Prostaglandin E₂ As an Inhibitory Modulator of Fibrogenesis in Human Lung Allografts

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Rationale: Donor mesenchymal stromal/stem cell (MSC) expansion and fibrotic differentiation is associated with development of bronchiolitis obliterans syndrome (BOS) in human lung allografts. However, the regulators of fibrotic differentiation of these resident mesenchymal cells are not well understood.

Objectives: This study examines the role of endogenous and exogenous prostaglandin (PG)E₂ as a modulator of fibrotic differentiation of human lung allograft-derived MSCs.

Methods: Effect of PGE₂ on proliferation, collagen secretion, and α -smooth muscle actin (α -SMA) expression was assessed in lung-resident MSCs (LR-MSCs) derived from patients with and without BOS. The response pathway involved was elucidated by use of specific agonists and antagonists.

Measurement and Main Results: PGE₂ treatment of LR-MSCs derived from normal lung allografts significantly inhibited their proliferation, collagen secretion, and α -SMA expression. On the basis of pharmacologic and small-interfering RNA approaches, a PGE₂/EP2/prostanoid (EP)2/adenylate cyclase pathway was implicated in these suppressive effects. Stimulation of endogenous PGE₂ secretion by IL-1 β was associated with amelioration of their myofibroblast differentiation *in vitro*, whereas its inhibition by indomethacin augmented α -SMA expression. LR-MSCs from patients with BOS secreted significantly less PGE₂ than non-BOS LR-MSCs. Furthermore, BOS LR-MSCs were found to be defective in their ability to induce cyclooxygenase-2, and therefore unable to up-regulate PGE₂ synthesis in response to IL-1 β . BOS LR-MSCs also demonstrated resistance to the inhibitory actions of PGE₂ in association with a reduction in the EP2/EP1 ratio. **Conclusions:** These data identify the PGE₂ axis as an important autocrine-paracrine brake on fibrotic differentiation of LR-MSCs, a failure of which is associated with BOS.

Keywords: mesenchymal stem cell; bronchiolitis obliterans syndrome; lung transplant; prostaglandin E₂

The major cause of long-term mortality in lung transplant recipients, bronchiolitis obliterans (BO), is a graft response marked by fibrosis and obliteration of the terminal airways (1). The resulting obstructive ventilatory defect is termed "bronchiolitis

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Fibrotic obliteration of small airways by aberrant mesenchymal cell proliferation and differentiation leads to chronic allograft failure after lung transplantation. However, factors regulating mesenchymal cell phenotype in human lung allografts are poorly understood.

What This Study Adds to the Field

These data identify prostaglandin E₂ as an inhibitory modulator of fibrotic differentiation of mesenchymal progenitors residing in human lung allografts and establish loss of this autocrine brake in chronic allograft rejection in human lung transplants.

obliterans syndrome" (BOS). BO shares many common features with other fibroproliferative diseases including development of a profibrotic milieu and accumulation of myofibroblasts, a differentiated mesenchymal cell with an increased collagen secretory ability and α -smooth muscle actin (α -SMA) expression. However, the pathogenesis of this fibrotic remodeling and the regulators of fibroproliferative pathways in BOS are not well understood.

Adult mesenchymal progenitor cells have the potential to regulate the local microenvironment by their paracrine actions or transdifferentiation to other mesenchymal lineages (2). We have recently demonstrated the presence of multipotent donor-derived mesenchymal stem/stromal cells (MSC) in human lung allografts (3). Similar to other adult MSCs (4), allograft-derived lung-resident MSCs demonstrate an ability to inhibit T-cell proliferation *in vitro* (5). However, MSCs can also participate in fibrotic responses (6, 7) and several recent findings suggest an important pathogenic role for lung-resident MSCs (LR-MSCs) in fibroproliferative responses marking allograft remodeling. First, an increase in the number of LR-MSCs precedes BOS onset (8). Second, LR-MSCs can differentiate to myofibroblasts and LR-MSCs isolated from patients with BOS demonstrate an altered profibrotic phenotype (9). Third, like LR-MSCs, myofibroblasts in BO lesions demonstrate a local lung rather than recipient origin (9). However, the mechanism of altered LR-MSC phenotype in BO and the mediators driving the ultimate fate of these cells remains to be determined.

