Thrombin stimulates the expression of PDGF in lung epithelial cells

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Thrombin stimulates the expression of PDGF in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 279: L503–L510, 2000.—Several growth factors, including platelet-derived growth factor (PDGF), have been implicated in the mechanism of lung and airway remodeling. In the present study, we evaluated whether thrombin may promote lung and airway remodeling by increasing PDGF production from lung and airway epithelial cells. Conditioned medium (CM) was prepared by treating epithelial cells with increasing concentrations of thrombin; before use in the assays, CM was treated with hirudin until complete inhibition of thrombin activity. CM from epithelial cells stimulated the proliferation of lung fibroblasts and bronchial smooth muscle cells. Anti-PDGF antibody significantly inhibited this CM proliferative activity, implicating PDGF in this effect. Enzyme immunassay and RT-PCR demonstrated that thrombin induced the secretion and expression of PDGF from bronchial and alveolar epithelial cells. RT-PCR showed that epithelial cells express the thrombin receptors protease-activated receptor (PAR)-1, PAR-3, and PAR-4. The PAR-1 agonist peptide was also found to induce PDGF secretion from epithelial cells, suggesting that the cellular effect of thrombin occurs via a PAR-1-mediated mechanism. Overall, this study showed for the first time that thrombin may play an important role in the process of lung and airway remodeling by stimulating the expression of PDGF via its cellular receptor, PAR-1.

coagulation; lung fibrosis; bronchial asthma; platelet-derived growth factor receptor

Structural remodeling of the lung tissue is believed to be the consequence of repeated cycles of epithelial injury and repair process associated with chronic pulmonary disease (3). Remodeling of the alveolar compartment of the lung, such as that occurring in pulmonary fibrosis, is characterized by interstitial and intra-alveolar deposition of connective tissue and collapse and distortion of air spaces by scar contraction (5). Tissue remodeling is also a severe complication of airway disorders, particularly of bronchial asthma. It is believed that airway remodeling leading to thickening of the bronchial wall is responsible for the underlying irreversible airway hyperreactivity observed in patients with chronic bronchial asthma (23). The morphological changes of airway remodeling include epithelial sloughing, thickening of the epithelial basement membrane, edema, inflammatory cell infiltration of the submucosa, hyperplasia of mucus glands, accumulation of myofibroblasts, and thickening of medial smooth muscle layers (2, 7, 8). Several growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-β, epidermal growth factor (EGF), and insulin-like growth factor, secreted by inflammatory or resident structural cells, have been implicated in the mechanism of the structural changes associated with lung and airway remodeling (15, 16).

Thrombin is a serine protease that, besides its critical role in thrombosis and hemostasis, has also been described as inducing cellular and molecular events relevant to tissue remodeling. For example, thrombin may induce platelet aggregation and platelet release of thromboxane A2, serotonin, procoagulant, and growth factors. Thrombin may also stimulate chemotaxis of monocytes and lymphocytes, the motility and proliferation of fibroblasts and smooth muscle cells, and the secretion of growth factors and proteases from the vascular endothelium (9, 11, 22). These observations together with previous studies (10, 18, 19) showing increased generation of thrombin in pulmonary diseases suggest that thrombin may also play an important role in the pathogenesis of lung and airway remodeling. We hypothesized that thrombin may also indirectly promote lung and airway remodeling by increasing the production of growth factors from alveolar and bronchial epithelial cells. To demonstrate this possibility, in the present study, we evaluated the effect of thrombin on the secretion of PDGF from alveolar and bronchial epithelial cells and the effect of conditioned medium (CM) of these cells stimulated with thrombin on the proliferation of lung fibroblasts and bronchial smooth muscle cells. It is known that the cellular effects of thrombin depend on stimulation of the pro-

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tease-activated receptor (PAR)-1, PAR-3, or PAR-4 (6). In the present study, we also evaluated the expression of these receptors in alveolar and bronchial epithelial cells.

