Induction of the Plasminogen Activator System by Mechanical Stimulation of Human Bronchial Epithelial Cells

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Mechanical stimulation of the airway epithelium, as would occur during bronchoconstriction, is a potent stimulus and can activate profibrotic pathways. We used DNA microarray technology to examine gene expression in compressed normal human bronchial epithelial cells (NHBE). Compressive stress applied continuously over an 8-h period to NHBE cells led to the upregulation of several families of genes, including a family of plasminogen-related genes that were previously not known to be regulated in this system. Real-time PCR demonstrated a peak increase in gene expression of 8.0-fold for urokinase plasminogen activator (uPA), 16.2-fold for urokinase plasminogen activator receptor (uPAR), 4.2-fold for plasminogen activator inhibitor-1 (PAI-1), and 3.9-fold for tissue plasminogen activator (tPA). Compressive stress also increased uPA protein levels in the cell lysates (112.0 versus 82.0 ng/ml, $P = 0.0004$), and increased uPA (4.7 versus 3.3 ng/ml, $P = 0.02$), uPAR (1.3 versus 0.86 ng/ml, $P = 0.007$), and PAI-1 (50 versus 36 ng/ml, $P = 0.006$) protein levels in cell culture media. Functional studies demonstrated increased urokinase-dependent plasmin generation in compression-stimulated cells (0.0090 versus 0.0033 OD/min, $P = 0.03$). In addition, compression led to increased activation of matrix metalloproteinase (MMP)-9 and MMP-2 in a urokinase-dependent manner. In postmortem human lung tissue, we observed an increase in epithelial uPA and uPAR immunostaining in the airways of two patients who died in status asthmaticus compared with minimal immunoreactivity noted in airways from seven lung donors without asthma. Together these observations suggest an integrated response of airway epithelial cells to mechanical stimulation, acting through the plasminogen-activating system to modify the airway microenvironment.

Keywords: plasminogen activator system; matrix metalloproteinase 9; bronchial epithelial cells; mechanical stimulation; DNA microarrays

One of the features of the asthmatic airway is epithelial upregulation of a number of pro-phlogistic pathways (1, 2). While many consider the inflammatory process in asthma to derive from products of inflammatory cells, such as mast cells, eosinophils, and lymphocytes, we have previously shown that mechanical stimulation of the airway epithelium, as would occur during

bronchoconstriction, is itself a potent stimulus and can activate profibrotic pathways (3, 4).

Airway epithelial cells experience substantial compressive mechanical stress as a result of bronchoconstriction (5). Prior work has shown that compressive stress applied to human bronchial epithelial (NHBE) cells initiates the release and activation of mediators, including endothelin and transforming growth factor- β , traditionally associated with inflammatory stimuli (3, 4). These profibrotic genes were examined because of their known mechanoresponsive properties in other cell types, such as endothelium and cardiomyocytes (6–11). However, they may represent only a subset of the mechanoresponsive genes in airway epithelial cells. We used DNA microarray technology to examine expression of over 14,500 human genes in NHBE cells in air-liquid interface culture. We show herein that compressive stress of airway epithelial cells leads to expression of a group of plasminogen activator genes, the respective protein entities, and functional activity of downstream molecules capable of modifying the airway microenvironment.

MATERIALS AND METHODS

Cell Culture

NHBE cells were obtained from Clonetics-BioWhittaker (San Diego, CA) and cultured at an air-liquid interface, as previously described (12). In brief, passage 2 cells were expanded on tissue culture-treated plastic, in an atmosphere of 5% CO₂ in air at 37°C in bronchial epithelial growth medium (BEGM; Clonetics) supplemented with bovine serum albumin (1.5 μ g/ml) and retinoic acid (50 nM). Passage 3 cells were then plated on uncoated nucleopore membranes (25 mm diameter, 0.4 μ m pore size, Transwell Clear; Costar, Cambridge, MA) at 100,000 cells/well. The cells were fed as previously detailed (13) with a 1:1 mixture of BEGM and Dulbecco's Modification of Eagle's Medium (DMEM; Media Technologies, Herndon, VA) as recommended by the supplier (Clonetics). Culture medium was applied both apically and basally until cells were confluent and then basally after an air-liquid interface was established, after about a fortnight of culture. The cells were maintained until a uniform, differentiated cell population, with prominent cilia and mucus-secreting capabilities, was visually present; all cells were fed 18 h before each experiment.

Experimental Apparatus

As previously described, cells grown on Transwells were exposed to transcellular compression by connecting the top of each Transwell to an air/CO₂ compression source. Silicon plugs with an access port for compression application were press fit in the top of each Transwell 18 h before the experiment, creating a sealed pressure chamber over the apical surface of the NHBE cells (14). The basal surface and medium were left exposed to atmospheric pressure. Each plug was connected, in parallel, to a 5% CO₂ (balance air) pressure cylinder via a humidified chamber maintained at 37°C. At the onset of the experiment compressed cells were exposed to a transcellular gradient of 30 cm H₂O; control cells were treated similarly but exposed to no pressure gradient. Cell lysates were collected for RNA or protein analysis by the addition of the appropriate lysis buffer (Rneasy [Qiagen, Valencia, CA] for

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RNA or 1% Triton X-100 at pH 8.5 for protein studies) and collecting the solution with cell scrapers. Samples were freeze-thawed once to solubilize membrane proteins so that we could study membrane-associated urokinase plasminogen activator (uPA) bound to uPA receptor (uPAR), which is likely to be a biologically important form of uPA. The samples were then spun down at 14,000 rpm and the supernatant was isolated for protein analysis.

Protocols

For detailed illustration of the protocols, *see* Figure 1.

(1) DNA microarray experiment. mRNA expression was examined immediately before and over an 8-h period after the onset of compressive stress. Cellular extracts for RNA isolation were collected at 30-min intervals up to 4 h, and again at 8 h during continuous application of compressive stress. Five replicate experiments were performed and total RNA samples from each time point were pooled (by equivalent mass) for hybridization to DNA microarrays.

(2) Protein expression and plasminogen activation studies. Cells were either stimulated with compression (30 cm H₂O) or not stimulated (controls) over an 8-h period. Cell lysates and media were collected and examined in several groups: (1) 8 h after the onset of compressive stress, (2) unstimulated cells at 8 h after the onset of the experiment (time matched control), (3) at 24 h after the onset of 8 h of compression, and (4) unstimulated cells at 24 h after the onset of the experiment (time matched control).

