Pivotal Role of Cathepsin K in Lung Fibrosis

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The paramount importance of the homeostasis of the extracellular matrix for pulmonary function is exemplified by two opposing extremes: emphysema and pulmonary fibrosis. This study examined the putative role of cathepsin K (catK) in the pathology of lung fibrosis in mice and its relevance to the human disease activity. We compared the induction of lung fibrosis by administration of bleomycin. CTSK^{-/-} mice deposited significantly more extracellular matrix than control mice. Primary lung fibroblasts derived from CTSK^{-/-} mice showed a decreased collagenolytic activity indicating the role of catK in collagen degradation. Interestingly, CTSK^{+/+} control mice revealed an increased expression of catK in fibrotic lung regions suggesting a protective role of catK to counter the excessive deposition of collagen matrix in the diseased lung. Similarly, in lung specimens obtained from patients with lung fibrosis fibroblasts expressed larger amounts of catK than those obtained from normal lungs. Activation of human pulmonary fibroblasts in primary cell cultures led to an increased activity of catK through enhanced gene transcription and protein expression and to increased intracellular collagenolytic activity. We believe that this is the first study to show that catK plays a pivotal role in lung matrix homeostasis under physiological and pathological conditions. (*Am J Pathol* 2004, 164:2203–2216)

Homeostasis of deposition and recycling of extracellular matrix (ECM) proteins is of general importance for the physiological function of almost all organs and especially that of the lung. The lung provides an excellent example of the hazardous effects of two opposing extremes: predominant matrix degradation leading to emphysema and predominant matrix deposition leading to pulmonary fi-

brosis.^{1–3} Interstitial fibrosis of the lung is a final common pathway of many different lung diseases, such as idiopathic interstitial pneumonitis (idiopathic pulmonary fibrosis) and granulomatous diseases (sarcoidosis).^{1,4,5} Fibrotic areas are also commonly found in patients suffering from chronic obstructive pulmonary disease⁶ and, therefore, lung fibrosis is an urgent clinical problem.

Fibrosis is characterized by interstitial accumulation of matrix proteins produced by activated fibroblasts.¹ Depending on the localization and amount of fibrosis, organ integrity and function can be seriously disrupted.⁷ A continuous ECM turnover exists under physiological conditions.⁸ The dynamic equilibrium between synthesis and degradation maintains the physiological balance and is tightly controlled by *de novo* synthesis and deposition of ECM, and proteolytic degradation of existing ECM.⁹ Under pathological conditions, the balance between ECM production and degradation may deteriorate, resulting in an increased amount of ECM, which then progressively impairs organ function. Whereas the signals leading to an increased secretion of ECM proteins are well understood^{10,11} the molecular mechanisms limiting matrix deposition are poorly defined.

A number of studies addressed the function of matrix metalloproteases (MMP) in ECM turnover of the lung. They have shown that the MMPs 2, 7, 9, and 12 and the tissue inhibitors of metalloproteases play an important role in the pathogenesis of lung fibrosis.^{12–18} It was suggested that there is a higher expression of tissue inhibitors of metalloproteases compared with MMPs in idiopathic pulmonary fibrosis and in mouse models where fibrosis is induced by transforming growth factor- β 1 overexpression. This generates a nondegrading microenvironment and thus leads to development of fibrosis.^{15,18} On the other hand, it has been shown that MMP7 knockout mice are protected from pulmonary fibrosis in response to bleomycin. The MMP inhibitor Batimastat prevented the development of bleomycin-induced lung fibrosis. This was accompanied by a suppression of the inflammatory reaction after Batimastat administration.^{16,17} In summary these data suggest that MMPs play a dual role in the degradation of deposited ECM and in

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the regulation of chronic inflammatory reactions that are associated with the development of lung fibrosis.

Cysteine proteases have been linked with matrix turnover: they degrade existing ECM¹⁹ and limit the release of newly synthesized ECM from fibroblasts.²⁰ Within the papain-cysteine proteinase family, three proteases show significant matrix-degrading activities, ie, cathepsin (cat)K, catL, and catS. Among these, catK is the most potent mammalian elastase yet described and it has been shown to possess a unique collagenolytic activity.^{21,22} This activity does not depend on destabilization of the triple helix, but rather it cleaves type I and II collagen at the ends (telopeptide) and at multiple sites within the native triple helix. The role of catK in bone turnover is well established; mutations in the catK gene lead to pycnodysostosis and catK knockout (CTSK^{-/-}) mice develop osteopetrosis.^{23,24} However, evidence is increasing that catK is also involved in pathological processes unrelated to bone remodeling, eg, granulomatous diseases, amyloidosis, and atherosclerosis.^{25–30}

The pathophysiological significance of proteases in lung disease is exemplified by α 1-anti-trypsin deficiency: the loss of protease-inhibiting function leads to emphysema. Among others, elastase and cathepsins may contribute to the pathogenesis of emphysema.³¹ However, the pathophysiological role of proteases in lung fibrosis is less well understood.

Based on our initial observation that lung specimens obtained from fibrotic lung tissues in mice and man contained larger amounts of catK than normal lung specimens, we sought to determine whether catK is involved in the pathogenesis of lung fibrosis. CTSK^{-/-} mice showed increased alveolar wall thickness after bleomycin treatment and deposited significantly more collagen compared with control mice. CatK-deficient fibroblasts derived from CTSK^{-/-} mice showed decreased collagenolytic activities. We believe that this is the first study to show that catK plays a pivotal role in lung matrix homeostasis under physiological and pathological conditions.

Materials and Methods

Human Tissues

Tissue samples from patients with ($n = 7$) and without ($n = 15$) lung fibrosis were obtained from diagnostic open lung biopsies (fibrotic samples) or from healthy tissue areas during pneumonectomy for tumor resection (nonfibrotic samples). The fibrotic samples contained the following entities: usual interstitial pneumonia (UIP, three patients), nonspecific interstitial pneumonia (NSIP, one patient), bronchiolitis obliterans-organizing pneumonia (BOOP, three patients).¹ Although these diseases were different in clinical behavior and outcome they are characterized by increased amounts of activated fibroblasts and increased matrix deposition. The histopathological classification of the tissue samples was performed by an experienced pathologist (CR). Tissue samples were obtained immediately after surgery, snap-frozen in liquid

nitrogen, and stored at -80°C until required. For histological processing, tissue samples were fixed in 10% neutralized formalin and embedded in paraffin.