MATERIALS AND METHODS

Reagents. Dulbecco’s modified Eagle’s medium (DMEM), L-glutamine, vitamin solution, sodium pyruvate, nonessential amino acids, transferrin, bovine pituitary extract, TRIzol reagent, and Superscript preamplification system were purchased from Gibco BRL (Life Technologies, Grand Island, NY). Fetal bovine serum (FBS) was from BioWhitaker (Walkersville, MD); and penicillin, streptomycin, and dexamethasone were from Nacalai Tesque (Kyoto, Japan). BSA, Ham’s F-12 medium, epinephrine, gentamicin, amphotericin B, insulin, triiodothyronine, cholela toxin, endothelial growth supplement, l-leucine, l-lysine, l-methionine, HEPES, and nonspecific protease from Streptomyces griseus were from Sigma (St. Louis, MO). Human EGF was from Higeta Shouyu (Tokyo, Japan). Hydrocortisone and retinoic acid were from Sigma (St. Louis, MO). Human EGF was from Streptomyces griseus. bFGF, cDNA probes of von Willebrand factor. The cells were cultured with a culture kit (Clonetics) containing a modified MCDB131 medium supplemented with 5% FBS, 50 μg/ml of gentamicin, 50 ng/ml of amphotericin B, 0.5 ng/ml of human EGF, 5 μg/ml of insulin, and 2 ng/ml of human FGF. All cells were cultured in 10-cm dishes in an atmosphere composed of 5% CO₂ and 95% air. Confluent cells were harvested by a brief exposure to 0.025% trypsin-0.02% EDTA in HEPES-buffered saline (50 mM HEPES and 150 mM NaCl, pH 7.4) and passaged after 5–7 days.

Preparation of CM. A549, NHBE, and BEAS-2B cells were grown to 80–90% confluence in 10-cm dishes, and human nasal epithelial cells were grown in 12-well tissue culture plates. After incubation for 24 h in basal medium without supplements, the cells were washed and incubated for 48 h in the presence of varying concentrations of thrombin. The CM was harvested, centrifuged at 1,200 g for 15 min, and stored at −80°C until used. To evaluate the participation of the thrombin receptor (PAR-1), after preincubation in FBS- and supplement-free medium for 24 h, NHBE cells were treated with the PAR-1 agonist peptide (SFLRNP) or its negative control (YFLRRNP) for 48 h, and then the CM was collected and used in the assays.

Proliferation assay. CM was used in the proliferation assays after it was treated with hirudin (250 antithrombin units) at 37°C for 30 min and after confirmation of the absence of thrombin activity in CM with the chromogenic substrate S2238 for thrombin (Chromogenix, Mölndal, Sweden) by amidolytic assay. The proliferative effect of CM on fibroblasts and bronchial smooth muscle cells was assessed by a colorimetric method as previously described (21). Briefly, after the cells were cultured in 96-well plates up to 70–80% of confluence, their growth was arrested by culturing overnight in medium without FBS and growth factors. After the cells were washed, 100 μl of hirudin-treated CM was applied to each well and incubated for 48 h under an atmosphere of 5% CO₂ and 95% air. After being appropriately washed, the cells were fixed by treatment with 10% Formalin for 30 min; the wells were then washed, and 1% (wt/vol) methylene blue in 0.01 M borate buffer was added. After the wells were washed with borate buffer, a 1:1 dilution of ethanol in 0.1 M HCl was added, and the absorbance at 650 nm was measured with a microplate reader. This method has been previously validated with a number of characterized growth factors (21). In addition, proliferative assays were also carried out with a cell-counting kit purchased from Dojindo (Kumamoto, Japan); this kit contains a tetrazolium compound, WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2-H-5-tetrazolio]-1,3-benzene disulfonate, which is used for the colorimetric determination of cell number.

In separate experiments to evaluate whether the proliferative effect of CM depends on the presence of PDGF, the CM prepared from cells stimulated with the same concentration of thrombin (250 nM) was treated with varying concentrations of anti-human PDGF antibody at 37°C for 30 min. The anti-PDGF antibody-treated CM was then used in proliferation assays performed as described above.

Enzyme immunassay. Enzyme immunassays were carried out to assess the concentration of growth factors in CM. Commercial immun assay kits (described in Reagents) were used to measure the concentrations of PDGF-AB, bFGF, and EGF.