Prostaglandins (PGs) are lipid mediators formed by the sequential actions of cyclooxygenase (COX) and specific PG synthases on arachidonic acid. MSCs constitutively produce PGE₂ and PGE₂ production by MSCs can be potentiated by induction of the COX-2 enzyme in response to various mediators (4, 10). PGE₂ is a well-established inhibitor of T-cell proliferation (11, 12) and recent studies have demonstrated that PGE₂ secreted by MSCs is important in mediating their T-cell suppressive ability (4, 5, 13, 14). It is also pertinent to note that PGE₂ is a well-characterized down-regulator of fibrogenesis.

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The relevant *in vitro* actions of PGs include inhibition of fibroblast migration (15), proliferation (16, 17), collagen synthesis (18), and myofibroblast differentiation (19). In animal models, PGE₂ has been demonstrated to protect against inflammation-induced fibrotic remodeling of the airway and parenchyma (20, 21). However, the modulation of LR-MSC fibroproliferative responses by PGE₂ and the role of this lipid mediator in the pathogenesis of BOS have not been evaluated.

Here we study LR-MSCs derived directly from lung allografts with and without BOS and demonstrate that PGE₂ is an important autocrine–paracrine brake on fibrotic differentiation of LR-MSCs. Importantly, dysregulation of PGE₂ synthesis and signaling is shown to be associated with BOS. Some of the results of these studies have been previously reported in the form of an abstract (22).

METHODS

Isolation and Culture of LR-MSCs

The MSCs were derived from the bronchoalveolar lavage (BAL) fluid of lung transplant recipients by plastic adherence and subsequently characterized by cell surface analysis and differentiation under a protocol approved by the University of Michigan Institutional Review Board, as previously described (3, 5, 8, 23). LR-MSCs obtained from individual BAL samples were treated as separate cell lines and cells were studied at passages two through six. Patients were diagnosed with BOS according to the International Society of Heart and Lung Transplantation guidelines (24) on the basis of a persistent decrease in the FEV₁ by 20% or more of the peak predicted value after transplantation in the absence of confounding variables, as previously described (25, 26). Patients with pathologic findings of acute rejection based on transbronchial biopsy or evidence of infection on microbiologic cultures at the time of BAL were excluded from the study. No significant difference was noted in time post-transplant ($P = 0.20$ between BOS and non-BOS subjects included in the study).

Reagents and Conditions

For proliferation assays and protein analysis, the following modulation concentrations were used: 1- μ M PGE₂; 1- μ M butaprost (E prostanoid [EP]₂ receptor agonist); 4- μ M EP₁ receptor agonist (ONO-004); 1- μ M EP₃ receptor agonist (ONO-248); 1- μ M EP₄ receptor agonist (ONO-329); 50- μ M adenylate cyclase agonist (forskolin); 50- μ M nonselective phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine [IBMX]); and 100- μ M adenylyl cyclase inhibitor (SQ22536). PGE₂ and butaprost were purchased from Cayman Chemical (Ann Arbor, MI). SQ22536 was purchased from Biomol/Enzo Life Sciences (Farmingdale, NY). ONO-004, ONO-AE3-248, and ONO-AE1-329 were provided as a generous gift by Ono Pharmaceuticals (Osaka, Japan). The rest of the reagents were obtained from Sigma (St. Louis, MO). Myofibroblast differentiation was induced by transforming growth factor (TGF)- β at 2 ng/ml and IL-13 at 10 ng/ml, both from R&D Systems (Minneapolis, MN).

Western Blotting

Protein lysates were collected and Western blot analysis for α -SMA and collagen I performed using 1:1,000 dilution of monoclonal α -SMA antibody (Dako, Carpinteria, CA) or 1:500 dilution of rabbit polyclonal to Collagen I (Cedarlane, Ontario, Canada) as previously described (27, 28).

Proliferation Assays

The 1×10^4 LR-MSCs were plated per well in 96-well flat-bottom culture-treated plates, serum starved overnight, and then subjected to various treatment conditions. Twenty-four hours post-treatment, [³H]thymidine (1 μ Ci per well) was added to wells and allowed to incubate for 20 hours before harvesting. [³H]thymidine incorporation was expressed as the mean of six replicates in counts per minute.

