The Milieu of Damaged Alveolar Epithelial Type 2 Cells Stimulates Alveolar Wound Repair by Endogenous and Exogenous Progenitors

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Alveolar epithelial integrity is dependent upon the alveolar milieu, yet the milieu of the damaged alveolar epithelial cell type 2 (AEC2) has been little studied. Characterization of its components may offer the potential for *ex vivo* manipulation of stem cells to optimize their therapeutic potential. We examined the cytokine profile of AEC2 damage milieu, hypothesizing that it would promote endogenous epithelial repair while recruiting cells from other locations and instructing their engraftment and differentiation. Bronchoalveolar lavage and lung extract from hyperoxic rats represented AEC2 *in vivo* damage milieu, and medium from a scratch-damaged AEC2 monolayer represented *in vitro* damage. CINC-2 and ICAM, the major cytokines detected by proteomic cytokine array in AEC2 damage milieu, were chemoattractive to normoxic AECs and expedited *in vitro* wound healing, which was blocked by their respective neutralizing antibodies. The AEC2 damage milieu was also chemotactic for exogenous uncommitted human amniotic fluid stem cells (hAFSCs), increasing migration greater than 20-fold. hAFSCs attached within an *in vitro* AEC2 wound and expedited wound repair by contributing cytokines migration inhibitory factor and plasminogen activator inhibitor 1 to the AEC2 damage milieu, which promoted wound healing. The AEC2 damage milieu also promoted differentiation of a subpopulation of hAFSCs to express SPC, TTF-1, and ABCA3, phenotypic markers of distal alveolar epithelium. Thus, the microenvironment created by AEC2 damage not only promotes autocrine repair but also can attract uncommitted stem cells, which further augment healing through cytokine secretion and differentiation.

Keywords: AEC2; amniotic fluid stem cells; epithelial damage; CINC-2; ICAM

The functional role of the alveolar epithelial cell type 2 (AEC2) has expanded over the years from a surfactant factory to an immunomodulator of the alveolus (1). AEC2s express toll receptors, which play an important role in innate host defense of the lung (2). Surfactant secreted by AEC2s not only lowers surface tension and promotes compliance but also acts as a barrier and as a source of proteins that modify inflammatory signaling in the alveolus (3). Human lung diseases, such as fatal respiratory distress syndrome in the neonatal period and interstitial lung

CLINICAL RELEVANCE

Characterization of the soluble factors in the alveolar epithelial milieu that are required for AEC2 maintenance and repair would have important translational clinical and bioengineering applications. The AEC2 damage milieu is shown to promote endogenous healing of an epithelial wound through autocrine cytokines, and also can attract uncommitted human progenitor cells, which augment healing through secretion of healing cytokines. The AEC2 damage milieu is sufficient to induce differentiation of uncommitted stem cells into epithelial cells expressing SPC, TTF-1 and ABCA3, markers of distal pulmonary alveolar epithelium, thus further establishing its reparative potential.

disease of later onset, have been linked to mutations in surfactant protein-C (SP-C) (4). In addition, AEC2s are the putative resident alveolar progenitors that can replace damaged AEC1s after injury (5). Maintenance of a functional population of AEC2s is therefore critical for normal lung alveolar function, homeostasis, and repair.

The observation that damaged AEC2 monolayers can repair themselves *in vitro* in the absence of serum or exogenous growth factors (6) suggests that autocrine modification of the AEC2 milieu promotes alveolar epithelial repair. We have shown previously that exogenous human amniotic fluid stem cells (hAFSCs), when delivered by tail vein injection to mice, can target damaged pulmonary alveolar epithelium, where they are induced to express markers of distal pulmonary alveolar epithelium (7). Thus, the AEC2 damage milieu may be capable of recruiting undifferentiated cells from other locations, including the local alveolar bloodstream, and directing their engraftment and differentiation as well as promoting autocrine repair. Bone marrow stem cells also track to damaged alveolar epithelium (8), where they can expedite repair without engrafting (9), perhaps by contributing growth factors or cytokines that augment endogenous epithelial healing. A threshold of lung injury seems to be required for the appearance of significant numbers of stem cell-derived lung epithelia (10), suggesting that cells administered systemically are homing to local chemotactic signals released by alveolar epithelial damage. However, the niche, or milieu, of damaged AEC2 cells and its complement of autocrine and exocrine factors has been little studied.

AEC2s and other cell types in the alveolus, together with the underlying matrix, contribute to the soluble components of the AEC2 milieu, which is modified by damage as matrix is exposed and infiltrating cells are recruited. In this study, we examined the milieu of AEC2s using two damage models, with an emphasis on cytokine/chemokine production. Hyperoxia was used as an *in vivo* epithelial damage model because subacute hyperoxia

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in rodents is a relevant model for human acute lung injury associated with oxidant stress without inflammation (11). BAL and lung extract from hyperoxic rats were used to represent AEC2s *in vivo* damage milieu and are reflective of autocrine contributions from many cell types. In contrast, conditioned medium from a scratch-damaged AEC2 monolayer reflects AEC2-specific cytokines released after damage, and this model was used as a convenient closed system to examine the AEC2 *in vitro* damage milieu. A template system that generated wounds with minimal damage was used as a model of passive epithelial denudation to compare with scratch-generated damage denudation. We present novel evidence that the AEC2 damage milieu not only promotes endogenous healing through autocrine cytokines but also induces uncommitted human progenitor cells to actively participate in epithelial repair by contributing healing cytokines into the AEC2 milieu. The AEC2 damage milieu *per se* is sufficient to induce subsets of these uncommitted stem cells to acquire phenotypic markers of distal alveolar epithelium, thus further establishing its reparative potential.

METHODS

Cell Culture

AEC2s were isolated as described by Dobbs (12). Human amniotic fluid from normal male fetuses (17–18 wk gestation) was obtained from Genzyme Genetics Corporation (Monrovia, CA) and sorted for hAFSCs (c-kit positive cells) using magnetic-activated cell sorting (7). hAFSC details are provided in the online supplement.

