Formyl Peptide Receptor Ligands Promote Wound Closure in Lung Epithelial Cells

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Mitochondrial antigens released from damaged cells act as “danger signals” capable of promoting innate immune cell migration and activation via formyl peptide receptors (FPRs). Lung epithelial cells are equipped to migrate and mount innate immune responses in the context of acute lung injury. The goal of this study was to determine whether lung epithelial cells express FPRs, which are capable of responding to mitochondrial antigens to promote wound closure and inflammation. Using human Beas2B lung epithelial cells grown to confluency and subjected to linear scratch injury, it was found that mitochondrial antigens enhanced epithelial wound closure, and this phenomenon was inhibited by cyclosporin H, a selective inhibitor of FPR. Although mitochondrial antigens also promoted IL-8 release, this release was not FPR dependent and was unrelated to FPR-induced lung epithelial cell wound closure. The expression of functional FPR was confirmed in Beas2B and primary human tracheobronchial epithelial cells, particularly in lamellipodia at the leading edge of the closing wound. The expression of FPR was increased in response to TNF- α , LPS, scratch injury, and mitochondrial antigen treatment. Considered together, these data confirm that human lung epithelial cells express functional FPRs, which are capable of responding to endogenous mitochondrial danger signals, to promote wound closure.

Keywords: mitochondria; cell migration; Beas2B; IL-8; acute lung injury

The lung epithelium is often damaged in the context of human disease, and rapid restoration of an intact epithelial barrier is required to prevent serious complications, such as pulmonary edema and pneumonia (1, 2). Epithelial cell wound closure is an important first step in this regeneration process; however, the mechanisms responsible for lung epithelial wound closure remain unclear. In this context, the high-affinity formyl peptide receptor (FPR), a small G-protein-coupled receptor commonly expressed on immune cells, specifically recognizes bacterial and mitochondrial N-formyl peptides to promote inflammatory cell migration (3), and FPR agonists promote gut epithelial wound closure (4). The role of FPR in facilitating lung epithelial wound closure is unknown.

CLINICAL RELEVANCE

These studies are the first to show that lung epithelial cells express functional formyl peptide receptors, which respond to mitochondrial danger signals to promote lung epithelial wound closure. Thus, lung epithelial formyl peptide receptors may participate in lung epithelial repair in the context of acute lung injury.

Recent studies have shown that various inflammatory mediators, released in response to epithelial injury, contribute to epithelial migration. In this context, increased IL-8 expression is temporally associated with the lung epithelial migration and proliferation in the initial phases of airway epithelial repair (5). Under optimal wound healing conditions, IL-8 expression declines after cell migration is complete, at which time the expression of matrix metalloproteinases and other growth factors increases, conditions favoring pseudostratification and differentiation of epithelial cells (5, 6). *In vitro* studies have demonstrated that IL-8 is capable of stimulating human keratinocyte migration (7), is highly expressed at the leading edge of damaged epithelial layers, and enhances wound healing *in vivo* (8, 9). IL-8 and CXCR2, the receptor for IL-8, promote matrix metalloproteinase expression (10, 11). Thus, IL-8 is considered by some investigators to be a proximal regulator of epithelial wound repair (6).

Recent investigations indicate that endogenous antigens, or “danger signals,” are liberated from damaged cells to promote immune cell migration and innate immune responses, including IL-8 release. For instance, mitochondrial N-formylated peptides, which are encoded by mitochondrial DNA and specialized for electron transport, are potent chemotaxins for neutrophils (3). We have recently shown that mitochondrial antigens released from damaged cells, particularly N-formyl peptides, contribute to monocyte IL-8 release by interacting with the high-affinity FPR (12). Furthermore, bacterial N-formyl peptides are reported to regulate intestinal epithelial cell migration *in vitro* (4). Here, we investigate the role of mitochondrial N-formyl peptides as potential mediators of lung epithelial wound closure. We hypothesized that mitochondrial antigens released from damaged cells would trigger lung epithelial wound closure through FPR. Given the circumstantial evidence linking IL-8 to epithelial cell and immune cell migration (7–9) and previous studies indicating that FPR ligands promote IL-8 production (5, 12), the specific role of this chemokine was considered. Some of the results of these studies have been previously reported in abstract form (13).

