Endothelin-1 Signaling Promotes Fibrosis In Vitro in a Bronchopulmonary Dysplasia Model by Activating the Extrinsic Coagulation Cascade

Konstantinos Kambas,*,1 Akrivi Chrysanthopoulou,*,1 Ioannis Kourtzelis,*,† Marianna Skordala,‡ Ioannis Mitroulis,† Stavros Rafail,‡ Stergios Vradelis,*, Ioannis Sigalas,‡ You-Qiang Wu,† Matthias Speletas,§ George Kolios,§ and Konstantinos Ritis*

Neonatal respiratory distress syndrome can progress to bronchopulmonary dysplasia (BPD), a serious pulmonary fibrotic disorder. Given the involvement of the extrinsic coagulation cascade in animal models of lung fibrosis, we examined its role in BPD. We observed a higher number of neutrophils expressing tissue factor (TF) in bronchoalveolar lavage fluid (BALF) from infants with BPD than from those with uncomplicated respiratory distress syndrome together with a parallel decrease in TF and connective tissue growth factor (CTGF) in BALF supernatants during the disease course. The involvement of coagulation in the fibrotic process associated with BPD was further evaluated by treating primary human colonic myofibroblasts with BALF supernatants from infants with BPD. These human colonic myofibroblasts demonstrated an enhanced C5a- and thrombin-dependent migration. Moreover, they expressed TF in an endothelin-1-dependent manner, with subsequent activation of the extrinsic coagulation cascade and CTGF production mediated by protease-activator receptor-1 signaling. These data provide a novel mechanism for the development of BPD and indicate that endothelin-1 signaling contributes to fibrosis by upregulating a TF/thrombin amplification loop responsible for CTGF production, and offer novel and specific therapeutic targets for pulmonary fibrotic disease.

The Journal of Immunology, 2011, 186: 6568–6575.

Cross talk between inflammation and the activation of the extrinsic coagulation cascade has been described in a number of disease models (1). In addition to activated endothelium and monocytes, neutrophils have been recently shown to express tissue factor (TF) (2–7), the in vivo initiator of coagulation. Importantly, the inflammatory mediators C5a (2–4, 6–8), TNF-α (5, 6, 9), and IL-1β (10) are all inducers of TF. Furthermore, a recent study has demonstrated a correlation between coagulation and the inflammatory response in patients with acute respiratory distress syndrome (ARDS), indicating that the expression of TF by neutrophils in the alveolar microenvironment is C5a- and TNF-α dependent (6).

The coagulation system is actively involved in several fibrotic models (11–15) through the production of thrombin. Moreover, thrombin has been linked to the production of procollagen (11) and the expression of connective tissue growth factor (CTGF), a crucial fibrotic factor, in lung fibroblasts via the protease-activated receptor 1 (PAR-1) (11, 12). One of the main cell types that contribute to fibrosis via collagen production is myofibroblasts (16, 17). Although they originate from the differentiation of several cell populations in different tissues, they share a common function (18), producing profibrotic mediators.

In an attempt to further elucidate the interplay between inflammation and fibrosis mediated by the extrinsic coagulation system, we have used the pulmonary fibrotic disease bronchopulmonary dysplasia (BPD) as a model. This is a life-threatening, rapidly progressing fibrotic disorder in infants that occurs as a complication of neonatal respiratory distress syndrome (ARDS) (19). BPD is thought to be the result of an excessive inflammatory response (20), and earlier studies have established the significance of neutrophils in the alveolar microenvironment in this process (21, 22). Furthermore, the levels of several mediators, such as IL-6 (23–25), IL-8 (23, 25), TNF-α (25), and endothelin-1 (ET-1) (26), have been found to be increased in tracheal aspirates from infants with BPD. Especially, ET-1 signaling, which is implicated in the
pathogenesis of various pulmonary fibrotic models (27, 28), was identified as a target for antifibrotic therapeutic interventions (29). However, the possible mechanism linking ET-1 and the inflammatory response to the development of fibrosis is not yet clear.

The relatively rapid progression toward fibrosis during the course of BPD makes this disease a particularly useful model for analyzing the link between inflammation and extrinsic coagulation. In this report, we provide evidence that in BPD, the extrinsic coagulation pathway, together with C5a, promotes the migration of myofibroblasts to the alveolar microenvironment. These cells produce collagen and CTGF via PAR-1 signaling, thereby contributing to fibrosis. Moreover, ET-1 induces TF expression in myofibroblasts, further amplifying the thrombin generation and the subsequent progression to fibrosis.