Determination of PGE₂ Synthesis

LR-MSCs were cultured in the absence or presence of IL-1 β (10 ng/ml) for 24 hours and PGE₂ quantified in culture supernatants using a highly sensitive and specific enzyme immunoassay kit from Cayman Chemicals.

Figure 1. Prostaglandin (PG)E₂ inhibits proliferation and profibrotic differentiation of lung-resident mesenchymal stromal cells (LR-MSCs) isolated from normal human lung transplant recipients. LR-MSCs derived from lung transplant recipients without evidence of acute rejection, infection, or bronchiolitis obliterans syndrome were studied for their response to exogenous PGE₂ in the presence and absence of profibrotic stimuli. (A) Proliferation of LR-MSCs in the presence of PGE₂ (1 μ M) assessed by [³H]thymidine incorporation. Data are presented as percent of untreated control. $n = 10$. (B) Collagen I protein expression in LR-MSCs treated with transforming growth factor (TGF)- β 1 (2 ng/ml) in the presence and absence of 1- μ M PGE₂ was measured by Western blot analysis. Densitometric analysis is presented as percent of untreated control. $n = 10$. (C) α -Smooth muscle actin (α -SMA) protein expression in LR-MSCs treated with TGF- β 1 (2 ng/ml) in the presence and absence of 1- μ M PGE₂ is shown. Densitometric analysis is presented as percent of untreated control. $n = 10$. (D) α -SMA protein expression in LR-MSCs treated with IL-13 (10 ng/ml) in the presence and absence of 1- μ M PGE₂ is shown. Densitometric analysis is presented as percent of untreated control. $n = 5$, *** $P < 0.001$, ** $P < 0.01$.

Real-Time Polymerase Chain Reaction Analysis

Total LR-MSC mRNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA). Primers and probes for each prostanoid receptor were designed and then purchased from Applied Biosystems (Foster City, CA), and sequences are as follows: EP1 forward: 5'-TGG GCC AGC TTG TCG G-3', EP1 reverse: 5'-CTG CAG GGA GGT AGA GC-3', EP1 probe: 5'-6FAM-TCA TGG TGG TGT CGT GCA TCT GCT GGA-TAMRA-3', EP2 forward: 5'-GGT GCT CGC CTG CAA CTT C-3', EP2 reverse: 5'-TCC GCA GCG GCT TCT C-3', EP2 probe: 5'-6FAM-TCC GCA TGC ACC GCC GAA TAMRA-3', EP3 forward: 5'-CCA CCT CTA CTG TGA TTG ATC C-3', EP3 reverse: 5'-CTC AGC CCT TCC GTT GGT T-3', EP3 probe: 5'-

6FAM-TGG ATT TGT CCT TTC CCG CCA TGT C-TAMRA-3', EP4 forward: 5'-CAT GTA CGC GGG CTT CAG-3', EP4 reverse: 5'-GCG AGG TGC GGC GCA TGA AC-3', EP4 probe: 5'-6FAM-TCG CCA CCG TCC TCT GCA ACG T-TAMRA-3'. All polymerase chain reactions (PCR) were performed on an Applied Biosystems ABI Prism 7000 Thermocycler (Applied Biosystems).

Small-interfering RNA

Small-interfering RNA (siRNA) targeting EP2 receptor and nontargeting scrambled siRNA were purchased from Applied Biosystems. LR-MSCs (60% confluence) were transiently transfected with siRNA using Oligofectamine and OPTI-MEM medium (Invitrogen, Carlsbad, CA).