In Vitro AEC2 Active Damage Model: Scratch Wound

Confluent AEC2 monolayers were damaged as described previously (6). Cytokines, BAL, lung extract, exogenous cells, or neutralizing antibodies were added immediately after damage. The inhibitors PD98059 (50 μ M) (14) and SB 505124 (1 μ M) (15) (Sigma, St. Louis, MO) were incubated with the freshly damaged monolayer for 2 hours before the addition of the test cytokine.

In Vitro AEC2 Passive Damage Model: Wound Template

A strip of passive denudation of the same size as the scratch gap, but with minimal cell damage, was generated using a wound template (Cyto-select Wound Healing Assay; Cell Biolabs, San Diego, CA).

In Vivo AEC2 Damage Model: Hyperoxia

Adult male Sprague-Dawley rats were exposed to short-term hyperoxia (16) and were used for AEC2 isolation with no *in vivo* recovery period for maximal AEC2 damage (17).

Collection of BAL

Normoxic and hyperoxic rat lungs were lavaged to capacity six times with Hepes-buffered DMEM. The BAL was centrifuged to remove cells, aliquoted, and frozen at -20°C .

Preparation of Lung Extract from Normoxic and Hyperoxic Lungs

Minced, unfiltered, elastase-digested lung (11) was incubated in Hepes-buffered DMEM at 37°C , with gentle agitation, for 3 hours (1 minced lung/50 ml DMEM), filtered through 100- μ m nitex, centrifuged to remove cells, aliquoted, and frozen at -20°C .

Cytokine Proteomic Microarray

Conditioned medium (undiluted) and BAL was screened using proteomic cytokine arrays (R&D, Minneapolis, MN). The blots were scanned, and spot densities were compared after correction for internal controls.

CINC-2 and ICAM ELISAs

CINC-2 and ICAM were measured in cell-conditioned medium and BAL using rat-specific Quantikine Rat ELISAs (R&D Systems) according to the manufacturer's instructions.

Cell Migration

Migration was measured using the Boyden chamber method (18). Migrating cells were stained and quantitated as previously described (19).

CM-DiI Fluorescent Labeling of Exogenous Cells

AEC2s grown in a separate flask in parallel with the monolayer plate or hAFSCs cultured as described previously (7) were labeled with cell tracker CM-DiI (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Immunostaining

Cells were fixed in 4% paraformaldehyde or 1:1 ice cold methanol acetone, depending on the antibodies used (*see figure legends*).

RNA extraction and RT-PCR details, including human SPC and TTF-1 primer sequences, are provided in the online supplement.

Western Blot

Western blot analysis was performed on cell lysates as previously described (20). Antibodies and cytokines are listed in the online supplement.

Data Analysis

Scanned blots were analyzed using Scion Image software (NIH). Students *t* test was used to compare treated versus untreated groups using Graph Pad software. Data are expressed as mean $\pm$ SEM. A *P* value $\leq$ 0.05 was considered significant.

RESULTS

The Milieu of Damaged Lung Promotes AEC2 Cell Growth

Freshly isolated normoxic AEC2s are essentially nonproliferative, with greater than 90% of the population in G1 phase of the cell cycle, and remain quiescent in culture (20). In contrast, AEC2s isolated from hyperoxic rats are more proliferative, suggesting that the hyperoxic milieu within the lung is conducive to AEC2 growth. We obtained BAL and lung extracts from hyperoxic rats, taken to be representative of the AEC2's *in vivo* damage environment, and compared their effects with BAL and lung extract from normoxic animals when added to 24-hour cultured, attached, nonconfluent normoxic AEC2s. The hyperoxic BAL and lung extracts increased the cell numbers by 2-fold in 24 hours (Figures 1A and 1B). In contrast, normoxic BAL and lung extract did not significantly affect AEC2 proliferation. Thus, the AEC2 cell *in vivo* hyperoxic milieu is sufficient to recapitulate the proliferation induced by *in vivo* hyperoxia when added to normoxic AEC2s *ex vivo*.

The AEC2 Damage Milieu Promotes *In Vitro* Migration and Wound Healing of AEC2s

We used the scratch damage model to determine whether the damage cues present in the microenvironment of damaged AEC2s could potentiate alveolar epithelial repair. Hyperoxic BAL and lung extract promoted significantly faster healing of an AEC2 monolayer scratch wound (Figure 1C). The AEC2 is normally unmigratory (18) but can be induced to migrate by *in vivo* hyperoxia (19). To determine whether AEC2 cell damage milieu promoted chemotaxis of AEC2s, we used an *in vitro* Boyden chamber approach (18, 19) to measure migration of AEC2s toward AEC2 damage milieu. *In vivo* and *in vitro* AEC2 damage milieu stimulated migration in the normally unmigratory AEC2s (Figure 1D). These

migration experiments were also repeated using primary cultures of AEC1s with similar results (data not shown), suggesting that the whole alveolar epithelium is responsive to signals resulting from AEC2 damage.

Exogenous AEC2 Attach within the Area of Damage on a Wounded AEC2 Cell Monolayer

Because AEC2 *in vitro* damage milieu stimulated AEC2 migration, we tracked exogenous fluorescent-labeled AEC2s added to a fresh monolayer scratch wound. We added 10^5 exogenous cells to a damaged monolayer in a 5-cm² tissue culture well, replacing the estimated 5% loss caused by wounding and washing. A wound generated by growing the monolayer around a removable template served as a passive (minimally damaged) denudation control, and exogenous cells were added to nonwounded confluent monolayers in parallel to assess the pattern of engraftment to nondamaged cells. The size of the wounds generated by scratch and template was equivalent ($952 \pm 208 \mu\text{m}$ versus $988 \pm 33 \mu\text{m}$, respectively; $n = 7$). After 24 hours, exogenous fluorescent AEC2s had efficiently attached to a scratch wound (Figure 2A, left panel) and to a lesser extent to a template wound (Figure 2A, middle panel), where they were lightly attached and more likely to be removed by washing. Exogenous cells added to undamaged monolayers were diffusely scattered over the monolayer with much less efficient attachment (Figure 2A, right panel). The scratch wound had healed by 24 hours in the presence of