Materials and Methods

Patient groups

The study subjects were 12 newborn infants admitted to the Neonatal Department and Neonatal Intensive Care Unit of University General Hospital of Alexandroupolis, Greece, who required mechanical ventilation at birth because of RDS. They were divided into two groups on the basis of disease course: 1) the RDS group consisted of six infants with acute respiratory failure and x-ray findings in the lungs consistent with RDS, who subsequently recovered, were breathing freely, and had a normal chest radiograph on the 28th day of life; and 2) the BPD group consisted of six infants with RDS who subsequently experienced development of BPD, according to established clinical criteria (30), and an abnormal chest radiograph. The clinical parameters of all 12 patients are described in Supplemental Table I. Moreover, four healthy infants were also recruited for the collection of peripheral blood for the isolation of neutrophils. All experimental procedures were approved by the Institutional Review Board and were in compliance with institutional guidelines and the Helsinki Declaration. Written consent was provided by the guardians of all infants in this study.

Bronchoalveolar lavage fluid collection and preparation

Bronchoalveolar lavage fluid (BALF) was collected using a nonbronchoscopic technique, according to the recommendations of the European Respiratory Society Task Force committee (31). BALF collection was performed on day 3, because the highest level of cell migration into the alveolar microenvironment was observed at this point in the disease process, according to our preliminary data and previous findings (22). In the case of the BPD infants, further BALF collection was performed on day 12, the earliest day on which two of the BPD infants were extubated. BALF was processed as previously described (6). BALF supernatants from three patients with either BPD or RDS were pooled for further studies, after assessing the potential of all samples to induce collagen production in myofibroblast cultures.

Cell preparation and culture

In vitro studies were performed on human colonic myofibroblasts (HCMFs) isolated, as previously described (32), from normal tissue in endoscopic biopsy samples obtained from four healthy donors at the Academic Hospital of Alexandroupolis, Greece. Primary human lung myofibroblasts were also isolated and cultured from a single lung lobectomy sample. Samples were collected in compliance with institutional guidelines. All experiments were carried out on cells at passage 2 to 3. Myofibroblasts were characterized by immunocytochemical staining with the following Abs: mouse anti-actin (smooth muscle; Invitrogen, Carlsbad, CA), mouse anti-vimentin (Invitrogen), and mouse anti-desmin mAbs (Invitrogen; Supplemental Fig. 1). Peripheral polymorphonuclear cells (PMNs) from infants of the two groups were isolated as previously described (6).

Stimulation and inhibition studies in cultured cells

Myofibroblasts were seeded into six-well plates (C25) or in four-well chamber slides (C5a) and cultured for 7–10 d in complete DMEM (Life Technologies BRL, Carlsbad, CA). Cells were cultured in serum-free medium for 24 h before use in stimulation/inhibition studies.

The majority of in vitro stimulation and inhibition studies were performed in HCMFs because of abundance of cells, whereas some key experimental procedures were further reproduced in lung myofibroblasts. Myofibroblasts were incubated with 40 μl pooled BALF supernatant at 37°C in serum-free DMEM medium (Life Technologies BRL) in the presence of various inhibitors or their respective controls. More specifically, a neutralizing TF mAb (10 μg/ml; American Diagnostica, Greenwich, CT), monoclonal mouse IgG1 (DAKO, Denmark), neutralizing CTGF mAb (10 μg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA), or anti-thrombin III (ATIII) 0.5 U/ml; Kybernin ZLB Behring, Germany) was applied to inhibit crucial components of the extrinsic coagulation cascade. The latency-associated peptide mAb (TGF-B1, 1 μg/ml; R&D Systems, Minneapolis, MN) was used to inhibit TGF-B1 signaling in BALF supernatants. To block PAR-1, we pretreated HCMFs for 30 min with the small molecule PAR-1 antagonist Lcn2 (2.5 μg/ml) (Resol proportion of cell concentration; Anaspec, Freemont, CA). HCMFs were treated with ET-1 peptide (70 pmol final concentration; Sigma-Aldrich), TNF-α (Sigma-Aldrich) was used in a concentration of 5 ng/ml, whereas blocking of TNF-α in BALF was performed with recombinant anti-human TNF-α Ab (HUMIRA, 0.2 μg/μl; Abbott Laboratories, North Chicago, IL). ET-1 receptors A and B were blocked with bosentan (10 μM; Actelion, Switzerland). Recombinant human C5a (10 nm) and C5a receptor a (C5aRa; 5 μM) (PMX-53) were also applied. Pooled blood serum (2 μl) from adult healthy donors (hereafter referred to as normal serum) was used in cell cultures requiring coagulation factors for thrombin generation. The final volume was adjusted to 2 ml in the assay using six-well plates and 300 μl in the assay that was carried out in four-well chamber slides. To isolate mRNA and protein samples, we harvested cells 3 and 4 h after incubation, respectively, because optimization experiments had yielded the highest expression at these time points. All the reagents used in the study were endotoxin-free (Limulus amoebocyte assay; Sigma-Aldrich).

Relative quantification of TF and CTGF mRNA synthesis

Isolation of total RNA and cDNA synthesis were conducted as previously described (6). Real-time RT-PCR for the full-length TF isoform (referred hereafter as TF), CTGF, PAR-1, and C5aR1 (C5a receptor 1) was performed under the conditions described in Supplemental Table II. The GAPDH housekeeping gene was used to normalize the expression levels of target genes, and the 2-ΔΔCt method (33) was applied to quantify their relative expression levels.

