TGF-β signaling promotes survival and repair in rat alveolar epithelial type 2 cells during recovery after hyperoxic injury

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Submitted 26 July 2007; accepted in final form 31 January 2008

Buckley S, Shi W, Barsky L, Warburton D. TGF-β signaling promotes survival and repair in rat alveolar epithelial type 2 cells during recovery after hyperoxic injury. Am J Physiol Lung Cell Mol Physiol 294: L739–L748, 2008. First published February 1, 2008; doi:10.1152/ajplung.00294.2007.—Hyperoxic rats treated with inosine during oxygen exposure have increased levels of active transforming growth factor (TGF)-β in the bronchoalveolar lavage (BAL), yet alveolar epithelial type 2 cells (AEC2) isolated from these animals demonstrate less hyperoxia-induced DNA damage and increased expression of active Smad2. To determine whether TGF-β1 signaling promotes survival and repair in rat alveolar epithelial type 2 cells (AEC2) isolated from these animals treated with inosine during oxygen exposure, we incubated AEC2 from hyperoxic rats with TGF-β1 for 24 h and assayed for DNA damage by fluorescein-activated cell sorter analysis of TdT-mediated dUTP nick end labeling. TGF-β1 was protective over a concentration range similar to that in BAL of inosine-treated hyperoxic animals (50–5,000 pg/ml). TGF-β1 also augmented hyperoxia-induced DNA repair activity and cell migration, stimulated autocrine secretion of fibronectin, accelerated closure of a monolayer scratch wound, and restored hyperoxia-depleted VEGF secretion by AEC2 to normoxic levels. The TGF-β1 receptor type I activin-like kinase-4, -5, and -7 inhibitor peptide SB-505124 abolished the protective effect of TGF-β on hyperoxic DNA damage and increased TdT-mediated dUTP nick end labeling in normoxic cells. These data suggest that endogenous TGF-β-mediated Smad signaling is required for AEC2 homeostasis in vitro, while exogenous TGF-β1 treatment of hyperoxia-damaged AEC2 results in a cell that is equipped to survive, repair, migrate, secrete matrix, and induce new blood vessel formation more efficiently than AEC2 primed by hyperoxia alone.

TdT-mediated dUTP nick end labeling; Ku 70; VEGF; migration; fibronectin; activin-like kinase-5 inhibitor; in vitro wound healing

TRANSFORMING GROWTH FACTOR-β (TGF-β) is a well-studied growth factor that transduces signals through downstream receptor-activated Smad-dependent and -independent pathways and, thereby, impacts many cell functions, including proliferation, apoptosis, and extracellular matrix deposition (27). In a previous study, we found that administration of inosine to hyperoxia-exposed adult rats in vivo protected alveolar epithelial type 2 cells (AEC2) from hyperoxic damage. AEC2 that were freshly removed from inosine-protected hyperoxic animals and immediately fixed or lysed were blocked in the G1 phase of the cell cycle and expressed increased activated Smad2 compared with AEC2 from animals exposed to hyperoxia alone, suggesting increased exposure to TGF-β within the lung. Accordingly, levels of active TGF-β were significantly higher (~3-fold) in lavage fluid from the inosine-protected animals than animals subjected to hyperoxia alone (12). These data were somewhat unexpected and led us to hypothesize that TGF-β may actually protect AEC2 against hyperoxic damage.

A nonredundant role for TGF-β-mediated Smad signaling in development is demonstrated by the embryonic lethality of the Smad2-knockout mouse (38), whereas the Smad3-knockout mouse, although viable, has impaired alveolarization and emphysema in later life (14). Active TGF-β levels increase in bronchoalveolar lavage (BAL) of rodents during alveolarization in the 1st wk of life (11) and during the repair phase after hyperoxic injury to neonate and adult rodents (7). Taken together, these observations suggest that TGF-β is essential for normal lung development and homeostasis, and systemic TGF-β has a clinical potential for expediting wound healing (34). On the other hand, excess TGF-β signaling during the neonatal period in rodents is associated with a lung phenotype similar to bronchopulmonary dysplasia (19), and increased TGF-β in BAL of low-birth-weight human neonates is associated with a poor outcome (21). In the adult lung, excessive TGF-β-mediated Smad3 signaling, as seen after bleomycin administration, is associated with extensive fibrosis. Mice lacking αvβ3 integrin, which can activate latent TGF-β, are protected from bleomycin-induced pulmonary edema (31). Although TGF-β is essential for normal lung development and homeostasis, levels of TGF-β activity within the lung must be kept within a critical range.

At the cellular level, TGF-β elicits responses in AEC2 that are dependent on dose, state of the cell, timing of the addition, and duration of treatment. These responses include transdifferentiation (5, 18, 36), GSH depletion, decreased transmembrane permeability (31), decreased surfactant protein C (SPC) mRNA, and increased fibronectin secretion (24). Bhaskaran et al. (5) showed that the proliferative status of the AEC2 at the time of addition of TGF-β is a critical determinant for transdifferentiation. In the present study, we use an in vitro system as a tractable way to deliver known levels of TGF-β directly to the cell and to measure and quantitate direct AEC2 responses to TGF-β. This system rules out indirect effects mediated through other cell types, which is an advantage and a caveat, since in vivo the AEC2 may also respond to TGF-β indirectly through other cell types in the AEC milieu. We demonstrate that, in vitro, TGF-β indeed protects AEC2 cultured from adult hyperoxic rats from hyperoxia-induced DNA damage through Smad signaling. In addition, TGF-β promotes active DNA repair, fibronectin secretion, and a more migratory and angiogenic phenotype. Thus TGF-β appears to play a key role in protecting the hyperoxic AEC2 against oxidative damage incurred in vivo.

