Bovine Lactoferrin Stimulates Human Corneal Epithelial Alkali Wound Healing In Vitro

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PURPOSE. The purpose of this study was to investigate the effect of bovine lactoferrin (BLF) on human corneal epithelial wound healing using an in vitro alkali-induced wound model and to understand its role in promoting wound healing.

METHODS. Confluent human corneal limbal epithelial (HCLE) cells wounded using 0.5 μL of 0.1 M sodium hydroxide were treated with BLF (0, 0.1, 1, 2.5, and 5 mg/mL) or anti-human interleukin-6 (IL-6) receptor neutralizing antibody (anti-IL-6 antibody; 1, 10, and 50 μg/mL) or tyrphostin AG1295 (an inhibitor of platelet-derived growth factor [PDGF] receptor kinase; 1 and 10 μM), IL-6, or PDGF-BB. The conditioned medium collected for BLF treatment (0 and 5 mg/mL) was analyzed using a protein array for a number of cytokines/growth factors involved in corneal wound healing. A preliminary animal study using mice was carried out to determine the effect of BLF on alkali wounds.

RESULTS. BLF at 2.5 and 5 mg/mL promoted wound healing (P < 0.01). During wound closure, BLF upregulated PDGF-BB 180-fold and IL-6 10-fold compared with control. Treatment with tyrphostin AG1295 (10 μM; P < 0.01) or anti-IL-6 antibody (50 μg/mL; P < 0.01) in the presence of BLF inhibited wound closure, whereas the addition of exogenous IL-6 and PDGF-BB promoted wound closure. Preliminary animal studies have shown that BLF (5 mg/mL) promotes alkali wound healing in vivo.

CONCLUSIONS. These results suggest that BLF at ≥2.5 mg/mL stimulates HCLE wound healing, and this stimulation is mediated through the upregulation of PDGF or IL-6. (Invest Ophthalmol Vis Sci. 2009;50:1636–1643) DOI:10.1167/iovs.08-1882

Damage to the corneal epithelium can be caused by trauma, microbial insult, or chemical insult, during contact lens wear or by surgery such as photorefractive keratectomy or laser in situ keratomileusis. Most corneal epithelial wounds heal promptly. However, under certain clinical conditions, such as chemical injury, healing of the corneal epithelium is delayed, leaving the underlying stroma vulnerable to infection and ulceration. In the United States alone, ocular chemical injuries represent 7% to 18% of ocular traumas. Alkali injuries are of particular concern and cause acute inflammation characterized by rapid infiltration of neutrophils into the cornea followed by chronic inflammation involving the migration and recruitment of inflammatory cells over extended periods, further damaging the corneal surface. In serious cases this leads to corneal ulceration, perforation, scar formation, and permanent loss of vision. Prompt corneal epithelial healing is necessary to maintain corneal epithelial integrity and to preserve vision.1–3 Growth factors and cytokines play important roles in corneal wound healing. Epidermal growth factor (EGF), keratinocyte growth factor, and PDGF are some of the growth factors known to stimulate corneal wound healing.4–6 Interleukin (IL)-1α and IL-6 have also been found to be strongly induced early after corneal alkali burn by the regenerating epithelium, suggesting that they may play an important role in regenerating corneal epithelium.7 Increased IL-1 and IL-6 in wounded corneas can initiate a cascade of epithelial wound-healing events. IL-6 has been shown to promote corneal epithelial cell migration and wound closure in vivo.8,9 Various exogenous growth factors, cytokines, and matrix proteins have been used to treat corneal epithelial wounds.1 However, treatment for corneal epithelial defects is still not effective; consequently, the development of novel therapies for corneal epithelial defects is of great clinical importance.