Animals

CatK-knockout mice (CTSK^{-/-}) and their catK-positive littermates (CTSK^{+/+}) were used.²⁴ The mice were on mixed Bl6/J129 background. For the experiments we used homozygote siblings from mating of heterozygous animals. Experimental animals were 8 to 12 weeks old and included males and females. Altogether 24 CTSK^{-/-} and 24 CTSK^{+/+} mice were included. These animals were bleomycin treated for 10 or 14 days or served as controls (eight animals per group). All animal experiments were performed in accordance with protocols approved by the local animal care advisory committee.

Bleomycin Treatment

A single dose of 0.075 U bleomycin (Canceronova GmbH, Reute, Germany) in 50 μl of sterile Hanks' solution (Biochrom, Berlin, Germany) was administered by tracheal puncture under intraperitoneal Avertine anesthesia (0.5 mg/g body weight). This procedure and dose of bleomycin has been previously shown to produce pulmonary fibrosis in mice of similar genetic background.³² The mice were anesthetized with pentobarbital immediately or 10 and 14 days after bleomycin application, and lung vasculature was perfused free of blood by slowly injecting 2 ml of Hanks' solution into the right ventricle. Lungs were removed and the right lung was shock-frozen and stored in liquid nitrogen until further use. The left lung was fixed by infiltrating 4% saline-buffered formalin and embedded in paraffin.

Quantitative Image Analysis

Deparaffinized serial sections obtained from murine lung tissue were stained with hematoxylin and eosin (H&E) or a modified trichrome stain.³³ Wall thickness in H&E-stained sections (alveolar wall fraction) and the amount of ECM deposited in the trichrome stain (fibrosis fraction) were quantified using the MetaVue Image analysis software (Universal Imaging Corp., Downingtown, PA). One entire tissue section of each mouse lung was systematically captured by a video camera (Spot RT; Diagnostic Instr. Corp., Burroughs, MI) applying a $\times 20$ objective lens (18 to 32 fields per tissue section). Overall 952 fields were analyzed. The image analysis software was configured to measure the area covered by H&E-stained tissue (alveolar wall fraction) or deposited ECM (fibrosis fraction) and to divide these values by the constant field of interest, thereby deriving a percentage area value for each slide. Percentage alveolar wall area fraction and fibrosis fraction data are presented as mean $\pm$ SEM values for each animal group.

Hydroxyproline Quantification

The collagen content of lung tissue was determined as previously described by assay of lung hydroxyproline content after hydrolysis in 6 N of HCl.³⁴ Minced lung tissue was hydrolyzed overnight at 110°C, neutralized using 5 N of NaOH, and desiccated. Next, the hydrolysate was resuspended in 200 μ l of deionized water. Triplicates of 60 μ l of supernatant were transferred to a 96-well plate and 20- μ l assay buffer (*n*-propanol, deionized water, and stock puffer in a ratio of 3:2:10, with the stock buffer consisting of 0.24 mol/L citric acid, 0.88 mol/L sodium acetate, 0.21 mol/L acetic acid, 0.85 mol/L sodium hydroxide, pH 6.1) were added. The colorimetric assay was performed by addition of chloramine-T reagent (28 mg chloramine-T, 100 μ l *n*-propanol, 800 μ l deionized water) for 15 minutes at room temperature and subsequently of 80 μ l DMBA reagent (2 g dimethylaminobenzaldehyde in 1.25 ml *n*-propanol and 2.75 ml perchloric acid) for 20 minutes at 60°C. The extinction was read at 570 nm. The collagen content of the tissues was estimated from a collagen I standard curve made up in deionized water.

Immunohistochemistry

Immunostaining was performed on serial sections with monoclonal antibodies directed against human catL (33/2, 40 ng/ml),³⁵ human vimentin (70 ng/ml; DAKO, Glostrup, Denmark,) and with polyclonal antibodies directed against human or mouse catK (dilution, 1:1500).³⁶ The amount of infiltrating granulocytes as a measure of the inflammatory reaction was quantified after myeloperoxidase staining using a rabbit polyclonal antibody (NeoMarkers, Fremont, CA).³⁷ Biotin-conjugated anti-mouse or anti-rabbit sera (1:200, both from Vector Laboratories, Burlingame, CA) were used as secondary antibodies. Before immunostaining, the specimens were boiled in citrate buffer for 5 minutes (CatL and vimentin). Immunoreactions were visualized using the avidin biotin complex method applying Vectastain ABC peroxidase or alkaline phosphatase kits (Vector Laboratories). 3,3'-Diaminobenzidine or FastRed were used as substrates. The sections were counterstained with hematoxylin. The specificity of immunostaining was tested by substitution of primary antibodies with unrelated antibodies or preimmune serum or preincubating the antiserum with saturating amounts of purified antigen. The specificity of the antiserum against mouse catK was evaluated using lung tissues of catK knockout mice.

Immunofluorescence Labeling

Fibroblasts were grown on cover slides and immunolabeled using a monoclonal antibody directed against the lysosomal Lamp-1 antigen (BD, Heidelberg, Germany), a rabbit anti-catK antibody, and the DNA-stain 4,6-diamidino-2-phenylindole (Partec GmbH, Münster, Germany). Immunoreactivity was detected using fluorescein-labeled anti-mouse antibody and phycoerythrin-labeled

anti-rabbit antibody (both Dianova, Hamburg, Germany). The immunostaining was analyzed using a confocal laser-scanning microscope (Leica TCS SP2; Leica Microsystems, Mannheim, Germany).

