Interleukin-1β augments in vitro alveolar epithelial repair

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Geiser, Thomas, Pierre-Henri Jarreau, Kamran Atabai, and Michael A. Matthay. Interleukin-1β augments in vitro alveolar epithelial repair. *Am J Physiol Lung Cell Mol Physiol* 279: L1184–L1190, 2000.—Biologically active interleukin (IL)-1β is present in the pulmonary edema fluid obtained from patients with acute lung injury and has been implicated as an important early mediator of nonpulmonary epithelial wound repair. Therefore, we tested the hypothesis that IL-1β would enhance wound repair in cultured monolayers from rat alveolar epithelial type II cells. IL-1β (20 ng/ml) increased the rate of in vitro alveolar epithelial repair by 118 ± 11% compared with that in serum-free medium control cells (P < 0.01). IL-1β induced cell spreading and migration at the edge of the wound but not proliferation. Neutralizing antibodies to epidermal growth factor (EGF) and transforming growth factor-α or inhibition of the EGF receptor by tyrphostin AG-1478 or genistein inhibited IL-1β-induced alveolar epithelial repair, indicating that IL-1β enhances in vitro alveolar epithelial repair by an EGF- or transforming growth factor-α-dependent mechanism. Moreover, the mitogen-activated protein kinase pathway is involved in IL-1β-induced alveolar epithelial repair because inhibition of extracellular signal-regulated kinase activation by PD-98059 inhibited IL-1β-induced alveolar epithelial repair. In conclusion, IL-1β augments in vitro alveolar epithelial repair, indicating a possible novel role for IL-1β in the early repair process of the alveolar epithelium in acute lung injury.

alveolar type II epithelial cells; wound repair; epidermal growth factor; transforming growth factor-α

ACUTE LUNG INJURY is usually characterized by significant damage to the alveolar epithelial barrier (2). The efficient regeneration of an intact alveolar epithelium is crucial to restore normal function of the alveolar barrier (19). Alveolar type II epithelial cells are primarily responsible for reepithelialization and restoration of the normal alveolar architecture (1). This process requires spreading, migration, and proliferation of alveolar type II cells, which differentiate and replace necrotic or apoptotic alveolar type I cells. Although extracellular matrix, especially fibronectin, probably plays an important role in the alveolar repair process (9), growth factors such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have also been shown to augment alveolar epithelial repair in vitro (15).

During the early phase of acute lung injury, a variety of inflammatory mediators are released into the alveolar space. Interleukin (IL)-1β and tumor necrosis factor-α are markedly elevated in edema fluid (24) and bronchoalveolar lavage fluid (23) from patients at an early stage of acute respiratory distress syndrome (ARDS). In both bronchoalveolar lavage fluid and pulmonary edema fluid, IL-1β was biologically active and primarily responsible for the inflammatory activity in the pulmonary edema fluid from patients with ARDS, whereas very little biologically active tumor necrosis factor-α was found (23, 24). It is not known, however, if the early-response cytokine IL-1β plays a role in epithelial repair in the lung. Studies (5, 13, 27, 31) in epidermal, corneal, and colonic epithelial wound repair indicated that IL-1β is expressed at very early stages in the wound edges and might be important for mediating epithelial repair.

Therefore, our first objective was to test the hypothesis that IL-1β modulates alveolar epithelial repair using our in vitro wound healing model. The results indicated that IL-1β markedly increased alveolar epithelial repair. Our second objective was to examine the mechanisms that accounted for the stimulatory effect of IL-1β on alveolar epithelial repair, including the potential contribution of cell proliferation and cell spreading, and the potential role of integrins that have been previously implicated in alveolar epithelial cell migration (16). In addition, we studied the contribution of EGF, TGF-α, and their common receptor (EGFR) on IL-1β-induced alveolar epithelial repair to test the hypothesis that IL-1β enhances alveolar epithelial repair by an EGF- or TGF-α-dependent mechanism. Finally, we studied the importance of the mitogen-activated protein kinase (MAPK) signal transduction pathway in IL-1β-induced alveolar epithelial repair because it was previously shown that extracellular signal-regulated kinases (ERKs) were activated in epithelial injury and repair in vivo (22) and in vitro (11).