Lactoferrin, an 80-kDa glycoprotein found in body secretions, is a protein with a number of functions, including inflammation reduction, immune response modulation, and antibacterial activity.10 Several studies have examined the effects of lactoferrin on wound healing in vitro and in vivo, the results of which suggest lactoferrin may have potential as a treatment for corneal wounds. It has been found that treatment with recombinant lactoferrin promotes skin wound closure in healthy and diabetic mice with full-thickness wounds.11,12 Further, lactoferrin has been shown to promote the migration of skin fibroblasts, a crucial step in wound healing.13 and to facilitate the contractile activity of fibroblasts in vitro.14 Lactoferrin has also been shown to stimulate epithelial cell proliferation in other organs.15 Its application to the cornea is suggested by its ability to protect corneal cells from damage by ultraviolet B radiation.16

In this study we explored the effects of bovine lactoferrin (BLF) on the healing of corneal epithelial cells in vitro after sodium hydroxide chemical injury and investigated the mechanisms by which BLF promotes corneal epithelial wound healing.

METHODS

Cell Culture

The immortalized human corneal limbal epithelial (HCLE) cell line (a kind gift of Ilene Gipson, The Schepens Eye Research Institute, Boston, MA) was used. HCLE cells were cultured as previously described.17 Briefly, HCLE cells were maintained on plastic at 2 × 10⁴ cells/cm² in a keratinocyte serum-free medium (K-SFM; Invitrogen-GIBCO, Carlsbad, CA), supplemented with 25 μg/mL bovine pituitary extract, 0.2

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ng/mL EGF (Invitrogen, Mount Waverley, VIC, Australia) and 0.4 mM CaCl₂ and grown at 37°C in a 5% carbon dioxide atmosphere. The cultures were switched at approximately 50% confluence to a 1:1 mixture of K-SFM/low-calcium Dulbecco’s modified Eagle’s medium (DMEM)/Ham F12 (Invitrogen) to achieve confluence. All experiments involving the culture of HCLE cells were carried out at 37°C in a 5% carbon dioxide atmosphere unless otherwise indicated.

Cell Proliferation

Before studying its effect on cell proliferation, BLF (Sigma-Aldrich, St. Louis, MO) was analyzed for endotoxin content using a limulus amebocyte lysate assay (ACL-1000 kit; Bio-Whittaker, Walkersville, MD). The effect of BLF on HCLE cell proliferation was determined with a cell proliferation assay (CyQuant Kit; Invitrogen-Molecular Probes, Carlsbad, CA) in which a fluorescent dye exhibits strong fluorescence enhancement when bound to DNA. Briefly, HCLE cells were seeded at a density of 1 × 10⁴ cells per well in K-SFM medium into 96-well tissue culture plates and cultured for 24 hours. At the end of 24 hours, K-SFM was removed, and cells were washed with phosphate-buffered saline (PBS) before the addition of 1:1 K-SFM/low-calcium DMEM/F12 containing different concentrations of BLF (0.1, 1, 2.5, or 5 mg/mL; n = 6) and BSA (62.5 μM; same molarity as 5 mg/mL BLF) as control were incubated for another 8, 24, and 48 hours. After the medium was removed, 200 μL dye-cell lysis buffer (CyQuant GR; Invitrogen-Molecular Probes) was added to each well and was incubated for 5 minutes at room temperature. Fluorescence intensity, related to the number of viable cells, was measured at 485/535 nm.

Cell Migration

The effect of BLF on the cell migration of HCLE cells was determined with an in vitro chemotaxis 96-well cell migration kit (QCM; Chemicon, Billerica, MA) according to the manufacturer’s protocol. Briefly, HCLE cells (5 × 10⁵ cells in 100 μL culture medium) were seeded into each of the upper migration chambers. The lower migration chambers contained 150 μL culture medium containing BLF at concentrations of 0, 0.1, 2.5, or 5 mg/mL (n = 6). Cells were incubated overnight. Those on the upper surface of the membrane (8-μm pore size) were gently removed. Migratory cells on the lower surface of the membrane were dissociated from the membrane using cell detachment buffer. These cells were lysed and stained with a fluorescent dye (CyQuant GR; Invitrogen-Molecular Probes) that exhibits strong fluorescence enhancement when bound to cellular nucleic acid. Cell migration was determined by measurement of the fluorescence of cell solutions at 485/535 nm.