Western blot analysis

For TF detection, Western blotting was performed as previously described (6). To detect CTGF production, we used a polyclonal goat anti-human Ab (1/250 dilution; Santa Cruz Biotechnologies) and mouse IgG1 (1/500 dilution; 70 SeraLab, Abingdon, U.K.) overnight at 4°C. A CTGF blocking peptide was used (1.5 μg/ml; Santa Cruz Biotechnologies) to ensure the specificity of Ab binding. For Western blotting, protein precipitates from identical volumes of BALF supernatant were used, and equal amounts of protein were loaded onto the gel for each sample to obtain comparable results in the absence of a reference protein. In addition, TF and CTGF were blotted in parallel to eliminate any loading errors. Integrated OD analysis of the scanned films was performed using Gel Pro 3.1.

TF and CTGF immunostaining

Immunocytochemical staining was performed using a LSAB + System AP kit (DAKO), according to the manufacturer’s instructions. TF and CTGF were detected using a streptavidin/biotin method, with an IgG1 mouse anti-human TF mAb (1/100 dilution; American Diagnostica) or IgG goat antihuman polyclonal CTGF Ab (1/100 dilution; Santa Cruz Biotechnologies). An IgG1 anti-CD19 mAb (DAKO) was used as a negative control.

ET-1 immunoreactivity assays

ET-1 levels in BALF supernatants from infants were determined using an ELISA kit (R&D Systems), according to the manufacturer’s instructions. ET-1 was measured in three BALF supernatants from either BPD or RDS infants; the supernatants were then pooled and used in stimulation studies. The detection range of the assay was <1–200 pg/ml.

Collagen assays

The soluble collagen types released into culture medium by treated myofibroblasts (I–V) were measured using the Sircol Collagen Assay kit (Biocolor, Belfast, U.K.), according to the manufacturer’s instructions and as previously described (34). Collagen measurement was performed in both cell lysates (100 μg total protein) and culture supernatants (after
concentration of the collagen using the manufacturer’s isolation and concentration reagent). Collagen production was measured in culture supernatants and myofibroblast lysates after 24 and 12 h of stimulation, respectively, the time points at which the highest collagen production was observed. Our analysis produced similar results for culture supernatants and cell lysates, and consequently only results from culture supernatants are presented in this article.

**Fluorescence microscopy**

To detect the presence of C5aRs on HCMFs, we cultured cells on chamber slides and incubated them with 50 nM recombinant human C5a-GFP for 30 min at 37˚C. To verify the specificity of the C5a-GFP binding, we pre-treated cells with a highly specific antagonist for the C5aR, C5aRa (PMX-53), or its inactive control (both at 5 μM, for 30 min at 37˚C before incubation with C5a-GFP). HCMFs were also stained with a PE-conjugated anti-C5aR1 mAb (BD Pharmingen) in the presence of C5aRa or its inactive control. PE-conjugated mouse IgG1 was used as the control Ab. Cells were subsequently fixed in 4% PFA and visualized by reverse fluorescence microscopy (Axiovert 25, filter set 09, 488009; Zeiss).

**HCMF migration assays**

To assess the migration of myofibroblasts, an in vitro wound healing assay was carried out according to the manufacturer’s instructions (Cell Biolabs, San Diego, CA). Concentrations of reagents were identical to the ones used in the stimulation and inhibition studies section. Cells were visualized by reverse light microscopy (Axiovert 25; Zeiss). The migration rate was measured according to the manufacturer’s instructions.

**Statistical analysis**

Data are presented as means ± SD. Because of the limited sample size, a Mann–Whitney nonparametric test (unpaired, for six or more samples) or a Student t test (for fewer than six samples) was used to compare the differences in means. The level of significance was set to p ≤ 0.05. Statistical analysis was performed using the SPSS 8.0 statistical program for Windows.

**Results**

**TF and CTGF coexpression in BALF supernatants from infants with BPD**

To assess the inflammatory status of infants with BPD, we examined the cell composition in BALF obtained at selected time points during mechanical ventilation (Supplemental Table III). PMNs were the main population present in the lavage from infants with BPD (80.8 ± 8.5%); in contrast, the percentage of PMNs was lower (47.8 ± 6.0%) in the BALF from infants with RDS. Given the fact that inflammation and coagulation can be tightly correlated in various diseases and our recent description of TF upregulation in BALF neutrophils from patients with ARDS (6), an analogous condition in adults, we used immunostaining to examine the expression of TF in BALF cells from infants. Neutrophils from BPD BALF showed TF expression (Fig. 1A), whereas those from infants with uncomplicated RDS did not (Fig. 1AII). Most nonneutrophil BALF cells (e.g., atypical pneumocytes, macrophages) from either group stained negative for TF (data not shown). The results of our real-time RT-PCR (Fig. 1B, bars 2 and I, respectively) and Western blot analyses (Fig. 1Ca, lanes I and II, respectively) were consistent with the immunostaining findings.