(3) Matrix metalloproteinase activity studies. Cell lysates were collected for measurement of matrix metalloproteinase (MMP)-9 and MMP-2 activity from cells stimulated with compression applied continuously for up to 8 h at several time points: (1) before compression, (2) after 4 h of compression, (3) after 8 h of compression, (4) 12 h after the onset of 8 h of compression, and (5) 24 h after the onset of 8 h of compression. In addition, to demonstrate a link between MMP-9/MMP-2 and uPA, MMP-9/MMP-2 activity was determined in compression stimulated cell lysates in the presence of an uPA inhibitor and a

plasmin inhibitor. Cells were treated with one of five protocols: (1) control; (2) compression applied for 8 h; (3) 1 h pre-incubation at 37°C with aprotinin (0.5 µg/ml), an inhibitor of serine proteinases including plasmin, followed by 8 h of compressive stress; (4) 1 h pre-incubation at 37°C with a mouse IgG₁ control antibody followed by 8 h of compressive stress; or (5) 1 h pre-incubation at 37°C with a monoclonal mouse IgG₁ inhibitory antibody against the uPA B chain (#394; American Diagnostica, Stamford, CT) followed by 8 h of compressive stress. In each case, cell lysates were collected 24 h after the initiation of the experiment.

DNA Microarray

The RNA from cell extracts was collected from five 8-h compression experiments, and time points from each replicate were validated by examining gene expression by quantitative real-time PCR on a panel of known mechanoresponsive genes, including heparin-binding epidermal growth factor-like growth factor, *c-fos*, and early growth response-1 (data not shown). RNA from the five experiments was pooled, then processed and hybridized to Affymetrix Human 133A DNA microarrays (Affymetrix, Santa Clara, CA) by a core facility, the Harvard Medical School Partners Healthcare Center for Genetics and Genomics. The resultant gene expression image files were processed using the Robust Multi-array Average (RMA) method (15, 16). This technique employs a quantile normalization of “perfect match” expression values from raw image files. Overall quality of the gene expression data from each array was confirmed by examining several housekeeping genes (representing multiple probe sets) that had been confirmed not to change with compressive stress, as well as several “positive-control” genes that are known to be regulated by compression (HBEGF, endothelin-2, *c-fos*, *Egr-1*). One of the 10 DNA microarrays, the 2.5 h time-point, failed the above criteria and was excluded from further analysis.

From the 22,283 probe sets investigated, we identified those regulated by the compressive stimulus according to the following criteria.

Figure 1. Protocols for compressive stress. Black triangles indicate harvest of samples for analysis.

For each probe set, we identified the two highest and two lowest RMA signal intensity values. These values were tested for differential expression using Significant Analysis of Microarrays (SAM) (17). This identified 1,024 probe sets to be regulated as defined by differential expression ($d = 1.215$, False Discovery Rate = 0.008). Hierarchical clustering (using centroid linkage and correlation coefficient) by gene expression profile over time was performed using Cluster 3.0 (18). Further analysis with gene ontology using the Database for Annotation, Visualization and Integrated Discovery (DAVID) applied to the 1,024 filtered probe sets and to sub-clusters of probe sets, identified several groups of significantly over-represented ontological categories (19).

Reverse Transcription and Real-Time PCR

Total RNA was purified from cell lysates with a commercial kit (Rneasy; Qiagen). Equal amounts of RNA (2 μ g) were reverse transcribed using Ready-to-Go RT-PCR beads (Amersham, Piscataway, NJ) by incubation at 42°C for 30 min (Mastercycler; Eppendorf AG, Hamburg, Germany). Real-time PCR reactions were performed using SYBR Green Master Mix (Bio-Rad, Hercules, CA) in an iCycler PCR System (Bio-Rad). Fold changes were calculated using the “delta delta Ct” method (20). As expected, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was unchanged during compressive stress (data not shown) and was used as the reference standard. Real-time PCR primers (Invitrogen, Carlsbad, CA) targeting uPA, uPAR, plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (tPA), and GAPDH were designed using Primer Express software (Applied Biosystems, Foster City, CA) with similar melting point temperatures, primer lengths, and amplicon lengths to obtain similar PCR efficiency (Table 1). Each primer was tested against GAPDH over a range of concentrations to ensure similar PCR efficiency.

Analysis of uPA, uPAR, and PAI-1 by ELISA

Cell lysates and conditioned media were obtained at 8 h and 24 h after initiation of compressive stress. Levels of uPA, uPAR, and PAI-1 in the conditioned media and cell lysates were quantified by ELISA according to the manufacturer's specification (American Diagnostica).

Coupled Colorimetric Assay for Plasminogen Activation

Plasminogen activator activity was measured by the addition of 80 μ l of colorimetric reaction buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 0.2 μ g/ml leupeptin, 0.32 μ M Glu-plasminogen [American Diagnostica], 0.2 mM Spectrozyme PL [American Diagnostica], and 0.1% Triton X-100) to 20 μ l of cell lysates from NHBE cells stimulated with compressive stress or unstimulated time-matched controls (21). The photometric absorbance of the reaction mixtures at 405 nm was monitored at 37°C using a Bio-Rad 680 microplate reader. Reaction velocity was calculated as the rate of optical density over time; plasmin generation was expressed as the maximal reaction velocity. We confirmed that this reaction is driven by urokinase-dependent plasminogen activation by the addition of a specific inhibitory anti-uPA antibody (product #394; American Diagnostica) at various doses (2.5 μ g/ml or 25 μ g/ml) to a mixture of compression-stimulated cell lysates (20 μ l)

and colorimetric reaction buffer (80 μ l), and then measuring subsequent plasmin generation.