The effect of IL-6 and PDGF on HCLE cell migration was also determined, as described. IL-6 (4 ng/mL) and PDGF-BB (5 ng/mL) in the presence or absence of fibronectin (50 μg/mL; Sigma-Aldrich, MO; n = 6) was placed in the lower chamber of the cell migration kit, and assay was performed according to the manufacturer’s protocol.

Immunocytochemistry

For immunocytochemical detection of fibronectin, HCLE cells were cultured to confluence in K-SFM medium in eight-well chamber slides. Cells were wounded using 0.1 M NaOH and were then washed three times with K-SFM. Wounded and unwounded cells were incubated with K-SFM for 4 hours. Medium was removed; cells were washed three times with PBS, and 0.8 mL of 5.7% formaldehyde was added to each well. Cells were fixed for 15 minutes at room temperature and were blocked with 3% BSA for 1 hour. Rabbit anti-human fibroectin antibody (Sigma-Aldrich) or rabbit IgG (R&D Systems, Minneapolis, MN) was applied and incubated at 4°C overnight. Slides were washed with PBS and incubated 1 hour at room temperature with anti-rabbit IgG antibody conjugated with FITC (Dako, Glostrup, Denmark). Slides were rinsed with PBS, counterstained with DAPI, and mounted with mounting medium with DAPI nuclear stain (Vec-tashield; Vector Laboratories, Burlingame, CA).

Effect of BLF on HCLE Wound Healing of Alkali-Induced Wound Model

To determine the effect of BLF on healing of an alkali-induced wound, confluent monolayers of HCLE cells were wounded using 0.5 μL of 0.1 M sodium hydroxide delivered carefully using a 10-μL pipette. The distance and force with which NaOH was delivered onto confluent cells was kept constant to create a uniform circular wound. After wounding, cells were immediately washed twice with culture medium (1:1 K-SFM/low Ca²⁺ DMEM/F12) to remove cellular debris. Culture medium, containing 0, 0.1, 1, 2.5, or 5 mg/mL BLF or BSA (62.5 μM; same molarity as 5 mg/mL BLF), was added to the wells and incubated for 24 hours at 37°C in 5% CO₂. Wounds were assessed visually to ensure that those with the same wound area were compared, and wound closure in response to the treatments was monitored visually every few hours. Conditioned medium was collected 24 hours after wounding for cytokine and growth factor analysis. At the end of the experiment, cells were fixed and stained (Diff-Quick; Becto Laboratories, Liverpool, NSW, Australia). Wounds were photographed at 40× magnification, and the area of the wound was determined using an image analysis system (Image J version 1.33; National Institutes of Health, Bethesda, MD). Degree of wound closure in response to the BLF treatment (n = 5) was determined as the percentage reduction of wound area compared with that at the time of wounding (0 hour).

Protein Array and ELISA

A multiplex bead-based assay designed to quantitate multiple cytokines and growth factors from a single sample (Bio-Plex; Bio-Rad, Hercules, CA) was used. Conditioned medium of wounded HCLE cells treated with 5 mg/mL BLF and control (no BLF treatment; n = 5) was collected as described. Samples were centrifuged to remove cellular debris and stored at −80°C until further use. Samples were analyzed with the multiplex bead-based assay (Bio-Plex; Bio-Rad) according to the manufacturer’s protocol for the following cytokines and growth factors known to be involved in wound healing: IL-1α, IL-1β, IL-6, IL-8, IL-4, IL-10, IL-18, tumor necrosis factor-α (TNF-α), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), basic fibroblast growth factor (bFGF), PDGF-BB, and hepatocyte growth factor (HGF). Conditioned media of wounded and unwounded HCLE cells treated with BLF and BSA (control) at the same molar concentrations and control medium (n = 5) were analyzed for IL-6 and PDGF expression by ELISA according to the manufacturer’s protocol (R&D Systems).

