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LABORATORY STUDY

Immunolocalisation of leukaemia inhibitory factor in the cornea

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Abstract

Aim **Leukaemia inhibitory factor (LIF) is a pleotrophic cytokine expressed in a variety of cell types, and have shown to regulate stem cell proliferation, vascular genesis, inflammation, and immunity in various locations. Expression of LIF and its role in the cornea have not been studied previously. In this study, we examined the expression of LIF in the cornea.**

Materials and method **Immunohistochemistry was performed using polyclonal LIF antibodies, and Avidin–Biotin ABC complex on cultured human corneal epithelium corneal fibroblasts and wild-type murine corneal epithelium.**

Results **LIF was detected in the cytoplasm of murine corneal epithelium, cultured human corneal epithelium, and fibroblasts. The expression of LIF was mainly cytoplasmic.**

Conclusion **LIF is expressed in the corneal epithelium and fibroblasts. It may have an important role in the maintenance of homeostasis of the corneal epithelium and cornea stroma. Further studies are necessary to elucidate the role of LIF in the cornea.**

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Introduction

Leukaemia inhibitory factor (LIF) is a pleotrophic cytokine expressed in a variety of cell types *in vitro*, which include activated T lymphocytes, monocytes, mast cells neuronal cells, dermal keratinocytes, fibroblasts and endothelial cells, intestinal mucosal, respiratory mucosal, and retinal pigment epithelial cells.[1–5] LIF belongs to a group of cytokines that includes ciliary neurotropin factor, IL-6, Ct-1, OM, and IL-11.[5] All the groups members elicit different biological effects through the GP-130 signal transducing subunit as a common receptor.[5]

Li *et al*[6,7] first reported the expression of LIF mRNA in cultured human corneal epithelial cells and keratocytes. The regulatory function of LIF in a variety of locations including stem cell proliferation, vascular genesis, inflammation, and immunity has been recently elucidated.[8–11] However, the role played by LIF in the cornea is largely unexplored. In this study, we examined the corneal expression of LIF.

Material and methods

Culturing human corneal epithelial cells

Corneal epithelial cells were cultured according to accepted previous methods.[12,13] Briefly, donor human corneal buttons maintained in organ culture media were obtained from the Manchester Eye bank. A measure of 2 mm explants from three different donors were prepared after removing central 8 mm of cornea. Explants were incubated with 0.1% trypsin and EDTA for 2 h at 37°C in 5% CO_2. Single cells were isolated and plated on growth-arrested 3T3-J2 feeder layer containing Green's media. The medium was changed every third day. The culture reached 70–80% confluence on day 14. 3T3 cells were removed by vigorous pipetting. The remaining 3T3 cells were removed with 0.01% trypsin for 15 s. The epithelial cells were then allowed to reach confluence. After 3 weeks, the epithelial cells became stratified. Epithelial sheets were separated from the culture dish with dispase (2 mg/ml in DMEM-Ca free and 10FCS) and fixed in 4% paraformaldehyde for 4 h.

Culturing human corneal fibroblasts

Corneal fibroblasts were cultured according to accepted previous methods.[6,14] Human corneas

were obtained from Manchester Eye Bank. Central 8 mm corneal buttons from three different donors were fashioned after removing the endothelium and the epithelium. The corneal stroma was cut into 2 × 2 mm pieces and incubated in fibroblast growth medium containing DMEM, 2 mM L-glutamine, 10% foetal calf serum and 100 IU/ml penicillin and streptomycin (all from Sigma). Upon confluence, the cells were passaged into cell culture flasks. Human corneal fibroblasts (passage 4) were cultured on cover slips (Sigma Grace Bio-Labs) according to manufacturer's instructions and incubated at 37°C in a CO_2 incubator. After 48 h, the cells were washed in PBS twice and fixed in 4% paraformaldehyde at room temperature for 15 min. After two washes in PBS, the cells were dehydrated through graded concentrations of ethanol.