Cell Culture

Fibroblasts were obtained by mincing freshly excised lung parenchyma into $\sim 1\text{-mm}^3$ pieces, followed by digestion with collagenase IV (1 mg/ml; Sigma, Deisenhofen, Germany) for 30 minutes at 37°C. Fibroblasts were cultured in 75-mm³ tissue culture flask in Iscove's modified Dulbecco's medium with 10% (w/v) fetal calf serum (10^{-3} mol/L glutamine and antibiotics at 37°C and 5% (v/v) CO₂ until they reached confluence. Only fibroblasts between passages three and eight were used for the experiments.

Western Blot Analyses

Fibroblasts were lysed using 0.5% Triton X-100 in phosphate-buffered saline (PBS, pH 7.5) on ice for 20 minutes and centrifuged at 12,000 $\times g$. The supernatant was removed and stored at -80°C until further use. Aliquots (3 μ g of protein) of fibroblast lysates were separated using 4 to 12% NuPage Bis-Tris Gel (Novex/Invitrogen, Carlsbad, CA) and blotted to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Little Chalfont, UK). The membranes were blocked using nonfat dry milk (16 hours, room temperature) and incubated with affinity-purified catK antiserum (1:50) or anti-catL antibody (33/2, 10 ng/ml). The immunoreaction was detected using polyclonal antisera directed against rabbit or mouse IgG conjugated to alkaline phosphatase (1:5000; Dianova, Hamburg, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, Madison, WI) as substrate.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For RT-PCR, total RNA was isolated from cells using the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed by the First Strand DNA Synthesis kit (Amersham Pharmacia Biotech, Freiburg, Germany). The PCR reaction was optimized with the following primer pairs (5'-3'): catL sense, CAG GCA GGT GAT GAA TGG CT; catL anti-sense, CAG GCC TCC ATT ATC CTG AA; catK sense, GAA CCG GGG TAT TGA CTC T; catK anti-sense, CAG GCG TTG TTC TTA TTT C; β -actin sense, TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA; and β -actin anti-sense, CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG. DNA amplification was performed in 50 mmol/L Tris-HCl, 3 mmol/L MgCl₂, 25 μ g/ml bovine serum albumin, 0.5 μ mol/L of each primer, 0.2 mmol/L dNTP, and 0.4 U of *Taq* polymerase (Invitex, Berlin, Germany) using the LightCycler (Idaho Technologies, Idaho Falls, ID). Reactions were cycled 40 times in glass capillaries (95°C denaturation, 66°C annealing for 3 seconds,

and 72°C extension for 13 seconds). At the end of each extension phase, product accumulation was monitored at a temperature 5°C below the product melting temperature. PCR product specificity was verified by taking a melting curve and by agarose gel electrophoresis. Data were analyzed using the LightCycler software. Serial dilutions of plasmid DNA containing the cloned amplicons were used as calibration standards with calculated absolute number of copies in tissue extracts. Results for cathepsin mRNA expression in fibroblasts were calculated in relation to β -actin mRNA expression.

Measurement of Enzymatic Activities

The surplus in endogenous inhibitors prevents the measurement of catK and catL activities in complete cell lysates. Therefore, the cellular endosomal/lysosomal fraction was prepared as described by Tournu and colleagues.³⁸ Cells (2×10^6) were homogenized with 0.25 mol/L saccharose containing 25 mmol/L HEPES and 2 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4, using a needle and syringe. The homogenate was centrifuged at $2000 \times g$ for 5 minutes. The resulting supernatant was centrifuged at $33,000 \times g$ for 20 minutes. The pellet, referred to as endosomes/lysosomes, was resuspended in 20 mmol/L of sodium acetate buffer, pH 5.5, containing 2.5 mmol/L EDTA and 2.5 mmol/L dithiothreitol. For release of the lysosomal content, the endosomes/lysosomes were shock-frozen and stored at -80°C for at least 2 hours. Enzyme activities of catK and catL were determined as described elsewhere^{39,40} with minor modifications. The activity of catK was measured against the substrate Z-GPR-MCA (20 $\mu\text{mol/L}$) in the presence of a specific catB inhibitor (CA-074, 20 $\mu\text{mol/L}$; Bachem, Heidelberg, Germany). The catL-like activity was assessed by the hydrolysis of Z-FR-MCA (5 $\mu\text{mol/L}$; Bachem) in the presence of the catB-specific inhibitor.

Collagenolytic activities were measured using the Enz-Check collagenase assay (Molecular Probes, Leiden, The Netherlands) with minor modifications. For the detection of cathepsin-mediated collagenolytic activity the lysosomal/endosomal fractions were incubated with fluorescein-labeled collagen for 16 hours at 37°C . The assay buffer provided by the manufacturer was substituted by sodium acetate buffer (pH 5.5). EDTA (5 mmol/L) was added to block the activity of metalloproteases.³⁹ MMP-mediated collagenolytic activities were determined in complete cell lysates or in cell culture supernatants. For generation of cell culture supernatants the cells were grown to confluence. Next, the growth medium was replaced by medium without serum (Ham's F12, Invitrogen) and the cells were cultured for another 48 hours. After this time the cell-free supernatants were used for the measurement of the collagenolytic activities. The assay was performed as suggested by the manufacturer in the presence of the cathepsin inhibitor E64 (20 $\mu\text{mol/L}$; Sigma). The resulting activities were normalized to the amount of protein in the reaction mixture (cell lysates) or to the protein concentration in the lysates of the cells that were used for the generation of culture supernatants. In control

experiments the cathepsin activity was suppressed by E64 and the MMP activity by 1,10-phenantroline (100 $\mu\text{mol/L}$, Sigma).