The fibrotic nature of BPD and the previously reported correlation between exogenous coagulation cascade and fibrosis (11, 12) led us to examine the levels of CTGF in BALF supernatants. The protein concentration of CTGF was higher in the BALF supernatants from the BPD group (Fig. 1Cb, lane II) than from the RDS group (Fig. 1Cb, lane I). TF protein levels were also higher in BALF supernatants from the BPD group (Fig. 1Cb, lanes I and II, respectively). TF expression in BALF cells decreased on the 12th day of life in three infants who experienced development of BPD (Fig. 1D), whereas both TF and CTGF in supernatants also decreased over time (Fig. 1E), suggesting a linkage between them.

**FIGURE 1.** TF and CTGF expression in BALF from infants with BPD and RDS. A, TF immunostaining in BALF cells obtained from infants with BPD (I) or RDS (II) (n = 3 for each group). An anti-CD19 Ab was used as a negative control (III). Original magnification ×1000. A single representative experiment is shown. B, Relative expression of TF in BALF-derived cells from infants with BPD (bar 2; TF, 15.99 ± 0.871; *p < 0.01) as compared with those with RDS (bar I) during the third day of disease manifestation. All experiments were performed in triplicate. Data are presented as mean relative expression (fold increase) ± SD of DCt values using the 2−ΔΔCt equation (n = 6 for each group). C, Western blot analysis of TF and CTGF protein levels in (a) BALF cell lysates and (b) BALF supernatants from infants with RDS (lane I) or BPD infants (lane II) on the third day of disease (n = 3). One representative experiment of three independent experiments is shown. D, Kinetic study of the relative quantitation of TF mRNA in BALF cells obtained from infants with BPD (n = 3) during the 3rd and 12th day after the onset of the disease. Expression levels are shown as fold expression compared with the values for infants with RDS on the third day. Squares, triangles, and circles represent the three different infants used in this analysis. E, TF and CTGF protein levels in BALF supernatants from infants with BPD, as detected by immunoblotting on the 3rd (lane a) and 12th (lane b) day.

Moreover, BALF cells from BPD infants exhibited increased TF mRNA levels when compared with peripheral PMNs from the two infant populations (Supplemental Fig. 2), suggesting that its upregulation is a localized pulmonary phenomenon.

**C3a and thrombin induce HCMF migration into the BPD environment**

Myofibroblasts are found in fibrotic lesions from BPD infants on the fourth day of the disease course (35). Given that these cells are highly involved in fibrosis, we asked how the possible interplay between the TF pathway and the fibrotic nature of CTGF in these cells might contribute to the pathogenesis of BPD.

To examine their ability to proliferate and migrate toward the alveolar environment, we stimulated HCMFs with BALF supernatant from both infant groups. An increased proliferation and migration rate was observed after incubation with BPD BALF (Fig. 2AII, 2B, bar III), as compared with BALF from infants with RDS (Fig. 2AII, 2B, bar II). Moreover, HCMFs treated with BPD BALF indicated increased α-smooth muscle actin levels, as demonstrated by immunostaining (Supplemental Fig. 1C).

The previously described effect of thrombin on myofibroblast migration (17) and its presence in BALF (36) prompted us to assess the effect of ATIII on the HCMF migration induced by BPD BALF. The migration rate was significantly reduced (Fig. 2AIV, 2B, bar IV) after exposure to ATIII, indicating that the extrinsic coagulation cascade plays a key role in this phenomenon.
FIGURE 2. HCMFs express C5aRs and migrate in response to C5a/ thrombin activity. C5a- and thrombin-dependent migration (A) and migration rate (B) of HCMFs in the BPD environment. In vitro migration, as assessed by wound healing assay, of HCMFs treated with culture medium (I); BALF supernatant from infants with RDS (II) or BPD infants (III); BPD BALF supernatants preincubated with ATIII (0.5 U/ml) (IV); BPD BALF supernatants after preincubation of cells with C5aRa (5 μM) (V) or bosentan (10 μM) (VI); or simultaneous stimulation with C5a and thrombin (VII). C5a alone (10 nM) (VIII); thrombin alone (5 U/ml) (IX); or simultaneous stimulation with C5a and thrombin (X). Duplicate wells were prepared for each sample. Original magnification ×50. One representative experiment of three independent experiments is shown in A, B. Data are presented as means ± SD (n = 3).

In an attempt to identify other migratory stimuli, we also investigated the effect of C5a anaphylatoxin, a strong chemotactic (37) that is involved in the development of ARDS in adults (6). We initially demonstrated the expression of C5aR on the surface of HCMFs by detecting the binding of GFP-labeled recombinant human C5a (Supplemental Fig. 3B); this observation was verified by assessing mRNA and protein levels of C5aR (Supplemental Fig. 3A, 3C). We observed that C5a caused an increase in the migration rate of HCMFs in the BPD environment, and this property was abolished by the addition of a C5aR antagonist (Fig. 2A, 2B, bar V). The induction of migration in HCMFs treated with pure thrombin (Fig. 2AIX, 2B, bar IX) or recombinant human C5a (Fig. 2AVIII, 2B, bar VIII) further supported these findings. Moreover, thrombin and recombinant C5a had a synergistic effect on the rate of HCMF migration (Fig. 2AX, 2B, bar X).

