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Cysteine cathepsins in human silicotic bronchoalveolar lavage fluids

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Abstract

Mature, active cysteine cathepsins (CPs) were identified in human inflammatory bronchoalveolar lavage fluid (BALF) supernatants from patients suffering from silicosis by both western blot and surface plasmon resonance analyses. BALFs are not a reservoir of activatable proforms, since no autocatalytic maturation at acidic pH occurs. Cathepsin H is the most profuse among studied CPs (median value: 36.5 nM), while cathepsins B and L are the two most abundant thiol-dependent endoproteases. The overall concentration of active cathepsins B, H, K, L, and S is ~10-fold lower than their concentration in BALF supernatants from patients suffering from inflammatory acute lung injuries (962 ± 347 nM).The cathepsins (approximately 70 nM)/cystatin-like inhibitors (approximately 9 nM) ratio is unbalanced in favor of enzymes (~8-fold). This presence of uncontrolled CPs suggests that they may contribute, in addition to matrix metalloproteases, to the lung tissue breakdown/remodeling occurring during silicosis, although their exact contribution to interstitial inflammation remains to be evaluated.

Keywords: Activity-based probe; Bronchoalveolar lavage; Cathepsin; Cysteine protease; Protease inhibitor; Silicosis

1. Introduction

Silicosis is an occupational lung disease that is characterized by chest X-rays showing rounded opacities. Patients suffer from fatigue, dry cough, weight loss, decreased pulmonary function, compromised gas exchange, and often develop cyanosis (as reviewed in [1]). Pulmonary hypertension and chronic hypoxemia may cause death in severe forms of the disease. Even though this pneumoconiosis may be prevented by environmental dust control, it occurs still frequently and peculiarly among workers in developing countries. Silicosis is caused by inhalation of crystalline silica, particularly in high-hazard occupations (e.g., mining, sandblasting, tunneling, or rock grinding) in which silica dust exposures are frequent [2]. In addition to a possible link with lung carcinoma, silicosis is also associated with systemic and autoimmune diseases such as scleroderma and rheumatoid arthritis [3]. Despite the pathophysiological mechanisms remain unclear, it is established that the lung responds to silica by triggering an inflammatory cascade of reactions. Alveolar macrophages release reactive nitrogen and oxygen species, and inflammatory cytokines [4]. Upregulation of antioxidant enzymes, induction of inflammatory factors (IL-1, IL-6, TNF-alpha, and TGF-beta), activation of NF-κB, and of lipid peroxidation are observed [5]. Furthermore silicotic lungs showed increased collagenase and gelatinase activity. Silica deposition also resulted in apoptosis associated with inflammatory infiltrates in lung parenchyma [6]. Most studies on the lung proteolytic enzymes involved in degradation of collagen have focused primarily on MMPs (see, for review, [7]). Early granulomas exhibit intense staining for matrix metalloproteinases in experimental silicosis [8], and MMP-2, MMP-9, and stromelysin activities increase progressively in alveolar macrophages during fibrotic responses [9]. Conversely, their inhibitors (TIMP-1 and TIMP-2) showed a moderate reduction in late silicotic nodules, suggesting that an

Abbreviations: AMC, 7-amino-4-methyl coumarin; BALF, bronchoalveolar lavage fluid; Biot, biotinyl; CP, cysteine protease; CPI, cysteine protease inhibitor; DTT, Dithiothreitol; E-64, L-3-carboxy-trans-2,3-epoxy-propionyl-leucylamide-(4-guanido)-butane; ECM, extracellular matrix; MMP, matrix metalloprotease; MMTS, methylmethane-thiosulfonate; SPR, surface plasmon resonance; Z, benzyloxycarbonyl