Figure 1. Alveolar epithelial cell type 2 (AEC2) damage milieu promotes AEC2 proliferation, wound healing, and migration. (A) Freshly isolated AEC2s from control rats were allowed to attach overnight, incubated a further 24 hours in DMEM with 10% FBS $\pm$ normoxic and hyperoxic bronchoalveolar lavage (BAL) or lung extracts, and examined under phase contrast light microscopy at 20 $\times$ magnification (bar = 250 μm). Hyperoxic BAL or lung extracts induced pronounced proliferation of AEC2s, as confirmed by cell counts in panel B. (B) AEC2 numbers from the conditions described in panel A are expressed as fold increase over untreated. Hyperoxic BAL and lung extract increased cell numbers of AEC2s ($P < 0.004$ and $P < 0.003$, respectively; $n = 5$). The hyperoxic BAL and lung extract were significantly different from normoxic BAL and lung extract ($P < 0.001$ and $P < 0.02$, respectively). (C) A confluent AEC2 monolayer was damaged by micropipet tip, washed to remove damaged cells and serum, and incubated in DMEM $\pm$ BAL or lung extract from hyperoxic rats. After 24-hour incubation, the cells were stained with crystal violet, and the damage area was photographed at 20 $\times$ (bar = 250 μm). This composite, which shows that hyperoxic BAL and damaged lung extract promote AEC2 wound healing, is representative of several experiments. Wound width at 24 hours: untreated, $552 \pm 109 \mu\text{m}$; hyperoxic lung extract-treated, 0 (100% healed); hyperoxic BAL-treated, $212 \pm 32 \mu\text{m}$ ($P < 0.04$ versus untreated; $n = 3$). (D) The chemoattraction of AEC2s toward milieu from undamaged and damaged AEC2s was measured by plating AEC2s on porous (8 μm) filters atop wells containing AEC2 *in vitro* milieu (conditioned medium [CM] from undamaged and scratch damaged AEC2s) or *in vivo* milieu (normoxic and hyperoxic BAL or lung extract). After 24 hours, migrating cells on the underside of the filter were stained and quantitated by spectrophotometric analysis of eluted cell stain (x axis, A600 nm). Migration toward the AEC2 milieu versus basal migration toward DMEM alone: hyperoxic BAL ($n = 7$), $P < 0.0001$; hyperoxic lung ($n = 7$), $P < 0.02$; control BAL ($n = 6$), $P < 0.01$; and CM from *in vitro* damaged AEC2 ($n = 3$), $P < 0.01$; CM from undamaged AEC2 ($n = 3$), NS. Hyperoxia significantly increased the chemoattraction of the BAL and lung extracts: hyperoxic versus control BAL, $P < 0.002$; hyperoxic versus control lung, $P < 0.03$.

exogenous AEC2 cells, in contrast to the template wound, which remained unhealed (Figure 2B, left panels). Endogenous wound healing was also more efficient in the scratch wound than in the template wound (Figure 2B, right panels). To determine whether exposed matrix was responsible for the increased wound healing seen when cells were damaged versus denuded, we compared scratch and template wounds immediately after damaging for protein, fibronectin, laminin, and collagen deposition. There were similar small remnants of protein detected in template and scratch wounds, mainly adjacent to the wound edge. Laminin was detected similarly (Figure 2C), but no fibronectin or collagen staining was detected in the wounds or at the wound edges of either damage model (data not shown). Although it is possible that endogenous and exogenous AEC2s are attracted to traces of laminin or other proteins left in the wound after damage, this is unlikely to account for the difference in healing properties between scratch-damage milieu and template-damage milieu because both wound types showed similar trace amounts of protein staining. Taken together, the data suggest that soluble autocrine factors released by damaged AEC2s are sufficient to expedite endogenous repair of a wounded alveolar epithelial membrane but can also attract exogenous cells to augment endogenous wound healing. In contrast, denudation without damage is a less effective stimulus for repair.

Increased CINC-2 and ICAM Levels Are Detected in the Milieu of Damaged AEC2s

To examine cytokine/chemokine release into the AEC2 milieu after a breach of alveolar epithelial integrity, we screened

A Exogenous AEC2 deposition on AEC2 monolayer

B AEC2 monolayer wound healing

C Protein in wound

Figure 2. Exogenous AEC2s expedite healing at the site of damage in an AEC2 monolayer. (A) Exogenous CM DiI-labeled AEC2s (2×10^4 exogenous cells/cm²) were added to AEC2 monolayers freshly wounded by scratch (active denudation) or template (passive denudation) and to a nonwounded monolayer in parallel and cultured for 48 hours. The initial sizes of wounds were equivalent (952 ± 208 μ m scratch versus 988 ± 33 μ m template; $n = 7$). Photographs of live cultures at 48 hours show that exogenous AEC2s attached within the damage area (arrows) of both wounds but with increased deposition in the scratch. In contrast, exogenous cells added to an undamaged monolayer show limited and diffuse attachment (original magnification, $\times 10$; bar = 1,000 μ m). (B) Exogenous and endogenous AEC2-mediated wound healing at 24 hours is more efficient in a scratch-wounded versus template wounded AEC2 monolayer. Wound width, scratch versus template at 24 hours: exogenous, 0 (100% healed) versus 535 ± 106 μ m; endogenous, 313 ± 53 μ m versus 742 ± 130 μ m ($P < 0.02$; $n = 4$) (original magnification, $\times 4$; bar = 1,000 μ m). (C) Small remnants of protein remaining in the wound were seen predominantly near the wound edges of scratch and template wounds that were fixed in 4% paraformaldehyde immediately after damaging and washing. Similar levels of protein (Coomassie Blue staining) and laminin (immunostaining) were detected in both wound types (original magnification, $\times 10$; bar = 400 μ m). No collagen (Sirius red) or fibronectin immunostaining was detected (data not shown).