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MATERIALS AND METHODS

Additional methodological details are provided in the online supplement.

HepG2 Cell Fractionation

HepG2 cell necrosis was induced by freeze/thaw and confirmed by the lactate dehydrogenase assay, as detailed previously (12, 14). Cell and mitochondrial subfractions were prepared from HepG2 cells using differential centrifugation as described by Nair and colleagues (15).

Beas2B Cell Culture and Treatment

After scratch wound generation in Beas2B cells (16) and replacement with fresh media, cells were treated with necrotic HepG2 cells or mitochondrial subfraction proteins, 2% FBS, or the FPR agonist ND6. In some instances, preincubation with FPR inhibitors, cyclosporin H (CsH) (1 μ M) or Boc-Phe-Leu-Phe-Leu-Phe (BOC; 10 μ M), or an IL-8 blocking antibody (1 μ g/ml) (R&D Systems, Minneapolis, MN) were made. Cell supernatants and pellets were then collected 18 to 20 hours after treatment and stored at -80°C for later analyses. Given that the results may be affected by the use of older Beas2B cell lines, these studies were limited to cell lines having no more than 15 passages to avoid changes in wound closure relating to cell line senescence.

Human Primary Lung Cell Preparations

Human lungs were collected with approval from The Ohio State University Institutional Review Board and with patient consent. Normal, healthy adult donor lungs ($n = 3$) were obtained, and fully differentiated primary human tracheobronchial epithelial cells (hLEC) were isolated, seeded, and grown at an air-liquid interface as described elsewhere (17, 18).

Mechanical Wound Model for Differentiated hLECs

After wound generation in hLECs and removal of resultant cell debris, transepithelial electrical resistance (TEER) was evaluated over time as previously detailed (16). hLECs were treated basolaterally with mitochondrial protein preparations in the absence or presence of CsH and followed for 5 days after treatment.

RT-PCR

Beas2B cells were exposed to BSA (5 μ g/ml), mitochondrial protein (5 μ g/ml), and linear scratch injury alone or in combination with mitochondrial antigen or in the presence of inflammatory mediators (LPS [1 μ g/ml] and TNF- α [100 ng/ml]) for 24 hours. Total Beas2B cell RNA was then isolated, and semiquantitative RT-PCR was performed as described previously (12). hLEC RNA, postwound, was evaluated to confirm *FPR* expression.

Fluorescence Microscopy

After blockade with 10% goat serum/1% FBS in Tris-buffered saline with 0.5% Tween for 1 hour at room temperature, fixed hLECs or Beas2B cells were incubated with a rabbit anti-human FPR antibody (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour, washed, and then followed by a goat anti-rabbit Alexa Fluor 488-labeled secondary antibody (1:500) (Molecular Probes, Inc., Carlsbad, CA) for 1 hour at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (100 ng/ml) (Sigma-Aldrich, St. Louis, MO), and normal rabbit IgG (Santa Cruz Biotechnology, Inc.) was used as a negative control. Immunofluorescence was evaluated using confocal fluorescence microscopy at 488 nm (FPR) and 350 nm (DAPI).

Statistical Analyses

The data were derived from at least five independent experiments and are expressed as mean $\pm$ SEM. Statistical significance was based on a value of $P \leq 0.05$. SigmaPlot 10.0 and SYSTAT 12.0 software (Systat Software, Inc., San Jose, CA) was used to plot the data and carry out the statistical analyses, respectively.

RESULTS

The Mitochondrial Fraction of Necrotic Cells Induces Lung Epithelial Cell Wound Closure

After establishing a linear scratch wound in an otherwise confluent monolayer of Beas2B cells and removing those that were floating along with resultant cell debris, cells were treated with fresh media containing cell lysate or cellular subfraction proteins derived from necrotic HepG2 cells. The mitochondrial fraction of the cell, particularly the mitochondrial membrane, was most potent in terms of promoting Beas2B cell wound closure (Figure 1A), and this effect was demonstrated to be dose dependent for both mitochondrial protein treatments (Figure 1B).

