Endothelin-1 Induces Alveolar Epithelial–Mesenchymal Transition through Endothelin Type A Receptor–Mediated Production of TGF- β 1

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Endothelin-1 (ET-1) is implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF), but the cellular mechanisms underlying the role it plays in this disease are not well characterized. Epithelial–mesenchymal transition (EMT), which was recently demonstrated in alveolar epithelial cells (AEC), may play an important role in the pathogenesis of IPF and other forms of pulmonary fibrosis. Whether ET-1 contributes to the induction of EMT in AEC is unknown. The aims of this study were to evaluate AEC production of ET-1 and to determine if ET-1 induces EMT in AEC. We demonstrate that ET-1 is produced at physiologically relevant levels by primary AEC and is secreted preferentially toward the basolateral surface. We also demonstrate that AEC express high levels of endothelin type A receptors (ET-A) and, to a lesser extent, type B receptors (ET-B), suggesting autocrine or paracrine function for alveolar ET-1. In addition, ET-1 induces EMT through ET-A activation. Furthermore, TGF- β 1 synthesis is increased by ET-1, ET-1 induces Smad3 phosphorylation, and ET-1–induced EMT is attenuated by a TGF- β 1–neutralizing antibody. Thus, ET-1 is an important mediator of EMT in AEC, acting through ET-A–mediated TGF- β 1 production. These findings increase our basic understanding of the role of ET-1 in pulmonary fibrosis and suggest potential roles for AEC-derived ET-1 in the pathogenesis of other alveolar epithelial–mediated lung diseases.

Keywords: pulmonary fibrosis; alveolar epithelial cells; polar secretion; lung injury

Endothelin-1 (ET-1) is a 21–amino acid peptide that was first described in endothelial cells (1). ET-1 is most well known for the potent vasoconstrictor activity it displays, but it also serves as a bronchoconstrictor, chemoattractant, fibroblast and smooth muscle cell mitogen, and stimulant of collagen synthesis (2). ET-1 has been extensively implicated in the pathogenesis of pulmonary fibrosis. Patients with scleroderma have elevated levels of ET-1 in plasma, bronchoalveolar lavage (BAL) fluid, and lung tissue (3, 4). In patients with idiopathic pulmonary fibrosis (IPF), plasma ET-1 levels are also elevated (5), and relative histologic expression of ET-1 correlates with disease severity (6). In an experimental model of pulmonary fibrosis induced by intratracheal administration of bleomycin, ET-1 levels increase

CLINICAL RELEVANCE

Treatments for idiopathic pulmonary fibrosis (IPF) are not yet known. Our findings that endothelin-1 is produced by alveolar epithelial cells and induces alveolar epithelial–mesenchymal transition may lead to novel treatments for pulmonary fibrotic diseases such as IPF.

in the lung before the development of fibrosis (7). Importantly, transgenic mice overexpressing ET-1 develop profound pulmonary and renal fibrosis (8). Finally, the endothelin receptor antagonist bosentan attenuates bleomycin-induced lung injury in rats (9). However, the specific mechanisms underlying the induction of pulmonary fibrosis by ET-1 are unknown.

The primary pathogenic cellular mediators of pulmonary fibrosis are myofibroblasts, which secrete extracellular matrix components and fibrogenic growth factors, migrate to sites of injury, and cause wound contraction (10). Classically it was believed that resident pulmonary fibroblasts differentiate into myofibroblasts in response to inflammation. However, anti-inflammatory treatments have been unsuccessful in treating diseases such as IPF, suggesting that alternative mechanisms may be involved (11). Recently new hypotheses regarding the origin of pulmonary myofibroblasts have been raised (12). Bone marrow–derived circulating progenitor cells or “fibrocytes” home to the lungs, differentiate into fibroblasts, and contribute to extracellular matrix deposition (13, 14). In addition to circulating fibrocytes, our group demonstrated the potential for epithelial–mesenchymal transition (EMT) of alveolar epithelial cells (AEC) to contribute to the pool of pulmonary myofibroblasts (15). During EMT epithelial cells lose their cell–cell interactions and polarity, undergo cytoskeletal remodeling, and take on a mesenchymal phenotype (16). We demonstrated a causal role for transforming growth factor (TGF)- β 1 in alveolar EMT, but additional underlying mechanisms are yet to be elucidated.