MMP-2/MMP-9 Zymogram

MMP-9 activity was measured by gelatin zymography on lysates of NHBE cells stimulated under various conditions as outlined above. Cell lysates were loaded (30 μ g of total protein) into 10% gelatin zymogram gels (Bio-Rad) and proteins were separated by electrophoresis. Gels were renatured in 2.5% Triton X-100 for 30 min at room temperature, then incubated in developing buffer (50 mM Tris-HCl, 0.2M NaCl, 5 mM CaCl₂, 0.02% Brij 35) for 22 h at 37°C. Gels were stained (2.5% Coomassie Blue, 45.4% methanol, 9.2% acetic acid) and destained (4.5% methanol, 6.8% acetic acid), then imaged with a Bio Imaging System (Syngene, Frederick, MD) under ultraviolet light.

Immunostaining for uPA and uPAR in Human Lung Tissue

The protocols used to obtain the discarded lung tissue used in this study were approved by the BWH Human Research Committee and the New England Organ Bank. Samples of discarded lung tissue obtained from postmortem examinations of patients dying from status asthmaticus ($n = 2$) and unused lung donor tissue ($n = 9$) were fixed in 4% paraformaldehyde, routinely processed, and embedded in paraffin. Five-micron-thick sections were cut, and the samples were screened using a hematoxylin and eosin stain to identify the presence of suitable airways for each case. On the basis of this review, between one and three blocks were chosen from each case. Potentially usable samples were then screened for possible overfixation by staining for the smooth muscle marker desmin, using murine antibody of the same IgG subclass as those present in the uPA and uPAR antibodies. Of the nine donor lung tissue samples, seven demonstrated abundant desmin immunostaining. Only lung tissue samples that both had an adequate number of airways by hematoxylin and eosin stain and showed abundant desmin immunopositivity were used in this study. Thus, seven lung donor samples were used for the analysis of uPA and uPAR.

Tissue sections were dewaxed in xylenes and rehydrated in graded alcohols. Immunohistochemical analysis was then performed using a modified avidin-biotin complex (ABC) technique. Mouse monoclonal antibodies against uPA (1:50 dilution, #394; American Diagnostica), uPAR (1:50 dilution, #3937; American Diagnostica), desmin (1:100; Dako Cytomation, Carpinteria, CA), and nonspecific murine IgG (1:25; Sigma, St. Louis, MO) were used as primary antibodies. For all antibodies endogenous peroxidase activity was quenched using a peroxidase blocking reagent (Dako Cytomation) according to the manufacturer's instructions prior to the primary antibody and then 1% hydrogen peroxide in methanol for 10 min following the secondary antibody. Nonspecific immunoglobulin binding was blocked with 10% horse serum (GIBCO-Invitrogen, Carlsbad, CA) in PBS with 2% bovine serum albumin (BSA). The primary antibodies, diluted as above in PBS with 2% BSA, were applied to tissue sections and incubated at 4°C overnight in a humidified chamber. The slides were subsequently incubated with biotinylated donkey anti-mouse IgG secondary antibody (1:500; Jackson Laboratories, West Grove, PA) at room temperature for 1 h. Tissue sections were then incubated with streptavidin-horseradish peroxidase diluted 1:1,000 in PBS for 30 min at room temperature. Immunopositivity was visualized using the chromagen diaminobenzidine (0.025%) in PBS and 0.1% hydrogen peroxide. All samples were counterstained with 2% methyl green (Sigma).

Statistics

Data are presented as mean fold changes and standard error. A two-sided *t* test was performed for comparisons, and where appropriate a paired two sample for means *t* test was used. *P* values < 0.05 were considered significant.

RESULTS

DNA Microarray Analysis of NHBE Cells Exposed to Compressive Stress Exhibit Different Patterns of Gene Expression

To examine the effects of compressive stress on gene expression, NHBE cells were exposed to an apical to basolateral compressive

TABLE 1. REAL-TIME PCR PRIMER SEQUENCES

Gene	Sequence	Primer (Forward/Reverse)
GAPDH	5' TGGGCTACACTGAGCACCAG 3'	Sense
	5' GGGTGTCTGCTGTGAAGTCA 3'	Antisense
uPA	5' AAGCCAGGCGTCTACACGAG 3'	Sense
	5' ACTGCCGATCCAGGTAAGA 3'	Antisense
uPAR	5' CGAGGCCCCATGAATCAAT 3'	Sense
	5' TTTTCGGTTCGTGAGTGCC 3'	Antisense
PAI-1	5' TCTGCAGACCTGGTTCCAC 3'	Sense
	5' AGCCCGTAGTTCATCCTG 3'	Antisense
tPA	5' TTCAGCTAAAGCCCAACCTCC 3'	Sense
	5' CAAAGCTGCTCACGGTGACA 3'	Antisense

Definition of abbreviations: GAPDH, glyceraldehyde 3'-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

TABLE 2. MICROARRAY GENE EXPRESSION VALUES IN RMA, MAXIMAL FOLD CHANGE, AND SAM SCORES FOR THE PLASMINOGEN-RELATED GENES

Gene	RMA Expression Value		Maximum Fold Change	SAM Score
	Minimum	Maximum		
uPA	8.30	10.18	3.61	4.55
uPAR	8.58	11.04	4.97	6.19
PAI-1	6.77	8.10	2.52	2.32
tPA	9.08	10.33	2.27	3.58

Definition of abbreviations: PAI-1, plasminogen activator inhibitor-1; RMA, robust multi-array average; SAM, significant analysis of microarrays; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

stimulus over an 8-h period (Figure 1). As detailed in the methods, RNA from cell extracts was collected, pooled, and used to probe the transcriptional activity of 14,500 genes (22,283 probe sets) using Affymetrix Human 133A DNA microarrays. After filtering for the most differentially regulated genes (1,024 probe sets, as detailed in MATERIALS AND METHODS), several functional groups of genes coordinately regulated by compressive stress were identified (Figure 2). This report focuses on gene expression among members of the plasminogen activator family (Table 2).

Ontologic Analysis of Gene Clusters Identified a Novel Group of Compression-Stimulated Plasminogen Activator Genes

We employed DAVID, a tool that analyzes lists of genes and provides statistical measures for overrepresented ontology groups (19), to examine individual clusters of 50 to 100 genes with similar expression patterns as identified by hierarchical clustering (see MATERIALS AND METHODS). Functional groups of genes regulated by compression included those involved in growth factor activity, mitogen-activated protein kinase (MAPK) regulation, transcriptional regulation, cytoskeletal protein binding, and plasminogen activator activity (which were induced), and metalloendopeptidase activity, heat shock protein activity, and DNA replication and mitosis (which were repressed) (Table 3). These studies confirmed the coordinated induction of a cluster of 89 probe sets containing numerous members of the “plasminogen activator activity” gene family (Expression Analysis Systematic Explorer [EASE] score < 0.001, Fisher’s Exact test < 0.001) (19).