conditioned medium from undamaged versus scratch-damaged versus template-damaged AEC2s using a proteomic cytokine array of 30 rat-specific cytokines. CINC-2 and ICAM were the only cytokines significantly elevated in the medium collected from scratch-damaged cells at levels greater than 1.5-fold higher than in control medium (Figure 3A), and this difference had resolved by 48 hours (data not shown). A template wound with minimal cell damage generated 25% less ICAM and 66% less CINC-2 than scratch-damaged wounds. The damage-mediated CINC-2 and ICAM release was confirmed using rat-specific ELISAs (Figures 3A and 3B). CINC-2 was increased 1.5-fold during the first 24 hours of healing. Basal levels of ICAM were relatively high in control AEC2 conditioned medium (CM) ($4,942 \pm 50$ pg/ml), with a 1.6-fold increase in ICAM secretion

Figure 3. CINC-2 and ICAM are released by AEC2 damage and promote cell migration. (A) Cytokines induced by AEC2 damage were detected by screening conditioned medium from AEC2 wounded by scratch or template using a rat specific protein cytokine array of 30 cytokines. The CM was collected 24 hours after damage and compared with 24-hour CM from undamaged cells. Of the seven cytokines detected with high expression in AEC2 CM, only CINC-2 and ICAM were increased by AEC2 damage (scratch wounding) by greater than 1.5-fold. Template wounds, which involve minimal cell damage, did not result in increased CINC and ICAM secretion. (B and C) CINC-2 and ICAM levels were quantitated in CM from undamaged and scratch damaged AEC2s (*in vitro* damage) and BAL from normoxic and hyperoxic rats (*in vivo* damage) by rat-specific ELISA. CM was collected 24 hours after damage for CINC-2 (B) and 0 to 3 hours and 3 to 24 hours after damage for ICAM (C). CINC-2 levels were elevated in CM from damaged versus undamaged AEC2s ($P < 0.05$; $n = 3$) and in hyperoxic BAL versus control BAL ($P < 0.02$; $n = 3$ control BAL, $n = 6$ hyperoxic BAL). ICAM levels were elevated in damaged AEC2 CM in the first 3 hours after damage ($P < 0.02$) and in hyperoxic versus normoxic BAL ($P < 0.02$; $n = 3$ control BAL, $n = 6$ hyperoxic BAL). (D) AEC2 migration toward serum-free medium $\pm$ ICAM or CINC-2 at levels covering the range seen in damaged AEC2s was measured using 8- μ m-pore filters. Migrating cells were stained and quantitated by spectrophotometric analysis of eluted cell stain. Data are expressed as fold increase over medium control (DMEM with 0.1% BSA). ICAM and CINC-2 induced migration at doses of 5 ng/ml (each $P < 0.02$) and 50 ng/ml ($P < 0.01$ and $P < 0.02$, respectively; $n = 3$).

in the first 3 hours after damage, although we were not able to detect a significant difference by 24 hours after damage. Up-regulation of these cytokines has been reported previously in models of acute lung injury *in vivo* (21, 22). Indeed, we detected a 3.4-fold increase in CINC-2 and a 1.8-fold increase in ICAM-1 in hyperoxic BAL versus normoxic BAL (Figures 3B and 3C). To demonstrate that CINC-2 and ICAM-1 secretion was a response to damage and not due to proliferation *per se*, conditioned medium from undamaged proliferating and nonproliferating AEC2s was screened by protein microarray (see Figure E2 in the online supplement). Proliferation was induced by 25 ng/ml KGF treatment for 24 hours and confirmed by PCNA Western blot. There was no difference in CINC-2 expression between proliferating and nonproliferating AEC2s, and ICAM was barely detectable in CM from either population. This is in contrast to the elevated CINC-2 and ICAM levels secreted by damaged AEC2s shown in Figure 3A and confirms that release of CINC-2 and ICAM reflects an autocrine response to cell damage.

CINC-2 and ICAM-1, the Major Cytokines Detected in AEC2 Damage Milieu, Promote Migration and Play a Functional Role in AEC2 Epithelial Repair

We measured *in vitro* migration of AEC2s through porous 8- μ m filters toward DMEM $\pm$ CINC-2 and ICAM at concentrations at and above the level seen in damage milieu and found significantly increased migration at 5 ng/ml or greater (Figure 3D). Both cytokines also expedited *in vitro* wound healing, whereas

endogenous wound healing was delayed by their respective neutralizing antibodies (Figures 4A and 4B), confirming a functional role for both cytokines in AEC2 autocrine repair *in vitro*. CINC-2 and ICAM neutralizing antibodies also delayed wound healing stimulated by AEC2 *in vivo* damage milieu since delayed wound healing was also seen in the presence of hyperoxic BAL (Figure E3). CINC-2 or ICAM did not promote proliferation of AEC2s when added at the time of plating or to already attached cells for a period of 24-hours, as measured by cell counts. However, when added to freshly damaged AEC2s, both cytokines stimulated proliferation significantly by 24 hours (CINC-2: 1.4 ± 0.1 -fold versus untreated [$P < 0.03$]; ICAM: 1.6 ± 0.3 -fold versus untreated [$P < 0.04$; $n = 4$]).

CINC-2 and ICAM Activate Cell Signaling Pathways in AEC2s that Promote Autocrine Wound Healing

To determine mechanisms of CINC and ICAM-mediated AEC2 wound healing, we examined the effect of CINC and ICAM on the activation of common signaling pathways in AEC2s. CINC-2 and ICAM were added to fresh isolates of AEC2 for 5 minutes or 1 hour or added at the time of plating for 24-hour culture. The dosage used was 5 ng/ml, which was previously shown to promote migration and expedite wound healing. The cells were lysed after 5 minutes, 1 hour, or 24 hours of exposure to the cytokines. Although short-term exposure to CINC-2 and ICAM did not induce ERK or Smad2 activation (data not shown), both cytokines induced sustained ERK and Smad2 phosphorylation after 24 hours of treatment (Figure 4C). The delayed activation