In normal lung ET-1 is produced by endothelial cells, fibroblasts, alveolar macrophages, and bronchial epithelial cells (2). ET-1 is a potent modulator of the transdifferentiation of fibroblasts into myofibroblasts, and it increases the production of TGF- β 1 by lung fibroblasts (17). However, it is not known whether ET-1 is produced by AEC, or whether ET-1 is involved in alveolar EMT. The purpose of the current study was to delineate the specific role of ET-1 in alveolar EMT. Using primary rat AEC and an immortalized AEC line, we tested the hypotheses that ET-1 is produced by AEC, that ET-1 synthesis is induced by TGF- β 1, and that ET-1 causes EMT in AEC. Experiments were further designed to determine the specific receptor subtype that mediates ET-1–induced EMT and the downstream mechanisms underlying of ET-1–induced EMT. This work was

(Received in original form September 19, 2006 and in final form March 8, 2007)

This work was supported by NIH UO1 HL63399 (to P.W.S.), NIH RO1 HL38578 and HL62569 (to Z.B.), and NIH K12 HD047349 (Pediatric Critical Care Scientist Development Program) (to B.C.W.).

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Am J Respir Cell Mol Biol Vol 37, pp 38–47, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2006-0353OC on March 22, 2007

Internet address: www.atsjournals.org

presented in part at the 2006 American Thoracic Society International Conference (18).

MATERIALS AND METHODS

Materials

ET-1 (Phoenix Pharmaceuticals, Belmont, CA); endothelin type A receptor (ET-A) antagonist (BQ-123) and ET-B antagonist (BQ-788) (Calbiochem, San Diego, CA); recombinant human TGF- β 1, and monoclonal anti-TGF- β 1 antibody (anti-TGF- β 1) (R&D Systems, Minneapolis, MN) were obtained from commercial sources. The following antibodies were used for immunofluorescent staining and immunoblotting: mouse monoclonal anti-p180 (lamellar protein antibody) (Covance, Berkeley, CA); rabbit polyclonal anti-ET-1 (recognizing mature ET-1 only) (Calbiochem); rabbit polyclonal anti-pro-surfactant protein B (pro-SP-B) (Neomarkers, Fremont, CA); rabbit polyclonal anti-aquaporin 5 (AQP5) (Chemicon International, Temecula, CA); mouse monoclonal anti-von Willebrand factor (vWF), anti-ET-A, anti-ET-B, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA); mouse monoclonal anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1) (Novus Biologicals, Littleton, CO); mouse monoclonal anti-phospho-Smad3 and total Smad 2/3 (Cell Signaling, Danvers, MA); and mouse monoclonal anti- α -smooth muscle actin (α -SMA) (Sigma, St. Louis, MO).

Cell Culture

Primary rat alveolar type II cells (AT2) were isolated from adult male Sprague-Dawley rats by elastase disaggregation (2.0–2.5 U/ml) followed by differential adherence on IgG-coated bacteriological plates as previously described (19). All animals were treated in accordance with the guidelines of and with the approval of the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Cell purity was 85–90%. Analysis by immunofluorescence for vWF and PECAM-1 performed immediately after isolation revealed no endothelial cell contamination (data not shown). AT2 cells were then plated in a minimal defined serum-free medium (MDSF) (19) on 1.1 cm², 0.4 μ m pore size uncoated polycarbonate filter cups (Transwell, Corning Costar, Cambridge, MA) at a density of 1×10^6 cells/cm². For the first 24–48 h in culture, media were supplemented with 100 μ g/ml cis-OH-proline (Sigma) to selectively eliminate fibroblasts from cultures (20). The efficacy of this strategy to fully eliminate fibroblasts has been previously confirmed (15). Cultures were then maintained in a humidified 5% CO₂ incubator at 37°C for up to 6 d. Media were changed daily and supplemented with combinations of TGF- β 1 (1.0 ng/ml), ET-1 (150 nM), the ET-A receptor antagonist BQ-123 (10 μ M), or the ET-B receptor antagonist BQ-788 (10 μ M).

RLE-6TN cells derived from rat AT2 cells were obtained from American Type Culture Collection (Manassas, VA), and were used for all assessments of EMT to eliminate the possibility of contamination with mesenchymal cell elements. We have previously demonstrated that RLE-6TN cells exhibit an EMT response to TGF- β 1 that is comparable to that of primary cells but occurs over a shorter time frame (15). Cells were cultured in Dulbecco's Modified Eagle's media (DMEM), nutrient mixture F-12 Ham (Sigma) supplemented with 10% fetal bovine serum (FBS), 40 mmol/liter HEPES, 10 ml glutamine, and 100 μ g/ml Primocin antimicrobial agent (Invivogen, San Diego, CA). Cells were treated daily by supplementing media with combinations of TGF- β 1 (1.0 ng/ml), ET-1 (150 nM), BQ-123 (10 μ M), BQ-788 (10 μ M), or anti-TGF- β 1 mAb (4 μ g/ml) (21–23). Before use in studies on ET-1-induced EMT, the activity of the anti-TGF- β 1 antibody was demonstrated through effective attenuation of TGF- β 1-induced EMT in AEC, and 4 μ g/ml was noted to be the minimal effective concentration (data not shown). Nonspecific mouse IgG used as a control had no effect at similar concentrations. The concentration of 10 μ M used for the ET-1 receptor-specific antagonists was based on prior studies (24, 25), and there is minimal cross-reactivity between receptor subtypes at this concentration (25, 26). Primary bovine aortic endothelial cells (EC) were harvested as previously reported and maintained in endothelial growth medium-2 (Cambrex Bioscience, Baltimore, MD) supplemented with 5% FBS (27). Rat lung fibroblasts (RLF) (Cell Applications, Inc., San Diego, CA) were maintained in DMEM nutrient mixture F-12 Ham supplemented with 10% FBS, 40 mmol/liter HEPES, 10 ml

glutamine, and 100 μ g/ml streptomycin. All cultures were maintained in a humidified 5% CO₂ incubator at 37°C for up to 6 d.

