultraviolet irradiation in pterygium-derived epithelium cells (PECs) and pterygium fibroblasts (PFs).

METHODS. PECs and PFs were incubated with various concentrations of HB-EGF. Cell proliferation was evaluated by measurement of [3H]thymidine incorporation. The potential chemotactic effect of HB-EGF on these two cell lines was assessed with migration assays, using modified Boyden chambers and checkerboard analysis.

RESULTS. Incubation of PECs and PFs with HB-EGF resulted in a significant increase in [3H]thymidine incorporation. HB-EGF stimulated chemotaxis of both PECs and PFs. Maximum stimulation occurred at 1 ng/mL for PFs and 10 ng/mL for PECs. These effects were abolished by the addition of a neutralizing antibody to HB-EGF.

CONCLUSIONS. The findings demonstrate the potential proliferative and chemotactic effects of HB-EGF on both PECs and PFs. This is the first study to illustrate the positive effect of a specific growth or chemotactic factor on the cellular elements of a pterygium. (Invest Ophthalmol Vis Sci. 2004;45:110–113) DOI:10.1167/iovs.03-0046

A pterygium is a disorder involving epithelial hyperplasia and accompanying fibrovascular proliferation that extends into the cornea. This growth is thought to originate at the corneal–conjunctival junction, where it is proposed that altered limbal stem cells migrate centripetally to encroach on the central cornea.

The role of UV light as an etiological agent for pterygia remains unsubstantiated, although there is strong epidemiologic evidence for UV as a possible causative factor. Several studies have demonstrated a significant association between pterygia and UV exposure. Peripheral focusing of scattered light entering the anterior chamber laterally provides a possible explanation and establishes a link between UV irradiation and the predilection for the formation of a pterygium on the medial cornea.

UV light has long been implicated as the etiological agent for cutaneous malignancies, such as melanoma, basal cell carcinoma, and squamous cell carcinoma. The mechanism by which UV light achieves this has been extensively studied, and it appears that cytokines and growth factors play a central role in the process. Despite the long-standing supposition of an association between UV exposure and pterygium, only recently has there been a link between UV irradiation and cytokine and growth factor expression been established in pterygium epithelial cells (PECs). Recently, we conducted several studies outlining the growth factors that were upregulated in PECs exposed to UVB irradiation. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) was shown to be upregulated in PECs and was localized to the epithelium and vasculature of pterygium specimens by immunohistochemical assays.

HB-EGF was originally identified as a 20- to 22-kDa soluble glycoprotein that is structurally a member of the EGF family. HB-EGF is targeted to certain cells, particularly fibroblasts, smooth muscle cells (SMCs), and keratinocytes. It is a potent mitogen for many cell types, including mouse 3T3 fibroblasts, bovine aortic SMCs, human epithelial cells, mouse keratinocytes, human keratinocytes, and breast and ovarian tumor cells.

Although previous studies have demonstrated the presence of growth factors in pterygia, none have shown an altered expression of growth factors in response to UV light. In this study we investigated the effects of HB-EGF on PEC and PF proliferation and migration–chemotaxis.

METHODS

Donor Ocular Tissue and Cell Culture

PTERYGIUM EPITHELIAL CELLS. Epithelial cells were cultured and characterized from explants of fresh pterygium tissue (1–2 mm²), as previously reported. PECs were subsequently expanded in 75-cm² tissue culture flasks (Nunc, Roskilde, Denmark) in Eagle’s minimum essential medium (EMEM; Trace Biosciences, Sydney, Australia) supplemented with 10% fetal bovine serum (FBS; Trace Biosciences), 100 U/mL penicillin, and 100 mg/mL streptomycin (Trace Biosciences). Cells in the third to seventh passages were used for the experiments described in the present study. All data presented were obtained from the PECs derived from one pterygium, but similar results were obtained with cells derived from two additional pterygia from different patients. Informed consent was obtained from each subject. 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.

PTERYGIUM FIBROBLASTS. Fibroblasts were cultured from explants of fresh pterygium tissue (1–2 mm²), as were the PECs, but with the following difference: The pterygium body, containing the PFs, was separated from the epithelium at the time of surgery and cultured separately. Culture medium and passages were the same as for PECs. As with PECs, all data presented were obtained with the PFs derived from one pterygium, but similar results were obtained with cells derived from two additional pterygia from different patients. PFs were characterized by their site of origin, their distinctive morphology, and flow
cytometric analysis. Because of the well-documented ability of fibroblasts to express otherwise foreign proteins in culture,\textsuperscript{27} flow cytometry was performed with anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody stains epithelium exclusively in pterygium tissue (Nolan et al., unpublished data, 2003). Negative immunoreactivity suggests that these cells constituted a pure population of fibroblasts. PECs were characterized by morphology and by flow cytometric analysis, which revealed positive p65 immunoreactivity in 96% of the cells, suggesting that they constituted a pure population of epithelial cells.

**Flow Cytometric Analysis**

Flow cytometric analysis was performed on cultured cells at passage 4 according to an immunofluorescence technique previously described.\textsuperscript{24} Briefly, after trypsin digestion, cells were allowed to reestablish protein expression for 1 hour at 37°C in (2% FBS/PBS). The cells were counted, centrifuged at 1600 rpm for 5 minutes, washed in PBS, resuspended in cold 100% methanol, and permeabilized for 10 minutes at −20°C. They were centrifuged to remove the methanol, resuspended in cold 0.1% Triton X-100 (Sigma-Aldrich, Castle Hill, Australia), immediately centrifuged at 400g for 5 minutes, diluted in 300 μL of PBS, and aliquoted into each of three tubes for direct immunofluorescence. Cells were incubated with the anti-p65 antibody for 30 minutes, washed three times in 2% BSA/PBS, blocked with human serum, incubated with a secondary FITC-conjugated Fab fragment-specific antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 minutes, washed three times in 2% BSA/PBS, and resuspended in 1% paraformaldehyde. Data were acquired with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) and processed with the accompanying system software program (Cell Quest; BD Biosciences).