Figure 4. CINC-2 and ICAM contribute to endogenous AEC2 wound healing. (A) Confluent cultures of AEC2s were scratch-damaged and incubated for 24 hours with basal medium (DMEM with 0.1% BSA) $\pm$ 5 ng/ml CINC-2 or 5 ng/ml ICAM. Optimal doses of cytokines had been determined in previous dose-response experiments. The cultures were then stained with crystal violet and photographed under low power (original magnification, $\times 10$; bar = 500 μ m). CINC-2 and ICAM promoted AEC2 wound healing within 24 hours, whereas untreated wounds remained unhealed. (B) Confluent cultures of AEC2s were scratch damaged and incubated with basal medium (DMEM with 0.1% BSA) $\pm$ an excess of CINC or ICAM neutralizing antibodies. The control well received antibody vehicle (PBS with 5% trehalose). Incubation time was 48 hours, the time required for endogenous healing. CINC and ICAM neutralization delayed endogenous healing, as shown by noticeably less compacted cells within the wound compared with the untreated wound. (C) Undamaged AEC2s were cultured for 24 hours $\pm$ CINC-2 or ICAM (5 ng/ml). The cells were lysed, and the cellular proteins were analyzed by Western blot. Both cytokines resulted in ERK activation at 24 hours, as measured by the appearance of the phospho (active) form, which was not readily detected in untreated cells. Increased expression of the active, phosphorylated form of Smad2 was also apparent after 24-hour treatment with CINC or ICAM (1.8 ± 0.4 - and 1.7 ± 0.4 -fold basal, respectively, by densitometry; $n = 3$ blots). Short-term exposure of AEC2s to CINC-2 and ICAM for 5 minutes or 1 hour did not result in significant activation of ERK or

Smad-2 (data not shown). (D) Freshly damaged AEC2 monolayers were cultured $\pm$ MAP kinase kinase (MAPKK) inhibitor PD 98059 (50 μ M) or TGF- β 1 receptor kinase inhibitor SB505124 (1 μ M) for 2 hours before the addition of CINC-2 and ICAM (5 ng/ml). All wells contained carrier DMSO (0.1% final volume). After incubation for 24 hours, the cultures were stained with crystal violet and photographed under low power (original magnification, $\times 4$; bar = 1,000 μ m). Endogenous healing was delayed in the presence of either inhibitor, with less defined wound margins (wound widths: untreated, 250 ± 11 μ m; SB 505124-treated, 467 ± 37 μ m [$P < 0.003$ versus untreated]; PD 98059-treated, 413 ± 37 μ m [$P < 0.006$ versus untreated]). CINC-2 and ICAM expedited wound closure (CINC-2 wound width, 66.7 ± 63 μ m [$P < 0.05$] versus untreated; ICAM wound 88.6 ± 44 μ m [$P < 0.02$] versus untreated; $n = 3$), while inhibition of MAP kinase activation with PD 98059, or TGF- β 1 receptor kinase activity by SB 505124 decreased CINC-2 and ICAM-mediated wound healing to endogenous levels.

of both pathways may be secondary to cytokine-mediated attachment *per se* or to induction and release of growth factors or cytokines. In contrast, we could not detect CINC-2- or ICAM-mediated changes in the phosphorylation of the other key signaling members of the MAP kinase family, p38 and JUNK (data not shown). Inhibition of MAP kinase kinase (MAPKK) activation by PD98059 or TGF- β 1 receptor kinase activity by SB505124 slowed endogenous wound healing and decreased CINC-2 and ICAM-mediated wound healing to endogenous levels (Figure 4D). These data suggest that CINC- and ICAM-mediated epithelial repair is mediated through indirect stimulation of MAPK and TGF- β signaling.

Amniotic Fluid Stem Cells Attach within the Site of AEC2 Damage and Participate in Repair

We have previously shown that in systemically delivered uncommitted human amniotic fluid stem cells, c-kit + hAFSCs can be detected in murine lung epithelium after hyperoxic injury *in vivo*, albeit in low numbers (7). To see if the milieu of damaged AEC2 *per se* could provide an attractive environment for uncommitted stem cells, we added CM-DiI-labeled hAFSCs to AEC2 monolayers freshly damaged by scratch or by template. The AEC2 milieu generated by the damage model was more attractive to engraftment of hAFSCs than the passive denudation model since hAFSCs attached more efficiently to a scratch wound than to a template wound (Figure 5A, *upper panels*). Wound healing at 24 hours in the presence of hAFSCs was also

more efficient in the scratch wound versus the template wound (Figure 5A, *lower panels*).

Conditioned Medium from Damaged AEC2s Is Attractive to Amniotic Fluid Stem Cells

To confirm that the microenvironment of damaged AEC2s was attractive to hAFSCs, we compared the migration of hAFSCs toward AEC2-conditioned medium from undamaged and *in vitro* scratch-damaged AEC2s as well as BAL from control and hyperoxic animals (Figure 5B, *upper panels*). hAFSCs migrated toward conditioned medium from *in vitro* damaged AEC2s with an approximately 5-fold increase over basal medium. The migratory response of hAFSCs toward hyperoxic BAL had a greater than 20-fold increase in migration compared with basal medium and was approximately 10-fold higher than the response to BAL from normoxic animals, which was also stimulatory. The cells migrating toward hyperoxic BAL had a predominantly fibroblast-like phenotype, in contrast to the cells migrating toward damaged AEC2 CM, which had an epithelial phenotype, or control BAL, where tight spheres of small cells were seen, only a few of which had recognizably epithelial and fibroblast morphology (Figure 5B, *lower panels*). Unlike rat AEC2s, hAFSCs did not migrate significantly toward CINC-2 or ICAM, the major cytokines released by AEC2 damage (data not shown). Thus, the chemotactic signals originating from damaged AEC2 milieu that are attractive to hAFSCs remain to be identified.