Myofibroblasts produce CTGF in the BPD microenvironment by activating the extrinsic coagulation pathway

Because myofibroblasts migrate and express CTGF (Fig. 3A, gray bar 3, 3B, 3CIII, 3D, bar 1) in response to BPD BALF, we searched for components of the BALF that could initiate this fibrotic process. Given the reported involvement of the extrinsic coagulation cascade in pulmonary fibrotic models (13–15), we incubated HCMFs with BPD BALF after treatment with either ATIII or a neutralizing mAb against TF. The CTGF gene overexpression was abolished by treatment with ATIII (Fig. 3A, gray bar 4, 3BIV, 3CVI, 3D, bar 4) and was partially inhibited by anti-TF Ab (Fig. 3A, gray bar 5, 3CIII, 3D, bar 5). These findings were further observed in lung myofibroblast stimulations (Supplemental Fig. 4A, 4B). Moreover, a marked increase in collagen production was observed in culture supernatants from HCMFs treated with BPD BALF (Fig. 3E, bar 3), compared with cells treated with RDS BALF (Fig. 3E, bar 2). Similar results were also obtained from lung myofibroblast cultures (Supplemental Fig. 4C). Treatment with ATIII inhibited collagen production (Fig. 3E, bar 4), whereas the anti-TF mAb had only a partial inhibitory effect (Fig. 3E, bar 5). Moreover, neutralization with CTGF polyclonal Ab attenuated the production of collagen, addressing the involvement of CTGF in collagen production in myofibroblasts (data not shown). Blocking of the fibrosis-related factor TGF-β1 with neutralizing mAb in BPD supernatant from BPD infants failed to abolish CTGF expression and collagen production (data not shown).

To further verify the involvement of the extrinsic coagulation cascade in the upregulation of CTGF in HCMFs, we blocked PAR-1 in cells before treating them with BPD supernatant from infants with BPD. Pretreatment with the PAR-1 antagonist resulted in...
a significant decrease in both CTGF mRNA levels in HCMFs (Fig. 3A, gray bar 6) and collagen levels in culture supernatants (Fig. 3E, bar 6). Furthermore, PAR-1 mRNA expression levels were significantly increased in HCMFs incubated with BALF from infants with BPD (Fig. 3A, black bar 3), as compared with cells stimulated with BALF from infants with RDS (Fig. 3A, black bars 1 and 2, respectively). This effect was abolished by ATIII (Fig. 3A, gray bar 4) or the PAR-1 antagonist (Fig. 3A, black bar 6) and partially inhibited by anti-TF treatment (Fig. 3A, black bar 5).

The BPD alveolar microenvironment induces TF expression in myofibroblasts

The partial inhibitory effect of anti-TF Ab on CTGF and collagen production by HCMFs suggested a potential production of TF by these cells. Indeed, HCMFs stimulated with BALF supernatants from infants with BPD exhibited a significant increase in TF mRNA transcripts (Fig. 4A, bar 2) when compared with HCMFs stimulated with BALF supernatants from the RDS group (Fig. 4A, bar 3). This finding was further corroborated on the protein level by Western blotting (Fig. 4B, lane II) and immunocytochemical staining (Fig. 4CII). TF immunostaining also verified these findings in lung myofibroblasts (Supplemental Fig. 4D). The production of TF by myofibroblasts suggests the existence of an amplification loop, which produces further activation of the TF pathway and subsequent CTGF induction.

ET-1 signaling mediates the induction of TF in myofibroblasts

To identify mediators that might be responsible for the upregulation of TF expression in myofibroblasts, we investigated the role of C5a and TNF-α, well-established TF inducers that have previously been described in analogous pulmonary models (ARDS) (6). Treatment of HCMFs with C5a or a neutralizing anti–TNF-α Ab in BPD BALF failed to affect the TF expression levels (data not shown).

Furthermore, because ET-1 and the extrinsic coagulation cascade are involved in the fibrotic process and given that HCMFs express ET-1 receptors (38, 39), we examined the potential effect of ET-1 on TF expression. We initially assessed ET-1 levels in BALF supernatants from both infant groups. ET-1 levels were higher on the third day in BALF supernatants from infants with BPD than those with RDS (Fig. 5A). However, treatment of HCMFs with BALF supernatant had no effect on the mRNA levels of ET-1 receptors (ETA and ETB; Supplemental Fig. 5).