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MATERIALS AND METHODS

Oxygen treatment. Adult male Sprague-Dawley rats were exposed to short-term hyperoxia, as described previously (8), using a protocol approved by Children’s Hospital Los Angeles Institutional Animal Care and Use Committee. Briefly, rats were placed in an oxygen chamber (Terra Universal, Fullerton, CA) and exposed to humidified 95% oxygen for 48 h, which induces reproducible damage to the alveolar epithelium with minimal mortality. In some experiments, exposure period, the rats were anesthetized by intraperitoneal injection of pentobarbital sodium. After complete exsanguination by saline perfusion via the pulmonary artery, the lungs were lavaged for removal of macrophages. BAL was centrifuged to pellet any cells, and the supernatant was frozen in aliquots at −70°C. The lavaged lungs were then used for AEC2 isolation and culture. Cells were cultured in the presence of 10% serum for ≤24 h, except for migration and matrix metalloproteinase (MMP) studies, which were performed in serum-free medium for ≤48 h culture.

Isolation and culture of adult AEC. AEC2 were isolated from lavaged lungs by elastase digestion and then differentially adhered on IgG-coated plates as described by Dobbs et al. (16). The cells were cultured with and without TGF-β1 in HEPES-buffered DMEM with 10% FBS on Primaria tissue culture plates for 24 h to achieve confluent monolayers. The cells were then used for AEC2 isolation and culture. Cells were cultured in the presence of 10% serum for ≤24 h, except for migration and matrix metalloproteinase (MMP) studies, which were performed in serum-free medium for ≤48 h culture.

In vitro inhibition of activin-like kinase-5. SB-505124 (Sigma, St. Louis, MO) is a selective inhibitor of TGF-β-mediated Smad signaling that works by inhibiting activin-like kinase (ALK)-4, -5, and -7 (15). SB-505124 (1 μM) was added to freshly isolated normoxic and hyperoxic AEC2 in the absence of serum for 1 h at 37°C, before the addition of serum to a final concentration of 10%, with and without 5 ng/ml TGF-β1. The cells were incubated for 24 h and then fixed in 1% paraformaldehyde for TdT-mediated dUTP nick end labeling (TUNEL). Parallel wells were lysed for Western blot. Successful blockade was confirmed by phosphorylated Smad2 immunoblot.

TUNEL. DNA strand breaks were measured in 1% paraformaldehyde-fixed AEC2 by labeling with FITC-dUTP using an APO-DIRECT kit from Becton Dickinson/PharMingen (San Diego, CA) according to the manufacturer’s instructions. FITC-labeled cells were counterstained with propidium iodide and analyzed with a FACSCalibur (Becton Dickinson) system and Cellquest software, as previously described (8). Control cells that were positive and negative for apoptosis were assayed by TUNEL in parallel with the AEC2.

Cell cycle analysis. AEC2 were fixed in 70% ethanol, stained with propidium iodide as previously described (13), and sorted by flow cytometer (FACS) using the FACSCalibur system and Cellquest software. ModfitLT (version 3.1) software was used for the cell cycle analysis.

Kn 70 DNA repair enzyme assay. Nuclear extracts were prepared from AEC2 isolated from control and hyperoxic animals that had been cultured on plastic for 24 h with and without 5 ng/ml TGF-β1. This dose had previously been shown to be the most effective in protecting against hyperoxia-induced DNA damage. An Active Motif (Carlsbad, CA) nuclear extraction kit and a Ku 70 DNA repair kit were used according to the manufacturer’s instructions. Nuclear extracts were prepared using hypotonic lysis, subjected to detergent extraction, and frozen at −70°C until assay. Nuclear extracts from AEC2 containing <10 μg of nuclear protein were incubated with immobilized, blunt-ended linear nucleotide in wells of a 96-well plate for 1 h at room temperature. Bound Ku 70 was detected using a Ku 70 antibody that cross-reacts with rat, followed by detection with horseradish peroxidase-linked secondary antibody. Each sample was assayed with Ku competitor oligonucleotide to ensure specificity, and a positive control consisting of a nuclear lysate of Raj cells, which are provided with the kit, was included in every run. The color product was detected at 450 nm with use of a Perkin Elmer Victor plate reader, and absorbance values were corrected for assay blank (activity in the absence of sample) and nonspecific bands (activity in the presence of competitor).

Cell migration. Freshly isolated AEC2 in DMEM with 0.25% FBS and without 5 ng/ml TGF-β1 were plated into Boyden chambers (8-μm pores) coated with fibronectin or BSA (QCM-FN Quantitative Cell Migration Assay, Chemicon, Temecula, CA). (We have found more consistent results when we used commercially coated chambers.) Cells (2.5 × 106 per 500 μl per chamber) were plated over 400 μl of DMEM with 0.25% BSA and incubated at 37°C for 48 h, the time required to achieve measurable migration of AEC2 (23). TGF-β was included in the medium at the time of plating and was present in upper and lower chambers in experiments measuring unstimulated migration and in lower chambers only in experiments measuring migration toward a TGF-β gradient. After 48 h of culture, the cells were processed as follows: the filters were removed from the wells, and cotton swabs were used to remove cells from the upper surfaces. Complete removal of cells was confirmed microscopically. The cells that had migrated through the pores and were adherent to the lower surface of the chamber were stained for 30 min in crystal violet, which was provided with the kit, and then washed with water. The stained cells were examined for morphology using a phase contrast microscope to assess the phenotype of migrated cells, and the stain was then eluted and quantitated spectrophotometrically using a plate reader.

Cell-free filters were processed in parallel and served as blanks. Migration through fibronectin was compared with migration through BSA for each condition.

In vitro scratch wound. Freshly isolated hyperoxic AEC2 were plated densely on uncoated plastic and incubated overnight in the presence of 10% serum with and without TGF-β1. On the next day, the confluent monolayers were washed to remove unattached cells and isolation debris. Then a pipette tip was drawn across the monolayer, which was washed to remove damaged cells. The damaged area was examined microscopically to confirm complete removal of cells; then the cultures were incubated in DMEM with 0.25% BSA with and without TGF-β1. At various times after damage, the medium was removed and centrifuged to pellet cells, and the supernatant was frozen at −70°C for later analysis of MMPs and fibronectin. The cells were stained with crystal violet for 30 min, washed, examined microscopically, and photographed, or were lyzed for Western blot.

