Bovine Lactoferrin Structures Promoting Corneal Epithelial Wound Healing In Vitro

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PURPOSE. To use an in vitro alkali-induced wound model to identify structures of bovine lactoferrin (BLF) that contribute to the promotion of human corneal epithelial healing.

METHODS. BLF N-lobe and C-lobe were separated using limited proteolysis and purified by preparative chromatography. Isoforms of BLF were separated according to serine protease activity. Catalytic activities of isoforms and lobes were quantified by hydrolysis of a synthetic serine protease substrate. The promotion of healing by cognate moieties, lactoferricin-B, BLF isoforms, and BLF in various forms—iron-free, iron-saturated, deglycosylated, zwitterionic detergent exposed, chaotrope denatured, disulfide reduced—was assessed on alkali wounded confluent monolayers of human corneal epithelial cells.

RESULTS. The C-lobe of BLF (6.4-128 μM) promoted greater wound healing than native-BLF or N-lobe. BLF (12.8 μM) promoted wound closure in an iron-free, iron-bound, or deglycosylated state or after exposure to zwitterionic detergent. Healing was not stimulated by chaotropically denatured or disulfide reduced BLF (12.8 μM) or by lactoferricin-B (12.8 μM). Proteolytically active BLF (0.6 μM) promoted wound closure at a lower concentration than proteolytically inactive BLF (12 μM). This proteolytic activity was localized to the N-lobe.

CONCLUSIONS. The C-lobe is the primary promoter of BLF-stimulated corneal epithelial wound closure in vitro and is effective at concentrations ≥6.4 μM. Increased healing from BLF occurs with the native conformation and is unaffected by glycosylation or iron saturation. To a lesser extent, proteolytic activity of the N-lobe also improves healing rates. The BLF C-lobe may be a novel treatment for corneal lesions with delayed healing. (Invest Ophthalmol Vis Sci. 2011;52:2719–2726) DOI:10.1167/iovs.10-6352

The corneal epithelium can be compromised by a wide array of stimuli, including trauma, contact lens wear, infection, noxious chemicals, and surgery. Fortunately, the surface of the eye is highly adept at self-repair; therefore, for most injuries, healing occurs rapidly and without lasting consequences. Any delay in wound closure, however, leaves the cornea vulnerable to microbial assault and associated inflammatory infiltration that may result in permanent visual impairment. Thus, by accelerating the restoration of epithelial integrity, the risk of unfavorable sequelae is minimized. 1 To achieve this outcome, numerous matrix proteins, growth factors, and cytokines have been studied, 2 but none have proven to be entirely effective. Thus, the development of new agents to promote epithelial healing is of great clinical importance.

Alkali burns are a particularly severe corneal injury that account for up to 18% of ocular trauma admissions across the world, 3 with 50% of severe cases resulting in permanent visual impairment. 4 These burns are characterized by a heightened inflammatory response and delayed wound healing, prolonging the risk period for sight-threatening secondary complications. 5 One potential approach to improving the clinical outcome is enhancement of epithelial resurfacing over the exposed stroma.

Recently, we reported that bovine lactoferrin (BLF) can increase rates of wound closure in alkali-induced corneal lesions by promoting epithelial cell migration. 6 BLF is an abundant milk protein available in substantial quantities, frequently as a byproduct from the dairy industry. Tissue repair, only recently identified for LF, adds to the many documented activities of this protein as an antimicrobial, 7 anticancer, 8 and anti-inflammatory 9 agent. The structural basis for these and other functions of LF have been attributed to iron-binding sites, lactoferricin B (LFcin B) peptides, proteolytic activities, lobal differences, and, to a lesser extent, glycan chains. 10

The migration of epithelial cells across the ocular surface is influenced both by the nature of the extracellular matrix and by signaling growth factors and cytokines. 2 Such augmentation is observed in vitro with interleukin-6 (IL-6) and platelet-derived growth factor-ββ, both of which are up-regulated by BLF and increase corneal epithelial migration in the presence of the provisional matrix glycoprotein fibronectin. 11,12 Cell migration is also facilitated by other richly glycosylated constituent membrane and extracellular matrix proteins, including integrins 13 and the sulfated glycosaminoglycan (GAG)-linked lumican. 14