Figure 2. Prostaglandin (PG) E₂ elicits its antifibrotic effects on lung-resident mesenchymal stromal cells (LR-MSCs) by E prostanoid (EP) receptor 2 cAMP-mediated signaling. (A) Real-time quantitative PCR of EP receptors in LR-MSCs derived from normal lung allografts demonstrating a significant up-regulation of EP2 compared with other EP receptors. Data represent the mean $\pm$ SEM of 10 separate cell lines. (B) Effect of selective EP receptor agonists on proliferation of LR-MSCs. Proliferation was measured by [³H] thymidine incorporation. Only butaprost, a selective EP2 agonist, demonstrated an ability to inhibit LR-MSC proliferation, similar to that seen in the presence of PGE₂. Values are represented as percent of untreated control, with data representing the mean $\pm$ SEM of five separate experiments. (C) Effect of selective EP receptor agonists on myofibroblast differentiation of LR-MSC. α -Smooth muscle actin (α -SMA) protein expression analysis by Western blot in LR-MSCs treated with transforming growth factor (TGF)- β 1 (2 ng/ml) in the presence and absence of selective EP receptor agonists is demonstrated. n = 5. (D) Effect of EP2 small-interfering RNA (siRNA) on fibrotic differentiation of LR-MSCs. LR-MSCs were transfected with EP2 siRNA oligonucleotides for 24 hours and then treated with TGF- β 1 (2 ng/ml) in the presence and absence of PGE₂. n = 5. (E and F) Elevations in cAMP inhibit LR-MSC proliferation and α -SMA expression. Effect of cAMP up-regulation

by direct treatment with direct adenylate cyclase activator forskolin (50 μ M) and phosphodiesterase inhibitor IBMX (50 μ M) on LR-MSC proliferation (E) and α -SMA expression (F) is shown. Treatment with adenylate cyclase inhibitor SQ22536 (100 μ M) was associated with abrogation of PGE₂ antiproliferative effects, demonstrating cAMP as the second messenger in this signaling pathway. n = 5, * P = 0.02, ** P < 0.01, *** P < 0.0001.

Cells were incubated with the transfecting media for 24 hours. Transfecting media were replaced with serum-free media for 48 hours before cells were treated or collected for experiments. EP2 receptor mRNA expression levels were tested to confirm silencing efficiency. Real-time PCR demonstrated a $56.5 \pm 18.22\%$ reduction in EP2 receptor mRNA expression level after silencing.

Statistical Analysis

Data are presented as mean values $\pm$ SEM. Statistical significance was analyzed using GraphPad Prism 3 software (La Jolla, CA). Significance was assessed with a Student *t* test for comparisons of two groups, or with analysis of variance and a *post hoc* Neuman-Keuls test for three or more groups. *P* less than 0.05 was considered significant.

RESULTS

LR-MSc Proliferation and Myofibroblast Differentiation Are Inhibited by Exogenous PGE₂

We have previously demonstrated that human lung allograft-derived MSCs can differentiate to myofibroblasts *in vitro* in response to profibrotic mediators TGF- β and IL-13 (9). To determine if PGE₂ can modulate the profibrotic activation of human lung allograft-derived MSCs, the effect of exogenous PGE₂ on proliferation and myofibroblast differentiation of LR-MSCs isolated from normal lung allografts was investigated.

LR-MSCs isolated from lung allografts without evidence of acute rejection, infection, or BOS (*n* = 10) treated overnight with PGE₂ demonstrated an approximately 70% inhibition of proliferation as measured by [³H]thymidine incorporation (Figure 1A). To examine if PGE₂ could also inhibit the transition of LR-MSCs to myofibroblasts, LR-MSCs were incubated with TGF- β 1 $\pm$ PGE₂ for 24 hours, and their α -SMA and collagen I protein expression measured by Western blot analysis. Significantly lower collagen I and α -SMA protein expression was also noted in TGF- β 1-stimulated LR-MSCs in the presence of PGE₂ (Figures 1B and 1C). PGE₂ also led to a decrease in myofibroblast differentiation induced by IL-13 (Figure 1D). All experiments shown were performed using a 1- μ M dose of PGE₂. This dose of PGE₂ was chosen based on previous literature (27, 29) and preliminary experiments demonstrated a dose-dependent effect of PGE₂ in the range of 100 nM to 10 μ M.

Inhibition of Profibrotic Properties of LR-MSCs by PGE₂ Is Mediated by EP2 Receptor Ligation and cAMP Signaling

The cellular actions of PGE₂ are transduced on its ligation of one of four 7-transmembrane G-protein-coupled EP receptors, designated EP1–4 (30). Examination of the relative expression of EP receptors by real-time PCR demonstrated a relative abundance of the G α_s -coupled receptor EP2, suggesting that the inhibitory effect of PGE₂ was likely mediated by this receptor (Figure 2A). To investigate this further, the effect of selective EP receptor agonists on LR-MSc proliferation and differentiation was assessed. As shown in Figure 2B, the EP2 receptor-selective agonist butaprost was able to suppress LR-MSc proliferation in a manner similar to PGE₂, whereas agonists selective for EP1, 3, and 4 could not (EP4 data are not shown because of the little to no EP4 receptor expression on LR-MSCs). Butaprost also uniquely demonstrated inhibition of TGF- β -induced α -SMA expression in a similar manner to that of PGE₂ (Figure 2C).