Transmission Electron Microscopy

Human fibroblasts were grown on thick collagen layers for 5 days (1 mg/ml of collagen I). Then the collagen clots containing the fibroblasts were fixed in a 0.1 mol/L cacodylate-buffered mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde, pH 7.35. Processing of collagen clots was performed as described.⁴¹ Briefly, after six rinses throughout a 30-minute period in 0.1 mol/L cacodylate buffer, tissue blocks were osmicated in 1% OsO_4 in 0.1 mol/L cacodylate buffer for 2 hours, washed again in 0.1 mol/L cacodylate buffer (four rinses throughout a 20-minute period), rinsed in twice-distilled water (two rinses throughout a 10-minute period), and transferred to half-saturated aqueous uranyl acetate for *en bloc* staining overnight. All steps were performed at 8°C . After washing in twice-distilled water (six changes throughout a 30-minute period), samples were dehydrated through an ascending series of ethanol, transferred to Araldite via propylene oxide and a 1:1 mixture of propylene oxide-Araldite, embedded in Araldite, and polymerized at 60°C for 3 days. From each block, ultra thin sections were cut and counterstained with lead citrate, using an Ultrastainer (Leica, Bensheim, Germany). Qualitative and stereological analysis by transmission electron microscopy was performed using an EM 902 (LEO, Oberkochen, Germany).

Determination of Collagen and ECM Deposition

Collagen secretion and deposition into ECM was assessed by proline incorporation assays originally developed by Peterkofsky and Diegelmann⁴² and described in detail earlier.^{7,43} All assays were performed in triplicates. Briefly, 5×10^4 fibroblasts were seeded into 24-well plates (Falcon, Heidelberg, Germany) in culture medium containing 10% fetal calf serum. After 16 hours the medium was exchanged by low-serum medium (Dulbecco's modified Eagle's medium supplemented with 0.1% fetal calf serum, 100 $\mu\text{g/ml}$ L-ascorbic acid) containing $[2,3,4,5\text{-}^3\text{H}]\text{-L-proline}$ (2 $\mu\text{Ci/ml}$; NEN, Boston, MA). When indicated, E64d was added (10 $\mu\text{mol/L}$). After 72 hours the culture medium was removed and remaining fibroblasts were lysed with distilled water (10 minutes, room temperature). ECM was ethanol-fixed (70% ethanol, 15 minutes, room temperature). One half of the wells was incubated with 30 U/ml of collagenase (*Clostridium histolyticum*; Sigma) in collagenase assay buffer (50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L CaCl_2 , 2.5 mmol/L *N*-ethylmaleimide) for 4 hours at 37°C . The remaining wells were incubated with assay buffer. The supernatants were removed and residual ECM was solubilized by overnight incubation in 0.3 mol/L of NaOH-1% sodium dodecyl sulfate. Equal aliquots of supernatants after collagenase digestion and supernatants containing the residual ECM were subjected to liquid scintillation counting. The counts measured in supernatants after collagenase treatment

Figure 1. CTSK^{-/-} mice developed more lung fibrosis than CTSK^{+/+} mice. **a:** Quantitative image analysis of lung tissue sections expressed as alveolar wall fraction. The alveolar wall fraction was higher after bleomycin treatment in CTSK^{-/-} mice than in CTSK^{+/+} littermates. **b:** Increased collagen concentration in lung tissues after bleomycin challenge. The collagen concentration was determined by hydroxyproline assay. **c:** Number of myeloperoxidase-positive inflammatory cells in the lungs of control and knockout animals. There was no significant difference between knockout and control animals. Values of eight mice in each group are presented as means $\pm$ SEM. **Solid circles,** CTSK^{-/-} mice; **open squares,** CTSK^{+/+} mice. *, $P < 0.05$.

represent the collagen content. [³H]-proline measured after solubilization of the remaining ECM represents non-collagenous ECM. The total of both counts was equal to the counts from solubilized ECM without collagenase treatment and represents the total proline incorporation. Relative ECM synthesis can be calculated by the established formula:⁴³ ECM = cpm in collagen + 5.4*cpm in noncollagen ECM. The formula contains the factor 5.4 to correct the 5.4-fold higher proline or hydroxyproline content of collagens compared with that of other proteins.

Statistical Analysis

All statistical analyses were performed with SPSS 10.0 for Windows (SPSS, Chicago, IL). Results were presented as mean values $\pm$ SE. Mean values were compared by Student's *t*-test. In addition the data were analyzed using the nonparametric Mann-Whitney *U*-Test. Differences were considered to be significant if the *P* values were <0.05 in both tests.

Results

We have previously shown that catK expression is not restricted to bone tissue. A major extraskelletal site of catK expression is the lung.²⁷ CatK expression was found in bronchial epithelial cells and in multinucleated giant cells but not in alveolar macrophages.²⁸ To investigate the role of catK in lung fibrosis, we compared the development of fibrotic changes after bleomycin administration in the lungs of CTSK^{-/-} and CTSK^{+/+} mice and analyzed the expression of catK in mouse and human lung tissues.

CTSK^{-/-} Mice Develop More Lung Fibrosis than Wild-Type Mice

After administration of bleomycin, CTSK^{-/-} mice developed a much more pronounced alveolar wall remodeling:

The alveolar wall fraction was increased by 18% in CTSK^{-/-} mice *versus* 6% in CTSK^{+/+} mice (Figure 1a). The collagen concentration was increased 3.1-fold in CTSK^{-/-} mice *versus* 2.1-fold in CTSK^{+/+} mice (Figure 1b). In addition, the fibrosis fraction was increased significantly (Figure 2, B and C). At the same time there was no significant difference in the amount of infiltrating myeloperoxidase-positive granulocytes in CTSK^{-/-} mice compared to CTSK^{+/+} mice (Figure 1c). These findings indicate that catK plays a pivotal role in preventing the development of lung fibrosis and that it has no direct influence on the intensity of the inflammatory reaction.

In addition, the percentage of the alveolar wall fraction was increased by ~20% and the collagen content was 1.5-fold higher in untreated CTSK^{-/-} mice compared with CTSK^{+/+} mice, suggesting that catK is important for ECM homeostasis of the lung under physiological conditions (Figure 2, C and D). The molecular mechanisms that cause these changes remain to be elucidated.