A hAFSC deposition on AEC2 monolayer

B hAFSC migration towards AEC2 damage milieu

Figure 5. Human amniotic fluid stem cells (hAFSCs) are attracted to damaged AEC2s and damaged AEC2 milieu. (A) AEC2 monolayers, undamaged or damaged by scratch (active denudation) or template (passive denudation), were cultured for 24 hours with DiI-labeled hAFSCs (*upper panels*; original magnification, $\times 10$; bar = 500 μ m). hAFSCs attached sparsely and randomly to an undamaged monolayer but attached within the scratch damage site and to a lesser extent to the template damage site. The wound healing at 24 hours in the presence of exogenous hAFSCs was more efficient in the scratch wound than in the template wound ($175 \pm 35 \mu$ m versus $545 \pm 20 \mu$ m, respectively; $P < 0.001$; $n = 3$), as shown by crystal violet staining (*lower panels*; original magnification, $\times 4$; bar = 1,000 μ m). (B) Migration of hAFSCs toward *in vitro* (left panel) and *in vivo* (right panel) AEC2 damage milieu was measured and compared with migration toward medium alone. Migration was expressed as absorbance of crystal violet stain eluted from migrated cells (A_{600nm}). Photographs show the phenotype of stained cells on filters at $\times 10$ magnification (bar = 500 μ m). AEC2 CM versus medium (DMEM + 0.1% BSA), $P < 0.002$; damaged AEC2 CM versus medium, $P < 0.001$; damaged versus undamaged CM, $P < 0.05$; control BAL versus medium, $P < 0.02$; hyperoxic BAL versus medium, $P < 0.0001$; hyperoxic BAL versus normoxic BAL, $P < 0.0001$. Control med, $n = 4$; AEC2 CM, $n = 3$; AEC2 dam CM, $n = 3$; control BAL, $n = 3$; hyperoxic BAL, $n = 4$.

Figure 6. hAFSCs contribute healing cytokines in response to AEC2 damage. (A) A human-specific array of 36 cytokines and chemokines was used to compare the medium from hAFSCs incubated 24 hours in the presence of AEC2 *in vivo* or *in vitro* (scratch) damage milieu with AEC2 damage milieu alone. Only five human cytokines from a panel of 36 were detected at high levels. MIF and PAI-1 were highly expressed in the presence of *in vitro* AEC2 damage. No spots were detected in AEC2 damage milieu in the absence of hAFSCs using the human array. (B) Confluent cultures of AEC2s were scratch damaged and incubated for 24 hours with medium (DMEM with 0.1% BSA) ± MIF or PAI-1 at 5 ng/ml. The cultures were then stained with crystal violet and photographed under low power (original magnification, $\times 4$; bar = 1,000 μ m). MIF and PAI-1 healed the wound in 24 hours, whereas untreated wounds remained unhealed. (C) Confluent cultures of AEC2s were scratch-damaged and preincubated for 2 hours ± MIF antagonist ISO-1 (50 μ M) and PAI-1 neutralizing antibody before the addition of exogenous hAFSCs. As ISO-1 was dissolved in DMSO, an equivalent volume was added to control and PAI-1 neutralizing antibody-treated cultures. After 24-hour culture, the cells were stained with crystal violet and photographed (original magnification $\times 4$; bar = 1,000 μ m). hAFSCs significantly promoted AEC2 repair: gap width $133 \pm 66 \mu$ m (+hAFSCs) versus $342 \pm 47 \mu$ m (–hAFSCs) ($n = 3$; $P < 0.01$) but could not promote repair when MIF or PAI-1 were inhibited.

Amniotic Fluid Stem Cells Modulate the AEC2 Damage Milieu

hAFSCs can ameliorate tissue damage by modulating cytokines in the damage milieu, as seen in a rodent model of acute tubular necrosis (23). Therefore, we screened for human cytokines released when hAFSCs were incubated in the presence of damaged AEC2 milieu using a human-specific cytokine proteomic screen of 36 cytokines (Figure 6A). Although no human cytokines were detected in damaged AEC2 milieu in the absence of hAFSCs, five human cytokines were seen at readily detectable levels when hAFSCs were cultured with AEC2 *in vivo* damage milieu: growth regulated protein- α , IL-6 and IL-8, macrophage migration inhibitory factor (MIF), and plasminogen activator inhibitor 1 (PAI-1), whereas MIF and PAI-1, and to a lesser

extent IL-6, were detected after culture of hAFSCs with AEC2 *in vitro* scratch damage medium. PAI-1 and MIF were endogenously secreted by hAFSCs that were cultured in the absence of damage milieu but were strongly induced by AEC2 damage. MIF, a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation, plays a role in the regulation of macrophage function in host defense and may be involved in integrin signaling pathways. AEC2s proliferate in response to MIF and express high surface levels of CD74, which binds MIF (24). PAI-1 is a major negative regulator of pericellular plasmin and accompanies wound repair *in vitro* and *in vivo* (25), including alveolar epithelial wound healing (26). Because MIF and PAI-1 were likely candidates to promote wound healing and were strongly induced by *in vivo* and *in vitro* damage milieu, we focused on these cytokines.

MIF and PAI-1, Detected in the Milieu of Damaged AEC2s when hAFSCs Are Present, Promote Wound Healing

To confirm a functional role for MIF and PAI-1 in alveolar epithelial wound healing, we added human recombinant MIF and PAI-1 (5 ng/ml) to freshly scratch-damaged monolayers of AEC2s in the absence of hAFSCs. After 24 hours, the scratch wounds treated with MIF and PAI-1 were healed (Figure 6B), in contrast to the control scratch. Proliferation of damaged AEC2s, as measured by cell numbers, was increased 2.1 ± 0.1 -fold by MIF and 1.6 ± 0.1 -fold by PAI-1, suggesting that expedited wound closure in the presence of the cytokines was due to increased cell proliferation. In contrast, AEC2s did not migrate toward MIF or PAI-1 at doses that promoted wound healing (data not shown). Inhibition of PAI-1 or MIF, using MIF antagonist ISO-1 (50 μ M) or an excess of PAI-1 neutralizing antibody, delayed hAFSC-mediated AEC2 wound healing (Figure 6C), confirming that the major cytokines secreted by hAFSCs in the presence of damaged AEC2s contribute to a prohealing milieu.