RNA isolation and RT-PCR. RT-PCR was carried out to evaluate the expression of thrombin receptors (PAR-1, PAR-3, and PAR-4) and PDGF receptors (α and β) in alveolar and airway epithelial cells, lung fibroblasts, and bronchial
smooth muscle cells. Total RNA was extracted from confluent cells by the guanidine isothiocyanate procedure with the TRIZOL Reagent. Two micrograms of total RNA were reverse transcribed with oligo(dT) primer and the Superscript preamplification system kit following the manufacturer’s instructions. The primers used in the PCR are listed in Table 1. The PCR mixture contained 100 mM Tris-HCl, pH 8.3, and 200 mM KCl, 25 mM MgCl2, 100 μM deoxyribonucleotides (dATP, dGTP, dCTP, and dTTP), 10 μM each forward and reverse primer, an appropriate amount of denatured salmon sperm DNA, and 50% formamide, 1 M ammonium acetate and 20 mM NaOH at room temperature for 30 min. The cDNA was then electrophoresed on a 2% agarose gel, the gel was incubated in a solution containing 1.5 M NaCl and 500 mM NaOH for 30 min at room temperature cycler (Astec, Fukuoka, Japan). The PCR products were purified from the gel using a PCR product purification kit (Amersham Pharmacia Biotech, Tokyo, Japan) and sequenced directly by the dideoxy chain termination method using an automatic DNA sequencer (Pharmacia, Uppsala, Sweden) with [α-32P]dCTP with a DNA labeling kit (Takara Shuzo, Kyoto, Japan). Sequal analysis. All data are expressed as means ± SE unless otherwise specified. The difference between three or more variables was calculated by analysis of variance. Statistical analyses were carried out with the StatView 4.1 package software for the Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

Effect of CM on proliferation of lung fibroblasts and bronchial epithelial cells. To evaluate the role of thrombin in the secretion of PDGF from bronchial and alveolar epithelial cells, the proliferative activity of CM from these cells stimulated with varying concentrations of thrombin on lung fibroblasts and bronchial smooth muscle cells was assessed after complete inhibition of residual thrombin activity in the CM with hirudin. In the methylene blue-based proliferation assays, CM from the primary bronchial epithelial cells (NHBE cells) significantly stimulated the proliferation of fibroblasts and bronchial smooth muscle cells in a dose-dependent manner (Fig. 1A). Similarly, CM from the alveolar epithelial cell line A549 significantly stimulated the proliferation of fibroblasts and bronchial smooth muscle cells in a dose-dependent fashion (Fig. 1B). These findings were in good agreement with the results obtained with the cell-counting kit containing the tetrazolium salt WST-1. For preparation of the control sample, aliquots of culture medium were incubated in the presence of the same concentrations of thrombin but in the absence of epithelial cells; after treatment with hirudin, the control medium was also used in the proliferative assays. These control media did not show proliferative activity on lung fibroblasts (data not shown), further ensuring that hirudin completely inhibited residual thrombin activity in our assays.

Effect of anti-PDGF antibody on proliferative activity of CM from airway epithelial cells. To assess whether the proliferative activity of CM depends on the secretion of PDGF from epithelial (NHBE) cells, the inhibitory effect of anti-PDGF antibody that recognizes PDGF-AA, PDGF-BB, and PDGF-AB was evaluated. Compared with control samples (0 μg/ml of anti-PDGF antibody), the anti-PDGF antibody significantly inhibited the proliferative activity of CM prepared with NHBE cells stimulated with 250 nM thrombin in a

Table 1. Primer sequences for RT-PCR

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<tr>
<th>mRNA</th>
<th>Species</th>
<th>5’ Primer Nucleotides</th>
<th>3’ Primer Nucleotides</th>
<th>Annealing Temperature, °C</th>
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<td>5’ 829-949</td>
<td>59-949</td>
<td>59</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>5’ 949-970</td>
<td>5’ 970-1075</td>
<td>949-1075</td>
<td>55</td>
</tr>
<tr>
<td>PDGF-R</td>
<td>5’ 1388-1409</td>
<td>5’ 1409-1935</td>
<td>1388-1935</td>
<td>55</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>5’ 743-780</td>
<td>720-780</td>
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<tr>
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<td>5’ 527-551</td>
<td>505-551</td>
<td>55</td>
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<tr>
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<td>5’ 152-172</td>
<td>5’ 172-191</td>
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<td>55</td>
</tr>
<tr>
<td>PAR-3</td>
<td>5’ 949-970</td>
<td>5’ 970-1096</td>
<td>949-1096</td>
<td>55</td>
</tr>
</tbody>
</table>
| PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAR, protease-activated receptor.
dose-dependent manner (Fig. 2). This inhibition of fibroblast proliferation was significant above 10 μg/ml of the anti-PDGF antibody.

Secretion of PDGF and other growth factors by epithelial cells treated with thrombin. To assess whether thrombin stimulation increases the secretion of PDGF and other growth factors from epithelial cells, the concentration of these growth factors in CM was measured. As shown in Fig. 3, thrombin significantly and specifically stimulated the secretion of PDGF-AB but not that of bFGF or EGF from NHBE cells. The increase in the concentration of PDGF-AB in CM stimulated with thrombin was significant from a thrombin concentration of 62.5 nM for BEAS-2B cells (Fig. 4A); in A549 cells (Fig. 4B), thrombin stimulated the secretion of PDGF-AB at a thrombin concentration of 62.5 nM and reached a plateau with 125 nM thrombin.