Tissue repair progresses by sliding of the epithelial sheet across the wound in conjunction with progressive plasmin degradation of the fibrin provisional matrix. When the plasmin activation system is absent, corneal mending is retarded. 15 However, a fine balance must be maintained because the persistence of this protease system can also contribute to delayed wound healing as occurs with alkali burns. 16 Similarly, free radical tissue damage also impairs healing, and improved corneal recovery is reported for alkali injuries treated with inhibitors and scavengers of the hydroxyl radical. 17

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BLF is a 78-kDa, iron-binding, bilobal (N and C lobes) glycoprotein with a highly cationic N terminus and serine protease activity. Both plasmin and BLF hydrolyze peptide bonds on the C-terminal side of arginine residues, suggesting BLF may make a similar contribution to wound healing. The antioxidant action of BLF, through the chelation of free iron that catalytically decomposes superoxide to hydroxyl radicals, also potentially reduces cell damage from alkali injury. LF in B cells, located near the amino terminus of the N-lobe, promote antibacterial and immunomodulation functions by charge- and polarity-based mechanisms. This region interacts with proteoglycans and sulfated GAGs, resulting in a high-density but low-affinity surface binding of lactoferrin to cell and extracellular matrix components important to migration. Membrane-binding affinities are also affected by glycosylation of LF as observed for intestinal epithelia. The proliferation of osteoblasts and increased motility in fibroblasts have been demonstrated to be promoted more strongly by the C-lobe of LF than the N-lobe. Investigation of these structures will be of pharmaceutical value and of clinical importance to the development of novel therapeutic strategies for healing corneal defects.

The present study was conducted to determine the influence of these regions on BLF-promoted human corneal epithelial wound healing in vitro.

**METHODS**

**BLF Lobe Purification**

Separation of BLF (a gift from Andrew Brown, Murray Goulburn Cooperative, Cobram, VIC, Australia) into N-lobe and C-lobe fragments was modified from the method of Legrand. A 0.2% solution of BLF in 0.1 M tris-HCl buffer, pH 8.2, containing 25 mM CaCl₂ was digested with 25 TAME units of immobilized trypsin (Pierce, Rockford, IL) per milligram substrate at 37°C with moderate agitation. Incubation times of 0.5 and 4 hours were used to maximize the yield of the N-lobe and C-lobe, respectively. The reaction was terminated by centrifugal separation of trypsin gel from the sample in accordance with the manufacturer’s directions.

Lobes were then purified by cation exchange chromatography using a column (Mono S 5/50 GL; GE Healthcare, Piscataway, NJ) equilibrated in 50 mM HEPES, pH 8.0. Elution was carried out by a linear gradient up to 1 M NaCl in the same buffer. The isolated peaks were applied to a size exclusion column (Bio-Gel P-60 26/1000; Bio-Rad Laboratories, Hercules, CA) in 10% acetic acid and 150 mM NaCl at 0.4 mL/min. BLF and fragments were visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with the Laemmli system on 12% tris-HCl gels with Coomassie stain (Blue R-250; Bio-Rad Laboratories). Gels were scanned on a densitometer (GS-800; Bio-Rad Laboratories). Gels were rehydrated with 10 mM dithiothreitol for 1 hour at 4°C and bands were excised from the gel for further analysis.

**Endotoxin Content**

BLF endotoxin content was analyzed with *Limulus* amoebocyte lysate assay (QCL-1000; Lonza, Walkersville, MD) in accordance with the manufacturer’s instructions.

**Serine Protease Isolation, Activity, and Characterization**

Fractions of BLF with proteolytic activity were purified with a benzamidine-serine protease affinity column (GE Healthcare, Uppsala, Sweden) according to the manufacturer’s protocol. Briefly, BLF was loaded onto the column in 50 mM Tris-HCl buffer with 0.5 M NaCl at pH 7.4 and the bound fractions were eluted at pH 2.0 into a collection buffer, restoring pH to physiological levels. Total protein content of these fractions was determined by bicinchoninic acid assay (BCA; Pierce) in accordance with the manufacturer’s instructions using native BLF as the protein standard.