ET-1 Enzyme-Linked Immunosorbent Assay

Precoated enzyme-linked immunosorbent assay (ELISA) plates (Immunobiological Laboratories, Minneapolis, MN) were incubated overnight at 4°C with 100 μ l of supernatant from primary AEC treated with and without TGF- β 1 for 2, 4, or 6 d. All supernatant samples were collected after 24 h of culture after a fresh media change. Before sample application, ELISA plates were washed with 0.05% Tween-20 in PBS, 100 μ l of labeled antibody was applied for 30 min, and the plates were washed again. Color was developed and measurements were obtained at 450 nm on a standard ELISA DXT-100 Beckman Coulter plate reader (Beckman Coulter, Fullerton, CA).

ET-1 Localization

Cytospins of primary AEC were immediately fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton-X100. Cell preparations were then blocked using a casein-based protein solution (CAS block; Zymed Laboratories, South San Francisco, CA) and incubated with monoclonal anti-lamellar protein Ab (p180; 1:1,000) in Tris-buffered saline with 0.1% Tween (TBS-T) at room temperature (RT) for 2 h. After extensive rinsing with TBS-T, cells were incubated with anti-mouse IgG conjugated to Alexa 488 (Molecular Probes, Invitrogen, Carlsbad, CA) diluted in CAS block for 1 h at RT. After further rinsing, cells were incubated with rabbit polyclonal anti-ET-1 antibody (1:500) overnight at 4°C. Cells were then rinsed once again with TBS-T and incubated with anti-rabbit IgG conjugated to Alexa 594 (Invitrogen) diluted in CAS block at RT for 1 h. Cells were rinsed and coverslipped with Prolong Gold Anti-Fade fluorescent mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Image stacks (consisting of a series of images at 0.1- μ m intervals over a total of 2–3 μ m thickness from single cells) were taken with a Deltavision RT deconvolution microscope (Applied Precision, Issaquah, WA). Image processing and deconvolution were then performed using SoftwoRx image acquisition and analysis software (Applied Precision).

RNA Isolation and Real-Time RT-PCR

Primary AEC monolayers incubated with and without TGF- β 1 were washed with PBS, and RNA was isolated by guanidium thiocyanate/phenol/chloroform extraction. Random priming was used to generate cDNAs from isolated RNA in reverse transcriptase reactions and real-time PCR was then performed (28). Specific primer sequences (sense/antisense) were designed as follows: ET-1: 5'-TGTTGGAGGGAA-GAAAATAAGAAG-3'/5'-ACTCGAAAGGAGGTCTTGATGCT-3'; ET-A: 5'-CCCTCAGCGAACACCTCAA-3'/5'-CCAAGCAGAAGACGGTCTTTG-3'; ET-B: 5'-GATTGCCTTGAATGACCACTTAA-A-3'/5'-AGACCAGGCAGAATACTGTCTTG-3'. A 20- μ l Sybr PCR reaction mixture was used containing 2 μ l of cDNA, 2 μ l of forward primer, 2 μ l of reverse primer, and 14 μ l of Sybr master mix (Applied Biosystems, Foster City, CA). PCR data analysis was performed using standard curves generated with serial dilutions of known quantities of control cDNA gene template. All results were normalized to the relative expression levels of 18S RNA.

Immunoblot Analysis

Cell cultures were washed with PBS and lysed in 2% SDS sample buffer on ice for 30 min. Cell lysates were heated to 100°C for 1 min and sonicated. Lysates were centrifuged at 13,000 $\times$ g for 15 min and supernatant protein content was quantified using a commercial protein quantification assay (Bio-Rad, Hercules, CA). Equal amounts of protein (10 μ g) were separated by 10% SDS-PAGE under reducing conditions and electrophoretically transferred to Immobilon-P nylon membranes (Millipore, Medford, MA). Membranes were blocked for 2 h with 5% nonfat dry milk in TBS with 0.1% Tween and probed for pro-SP-B, ET-A receptor, ET-B receptor, or α -SMA. Anti-GAPDH antibody was used as a loading control. Membranes were subsequently incubated with horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit secondary antibodies. Antibody complexes were visualized using Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) and an image processing station (UVP

Bioimaging Systems, Upland, CA) equipped with Labworks imaging software (UVP).