To further establish that EP2 is responsible for PGE₂-induced inhibition of LR-MSc myofibroblast differentiation, we used gene-specific knockdown by siRNA. Transfection of the siRNA targeting EP2 blocked the ability of PGE₂ to inhibit TGF- β -induced α -SMA expression (Figure 2D), thus verifying that the inhibitory effects of PGE₂ on LR-MSCs are mediated by EP2.

The EP2 receptor couples to G_s, which stimulates adenylate cyclase, resulting in increased cAMP. To validate that cAMP signaling is capable of mediating antifibrotic actions, LR-MSCs were treated with the direct adenylate cyclase activator forskolin in the presence of the phosphodiesterase inhibitor IBMX (to prevent cAMP degradation). Forskolin suppressed both proliferation (Figure 2E) and α -SMA expression (Figure 2F) of LR-MSCs in a similar manner to PGE₂. To further pinpoint cAMP as the second messenger eliciting PGE₂ antiproliferative effects, LR-MSCs were treated with PGE₂ in the presence of SQ22536, an adenylate cyclase inhibitor. Inhibition of adenylate cyclase with SQ22536 was able to abrogate PGE₂ effects on LR-MSc proliferation (Figure 2E). These data suggest that EP2 receptor cAMP-mediated signaling is the likely mechanism by which PGE₂ elicits antifibrotic effects in LR-MSCs.

Figure 3. Endogenous prostaglandin (PG)E₂ is important for maintaining an antifibrotic phenotype of lung-resident mesenchymal stromal cells (LR-MSCs). (A) α -Smooth muscle actin (α -SMA) expression in LR-MSc-treated indomethacin (10 μ M) was studied by Western blot analysis. Blocking endogenous PGE₂ production by this nonselective cyclooxygenase (COX) inhibitor promoted up-regulation of baseline α -SMA expression. Comparison of this myofibroblast differentiation with that induced by profibrotic mediator, transforming growth factor (TGF)- β 1 (2 ng/ml), is shown. *n* = 8, **P* < 0.05. (B) Effect of endogenous PGE₂ synthesis on the ability of LR-MSCs to undergo myofibroblast differentiation in response to TGF- β was further investigated by pretreating cells with IL-1 β and indomethacin. Presence of COX-2 inducer, IL-1 β (10 ng/ml), prevented TGF- β -induced α -SMA expression. This beneficial effect of IL-1 β was abrogated in the presence of the nonspecific COX inhibitor indomethacin (*n* = 10). ***P* < 0.01, ****P* < 0.0001.

Figure 4. Dysregulation of lung-resident mesenchymal stromal cells (LR-MSCs) prostaglandin (PG)E₂ synthetic function in bronchiolitis obliterans syndrome (BOS). (A) Quantitation of PGE₂ synthetic capacity of LR-MSCs from patients $\pm$ BOS by ELISA. LR-MSCs isolated from bronchoalveolar (BAL) fluid of patients with BOS demonstrated lower baseline PGE₂ secretion (103.3 ± 24.3 pg/ml) compared with non-BOS LR-MSCs (486.7 ± 151 pg/ml) ($P = 0.03$). PGE₂ secretion in response to IL-1 β BOS LR-MSCs was also significantly impaired compared with BOS-free control LR-MSCs (540.3 ± 154.5 pg/ml vs. $11,930 \pm 3,738$ pg/ml; $P < 0.01$). $n = 6$ for each group, $*P < 0.05$. (B) Cyclooxygenase (COX)-2 protein expression by Western blot analyses exhibiting diminished COX-2 up-regulation in BOS LR-MSCs compared with non-BOS LR-MSCs. Treatment with IL-1 β (10 ng/ml) induced an 11.66 ± 4.75 -fold expression of COX-2 in normal non-BOS LR-MSCs ($P = 0.004$). In contrast, no significant induction was noted in BOS LR-MSCs in response to IL-1 β ($P = 0.49$). $n = 5$ for each group, $**P < 0.01$.