We then used Principal Component Analysis (PCA) to examine these clusters of genes relative to the 1,024 filtered probe

sets and found that by PCA analysis the plasminogen genes grouped together (Figure 3).

Compressive Stress Increases mRNA Expression of Plasminogen-Related Genes

Given the potential role of plasminogen-related genes in regulating extracellular matrix remodeling, we chose to examine these genes in more detail. These genes which include the uPA, uPAR, PAI-1, and tPA were examined by quantitative RT-PCR on an aliquot of the original five RNA samples before pooling (Figure 4). RT-PCR confirmed replication among the five experiments and that compressive stress increases expression of these genes (Figure 4). There was a significant increase in uPA expression at 2 and 3 h ($P < 0.05$), peaking 8.0-fold above baseline at 4 h and 8 h after the onset of compression. uPAR expression was significantly increased at all time points ($P < 0.05$) and peaked at 16.2-fold above baseline at 4 h. PAI-1 was significantly increased at 1, 2, 3, and 4 h ($P < 0.05$) after the onset of compression, peaking at 3.9-fold at 4 h. tPA was significantly increased 4 h after the onset of compression ($P < 0.05$) at 4.2-fold above baseline. We confirmed that the temporal changes in gene expression were dependent on the application of compressive stress by using quantitative PCR analysis of time-matched controls (see online supplement).

Compressive Stress Increases Expression of Plasminogen-Related Proteins

Protein expression of uPA, uPAR, and PAI-1 was quantified in the cell lysates and media of compressed NHBE cells and unstimulated time-matched controls after 8 h and 24 h. Compressive stress increased protein levels of uPA in cell lysates (101.8 ± 38 versus 140 ± 34 ng/ml at 8 h, $P = 0.05$, and 82 ± 14 versus 112 ± 19 ng/ml at 24 h, $P = 0.0004$). In the cell culture media compressive stress increased uPAR protein levels at 8 and 24 h after the onset of compression (0.64 ± 0.09 versus 1.04 ± 0.14 ng/ml, $P = 0.053$, and 0.86 ± 0.07 versus 1.3 ± 0.08 ng/ml, $P = 0.007$, respectively). uPA and PAI-1 protein secretion into the media was increased 24 h after the onset of compressive stress (3.3 ± 0.86 versus 4.7 ± 1.1 ng/ml, $P = 0.02$, and 36 ± 10 versus 50 ± 13 ng/ml, $P = 0.006$, respectively) (Figure 5). uPAR and PAI-1 protein levels in the cell lysates were not measurably altered by compressive stress (data not shown).

Compressive Stress Increases Plasminogen Activator Activity in NHBE Cells

Because we found increased uPA in the cellular fraction, but not its endogenous inhibitor PAI-1, and because cell membrane-associated uPA bound to uPAR is biologically active, we

TABLE 3. SELECTED OVERREPRESENTED GENE ONTOLOGY GROUPS IN COMPRESSION-STIMULATED CELLS AND THEIR RESPECTIVE EASE AND FISHER’S EXACT STATISTICS INDICATING DEGREE OF OVERREPRESENTATION

Ontology	Regulation of Genes	EASE Statistic	Fisher’s Exact Statistic
Growth factor activity	Upregulated	0.00000179	0.000000129
Actin binding	Upregulated	0.0000118	0.00000138
Cytoskeletal protein binding	Upregulated	0.0000702	0.0000106
Plasminogen activator activity	Upregulated	0.000221	0.000000928
MAPK phosphatase activity	Upregulated	0.000832	0.00000981
Transcription cofactor activity	Upregulated	0.00772	0.00155
DNA replication	Downregulated	0.00000000000362	0.0000000000003
Heat shock protein activity	Downregulated	0.000172	0.00000443
Metalloendopeptidase activity	Downregulated	0.0225	0.00174

Definition of abbreviations: EASE, Expression Analysis Systematic Explorer; MAPK, mitogen-activated protein kinase.

Figure 2. Hierarchical clustering of 1,024 differentially regulated genes in response to compressive stress. Gene expression patterns are listed from *top to bottom*, and the time course of compression is presented as time zero (before compression) on the *left* with increasing durations of compression up to 8 h to the *right*. Upregulated genes are indicated in *red* and downregulated genes are indicated in *green*.

examined the net plasminogen activator activity in cell lysates from control and compressive stress-exposed NHBE cells. Membrane-associated proteins were prepared by detergent extraction and a single freeze-thaw cycle. There was increased plasminogen activator activity in response to compressive stress after 8 h and

Figure 3. PCA performed on the 1st and 2nd principal components of the 1,024 most differentially regulated genes. Plasminogen activator genes are indicated by red circles (uPA, UPAR [2 probes], PAI-1, tPA).

24 h of stimulation (0.011 ± 0.002 versus 0.0079 ± 0.002 OD/min, $P = 0.06$, and 0.0090 ± 0.003 versus 0.0033 ± 0.0008 OD/min, $P = 0.033$, respectively) (Figure 6, *bottom*). In addition, the plasmin generation activity of the compressed cell lysates was specific for urokinase as demonstrated by an anti-uPA antibody that inhibited plasmin generation (0.0070 ± 0.0012 OD/min versus 0.0009 ± 0.0004 OD/min, $P = 0.00039$ for 25 $\mu\text{g/ml}$ of anti-uPA, and 0.0070 ± 0.0012 OD/min versus 0.0016 ± 0.0002 OD/min, $P = 0.0058$ for 2.5 $\mu\text{g/ml}$ of anti-uPA).