α -SMA Immunofluorescence Staining

RLE-6TN cells were cultured on 4-well BD Falcon chamber culture slides (BD Biosciences, San Jose, CA) for 6 d in media supplemented with combinations of ET-1, TGF- β 1, BQ-123, BQ-788, or anti-TGF- β 1 mAb. Cells were rinsed with TBS, fixed with 2% paraformaldehyde, and permeabilized with 0.2% Triton-X-100. Cells were blocked with CAS block (Zymed) and incubated with monoclonal anti- α -SMA mAb. After extensive washing with TBS and TBS with 0.1% Tween, cells were incubated with anti-mouse Alexa 488-conjugated IgG (Invitrogen) for 1 h at RT. Cells were washed, post-fixed with 3.7% formalin, and coverslipped with Vectashield fluorescent mounting medium with DAPI (Vector Laboratories, Burlingame, CA) to stain the nucleus. Immunofluorescence was visualized using a Zeiss Axioskop 40 microscope equipped with epifluorescent optics. Images were captured and processed using an AxioCam HRC color digital camera and Axiovision software (Zeiss, Gottingen, Germany).

TGF- β 1 ELISA

TGF- β 1 was measured on Day 6 in cell culture supernatant samples from primary AEC treated with and without ET-1. Conversion of total TGF- β 1 to active TGF- β 1 was performed by acidifying the samples with 1 N HCl followed by subsequent neutralization with 1.2 N NaOH/0.5 M HEPES. One hundred microliters of cell supernatant, standards, positive controls, or medium used as a negative control were incubated on a pre-coated ELISA plate along with 100 μ l of buffered protein solution for 2 h (Quantikine Mouse/Rat/Porcine/Canine TGF- β 1 Immunoassay; R&D Systems). Plates were washed four times and 100 μ l of TGF- β 1 conjugate was added. After incubating for 2 h, plates were washed and substrate and stop solution were added. Color absorbance

at 450 nm was measured using a DXT 100 Beckman/Coulter ELISA plate reader.

Statistical Analysis

Data shown are mean $\pm$ SEM. Experimental data were analyzed using an unpaired Student's *t* test for comparison of two groups and one-way ANOVA with Neuman-Keuls *post hoc* testing for comparisons between more than two groups. *P* values < 0.05 were considered to be significant.

RESULTS

Primary AEC Produce ET-1

ET-1 secretion into the apical and basolateral supernatant of primary AEC monolayers on Days 2, 4, and 6 of culture was measured by ELISA (Figure 1A). ET-1 was detectable in the apical and basolateral supernatant of AEC at all three time points, and amounts increased progressively from Day 2 to Day 6. The vast majority of AEC-derived ET-1 was secreted into the basolateral compartment at all time points, resulting in a significant concentration difference between the apical and basolateral compartments (50 ± 18 pg/ml and 166 ± 28 pg/ml, respectively; *P* < 0.01) at the time of sampling of the supernatant. The presence of ET-1 production in AEC was also confirmed using RT-PCR. Pre-pro-ET-1 mRNA was detected at both Day 2 and Day 6 of culture (results not shown).

Having demonstrated polar secretion of ET-1 to the basolateral surface of primary AEC, we wished to determine the intracellular location of ET-1 relative to a protein secreted to the apical surface. Such localization would provide additional

Figure 1. AEC produce ET-1 and express ET-1 receptors. (A) ELISA analysis of supernatant from primary rat AEC monolayers demonstrates ET-1 production per 24 h at Days 2, 4, and 6 of culture. ET-1 production increased over time and was directed preferentially to the basolateral surface. Values are mean $\pm$ SEM (*n* = 3–5; **P* < 0.05 versus apical). (B) Primary AEC stained for lamellar protein (p180, green, Alexa 488) and ET-1 (red, Alexa 594) show ET-1 in the cytoplasm of AEC but in a distinct location from p180. Blue staining represents DAPI-stained nuclei. Deconvoluted high-power images were derived from stacks of images taken at 0.1- μ m intervals over a total of 2–3 μ m thickness of single cells. The Pearson's coefficient of correlation between p180 and ET-1 was 0.228. (C) Immunoblots for ET-A demonstrated high levels of ET-A in AEC relative to RLE. Immunoblots for ET-B revealed much greater expression of ET-B in endothelial cells (EC) than AEC. Findings by immunoblot analysis were confirmed by three to five independent experiments.

evidence that AEC are a cellular source of ET-1. Immediately after isolation, cells were co-immunostained for mature ET-1 and lamellar body protein (p180) (Figure 1B). High-power deconvolution microscopy revealed that ET-1 is localized in the cytoplasm of primary AEC. Some cells also demonstrated nuclear staining for ET-1 (Figure 1B, *second row*). Image analysis indicated that the correlation between the subcellular location of ET-1 and p180 was low (Pearson's coefficient of correlation = 0.23), suggesting that ET-1 and p180 do not reside in association with a common cytoplasmic organelle.