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imbalance in the expression of MMPs and TIMPs may be implicated in ECM remodeling and basement membrane disruption. At the noticeable exception of caspases (family C14) which are core effectors of apoptosis (as reviewed by Denault and Salvesen [10]), no study has been reported so far about the presence and the putative role of cysteine proteases. Papain-related cysteine cathepsins (family C1) participate to a variety of proteolytic processes (e.g., turnover of endocytosed proteins, prohormone processing, MHC-II antigen presentation) [11]. Cathepsins are also involved in diseases such as tumor metastasis, osteoporosis, or rheumatoid arthritis, and are drug targets of clinical interest [12,13]. While cathepsin H is preferentially found in type II pneumocytes, lung CPs are mainly expressed by macrophages, fibroblasts and epithelial cells [14,15]. Stimulated monocyte-derived macrophages can release CPs [16], that are found in bronchoalveolar lavage fluids (BALFs) of smokers [17]. Recently we identified also active forms of cysteine cathepsins B, H, K, L and S in cell-free supernatants of BAL fluids from patients suffering of infiltrative inflammatory disorders (e.g., sarcoidosis, alveolar proteinosis) [18]. Even though CP activity is regulated by their natural inhibitors (CPIs), including both cystatin C and kininogens, the CP/CPI ratio was significantly unbalanced in favor of proteolysis (3/5-fold), as confirmed by the CP-dependent degradation of exogenous kininogens by BALFs [18]. That supports that, in addition to neutrophile elastase and MMPs, elastolytic CPs are involved in the degradation and/or remodeling of ECM [19–21]. The aim of this work was to characterize the presence of enzymatically active forms of cysteine cathepsins in BALFs of patients suffering from silicosis, and evaluated the imbalance between CPs and their specific inhibitors. This was done by assaying mature cathepsins B, H, K, L and S, their proforms and their endogenous inhibitors (kininogens and cystatin C).

2. Materials and methods

2.1. Patient selection and biological samples

Eleven elligible patients (Department of Pneumology, Bretonneau University Hospital, Tours, France) that fulfilled the diagnostic criteria for silicosis as set out in the consensus American-European guidelines (i.e., with a history of exposure to silica and chest X-ray interpretation showing rounded opacities) were included in the study. Time inclusion was from 12-01-2002 to 12-01-2004. All were negative for emphysema and tuberculosis. Three of these subjects were smokers, but abstained from cigarette smoking for 72 h before bronchoscopy. They were negative for viral serologies (HVB, HIV, CMV). None of them were under long or short course treatment with heparin, corticosteroids, and non-steroidal anti-inflammatory agents. Bronchoalveolar lavages were performed according to recommendations and guidelines of the European Respiratory Society. Due to the French policies and ethical considerations, lavages were not applied to healthy (control) subjects. Briefly aliquots of sterile saline were infused at room temperature to the lingula or the middle lobe, and immediately gently reaspirated. The first aliquot was collected separately, and not used for further biochemical studies. Cytological data are reported in Table 1. BAL fluid samples were immediately filtered and buffered (final concentration: 0.1 M Na-acetate, pH 5.0, plus the peptidase inhibitors 0.4 mM PMSF, 0.1 mM EDTA, 0.04 mM pepstatin A, and 1 mM MMTS). After centrifugation (2000×*g*; 4 °C) for 15 min as reported elsewhere [18], the resulting cell-free supernatants were aliquoted, their protein content quantified using the Bradford reagent (BioRad, Hercules, USA), and frozen at −80 °C.

Table 1
Bronchoalveolar lavages: cell counts (expressed as %)

Patient no.	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)
1	93	5	2
2	98	2	<1
3	100	0	0
4	95	5	0
5	94	5	1
6	93	5	2
7	91	7	2
8	93	7	<1
9	91	7	2
10	98	2	<1
11	98	1	1

2.2. Enzymes and inhibitors

N-(4-Biphenylacetyl)-*S*-methylcysteine-(D)-Arg-Phe-β-phenethylamide and human high molecular weight kininogen were from Calbiochem (VWR International, Libourne, France). CA-074, E-64, PMSF, pepstatin A, EDTA and MMTS were from Sigma-Aldrich (Saint-Quentin Fallavier, France). DTT (DL-dithiotreitol) came from Bachem (Weil am Rhein, Germany). Human cathepsins B, L, and H, and bovine cathepsin S were supplied by Calbiochem. Recombinant human cathepsin K, expressed in *Pichia pastoris*, was a kind gift from Dieter Brömme (University of British Columbia, Vancouver, Canada). H-Arg-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC were purchased from Bachem and Z-Leu-Arg-AMC from ICN Biochemical (Aurora, USA). All other reagents were of analytical grade.