Fibroblasts from CTSK^{-/-} Mice Possess a Decreased Collagenolytic Activity

Fibroblasts play a crucial role in matrix turnover in the lung. Therefore we investigated whether the collagenolytic activity of lung fibroblasts depends on the catK expression. We used fibroblasts derived from the lung tissue of CTSK^{-/-} mice and compared their MMP- and cathepsin-related collagenolytic activity with that of fibroblasts of catK-positive littermates (CTSK^{+/+}). We found no difference in MMP-dependent collagenolytic activity and a 40% reduction in cathepsin-mediated collagenolytic activity in CTSK^{-/-} mice (Figure 3, a and b).

CatK Expression Is Up-Regulated During Bleomycin-Induced Lung Fibrosis

The high matrix-degrading potential of catK suggests a possible role in matrix remodeling in the lung. To investigate the putative role of catK in lung we investigated the expression of catK in the course of bleomycin-induced lung fibrosis. In the lung tissue of catK-positive mice we found a time-dependent up-regulation of catK expression after the induction of lung fibrosis (Figure 3c).

CatK Expression Is Up-Regulated in Fibrotic Lung Diseases

To determine whether the increased expression of catK is found also in human lung tissues derived from patients suffering from fibrotic lung diseases, we analyzed the amount of catK mRNA by quantitative RT-PCR. Generally, the mean amounts of catK mRNAs were elevated approximately threefold in patients with lung fibrosis compared to those without lung fibrosis. Mean mRNA levels for catL were similar in fibrotic and nonfibrotic lungs (Figure 4).

Figure 2. CatK knockout mice were characterized by an increased alveolar wall thickness and more matrix deposition after bleomycin application. The alveolar wall fraction measured after H&E staining was higher in untreated CTSK^{-/-} mice (**A**) than in untreated CTSK^{+/+} mice (**B**). Representative trichrome-stained tissue sections showed the increased matrix deposition after bleomycin treatment in CTSK^{-/-} mice (**C, E**) compared to CTSK^{+/+} mice (**D, F**). Original magnifications: $\times 40$ (**A–D**); $\times 25$ (**E, F**).

Fibroblasts Express CatK in Fibrotic Human Lung Tissue

Interstitial lung fibrosis in man is characterized, similar to bleomycin-induced lung fibrosis in mouse, by a predom-

inant matrix deposition. Therefore we hypothesized that catK overexpression may represent a protective mechanism to counter the excessive matrix deposition in the development of this disease. Looking for pathogenetic parallels between mice and man we studied the expres-

Figure 3. Induction of cathepsin expression after bleomycin application and reduced collagenolytic activity in lung fibroblasts of CTSK^{-/-} mice. **a:** No significant difference of MMP-catalyzed collagenolytic activity between CTSK^{+/+} and CTSK^{-/-} mice. The collagenolytic activities were measured in cell lysates (white bars) and cell culture supernatants (gray bars). **b:** The cathepsin-mediated collagenolytic activity of fibroblasts derived from CTSK^{-/-} mice (black bars) was lower than the activity derived from CTSK^{+/+} mice (white bars). Results of four independent experiments are given as means $\pm$ SEM. **c:** Induced catK expression in lung tissue of CTSK^{+/+} mice after bleomycin treatment. Values of three mice at each time point are presented as means $\pm$ SEM. *, $P < 0.05$.

sion pattern of catK in human and mouse lung tissue using immunohistochemistry by comparing specimens obtained from patients with lung fibrosis with those obtained from patients without fibrosis and from mice before and after bleomycin challenge. In human lung tissue catK was barely expressed by vimentin-positive fibroblasts in nonfibrotic lungs (Figure 5, A and B), whereas bronchial epithelial cells distinctly and strongly expressed catK, as shown previously.²⁸ Immunostaining for catL, another lysosomal cysteine protease, was found in epithelial cells, macrophages, and fibroblasts (Figure 5C). The expression pattern of catK was markedly different in fibrotic lungs. We found distinct and strong catK immunoreactivity in fibroblasts (Figure 5; D, G to J). Similar to nonfibrotic lung tissues catK was found also in bronchial epithelial cells. The intensity of the immunoreaction was unchanged in epithelial cells. Additionally, some catK-positive multinucleated giant cells were found (Figure 5K). Thus, we concluded that increased catK expression that

was found by quantitative PCR was mainly caused by increased amount of catK in fibroblasts.

The expression pattern of catL in lung fibrosis was not different from nonfibrotic lungs. No immunostaining was observed after incubation with preimmune serum, confirming the specificity of immunostaining (Figure 5F). The expression pattern in mouse lung tissues was slightly different. In untreated mice we found, concordantly to human tissue, a prominent immunoreactivity in bronchial epithelial cells (Figure 6A). In addition we found catK-positive alveolar macrophages (Figure 6B). After bleomycin challenge we found significant immunostaining in interstitial fibroblast-like cells (Figure 6C). No immunostaining was observed in lung tissues of catK knockout mice (Figure 6D).

Primary Cell Cultures of Human Lung Fibroblasts Express CatK

Previous studies have provided evidence of the ability of synovial fibroblasts to express catK and catL.^{8,44} Our observations made in lung tissue specimens (*in situ*) suggest that pulmonary fibroblasts may have the ability to synthesize catK and catL. Primary cell cultures of fibroblasts, obtained from the lungs of patients with and without fibrosis, were used to further characterize their capability and ability to express catK and catL.

In primary cell culture fibroblasts from patients with lung fibrosis expressed, similar to our observations made *in situ*, significantly more catK than fibroblasts from nonfibrotic tissues. In contrast to the *in situ* findings some immunostaining was found also in fibroblasts derived from patients without lung fibrosis (Figure 7, a and b). Expression of catL was similar in fibroblasts from patients with and without fibrosis (Figure 7, c and d).

Increased expression of catK was related to increased gene transcription because quantitative RT-PCR showed that fibroblasts from patients with lung fibrosis contained twofold more catK mRNA than fibroblasts from patients without lung fibrosis (Figure 8a). CatL mRNA levels were unchanged (Figure 8b). These observations showed that fibroblasts from fibrotic lungs synthesized more catK than isolated fibroblasts from nonfibrotic lung tissues and that lung fibroblasts retained their different expression profiles for catK and catL in primary cell culture. Small amounts of catK were found in isolated and primary cultured fibroblasts obtained from nonfibrotic lung tissue. This difference to immunohistochemical observations made *in situ* was considered as a consequence of isolation and cell culture.