We next evaluated the presence of ET-A and ET-B receptor subtypes in primary AEC at Day 6 (Figure 1C) using immunoblot analysis. ET-A receptor expression was greater in AEC than rat lung fibroblasts (RLF), cells known to express ET-A. ET-B receptor protein was minimally detected in primary AEC at levels far below those seen in endothelial cells (EC), cells known to express ET-B. RT-PCR also confirmed the presence of ET-A and ET-B receptor mRNA in primary AEC harvested at Days 2 and 6 of culture (data not shown). Together, these data demonstrate ET-1 production and polar basolateral secretion by primary AEC, the presence of ET-A, and to a lesser extent the presence of ET-B receptor subtypes in AEC.

TGF- β 1 Increases the Production of ET-1 by Primary AEC

To examine the relationship between ET-1 and TGF- β 1 in AEC, the effect of TGF- β 1 (1 ng/ml) on ET-1 production at Days 2,

4, and 6 was evaluated by ELISA (Figure 2A). TGF- β 1 increased total ET-1 production to nearly double that of control cells at Days 4 and 6. The effect of TGF- β 1 on polar ET-1 secretion was also evaluated (Figure 2B). On Days 4 and 6, TGF- β 1 abrogated the directional secretion of ET-1 to the basolateral surface that is observed in untreated AEC.

RT-PCR was used to evaluate the effect of TGF- β 1 on ET-1 mRNA abundance in primary AEC at Day 2 and day 6 (Figure 2C). Whereas TGF- β 1 increased ET-1 mRNA expression on Day 2, on Day 6 ET-1 mRNA expression was not changed. Finally, immunoblots were used to assess the effect of TGF- β 1 on ET-A and ET-B expression in primary AEC after 6 d (Figure 2D). TGF- β 1 did not change the relative expression of ET-A receptors in AEC. ET-B expression, however, increased dramatically with TGF- β 1 exposure. These data demonstrate that TGF- β 1 increases the production and secretion of ET-1 by AEC and the expression of one of the ET receptor subtypes.

ET-1 Induces EMT in AEC

Expression of the myofibroblast marker α -SMA, cell morphology, and the type II alveolar epithelial cell marker Pro-SpB were examined in RLE-6TN cells with and without exogenous ET-1 treatment to evaluate the impact of ET-1 on alveolar EMT. Using both immunoblot analysis and immunofluorescence staining, we found that ET-1 caused an increase in α -SMA expression (Figures 3A and 3B). Furthermore, BQ-123 prevented the

Figure 2. TGF- β 1 increases ET-1 production from AEC and attenuates its polar basolateral secretion. (A) Supernatant from AEC treated with and without TGF- β 1 showed increased total ET-1 production by ELISA at Days 4 and 6. (B) TGF- β 1 abrogated the basolateral directionality of the secretion of ET-1 by AEC. On Days 4 and 6 the apical:basolateral ratio of secretion increased from < 0.25 in control AEC to > 0.5 in TGF- β 1-treated AEC. (C) ET-1 mRNA abundance evaluated by RT-PCR increased at Day 2 in the presence of TGF- β 1 but was not elevated on Day 6. (D) Immunoblot evaluating ET-A receptor expression showed no difference between control versus TGF- β 1-treated AEC. Immunoblot evaluating ET-B receptor expression showed an increase in AEC ET-B with TGF- β 1. GAPDH was used as a loading control. Immunoblots shown are representative of four to five independent studies. Values are mean $\pm$ SEM. * P < 0.05 versus control. Lightly shaded bars, control; darkly shaded bars, TGF- β 1.

ET-1-induced increase in α -SMA expression, while BQ-788 had no effect. ET-1 also caused a change in cell shape from cuboidal to elongated and fibroblast-like, with prominent longitudinal stress-fibers, consistent with a myofibroblast-like phenotype. Co-treatment with BQ-123 preserved epithelial cell morphology, whereas co-treatment with BQ-788 did not. Finally, we found that ET-1 caused a dramatic decrease in the expression of the epithelial marker pro-SpB (Figure 3C). These results demonstrate that ET-1 induces EMT in AEC through ET-A.