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MATERIALS AND METHODS

Reagents

Elastase was purchased from Roche Diagnostics (Indianapolis, IN). Rat IgG, fibronectin, and DNase I were from Sigma (St. Louis, MO). Cell culture medium (minimal essential medium (MEM)), fetal bovine serum (FBS), penicillin, and streptomycin were from the Cell Culture Facility, University of California, San Francisco. Human IL-1β was purchased from R&D Systems (Minneapolis, MN). Monoclonal IL-1 receptor 1 antibody (rabbit anti-rabbit antibody) and its blocking peptide were from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing anti-EGF antibody was from R&D Systems, and anti-TGF-α antibody was from Calbiochem (San Diego, CA) (both mouse monoclonal IgG1). Murine anti-human β3 and α5 antibodies were from Chemicon, and murine anti-human α2 antibody was from GIBCO BRL. Murine IgG1 κ were from Sigma. Tyrophan AG-1478 (an EGF receptor kinase inhibitor), genistein, and PD-98059 (all species independent) were purchased from Calbiochem.

Isolation and Primary Cultures of Alveolar Epithelial Cells

Alveolar type II cells were isolated from pathogen-free male Sprague-Dawley rats as previously described (7, 15). Rats (120 g) were injected intraperitoneally with pentobarbital sodium (60 mg/kg body wt) and heparin sodium (400 U/kg body wt). After exsanguination, a tracheotomy was performed, and 25 ml of buffer A (HEPES-Krebs buffer; 133 mM NaCl, 5.2 mM KCl, 1.89 mM CaCl2, 1.29 mM MgSO4, 2.59 sodium phosphate buffer, and 10.3 mM HEPES) was infused through the air-filled lungs via the pulmonary artery to flush the blood out of the vascular bed. The lungs were then removed and lavaged 10 times with buffer B (buffer A without calcium and magnesium). Elastase solution (3.3 U/ml, 10 ml total volume) was instilled, and the lungs were incubated at 37°C for 10 min; then another 4 ml of elastase solution was instilled for another 10 min. The lungs were minced in the presence of DNase I, and FBS was added to stop the elastase digestion. The lungs were then sequentially filtered through 150-, 20-, and 10-μm filters (Sefar America, Kansas City, MO). The filtrate was centrifuged, and the cell suspension was plated on plastic dishes precoated with rat IgG (0.5 mg/ml in Tris buffer, pH 9.4, for 3 h at room temperature) for 45 min in the incubator (37°C, 5% CO2) to remove alveolar macrophages. Unattached cells were removed, centrifuged, and plated on 24-well culture plates in MEM containing 10% FBS and penicillin-streptomycin. Cell viability was >95% (trypan blue exclusion), and the yield was 15–25 × 106 cells/rat. Cell purity was assessed by Papanicolaou stain and was usually between 75 and 85%. A confluent monolayer was usually reached after 48 h in culture as in prior studies by Garat et al. (9) and Kheradmand et al. (15).

Wound Healing Assay

Wound healing over time was measured with an image analysis system as previously described (9, 15). Confluent cell monolayers were gently washed with serum-free MEM to remove the serum, and a linear wound was made with a pipette tip. After being wounded, the cells were washed to remove cell debris. Serum-free MEM containing 0.1% fatty acid-free bovine serum albumin with and without the stimulus was added to the wounded cells. Experiments were done in triplicate. The area of the denuded surface was measured immediately after wounding and after 18 h. The cells were placed on an inverted microscope (Axovert 35, Zeiss), and an image was obtained with the use of a digital camera (NEC, Hawthorne, CA) connected to the microscope. The image was subsequently captured by an image-analyzing frame-grabber (LG-3 scientific frame grabber, Scion, Frederick, MD) and analyzed with image analysis software (National Institutes of Health Image 1.55). The rate of wound repair was expressed as square micrometers per hour. Inhibition experiments were done by preincubating the wounded epithelial monolayers with the inhibitors (integrin antibodies, anti-EGF and anti-TGF-α antibodies, AG-1478, genistein, or PD-98059) for 30 min in serum-free MEM before the addition of IL-1β. We used IL-1β at a concentration of 20 ng/ml because preliminary experiments showed a maximal response at this dose.