Mitochondrial Antigens Engage FPR to Promote Beas2B Cell Wound Closure

The enhancement of wound closure by mitochondrial antigens derived from necrotic HepG2 cells was attenuated by treatment with selective (CsH) and nonselective (BOC) inhibitors of FPR (19, 20) (Figure 2A). Serum-induced wound closure was unaffected by FPR inhibition, indicating that the serum components responsible for this action were not FPR agonists and promoted wound closure through other mechanisms. Enhanced and dose-dependent cell migration in response to a synthetic mitochondrial peptide (ND6), which was CsH sensitive, provided further evidence that Beas2B cell migration involved FPR regulation (Figure 2B).

Wound Closure in Response to Mitochondrial Antigens Is Unrelated to IL-8 Release

As in cell wound closure, Beas2B cells were observed to produce IL-8 in response to selective treatment with the mitochondrial fraction of the cell, which was unaffected by pretreatment with FPR inhibitors, CsH, or BOC (Figure 3A). Pretreatment with an IL-8-blocking antibody quenched mitochondrial protein-induced IL-8 release signal (Figure 3A) but had no impact on the rate of wound closure (Figure 3B). Considering that FPR inhibition effectively blocked cell migration (Figure 2A) and that treatment with an IL-8-blocking antibody was ineffective at doing so, these data indicate that IL-8 was not primarily responsible for cell migration in response to mitochondrial antigens.

Functional FPR Expression Is Confirmed on hLECs

Primary, differentiated human tracheobronchial epithelial cells were demonstrated to express the *FPR* transcript (Figure 4A) and FPR protein, particularly along the margin of the scratch wound, within lamellipodia-like processes emanating from the margin of the wound (Figure 4B). *FPR* expression was enhanced in confluent Beas2B cell cultures in response to mitochondrial antigens, mechanical scratch injury, or proinflammatory cytokines (LPS, TNF- α) (*see* Figure E2 in the online supplement). The rate of closure of mechanical scratch wounds produced in confluent hLECs, as reflected by restoration of TEER, was enhanced in the presence of mitochondrial antigens, an effect that was inhibited by CsH (Figure 4C).

DISCUSSION

The current study demonstrates that lung epithelial cell wound closure and IL-8 release are enhanced by selective treatment with the mitochondrial component of necrotic cells (Figures 1A and 3A, respectively). Under these conditions, epithelial wound closure was attenuated in the presence of inhibitors of the high-

Figure 1. (A) Relative scratch wound closure in Beas2B cells 18 to 20 hours after linear injury, as a percentage of that measured at baseline, in the absence or presence (5 μ g/ml) of necrotic HepG2 cell lysate protein or corresponding HepG2 cellular subfractions. The mitochondrial fraction of the cell, particularly the mitochondrial membrane, was observed to most potently induce wound closure (* P < 0.05, compared with no treatment). Serum served as a positive control. (B) A dose-response relationship was demonstrated between the mitochondrial protein treatments and the extent of relative Beas2B cell scratch wound closure 18 to 20 hours after injury. * P < 0.05 relative to no treatment. † P < 0.05 compared with matching total mitochondrial protein dose.

affinity FPR and was not dependent on IL-8 (Figures 2A and 3B). These studies are the first to consider the role of FPRs in the context of lung epithelial wound repair.

In humans, the FPR family is limited to three subtypes: the high-affinity FPR, FPR like-1 (FPRL1), and FPR like-2 (FPRL2). The FPR family of receptors features G-protein-dependent signal transduction and is distinguished by their antigen selectivity and biological actions. As opposed to FPR, which is highly selective for N-formyl peptides, FPRL1 has relatively low affinity for N-formyl peptides and recognizes an