ET-1 Increases TGF- β 1 Secretion and Induces Smad3 Phosphorylation in Primary AEC through ET-A

ET-1 and TGF- β 1 production are reciprocally regulated in pulmonary fibroblasts (29). Therefore, we next evaluated the effects of ET-1 on TGF- β 1 production by AEC (Figure 4A). ET-1 increased total TGF- β 1 production by over 100%. The increase in TGF- β 1 by ET-1 from < 40 pg/ml to 80–100 pg/ml yielded a concentration similar to the EC₅₀ of TGF- β 1 necessary to induce

reductions in transepithelial resistance in AEC (30). Co-administration of the ET-A receptor antagonist, BQ-123, completely abrogated the increase in TGF- β 1 secretion caused by ET-1. In contrast, the ET-B receptor antagonist BQ-788 had no effect on ET-1-induced TGF- β 1 secretion. Together, these findings indicate that ET-1 stimulates TGF- β 1 production from primary AEC through the ET-A receptor. To demonstrate that the TGF- β 1 produced by treatment with ET-1 induced an autocrine response, we also examined the effects of ET-1 on Smad3 phosphorylation. After treatment with ET-1 for 24 h, primary AEC demonstrated Smad3 phosphorylation that was abrogated by treatment with BQ-123, but not by BQ-788 (Figure 4B).

ET-1-Induced EMT Is Mediated by TGF- β 1

Given the increase in TGF- β 1 production induced in AEC by ET-1, we next evaluated the effect of a TGF- β 1-neutralizing antibody on ET-1-induced alveolar EMT in RLE-6TN cells. As observed by both immunoblot analysis and immunofluorescence

Figure 3. ET-1 induces EMT in AEC via ET-A. (A) Immunofluorescence analysis demonstrated that ET-1 increases α -SMA expression, and that the increase is inhibited by BQ-123, but not BQ-788. AEC treated with ET-1 also changed morphology from a typical rounded shape to an elongated fibroblast-like morphology (phase images, right panels) with prominent intracellular stress fibers (fluorescent images, middle panels). BQ-123 prevented this morphologic change, while BQ-788 did not. For all immunofluorescence images, green staining represents α -SMA and blue staining represents nuclei stained with DAPI. (B) Immunoblot analysis similarly revealed increased α -SMA expression with ET-1 (150 nM) treatment relative to control. BQ-123, an ET-A antagonist, completely abrogated this effect. BQ-788, an ET-B antagonist, had no effect. For immunoblot analysis, one representative blot is shown. Densitometric analysis (right panel) summarizes four separate experiments. $*P < 0.05$ compared with control and ET-1 + BQ-123. (C) Immunoblot analysis of the epithelial marker pro-SP-B revealed a dramatic decrease in expression with ET-1 treatment relative to control. BQ-123 prevented this decrease, while BQ-788 did not. Densitometric analysis (right panel) represents three separate experiments. $*P < 0.05$ compared with control and ET-1 + BQ-123.

Figure 3. (Continued).

staining, ET-1 increased α -SMA expression, and the addition of an anti-TGF- β 1 antibody (4 μ g/ml) attenuated ET-1-induced α -SMA expression (Figure 5). We also examined the effect of ET-A antagonism on alveolar EMT induced by TGF- β 1. Neither ET-A nor ET-B antagonism (with BQ-123 and BQ-788, respectively) had any effect on TGF- β 1-induced EMT (Figure 6). In contrast, addition of the anti-TGF- β 1 antibody (4 μ g/ml) in control experiments completely abrogated TGF- β 1-induced EMT (data not shown). These results demonstrate that TGF- β 1 is the downstream effector of ET-1 causing EMT in AEC.

DISCUSSION

ET-1 plays a key pathogenetic role in pulmonary fibrosis and in a variety of other pulmonary diseases including pulmonary hypertension, asthma, acute respiratory distress syndrome, and lung allograft rejection (2). In the present investigation we demonstrate that primary AEC produce physiologically relevant quantities of ET-1. Our findings are consistent with the previous observation that a transgene regulated by the ET-1 promoter in mice was highly expressed not only in endothelial cells, but also in type II pneumocytes (31). ET-1 mRNA expression and protein secretion were also previously demonstrated in a transformed AEC line (L2), but our current studies are the first to show ET-1 production by primary AEC (32).

Our results also demonstrate that AEC secrete ET-1 predominantly toward the basolateral cell surface. Such polar secretion occurs in endothelial cells (33), and is likely critical to the role of ET-1 as a paracrine/autocrine mediator. We found that AEC produce ET-1 at levels comparable to those of endothelial cells (34, 35). In addition, AEC-associated ET-1 was detected predominantly in the cytoplasm by immunocytofluorescence, and

some ET-1 immunoreactivity was also detected in the nucleus. This is consistent with previous findings of ET-1 signaling in the nucleus of oligodendrogloma cells and endocardial endothelial cells (26, 36). Given the intimate relationship of AEC to other ET-1-responsive pulmonary cell types including endothelial cells, fibroblasts, and smooth muscle cells, AEC-derived ET-1 may play a critical role in a host of physiologic and pathophysiologic pulmonary processes. For example, directional alveolar epithelial ET-1 secretion basolaterally may serve as an important component of pulmonary responses to hypoxia, with AEC serving as sensors and effectors (through ET-1 secretion) of local alveolar hypoxic responses. The extent to which AEC-derived ET-1 contributes to these processes is yet to be determined.