For tandem mass spectrometry (LC-MS/MS) analysis, native BLF and the fractions collected from the benzamidine column over five repeats were separately resolved on SDS-PAGE gels and stained as described. The protein bands were excised as 1-mm² cubes, washed with 100 mM NH₄HCO₃ for 20 minutes, and destained in 25 mM NH₄HCO₃ in 50% acetonitrile that was then replaced with 100% acetonitrile for dehydryation at 37°C under vacuum (CentriVap; Labconco, Kansas City, MO). The gel cubes were rehydrated with 10 mM dithiothreitol for 1 hour at 37°C to chemically reduce disulfide bonds, followed by alkylation with 25 mM iodoacetamide at room temperature for 1 hour. After three rinses in ultrapure water (MilliQ; Millipore, Bedford, MA), the gel cubes were again dehydrated as described. In-gel digestion was performed by the addition of 50 ng sequencing grade modified trypsin (ProMega, Madison, WI) per cube, in cold 50 mM NH₄HCO₃, followed by incubation at 37°C overnight. The supernatant of the digest was pooled and dried under vacuum with the gel extracts obtained from one 20-minute incubation with 20 mM NH₄HCO₃ and three 20-minute incubations with 50% acetonitrile/5% formic acid. Samples were further cleaned with zip tips (C18; Eppendorf, Hamburg, Germany) in accordance with the manufacturer’s instructions. These were analyzed with a mass spectrometer (LTQ-FT Ultra; Thermo Electron, Bremen, Germany). Peptides were first separated by nano-LC with a high-performance liquid chromatography (HPLC) system and autosampler (UltiMate 3000; Dionex, Amsterdam, The Netherlands). Samples were concentrated and desalted onto a precolumn (micro C18; 50 μm x 2 mm; Microm Bioresources, Auburn, CA) with 0.05% heptfluorobutyric acid at 20 μL/min. After a 4-minute wash, the precolumn was switched (Valco 10-port valve; Dionex) into line with an in-house built fritless native C18 column. The HPLC mobile phase consisted of a mixture of buffer A (2% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 20% formic acid). Peptides were eluted from the column along a linear gradient of 2% to 50% buffer B over 30 minutes followed by a 100% buffer B wash over 1 minute at a flow rate of 300 nL/min. LTQ-FT was operated, as described previously. Peak lists were generated using Mascot Daemon/extract_msn (Matrix Science, London, UK), with default parameters and were submitted to the database search program Mascot.

For analysis, peptide and fragment tolerances were set to ±4.0 ppm and ±0.05 Da, respectively. Variable modifications of methionine oxidation, propionamide cysteine, and carboxamidomethylation of cysteine were included, along with a maximum of one missed tryptic cleavage. The search was against the nonredundant National Center for Biotechnology Information database (October 2010). Identifications were accepted based on MOWSE and individual ion scores >44 indicating significant homology (P < 0.05). Recovered peptides were analyzed using Scaffold (Proteome Software, Portland, OR) to probabilistically validate identifications with a false discovery rate set to <5%.

A proportion of each collected fraction from the serine protease affinity column was subjected to irreversible serine protease inhibition by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF; Fluka Analytical, Buchs, SG, Switzerland) at a 10:1 molar excess and was removed by buffer exchange. Quantification of BLF proteolytic activity was adapted from Massucci et al. Serine protease activity measurements were made with the substrate Nα-benzoyloxycarbonyl-phenylal-
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anine-arginine-7-amido-4-methyl-coumarin (Z-Phe-Arg-AMC; Sigma-Aldrich, St. Louis, MO) at concentrations from 3 to 300 µM in 20 mM phosphate buffer, pH 7.0, with 100 mM NaCl at 25°C. Cleavage of the peptide and release of the AMC group by 0.1 µM of the eluted BLF fractions was monitored spectrophuorometrically at 465-nm excitation and 360-nm emission wavelengths to calculate the initial reaction velocity. The kinetic parameters \( k_{\text{cat}} \) and \( k_{\text{cat}} \) were extrapolated by linear regression of the Lineweaver-Burk plot. Comparisons of the reaction rates of 0.1 µM BLF serine protease affinity column fractions, BLF lobes, and serine protease inhibited BLF were also made using 50 µM Z-Phe-Arg-AMC.