Figure 2. (A) Relative scratch wound closure in Beas2B cells 18 to 20 hours after linear injury, as a percentage of that measured at baseline, in the absence or presence (5 μ g/ml) of mitochondrial protein treatments with or without pharmacological inhibitors of high-affinity formyl peptide receptors (FPR) (cyclosporin H [CsH, 1 μ M] or Boc-Phe-Leu-Phe-Leu-Phe [BOC, 10 μ M]) present. Mitochondrial protein treatments notably increased wound closure (* P < 0.05 relative to no treatment); FPR inhibition was partially, yet significantly, effective at attenuating this effect († P < 0.05 compared with matching mitochondrial protein treatment alone; ‡ P < 0.05 relative to no treatment and matching protein treatment alone). FPR inhibition had no effect upon the response to serum-induced wound closure. (B) A dose-response relationship was observed between synthetic mitochondrial N-formyl protein (ND6) treatment and the extent of relative Beas2B cell scratch wound closure 18 to 20 hours after injury, which was CsH sensitive (* P < 0.05 compared with no treatment; † P < 0.05, relative to matching protein treatment alone).

array of other exogenous (e.g., bacterial) and endogenous (e.g., annexin-1) antigens to promote inflammatory cell migration and activation. In contrast, FPRL2 is unresponsive to N-formyl peptides, and the function of this receptor in humans remains unclear. The interested reader is encouraged to consult the excellent review of N-FPRs by Rabi et al. and colleagues for more detailed information on this topic (21). Unpublished data from our laboratory indicates that mitochondrial antigens promote

FPRL1-dependent IL-8 release from human lung epithelial cells without influencing the rate of wound closure.

The lung represents a primary barrier against potential environmental pathogens, and, as such, is poised to detect diverse pathogens through an array of pattern-recognizing receptors, including Toll-like receptors and NODS (2); however, the role of FPRs in the lung has not been considered. Using mice deficient in FPR1 (the murine homolog of human FPR), Gao and colleagues demonstrated this receptor to be integral for mounting effective immune responses to pathogenic bacteria, thereby promoting bacterial clearance and host survival (22). In addition to bacterial defenses, previous studies, including our own (12), indicate that FPR-mediated responses to endogenous mitochondrial antigens promote neutrophilic inflammatory responses through FPR-mediated chemotaxis (3, 23) or through the enhancement of IL-8 release via FPR activation (12). In addition to its function as a neutrophil chemotaxin, IL-8 is also reported to promote nonpulmonary epithelial migration in the context of wound healing (9, 24). Thus, the finding of functional FPR receptors on lung epithelial cells has important implications for responses to exogenous (bacterial) and endogenous (e.g., mitochondrial) antigens.

Necrotic cell death is a common consequence of acute illnesses, such as thermal injury, trauma, acute lung injury, and severe infections (25, 26). In contrast to programmed cell death, wherein proteins, DNA, and lipids are compartmentalized within lysosomes and undergo thorough enzymatic degradation, necrotic cell death is associated with the liberation of intact cellular components. The degradation of endogenous “danger signals” explains, in part, why apoptotic cells fail to promote inflammatory responses, whereas necrotic cell exposure strongly promotes immune cell activation (27). In this context, mitochondrial antigens are commonly released from damaged tissues during acute illness (28, 29), and the mitochondrial component of necrotic cells has unique proinflammatory properties owing to features retained from their bacterial origins. Namely, mitochondria retain bacterial qualities such as circular DNA, enriched in CpG motifs, which encodes for highly abundant N-formylated peptides, which are well conserved among eukaryotes and considered essential for electron transport (30). These unique mitochondrial properties likely explain the relatively high potency of the mitochondrial fraction of necrotic cells compared with other cell fractions (e.g., cytoplasm, nucleus) in terms of inducing monocyte-mediated innate immune responses (12, 31). In keeping with these previous studies, we now show that lung epithelial cells release IL-8 in response to selective treatment with the mitochondrial component of necrotic cells (Figure 3A). FPR, however, is not required for IL-8 release under these conditions.