The mechanisms of ET-1 processing within AEC are not known at this time. We demonstrate in this report the secretion and intracellular localization of mature ET-1. Conversion of big-ET-1 into its mature form occurs either at the cell surface or at an intracellular location. Multiple isoforms of endothelin-converting enzyme-1 (ECE-1) are known to exist. Isoform 1a is primarily associated with the cell surface, while 1b and 1c are intracellular enzymes. No investigation of the relative expression of these enzymes or the mechanisms of processing of ET-1 in AEC has been performed to this point. However, A549 cells, a transformed cancer pulmonary epithelial cell line, express predominantly ECE-1b at an intracellular location (37, 38), produce mature ET-1 within the cytoplasm, and subsequently release it (39). In the only study of its kind, immunohistochemical analysis revealed ECE-1 within AEC in both control and fibrotic lungs from bleomycin-exposed animals, but the specific isoforms were not identified (9). Clearly, further investigation of these mechanisms in AEC is needed.

Figure 4. ET-1 increases total TGF- β 1 production from primary AEC and induces Smad3 phosphorylation through ET-A. (A) ELISA was used to quantify total TGF- β 1 in the supernatant of AEC treated with and without ET-1. ET-1 increased TGF- β 1 production by AEC. BQ-123, an ET-A receptor antagonist, prevented the ET-1-induced increase in TGF- β 1 production. BQ-788, an ET-B receptor antagonist, had no effect on TGF- β 1 secretion. (B) Immunoblot analysis demonstrated an increase in phosphorylated Smad3 (p-Smad3) by ET-1 after 24 h of treatment. BQ-123 abrogated this effect, and BQ-788 had no effect. Bar graphs represent mean $\pm$ SEM ($n = 4$). * $P < 0.05$ versus control (no treatment) and ET-1 + BQ-123.

Our demonstration of ET-A and ET-B receptor expression in AEC suggests that AEC-derived ET-1 may have important autocrine functions. ET-A receptors are expressed predominantly in vascular and airway smooth muscle cells, while ET-B receptors are found primarily on endothelial cells (2). Previous results evaluating ET-A receptor and ET-B receptor expression in AEC have been variable. Wendel and colleagues were unable to detect ET-A or ET-B mRNA expression in type I or type II AEC in rat lung after bleomycin injury (40). In contrast, ET-A receptor expression has been demonstrated in an immortalized AEC line (32) and in the alveolar epithelium of normal and scleroderma lung tissue by immunohistochemistry (41). Our results further demonstrate that in primary AEC ET-A receptors are expressed at relatively higher levels than ET-B. This is in contrast to the findings of Abraham and coworkers, who identified both ET-A and ET-B in the alveolar epithelium in both normal and scleroderma lung tissue, with a relative predominance of the ET-B receptor subtype (41). We also observed an increase in ET-B receptor expression with TGF- β 1 treatment

and this may reflect the transformation of AEC into myofibroblasts, as myofibroblasts are known to express ET-B (42), or it may represent a novel response of the cell to TGF- β 1 stimulation. Further investigation is needed to elucidate the importance of this finding. However, the presence of ET-A and ET-B receptors in primary AEC suggests the existence of important autocrine ET-1-mediated signaling pathways in AEC.

Further work will also be required to define the significance of the abrogation of the polar secretion of ET-1 by TGF- β 1 (Figure 2B). Loss of polarity is a manifestation of EMT (43), and as such the loss of polar secretion may simply reflect the complete loss of polarity of the cell. Alternatively, it may reflect the fact that AEC monolayers, when exposed to TGF- β 1 continue to secrete ET-1 basolaterally but become much more permeable (30), and so secreted ET-1 is better able to equilibrate between the apical and basolateral compartments.

Having demonstrated that AEC synthesize ET-1 and express ET-1 receptors, and recognizing that ET-1 has been extensively implicated as a mediator of lung injury and pulmonary fibrosis (2, 8), we investigated the role of ET-1 in alveolar EMT. We show that ET-1 induces alveolar EMT through the ET-A receptor. Although it has previously been shown that ET-1 induces EMT through the ET-A receptor in an ovarian carcinoma-derived cell line (44), our findings are the first to implicate ET-1 in EMT in AEC. Considering the key role of TGF- β 1 in alveolar EMT, we also examined the relationship between ET-1 and TGF- β 1 in AEC. We demonstrate that ET-1 production is dramatically increased by TGF- β 1 in AEC. Previously, it was shown that TGF- β 1 increases ET-1 mRNA expression in aortic endothelial cells (45). TGF- β 1 also increases ET-1 production in pulmonary artery smooth muscle cells (46) and pulmonary fibroblasts (17). In contrast, TGF- β 1 has had variable effects on ET-1 production by cardiac myocytes, liver presinusoidal stellate cells, and cirrhotic hepatocytes (24, 47).