Subcellular Localization of CatK and Engulfed Collagen in Lung Fibroblasts

CatK immunoreactivity was visualized in isolated fibroblasts by conventional light microscopy and by confocal microscopy. Light microscopy showed no significant differences in the immunoreactivity of single fibroblasts (Figure 9A). The majority of these cells expressed signif-

Figure 4. CatK and catL mRNA expression in human lung tissue derived from patients with or without lung fibrosis. Significantly higher catK mRNA (**a**) but not catL mRNA (**b**) concentrations were found in fibrotic lung tissues. Results are given as means $\pm$ SEM. *, $P < 0.05$.

Figure 5. CatK expression is increased in fibrotic lung tissue, whereas catL expression remains unchanged. **A, B:** CatK immunoreactivity in normal lung tissue showing a constitutive expression in bronchial epithelial cells but not in fibroblasts. **C:** CatL immunoreactivity in normal lung tissue showing ubiquitous expression. **D:** Vimentin immunoreactivity in fibrotic lung tissue showing fibroblasts in fibrotic lesions. **E:** CatK immunoreactivity in fibroblasts of fibrotic lung tissue. **F:** Negative control, anti-catK antibody was blocked by preincubation with saturating amount of recombinant catK. **G, G':** CatK expression in an intraluminal fibroblast accumulation in tissue derived from a patient with BOOP. **H:** CatK in large fibroblast clusters (patient with UIP). **I:** CatK in fibroblast foci and in epithelial cells of small bronchi (patient with UIP). **J:** CatK in bronchial epithelial cells and peribronchial fibroblasts (patient with BOOP). **K:** CatK in rarely detectable multinucleated giant cells (patient with UIP). Fibroblasts are indicated by **solid arrows**, epithelial cells by **small arrows**, multinucleated giant cells and macrophages by **arrowheads**. Original magnifications: $\times 100$ (**B, C, G', H, I, K**); $\times 40$ (**A, D–F**); $\times 25$ (**G, J**).

icant amount of catK that was localized in the vesicular compartment of human lung fibroblasts. As expected the enzyme was almost exclusively found to be co-localized with Lamp-I, a common lysosomal marker, in perinuclear areas and in the protrusion of fibroblasts (Figure 9; B to D). Some lysosomes were localized near the cell membrane suggesting a secretion of lysosomal proteins including catK. Using transmission electron microscopy, we could

demonstrate that human lung fibroblasts cultured on collagen clots have internalized collagen fragments that were in shape similar to the fragments of the surrounding matrix (Figure 10). The internalized collagen was localized in phagolysosomal structures (Figure 10, inset) suggesting that they will be subsequently degraded in lysosomes. We have previously shown that catK is essential for intracellular collagen fibril degradation in synovial fibroblasts.⁴⁵

(a)

non-fibrotic			fibrotic				
1	2	3	4	5	6	7	M
							- 38 kD - 28 kD - 17 kD - 6 kD

(b)

Condition	rel. protein amount, %
non-fibrotic	100
fibrotic	175

(c)

non-fibrotic			fibrotic				
1	2	3	4	5	6	7	M
							- 38 kD - 28 kD - 17 kD - 6 kD

(d)

Condition	rel. protein amount, %
non-fibrotic	100
fibrotic	100

Figure 7. Increased catK protein expression in fibrotic fibroblasts. Lung fibroblasts from patients without (lanes 1 to 3) or with lung fibrosis (lanes 4 and 5, UIP; lanes 6 and 7, BOOP) were isolated. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation the cell lysates were blotted. CatK was visualized using a specific polyclonal antibody (a), catL immunoreactivity was detected staining with a monoclonal antibody (c). The intensities of the bands corresponding to mature catK (b) or to pro-catL (d, 1) and mature catL (d, 2) were quantified.

Fibroblasts from Patients with Lung Fibrosis Contain More CatK Activity and Display Higher Lysosomal Collagenolytic Activity

Neither immunohistochemistry nor RT-PCR or high-resolution microscopy provided any information regarding the functional activities of catK and catL. Because accurate quantification of specific enzymatic activities seems to be impossible in live cells we separated the endosomal/lysosomal fractions of isolated human lung fibroblasts and assayed the specific enzymatic activities of catK and catL. Fibroblasts obtained from patients with lung fibrosis showed significantly higher catK activity than fibroblasts from patients without fibrosis (Figure 11a). To test whether the increased catK expression was actually associated with higher matrix degradation, we tested the collagenolytic activity in the lysosomal/endosomal fraction. Extracts from fibroblasts obtained from patients with lung fibrosis showed almost threefold higher collagenolytic activity than those from patients without lung fibrosis (Figure 11b). These studies provide evidence that increased

Figure 6. CatK expression in mouse lung. CatK was expressed in bronchial epithelial cells (**A**) and in alveolar macrophages (**B**) of unchallenged mice. **C**: After bleomycin application we found a significant immunoreactivity in interstitial fibroblasts. The specificity of the immunoreaction was controlled by using lung tissue of catK knockout mice. **D**: No immunostaining was observed. Fibroblasts are indicated by **solid arrows**, epithelial cells by **small arrows**, multinucleated giant cells and macrophages by **arrowheads**. Original magnifications, $\times 100$.

Figure 8. Human lung fibroblasts isolated from fibrotic tissues express more catK mRNA than fibroblasts from nonfibrotic tissues. Significant higher catK mRNA (a) but not catL mRNA (b) concentrations were found in fibroblasts derived from fibrotic lung tissues using quantitative RT-PCR. Primary fibroblast cultures were obtained and analyzed from all patients who were included in this study. Results are represented as means $\pm$ SEM. *, $P < 0.05$.

transcription and expression of catK in lung fibroblasts is associated with increased enzymatic activity.