Western blot. Western analysis was performed on cell lysates as described by Bui et al. (13) with 20 μg of protein per lane. Blots were repeated three times, and representative results are shown. Equal loading was confirmed by blotting with an antibody to actin. Blots using phosphorylated antibodies were compared with blots using the unphosphorylated form of the antibody. Primary antibodies were directed against rat epitopes where possible. Proteins of interest were detected using horseradish peroxidase-linked secondary antibodies and the enhanced chemiluminescence system following the manufacturer’s instructions (ECL, Amersham, Arlington Heights, IL). Psion Image software (National Institutes of Health) was used to scan blots for quantitation. Antibodies were obtained from the following sources: Smad2 and phosphorylated Smad2 from Cell Signaling Technology (Beverly, MA), smooth muscle actin from Sigma, pro-SPC from Seven Hills Bioreagents (Cincinnati, OH), GADD45α, proliferating cell nuclear antigen, ERK, and phosphorylated ERK from Santa Cruz Biotechnology (Santa Cruz, CA), MMP-9 and MMP-2 from Chemicon, and actin from ICN (Irvine, CA). T1-α was kindly supplied by Dr. Mary Williams. Secondary antibodies were obtained from Sigma. Composites represent blots from at least three independent experiments.
VEGF ELISA. VEGF in conditioned medium (CM) from 24-h cultured AEC2 and BAL was measured using the Quantikine rat VEGF ELISA (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Freshly isolated normoxic and hyperoxic AEC2 were plated at \( \sim 2 \times 10^4 \) cells/cm\(^2\) on plastic with and without 5 ng/ml TGF-\( \beta \)-1 and incubated for 24 h at 37°C. CM was removed, centrifuged to remove cells and isolation debris, and frozen at \(-70^\circ\)C until use. Undiluted CM was assayed for VEGF, with medium and assay controls included in every run. The corresponding cells were lysed in RIPA buffer, and the protein was measured to correct VEGF levels for cell attachment.

**Rat fibronectin ELISA.** CM from 24-h serum-free cultures of AEC2 with and without TGF-\( \beta \)-1 and BAL from control and inosine-treated hyperoxic rats in vivo were analyzed by ELISA relative to rat fibronectin standards as described by Garat et al. (18), except we used a peroxidase color substrate (R & D Systems) for detection. Rat fibronectin for the standards was obtained from Calbiochem (La Jolla, CA), and the rat-specific fibronectin antibody was purchased from Chemicon. CM and BAL were diluted 10-fold for analysis.

**Statistics.** Values are means ± SE and represent multiple independent experiments. Composite Western blots represent data from at least three independent experiments. Statistical significance between treated and untreated groups was calculated using Student’s \( t \)-test. \( P < 0.05 \) was considered significant.

**RESULTS**

**TGF-\( \beta \)-1 reduces hyperoxia-induced DNA damage to AEC2.** In vivo exposure of rats to 48 h of hyperoxia reproducibly results in DNA damage in AEC2 cultured from these rats, whereas TUNEL is negligible in AEC2 cultured from normoxic animals (8). TGF-\( \beta \)-1 was added to AEC2 from hyperoxic animals at the time of plating at physiological doses distributed around the levels found in BAL of inosine-protected hyperoxic animals (12). The cells were cultured for 24 h on plastic and then fixed and analyzed for DNA damage by FACS analysis of TUNEL. TGF-\( \beta \)-1 reduced TUNEL of hyperoxic AEC2 at all doses tested (Fig. 1). TGF-\( \beta \)-1 increased the number of cells attached in 24 h by 1.5 ± 0.12-fold. Because the number of cells in the S and G2/M phases did not increase in subsequent cell cycle analysis, the increased cell numbers were not a result of proliferation (Fig. 2C).

Fig. 1. Transforming growth factor (TGF)-\( \beta \) reduces hyperoxia-induced DNA damage to alveolar epithelial type 2 cells (AEC2). Freshly isolated AEC2 from hyperoxic rats were cultured for 24 h with and without addition of TGF-\( \beta \)-1 at the time of plating. Cells were fixed in paraformaldehyde, postfixed in 70% ethanol, labeled with FITC-dUTP, and analyzed by fluorescein-activated cell sorting (FACS) for TdT-mediated dUTP nick end labeling (TUNEL). *Significantly different from 0 TGF-\( \beta \)-1 (5,000 pg/ml). ■Significantly different from 0 TGF-\( \beta \)-1; \( P < 0.03 \) at 50 pg/ml (\( n = 10 \)), \( P < 0.05 \) at 500 pg/ml (\( n = 6 \)), and \( P < 0.002 \) at 5,000 pg/ml (\( n = 8 \)). TGF-\( \beta \)-1 did not affect very low TUNEL of normoxic AEC2 (\( \sim 10 \% \), data not shown).

Fig. 2. A: TGF-\( \beta \)-1 increases DNA repair in hyperoxic AEC2. Nuclear extracts from normoxic and hyperoxic AEC2 that had been cultured for 24 h with and without addition of 5 ng/ml TGF-\( \beta \)-1 at the time of plating were incubated with immobilized linear blunt-ended nucleotide in wells of a 96-well plate. Bound Ku 70 was detected using a Ku 70 antibody that cross-reacts with rat-specific horseradish peroxidase-linked secondary antibody. Ku 70 activity was present in hyperoxic AEC2 (0 TGF-\( \beta \)-1) and increased with TGF-\( \beta \)-1 (5,000 pg/ml). †Significantly different from 0 TGF-\( \beta \)-1 (\( P < 0.01 \), \( n = 5 \)). Ku70 activity in normoxic AEC was undetectable (data not shown). \( A_{\text{Abs}} \) absorbance at 450 nm. B: Western blot of cell lysates from 24-h cultures of hyperoxic AEC2 with and without addition of 5 ng/ml TGF-\( \beta \)-1 at the time of plating. Induction of GADD45α, a growth arrest and DNA damage-inducible protein, is apparent with TGF-\( \beta \)-1 treatment. UV-treated NIH-3T3 cells (3T3 + UV) served as a positive control for GADD45. Actin was included as loading control. C: freshly isolated AEC2 from hyperoxic rats were cultured for 24 h with (OT) and without (O) addition of 5 ng/ml TGF-\( \beta \)-1 at the time of plating. Cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed by FACS. TGF-\( \beta \)-1 increased the number of hyperoxic cells in the G0/G1 phase of the cell cycle. *Significantly different from hyperoxia alone (\( P < 0.01 \)). Percentage of normoxic AEC2 in the G0/G1 phase was \( \sim 90\% \) (90.4 ± 6.5%) and was not affected by TGF-\( \beta \)-1 (data not shown).