We next tested the effects of a specific blockade of ET-1 signaling using bosentan. This treatment significantly reduced TF levels at both the mRNA (Fig. 5D, bar 3) and protein (Fig. 5BIII, SCI, lane III) levels in HCMFs incubated with BALF supernatant from infants with BPD. Moreover, CTGF mRNA expression (Fig.
collagen deposition (Fig. 5E, bar 3), and the migration rate (Fig. 2B, bar VI) of these cells were also significantly reduced after treatment with bosentan. Inhibition of ET-1 signaling also attenuated TF induction and collagen production in lung myofibroblasts stimulated by BPD BALF (Supplemental Fig. 4DIV). This effect of ET-1 receptor blockade could be attributed to an inhibition of the additional TF production in HCMFs and the subsequent generation of thrombin.

We further exposed HCMFs to a specific ET-1 peptide, which resulted in an upregulation of TF protein (Fig. 5FIV, lane IV) and mRNA (Fig. 5D, bars 5 and 6) expression levels.

Notably, in the presence of serum from healthy individuals (normal serum), the ET-1 peptide induced HCMF migration (Fig. 5FIV), CTGF expression (Fig. 5D, bar 6), and collagen deposition (Fig. 5E, bar 6); no such increases occurred in the absence of serum (Fig. 5D, 5E, bar 5, 5FIII). Moreover, blocking with bosentan or pretreatment with ATIII abolished the cell migration (Fig. 5FIV and 5FVI, respectively), CTGF induction (Fig. 5D, bars 7 and 8, respectively), and collagen production (Fig. 5E, bars 7 and 8, respectively) in HCMFs incubated with ET-1 and normal serum. Migration of HCMFs was also inhibited by the PAR-1 antagonist (Fig. 5FVII), further supporting the involvement of TF/thrombin axis in this process. Taken together, these findings suggest a novel role for ET-1 in the fibrotic model of BPD by mediating the induction of TF in myofibroblasts (Fig. 6).

**Discussion**

This study was designed to provide novel insight into the development of fibrosis in BPD patients as a result of cross talk between inflammation and coagulation. Our experimental data have indicated that the inflammatory pulmonary environment in BPD patients contributes to the development of fibrosis by activating the extrinsic coagulation cascade through ET-1 signaling. We have demonstrated that C5a and thrombin can promote myofibroblast migration, and ET-1 signaling induces TF expression in these cells. As a result, an amplification loop is generated that augments the initial triggering of the extrinsic coagulation cascade, further activating myofibroblasts to produce CTGF and collagen. This course of events suggests that ET-1 plays a key role in the progression to fibrosis.

Other studies have reported an increased accumulation of PMNs in the alveolar space (21, 22) and noted a correlation between the number of neutrophils present in the alveoli of preterm infants and the development of BPD (21, 22). A role for these cells in the acute inflammatory response and tissue injury has also been documented (40). Similarly, neutrophils have been shown to be a significant source of TF expression in BALF from ARDS patients (6). This study has demonstrated that neutrophils from infants with BPD are the prevalent cell population in BALF, and they contribute, at least in part, to the progression of the disorder by eliciting TF expression and subsequent thrombin generation. The increased expression of TF in the BPD BALF PMNs as observed by immunostaining and the similar TF mRNA levels in peripheral PMNs from control infants and infants with either RDS or BPD indicates a localized phenomenon in the alveolar microenvironment. However, we cannot overlook the possible participation of microparticle-derived TF (1) produced by other cells and acquired by PMNs in the TF levels observed in PMNs from BPD BALF.

The extrinsic coagulation cascade is able to induce CTGF expression and collagen deposition via PAR-1 signaling in nonhuman pulmonary models, either through thrombin generation (11, 12) or through other proteases of the system (26). CTGF has been implicated in the pathogenesis of IPF through the activation of fibroblasts and alveolar epithelial type II cells (41), and it also plays a major role in bleomycin-induced lung inflammation in nonhuman models (12). Nevertheless, evidence for the involvement of CTGF in other human fibrotic respiratory syndromes such as BPD is still scarce. The higher levels of TF and CTGF in supernatants collected on the third day of the disease provide evidence for a correlation between these two molecules. Such a finding may be related to the previously reported accumulation of PMNs in the alveolar space and myofibroblasts in the fibrotic lesions of BPD (35). Despite the variability of TF expression in BPD BALF cells at day 3 (which could be attributed to the differential expression of inflammatory mediators in the alveolar microenvironment), we found a parallel reduction of TF levels and neutrophil numbers in BALF during the course of the disease. This finding is in accordance with previous studies that have indicated a decrease in thrombin concentration during the course of the disease (36).

The critical role of myofibroblasts in the fibrotic process (42) and their previously documented presence in BPD lesions in infants (35) prompted us to select this cell type for our in vitro studies. Given that these cells share common functions, even when derived from different tissues (18), we chose to perform the majority of our in vitro studies in primary cultures of myofibroblasts from human colon because of their availability, and to reproduce the key findings in primary lung myofibroblasts because of the rarity of sample.