Effect of IL-6 and PDGF Stimulated by BLF during Alkali-Induced Wound on Wound Healing

To determine the effect of PDGF-BB and IL-6 stimulated by BLF on alkali-induced wounds, wounds were treated with 1, 10, and 50 μg/mL anti-human IL-6 receptor neutralizing antibody (anti-IL-6 antibody; R&D Systems) and 1 and 10 μM tyrophostin AG1295 (6,7-dimethyl-2-phenylquinolinealine; Sigma-Aldrich), a selective inhibitor of tyrosine kinase in the PDGF receptor in the presence or absence of 5 mg/mL concentrations of BLF. Wounded HCLE cells were also treated with recombinant human (rh) IL-6 (4 ng/mL; R&D Systems) or rhPDGF-BB (5 mg/mL; Sigma-Aldrich), the concentrations found to be induced by 5 mg/mL BLF (Table 1; n = 5 for all treatments). Wound areas were measured as described.

Animal Studies

All procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Institutional ethics committee clearance was obtained before experiments. A preliminary study was carried out to test the efficacy of BLF on wound healing using a mouse model. Six- to 8-week-old male BALB/c strain mice (obtained from Biological Resources Centre, Perth, Western Australia) were used.
RESULTS

Effect of BLF on HCLE Cell Proliferation

BLF had no effect on cell proliferation at lower concentrations (≤2.5 mg/mL). However, BLF at a concentration of 5 mg/mL inhibited cell proliferation compared with respective controls at 24 and 48 hours (P < 0.01; Fig. 1). Comparison of 8-, 24-, and 48-hour time points with initial fluorescence intensity indicated that BLF did not induce apoptosis or sloughing of cells. This was also confirmed by visual microscopic inspection, during which sloughing of cells was not observed.

Effects of BLF, IL-6, and PDGF on HCLE Cell Migration

Figure 2 indicates that BLF at concentrations of 2.5 and 5 mg/mL inhibited the migration of HCLE cells when compared with control without BLF treatment (P = 0.002 and P = 0.004, respectively).

IL-6 and PDGF alone did not promote cell migration compared with control. However, IL-6 and PDGF significantly stimulated cell migration to 1.5-fold in the presence of fibronectin compared with IL-6 and PDGF alone (P = 0.001 and P = 0.003, respectively; Fig. 2). IL-6 and PDGF in the presence of fibronectin stimulated cell migration by approximately 20% compared

### Table 1. Cytokine and Growth Factor Production in Response to BLF Treatment

<table>
<thead>
<tr>
<th>Cytokine/Growth Factor</th>
<th>Control (pg/mL)</th>
<th>BLF (pg/mL)</th>
<th>Ratio of Cytokine Production between Control and BLF Treatment Groups</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-BB</td>
<td>23 ± 4</td>
<td>4202 ± 931</td>
<td>180</td>
<td>0.0015</td>
</tr>
<tr>
<td>IL-6</td>
<td>466 ± 158</td>
<td>4527 ± 1213</td>
<td>10</td>
<td>0.0045</td>
</tr>
<tr>
<td>IL-8</td>
<td>509 ± 84</td>
<td>252 ± 44</td>
<td>2</td>
<td>0.0093</td>
</tr>
<tr>
<td>IL-1β</td>
<td>61 ± 7.5</td>
<td>122 ± 23.4</td>
<td>2</td>
<td>0.0126</td>
</tr>
<tr>
<td>TNF-α</td>
<td>8 ± 0.8</td>
<td>25 ± 6</td>
<td>5</td>
<td>0.0127</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.7 ± 0.6</td>
<td>27 ± 5.6</td>
<td>4</td>
<td>0.0054</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>19 ± 10</td>
<td>87 ± 17</td>
<td>4</td>
<td>0.0094</td>
</tr>
<tr>
<td>IL-8</td>
<td>23 ± 0.5</td>
<td>44 ± 16</td>
<td>2</td>
<td>0.0855</td>
</tr>
</tbody>
</table>