Mouse cornea

Six wild-type mice were killed by cervical dislocation and their eyes were removed and fixed in 4% paraformaldehyde for 4 h. To facilitate sectioning, lenses were removed and the eyes were processed in paraffin wax. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histological and immunological techniques

Sections of eyes and the epithelial sheets cut at 7 μm were mounted on poly-L-lysine coated slides and deparaffinised sections were treated with 100% ethanol. For immunostaining, both the sections (eyes and the epithelial sheath) on the slides and the fibroblasts on the cover slips were treated as follows.[4] First, they were incubated with 3% hydrogen peroxide for 20 min to remove endogenous peroxidase activity, then rehydrated with 70% ethanol, and washed in PBS. The specimens were treated with normal rabbit serum for 30 min to block the nonspecific binding of antibodies and then incubated overnight at 4°C with primary LIF antibody (Santa Cruz Goat polyclonal IgG, stock diluted 1 : 500). The specimens were then treated for 30 min with secondary antibody (biotinylated, anti-goat IgG, Vectastain ABC Kit) diluted 1 : 200 in blocking serum, washed in PBS, and then incubated for 30 min with ABC complex. After washing in PBS, the binding antibody was visualised by 3,3′-diaminobenzidine (DAB, Sigma). All the specimens were then lightly counterstained with haematoxylin. Control slides and cover slips were incubated with normal rabbit serum, but otherwise treated in the same way.

Results

Mouse cornea

LIF expression was cytoplasmic and expressed in all layers of the corneal epithelium (Figure 1a and b). LIF expression was detected in all samples exmained. The superficial layers showed intense LIF expression. The basal layers showed weaker staining for LIF. LIF expression was present in limbal, paracentral, and central corneal epithelium. The endothelium and corneal stroma did not show LIF expression. Cultured corneal epithelial sheets expressed LIF in the cytoplasm and was detected in all layers of the corneal epithelium (Figure 1c and d). Similar to murine cornea, the superficial layers showed intense LIF expression. In the cultured corneal fibroblasts, LIF expression was detected in the cytoplasm (Figure 1e and f).

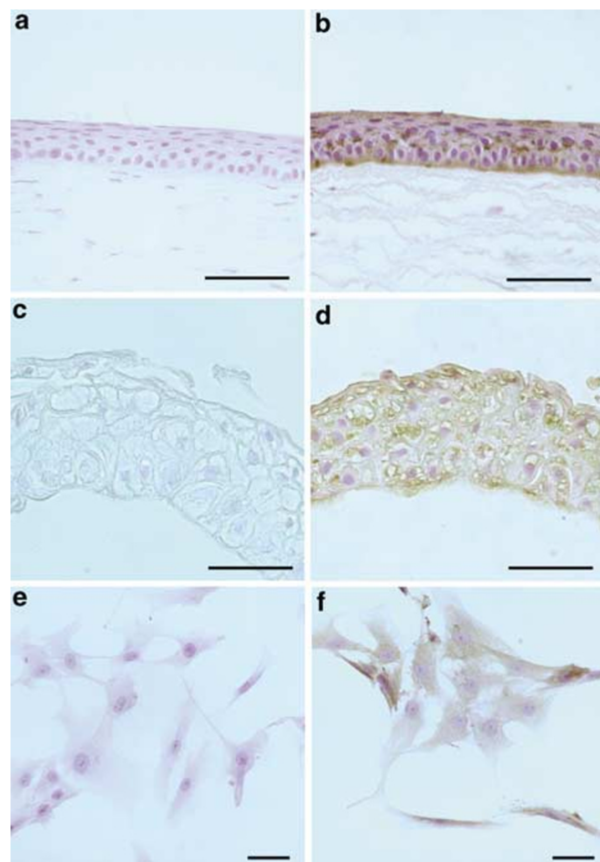

Figure 1 Immunolocalisation of LIF. ((a), (b)) Photo micrograph showing LIF expression in mouse corneal epithelium (b), the superficial layers stain stronger than basal layers. Endothelium and stoma are negative for LIF immunostaining, (a)-control. ((c), (d)) Photomicrograph showing expression of LIF cultured corneal epithelial sheets, (c)-control. ((e), (f)) Cultured corneal fibroblast expressing LIF (e), (f) control (Scale bar 50 μm).