We further demonstrate that TGF- β 1 production is reciprocally increased by ET-1 in AEC. The reciprocal stimulation of ET-1 by TGF- β 1 and TGF- β 1 by ET-1 during fibrosis has been suggested previously in studies of dermal and bronchial fibroblasts (29, 48). Our data also indicate that the induction of alveolar EMT by ET-1 is mediated by TGF- β 1, since ET-1-induced EMT was abrogated by a TGF- β 1-neutralizing antibody, ET-1 treatment induced Smad3 phosphorylation in primary AEC, and ET-1 receptor antagonism had no effect on the induction of alveolar EMT by exogenous TGF- β 1. This suggests that the ET-1-induced increase in AEC-derived TGF- β 1 may be a critical factor in the initiation of alveolar EMT by ET-1. In fact, AEC-derived TGF- β 1 is the predominant source of TGF- β 1 in the alveolus in IPF (49, 50), and TGF- β 1 stimulates the autoinduction of more TGF- β 1 production (51). Further, inflammation does not play a major role in the propagation of fibrosis and production of TGF- β 1 in diseases like IPF (12, 52, 53). Ongoing reciprocally up-regulated, self-perpetuating production of local fibrotic cytokines such as ET-1 and TGF- β 1 by AEC in response to injury may explain the chronic, unremitting, and self-perpetuating nature of epithelial injury and propagation of fibrosis seen in diseases like IPF. Importantly, EMT was recently implicated as the primary source of the majority of pulmonary myofibroblasts during pulmonary fibrosis *in vivo* (54). The demonstration of EMT as one of the predominant sources of fibroblasts is not unique to lung. Iwano and colleagues (55) demonstrated that $\sim 30\%$ of fibroblasts come from EMT after injury in kidney, and EMT of mature epithelia after injury has been demonstrated in multiple tissues, including lens and liver (56, 57). Although the mechanisms we now report are yet to be evaluated *in vivo*, our findings represent an important first step

Figure 5. The induction of EMT by ET-1 is mediated by TGF-β1. (A) Immunoblot analysis of AEC for α-SMA expression revealed ET-1 increased α-SMA expression relative to control. However, the addition of an anti-TGF-β1 monoclonal antibody (anti-TGF-β1 mAb) dramatically attenuated the effect of ET-1. GAPDH was used as a loading control. Immunoblots shown are representative of three independent studies. Densitometric values are mean ± SEM. * $P < 0.05$ compared with control and ET-1 + Anti-TGF-β1 mAb. (B) Immunofluorescence analysis similarly demonstrated that ET-1 increases α-SMA expression, and that the increase is inhibited by anti-TGF-β1 mAb. Green staining represents α-SMA, while blue staining represents nuclei stained with DAPI.

Figure 6. ET-1 receptors do not mediate alveolar EMT induced by exogenous TGF-β1. (A) Immunoblot analysis of AEC for α-SMA expression revealed that TGF-β1 (1 ng/ml) increased α-SMA expression dramatically relative to control. The addition of an ET-A (BQ-123) or ET-B (BQ-788) receptor antagonist had no effect on the increase in α-SMA expression. GAPDH was used as a loading control. Immunoblots shown are representative of five independent studies. Densitometric values are mean ± SEM. * $P < 0.05$ compared with control. (B) Immunofluorescence analysis similarly demonstrated that ET-1 receptor antagonism did not affect the TGF-β1-induced increase in α-SMA expression, or the change in cell morphology to a fibroblast-like shape. Green staining represents α-SMA, while blue staining represents nuclei stained with DAPI.

in understanding the combined roles of ET-1 and TGF- β 1 in EMT and pulmonary fibrosis.

The intracellular signaling mechanisms underlying ET-1- and TGF- β 1-induced alveolar EMT are yet to be elucidated. RhoA is a key intermediary in TGF- β 1-induced mammary and renal EMT, as a loss of TGF- β 1-induced EMT occurs with overexpression of dominant negative RhoA mutants (58). ET-1-induced intracellular events are also commonly Rho-dependent (59, 60). Thus, ET-1 and TGF- β 1 may converge on a common intracellular signaling pathway to induce EMT.

In summary, we demonstrate that ET-1 is produced by primary AEC at physiologically relevant levels, which may have important implications for the pathophysiology of a wide range of diseases of the lung. We also show that ET-1 induces alveolar EMT through ET-A via stimulation of endogenous TGF- β 1 production. These findings, which increase our basic understanding of the role of ET-1 in pulmonary fibrosis, may provide an important new focus of investigation for the development of future treatment strategies for fibrotic pulmonary diseases.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Katherine Luby-Phelps and Abhijit Bugde of the Live Cell Imaging Core at UT Southwestern for technical assistance with imaging.

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