BLF treatment consisted of 5 mg/mL during healing of alkali-induced wound (conditioned medium collected at the end of 24 hours after wounding). Data represent mean ± SD. Groups were compared using the Kruskal-Wallis test. The level of significance was adjusted for multiple comparisons. P < 0.01 was considered significant. Experiments were performed at least twice.
with the effects of fibronectin alone ($P = 0.009$ and $P = 0.032$, respectively; Fig. 2).

**Immunocytochemistry**

Wounded cells expressed more fibronectin than unwounded cells (Fig. 3), and large amounts of fibronectin were deposited near the wound edges after 4 hours of injury (Fig. 3B). Fibronectin was present near the cell boundaries and between cells in wounded and unwounded cells. No staining for fibronectin was present in the cells treated with serum alone (data not shown).

**BLF-Stimulated Closure of In Vitro Alkali-Induced Wound**

Treatment of chemical injury of confluent HCLE cells with 2.5 mg/mL BLF resulted in a 2.5-fold increase ($P = 0.004$ and $P = 0.005$; Fig. 4) in the percentage wound closure compared with control wounds (culture medium only or BSA in culture medium), whereas treatment with 5 mg/mL resulted in an approximately 3-fold increase ($P = 0.002$; Fig. 4) in the percentage wound closure than was observed in the control wounds (Fig. 4). Wounds treated with lower concentrations of BLF were not different from those in controls (Fig. 4).

**Cytokine Production in Response to BLF Treatment**

Conditioned media collected 24 hours after alkali wounding of HCLE cells and treated with 5 mg/mL BLF and control medium were analyzed for the presence of the following mediators using a protein array (Bio-Plex; Bio-Rad): IL-1α, IL-1β, IL-6, IL-8, IL-4, IL-10 and IL-18, TNF-α, GM-CSF, IFN-γ, bFGF, PDGF-BB, and HGF.

After alkali wounding of HCLE cells, modest increases in the production of IL-1α (2-fold) and IL-1β (3-fold) were observed (Table 1). Levels of IFN-γ and TNF-α were increased by approximately 4-fold compared with the untreated wounded HCLE cells. However, large increases in IL-6 and PDGF-BB of 10-fold and 180-fold, respectively, were observed (Table 1). In contrast, levels of the chemokine IL-8 were significantly downregulated ($P = 0.009$). There was no significant difference in the production of GM-CSF ($P = 0.084$). Other cytokines examined (IL-18, IL-4, IL-10, and HGF) were not detected.

BLF upregulated PDGF production to approximately 180-fold compared with control (BSA and culture medium) in wounded and unwounded cells ($P = 0.0003$). There was no significant difference in PDGF production in culture

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**FIGURE 3.** Immunocytochemical detection of fibronectin expression (green) on HCLE cells. (A, B) Fibronectin expression in unwounded and wounded cells at 20× magnification. Cell nuclei were stained with DAPI (blue).
medium and BSA-treated cells in wounded or unwounded cells.

The BLF used in these studies was analyzed for endotoxin content using the limulus amebocyte lysate assay. It was found that the endotoxin content was 1.73 EU/mg of BLF or 0.143 ng/mg of BLF. When HCLE cells were stimulated with 1 μg/mL lipopolysaccharide (LPS), the cytokine expression for IL-6 and IL-8 (proinflammatory cytokines) was not significantly different from that found in the absence of LPS (data not shown), demonstrating that levels of endotoxin in the BLF did not contribute to the induction of these cytokines.