Deglycosylation

Glycan chains were removed chemically according to the method of Sojar and Bahl.31 BLF in a 10% solution was incubated in anhydrous trifluoromethanesulfonic acid (TFMS; Sigma) with 10% anisole (Sigma) on ice for 30 minutes, followed by neutralization with 60% pyridine at −20°C and buffer exchanged to phosphate-buffered saline (PBS). Progress was monitored by the reduction in apparent molecular weight of the BLF bands with SDS-PAGE in 7.5% tris-HCl saline (PBS). Progress was monitored by the reduction in apparent molecular weight of the BLF bands with SDS-PAGE in 7.5% tris-HCl saline (PBS). Reduction and Alkylation

Iron-saturated (holo) bovine lactoferrin (h-BLF) was prepared by the addition of the iron complex ferric-nitrilotriacetate (Fe-NTA) by a method similar to that of Bates et al. A 1% solution of BLF in 20 mM tris-HCl buffer pH 7.4 was prepared with 5 mM bicarbonate added immediately before combination with a 2:1 molar excess of FeNTA and was incubated for 1 hour. The h-BLF was then buffer exchanged to PBS and concentrated as described. Occupation of iron-binding sites was confirmed spectrophotometrically by the ratio of 280 nm to 465 nm absorbance.

Reduction and Alkylation

A 1% solution of BLF in 0.6 M tris-HCl, pH 8.5, and 2% 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS; Sigma) with and without 6 M guanidine hydrochloride (Gdn-HCl; Sigma) was reduced by incubation with β-mercaptoethanol (Sigma) in a 50-fold molar excess to the disulfide bonds for 4 hours. Alkylation occurred by the addition of freshly prepared iodoacetamide (Sigma) to a concentration slightly above that of the reducing agent. The solution was protected from light during the 15-minute incubation before buffer exchange to PBS at 4°C.

Cell Culture

Immortalized human corneal-limbal epithelial (HCLE) cells (a gift from Ilene Gipson, Schepens Eye Research Institute, Boston, MA) were cultured as previously described. Briefly, cells were seeded at 2 × 10^4/cm² onto tissue culture-treated plates and were maintained in keratinocyte serum-free medium (K-SFM; Invitrogen-Gibco, Grand Island, NY), supplemented with 25 µg/mL bovine pituitary extract, 0.2 ng/mL recombinant epidermal growth factor, and 0.4 mM CaCl₂ at 37°C in a 5% CO₂ atmosphere. At 50% confluence, the cells were switched to a 1:1 mixture of K-SFM and low-calcium Dulbecco’s modified Eagle medium (DMEM)/Ham’s F12 (Inovitro) to achieve confluence.

Cell Viability

Cytotoxicity assay was performed as described by Mosmann. Briefly, at 50% confluence, HCLE cells were incubated in medium supplemented with BLF, benzamidine column fractions, or BSA, to a concentration of 0.6 µM or 12 µM, with or without PMSF pretreatment of the supplement. After 24 hours, the culture medium was replaced with one containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) and was further incubated for 4 hours. All wells were emptied and acid-isopropanol, 0.4 N HCl in isopropanol, was added at 100 µL/well, followed by 5 minutes of gentle agitation. The supernatant was transferred to a new plate, absorbance was measured at 570 nm, and the reference wavelength was 630 nm (Multiskan Spectrum; Thermo Scientific, Waltham, MA).

HCLE Alkali Burn Wound Healing Model

To determine the effect of BLF derivatives on healing of alkali-induced burns, confluent monolayers of HCLE cells were wounded using filter paper discs soaked in 0.1 M sodium hydroxide. Cells were immediately rinsed by three culture medium (1:1 K-SFM/low Ca2⁺ DMEM/F12) changes to restore pH and to remove cellular debris. The wound area was then photographed at 50× magnification and again after 24-hour incubation with the treatment solution. Areas of wounds were quantified using image analysis software (ImageJ software; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Results were expressed as relative wound closure, indicating a reduction in the wound area as a multiple of the control.

Treatment solutions for the alkali burn wound-healing model were prepared by diluting concentrated BLF (apo, holo, deglycosylated, CHAPS exposed, Gdn-HCl exposed, reduced and alkylated, and LFcin B; American Peptide, Vista, CA) to 12.8 µM in tissue culture medium. Benzamidine column fractions were reconstituted to the concentrations present in native BLF near its estimated peak wound healing concentration with and without PMSF pretreatment. BLF N-lobe and C-lobe were prepared to final concentrations of 1.28, 6.4, 12.8, 64, and 128 µM. Positive and negative controls of equimolar native BLF and bovine serum albumin (BSA; Bovogen Biologicals, Essendon, VIC, Australia), respectively, were included in each experiment. The LFcin B used, corresponding to BLF amino acids 20 to 31, was synthesized by conventional solid-phase peptide synthesis protocols at a purity of 98.8%, as quantified by the area of 215-nm absorbance peaks during reverse-phase HPLC.