Thrombin also significantly increased the concentration of PDGF-AB in CM prepared with primary cells from bronchial or nasal epithelium; thrombin increased PDGF-AB concentration from a concentration of 62.5 nM for NHBE cells (Fig. 5A) and 125 nM for nasal epithelial cells (Fig. 5B). The secretion of PDGF-AB from thrombin-treated BEAS-2B cells was also time dependent (Fig. 6).

Effect of thrombin on PDGF gene expression in epithelial cells. The effect of thrombin on the gene expression of PDGF-A and PDGF-B in NHBE cells was evaluated by RT-PCR and Southern blot analysis. As shown in Fig. 7, thrombin significantly increased the expression of the PDGF-B gene but not that of the
PDGF-A gene in NHBE cells. Similarly, Northern blot analysis also showed that thrombin stimulates the expression of the PDGF-B gene at a transcriptional level (data not shown).

**Effect of PAR-1 agonist peptide on PDGF secretion in epithelial cells.** To evaluate the participation of the thrombin receptor on PDGF secretion induced by thrombin, the effect of the PAR-1 agonist peptide on the concentration of PDGF in CM from NHBE cells was assessed. Like thrombin, the PAR-1 agonist peptide also induced PDGF-AB secretion from NHBE cells in a dose-dependent manner (Fig. 8). This effect was significant from a peptide concentration of 25 μM. The negative control peptide did not induce PDGF-AB secretion (data not shown). These results suggest that thrombin stimulation of PDGF-AB secretion from airway epithelial cells is mediated by PAR-1.

**Expression of PARs in epithelial cells.** To evaluate the type of thrombin receptors expressed by epithelial cells, mRNAs of PAR-1, PAR-3, and PAR-4 were analyzed by RT-PCR. RT-PCR showed that both alveolar (A549) and airway (NHBE) epithelial cells expressed not only PAR-1 but also PAR-3 and PAR-4; however, the bronchial epithelial cell line BEAS-2B did not express PAR-3 (Fig. 9).

**Expression of PDGF receptors in epithelial cells, lung fibroblasts, and bronchial smooth muscle cells.** Because it is known that the effect of PDGF depends on the presence of its receptors, α and β, on target cells, the expression of PDGF receptors was evaluated. RT-PCR showed that both PDGF receptors (α and β) are expressed by epithelial cells, lung fibroblasts, and bronchial smooth muscle cells (Fig. 10).

**DISCUSSION**

This study showed that CM from alveolar and bronchial epithelial cells treated with thrombin significantly stimulates the proliferation of lung fibroblasts and bronchial smooth muscle cells compared with that in the medium of untreated cells. Immunoassays and inhibition experiments revealed that the increased PDGF-A gene in NHBE cells. Similarly, Northern blot analysis also showed that thrombin stimulates the expression of the PDGF-B gene at a transcriptional level (data not shown).

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growth of these cells depends on the enhanced production and secretion of PDGF-AB induced by thrombin in epithelial cells.

It is well known that increased thrombin generation occurs in the lungs of most patients with inflammatory diseases of the pulmonary parenchyma and airways and that it plays a critical role in tissue remodeling (10, 18, 19). After injury, a tissue factor-activated factor VII complex is formed on lung and airway epithelial and/or endothelial cells, leading to activation of the extrinsic pathway of coagulation, thrombin generation, fibrin formation, and fibrosis (4, 12, 14). In the lung and airways, generated thrombin may modulate the tissue repair response by altering vascular permeability, by stimulating the secretion of proteases and the production or degradation of components of the connective tissue, and by promoting adhesion, spreading, and proliferation of fibroblasts and smooth muscle cells (1). In addition to its direct effects, thrombin may also play a role in lung tissue remodeling by stimulating the secretion of growth factors from several cells. For example, it has been reported that thrombin may induce increased expression of PDGF from lung fibroblasts, vascular smooth muscle cells, endothelial cells, and alveolar macrophages (13, 19, 20, 22, 26). In the present study, we hypothesized that thrombin may also participate in tissue remodeling by stimulating the secretion of growth factors from alveolar and bronchial epithelial cells.