Rat AEC2s within the Healing Wound Are Surrounded by hAFSCs

Cytokeratin staining of cells within an AEC2 wound, 24 hours after wounding and the addition of DiI-labeled hAFSC, showed islands of cytochrome-positive rat AEC2s in intimate proximity to cytochrome-negative, DiI-labeled hAFSCs (Figure 7A, upper panels). The hAFSC-modified milieu also attracted cytochrome-negative cells from the monolayer into the wound. These cells are likely fibroblasts, which are present as contaminants in the AEC2 cultures (usually $\sim 2\%$). Although SPC protein was not detected in hAFSC (DiI+) cells at this time (24 h), it was detected after 72 hours of culture of hAFSCs with damaged AEC2s by RT-PCR using human-specific primers (Figure 7A, bottom panel).

AEC2 Cell Damage Milieu Can Induce Expression of SPC, a Marker of Distal Alveolar Epithelium, in Uncommitted Human Stem Cells

To assess phenotypic changes mediated by AEC2 damage milieu in uncommitted hAFSCs, we cultured hAFSCs on collagen 1 with AEC2 control and damage milieu for various times and analyzed for SPC, a protein manufactured by AEC2s and a marker of distal alveolar epithelium, by immunostaining. Cells that stained for pro-SPC were not seen until after 4 days of culture with damage milieu (Figure 7B, upper panels). No SPC-staining cells were seen in hAFSCs cultured in basal medium (data not shown) or in cytopins of c-kit-positive hAFSCs assayed before each experiment (Figure E1). Human SPC was detected using RT-PCR with human primers

Figure 7. AEC2 damage milieu can induce SPC expression in a population of hAFSCs. (A) Cultures of damaged AEC2s, incubated for 24 hours with Dil-labeled hAFSCs, were fixed with 4% paraformaldehyde, immunostained for cytokeratin using an FITC-labeled secondary antibody, and counterstained with DAPI. Photographs show cells within the healing wound. Groups of cytokeratin positive (green), rat (Dil-negative) cells with the classical AEC2 phenotype are seen in intimate contact with Dil-positive (red), cytokeratin-negative hAFSCs (original magnification, $\times 20$; bar = 100 μ m). Although SPC protein costaining with Dil was not detected at this time (data not shown), human SPC, as detected by RT-PCR using human-specific primers, was induced in hAFSCs that had been cultured with damaged AEC2s for 72 hours (*bottom panel*). Human lung (*lane 2*) served as a positive control; rat AEC2s (*lane 3*) served as a negative control. Human SPC transcript was not detected in hAFSCs growing with damaged AEC2s for less than 72 hours (data not shown). (B) *Upper three panels*: hAFSCs were cultured with AEC2s *in vitro* damage milieu (CM from damaged AEC2) on chamber slides coated with collagen 1, fixed in methanol acetone 1:1 after various times in culture, immunostained with pro-SPC antibody and Alexa Fluor 488 secondary antibody, and mounted in DAPI mounting medium. Photograph shows

that SPC protein expression appeared after 96 hours; *right panels* of each pair are primary antibody controls (rabbit IgG). No positive pro-SPC protein staining was seen before 72 hours (data not shown). *Lower panel*: RNA was extracted from hAFSCs cultured for 24, 48, and 72 hours with AEC2 milieu (normoxic and hyperoxic lung extract) on chamber slides coated with collagen 1. Human SPC was detected in hAFSCs grown for 72 hours in AEC2 damage milieu by RT-PCR using human-specific primers. Coexpression of human TTF-1 reaffirmed an epithelial phenotype in SPC + hAFSCs. Human lung served as positive control, and rat AEC2s served as a negative control. Human SPC transcript was not detected before 72 hours (data not shown).

in hAFSCs grown with AEC2 damage milieu, confirming that pro-SPC-positive immunostaining was not due to endocytosis of rat surfactant from the damage milieu, whereas coexpression of human TTF-1 reaffirmed an epithelial phenotype in SPC+ hAFSCs (Figure 7B, *lower panel*). The differentiation rate, as measured by SPC immunostaining, was $13 \pm 1.4\%$ (range, 8–27%; $n = 11$) and was similar between the various types of AEC2 damage milieu tested (damaged AEC2 CM, hyperoxic BAL, and hyperoxic lung extract). SPC-staining hAFSCs were seen in 1 of 11 undamaged AEC2 milieu tested (a sample of normoxic BAL, where approximately 2% SPC + hAFSCs were detected). ABCA3, a transporter protein that plays an essential role in surfactant lipid metabolism and lamellar body biogenesis, was also induced in hAFSCs treated with AEC2 damage milieu (Figure E4 in the online supplement). SPC expression, together with TTF-1 and ABCA3 expression, confirms induction of a phenotype consistent with distal lung alveolar epithelium in hAFSCs treated with AEC2 cell damage milieu.

DISCUSSION

The local environment of a cell dictates cell fate, and bioengineering of complex organs like the lung will require a detailed knowledge of regional microenvironments to faithfully recapitulate regeneration. Similarly, the effectiveness of stem cell therapy to ameliorate tissue damage could likely be optimized if the specific damage niche was well characterized. After distal epithelial damage, the milieu of the damaged AEC2 will arguably determine the rate and nature of alveolar epithelial repair and is therefore of great interest.

Subacute hyperoxia was used as an *in vivo* damage model in this study because it elicits well documented and reproducible responses in rodent AEC2s and can serve as a model for human acute lung injury associated with oxidant stress without inflammation (11). Our data herein show that the AEC2 *in vivo*

hyperoxic milieu is sufficient to recapitulate the proliferation induced by *in vivo* hyperoxia when added to normoxic AEC2 *ex vivo* and can expedite alveolar epithelial wound healing through increased migration and proliferation. A prohealing milieu was specifically related to AEC2 cell damage rather than denudation because *in vitro* AEC2 wounding models showed that epithelial denudation without damage healed at a slower rate than damage denudation, was less attractive to exogenous cells, and induced less CINC and ICAM release. These differences are unlikely to arise from matrix exposed by damage because protein residue within wounds was minimal and was similar in composition between scratch and template wounds. These data, combined with the CINC-2/ICAM neutralization studies, suggest that soluble autocrine factors secreted by AEC2s in response to cell damage play a functional role in alveolar epithelial healing.