To obtain representative images, the cells were fixed with 1% formalin in PBS after three washes with medium and then were stained with Harris hematoxylin (Sigma) and eosin Y (Sigma).

Statistical Analysis

The data reported are pooled from experiments performed on a minimum of two separate occasions. Normal distribution of data was confirmed with the Kolmogorov-Smirnov test. For wound-healing experiments, data were summarized as mean ± SD of a sample size of at least 16 for each treatment at each concentration. Results of BLF (N-lobe, C-lobe, apo, holo, deglycosylated, CHAPS exposed, Gdn-HCl exposed, reduced and alkylated, and LFcin B) were assessed to determine differences between treatments at each concentration using one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons using Games-Howell correction because of unequal sample sizes and nonhomogeneous variance. Analysis of results for wound healing trials with benzamidine column fractions were analyzed as described with additional comparisons made between concentrations. Cell viability was compared to equimolar BSA, with or without the same PMSF pretreatment as in the group to be analyzed. Statistical significance was taken as \( P < 0.05 \). Analysis was performed using commercial statistical examination software (SPSS; SPSS Inc., Chicago, IL).
**RESULTS**

**BLF C-Lobe Promotion of Alkali-Induced Corneal Epithelial Wound Healing**

BLF, shown to have an endotoxin content <4 EU/mg, was subjected to limited tryptic digestion followed by ion-exchange and size exclusion chromatography. The resultant eluate was separated and purified into fractions with apparent molecular weights of 30 and 50 kDa as assessed by SDS-PAGE. N-terminal sequencing confirmed the identity of these fragments as the N-lobe and C-lobe of BLF, each accounting for more than 90% of the total protein present within their respective purified fractions as determined by optical densitometry of the bands visualized with Coomassie staining (Fig. 1).

The C-lobe promoted greater wound healing than equimolar levels of intact BLF or the N-lobe for concentrations 6.4 μM to 128 μM (P < 0.05 and P < 0.001, respectively; Fig. 2). At 6.4 μM, the C-lobe promoted a 3.5-fold increase in wound closure over BSA compared with 2.5-fold for native BLF (Fig. 2); this difference was sustained by the C-lobe for the higher concentrations. The N-lobe, however, promoted progressively reduced healing compared with intact BLF at concentrations from 6.4 μM to 128 μM (P < 0.01; Fig. 2), decreasing to the level of BSA at 12.8 μM (P = 0.098) and dropping below BSA at 128 μM (P = 0.017; Fig. 2).

**BLF Protease Activity in Corneal Epithelial Wound Healing**

Two fractions with distinct isolated peaks were separated from native BLF with the serine protease affinity column (Fig. 3). Comparison of the total protein content of the unbound (peak I; Fig. 3) and eluted (peak II; Fig. 3) fractions revealed approximately 5% of native BLF bound to the benzamidine substrate. All fractions were the same apparent molecular weight as BLF, as determined by SDS-PAGE, with no visible contaminating bands in the eluted fraction (Fig. 4).

The identity of the two fractions was confirmed by LC-MS/MS to be BLF, accession number gi 157830374, with Mascot protein scores of 2841 and 2517 for the unbound and eluted fractions, respectively. Within the BLF sequence recovered—55% for the unbound fraction and 53% for the eluted fraction—the peptide Ala-Pro-Val-Asp-Ala-Phe-Lys was detected.

**FIGURE 2.** (A) Closure of alkali-induced HCLE wounds treated with native BLF, BLF N-lobe, and BLF C-lobe at 1.28-, 6.4-, 12.8-, 64-, and 128-μM concentrations relative to equimolar BSA. Data are presented as mean ± SD (n = 16). *Statistically significant increase compared with equimolar native BLF (P < 0.05) and BLF N-lobe (P < 0.001). *Statistically significant decrease compared with equimolar native BLF (P < 0.01). **Statistically significant decrease compared with equimolar BSA (P < 0.05). (B) Alkali wounds before treatment and after 24-hour incubation with 12.8 μM BSA, BLF, BLF N-lobe, and BLF C-lobe.

in all native-BLF and unbound fraction samples but none from the eluted fraction. This peptide corresponds to the surface-exposed BLF amino acids 237–245 on the N-lobe.