Immunocytochemistry

After being coated with fibronectin (20 μg/ml) for 2 h at 37°C, eight-well chamber slides (Nunc, Naperville, IL) were washed with PBS. Precoating was necessary to obtain complete monolayers of alveolar epithelial cells within the same time period in which the wound healing experiments were being done (48 h after isolation). Isolated alveolar epithelial cells were plated at a density of 0.5 × 106 cells/well in MEM containing 10% FBS. The cells were wounded after confluence and fixed with methanol kept at −20°C. After saturation and permeabilization with a solution containing 0.7% gelatin and 0.016% saponin, the slides were incubated with the primary antibody (rabbit polyclonal IL-1 receptor type I antibody) for 60 min. After a wash, the second antibody (cyamine-3-labeled goat anti-rabbit IgG) was added for 30 min. Finally, the slides were washed in PBS containing 0.1% Triton, and an additional fixation was performed in 4% paraformaldehyde for 15 min. For controls, the primary antibody was preincubated with a blocking peptide for 30 min before addition to the cells.

Cell Spreading and Migration

Cell spreading and migration were determined with a modified protocol published before (15). Briefly, wounded alveolar epithelial monolayers were kept in culture in the presence and absence of IL-1β and fixed after 24 h. After the cells were stained with Diff-Quik (Dade Behring, Dudendingen, Switzerland), images were obtained with the computerized imaging system described in Wound Healing Assay, and 30 different intracellular distances in 3 randomly chosen high-power fields were measured in each condition at the edge of the wound and in the intact monolayer of the same well.

5-Bromo-2'-deoxyuridine Staining

5-Bromo-2'-deoxyuridine (BrdU) immunolabeling was done to determine the role of cell proliferation in IL-1β-induced alveolar epithelial wound healing. Cells were grown to monolayers on eight-well chamber slides coated with fibronectin (20 μg/ml). The monolayers were mechanically wounded with a pipette tip, and IL-1β was added to the culture medium. Twenty-four hours after the wounding, BrdU was added for 60 min at the end of the incubation period, and staining was performed according to the manufacturer’s protocol (Boehringer Mannheim, Indianapolis, IN). BrdU-positive cells were counted in five randomly chosen high-power fields at the edge of the wound.

Statistics

Data are presented as means ± SE or SD where appropriate. Statistical analysis was done by unpaired Student’s t-test or ANOVA where appropriate. The results were considered significant if P < 0.05.
RESULTS

Effect of IL-1β on In Vitro Alveolar Epithelial Repair

IL-1β (20 ng/ml) significantly increased the rate of wound closure in primary rat alveolar epithelial cells by 118 ± 11% compared with that in the serum-free medium control cells (P < 0.01; Fig. 1).

IL-1 Receptor Type 1 on Rat Alveolar Epithelial Cells

Two primary IL-1 receptors have been identified. IL-1 receptor type I is responsible for signaling after binding IL-1β, whereas IL-1 receptor type II does not transduce a signal and appears to act as a decoy receptor (6). By immunohistochemistry, there was staining specific for IL-1 receptor type I (Fig. 2A) in rat alveolar epithelial type II epithelial cells that could be blocked by a specific blocking peptide (Fig. 2B).

Mechanisms of IL-1β-Mediated Wound Closure

Cell spreading and migration. The effects of IL-1β on cell spreading were studied by measuring the internuclear distances of two adjacent cells at the edge of the wound and within the intact monolayer. IL-1β significantly increased the internuclear distance at the edge of the wound compared with that in the serum-free medium control cells, whereas it did not affect the internuclear distance in the intact monolayer compared with that in the medium control cells (Fig. 3). These results indicate that IL-1β induces cell spreading and migration at the edge of the wound, demonstrating that this mechanism contributes to the in vitro alveolar epithelial wound closure.