CINC-2 is a proinflammatory CXC chemokine that is a potent chemoattractant for neutrophils. AEC2s express CINC-2 and its receptor, CXCR2, and both are up-regulated after lung injury (21). Two variants of CINC-2 arise from alternative splicing, CINC-2 α and CINC-2 β , which differ in only three amino acids at the C-terminus (27). CINC-2 β is regulated differently from CINC-2 α and the other CXC chemokines in that its expression appears to be restricted to differentiated AEC2 cells and it is not increased by cytokines (28). In our studies, the proteome screen, ELISA, and neutralizing antibody recognized both CINC variants.

ICAM was also expressed at high levels in damaged AEC2 milieu, promoted migration of AEC2, and expedited *in vitro* AEC2 wound healing, which is consistent with the observation that ICAM-/- mice have delayed excisional wound healing (29). ICAM is a cell surface glycoprotein that is typically expressed on endothelial cells and cells of the immune system and is a member of the IgG superfamily. ICAM provides

adhesion between endothelial cells and leukocytes after stress or injury and increases planar chemotaxis of alveolar macrophages over the AEC surface, making phagocytosis more efficient (30). It is constitutively expressed in alveolar epithelium, particularly AEC1. In AEC2s, ICAM is associated with the cytoskeleton and provides an interface between mobile inflammatory cells and the alveolar surface (31). ICAM expression is induced during alveolarization as AEC1 differentiation takes place (32), and the increase of ICAM detected in the BAL after hyperoxia is consistent with recapitulation of this developmental process. Damage-induced soluble ICAM shedding by AEC has also been reported after FITC-mediated inflammatory lung injury, mechanical stress, and LPS/TNF- α stimulation (22, 33, 34). We also found significant ICAM levels in the BAL from normoxic animals, suggesting a physiological role for ICAM in alveolar homeostasis.

Postdamage decreases in the cytokines TMP-1, LIX, CINC-1, and CXCL3, detected by proteomic cytokine microarray in AEC2 damage milieu, are also consistent with a prohealing environment (19, 35). Taken together, the cytokine microarray data suggest that the AEC2s can contribute to a milieu conducive to healing through a balanced repertoire of autocrine cytokines while recruiting systemic cells to the site of damage. We speculate that the milieu of damaged AEC2s would be attractive to many cell types due to the inflammatory potential of the cytokines released. Because the rat cytokine screen was limited to only 30 cytokines or chemokines, healing of the alveolar epithelium is likely to involve other cell types that have yet to be unidentified.

The hAFSC is an intermediate type of stem or progenitor cell between embryonic stem cells and adult stem cells resident in differentiated organs and has been well characterized (13). hAFSCs are derived from a noncontroversial source and are pluripotent and nonteratogenic, making them potentially suitable for cell-based therapies. We have previously shown that systemically delivered uncommitted hAFSCs can target hyperoxia-damaged murine lung and integrate into the lung epithelium (7), suggesting that the damaged AEC2 milieu is chemoattractive to hAFSCs. Indeed, hAFSCs migrated toward hyperoxic BAL at rates 20-fold higher than basal migration toward medium alone and expedited endogenous AEC2 wound repair. hAFSCs were attracted to damaged AEC2s and could heal a scratch wound in 24 hours but were less effective in healing a wound generated by passive denudation, suggesting that interaction between the milieu of damaged AEC2s and hAFSCs promoted AEC2 repair. A human proteomic cytokine screen comparing CM from *in vitro* and *in vivo* damaged AEC2s, cultured in the presence and absence of hAFSCs, showed that human macrophage migration inhibitory factor (MIF) and plasminogen activator inhibitor-1 (PAI-1) proteins were secreted by hAFSCs and induced by exposure of hAFSCs to AEC2 damage milieu. Although MIF and PAI-1 are considered to be proinflammatory, they are also associated with epithelial wound repair (24, 25) and indeed were shown to expedite *in vitro* AEC2 wound healing in our study. Inhibition of MIF and PAI-1 slowed hAFSC-mediated AEC2 wound healing, which suggests that hAFSCs augment endogenous AEC2 repair by modifying the cytokine milieu as well as by their physical presence within the wound. The other human cytokines detected in CM from damaged AEC2s in the presence of hAFSCs, growth regulated protein- α , IL-6, and IL-8 are also proinflammatory cytokines that can play antiinflammatory or cytoprotective roles under specific conditions (36–38); thus, controlled induction of inflammation may be an important key to prompt alveolar epithelial wound repair. The prerequisite of damage to an organ for homing of exogenous stem cells to that organ (10) is consistent with this concept.

The AEC2 damage milieu could also induce expression of SPC, a protein manufactured by AEC2s and a marker of distal alveolar epithelium, in a subpopulation of hAFSCs. SPC protein was detected in engrafting hAFSCs within an AEC2 damage wound after 96-hour culture and in hAFSCs that had been cultured on collagen 1 in the presence of CM from *in vitro* damaged AEC2 cells or hyperoxic BAL or lung extract, after 96-hour culture. SPC transcript was also detected, using human-specific primers, suggesting that the protein detected was of human origin and arising from the hAFSCs rather than rat surfactant in the milieu that had been endocytosed by the hAFSCs. The frequency of SPC-staining cells ($13 \pm 1.4\%$; range, 8–27%) could potentially be increased by identification of the inducing factors, which would enable optimization of stem cells *ex vivo* before administration *in vivo*, by manipulating the milieu accordingly. The characterization of these SPC-expressing cells and the mechanism of differentiation will be the basis of future research.

Extension of this preliminary work into a rigorous proteomic characterization of the optimal cytokine/chemokine and growth factor balance that is required for alveolar epithelial cell maintenance and repair could have important translational clinical and bioengineering applications for patients with alveolar damage or disease.

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