Compressive Stress Increases MMP Activity in NHBE Cells in a Urokinase-Dependent Manner

Given the enhanced plasminogen activator activity in the pericellular environment of compressed NHBE cells, we sought to measure the presence and activity of MMPs in the same microenvironment. Gelatin zymography of NHBE cell lysates demonstrated increased generation of pro- and active forms of MMP-9 and MMP-2 from 8–24 h after the onset of compression (Figure 7A). Similar analysis of conditioned media failed to demonstrate active forms of either protease (data not shown). Pre-incubation of NHBE cells with an antibody to uPA, but not a nonspecific control antibody, and aprotinin (an inhibitor of plasmin activity) dramatically inhibited the generation of pro- and active MMP-9 and MMP-2 (Figure 7B). Together these results suggest that compressive stress drives increased MMP-2/MMP-9 expression

Figure 4. Real-time PCR results of gene expression of plasminogen activator genes in response to 8 h of continuous compressive stress. uPA, uPAR, PAI-1, and tPA were all significantly increased by compression. Significant increases compared with time zero are indicated by solid black symbols with the magnitude of the significance indicated respectively by the signs above: * $P < 0.05$, + $P < 0.01$, # $P < 0.001$.

and activation through a mechanism involving uPA-mediated plasmin generation.

Gelatin zymography demonstrated increasing generation of activated MMP-9 (88 kD) and activated MMP2 (62 kD) activity

from 8–24 h after the onset of compression (Figure 7A). Pre-incubation of NHBE cells with antibodies to urokinase and aprotinin (an inhibitor of plasmin activity) significantly inhibited the generation of the active forms of MMP-9 and MMP-2, and

Figure 5. uPA, uPAR, PAI-1 protein expression in response to compressive stress. (A) Compressive stress increases urokinase protein concentrations in cell lysates at 8 h and 24 h after the onset of compression. uPAR and PAI-1 concentrations are not altered by compression in the cell lysates (data not shown). Compressive stress increases cell culture media protein concentrations of: (B) soluble uPAR at 8 h and 24 h after the onset of compression, (C) uPA at 24 h after the onset of compression, and (D) PAI-1 at 24 h after the onset of compression.

Figure 5. (Continued).

of pro-MMP-9 and pro-MMP-2, suggesting that there is a link between compression and MMP-9/MMP-2 activity that is mediated through uPA-mediated generation of plasmin (Figure 7B).

Airways of Status Asthmaticus Patients Exhibit Pronounced uPA and uPAR Immunostaining

Our findings with primary bronchial epithelial cells in culture suggested that sustained airway constriction could modify the biology of the airway *in situ*. To test this, we examined preserved postmortem lung tissue from two patients who died from status asthmaticus, and unused lung tissue from seven lung donors without asthma. We performed immunostaining in the fixed tissue to visualize uPA and uPAR protein expression. We observed prominent immunostaining for both uPA and uPAR in the airways of the two patients with status asthmaticus, but little or no immunopositivity in the six lung donor samples (Figures 8A–8D). At higher magnification, notable immunostaining could be seen on the surface of airway epithelial cells in the status asthmaticus samples; again, little staining was observed in the airway epithelium of lung donor tissue (Figures 8E–8H). To verify the specificity of the observed immunostaining, we replaced the uPA and uPAR antibodies with a nonspecific isotype control, and found little staining in the status asthmaticus or lung donor tissues (Figures 8I and 8J). To control for any variation in

the samples, we screened samples with using a murine antibody against human desmin, to ensure that overfixation had not occurred (not shown).

DISCUSSION

We performed gene expression analysis in a cell culture model of bronchoconstriction using DNA microarrays. Among the functional groups of genes upregulated in compressed NHBE cells were a family of plasminogen-related genes that had not been previously recognized to participate in this response; these findings were confirmed using quantitative RT-PCR. We also demonstrated that there is enhanced expression of the protein products of these genes as determined both by immunoassays for the proteins and by uPA activity assays. uPA-blocking studies suggest a role for uPA in the MMP-9 and MMP-2 system, key matrix metalloproteinases in the pathogenesis of asthma (22).

Compressive stress applied to human airway epithelial cells mimics the mechanical effects of bronchoconstriction and initiates cellular responses that may contribute to the chronic changes in asthma (3, 4). Previously published studies of compressed NHBE cells demonstrate upregulation of profibrotic mediators (4), and the ability of this stimulus to alter the phenotype of co-cultured fibroblasts (3), as measured by collagen production. Both cell culture and animal model studies suggest that the

Figure 6. Plasminogen activator activity is increased in compressed cells. Colorimetric assay (405 nm) indicates increased plasmin generation over time (A) in NHBE cell lysates. Plasmin generation is greater in compression stimulated cells compared to unstimulated controls. (B) This effect is seen after both 8 and 24 h after the onset of compression, but only reaches statistical significance at 24 h. An antibody that blocks the activity of uPA confirms that the plasmin generation is due to uPA activity.

mechanotransduction occurs via activation of the EGFR and subsequently MAPK pathway (23, 24). Given the diverse downstream effects of the MAPK pathway, we postulated that this stimulus would lead to the regulation of additional key genes.

We used DNA microarrays to perform a survey of gene expression and confirmed the previously established expression of EGFR ligands (HB-EGF, amphiregulin, epiregulin) (25). We also found that the plasminogen activator group of genes was statistically overrepresented in our panel of surveyed genes as up-regulated in a time-dependent fashion after mechanical compression. PCA analysis showed that the members of this family—namely, uPA, uPAR, and PAI-1—have similar expression characteristics over time.

In addition to gene expression, we found that urokinase protein expression was upregulated in cell lysates and that the uPAR, uPA, and PAI-1 protein expression levels were increased in the media after 24 h. Given the opposing functional activities of uPA-uPAR versus PAI-1, we showed overall increased urokinase activity in compression-stimulated cells. Binding of inactive pro-uPA to uPAR facilitates its activation and therefore plasmin generation, thus focusing extracellular proteolysis to the cell surface (26). uPAR exists as a cell surface receptor anchored by a glycosphosphatidylinositol tail, and in its membrane-bound form may interact with integrins and vitronectin to promote cellular adhesion and migration or induction of paracrine intra-

cellular signaling (27). uPAR may also be cleaved by uPA, producing a soluble form of the receptor (28). In our study we found no mechanical compression-stimulated difference in the cell lysate-associated uPAR protein, but found increased soluble uPAR in the media as a result of compression. Soluble uPAR is a potent chemoattractant for monocyte-like cells; it also has been shown to act as a negative regulator of uPA by acting as a scavenger for uPA (29). Thus the increase in soluble uPAR in response to compression may act as a chemoattractant for other cells types, or may represent an endogenous method of downregulating the plasminogen activator response.