Endogenous PGE₂ Production and Signaling by EP2 Receptor Limits Profibrotic Differentiation of LR-MSCs

We have previously demonstrated that LR-MSCs secrete PGE₂ (5). Interestingly, an increase in baseline α -SMA was seen in LR-MSCs transfected with EP2 siRNA (Figure 2D), suggesting that endogenous PGE₂ generated by LR-MSC stimulation acts in an autocrine manner to limit myofibroblast differentiation. Blocking endogenous PGE₂ production by treatment with indomethacin, a nonselective COX inhibitor, was also found to increase basal α -SMA protein expression (Figure 3A), reiterating that endogenous PGE₂ production is important in maintaining the undifferentiated state of these cells. We have previously demonstrated that PGE₂ secretory ability of LR-MSCs is up-regulated in response to IL-1 β by induction of COX-2 (5). A significant decrease in TGF- β -induced α -SMA expression was

noted in the presence of IL-1 β (Figure 3B). This effect of IL-1 β was reversed in the presence of the indomethacin demonstrating that up-regulation of endogenous PGE₂ generation in LR-MSCs acts as a brake on myofibroblast differentiation induced by profibrotic signals.

LR-MSCs from Patients with BOS Demonstrate Altered PGE₂ Secretory Function

LR-MSCs from patients with BOS demonstrate a profibrotic phenotype marked by increased collagen secretory ability and α -SMA expression (9). Because PGE₂ secretory function of cells can also be altered in diseases characterized by tissue remodeling (20, 31), we measured PGE₂ synthetic capacity of BOS versus non-BOS LR-MSCs. Cells from patients with and without BOS were cultured for 18 hours in the absence or presence of IL-1 β , and the conditioned medium was analyzed for PGE₂. Baseline levels of PGE₂ secretion by BOS LR-MSCs were found to be almost fivefold less than those of normal LR-MSCs (Figure 4A). Furthermore, BOS cells elaborated 22-fold less PGE₂ than did non-BOS cells under stimulated conditions (Figure 4A). This failure of LR-MSCs from patients with BOS to up-regulate PGE₂ in response to IL-1 β suggested failure of induction of COX-2. When COX-2 protein expression was studied, LR-MSCs derived from patients with BOS indeed demonstrated a dramatically blunted induction of COX-2 in response to IL-1 β (Figure 4B).

LR-MSCs from Patients with BOS Demonstrate Impaired PGE₂ Responsiveness

To investigate if PGE₂ modulation of LR-MSC is altered in BOS, the effect of PGE₂ on TGF- β -induced α -SMA expression was compared between BOS and normal LR-MSCs. In LR-MSCs derived from normal lung allografts, PGE₂ inhibited TGF- β -induced α -SMA expression in 8 out of the 10 lines tested with a mean decrease to $52.95 \pm 10.64\%$ of the TGF- β -induced levels. By contrast, no significant decrease in α -SMA expression in TGF- β -stimulated BOS LR-MSCs was noted in the presence of PGE₂ (mean = $95.25 \pm 7.95\%$ of the TGF- β -induced levels) (Figure 5A). As demonstrated in the dot plot, variability in the response to PGE₂ was noted among LR-MSCs derived from individual patients with BOS. LR-MSCs from three patients with BOS demonstrated greater than 30% inhibition of TGF- β -induced α -SMA expression by PGE₂. However, in six BOS cell lines, PGE₂ actually augmented α -SMA expression.

To understand the mechanism of this altered responsiveness to PGE₂, relative EP receptor expression was examined by real-time PCR in the same non-BOS and BOS cell lines. As a group, the BOS cell lines demonstrated a relatively lower EP2/EP1 ratio than did the non-BOS cell lines ($P = 0.001$) (Figure 5B), and a significant correlation was noted between the EP2/EP1 ratio and PGE₂-induced α -SMA expression in the entire cohort ($P = 0.0057$; $R^2 = 0.34$) (Figure 5C). This alteration in ratio primarily reflected a relative increase in expression of EP1 receptors in BOS MSCs compared with non-BOS MSCs ($P = 0.008$).