The proteolytic activity of BLF eluted from the benzamidine was found to have a $K_m$ of 34 ± 4 μM and a $k_{cat}$ of 0.3 ± 0.08 minute$^{-1}$ for the serine protease substrate Z-Phe-Arg-AMC in pH 7.0 buffer at 25°C. This fraction of BLF (proteolytic [p-BLF]) at 0.1 μM and incubated with 30 μM substrate had substantially greater proteolytic activity than equimolar native BLF or the unbound, nonproteolytic (np-BLF) fraction (P < 0.001; Fig. 5). Hydrolysis of the serine protease substrate did not differ substantially between native BLF and the N-lobe (P = 0.94; Fig. 5),

**FIGURE 1.** Fractions from the tryptic digestion and purification of the BLF N-lobe and C-lobe. (A) Protein standard. (B) Tryptic digestion of BLF for 4 hours. (C) C-lobe purified from B by cation exchange and size exclusion chromatography. (D) BLF. (E) Tryptic digestion of BLF for 0.5 hour. (F-H) BLF and partially digested BLF containing C-lobe and N-lobe, respectively, as isolated peaks from size exclusion chromatography of (E). Visualized on 12% SDS-PAGE under reducing conditions and stained with Coomassie dye.

**FIGURE 3.** Affinity chromatography on benzamidine Sepharose column. Injection of 1 mL of 1% native BLF in 50 mM tris-HCl, 0.5 M NaCl, at pH 7.4. Peak (I) is the unbound fraction designated np-BLF. Peak (II) is step eluted with 10 mM HCl, 0.5 M NaCl, pH 2.0, and designated p-BLF.
and both were found to be more active than the C-lobe, np-BLF, and PMSF-inhibited BLF ($P < 0.001$; Fig. 5).

Wound closure by np-BLF and p-BLF was compared at the approximate levels found within native BLF (i.e., 1.20) at the estimated peak wound healing concentration of 12.8 μM (Fig. 2). Wounds incubated at concentrations of 0.6 μM p-BLF and 12 μM np-BLF produced a similar degree of wound closure ($P = 0.77$; Fig. 6) that exceeded equimolar BSA ($P > 0.001$; Fig. 6) by a factor of approximately 2.5, similar to 12 μM native BLF. However, when the concentrations of np-BLF and native BLF were lowered to the level at which p-BLF was effective, 0.6 μM, wound closure was no greater than with BSA ($P < 0.9$; Fig. 6). Conversely when p-BLF was increased to 12 μM, its promotion of wound closure was also no longer significantly different from BSA ($P = 0.16$; Fig. 6). At this concentration, p-BLF was found to be cytotoxic with a reduction in cell viability to 48% of the levels for equimolar BSA ($P < 0.001$).

Serine protease inhibition altered the promotion of wound healing only for p-BLF ($P < 0.001$; Fig. 6) with the effect of a loss of wound healing at the low concentration and a restoration of wound healing at the high concentration. Pretreatments with PMSF also abolished the cytotoxicity of p-BLF relative to BSA ($P = 1.0$). The reduction in wound healing with inhibition for native BLF at 12 μM did not reach significance ($P = 0.17$; Fig. 6), and no other treatments were found to be cytotoxic.

**Effect of BLF Glycosylation, Iron Saturation, Denaturation, and LFcin B on Wound Healing**

Chemical deglycosylation was complete after 30 minutes, with no further decrease in apparent molecular weight observed by nonreducing SDS-PAGE. An equivalent apparent molecular weight reduction of 9 kDa was observed for BLF enzymatically deglycosylated with peptide-N-glycosidase F under denaturing conditions (data not shown). Removal of glycans from BLF did not alter the promotion of wound healing; a 2.5- to 3-fold increase in closure of alkali-induced wounds was still observed for 12.8 μM deglycosylated BLF compared with BSA ($P < 0.001$; Fig. 7). This effect was not significantly different from that of native BLF ($P > 0.9$; Fig. 7).