Discussion

In this study, we were able to immunolocalise LIF in cultured human corneal epithelial cells and fibroblasts. The expression of LIF in epithelial cells and fibroblasts, which were not subjected to injury or other stimulatory cytokines, was intriguing. LIF was also expressed in organ-cultured corneal epithelium (results not shown). We then examined whether LIF is expressed in normal corneal epithelium. As we were not able to obtain fresh human corneal tissue, the mouse cornea was examined. This showed that LIF is expressed *in vivo* in the corneal epithelium. Recent studies have shown the regulatory importance of LIF in stem cell proliferation, vascular genesis, inflammation, and immunity.[8–11] However, the role played by LIF in the cornea is largely unexplored.

The sources of the adult corneal epithelial cells are the stem cell population residing at the limbus. The factors that regulate the corneal limbal stem cell have not been well elucidated. At lease two *in vitro* LIF culture systems were shown to enhance stem cell proliferation. In a coculture system containing murine fibroblast and embryonic stem cells, addition of LIF promotes the proliferation of ES cells by several fold.[15] This property is unique to LIF and only occurred in the presence of fibroblasts. Further addition of LIF, to human haematopoietic stem cells CD34(+) thy-1(+) and murine bone marrow-derived stromal cell coculture caused expansion of haematopoietic stem cells *in vitro*.[16] This stem cells expansion was specific to LIF and was observed only in the presence of fibroblast or stromal cells that are mesenchymal in origin. The action of LIF is indirect and mediated by mesenchymal cells. We observed that coculturing human limbal epithelial cells and limbal fibroblasts gave more prolific colonies in the presence of LIF (unpublished data). The interplay between corneal limbal stem cell and limbal fibroblast appears analogous to the interaction between haematopoietic stem cell and stromal cells, which is modulated under LIF. A stem cell expansion-promoting factor may be the possible link between the fibroblasts and corneal stem cell under the modulation of LIF.

The cornea is uniquely organised to discourage the induction and expression of inflammatory responses, particularly immune effector mechanisms that inflict significant injury to adjacent cells. The creation of such an immunological blind spot should render the cornea vulnerable to opportunistic infections and neoplasms. However, the conspicuous absence of neoplasm and the relatively low incidence of opportunistic infections suggest the existence of an effective immunological surveillance at the corneal surface. This property may be attributed to the presence of LIF in the corneal epithelial cells. Recently, the viral inhibitory potential of LIF has been demonstrated.[17,18] *In vitro* LIF exerted a potent, GP130 dependent, inhibition of HIV-I replication.[17] LIF also inhibits HIV-I in placenta and thymus tissue grown in *ex vivo* organ culture.[17] Further, LIF is upregulated in human marrow stromal cells infected with human cytomegalovirus.[18] In subacute sclerosing panencephalitis, LIF mRNA expression is upregulated in neurones infected with measles virus.[19] LIF may act as a pro- or anti-inflammatory factor depending on the profile of tissue and other cytokines. This inflammatory role has been documented in CNS tissues and in skin.[11,19–24]

New vessel formation in the cornea is a significant factor contributing to visual morbidity. Although factors such as VEGF, which modulates cornea, and new vascularisation has been characterised, the process of vascularisation is far from clear. Several studies have indicated that LIF could play a role in the regulation of blood vessel formation,[25–29] and LIF may have an implication in pathological vascularisation in ocular tissues. Depending upon the tissue origin or stage of differentiation, LIF has been shown to exert different actions. LIF stimulates adrenal cortex endothelial cells and embryonic cells, while it inhibit aortic endothelial cells.[25–27] Embryonic endothelial cell line can be optimally induced to differentiate into capillary-like structures *in vitro* by combined exposure to LIF plus bFGF.[26,28] During the formation of capillary structures from embryonic endothelial cell, line two—Stat3 and ^{P}41/43MAPK cascade systems are activated.[29] Stat3 acts as negative regulators on capillary development, and inhibition of this pathway is accompanied by increases capillary outgrowth, while inhibition of ^{P}41/43MAPK pathway dramatically reduces the capillary formation.[29] Fine tuning of the extent of capillary growth depends on the regulation of these pathways and the cytokine profile. LIF can activate both cascades, thereby acting as a negative as well as a positive regulator of vascular genesis.[28,29] Further studies examining the role of LIF in the corneal vascularisation may have therapeutic implications.

LIF is constitutively expressed in the corneal epithelium. It may play a role in modulating corneal stem cell dynamics, inflammation, and vascularisation. Further studies are necessary to determine the role of LIF in cornea during health and pathological processes.

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