Although the plasminogen system has been well studied in relation to cancer biology, wound healing, and tissue regeneration (26) with roles in cell adhesion, proliferation, and differentiation, there are fewer studies examining its role in asthma. The role of PAI-1 has been studied in a number of pulmonary diseases. Increased PAI-1 levels have been noted in BAL specimens from patients with adult respiratory distress syndrome (30). PAI-1-deficient mice are resistant to pulmonary fibrosis after bleomycin injury (31). In a PAI-1-deficient mouse used in an ovalbumin asthma model, collagen and fibrin deposition was less than that in wild-type mice, suggesting that the plasmin system is important in airway fibrosis in asthma (32). In a microarray study examining gene expression profiling in a nonhuman primate model of asthma, PAI-1 was noted to be up-regulated by antigen challenge

Figure 7. MMP-9/MMP-2 activity by Zymography. (A) pro-MMP-9/MMP-2 and active MMP-9/MMP-2 expression at various time points after the onset of compressive stress. Compressive stress was applied for up to 8 h, and cell lysates were examined for MMP-9/MMP-2 activity at baseline, 4, 8, 12, and 24 h after the onset of compressive stress. Pro-MMP-9 (92 kD) and pro-MMP-2 (72 kD) are present at all time points. Activated MMP-9 (82 kD) and MMP-2 (62 kD) are present from 8–24 h after the onset of compression, but were most apparent at 24 h. (B) MMP-9 and MMP-2 activities were examined in the presence of a urokinase inhibitor, a protease inhibitor that blocks plasmin (aprotinin), and a nonspecific control antibody (IgG₁) as shown in this representative zymogram. Both pro-MMP-9 (92 kD) and the activated MMP-9 (82 kD) are increased by compressive stress. Similarly pro-MMP-2 (72 kD) and the activated MMP-2 (62 kD) are also increased by compressive stress. Addition of an inhibitory antibody to urokinase as well as aprotinin reduced the activated MMP-9 and activated MMP-2 down to control levels.

and IL-4 treatment. Wagers and colleagues identified extravascular fibrin as a potential mediator of airway hyperresponsiveness in both mice and humans (33). They demonstrated increased immunostaining of thrombin in a patient who died from status asthmaticus and subsequently were able to reverse airway hyperresponsiveness in a mouse ovalbumin model by the administration of nebulized tissue plasminogen activator. Another group also found increased activation of the coagulation system in a mouse ovalbumin model of asthma. In this model, administration of activated protein C was able to reduce airway hyperresponsiveness, BAL eosinophils, and BALF IgE levels, as well as IL-4,

-5, and -13 levels (34). A commentary on one of these studies pointed out that the source of plasminogen activator activity was not identified, but it was speculated to derive from sources other than the epithelium (35). Our data provide unequivocal evidence that NHBE cells can be a source of this activity, although the relative contribution of this source to the total activity available *in vivo* is not known.

Our findings confirms previous work by Gerwin and coworkers (36), who reported a similar finding of increased uPA and PAI-1 gene expression in human bronchial epithelial cells stimulated with TGF- β . Furthermore, given the major function of the

Figure 8. Immunostaining for uPA and uPAR in human lung tissue. Representative images are shown from one of six lung donors, and one of two patients who died in status asthmaticus. Low-power fields (original magnification: $\times 10$) for uPA (A, B) and uPAR (C, D) reveal increased staining in status asthmaticus (B, D) relative to lung donor (A, C) tissue. Higher-power fields (original magnification: $\times 40$) highlight the increased epithelial staining for uPA (E, F) and uPAR (G, H) in status asthmaticus (F, H) relative to lung donor (E, G) airways. A relative absence of epithelial staining was found with a nonspecific isotype control antibody in both lung donor (I) and status asthmaticus (J) airway epithelium, although some nonspecific reactivity was seen in airway smooth muscle.

plasminogen activator system in fibrinolysis and the prothrombotic state of status asthmaticus, the induction of the plasminogen activator system could be a protective response aimed at minimizing detrimental fibrin deposition in the airways.

Our immunohistochemical analysis of uPA and uPAR expression in the airways further reinforces our finding that the airway epithelium can be a source of urokinase expression and activity. Moreover, the prominent expression of these proteins in the airways of status asthmaticus tissues, relative to lung donor tissues, suggests a disease-related role for these proteins. Clearly, the expression of uPA and uPAR in the airway is not restricted to the epithelium only. Moreover, multiple factors present in status asthmaticus airways likely contribute to their regulation. Nevertheless, the mechanical environment in these airways is profoundly different than under normal circumstances; our *in vitro* data strongly suggest that this altered mechanical environment contributes to the increased presence of urokinase system components.

Our findings are consistent with a recent study demonstrating increased gene expression of TGF- β 1 and PAI-1 that localizes to the epithelium in a chronic ovalbumin model of asthma (37). Given the role of plasmin in TGF- β activation, the findings of increased plasmin generation may also explain the mechanism of increased activated TGF- β 2 released by NHBE under compressive stress as we have previously demonstrated (4).

The importance of MMP-9 in asthma is well recognized (38–43). But the mechanism by which it is induced is not clear. We postulate that the uPA-plasmin system may activate MMP-9, thereby contributing to the pathology in asthma. Interestingly, uPA (but not plasmin) upregulates pro-MMP-9 expression and secretion by monocyte-like cell lines *in vitro* by increasing MMP-9 steady-state mRNA levels (44, 45). Furthermore, in an NHBE cell wound repair model, MMP-9 has been shown to be dependent on uPA (46). NHBE cellular migration and MMP-9 activation was shown to occur through uPA and the plasminogen system in both *ex vivo* and *in vitro* human bronchial epithelial cells. We demonstrate the generation of active MMP-9 in NHBE cells after mechanical compression of airway epithelial cells, and show that the MMP-9 system can be modulated by the uPA system. Thus bronchoconstriction leading to mechanical compression may contribute to airway remodeling by stimulating the expression of uPA, which activates the MMP-9.