Proliferation. To determine the contribution of cell proliferation to in vitro wound closure, wounded rat alveolar epithelial cell monolayers were stained for BrdU incorporation. With or without IL-1β, primary rat alveolar epithelial cells showed no BrdU incorporation either at the edge of the wound or in the monolayer (data not shown). Therefore, the IL-1β-induced increase in alveolar epithelial repair did not result from increased proliferation.

Differential Effects of Integrins on IL-1β-Induced Alveolar Epithelial Repair

Cell spreading is, in part, determined by cell-matrix interactions mediated by integrins. Because cell spreading and not cell proliferation was the major mechanism of IL-1β-induced alveolar epithelial repair, we studied the role of selected integrins (β1-, α2-, and αV-integrin) on IL-1β-induced alveolar wound closure. Antibodies blocking the β1-integrin subunit inhibited the stimulation of IL-1β on alveolar epithelial repair by 45 ± 5%, and blocking of the αV-subunit inhibited the stimulation of IL-1β by nearly 100% (Fig. 4). In contrast, blocking of the α2-integrin had no effect. As a control, the effect of these integrin antibodies was tested on alveolar epithelial repair in serum-free medium where they had no significant inhibitory effect. These results indicate that IL-1β-induced alveolar epithelial repair in vitro can be partially inhibited by blocking the αV- and, to a lesser extent, the β1-integrin subunits.

Effect of EGF, TGF-α, and EGFR Inhibition on IL-1β-Induced Alveolar Epithelial Repair

Because Kheradmand et al. (15) previously reported that EGF and TGF-α increased alveolar epithelial repair in our in vitro wound healing system, we hypothesized that autocrine release of EGF and TGF-α might mediate the IL-1β-induced increase in alveolar epithelial repair. Therefore, neutralizing antibodies specific for EGF and TGF-α were used to study the contribution of soluble EGF and TGF-α to IL-1β-induced alveolar epithelial repair. Both anti-EGF and anti-TGF-α antibodies inhibited IL-1β-induced alveolar epithelial repair in a concentration-dependent manner (Fig. 5). The most prominent inhibitory effect (78%) was achieved in the presence of both anti-EGF and anti-TGF antibodies (Fig. 5). However, we did not find a significant inhibition of the IL-1β-induced increase in alveolar epithelial repair in the presence of an isotype control antibody (mouse IgG1 κ).

To further confirm the contribution of EGF and TGF-α to IL-1β-induced alveolar epithelial repair, the signaling of the EGFR was blocked with a specific tyrosine kinase inhibitor, tyrphostin AG-1478. The IL-1β-induced increase in epithelial repair of rat alveolar epithelial cells was inhibited by AG-1478 in a concentration-dependent manner (Fig. 6). In the presence of 20 μM AG-1478, the rate of IL-1β-induced wound closure was similar to that in the medium control cells. The inhibitory effect of AG-1478 was not due to a toxic effect because trypan blue staining remained low (>95% viable cells), and wound closure was preserved. In addition to tyrphostin AG-1478, we used genistein, a tyrosine protein kinase inhibitor. Genistein inhibits EGFR phosphorylation and, therefore, EGF- and TGF-α-mediated cell activation. Genistein had a dose-dependent effect on IL-1β-induced alveolar epithelial re-

![Fig. 1. Effect of interleukin (IL-1β) on in vitro alveolar epithelial repair. Wounded monolayers of rat primary alveolar epithelial cells were incubated with IL-1β (20 ng/ml), and wound closure was measured over 18 h. Data are means ± SD from 8 different cell isolations. *P < 0.01 compared with serum-free medium control.](image-url)
pair, inhibiting 50% of the IL-1β-stimulatory effect at a concentration of 10 μM, similar to the effect of AG-1478 (data not shown).

**Effect of PD-98059, an Inhibitor of the ERK-Activating MAPK, on IL-1β-Induced Alveolar Epithelial Repair**

To determine whether the MAPK pathway is involved in IL-1β-induced alveolar epithelial repair, we studied the effect of IL-1β on alveolar epithelial repair in the presence of PD-98059, an inhibitor of ERK activation. PD-98059 inhibited IL-1β-induced alveolar epithelial repair in a concentration-dependent manner (Fig. 7), whereas the basal rate of alveolar epithelial repair was not affected by PD-98059 (0.1–10 μM) under serum-free conditions.