Spectroscopic analysis indicated iron saturation to be <10% for a-BLF and >90% for h-BLF. These differing levels of BLF iron saturation did not alter the promotion of wound closure after alkali injury to HCLE monolayers. A similar increase in wound closure was found for native BLF, a-BLF, and h-BLF compared with the BSA control ($P < 0.001$; Fig. 7), with a 2.5- to 3-fold increase in closure. There was no difference in promotion at a concentration of 12.8 μM between native BLF and a-BLF or h-BLF ($P > 0.95$; Fig. 7). BLF prepared using a chaotrope, 6 M Gdn-HCl, produced significantly less wound closure than did native BLF ($P < 0.001$; Fig. 7), whereas BLF prepared with the zwitterionic detergent 2% CHAPS continued to increase wound healing. The ability to promote wound healing was also lost after the reduction and alkylation ($P < 0.001$; Fig. 7) of BLF.

In isolation, the LFcin B peptide did not promote the closure of alkali-induced wounds in HCLE cells. Reduced wound healing was observed for LFcin B compared with BLF ($P <
although this is contrary to the effect on bone tissue in the LFcin peptides, from the N-lobe, reported on experiments becomes progressively lower as the concentration increases. 

explaining why a low level of promotion is observed that boring activity that is mildly antagonistic to wound closure, of the C-lobe wound healing site in addition to peptides harbors the greater efficacy of the C-lobe observed.

Thus, by removing the region comprising antagonistic activity, be responsible for diminishing the corneal healing response. A number of cytokines, their potential to promote wound closure, but, as yet, none has demonstrated sufficient efficacy to become part of a standard treatment regimen. Here we report that in vitro, the C-lobe of bovine lactoferrin is able to promote the closure of alkali-induced corneal epithelial wounds more potently than intact BLF. Increased efficacy of the C-lobe over native BLF and the N-lobe is interesting given the high degree of lobal homology. We propose this increase could be attributed to steric factors, greater substrate affinity, or an inhibitory effect from the target in the promotion of wound healing. This hypothesis is supported by the finding in hepatocytes that BLF lacking the N-terminal binds with higher affinity to a smaller number of sites than native BLF. 

Increased efficacy of the C-lobe over native BLF and the N-lobe is interesting given the high degree of lobal homology. A number of cytokines, growth factors and matrix proteins have been investigated for their potential to promote wound closure, but, as yet, none has demonstrated sufficient efficacy to become part of a standard treatment regimen. Here we report that in vitro, the C-lobe of bovine lactoferrin is able to promote the closure of alkali-induced corneal epithelial wounds more potently than intact BLF.

Use of therapeutic agents that accelerate epithelial coverage of corneal lesions would reduce exposure of the stroma to sight-threatening secondary infections. A number of cytokines, growth factors and matrix proteins have been investigated for their potential to promote wound closure, but, as yet, none has demonstrated sufficient efficacy to become part of a standard treatment regimen. Here we report that in vitro, the C-lobe of bovine lactoferrin is able to promote the closure of alkali-induced corneal epithelial wounds more potently than intact BLF.

Increased efficacy of the C-lobe over native BLF and the N-lobe is interesting given the high degree of lobal homology. We propose this increase could be attributed to steric factors, greater substrate affinity, or an inhibitory effect from the target in the promotion of wound healing. This hypothesis is supported by the finding in hepatocytes that BLF lacking the N-terminal binds with higher affinity to a smaller number of sites than native BLF. Lastly, the N-lobe may contain a repeat of the C-lobe wound healing site in addition to peptides harboring activity that is mildly antagonistic to wound closure, explaining why a low level of promotion is observed that becomes progressively lower as the concentration increases. This inhibitory activity might relate to the antimicrobial action of the LFcin peptides, from the N-lobe, reported on experiments with neoplastic cells and to our previous finding that native BLF reduces proliferation in the immortalized HCLE cell line, although this is contrary to the effect on bone tissue in which LFcin promotes a small degree of osteogenic activity. Reduced wound healing with high concentrations of N-lobe and p-BLF observed in the present study, and the localization of p-BLF proteolytic activity to the N-lobe, suggest this region may be responsible for diminishing the corneal healing response. Thus, by removing the region comprising antagonistic activity, improved closure rates could be expected and might explain the greater efficacy of the C-lobe observed.