suggests that TGF-\( \beta \) was promoting survival and/or attachment of hyperoxic AEC2, perhaps through protection or modulation of integrins. The population of cells that attached to plastic in the presence of TGF-\( \beta \) after 24 h did not differ in phenotype from the population that attached in the absence
of TGF-β and consisted of ~90–95% SPC-positive epithelial cells and ~5–10% fibroblasts. Addition of TGF-β1 to normoxic AEC2 did not affect TUNEL, which was usually ~10% (data not shown).

TGF-β1 promotes DNA repair activity in vitro and increases the percentage of hyperoxic AEC2 in the G0/G1 phase of the cell cycle. TUNEL in AEC2 cultured from hyperoxic animals varies from animal to animal and can be as high as 70–80% of the whole gated population, as measured by FACS (8). TUNEL must include some nonlethal, repairable DNA damage; otherwise, the bulk of the hyperoxic population would eventually detach and die, which is not the case. To assess the effect of in vitro TGF-β1 treatment on DNA repair, we prepared nuclear extracts from 24-h cultures of AEC2 with and without 5 ng/ml TGF-β1. The nuclear extracts were then incubated with immobilized damaged DNA for 1 h. DNA repair was measured as Ku 70 repair enzyme binding to strand breakage sites. Although Ku 70 activity was undetectable in cultures of normoxic AEC2 (data not shown), in hyperoxic AEC2, it was increased ~1.7 fold by TGF-β1 (Fig. 2A). GADD45, a growth arrest and DNA damage gene that induces DNA repair, is upregulated in rodent lung after short-term hyperoxia (29). Western blotting of lysates from 24-h cultures of hyperoxic AEC2 treated with TGF-β1, including a control lysate of UV-treated NIH-3T3 cells, showed that TGF-β1 induced expression of GADD45, further suggesting that TGF-β1 was augmenting the DNA repair process triggered by oxidative stress (Fig. 2B).

Although normoxic AEC2 are quiescent, with the majority of cells in the G0/G1 phase of the cell cycle, AEC2 cultured from hyperoxic rats show a limited period of proliferation during the recovery period (13). When freshly isolated hyperoxic AEC2 were treated with 5 ng/ml TGF-β1 and cultured for 24 h, a significant increase of cells in the G0/G1 phase of the cell cycle was observed compared with AEC2 exposed to hyperoxia alone, as measured by FACS analysis of propidium iodide-labeled cells (Fig. 2C). These data are consistent with a G1 cell cycle blockade, allowing time for DNA repair before the cells progress in the cell cycle for a limited period of hyperoxia-stimulated proliferation.

TGF-β1 promotes in vitro migration of hyperoxic AEC2 through fibronectin. AEC2 isolated from normoxic rats do not migrate through the pores of a Boyden chamber (10, 23), yet AEC2 from hyperoxic animals exhibit unstimulated migration after a recovery period in culture (10). We compared the migration of hyperoxic AEC2 with and without TGF-β1 after 48 h of culture on 8-μm porous filters coated with BSA or fibronectin, with TGF-β in the upper and lower wells. Fibronectin reduces hyperoxia-induced DNA damage to AEC2 (9) and has been shown on microarray analysis to be upregulated during in vitro wounding of respiratory epithelial monolayers (35). TGF-β1 significantly stimulated migration through fibronectin at all doses tested (Fig. 3). Migration through BSA-coated filters was not significantly increased by TGF-β1 (data not shown). Migration through fibronectin was stimulated at all doses tested. *Significantly different from hyperoxia alone (n = 5): P < 0.001 vs. hyperoxia + 50 pg/ml TGF-β1 (n = 4), P < 0.001 vs. hyperoxia + 500 pg/ml TGF-β1 (n = 3), and P < 0.05 vs. hyperoxia + 5,000 pg/ml TGF-β1 (n = 5). Hyperoxic AEC2 did not migrate toward a TGF-β1 gradient. Migration was not observed in untreated normoxic AEC2 or in response to TGF-β1 (data not shown).

Fig. 3. TGF-β1 increases migration of hyperoxic AEC2 through fibronectin. AEC2 isolated from normoxic rats do not migrate through pores in a Boyden chamber, yet AEC2 from hyperoxic animals exhibit unstimulated migration after a recovery period in culture (9). Migration of normoxic and hyperoxic AEC2 with and without TGF-β1 over 24–48 h of culture was compared using 8-μm porous filters coated with BSA or fibronectin (FN), with TGF-β1 in upper and lower wells. Migration of hyperoxic AEC2 through BSA-coated filters was not significantly increased by TGF-β1 (data not shown). Migration through fibronectin was stimulated at all doses tested. *Significantly different from hyperoxia alone (n = 5): P < 0.001 vs. hyperoxia + 50 pg/ml TGF-β1 (n = 4), P < 0.001 vs. hyperoxia + 500 pg/ml TGF-β1 (n = 3), and P < 0.05 vs. hyperoxia + 5,000 pg/ml TGF-β1 (n = 5). Hyperoxic AEC2 did not migrate toward a TGF-β1 gradient. Migration was not observed in untreated normoxic AEC2 or in response to TGF-β1 (data not shown).