**DISCUSSION**

The results of the study indicated that the inflammatory, early-response cytokine IL-1β enhances alveolar epithelial wound closure in vitro. This IL-1β-stimulated increase in alveolar epithelial wound closure was due to increased cell spreading at the edge of the wound and not to cell proliferation. EGF and TGF-α, which are both known to induce alveolar epithelial repair in vitro (15), contributed to the IL-1β-induced alveolar epithelial repair because inhibition of EGF,
TGF-α, or their common receptor markedly decreased the IL-1β-induced alveolar epithelial repair.

In previous studies (23, 24), IL-1β in the pulmonary edema fluid and bronchoalveolar lavage fluid of patients with acute lung injury has been shown to be biologically active and to contribute significantly to the inflammatory activity in the alveolar space. Moreover, IL-1β has been shown to be present in several in vivo studies of epithelial wounds. Hubner et al. (13) showed in a cutaneous injury model in vivo that IL-1β was detected as early as 7 h after injury, with maximal levels within 15–24 h after wounding. The expression of IL-1β declined after closure of the wound in this model, indicating that the expression of IL-1β is tightly regulated and might be important for normal wound healing (13). Another study showed enhanced, complete, and architecturally normal epithelial regeneration after IL-1 treatment of skin wounds in pigs (27).

Marked upregulation of IL-1β in vivo at the edge of the wound was also observed in a corneal wound healing model (28) and in a murine model of acute colitis (5). Although IL-1β appears to be important in wound healing, the functional role of IL-1β in alveolar epithelial repair has not been investigated.

We found a significant effect of IL-1β in enhancing alveolar epithelial repair. In addition, immunocytochemistry indicated that the IL-1 receptor is present on primary rat alveolar epithelial cells, extending the finding of a previous study (4) that reported the presence of the IL-1 receptor on A549 cells.

Fig. 3. Effect of IL-1β on cell spreading of rat alveolar epithelial cells. Internuclear distance was measured at the edge of the wound and within the monolayer in the presence and absence of IL-1β (20 ng/ml). Data are means ± SD. *P < 0.05, compared with serum-free medium control (wound edge plus monolayer).

Fig. 4. Effect of neutralizing integrin antibodies on IL-1β-induced alveolar wound closure. Anti-β1, anti-α2, or anti-αv antibody (each at 10 μg/ml) was added to the serum-free culture medium, and wound closure was measured in the presence and absence of IL-1β (20 ng/ml). Data are means ± SE from 2 independent experiments. An isotype control antibody (mouse IgG1 κ) did not show any effect on IL-1β-induced wound closure, and the integrin antibodies used had no inhibitory effect in serum-free medium alone (data not shown). Significant difference from IL-1β-induced wound closure: *P < 0.01; **P < 0.001.

Fig. 5. Effect of anti-transforming growth factor (TGF)-α and anti-epidermal growth factor (EGF) antibodies on IL-1β-induced alveolar wound closure. Rat alveolar epithelial cells were incubated with IL-1β together with anti-TGF-α, anti-EGF, or both antibodies at the indicated concentrations, and wound closure was measured. Data are means ± SD from 3 independent experiments. Anti-EGF and anti-TGF-α antibodies had no inhibitory effect in medium alone (data not shown), and an isotype control antibody (mouse IgG1 κ) did not show any effect on IL-1β-induced wound closure. Significant difference from IL-1β-induced wound closure: *P < 0.05. **P < 0.01. ***P < 0.001.

Fig. 6. Effect of AG-1478, a tyrphostin inhibiting EGF receptor kinase, on IL-1β-induced alveolar wound closure. Rat alveolar epithelial cells were incubated with AG-1478 at indicated concentrations, and IL-1β (20 ng/ml)-induced wound closure was measured. Data are means ± SD from 3 independent experiments. AG-1478 had no inhibitory effect in medium alone (data not shown). Significant difference from IL-1β-induced wound closure: *P < 0.01. **P < 0.001.
A recent study (16) indicated that migration of rat family of heterodimeric transmembrane glycoproteins, trax, an interaction that is mediated by integrins, an interaction between the cell and the extracellular matrix, and facilitates alveolar epithelial repair. Our data are therefore consistent with the data published by Kim et al. (16), who described the inhibitory effect of anti-αVβ3- and anti-β1-integrin antibodies on migration in the presence of substrate-bound fibronectin.