The serine protease plasmin is tightly regulated and plays an essential role in normal corneal epithelial wound healing, however, elevated tear plasmin levels are associated with defect formation during the repair process. Plasmin is not transformed from its zymogen, in tissue repair, until it is activated at the leading edge of the wound, where it begins to degrade the provisional fibrin matrix and to upregulate other migration-promoting factors. In the present study, a fraction purified from BLF, as described by Massucci et al., exhibited a very low level of serine protease activity and was able to promote HCLE wound closure at a concentration less than that of native BLF, yet at a high concentration p-BLF delayed wound closure because of cytotoxicity. This may reflect in vivo observations that plasmin is required for normal wound healing but that an excess becomes detrimental. However, the degree of proteolytic similarity between plasmin and BLF remains unclear because the substrate specificity of LF is confirmed only for Haemophilus influenzae IgA1 protease and Hap adhesin but not yet for human proteins.

Localization of the proteolytic activity of BLF to the N-lobe supports the conclusions of Qiu et al., who observed the degradation of bacterial proteins by this region with an activity that is halted by serine protease inhibitors. The absence of the BLF peptide sequence located at the N-lobe amino acid residues 237 to 243 in the p-BLF samples suggests this region could be structurally different in the proteolytically active variant. It was proposed by Hendrixson et al., based on site-directed mutagenesis studies, that serine 259 may be part of the catalytic site for LF, though pK\textsubscript{a}-shift calculations by Massucci et al. indicated this to be unlikely. An alternative sequence, however, over the region we have identified, may result in a conformation that creates the electrostatic potential required for deprotonation of serine 259 during substrate hydrolysis. The differing effect of serine protease inhibition on wound healing promotion by p-BLF compared with np-BLF, and their disparate effective concentrations, would seem to indicate the presence of two separate mechanisms (active sites) through which BLF increases rates of wound repair. If this is the case, there is also an antagonistic interaction between the two mechanisms because when they are used in conjunction, as occurs in native BLF, wound healing is not significantly greater than when they are used in isolation. This antagonism is further supported by the observation of no significant loss in wound healing when native BLF at 12 \(\mu\)M was serine protease inhibited; however, it is possible a reduction occurred below the sensitivity of our model. Further research into the structural basis of proteolytically active BLF may improve our understanding of these findings.

Of the 17 disulfide bonds stabilizing the secondary and tertiary structures of BLF, 10 are located on the C-lobe. That wound healing is lost with reduction and alkylation of BLF suggests an importance of the native conformation, as maintained by these bonds, to the region promoting this activity. Optical rotatory dispersion analysis of LF exposed to a chaotrope has demonstrated a conformational change. BLF re-folded in this manner has less activity, further highlighting the contribution of a stabilized native structure to the promotion of wound healing.

Occupation of the BLF iron-binding sites results in a marked conformational change whereby the domains of each lobe are drawn together, reducing exposure of the protein inner surface. Promotion of wound closure occurred equally with the apo or holo conformation, suggesting the peptide sequence inducing healing is located outside the inter-domain cleft. Furthermore, these observations suggest that the antioxidant ac-
tivity, driven predominantly by iron chelation, is not a factor in the promotion of in vitro corneal epithelial wound healing. There are five potential N-glycosylation sites on BLF, linked to either high mannose or complex glycans. Four are expressed strongly, and the other is present on only 30% of colostrum-derived BLF. In this study, deglycosylation was performed chemically rather than enzymatically to avoid the problem of steric interference at glycosidic asparagine 545,58 which is located in a deep cleft. Although this obstacle to endoglycosidase binding could be lessened by unfolding the protein, we found that BLF-mediated wound healing is lost with this form of denaturation. Although several carbohydrate-rich structures, such as fibronectin and carboxymethylcellulose, enhance corneal epithelial cell migration, for BLF, the glycans did not influence wound closure, indicating they neither obstruct the area that promotes healing nor promote it themselves.

In conclusion, the promotion of wound healing by BLF appears to rely on mechanisms independent of other ascribed functions that are mediated by the chelation of iron or intrinsic to the N-terminal peptide. The site promoting this activity is most likely located on the outer surface of the C-lobe, with a conformation that requires a degree of stabilization. This region of BLF warrants further investigation for its potential as a novel topical agent in the treatment of corneal epithelial lesions with delayed wound healing.

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References