TGF-β1 promotes epithelial wound healing in vitro. TGF-β is reported to promote airway epithelial wound repair through induction of MMP-2 in a scratch damage model using stably differentiated human nasal airway epithelial cells grown for 2 wk on collagen at the air-liquid interface (22). To determine whether TGF-β could promote repair of mechanical damage in addition to repair of hyperoxic damage, we used a simple wound model comprising a scratch across a monolayer. TGF-β1 promotes rapid closure of a scratch in a monolayer of hyperoxic AEC2 growing on uncoated plastic in the absence of growth factors or extracellular matrix. Complete closure occurs by 48 h at >50 pg/ml TGF-β1, whereas a narrowed gap, but incomplete closure, is observed in the untreated cells (Fig. 4A). The predominant cells migrating into the gap have an AEC2 phenotype. Early in the repair process, TGF-β induces expression of pro-MMP-9 and active MMP-9 in damaged AEC2 (Fig. 4B), whereas proliferating cell nuclear antigen is induced later, indicating DNA repair and/or proliferation. Fibronectin, which can protect AEC2 from hyperoxic damage (9), is increased 1.5-fold in TGF-β1-treated cells 24 h after damage (Fig. 4D).

TGF-β induces secretion of fibronectin from hyperoxic AEC2. TGF-β induces secretion of fibronectin from normoxic rabbit AEC2 (24). We analyzed CM from hyperoxic AEC2 with and without TGF-β and BAL from inosine-protected animals for fibronectin. Fibronectin is secreted by hyperoxic AEC2 in response to TGF-β (Fig. 5A), and BAL of inosine-protected hyperoxic animals, which contains increased levels of active TGF-β, also contains increased levels of fibronectin (Fig. 5B).

TGF-β1 restores hyperoxia-depleted autocrine VEGF secretion. TGF-β-mediated VEGF expression has been reported in lung epithelial cells (28). A cytokine protein array showed that increased VEGF protein was overexpressed by hyperoxic AEC2 from inosine-protected rats, which have increased active TGF-β in the BAL (data not shown). To determine whether TGF-β affected VEGF secretion, AEC2 from normoxic and hyperoxic rats were cultured for 24 h with and without 5 ng/ml TGF-β1. CM was assayed for VEGF with a rat-specific ELISA, and data were expressed per microgram of cellular protein to correct for differences in attachment. Normoxic
AEC2 constitutively secreted high levels of VEGF protein. Hyperoxia decreased VEGF secretion by ~40%, whereas addition of 5 ng/ml TGF-β1 restored VEGF secretion to normoxic levels (Fig. 6A). To determine whether the increased VEGF is associated with increased active TGF-β recovered from BAL of inosine-protected animals (12), we analyzed BAL from hyperoxic rats with and without inosine. BAL from inosine-treated hyperoxic animals showed a twofold increase in VEGF levels compared with animals exposed to hyperoxia alone. Since no VEGF secretion was detected in CM from cultured macrophages isolated from BAL of these animals (data not shown), we speculate that, in vivo, AEC2 may make a major contribution to VEGF in BAL after hyperoxia.

We found that VEGF itself was not a chemoattractant to hyperoxic AEC2 at doses distributed around the levels in BAL or CM (0.1–10 ng/ml), nor did it protect hyperoxic AEC2 from DNA damage (data not shown). Therefore, we speculate that AEC2-secreted VEGF serves a paracrine, rather than an autocrine, function that is perturbed by hyperoxia and restored by controlled amounts of TGF-β1.

Fig. 4. A: TGF-β1 expedites closure of a monolayer scratch wound in the absence of growth factors and extracellular matrix. Freshly isolated hyperoxic AEC2 were plated densely on uncoated plastic and incubated overnight with and without low (50 pg/ml) and high (5,000 pg/ml) doses of TGF-β1. On the following day, confluent monolayers were washed to remove unattached cells and isolation debris, damaged using a pipette tip, and incubated in serum-free DMEM with 0.1% BSA with and without TGF-β1. At 24 and 48 h after damage, cells were stained with crystal violet. By 24 h, both doses of TGF-β1 had reduced the gap compared with untreated cells, with the high dose being more efficient. By 48 h, the gap was completely sealed by TGF-β1.

B: TGF-β1 induces matrix metalloproteinase (MMP)-9 and proliferating cell nuclear antigen (PCNA) in AEC2 after scratch injury. Lysates were prepared from scratch-damaged hyperoxic AEC2 with and without 5 ng/ml TGF-β1 at intervals preceding complete closure of the scratch wound. Composite shows Western analysis of PCNA, MMP-9, and actin loading control, all from the same blot, which was reprobed for each antibody, and represents blots from ≥3 independent experiments (see C). Rat-specific MMP antibody recognizes active and propeptide (pro) forms of rat MMP-9. An increase in total MMP-9 expression (pro and active isoforms) can be detected in TGF-β1-treated cells by 4 h after damage and persists until ≥14 h, when an increase of PCNA can be detected. No change in MMP-2 expression was detected (data not shown). C: densitometric analysis of Western blots. MMP-9 was increased in TGF-β1-treated cells at 4, 8, and 14 h. PCNA was unchanged at 4 h but was increased at 8 and 14 h. *Significantly different from untreated cells: all P < 0.01 for MMP-9 (n = 3); P < 0.05 at 8 h and P < 0.01 at 14 h for PCNA (n = 4). D: conditioned medium (CM) from scratch-damaged hyperoxic AEC2 was analyzed for fibronectin by rat-specific ELISA after damage. TGF-β1-treated cells released 1.5-fold more fibronectin into CM after 24 h of culture.
TGF-β induces early and sustained Smad2 activation in hypoxic AEC2. When TGF-β was added to freshly isolated hypoxic AEC2, increased phosphorylation of Smad2 was seen 1 h after treatment (when very few cells had attached) and was still apparent after 24 h of culture. In contrast, TGF-β-mediated ERK phosphorylation was not seen after 1 h of TGF-β exposure, but TGF-β treatment maintained ERK activation during 24 h of culture (Fig. 7).