DISCUSSION

Graft resident donor-derived mesenchymal cells play a key role in fibrogenesis in the transplanted lung (8, 9). In this study we sought to examine the role of PGE₂ as an inhibitory modulator of fibrotic phenotype of human lung allograft-derived mesenchymal stem cells. We demonstrate that LR-MSCs derived from normal lung allografts demonstrate abundant expression of the G α_s -coupled receptor EP2, and acting by this

Figure 5. Bronchiolitis obliterans syndrome (BOS) lung-resident mesenchymal stromal cells (LR-MSCs) demonstrate impaired prostaglandin (PG)₂ responsiveness. (A) Ability of PGE₂ to inhibit transforming growth factor (TGF)-β-induced α-smooth muscle actin (α-SMA) expression in LR-MSCs from patients with BOS was compared with that of BOS-free controls by Western blot analyses. Data are presented as percentage of TGF-β-induced α-SMA expression. $n = 10$ normal LR-MSCs and $n = 11$ BOS LR-MSCs. (B) Ratio of E prostanoide (EP)₂ to EP₁ receptor expression in LR-MSCs derived from patients with and without BOS. Relative receptor expression was quantitated by real-time quantitative polymerase chain reaction (PCR). Individual cell lines are identified by different symbols. (C) Correlation of suppressive actions of PGE₂ with EP receptor expression. Circles represent LR-MSCs from normal controls; squares represent LR-MSCs from BOS cases. Pearson correlation $P = 0.0057$; $R^2 = 0.34$.

cAMP-dependent pathway, exogenous PGE₂ inhibits LR-MSC proliferation, collagen synthesis, and myofibroblast differentiation. Stimulation of endogenous PGE₂ secretion by IL-1β was associated with amelioration of their myofibroblast differentiation. By contrast, inhibition of endogenous PGE₂ synthesis by indomethacin or prevention of its action by EP2 receptor silencing was associated with augmented fibrotic differentiation. Furthermore, impairment in both PGE₂ synthesis and responsiveness was found in LR-MSCs isolated from patients with BOS, suggesting an *in vivo* phenotypic alteration favoring fibrogenesis. Our findings suggest that PGE₂, a secretory product of MSCs, acts as an autocrine brake on their fibrotic differentiation under normal circumstances and disruption of this homeostatic axis is associated with BOS. The identification of a dysregulated PG secretory and response pathway in LR-MSCs in association with a BOS provides the first demonstration of a potential role of PGE₂ in the pathogenesis of this disease.

Locally resident mesenchymal cells are important cellular participants in tissue repair responses. Lung-specific mesenchymal progenitors can serve important beneficial functions after allogeneic lung transplantation as promoters of epithelial growth (23) and inhibitors of T-cell responses (5). Indeed, by quantitating MSCs in the BAL of human lung allografts, we have demonstrated a significant increase in LR-MSC numbers in the first 3 months after lung transplantation, supporting a role for these cells in repair responses (8). However, an increase in LR-MSCs numbers later after transplant is associated with development of BOS (8), suggesting that a switch in LR-MSC phenotype or local milieu likely perpetuates fibrotic differentiation of these cells. The demonstration of an autocrine mechanism controlling LR-MSC fibrotic differentiation and its dysregulation in BOS, as suggested by the present work, offers a mechanistic explanation for this dual role of these cells in an allograft milieu.

Both exogenous and endogenous PGE₂ were shown to inhibit LR-MSC proliferation, collagen secretion, and myofibroblast differentiation *in vitro*. This suggests that PGE₂ secreted by LR-MSCs or from other cellular sources, such as the epithelium, can prevent their expansion and profibrotic differentiation, thus keeping the LR-MSCs recruited to the alveolar