2.3. Immunoblotting

The specific primary antibodies were: rabbit anti-human cathepsin B (Fitzgerald, Concord, USA), rabbit anti-human cathepsin H (Fitzgerald), rabbit anti-human cathepsin L (Fitzgerald), mouse anti-human cathepsin S (Krka, Ljubljana, Slovenia), rabbit anti-human cystatin C (Upstate, Lake Placid, USA), and rabbit anti-human high and low molecular weight kininogens [22]; the rabbit anti-human cathepsin K antibody was a kind gift from Dieter Brömme (University of British Columbia, Vancouver, Canada). The goat anti-rabbit IgG-peroxidase conjugate and the goat anti-mouse IgG-peroxidase conjugate were both from Sigma-Aldrich. Prestained molecular masses were from Cell Signaling (Saint Quentin en Yvelines, France). BAL fluids (7 μl)were diluted and boiled in reducing sample buffer (14 μl) prior separation by SDS-PAGE on 12% gels (1 h30, 20 mA) and electro transfer to a nitrocellulose sheet (1 h, 30 mA). After saturation in PBS, 0.1% Tween 20, 10% skim dry milk for 1 h at room temperature, the membrane was incubated with the primary antibody (1:1000, in PBS, 0.1% Tween, 3% skim dry milk) for 1 h at room temperature, then with a goat anti-rabbit IgG-peroxidase conjugate (1:5000) for 1 h at room temperature (or with a goat anti-mouse IgG-peroxidase conjugate (1:1000) for detection of the mouse anti-human cathepsin S). Revelation was done by using the Renaissance 4 CN plus (4-chloro-1-naphthol) system (NEN Life Science, Zaventem, Belgium), or alternatively by an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

2.4. In vitro labeling of cysteine cathepsins

Cathepsins B, H, K, L and S (1 μM) were incubated in the assay buffer with a molar excess (1:300) of a cystatin-derived probe, i.e., Biot-LVG-CHN2 [23,24], for 1 h at 37 °C. Samples were further subjected to a 12% (w/v) SDS-PAGE under reducing conditions. Proteins were blotted before the nitrocellulose sheet was saturated by incubation for 1 h at 37 °C with PBS containing 1% (w/v) BSA. After washing with PBS containing 0.1% Tween 20, and incubation with an extravidin-peroxidase conjugate (diluted 1:500) for 2 h at room temperature, the peroxidase activity was revealed in the presence of 4-chloro-1-naphthol. Control experiments were performed by pre-incubating cathepsins with E-64 (100 μM) prior adding the biotinylated probe.

2.5. BIAcore analysis of Biot-LVG-CHN$_2$ interaction with cysteine cathepsins from BAL fluids

BAL fluids were incubated in the assay buffer with Biot-LVG-CHN$_2$ (100 μM), for 4 h at 30 °C. The excess of activity-based probe was carefully removed by successive dialysis/concentration steps (Vivaspin, 4 000 rpm, 5 min, cut-off : 10,000) in order to avoid any further unwanted interactions between streptavidin-coated dextran and free residual Biot-LVG-CHN$_2$. A control experiment was done by using an unlabelled inhibitor, i.e., E-64 (5 μM). Samples were finally diluted in 0.1 M Na-phosphate, pH 6.0, 2 mM DTT, prior to analysis by surface plasmon resonance (SPR) using a BIAcore 1000 system (Uppsala, Sweden). Briefly, the streptavidin coated sensorchip SA5 (BIAcore International) was washed three times by injecting 5 μl of 1 M NaCl and 50 mM NaOH as indicated by the manufacturer. Immobilization of samples containing Biot-LVG-CHN$_2$-labeled cysteine cathepsins was performed as described previously with a few modifications [25], by using 0.1 M Na-phosphate, pH 6.0, 2 mM DTT as running buffer at a continuous flow rate of 5 μl/min. The specificity of the interaction was checked using BALF samples containing E-64-treated cysteine cathepsins. After 6 min of washing with running buffer, the strength (affinity) of the interaction was evaluated by injecting 5 μl NaOH 20 mM.