This study is the first to show that FPR regulates lung epithelial wound closure. Babbin and colleagues report FPR1 expression on murine intestinal epithelium, which is shown to promote epithelial wound closure in response to bacterial N-formyl peptides (4). We extend these observations to the human lung, demonstrating that endogenous mitochondrial antigens are capable of promoting lung epithelial wound closure. We further show that serum promotes wound closure through a distinct mechanism. In this regard, previous studies identified several poorly characterized components of serum, designated epibolin and coepibolin, which are responsible for epithelial migration (32). However, serum components apparently do not engage FPR, as indicated by a lack of response to the potent and selective FPR inhibitor, CsH (Figure 2A). Likewise, IL-8 is not essential for wound closure in response to mitochondrial antigens, as reflected by differential inhibition of wound closure without suppression of IL-8 in response to FPR inhibition and

Figure 3. (A) Supernatant IL-8 concentration 18 to 20 hours after scratch wound injury in Beas2B cell monolayers in the absence or presence (5 μ g/ml) of total mitochondrial protein. Significant IL-8 release induced by mitochondrial protein treatment was appropriately suppressed by coinubation with a blocking antibody specific for IL-8 (1 μ g/ml) (* P < 0.01 relative to no treatment; † P < 0.01 compared with total mitochondrial protein). Mitochondrial protein-induced IL-8 release was not affected by FPR inhibition (CsH [1 μ M]/BOC [10 μ M]). (B) Relative scratch wound closure in Beas2B cells 18 to 20 hours after linear injury, as a percentage of that measured at baseline, in the absence or presence (5 μ g/ml) of mitochondrial protein with or without a specific IL-8–blocking antibody (1 μ g/ml) present (* P < 0.05 relative to no treatment). Antibody blockade of IL-8 had no effect upon mitochondrial protein–induced wound closure.

the lack of inhibition of wound closure after treatment with an IL-8 blocking antibody (Figure 3B). Based on convincing evidence indicating that IL-8 promotes epithelial migration or wound closure under certain conditions (2, 5–9, 33) and the likelihood that serum promotes wound closure by yet another mechanism (32), FPR-mediated epithelial wound closure and barrier recovery appear to represent a novel mechanism. Although the observed expression of FPR in hLECs on the leading edge of the wound (Figure 4B), particularly within the lamellipodia-like processes that typically form during wound

Figure 4. (A) Expression of *FPR* in primary human tracheobronchial epithelial cells (hLEC) was verified in three different lung donors (1–3) by RT-PCR. The results were compared with a negative control wherein RT-PCR was performed in the absence of RNA (NC). *GAPDH* expression served as a loading control. (B) Expression of *FPR* protein was demonstrated (green) particularly within the vicinity of hLEC injury after wound generation in this representative fluorescent photomicrograph. *FPR* expression was most prevalent within lamellipodia-like processes extending from the wound margin (top left: hLECs from a representative donor; bottom left: negative control hLECs [blue, DAPI]; right: z-stack representation of *FPR* immunostaining in hLECs). (C) The rate of primary human tracheobronchial epithelial wound closure, as expressed by transepithelial electrical resistance (TEER), was enhanced in response to mitochondrial antigens (5 μ g/ml). The effect of mitochondrial antigens was attenuated by pretreatment with selective *FPR* inhibition (CsH, 1 μ M). * $P < 0.05$ compared with no treatment or CsH-pretreated groups. † $P < 0.05$ relative to the CsH-pretreated group.

closure (34), is of unclear significance, it suggests that *FPR* may regulate cytoskeletal events attendant to wound closure.

Limited access to hLECs necessitated the use of immortalized human epithelial (Beas2B) cell lines for some of these investigations. In this regard, Beas2B cells do not reliably form tight junctions (35), such that TEER is a poor indicator of wound closure. Moreover, the conventional scratch wound model used for the Beas2B cells was not feasible in the primary lung epithelial cells due to the sensitive nature of the unique growth conditions (i.e., collagen-coated, semipermeable membranes) (16). This explains why two different methods were used to measure the rate of wound closure in these studies.

In conclusion, this study is the first to demonstrate that lung epithelial cells express functional *FPR* receptors capable of

promoting wound closure in response to mitochondrial antigens. Epithelial wound closure induced by *FPR* is unrelated to IL-8 release and is mechanistically distinct from serum-induced wound closure. Thus, *FPR* represents a novel mechanism by which epithelial cell wound recovery is regulated. The importance of *FPR* and of other *FPR* family receptors in relation to acute and chronic lung diseases remain to be determined.

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