Our assessment of the ability of BALF supernatants from infants with BPD or RDS to induce migration, CTGF production, and collagen deposition in HCMFs indicated that the myofibroblasts exhibit significantly higher migration in the presence of BPD supernatant from BPD infants, and this migration is thrombin and C5a dependent. The involvement of complement was further verified by showing the functional presence of C5aR in these cells. The chemoattractant activity of thrombin has recently been reported (17). We suggest that the initial thrombin generation in BALF occurs as a result of the increased vascular permeability caused by inflammation and TF expression mediated by neutrophils and other alveolar cells, and that it constitutes a possible early chemoattractant stimulus for fibroblasts and myofibroblasts.
Thrombin then activates the attracted cells to proliferate and produce collagen. We found that in addition to their migratory capacity, myofibroblasts also express increased CTGF levels and produce collagen in the BPD environment, and that expression is mediated by the TF/thrombin/PAR-1 axis. This observation was consistent with our hypothesis that the activation of the extrinsic coagulation cascade contributes to the progression to fibrosis. The incubation of myofibroblasts with BALF supernatant from BPD infants also resulted in the production of significant amounts of TF. This novel finding suggests that these cells participate in the activation of the extrinsic coagulation pathway, which may lead to excess thrombin generation. Signaling via PAR-1 further induced CTGF production and collagen deposition. To identify the key mediator(s) that stimulate myofibroblasts to produce TF in BALF from infants with BPD, we examined candidate molecules that are known to induce TF expression in various cell populations (29). Stimulation and inhibition studies failed to implicate C5a or TNF-α in the TF-mediated generation of thrombin.

We also examined ET-1 because of its involvement in fibrotic disorders and BPD. To our knowledge, our results demonstrate for the first time that inhibition of both ET-1 receptors with bosentan in myofibroblasts abolished the BALF-dependent expression of TF and subsequent cell migration, as well as CTGF and collagen production. Furthermore, the ET-1 peptide was able to induce TF production. Moreover, it induced the production of CTGF and collagen, after adding normal serum, which provides coagulation factors, thus supporting the involvement of thrombin in the process. The involvement of ET-1 in the fibrotic response of infants with BPD was further supported by the presence of increased amounts of the protein in their BALF. Our findings and the previously reported ability of ET-1 signaling to induce the differentiation of lung fibroblasts into myofibroblasts suggest a role for ET-1 in the fibrotic process of BPD (43). These data provide further support for the importance of neutrophils in the alveolar space in the development of this syndrome, because these cells produce endothelin-converting enzyme, which allows them to convert big endothelin into the signaling ET-1 (44, 45).

Based on our findings, we have proposed a possible pathophysiologic mechanism for BPD (Fig. 6). In this model, the inflammation-driven chemoattractant activity in the alveolar environment (e.g., C5a and IL-8–rich), in conjunction with endothelial damage to the pulmonary microvasculature, cause peripheral neutrophils to migrate into the alveolar space. It also induces the expression of TF, resulting in thrombin generation, and other mediators that further enhance the inflammatory response (Fig. 6, section 1). C5a and the initial thrombin generation promote myofibroblast migration into the mesenchymal space (Fig. 6, section 2). Leukocytes convert big endothelin into ET-1, leading to further activation of the myofibroblasts via ET-1 signaling and the subsequent production of TF (Fig. 6, section 3). This process results in additional thrombin generation, initiating a vicious cycle (Fig. 6, section 3). TF/thrombin pathway proteases then stimulate myofibroblasts through PAR-1 signaling to produce CTGF, which induces collagen production via an autocrine mechanism. This process could apply equally well to other human fibrotic disorders in which inflammation and coagulation coexist.

In conclusion, this human ex vivo model demonstrates the involvement of the extrinsic coagulation cascade in the development of fibrosis. Our study indicates a novel role for ET-1 in fibrosis and suggests a mechanism for the amplification of thrombin generation. The development of molecules able to neutralize the mediators responsible for this process may form the basis for novel approaches for therapeutic intervention in fibrotic disorders.
21. Merritt, T. A., J. M. Puccia, and I. D. Stuard. 1981. Cytologic evaluation of pulmonary effluent in neonates with respiratory distress syndrome and bronchopulmonary dysplasia. *Acta Cytol.* 25: 631–639.

22. Ogden, B. E., S. A. Murphy, G. C. Saunders, D. Pathak, and J. D. Johnson. 1984. Neonatal lung neutrophils and elastase/protease inhibitor imbalance. *Am. Rev. Respir. Dis.* 130: 817–821.

23. Tullius, K., G. W. Noack, L. G. Burman, R. Nilsson, B. Wretlind, and A. Brauner. 1996. Elevated cytokine levels in tracheobronchial aspirate fluids from ventilator treated neonates with bronchopulmonary dysplasia. *Eur. J. Pediatr.* 155: 112–116.

24. Kotecha, S., L. Wilson, A. Wangoo, M. Silverman, and R. J. Shaw. 1996. Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatr. Res.* 40: 250–256.

25. Munshi, U. K., J. O. Niu, M. M. Siddiq, and L. A. Parton. 1997. Elevation of pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. *Br. J. Pharmacol.* 123: 965–970.