2.6. CP activity and CPI potential of bronchoalveolar lavage fluids

CPs from BALFs (20 μl/assay)were activated in their activity buffer (100 mM Na-phosphate buffer pH 6.0 containing 2 mM DTT and 2 mM EDTA) for 3 min at 30 °C, prior to making kinetic measurements in the presence of a panel of AMC-derived fluorogenic substrates. Hydrolysis was continuously recorded at 30 °C with a Kontron SFM 25 spectrofluorimeter (λ_{exc}=350 nm and λ_{em}=460 nm). Thiol-dependent endoproteases (i.e., cathepsins B+K+L+S) were active site-titrated with E-64 (0–10 nM final), using Z-Phe-Arg-AMC as substrate (5 μM), according to Barrett and coworkers [26]. Pre-incubation with a large excess (5 μM)of *N*-(4-Biphenylacetyl)-*S*-methylcysteine-(D)-Arg-Phe-β-phenethylamide, a specific inhibitor of cathepsin L [27] allowed us to deduce the average concentration of cathepsins B+K+S. Cathepsin B was titrated with *N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) (0–10 nM) using Z-Arg-Arg-AMC as substrate (5 μM) [28]. Increasing concentrations of titrated cathepsin S (0–1 nM) were incubated for 1 h at 37 °C in 100 mM Na-phosphate buffer pH 7.4, before addition of Z-Leu-Arg-AMC (5 μM) and monitoring of the residual enzymatic activity; concentration of BALF cathepsin S was further deduced from this calibration curve according to its unusual stability at mildly basic pH [29,30]. Concentration of cathepsin K was estimated from the difference between the concentration of E-64-titrated cathepsins B+K+S and the individual concentrations of both cathepsins B and S. Concentration of cathepsin L was deduced from the difference between the concentration of thiol-dependent endoproteases (i.e., cathepsins B+K+L+S) and the respective concentrations of cathepsins B, K and S. The aminopeptidase activity of cathepsin H was quantified by monitoring the enzymatic activity of increasing concentrations of titrated cathepsin H (0–40 nM), in presence of H-Arg-AMC (5 μM) at 30 °C in the assay buffer. Concentration of cathepsin H issued from BAL samples was deduced from this calibration curve. The experimental procedure to evaluate the BALF inhibitory potential was adapted from a previous report by Assfalg-Machleidt et al. [31]. According to that thiol-dependent endoproteases of BAL fluids are mostly represented by cathepsins B and L (≈86%), their specific CP activity was blocked by pre-incubation for 30 min by both CA-074 (1 μM) and *N*-(4-Biphenylacetyl)-*S*-methylcysteine-(D)-Arg-Phe-β-phenethylamide (1 μM). The BALF inhibitory potential was further deduced by addition of increasing amounts of BALF (0–16 μl) by using E-64 titrated papain (3 nM), in presence of Z-Phe-Arg-AMC (5 μM) as substrate.

3. Results and discussion

3.1. Immunodetection and labeling of cysteine cathepsins from BAL fluids

Cell-free supernatants of bronchoalveolar lavage fluids were buffered at acidic pH (0.1 M acetate buffer pH 5.0) in presence of a cocktail of protease inhibitors (1 mM PMSF, 1 mM EDTA, 0.04 mM pepstatin A, 1 mM MMTS) to stabilize and preserve cysteine cathepsins from pH inactivation and uncontrolled proteolysis. The median protein concentration, determined using the Bradford procedure, was 0.08 mg/ml (min. value: 0.02 mg/ml; max. value: 0.16 mg/ml). Immunoreactive cathepsins B, H, and L (approximately 25 kDa) (lines 1–9), but not cathepsins S (lines 10–12) and K (not shown), were detected in all BALF samples (Fig. 1). On the other hand, no bands corresponding to proforms of the enzymes were stained, indicating that cathepsins were fully processed into their mature forms or that the zymogene concentrations were too low to be detected under our experimental conditions. These results differ significantly from a previous study performed on BAL fluids from patients suffering from infiltrative inflammatory disorders (e.g., sarcoidosis, alveolar proteinosis) [18], that showed that cathepsins B, K and S are mainly detected as zymogens, whereas cathepsins H and L were predominantly mature forms. Alternatively, we have investigated the ability of Biot-LVG-CHN$_2$, a cystatin C-derived probe initially designed to target parasite cysteine proteinases from *Trypanosoma cruzi*, *T. congolense*, and *Plasmodium falciparum* [24–32], to bind to active forms of BALF cathepsins. By contrast with rat cathepsins B and L [23], this activity-based probe efficiently labelled purified human cathepsins B, L, K and H, and bovine cathepsin S (Fig. 2). Preincubation of cathepsins with E-64 abolished binding, confirming the specific targeting of the active site-reactive thiol by Biot-LVG-CHN$_2$. However after incubation of BALF samples with Biot-LVG-CHN$_2$, no reactive bands could be revealed using an extravidin-peroxidase conjugate (not shown). In order to outline this problem of detection limit, presence of active cysteine cathepsins was investigated by surface plasmon resonance with a BIAcore 1000 system. Biot-LVG-CHN$_2$-treated BALF samples showed a significantly increased signal (expressed as resonance units-RU on streptavidin-coated dextran). This corresponds to a stronger and specific interaction on the biosensor surface, compared to E-64-treated samples used as unlabelled controls (Fig. 3). The higher affinity of LVG-CHN$_2$-treated BALF samples to streptavidin is reinforced by a lower desorption induced by NaOH 20 μl injection after the dissociation phase compared to unlabelled controls. Overall, these results show that cysteine cathepsins from patients with silicosis are still enzymatically active in BALFs, as reported for patients