Blockade of Smad signaling. To determine whether direct intracellular TGF-β signaling was protective in hypoxic AEC2, we used an inhibitor of TGF-β receptor type 1, SB-505124. This small-molecule inhibitor selectively and concentration dependently inhibits TGF-β downstream Smad activation through ALK-4, -5, and -7 (15). Freshly isolated AEC2 were preincubated with and without 1 μM SB-505124 for 1 h, TGF-β (5 ng/ml) was added, and the cells were cultured for 24 h. The cells were fixed for FACS analysis of TUNEL, and parallel aliquots were lysed for Western blotting to confirm successful and sustained blockade of Smad 2 (Fig. 8A). SB-505124 effectively inhibited Smad2 activation and blocked TGF-β-induced protection of hypoxic AEC2, suggesting that TGF-β was protecting through Smad signaling (Fig. 8B).

Effect of TGF-β on phenotypic marker expression in hypoxic AEC2 under various culture conditions. To confirm that the cell eliciting the responses described above had an AEC2 phenotype and was not differentiating into AECl or myofibroblasts, we analyzed expression of marker proteins in lysates representative of all our experimental conditions (Fig. 9). Smooth muscle actin protein was not detected in any AEC2

![Figure 5](http://ajplung.physiology.org/)

**Fig. 5.** A: TGF-β1 increased secretion of fibronectin from hypoxic AEC2. AEC2 from hypoxic rats were cultured with and without addition of TGF-β1 at the time of plating. After attachment, serum-containing medium was removed, and cells were cultured for 24 h in the absence of serum. CM was collected, and fibronectin secretion was analyzed using ELISA for fibronectin relative to rat fibronectin standards. TGF-β1 stimulated fibronectin production at 500 and 5,000 pg/ml (*Significantly different at 500 pg/ml (P < 0.03, n = 5) and 5,000 pg/ml (P < 0.04, n = 8). B: fibronectin levels were increased in bronchoalveolar lavage (BAL) of inosine-protected hypoxic rats. Inosine + hyperoxia increases TGF-β1 in BAL while reducing DNA damage to the AEC2 (12). Rats were injected with inosine (200 mg/kg ip twice each day) for 48 h during exposure to normoxia or >90% hyperoxia, and lungs were lavaged to capacity 8 times with a buffered isotonic salt solution (16). BAL was analyzed by ELISA for fibronectin relative to rat fibronectin standards. Hyperoxia (O) increased fibronectin levels in BAL relative to BAL of control animals (C, O = 3); hyperoxia + inosine (OI, n = 6) increased fibronectin levels compared with hyperoxia alone (n = 6). *Significant difference between C and O (P < 0.04) and between O and OI (P < 0.03).

![Figure 6](http://ajplung.physiology.org/)

**Fig. 6.** A: TGF-β1 restores hyperoxia-depleted VEGF secretion from AEC2. AEC2 from normoxic and hyperoxic rats were cultured for 24 h with and without addition of 5 ng/ml TGF-β1 at the time of plating. CM was assayed for VEGF by rat-specific ELISA, and corresponding cells were lysed in the well for protein measurement. Normoxic AEC2 (C, n = 7) constitutively secreted high levels of VEGF protein. Hyperoxia (O, n = 6) decreased VEGF secretion by ~40% compared with normoxia. *Significant difference between O and C (P < 0.0001). Addition of 5 ng/ml TGF-β1 to hypoxic cells (OT, n = 11) restored VEGF secretion to normoxic values. CM from AEC2 isolated from inosine-protected hypoxic animals with increased TGF-β1 in BAL (OI, n = 4) secreted VEGF at normoxic levels. TGF-β1 did not significantly increase VEGF secretion from normoxic AEC2 (CT, n = 7). B: VEGF and TGF-β1 are increased in BAL from inosine-protected hyperoxic rats and their AEC2 have reduced hyperoxia-induced DNA damage upon culture compared with rats exposed to hyperoxia alone (12). BAL recovered from inosine-protected hypoxic animals has a 2-fold increase in VEGF compared with BAL from hyperoxic animals. *Significantly different from hyperoxia alone (P < 0.006, n = 4). No VEGF was detected in BAL from normoxic rats (data not shown).
lysate, whereas T1-α showed the characteristic induction with culture time on plastic but did not differ in the absence and presence of TGF-β. In contrast, pro-SPC showed the characteristic decrease with culture time on plastic, did not differ in the absence and presence of TGF-β in culture for 24 h with or without serum, and showed a slight reduction in protein expression in TGF-β-treated cells cultured for >48 h without serum.

Fig. 7. TGF-β1 sustains Smad activation in hyperoxic AEC2. Phosphorylation of Smad2 (pSmad2) was increased 1 h after addition of TGF-β1 to freshly isolated hyperoxic AEC2 (before cells have attached). After 24 h of culture, Smad2 signaling was still apparent in TGF-β1-treated cells. TGF-β1 did not increase ERK phosphorylation (pERK) at 1 h but maintained ERK activation through 24 h of culture.

Fig. 8. A: activin-like kinase (ALK)-5 inhibition in AEC2 results in sustained phosphorylated Smad 2 inhibition. Freshly isolated control (C) and hyperoxic (O) AEC2 were preincubated with and without 1 μM SB-505124 in DMEM for 1 h before addition of 10% serum with and without 5 ng/ml TGF-β1 (T) and then cultured for 24 h. Cells were lysed for Western blotting, which confirmed successful blockade of Smad2 activation. ERK activation was not inhibited by SB-505124 (data not shown). B: ALK-4, -5, and -7 inhibition in AEC2 blocks TGF-β1-mediated protection from hyperoxic DNA damage. TGF-β1 treatment (OT) of hyperoxic AEC2 (O) decreased hyperoxia-induced DNA damage (n = 5). Blockade of this protection by SB-505124 suggests that TGF-β1 protection was mediated through Smad signaling. *Significantly different from hyperoxia alone (P < 0.002, n = 5).