**BLF Promotion of Wound Healing by PDGF and IL-6**

Treatment of alkali-wounded HCLE cells with 5 mg/mL BLF resulted in a 3-fold increase in percentage wound closure compared with control without BLF (Fig. 5). When anti–IL-6 receptor antibody (1, 10, and 50 μg/mL) was added to the cells concurrently with 5 mg/mL of BLF, the percentage wound closure was not different from that found in the absence of BLF. Anti-IL-6 receptor antibody at a concentration of 50 μg/mL in the presence of 5 mg/mL BLF inhibited wound closure significantly compared with the wound treated with
BLF ($P = 0.008$). Anti–human IL-6 receptor neutralizing antibody alone, at concentrations of 1, 10, and 50 µg/mL, did not have any effect on wound closure compared with the control (data not shown). Treatment with exogenous IL-6 alone was sufficient to increase the percentage wound closure to that found by treatment with 5 mg/mL BLF (Fig. 5).

Tyrphostin AG1295 at a concentration of 10 µM inhibited wound closure significantly compared with the wound treated with 5 mg/mL BLF ($P = 0.006$). Tyrphostin AG1295 in the presence of 1 µM BLF did not show any effect on wound closure compared with the BLF-treated wound (Fig. 6). Tyrphostin AG1295 alone at concentrations of 1 and 10 µM did not show any statistically significant difference in wound closure compared with the control (data not shown), whereas treatment of exogenous PDGF resulted in levels of wound closure similar to those with 5 mg/mL BLF treatment (Fig. 6).

Effect of BLF on Corneal Alkali Wounds in Mice

In the mouse corneal alkali wound model, 3 of 4 (75%) mice treated with BLF healed completely 7 days after wounding, whereas only 1 out of 4 (25%) wounds healed in the control (PBS and BSA) treated groups. Representative photographs of corneal wounds in BLF and control (BSA and PBS) treated groups on days 0 and 7 are shown in Figure 7.

DISCUSSION

Corneal epithelial integrity is essential to the maintenance of a transparent, functional cornea. Chemical injuries to the cornea have many complications that interfere with the proper healing of the cornea and result in the formation of recurrent erosions and nonhealing defects, scarring, and chronic inflammation. In this study, BLF was found to promote healing of HCLE cells in an in vitro alkali-induced wounds. Further, this process appears to be mediated by the modulation of the cytokines produced by the epithelial cells.

Proliferation and migration are important processes in wound healing that have been reported to be influenced by lactoferrin in other systems. The effects of lactoferrin on cell migration are dependent on the cell type examined. Lactoferrin inhibits the migration of gastrointestinal and epidermal Langerhans cells in vitro but has been shown to promote the migration of fibroblasts in a wound-healing assay. Here we have found that BLF inhibited the migration of corneal epithelial cells (Fig. 2). However, in contrast to findings in rat intest-
tinal epithelial cells. Laetoferrin appeared to suppress HCLE cell proliferation.

The ability of laetoferrin to modulate cytokine responses in mucosal epithelia and inflammatory cells has been well established, and the studies reported here indicate that BLF exerts its effects on the healing of HCLE cells through alteration of the cytokine levels produced after wounding. Our results show large increases in the production of IL-6 and PDGF-BB in response to BLF treatment of wounds, and these increases correlated with increased percentage wound closure. These findings were confirmed by further investigations that revealed exogenous application of these cytokines also resulted in increased wound closure, comparable to that of BLF treatment, and that specific inhibition of the action of these cytokines abrogated the effect.