spaces in a nondifferentiated state. The microenvironment of a lung allograft milieu has been studied by BAL. The development of BOS, similar to other fibrotic diseases, is associated with a switch from a proinflammatory type 1 immune response to a type 2 immune response (32). Proinflammatory cytokines, such as IL-1β, tumor necrosis factor-α, and IFN-γ, predominate in ischemia reperfusion and acute rejection (33–37), whereas profibrotic mediators, such as IL-13 and TGF-β, are linked to development of BO (28, 38–41). IL-1β, tumor necrosis factor-α, and IFN-γ are also significant inducers of COX-2 and PGE₂ secretion (42). Thus, it can be postulated that LR-MSCs recruited to a proinflammatory milieu are prevented from undergoing fibrotic differentiation because of this inhibitory effect of PGE₂ on their proliferation, synthetic function, and α-SMA expression. Indeed, we report herein that IL-1β ameliorates the stimulatory effect of TGF-β on α-SMA expression of LR-MSCs by a COX-dependent mechanism. However, IL-13, a type 2 cytokine that enhances collagen secretion and α-SMA expression in LR-MSCs (9), is a known inhibitor of COX-2 induction and PGE₂ synthesis (43, 44). Thus, the profile of soluble mediators in the allograft environment could differentially modulate LR-MSC fibrotic differentiation by their contrasting effects on PGE₂ synthetic function.

LR-MSCs isolated from patients with BOS demonstrated a defect in their capacity to synthesize PGE₂. Diminished PGE₂ secretory ability was noted under basal and stimulated conditions; BOS LR-MSCs failed to demonstrate increases in COX-2 protein expression in response to agonist IL-1β. These findings are similar to those previously described in fibroblasts isolated from lung biopsies of patients with idiopathic pulmonary fibrosis (31). Because PGE₂ production by normal lung allograft-derived LR-MSCs has been demonstrated to be important in T-cell inhibition, decreased PGE₂ secretion in LR-MSCs from patients with BOS can potentially “switch” the function of these cells from immunoregulatory to profibrotic. PGE₂ has also been demonstrated to promote apoptosis by modulating multiple survival pathways in normal human lung fibroblasts (45). Furthermore, inhibition of COX-2 in such cells was shown to significantly increase resistance to FasL-induced apoptosis (46). Whether LR-MSCs demonstrate

resistance to apoptosis and whether PGE₂ has a role in inducing apoptosis of LR-MSCs recruited to alveolar airspaces remains to be investigated.

The four EP receptors transducing the cellular actions of PGE₂ are traditionally associated with specific G α subunits that activate varying signaling pathways (30): EP1 couples with G α_q , which results in increases in intracellular calcium and release of diacylglycerol (47); EP2 and EP4 couple with G α_s , which stimulates adenylyl cyclase, leading to increases in cAMP; and EP3 couples through G α_i , which inhibits adenylyl cyclase, leading to decreases in cAMP (48). The integrated response to PGE₂ often represents the net effect of additive or opposing signal transduction events, hence the effects of PGE₂ can be pleiotropic (49). Furthermore, if expression of EP receptors is altered in disease states, then PGE₂ might have opposing effects in normal and disease conditions. Indeed, although PGE₂ is a bronchodilator in healthy subjects with no asthma, it results in significant bronchoconstriction in some people with asthma (50). Similarly, contradictory effects of PGE₂ on cells isolated from diseased and nondiseased tissues have been described (51). EP2 was found to be the predominant receptor in LR-MSCs derived from normal lung allografts and the antifibrotic effects of PGE₂ on these cells were shown to be mediated by EP2-linked cAMP signaling, in keeping with previously published data in this field (19). However, exogenous PGE₂ failed to demonstrate significant suppression of myofibroblast differentiation in LR-MSCs isolated from patients with BOS. This resistance to the antifibrotic actions of exogenous PGE₂ in BOS is similar to what has been previously reported in tissue fibroblasts from patients with idiopathic pulmonary fibrosis (29) and seems to arise from a reversal of the ratio of inhibitory EP2 to stimulatory EP1 receptors. DNA hypermethylation was recently demonstrated to be responsible for diminished EP2 expression leading to PGE₂ resistance in pulmonary fibrosis (52). The mechanisms underlying dysregulated PGE₂ production and EP receptor expression in BOS needs further investigation. However, this documentation of defective PGE₂ production and response pathways in BOS LR-MSCs provides the first demonstration for a role for this mediator in the pathogenesis of fibroproliferation in human lung allografts.

In summary, by first determining the presence of an antifibrotic mechanism in LR-MSCs isolated from normal lung allografts and then demonstrating its dysregulation in BOS LR-MSCs, we establish a role for altered PGE₂ synthetic and response pathways in the pathogenesis of BOS.

Author disclosures are available with the text of this article at www.atsjournals.org.

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