The CM prepared by treating alveolar and bronchial epithelial cells with different concentrations of thrombin was found to stimulate the proliferation of lung fibroblasts and bronchial smooth muscle cells. This CM effect did not depend on the direct proliferative effect of thrombin because its activity in CM was blocked completely by hirudin before use in the proliferative assays. Measurement of growth factors in CM showed that thrombin upregulated the secretion of PDGF-AB from bronchial epithelial cells in dose- and time-dependent manners; RT-PCR analysis showed that this increased secretion of PDGF-AB depends on increased mRNA expression of PDGF-B. It is worth noting that upregulation of PDGF-AB was induced by thrombin not only in transformed epithelial cell lines but also in primary bronchial and nasal epithelial cells, suggesting that this effect is probably also observed in vivo at sites of inflammation. Inhibition assays carried out with anti-PDGF antibody confirmed that PDGF was responsible for the proliferative activity of CM from epithelial cells. Overall, these findings suggest that stimulation of PDGF secretion from epithelial cells may constitute another mechanism of thrombin participation in lung and airway remodeling. The biological relevance of PDGF in the mechanism of tissue remodeling is illustrated by the important role it plays in the pathogenesis of fibroproliferative processes such as wound healing, pulmonary fibrosis, atherosclerosis, myelofibrosis, scleroderma, and inflammatory joint diseases. Properties that implicate PDGF in the mechanism of tissue remodeling are its strong mitogenic and chemotactic activities on fibroblasts and smooth muscle cells and its ability to stimulate angiogenesis and the secretion of cytokines (e.g., transforming growth factor-β) that, in turn, may upregulate the expression of PDGF.
of extracellular matrix components (17, 24, 25). A previous study (24) has shown that the PDGF-B chain is the polypeptide that is mainly expressed in lung tissues from patients with lung fibrosis (24); the demonstration in the present study that thrombin upregulates the expression of PDGF-B but not that of PDGF-A reinforces the important role that thrombin plays in the process of lung fibrosis.

The cellular effects of thrombin have been found to depend on stimulation of the thrombin receptors PAR-1, PAR-3, and PAR-4 (6). After cellular activation by thrombin, a new NH2-terminal peptide, which functions as a tethered ligand for the receptor itself, is unmasked on PAR-1, PAR-3, or PAR-4. The tethered ligand of PAR-1 has been shown to promote proliferation of fibroblasts and vascular smooth muscle cells, cytokine secretion, and deposition of collagen in the extracellular matrix. To evaluate whether PAR-1 also mediates the effect of thrombin in primary airway epithelial cells (NHBE cells), the effect of the PAR-1 agonist peptide on PDGF-AB secretion was assessed. The PAR-1 agonist peptide induced PDGF-AB secretion from NHBE cells in a similar fashion as thrombin. RT-PCR analysis showed that besides the PAR-1 gene, PAR-3 and PAR-4 genes are also expressed by bronchial (NHBE and BEAS-2B) and alveolar (A549) epithelial cells. These findings suggest that PARs may play an important role in the mechanism of lung and airway remodeling by mediating the mitogenic and secretory activity of thrombin in epithelial cells.

PDGF is known to occur in the form of three dimers (PDGF-AA, PDGF-BB, and PDGF-AB) and to bind to specific high-affinity receptor subunits (αα, ββ, and αβ) on the surface of responsive cells (fibroblasts and smooth muscle cells). The effect of PDGF depends on the type of PDGF receptor subunit available on the cell surface. PDGF-BB can bind to any one of these receptor subunits; PDGF-AA can bind only to an αα-receptor; and PDGF-AB can bind either to an αα-receptor or to an αβ-receptor (24). In the present study, RT-PCR analysis showed that both the αα- and ββ-subunits were equally expressed by lung fibroblasts and bronchial smooth muscle cells. These findings explain the proliferative activity of PDGF-rich CM on fibroblasts and bronchial smooth muscle cells observed in the present study. In addition, airway epithelial cells express both subunit receptors, suggesting that they may also play an important role in epithelial regeneration in the lung and airways.

In conclusion, the results of this study showed for the first time that 1) thrombin regulates PDGF expression and secretion from nasal, bronchial, and alveolar epithelial cells; 2) the cellular effect of thrombin on PDGF secretion by epithelial cells is mediated by its receptor PAR-1; 3) airway epithelial cells express the thrombin receptors PAR-1, PAR-3, and PAR-4; and 4) the thrombin-induced secretion of PDGF is sufficient for stimulating the proliferation of fibroblast and bronchial smooth muscle cells. Overall, these findings suggest that thrombin may play an important role in the process of lung and airway remodeling by stimulating the secretion of PDGF via its PAR-1 receptor from alveolar and bronchial epithelial cells.
REFERENCES