26. Niu, J. O., U. K. Munshi, M. M. Siddiq, and L. A. Parton. 1998. Early increase in endothelin-1 in tracheal aspirates of preterm infants who develop bronchopulmonary dysplasia. *Pediatr. Pulmonol.* 24: 331–336.

27. Hocher, B., A. Schwarz, K. A. Fagan, C. Thöne-Reineke, K. El-Hag, H. Kasserow, S. Ettok, C. Bauer, H. H. Neumayer, D. M. Rodman, and F. Theuring. 2000. Pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. *Am. J. Respir. Cell Mol. Biol.* 23: 19–26.

28. Ross, B., P. D’Orléans-Juste, and A. Giaid. 2005. Role of endothelin in fibrosis and anti-fibrotic potential of bosentan. *Am. J. Respir. Crit. Care Med.* 171: 2173–2179.

29. de Blic, J., F. Midulla, A. Barbato, A. Clement, I. Dab, E. Eber, C. Green, J. Grigg, S. Kotecha, G. Kurland, et al; European Respiratory Society. 2000. Bronchopulmonary dysplasia. *Am. J. Respir. Crit. Care Med.* 163: 1723–1729.

30. Blic, J., F. Midulla, A. Barbato, A. Clement, I. Dab, E. Eber, C. Green, J. Grigg, S. Kotecha, G. Kurland, et al; European Respiratory Society. 2000. Bronchopulmonary dysplasia. *Am. J. Respir. Crit. Care Med.* 163: 1723–1729.

31. Kouroumalis, A., R. J. Nibbs, H. Apelt, K. L. Wright, G. Kolios, and S. G. Ward. 2005. The chemokines CXCL9, CXCL10, and CXCL11 differentially stimulate G alpha i-independent signaling and actin responses in human intestinal myo-fibroblasts. *J. Immunol.* 175: 5403–5411.

32. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-(Delta Delta C(T)) method. *Methods* 25: 402–408.

33. Huang, M., S. Sharma, L. X. Zhu, M. P. Keane, J. Luo, L. Zhang, M. D. Barwick, Y. Q. Lin, M. Dohadwala, B. Gardner, et al. 2002. IL-7 inhibits fibroblast TGF-beta production and signaling in pulmonary fibrosis. *J. Clin. Invest.* 109: 931–937.

34. Töti, P., G. Buonocore, P. Tanganelli, A. M. Catella, M. L. Palmeri, R. Vatti, and T. A. Seemayer. 1997. Bronchopulmonary dysplasia of the premature baby: an immunohistochemical study. *Pediatr. Pulmonol.* 24: 22–28.

35. Dik, W. A., L. J. Zimmermann, B. A. Naber, D. J. Janssen, A. H. van Kaam, and M. A. Versnel. 2003. Thrombin contributes to bronchoalveolar lavage fluid mitogenicity in lung disease of the premature infant. *Pediatr. Pulmonol.* 35: 34–41.

36. Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 11: 785–797.

37. Egidi, G., L. Jullierat-Jeanneret, P. Korth, F. T. Bosman, and F. Pinet. 2000. The endothelin system in normal human colon. *Am. J. Physiol. Gastrointest. Liver Physiol.* 279: G211–G222.

38. Kojima, N., M. Hori, T. Murata, Y. Morizane, and H. Ozaki. 2007. Different profiles of Ca2+ responses to endothelin-1 and PDGF in liver myofibroblasts during the process of cell differentiation. *Br. J. Pharmacol.* 151: 816–827.

39. Gando, S. 2010. Microvascular thrombosis and multiple organ dysfunction syndrome. *Crit. Care Med.* 38(2 Suppl):S35–S42.

40. Allen, J. T., R. A. Knight, C. A. Bloor, and M. A. Spiteri. 1999. Enhanced insulin-like growth factor binding protein-related protein 2 (connective tissue growth factor) expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. *Am. J. Respir. Cell Mol. Biol.* 21: 693–700.

41. Scotton, C. J., and R. C. Chambers. 2007. Molecular targets in pulmonary fibrosis: the myofibroblast in focus. *Crit. Care Med.* 132: 212–213.

42. Shi-Wen, X., Y. Chen, C. P. Denton, M. Eastwood, E. A. Renzoni, G. Bou-Gharios, J. D. Pearson, M. Dashwood, R. M. du Bois, C. M. Black, et al. 2004. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a racc/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibroblastic fibroblasts. *Mol. Biol. Cell* 15: 2707–2719.

43. Valdenaire, O., E. Rohrbacher, and M. G. Mattei. 1995. Organization of the gene encoding the human endothelin-converting enzyme (ECE-1). *J. Biol. Chem.* 270: 29794–29798.

44. Massai, L., N. Volpi, P. Carbotti, M. Fruscelli, M. Mencarelli, A. Pecorelli, M. Muccitella, M. Agliano, C. Alessandrini, and G. Grasso. 2007. Endothelin-1 and endothelin-converting enzyme-1 in human granulomatous pathology of eyelid: an immunohistochemical and in situ hybridization study in chalazia. *Histol. Histopathol.* 22: 1343–1354.