We focused our studies on plasmin-mediated activation of pro-MMP-9 in the cell lysates fraction. In preliminary studies we were unable to detect active forms of MMP-9 in the supernatant samples from cells, but did find prominent active MMP-9 in cell lysate samples. This may be due to the activity of tissue inhibitors of MMPs (TIMPs) 1–4, which are high-affinity inhibitors of MMPs present in extracellular fluids at micromolar concentrations that can rapidly inactivate soluble forms of active MMPs generated in the extracellular space (47). However, MMP activity is readily detected in pericellular environments, even in the presence of fluids containing TIMPs (48, 49). Recent work from our laboratories has shown that MMP-9 is expressed in an active, TIMP-resistant form on the surface of polymorph nuclear cells, and cell membrane-associated MMP-9 has catalytic activity and efficiency similar to that of the soluble form of MMP-9 (48). For this reason, we focused our studies on plasmin-mediated activation of pro-MMP-9 in the cell lysates fraction.

Although MMP-9 is the major gelatinase produced by epithelial cells, we have demonstrated that compression also induced activation of pro-MMP-2 associated with epithelial cells. Lung epithelial cells are known to produce pro-MMP-2 (50, 51) and MMP-2 has a very similar spectrum of catalytic activity when compared with MMP-9 (gelatinase B). Pro-MMP-2 binds to the surface of tumor cells, fibroblasts, and endothelial cells, where

it is activated in a ternary complex with TIMP-2 and MT1-MMP (52, 53), and plasmin has been shown to act synergistically with these molecules in generating the fully active, mature 62-kD MMP-2 on cell surfaces (54). It is also noteworthy in this respect that MMP-2 regulates airway inflammation in a murine model of airway hyperresponsiveness (55). It is likely that the generation of active MMP-9 and active MMP-2 by epithelial cells that are compressed have additive effects in the remodeling processes occurring in the airways of individuals with asthma.

In summary, we identified several groups of mechanoresponsive genes using DNA microarrays. One group of plasminogen activator genes was regulated at a protein level and functional level by compressive stress. The activation of this pathway may have important downstream effects, such as activating MMP-9, that could lead to an altered airway phenotype. These findings highlight the ability of a noninflammatory stimulus to contribute to the pathogenesis of asthma.

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References

1. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003;111:291–297.
2. Davies DE, Wicks J, Powell RM, Puddicombe SM, Holgate ST. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 2003; 111:215–225; quiz 226.
3. Swartz MA, Tschumperlin DJ, Kamm RD, Drazen JM. Mechanical stress is communicated between different cell types to elicit matrix remodeling. *Proc Natl Acad Sci USA* 2001;98:6180–6185.
4. Tschumperlin DJ, Shively JD, Kikuchi T, Drazen JM. Mechanical stress triggers selective release of fibrotic mediators from bronchial epithelium. *Am J Respir Cell Mol Biol* 2003;28:142–149.
5. Wiggs BR, Hrousis CA, Drazen JM, Kamm RD. On the mechanism of mucosal folding in normal and asthmatic airways. *J Appl Physiol* 1997;83:1814–1821.
6. Ruwhof C, van Wamel AE, Egas JM, van der Laarse A. Cyclic stretch induces the release of growth promoting factors from cultured neonatal cardiomyocytes and cardiac fibroblasts. *Mol Cell Biochem* 2000;208: 89–98.
7. Malek AM, Greene AL, Izumo S. Regulation of endothelin 1 gene by fluid shear stress is transcriptionally mediated and independent of protein kinase C and cAMP. *Proc Natl Acad Sci USA* 1993;90:5999–6003.
8. van Wamel AJ, Ruwhof C, van der Valk-Kokshoom LE, Schrier PI, van der Laarse A. The role of angiotensin II, endothelin-1 and transforming growth factor-beta as autocrine/paracrine mediators of stretch-induced cardiomyocyte hypertrophy. *Mol Cell Biochem* 2001; 218:113–124.
9. Wang DL, Tang CC, Wung BS, Chen HH, Hung MS, Wang JJ. Cyclical strain increases endothelin-1 secretion and gene expression in human endothelial cells. *Biochem Biophys Res Commun* 1993;195:1050–1056.
10. Ohno M, Cooke JP, Dzau VJ, Gibbons GH. Fluid shear stress induces endothelial transforming growth factor beta-1 transcription and production: modulation by potassium channel blockade. *J Clin Invest* 1995;95:1363–1369.
11. Kurihara H, Yoshizumi M, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, Hamaoki M, Kato H, Yazaki Y. Transforming growth factor-beta stimulates the expression of endothelin mRNA by vascular endothelial cells. *Biochem Biophys Res Commun* 1989;159:1435–1440.