ECM Production by Isolated Fibroblasts

The next set of experiments was designed to test whether the expression of catK by lung fibroblasts influences matrix deposition. [3 H]-Proline incorporation assays were performed to characterize the ECM production of fibroblasts in primary cell cultures from both patient groups. These analyses showed that fibroblasts from patients with lung fibrosis synthesized 1.7-fold more ECM and twofold more collagen (Figure 12a). Because cathepsins have been implicated in the regulation of collagen secretion we measured ECM production in the presence of the cell permeable cathepsin inhibitor E64d (Figure 12b). The higher collagen and ECM deposition after E64d treatment (1.6-fold and 1.8-fold, respectively) indicates that cysteine proteinases can reduce the release of newly synthesized ECM by fibroblasts.

To address the functional role of catK more specifically, we assessed the ability of CTSK $^{-/-}$ fibroblasts to produce ECM. We found that the ECM deposition by CTSK $^{-/-}$ fibroblasts was similar to that of CTSK $^{+/+}$ fibro-

Figure 10. Localization of internalized collagen fibrils using transmission electron microscopy. Fibroblasts were cultured in collagen clots. Transmission electron microscopy showed similar collagen fragments inside the fibroblasts and in the surrounding collagen clot. The collagen fragments are localized in phagolysosomes (inset). The fragments are labeled by arrows. Original magnifications: $\times 7000$; $\times 12,000$ (inset).

blasts (Figure 12c). Thus, catK does not regulate the release of newly synthesized ECM proteins, but rather it is involved in degradation of existing ECM. The increased matrix production of fibroblasts after E64d treatment is probably mediated by another, as yet unidentified, cysteine protease.

Discussion

Pulmonary fibrosis remains a devastating clinical disorder with very limited therapeutic options. A number of experimental approaches have been investigated in clinical trials including the modulation of key cytokines and growth factors, and treatment with corticosteroids or immunosuppressants. In a number of patients these have little effect on clinical outcome.^{46–48} Therefore, alternative approaches to prevent the development of lung fi-

Figure 9. Immunolocalization of catK in isolated fibroblasts using immunohistochemistry and confocal laser-scanning microscopy. **A:** Fibroblasts were grown on chamber slides and immunolabeled using a rabbit anti-catK antibody. No difference in the immunoreactivity between fibroblast subpopulations was found. **B–D:** Fibroblasts were grown on cover slides and immunolabeled using a monoclonal antibody directed against the lysosomal Lamp-1 antigen (green), a rabbit anti-catK antibody (red), and the DNA stain 4,6-diamidino-2-phenylindole (blue). The overlay of the sections shows a lysosomal localization of catK immunoreactivity in perinuclear regions, in cellular extensions, and near the cell membrane (orange). Original magnifications: $\times 25$ (A); $\times 40$ (B–D).

Figure 11. Increased catK activity in fibroblasts from fibrotic lung tissues. Significant higher catK (a) and collagenolytic activities (b) were found in fibroblasts derived from patients with lung fibrosis. The lysosomal/endosomal fraction of isolated fibroblasts from fibrotic lung tissues (gray) and nonfibrotic lung tissues (white) were incubated with the catK-specific substrate Z-GPR-MCA or fluorescein-labeled collagen. Results are given as box blots indicating median (solid line), mean (square), 25th and 75th percentile (box), and 5th and 95th percentile (error bars). *, $P < 0.05$.

brosis should be explored.⁴⁹ One drawback of most of the approaches used to date was that they targeted inducers of *de novo* ECM formation and deposition. In this study we focused for the first time on cathepsin-mediated matrix-degrading mechanisms in the lung, which prevent or reverse matrix deposition. Our results show that the expression of catK can protect against an exaggerated deposition of ECM proteins, and thus may play an essential role in the development of lung fibrosis. Thus catK may be a new therapeutic for the treatment of pulmonary fibrosis.

Because our study was designed to investigate the role of catK in the pathogenesis of lung fibrosis, we needed to determine whether the expression of catK is regulated in the course of lung fibrosis. Our quantitative RT-PCR results and qualitative immunohistochemical analyses demonstrated a significant up-regulation of catK expression in lung tissue derived from mice after

bleomycin application as well as from fibrotic lung areas of patients with different types of lung fibrosis. The increased immunoreactivity could be assigned to fibroblasts. We found no significant changes in the catK immunoreactivity of bronchial epithelial cells. In line with our previous studies we found no increase in catK immunoreactivity of human alveolar macrophages but a significant expression in rarely detectable multinucleated giant cells.²⁷ In contrast, mouse alveolar macrophages expressed catK before and after bleomycin challenge. Analyses of catL expression revealed no significant difference between fibrotic and nonfibrotic lung areas. Thus, our data imply a specific role for catK in the development of lung fibrosis.

One possible explanation for the increased catK expression in fibrotic lung tissues is that it is up-regulated in the course of chronic inflammation to prevent a surplus in matrix deposition. The surplus is prevented either by catK activity degrading ECM proteins before their secretion or after phagocytosis of deposited ECM proteins.^{19,45,50}

Addressing these mechanisms we established primary cell cultures of lung fibroblasts. Fibroblasts from catK-knockout mice showed a decreased cathepsin-mediated collagenolytic activity, whereas the MMP-catalyzed collagenolytic activity was unchanged. The proliferative capacity of these fibroblasts was similar to that of wild-type fibroblasts (not shown). Fibroblasts derived from human fibrotic lung tissues retained their phenotype of increased matrix deposition and catK expression. We found a higher cathepsin-mediated collagenolytic activity in fibroblasts of fibrotic lungs indicating that catK is functionally active and is crucial to degrade ECM proteins.