**Measurement of \[^{3}H\]Thymidine Incorporation**

PFs and PECs (1 × 10\textsuperscript{5} cells per well) were cultured in 96-well plates (Nunc, Roskilde, Denmark) in EMEM containing 0.1% BSA\textsuperscript{26} (Sigma Aldrich). Recombinant human HB-EGF (R&D Systems, Minneapolis, MN) was added to the wells in concentrations ranging from 1 to 100 ng/mL. Cells were cultured at 37°C in a 5% CO\textsubscript{2} atmosphere for 48 hours before the addition of \[^{3}H\]thymidine (1 kBq per well) to the culture medium. After 16 hours, cells were snap frozen and the associated radioactivity measured with a scintillation counter (TopCount NXT; PerkinElmer, Boston, MA).

**Chemotaxis**

Measurements of chemotaxis, by using a modified Boyden chamber assay and checkerboard analysis, were performed according to methods previously described.\textsuperscript{27–28} The chemotactic potential of HB-EGF (R&D Systems) for PECs and PFs was analyzed at concentrations ranging from 0 to 100 ng/mL. Cells were incubated for 6 hours at 37°C, fixed in 100% methanol, and counterstained with hematoxylin. Cells per high-power field (HPF) were counted five times per triplicate well and the results expressed relative to cell migration without HB-EGF. Inhibition studies were performed by adding 5 μg/mL neutralizing anti-human HB-EGF antibody (R&D Systems) to cell suspensions before addition of the cells to the Boyden chamber wells.

**Statistical Analysis**

The data were obtained from PECs and PFs derived from one pterygium. These results are representative of data obtained from two additional PEC and PF cell lines. The proliferation and chemotaxis data presented are the mean ± SD of triplicate values in three separate experiments. Two sample t-tests were performed to determine significant differences among control versus treatment groups.

**RESULTS**

**Effects of HB-EGF on Pterygium-Derived Cells**

We investigated the effects of HB-EGF on the proliferation of PECs and PFs by measuring the incorporation of \[^{3}H\]thymidine into cells incubated with various concentrations of the recombinant human form of HB-EGF.

Incubation of PFs and PECs with HB-EGF resulted in a significant increase in \[^{3}H\]thymidine incorporation, compared with that in control cells (Fig. 1A, B respectively; P < 0.05). This effect plateaued at concentrations greater than 1.0 ng/mL.

**Effects of HB-EGF on Migration of Pterygium-Derived Cells**

HB-EGF stimulated chemotaxis in both PECs and PFs. The maximum effect was observed at 1 ng/mL in PFs (Fig. 2A) and 10 ng/mL in PECs (Fig. 2B). These effects were obliterated by the addition of a neutralizing antibody to HB-EGF (P < 0.05).
to induce a mitogenic response in many cell types. A recent study revealed that EGF receptor 1 (EGFR-1) is expressed diffusely on the cell surface of PFs. The investigators demonstrated activation of signaling components downstream from the EGFR in non-stimulated control cells and in PFs exposed to EGF, showing that these receptors are functionally active in PFs.

The interaction between HB-EGF, MMP, and TIMP in pterygia is likely to result in the liberation of the soluble form of HB-EGF from pterygium epithelium. Given the mitogenic effects of HB-EGF and its ability to stimulate cell migration, it is a likely candidate to promote the cellular growth and migration that is characteristic of this lesion.

This study lends further weight to the theory that UV induction of HB-EGF in pterygium epithelium drives the fibroblastic growth in this disorder. HB-EGF exerts a chemotactic effect for both PFs and PECs. The concentration for maximum chemotactic effect in PECs was 10 ng/mL, whereas the optimum concentration for PFs was lower at 1 ng/mL. Given that these experiments show that HB-EGF is produced in the epithelium of pterygia, one would expect that the fibroblasts in this disease would migrate preferentially toward lower concentrations of HB-EGF, given that they are farther from the source of growth factor production. These concentrations are similar to those in human peritoneal mesothelial cells and fibroblast transfectants expressing HER-1 and HER-4.

This is the first study to investigate the effects of growth factors on the cellular elements contained within pterygium and therefore is the first study to demonstrate the positive effects of a growth factor on growth and chemotaxis in PFs and PECs. Furthermore, HB-EGF and IL-6 have recently been shown to be induced in PECs by UV irradiation and have been shown to act synergistically in promoting growth and cell survival in some myelomas.

These effects induced by HB-EGF are an important step forward in understanding the pathogenesis of this disease. Pterygia are characterized as a disorder involving epithelial hyperplasia and accompanying fibrovascular growth. This study reveals a growth factor that is induced by UV irradiation and is capable of producing the epithelial and fibroblastic growth that characterizes this disease. These experiments support a role for the pterygium epithelium as harboring the altered cell line in the disease and indeed for the pterygium epithelium to induce the secondary recruitment and growth of fibroblasts in the pathogenesis of this disease. Furthermore, HB-EGF is a potent growth factor capable of stimulating altered cell growth and anchorage independence. Therefore, HB-EGF may stimulate PF proliferation and growth in this disease.

**References**