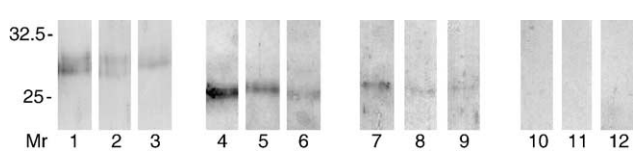

Fig. 1. Immunodetection of cysteine cathepsins. Western blot analyses of bronchoalveolar lavage fluids were performed using rabbit polyclonal antibodies against human cathepsins B (lanes 1–3), L (lanes 4–6), H (lanes 7–9), and a mouse monoclonal antibody against human cathepsin S (lanes 10–12). Three representative silicotic BALF samples are shown for the sake of clarity: lanes 1, 4, 7, 10, BALF #1, lanes 2, 5, 8, 11, BALF #2, lanes 3, 6, 9, 12, BALF #3.

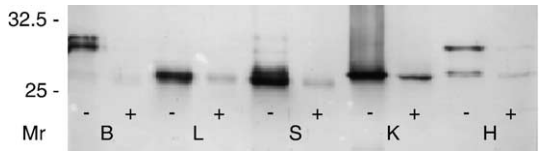

Fig. 2. Specific labeling of cysteine cathepsins by a cystatin-derived probe. Titrated cathepsins B, H, K, L and S (1 μM) were incubated for 1 h at 37 °C in the activity buffer with Biotinyl-Leu-Val-Gly-CHN2 (300-fold molar excess) (−), prior separation by a 12% (w/v) SDS-PAGE under reducing conditions [23]. Following electroblotting onto a nitrocellulose sheet, enzymes were incubated with an extravidin-peroxidase conjugate (diluted 1:500) for 2 h at room temperature. The peroxidase activity was further revealed in the presence of 4-chloro-1-naphthol (Renaissance 4 CN plus system). Control experiments (+) were performed by pre-incubating cysteine cathepsins with unlabelled E-64 (10 μM) prior addition of Biotinyl-Leu-Val-Gly-CHN_2 (300 μM).

suffering from emphysema [33], infiltrative inflammatory disorders [18] or cystic fibrosis [34].

3.2. Proteolytic activities of cysteine cathepsins in silicotic BAL fluids

CP activities were then measured using differential substrate specificity and stability at neutral pH, and selective inhibition by small synthetic inhibitors to distinguish between individual cathepsins (see the Materials and methods section). All samples hydrolyzed Z-Phe-Arg-AMC, Z-Arg-Arg-AMC, Z-Leu-Arg-AMC and H-Arg-AMC, confirming the presence of active CPs in BAL fluids, as deduced from BIAcore analysis. Individual concentrations of BALF cysteine cathepsins were reported in Fig. 4 (the horizontal bars represent the calculated median for each enzyme). No difference of concentrations was observed in the three samples corresponding to silicotic patients with a smoking history. Cathepsin H is the most abundant among studied CPs (median value: 36.5 nM), while cathepsins B (median value: 21.5 nM) and L (median value: 8 nM) are the most abundant thiol-dependent endoproteases

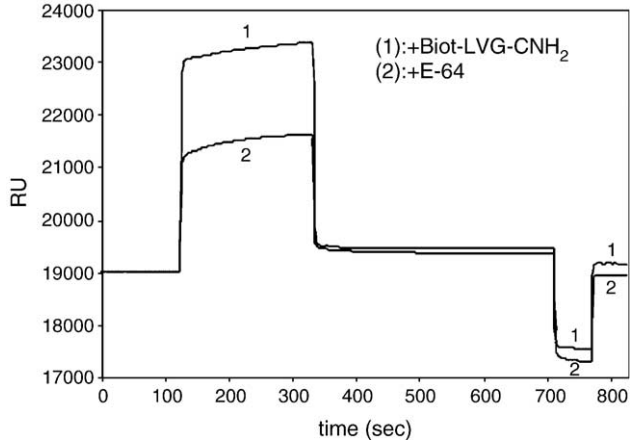

Fig. 3. Specific detection by SPR of active cysteine cathepsins in BAL fluids. (1) BAL fluids from silicotic patients were incubated in the assay buffer with Biot-LVG-CHN_2 (100 μM) for 4 h at 30 °C, as described in Materials and methods, prior analysis by plasmon resonance according to the manufacturer (BIAcore International). (2) The same experience was repeated by treatment of BALF samples by the unlabelled CP-specific inhibitor E-64 (45 μM). One representative BALF sample is shown for the sake of clarity.