ECM degradation is a multistep process, which involves: 1) recognition of the fibril by membrane-bound receptors, 2) segregation of the fibril, 3) partial digestion of the fibril and/or its surrounding noncollagenous proteins, and finally 4) lysosomal digestion after phagocytosis.¹⁹ Both insufficient extracellular or intracellular processing may lead to matrix accumulation and fibrosis. Fibroblasts internalize extracellular collagen very efficiently after binding of collagen to collagen receptors (eg, $\alpha_2\beta_1$ integrins).⁵¹ The functional importance of the intracellular collagen degradation by catK was shown in our previous studies documenting that inhibition of catK leads to intralysosomal collagen accumulation in synovial fibroblasts.⁴⁵ Using high-resolution microscopy, we have now shown that lung fibroblasts engulf collagen fibers and that catK is localized within the endosomal/lysosomal compartment in the perinuclear region, in cellular protrusions, and in part near the cell membrane. Noteworthy, using immunohistochemistry and flow cytometry (not shown) we found no significant difference in the catK expression levels in different fibroblast subpopulations. From this we conclude that collagen could be produced and degraded by the same cells. In contrast to human fibroblasts we found no collagen accumulation in mouse fibroblasts *in vivo* or *in vitro* (not shown). This finding is in agreement with data published by Everts and colleagues.⁵² On the other hand we found a decreased collagenolytic activity in catK knockout fibroblasts. Therefore we suggest that collagen fragments are rapidly

Figure 12. Regulation of matrix deposition by cysteine proteases. **a:** Isolated fibroblasts from patients with lung fibrosis secrete more ECM proteins including collagen than fibroblasts from patients without lung fibrosis. **b:** Incubation of fibroblasts with the cysteine protease inhibitor E64d augmented the deposition of ECM and collagen. **c:** Similar matrix deposition was shown by isolated fibroblasts from CTSK^{-/-} and CTSK^{+/+} mice. Results are given as means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$.

cleaved by additional enzymes. An alternative explanation could be that catK is secreted and mediates collagen cleavage in the extracellular environment. However, less data exist with respect to extracellular functions of catK in fibroblasts. The cathepsin-mediated collagenolytic activity was measured in the endosomal/lysosomal fraction because of a surplus of endogenous cathepsin inhibitors, cystatins, in the cytoplasm of all cells. These inhibitors control the extralysosomal activity of cathepsins very efficiently. Therefore, the measurement of catK activity is complicated in supernatants and complete cell lysates. Previous studies have shown, that monocyte-derived macrophages were capable to secrete catK, to acidify the pericellular milieu, and thus to maintain the enzyme in its active form.²⁵ Therefore, an extracellular function of catK is conceivable. Whether lung fibroblasts can use similar mechanisms for collagen and/or elastin degradation remains to be investigated.

Regarding the suggested role of catK in the degradation of newly synthesized collagen, it is of particular interest that inhibition of cysteine proteinases in human fibroblasts leads to increased matrix deposition and thus contributes to the development of pulmonary fibrosis. At the other hand, we ruled out the possibility that in mouse fibroblasts catK plays a crucial role in these processes by showing that the amount of ECM deposition was similar in fibroblasts derived from CTSK^{-/-} and CTSK^{+/+} mice. This was not unexpected because catK and other more ubiquitous cathepsins differ mainly in their capacity to cleave collagen fibers, which are assembled after secretion of procollagen molecules. We cannot rule out the possibility that the function of catK in human and mouse fibroblasts is similar but not identical. Therefore, the enzyme could influence the collagen production in human but not in mouse fibroblasts.

By analyzing CTSK^{-/-} mice and their catK-positive littermates, we sought to gain insight into the *in vivo* function of catK. Because CTSK^{-/-} mice developed significantly more lung fibrosis after bleomycin challenge than CTSK^{+/+} mice, we suggest that catK has in summary a protective function by preventing exaggerated matrix deposition and hence pulmonary fibrosis. The increased matrix deposition on knockout mice could be mediated by increased intracellular or ECM degradation or by a mixture of both.

Preliminary data show that even untreated CTSK^{-/-} mice were characterized by increased collagen content and enhanced alveolar wall thickness. Further studies have been initiated to investigate whether this represents a yet unknown phenotype of these knockout mice and whether other cells expressing catK under physiological conditions are involved in the regulation of constitutive matrix turnover.

In summary we report that one of the most potent matrix-degrading proteases is differentially expressed with increased proteolytic activity in fibroblasts of fibrotic lungs. CatK is a crucial enzyme for intralysosomal collagen degradation. The absence of catK aggravates lung fibrosis after bleomycin challenge. Therefore, we document for the first time a protective function of catK for the development of fibrosis. Considering previous studies

documenting the association of catK overexpression and lung emphysema,³¹ these data show that tight regulation of this powerful protease is necessary to maintain pulmonary matrix homeostasis. Both, overexpression and deficiency of catK may provoke structural reorganization of the lung tissue.

Summarizing our data and the findings of Zheng and colleagues³¹ we propose the following model of the catK function in the regulation of ECM homeostasis: both catK expression and ECM production are up-regulated during (chronic) inflammatory reactions. In our study this is reflected by the up-regulation of catK expression in fibrotic lung tissues and in CTSK^{+/+} mice after bleomycin challenge. The up-regulation of the protease could represent the anti-fibrotic reaction of the organism (human or mouse) in response to profibrotic signals. Under normal conditions this prevents the development of fibrosis. We have shown in our study that the lack of catK is followed by the enhanced development of fibrosis. This could be a model for an insufficient anti-fibrotic response. Furthermore, we suggest that, for example, the extraordinary up-regulation of matrix production may overcome the anti-fibrotic response (eg, the up-regulation of protease expression) and lead to the development of fibrosis. This could explain the development of fibrosis in patients and in wild-type mice irrespective of the catK up-regulation. Zheng and colleagues³¹ showed on the other hand that predominant expression of cathepsins (catS, catK) leads to the development of emphysema. Thus, we suggest that the course of the disease depends on the balance between ECM production and protease (and protease inhibitor) expression.

In this study we focused on the function of catK relying on its high matrix-degrading activity. Thus, we cannot rule out the possibility that other proteases have similar functions. However, in the development of new therapeutic strategies using catK inhibitors the potentially harmful side-effects of catK inhibitors applied in high doses in the therapy of osteoporosis and rheumatoid arthritis should be considered.⁵³ On the other hand, modulating the expression and activity of catK, eg, by cytokines or chondroitin sulfate,⁵⁴ may be a new approach for the treatment and/or prevention of lung fibrosis.

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