Fig. 9. A: phenotypic marker expression of hyperoxic AEC during culture with TGF-β1. Hyperoxic AEC2 cultured in the presence of serum for 24 h with and without addition of 5 ng/ml TGF-β1 at the time of plating were probed for phenotypic marker expression by Western blot. These lysates reflect cells used in all experiments, except fibronectin ELISA, migration, and scratch experiments. Freshly isolated cells (time 0) were a control for propeptide surfactant protein C (pro-SPC); NIH-3T3 cells were a control for smooth muscle actin (SMA). Pro-SPC antibody recognizes a major band at ~21 kDa, as well as some smaller (~14- and 12-kDa) processing intermediates in rat AEC2 lysates. No SMA was detected in AEC2 lysates. B: hyperoxic AEC2 cultured for various times with and without TGF-β1 in the absence of serum were probed for phenotypic marker expression by Western blot. Lysates reflect cells used in fibronectin ELISA, migration assays, and scratch experiments. Freshly isolated cells (time 0) were a control for pro-SPC; NIH-3T3 cells were a control for SMA. T1-α expression increased with time in culture but was unaffected by TGF-β1 over the period studied. Pro-SPC expression decreased with time in culture; only smaller processing intermediates were seen after 24 h without serum. TGF-β1 treatment for 48 h decreased expression of pro-SPC by ~30%, as measured through scanning densitometry. SMA was not detected in AEC2 lysates.
DISCUSSION

The role of the AEC2 as the surfactant-producing cell of the lung has been expanded over the years to include many other essential functions. The AEC2 is a putative progenitor of the alveolar epithelium, having the capacity to differentiate into AEC1 (1). AEC2 also have the potential for transdifferentiation into mesenchyme (20, 39, 42). AEC2 express toll-like receptors, contribute to innate immunity (2, 26), and secrete VEGF (28). Thus, survival of the AEC2 is critical, not only as the source of surfactant, but also as the source of other cell types required for homeostatic functions, including gas exchange or matrix maintenance, as well as for protection against pathogens.

Short-term hyperoxic injury to the adult rodent lung provides a convenient model for alveolar damage and repair, with injury, repair, and recovery occurring in a reproducible sequence. In the alveolar epithelium, ablation of the gas-exchanging AEC1 cells after hyperoxia is followed by a limited period of proliferation of the more oxygen-resistant AEC2 (37). However, AEC2 are still susceptible to hyperoxia, and DNA damage can be seen in vivo as punctate 8-oxoguanine staining in >60% of the total AEC2 population and in vitro by comet staining and FACS analysis of TUNEL (8, 32). AEC2 assayed for TUNEL immediately on removal from hyperoxic rats show negligible DNA damage, which becomes measurable after 24 h of culture on plastic, compared with normoxic AEC2 (8). Biological substrates such as fibronectin (9) or growth factors such as keratinocyte growth factor (8) can prevent or minimize hyperoxic DNA damage to AEC2. Thus it appears that minimal culture conditions lack factors protective to cells in which oxidant injury is already initiated. However, once DNA damage is incurred, the hyperoxic AEC2 are capable of DNA repair, even under less-than-optimium in vitro conditions: AEC2 cultured from hyperoxic animals can exhibit as much as 80% TUNEL (8), yet 80% of the cultures do not die. It appears that the AEC2 has already been primed in vivo to repair nonlethal DNA damage, even when isolated from its alveolar niche. Similarly, in vivo, the AEC2 must be capable of efficient DNA repair after short-term hyperoxia, since normal alveolar epithelial morphology is restored after a recovery period (36).

The AEC2 expresses TGF-β receptors and also constitutively secretes TGF-β (7). After short-term hyperoxic injury in the rat, AEC2 are exposed to increased levels of active TGF-β within the lung, since increased macrophage-derived active TGF-β is recovered by lavage (7). Levels of active TGF-β are significantly higher (~3-fold) in BAL from inosine-protected hyperoxic rats than animals subjected to hyperoxia alone, and AEC2 freshly removed from inosine-protected hyperoxic animals are blocked in the G1 phase of the cell cycle, express increased activated Smad2, and have less DNA damage than cells from hyperoxic rats (12). This suggests that increased exposure to TGF-β within the lung may limit the hyperoxic damage to the AEC2. The TGF-β-mediated reduction in TUNEL described here is accompanied by increased cell attachment, delayed reentry into the cell cycle, and active DNA repair activity. Growth arrest in the G1 phase, associated with reduced DNA damage and enhanced survival, has been also been reported in Mv1Lu lung epithelial cells (32).

In the present study, we show that Smad signaling through TGF-β is required for cellular homeostasis in vitro, as evidenced by the decreased attachment and increased DNA damage observed after Smad signaling blockade. A role for TGF-β in maintaining homeostasis of the adult human lung was suggested by early observations that active TGF-β is constitutively present in the epithelial lining fluid of the human lower respiratory tract at levels 15-fold higher than in serum (41) and that human alveolar macrophages constitutively secrete active TGF-β1 (3). Lung injury leads to an induction of TGF-β that limits inflammation and mediates tissue remodeling and repair yet results in fibrosis if the reparative processes are exaggerated and not adequately localized (4).

AEC2 acquire a migratory phenotype as a response to hyperoxic lung injury, as measured in vitro by migration assays through porous filters (10). Damage-mediated induction of AEC2 migration is also seen in vivo after chemical injury, where rapid reepithelialization of AEC1-depleted epithelium by AEC2 occurs even in the absence of AEC2 cell division (40). In our study, TGF-β1 augments hyperoxia-induced migration of AEC2 through fibronectin by almost twofold, but not migration through BSA. It is possible that the increased number of cells migrating through the filters reflect a more efficient initial attachment and/or survival of damaged AEC2, rather than an increased capacity for migration per se, since extracellular matrix proteins, including fibronectin, promote survival of hyperoxic AEC2 (9).