12. Randell SH, Walstad L, Schwab UE, Grubb BR, Yankaskas JR. Isolation and culture of airway epithelial cells from chronically infected human lungs. *In Vitro Cell Dev Biol Anim* 2001;37:480-489.
13. Yoon JH, Gray T, Guzman K, Koo JS, Nettekheim P. Regulation of the secretory phenotype of human airway epithelium by retinoic acid, triiodothyronine, and extracellular matrix. *Am J Respir Cell Mol Biol* 1997;16:724-731.
14. Ressler B, Lee RT, Randell SH, Drazen JM, Kamm RD. Molecular responses of rat tracheal epithelial cells to transmembrane pressure. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1264-L1272.
15. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003;31:e15.
16. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185-193.
17. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001;98:5116-5121.
18. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998;95:14863-14868.
19. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003;4:3.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-408.
21. McGowan R, Biliran H Jr, Sager R, Sheng S. The surface of prostate carcinoma DU145 cells mediates the inhibition of urokinase-type plasminogen activator by maspin. *Cancer Res* 2000;60:4771-4778.
22. Chapman HA. Disorders of lung matrix remodeling. *J Clin Invest* 2004;113:148-157.
23. Tschumperlin DJ, Shively JD, Swartz MA, Silverman ES, Haley KJ, Raab G, Drazen JM. Bronchial epithelial compression regulates MAP kinase signaling and HB-EGF-like growth factor expression. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L904-L911.
24. Tschumperlin DJ, Dai G, Maly IV, Kikuchi T, Laiho LH, McVittie AK, Haley KJ, Lilly CM, So PT, Lauffenburger DA, et al. Mechanotransduction through growth-factor shedding into the extracellular space. *Nature* 2004;429:83-86.
25. Chu EK, Foley JS, Cheng J, Patel AS, Drazen JM, Tschumperlin DJ. Bronchial epithelial compression regulates EGFR family ligand expression in an autocrine manner. *Am J Respir Cell Mol Biol* 2005;32:373-380.
26. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol* 2002;3:932-943.
27. Preissner KT, Kanse SM, May AE. Urokinase receptor: a molecular organizer in cellular communication. *Curr Opin Cell Biol* 2000;12:621-628.
28. Montuori N, Carriero MV, Salzano S, Rossi G, Ragno P. The cleavage of the urokinase receptor regulates its multiple functions. *J Biol Chem* 2002;277:46932-46939.
29. Jo M, Thomas KS, Wu L, Gonias SL. Soluble urokinase-type plasminogen activator receptor inhibits cancer cell growth and invasion by direct urokinase-independent effects on cell signaling. *J Biol Chem* 2003;278:46692-46698.
30. Bertozzi P, Astedt B, Zenzius L, Lynch K, LeMaire F, Zapol W, Chapman HA Jr. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. *N Engl J Med* 1990;322:890-897.
31. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 1996;97:232-237.
32. Oh CK, Ariue B, Alban RF, Shaw B, Cho SH. PAI-1 promotes extracellular matrix deposition in the airways of a murine asthma model. *Biochem Biophys Res Commun* 2002;294:1155-1160.
33. Wagers SS, Norton RJ, Rinaldi LM, Bates JH, Sobel BE, Irvin CG. Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. *J Clin Invest* 2004;114:104-111.
34. Yuda H, Adachi Y, Taguchi O, Gabazza EC, Hataji O, Fujimoto H, Tamaki S, Nishikubo K, Fukudome K, D'Alessandro-Gabazza CN, et al. Activated protein C inhibits bronchial hyperresponsiveness and Th2 cytokine expression in mice. *Blood* 2004;103:2196-2204.
35. Matthay MA, Clements JA. Coagulation-dependent mechanisms and asthma. *J Clin Invest* 2004;114:20-23.
36. Gerwin BI, Keski-Oja J, Seddon M, Lechner JF, Harris CC. TGF-beta 1 modulation of urokinase and PAI-1 expression in human bronchial epithelial cells. *Am J Physiol* 1990;259:L262-L269.
37. Kelly MM, Leigh R, Bonniaud P, Ellis R, Wattie J, Smith MJ, Martin G, Panju M, Inman MD, Gaudie J. Epithelial expression of profibrotic mediators in a model of allergen-induced airway remodeling. *Am J Respir Cell Mol Biol* 2005;32:99-107.
38. Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 2003;28:12-24.
39. Tonnel AB, Gosset P, Tillie-Leblond I. Characteristics of the inflammatory response in bronchial lavage fluids from patients with status asthmaticus. *Int Arch Allergy Immunol* 2001;124:267-271.
40. Lemjabbar H, Gosset P, Lamblin C, Tillie I, Hartmann D, Wallaert B, Tonnel AB, Lafuma C. Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med* 1999;159:1298-1307.
41. Mautino G, Henriquet C, Jaffuel D, Bousquet J, Capony F. Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *Am J Respir Crit Care Med* 1999;160:324-330.
42. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998;102:783-788.
43. Cataldo DD, Tournoy KG, Vermaelen K, Munaut C, Foidart JM, Louis R, Noel A, Pauwels RA. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol* 2002;161:491-498.
44. Menshikov M, Elizarova E, Plakida K, Timofeeva A, Khaspekov G, Beabealashvili R, Bobik A, Tkachuk V. Urokinase upregulates matrix metalloproteinase-9 expression in THP-1 monocytes via gene transcription and protein synthesis. *Biochem J* 2002;367:833-839.
45. Menshikov MY, Elizarova EP, Kudryashova E, Timofeyeva AV, Khaspekov Y, Beabealashvili RS, Bobik A. Plasmin-independent gelatinase B (matrix metalloproteinase-9) release by monocytes under the influence of urokinase. *Biochemistry (Mosc)* 2001;66:954-959.
46. Legrand C, Polette M, Tournier JM, de Bentmann S, Huet E, Monteau M, Birembaut P. uPA/plasmin system-mediated MMP-9 activation is implicated in bronchial epithelial cell migration. *Exp Cell Res* 2001;264:326-336.
47. Owen CA, Campbell EJ. The cell biology of leukocyte-mediated proteolysis. *J Leukoc Biol* 1999;65:137-150.
48. Owen CA, Hu Z, Barrick B, Shapiro SD. Inducible expression of tissue inhibitor of metalloproteinases-resistant matrix metalloproteinase-9 on the cell surface of neutrophils. *Am J Respir Cell Mol Biol* 2003;29:283-294.
49. Owen CA, Hu Z, Lopez-Otin C, Shapiro SD. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. *J Immunol* 2004;172:7791-7803.
50. d'Ortho MP, Clerici C, Yao PM, Delacourt C, Delclaux C, Franco-Montoya ML, Harf A, Lafuma C. Alveolar epithelial cells in vitro produce gelatinases and tissue inhibitor of matrix metalloproteinase-2. *Am J Physiol* 1997;273:L663-L675.
51. Yao PM, Buhler JM, d'Ortho MP, Lebarry F, Delclaux C, Harf A, Lafuma C. Expression of matrix metalloproteinase gelatinases A and B by cultured epithelial cells from human bronchial explants. *J Biol Chem* 1996;271:15580-15589.
52. Murphy G, Stanton H, Cowell S, Butler G, Knauper V, Atkinson S, Gavrilovic J. Mechanisms for pro matrix metalloproteinase activation. *APMIS* 1999;107:38-44.
53. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase: isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 1995;270:5331-5338.
54. Baramova EN, Bajou K, Remacle A, L'Hoir C, Krell HW, Weidle UH, Noel A, Foidart JM. Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. *FEBS Lett* 1997;405:157-162.
55. Corry DB, Rishi K, Kanellis J, Kiss A, Song LZ, Xu J, Feng L, Werb Z, Kheradmand F. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. *Nat Immunol* 2002;3:347-353.