When HCLE cells were exposed to IL-6 and PDGF, they did not induce cell migration (Fig. 2). However, in the presence of fibronectin, there was a statistically significant increase in cell migration of approximately 20% compared with the effects of fibronectin alone. This finding is consistent with the findings of others in that IL-6 and PDGF did not enhance the migration of rabbit corneal epithelial cells alone but were able to enhance the effects of fibronectin on cell migration. Consequently, in unwounded cells, though these cytokines increased in response to BLF, no corresponding increase occurred in migration. However, in wounded cells, fibronectin is expressed in response to initial wounding, which acts as a provisional matrix for cell migration. A number of studies have shown that fibronectin expression increases after injuries such as alkali burn, mechanical epithelial debridement, keratectomy, and excimer laser keratectomy. Here we have found that fibronectin expression was greater in wounded corneal epithelial cells than in the unwounded cells (Fig. 3) and that the distribution is consistent with that of previously reported findings. A large amount of fibronectin was deposited into the wound edges to promote cell migration to the wounded cells. This increase in fibronectin expression in the cornea has also been reported in vivo in response to wounding. Therefore, an increase in wound closure was observed in response to BLF treatment, presumably resulting from the synergistic activity of IL-6 and PDGF with fibronectin.

It has long been recognized that IL-6, a regulator of epithelial cell growth and cell-cell adhesion, is involved in corneal wound healing. Topical application of IL-6 to wounded rabbit corneas has been shown to facilitate epithelial wound closure possibly by upregulating the expression of integrins. The ability of laetoferrin to modulate IL-6 expression in other systems has also been reported in which the administration of laetoferrin was found to increase the production of IL-6 in peritoneal and alveolar cells or to decrease serum IL-6 in vivo during endotoxemia with complex temporal regulation. These findings suggest that the ability of laetoferrin to regulate IL-6 production may be dependent on type of cells and type of injury.

The role of PDGF and other growth factors in corneal wound healing is also well known. Our data, showing the large upregulation (180-fold) of PDGF after the administration of BLF, coincident with an increased percentage wound closure abrogated by the addition of specific inhibitors, is consistent with the current research and suggests a mechanism by which BLF might act.

In our studies, BLF modulated proinflammatory and anti-inflammatory cytokine responses; of particular interest in an alkali wound model is the decrease in the major neutrophil chemokine IL-8 given that prolonged recruitment of polymorphonuclear leukocytes into the cornea is a major source of damage during alkali injury and corneal infection. This down-regulation is consistent with the findings of various researchers in other models of inflammation.

Slight upregulation of IL-1 and TNF-α was also found in our study in response to BLF treatment during wound healing, but these factors did not appear to impair wound healing. IL-1 is reported to be essential for early wound closure in alkali-burned corneas, and the absence of TNF-α was also reported to result in an impaired wound-healing response. However, excessive production of TNF-α can be associated with uncontrolled inflammation. Therefore, the successful treatment of corneal defects, in particular alkali wounding, could depend on the balance and timing of cytokines produced.

In our preliminary in vivo studies, BLF stimulated the healing of alkali-induced corneal wounds, which is consistent with our in vitro findings. Possible mechanisms through which laetoferrin exerts its effects are thought to be the low-density lipoprotein receptor-related protein (LRP) and toll-like receptor-4 (TLR4). Binding of laetoferrin to LRP has been reported to promote cell growth and wound healing through the extracellular regulated kinase pathway in other organs. In addition, LRP has been found to be involved in PDGF-signaling pathways. Lactoferrin also regulates IL-6 expression through interaction with TLR4, which signals through nuclear factor κB, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase (p38 MAPK) pathways. Based on this evidence, BLF may upregulate IL-6 and PDGF with either LRP or TLR4 or in combination through the MAPK-signaling transduction pathways.

Our results presented here suggest that BLF may represent an excellent therapeutic intervention for corneal defects created by chemical injury. BLF acts by modulating cytokine production, upregulating factors implicated in corneal wound healing, and downregulating the key neutrophil chemokine in the cornea, IL-8. It is also compatible with the delicate ocular surface found in the tear film.

In conclusion, BLF at concentrations of ≥2.5 mg/ml promoted in vitro and in vivo wound healing of an alkali-induced HCLE wound. IL-6 and PDGF, which are known to be involved in corneal wound healing, were upregulated by BLF. It was demonstrated that BLF promoted wound healing through IL-6 and PDGF-BB. These results indicate that BLF may have clinical applications in the treatment of persistent corneal epithelial defects.

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