Fibronectin can also increase alveolar epithelial wound healing in vitro (18). TGF-β-mediated autocrine secretion of fibronectin could contribute to the accelerated wound healing of hyperoxic AEC2 grown on plastic in the absence of endogenous growth factors and extracellular matrix by stimulating migration and proliferation. It has been reported that 5 ng/ml TGF-β1 increases repair of scratch-damaged human nasal epithelial cells growing on collagen through upregulation of MMP-2 activity (22). Although we found that TGF-β induced pro-MMP-9 and active MMP-9 expression during the early repair period after scratch damage to hyperoxic AEC2 on plastic, we found no similar induction of MMP-2 in our scratch-damaged hyperoxic AEC2, either by gelatin zymography of CM or by immunoblots of cell lysates.

AEC2 are the major source of VEGF in the lung and express both receptors, Flt-1 (VEGF-R1) and Flk-1 (VEGF-R2) (28). We found that in vivo hyperoxia reduced constitutive secretion of VEGF by cultured AEC2 by ~40%. This may reflect in vivo events, since Maniscalco et al. (25) also report a reduction in AEC-associated VEGF after hyperoxia in neonatal rabbits measured in vivo at RNA and protein levels. TGF-β-mediated VEGF expression has also been reported in A549 lung epithelial cells and primary human bronchial epithelial cells (6, 30). We found that 24 h of incubation of AEC2 with 5 ng/ml TGF-β could restore hyperoxia-depleted VEGF secretion to normoxic levels. AEC2 isolated from inosine-treated hyperoxic rats, which have less DNA damage (12), secrete VEGF at normoxic levels. Interestingly, BAL from these rats, which contains a threefold increase in TGF-β compared with BAL from rats exposed to hyperoxia alone, also has a twofold increase in VEGF. We speculate that, in vivo, increased levels of TGF-β in BAL after hyperoxia increase VEGF secretion by AEC2. Our observations that VEGF per se does not protect AEC2 against hyperoxic injury and that AEC2 do not migrate toward from DNA damage but is also required for cellular homeostasis in vitro, as evidenced by the decreased attachment and increased DNA damage observed after Smad signaling blockade. A role for TGF-β in maintaining homeostasis of the adult human lung was suggested by early observations that active TGF-β is constitutively present in the epithelial lining fluid of the human lower respiratory tract at levels 15-fold higher than in serum (41) and that human alveolar macrophages constitutively secrete active TGF-β1 (3). Lung injury leads to an induction of TGF-β that limits inflammation and mediates tissue remodeling and repair yet results in fibrosis if the reparative processes are exaggerated and not adequately localized (4).
VEGF suggest a nonautocrine role for AEC2-secreted VEGF, perhaps in endothelial repair.

With the important caveat that the data here were obtained in vitro under conditions that may well be different from in vivo conditions, addition of physiological doses of TGF-β1 at the time of plating to hyperoxia-damaged AEC2 results in a cell that is equipped to survive, repair, migrate, secrete matrix, and induce new blood vessel formation more efficiently than AEC2 primed by hyperoxia alone. We speculate that the increased migration (10) and DNA repair (12) in hyperoxic AEC2 in the absence of exogenous TGF-β compared with normoxic AEC2, which do not migrate or express DNA repair proteins, may be a result of exposure to increased levels of TGF-β and fibronectin within the hyperoxic lung. Pittet et al. (31) showed decreased GSH levels and epithelial permeability in normoxic AEC2 treated with TGF-β 24 h after plating, although these cells remained viable and had no TUNEL. It may be that plating freshly isolated AEC2 in the presence of TGF-β selects for the attachment of a more robust cell population or that the hyperoxic AEC2, which already has lowered GSH (9), responds differently to TGF-β. It is also possible that GSH levels do fall in our cells but trigger a repair response, although GSH levels are normal in AEC2 isolated from hyperoxic inosine-treated rats, which are exposed to increased active TGF-β in BAL, and lowered in hyperoxic AEC2 (12). Maniscalco et al. (24) showed that addition of TGF-β to rabbit AEC2 after 24 h of culture results in decreased SPC mRNA and increased fibronectin expression. In situ hybridization revealed a markedly heterogeneous response to TGF-β, which the authors speculated could be due to variations in transdifferentiation status or cell cycle at the time of addition. Bhaskaran et al. (5) showed that although TGF-β signaling is involved in transdifferentiation of normoxic AEC2 to AEC1, an initial proliferation phase is essential for subsequent differentiation processes and that addition of exogenous TGF-β after the proliferative phase did not affect transdifferentiation. This may explain why we saw no TGF-β-mediated transdifferentiation (5, 20, 39, 42) in our experiments toward myofibroblast or AEC1 phenotype, since we administered TGF-β at the time of plating, effectively synchronizing the cells in the G1 phase.

In summary, endogenous TGF-β1-mediated Smad signaling is required for adult AEC2 homeostasis in culture, and addition of exogenous TGF-β to cultures of AEC2 from hyperoxic animals protects against hyperoxia-induced DNA damage through TGF-β-mediated Smad signaling while inducing migration, DNA repair, fibronectin and VEGF secretion, and accelerated wound healing. Fibronectin and VEGF are associated with increased TGF-β in BAL of inosine-protected hyperoxic rats. Definitive conclusions regarding the in vivo relevance, if any, of the specific AEC2 responses to TGF-β described here are beyond the scope of the present study because of the inherent limitations of the rat model. Future studies using hyperoxic exposure of genetically manipulated mice in which TGF-β signaling is ablated in the AEC2 would address this issue.

ACKNOWLEDGMENTS

We acknowledge the resources of the Gene, Immune, and Stem Cell Therapy Program Flow Cytometry Laboratory at Children’s Hospital Los Angeles for expert FACS analysis.