Because we wanted to study alveolar epithelial cells grown on their own matrix, we did not coat the plates before the experiment. However, it is known from previous experiments (8, 9) that wounded alveolar epithelial cells produce and secrete fibronectin in high amounts, which is a major compound of the extracellular matrix and facilitates alveolar epithelial repair. Our data are therefore consistent with the data published by Kim et al. (16), who described the inhibitory effect of anti-αVβ3- and anti-β1-integrin antibodies on migration in the presence of substrate-bound fibronectin.

There is increasing evidence that EGF, TGF-α, and their common receptor EGFR can regulate epithelial repair in vivo (17, 22) and in vitro (15, 21). EGF is overexpressed and activated in response to bronchial epithelial injury and plays a crucial role in epithelial repair (32). An increase in both TGF-α and EGF has been shown in bleomycin-injured rat lungs compared with control lungs (17). In addition, TGF-α has been identified in the bronchoalveolar lavage fluid and edema fluid from patients with acute lung injury (3, 18). Our results indicate that EGF, TGF-α, and their common receptor EGFR are involved in IL-1β-induced alveolar repair. In our studies, antibodies to EGF and TGF-α decreased IL-1β-induced alveolar epithelial repair, and blocking EGF with AG-1478 or genistein inhibited the effect of IL-1β almost completely. We selected different inhibitors of the EGF/TGF-α pathway to provide convincing evidence that the inhibitory effects were not due to unspecific effects of the reagents used. Our results indicate that IL-1β increases the secretion of EGF and TGF-α by alveolar epithelial cells. Soluble EGF and TGF-α can then act in an autocrine/paracrine fashion. In addition, we cannot rule out that IL-1β induces EGF upregulation in alveolar epithelial cells, leading to a higher susceptibility of the epithelial cells to EGF and TGF-α as others have reported (17, 32).

Alveolar macrophages are the main source of IL-1β in the alveolar space in acute lung injury (14) and may therefore stimulate alveolar epithelial repair. Also, IL-1β stimulates alveolar macrophages to release a variety of cytokines and growth factors including EGF and TGF-α (25). Stimulated macrophages may, therefore, contribute to epithelial repair as has been shown in a variety of different tissues (26) including the alveolar epithelium (10). Conceivably, a few contaminating macrophages might have been a source of TGF-α and EGF in addition to the alveolar epithelial cells themselves.

Several studies (11, 30) indicated the importance of EGFR signal transduction pathways in epithelial injury and repair. EGFR signaling in epithelial cells is primarily mediated by the MAPK pathway (30). We found that the MAPK pathway is involved in IL-1β-induced repair of the alveolar epithelium because blocking the activation of ERK with PD-98059 partly inhibited II-1β-induced alveolar epithelial repair. However, because the inhibition of alveolar epithelial repair with PD-98059 was not complete, we assume that other cellular signaling pathways are involved as well in IL-1β-induced alveolar epithelial repair.

In summary, this study demonstrated that the inflammatory cytokine IL-1β increased alveolar epithelial repair in vitro in primary rat alveolar epithelial cells. IL-1β increased alveolar epithelial repair primarily by increasing cell spreading at the edge of the wound, not by increasing cell proliferation and migration. The effect of IL-1β was, at least in part, mediated by EGF and TGF-α. These findings may be of relevance to the resolution of acute lung injury, providing evidence that IL-1β may play a role in initiating the
repair of damaged alveolar epithelium by enhancing the capacity of alveolar epithelial type II cells to reepithelialize a denuded alveolar epithelial barrier. Although some clinical studies (12, 20, 29) have focused on the potential deleterious effects of IL-1β in the early phase of sepsis-induced lung injury, these data suggest that IL-1β may have beneficial effects in the injured alveolus.

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