(35 nM). Conversely cathepsin S is poorly expressed (median value: 0.8 nM). The overall concentration is approximately 10-fold lower than the average concentration of active CPs (962±347 nM; median: 885 nM) found in BALFs from patients with acute lung infiltrative inflammatory disorders [18]. Taken together that CPs are secreted by differentiated macrophages, that cathepsin K is possible marker of inflammation infiltrative inflammatory disorders [35–37], and that silicosis is characterized by an important recruitment of macrophages, the level of extracellular CPs in BAL fluids may partly reflect the severity of the inflammation. In addition, the enzymatic activity on Z-Phe-Arg-AMC was not increased by incubating BAL fluids under acidic conditions (100 mM Na-acetate buffer pH 4.0) at 37 °C for 0 to 5 h. This absence of autocatalytic activation confirms western blot analysis, and show that BAL fluids from patient with silicosis do not represent a reservoir of activatable CPs, due to the lack of CP precursors.

3.3. Inhibitory potential of silicotic BAL fluids

Specific circulating inhibitors of CPs have been reported in numerous biological fluids, including epithelial lining fluids (see, for review, [38]). In the present study, western blot analysis indicated the presence of weak immunoreactive bands corresponding to both degraded forms of high and/or low molecular weight kininogens (including a major fragment of ~32 kDa) and of cystatin C (~13 kDa) (data not shown). The residual CP inhibitory capacity (expressed as inhibitory site equivalents) was evaluated by measuring the ability of BAL fluids to inhibit E-64-titrated papain. A median CPI value of 9 nM was found, that indicates that the CP/CPI ratio (~8-fold) was unbalanced in favor of enzymes. Taken together that CPs are potent elastolytic and/or collagenolytic enzymes infiltrative inflammatory disorders [12,16], and the imbalance in the expression of CPs and their specific inhibitors, it could be assumed that cysteine cathepsins – in addition to the newly described cathepsin V [21], neutrophile elastase and matrix metalloproteases (MMP 2=gelatinase A, MMP 9=gelatinase B, MMP 12=macrophage metalloelastase) [8] – may partic-

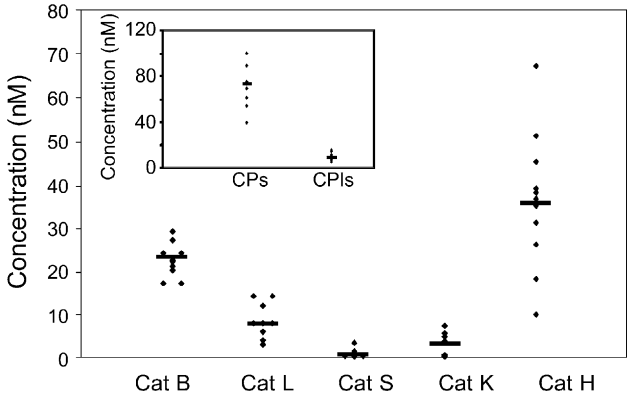

Fig. 4. Individual active forms of cathepsins B, H, K, L and S and CP/CPI balance in silicotic bronchoalveolar fluids. The horizontal bars represent the calculated median value. CPI: expressed as inhibitory site (cystatin-like) eq.

ipate in the basement membrane disruption and in the degradation of the major components of the extracellular matrix (collagen, laminin, and gelatin) and its remodeling that occur during progression of silicosis. Interestingly, a comparable CP/CPI balance (3- to 5-fold) was found in favor of cysteine cathepsins for patients suffering from acute lung injuries, despite both CPs (962 ± 347 nM) and CPIs (147 ± 23 nM) exhibit higher concentrations [18]. This observation raises the question of the upregulation/over expression of lung cysteine cathepsins under acute inflammatory episodes vs. chronic inflammations as reported for better documented peptidases (reviewed by Owen and Campbell, [39]). Unfortunately, as recently claimed by Bogyo and co-workers [40], the understanding of the contribution of individual cathepsins in the context of complex proteolytic events remains in its infancy and has to be clarified.

In conclusion, we report for the first time the presence of mature active forms of cathepsins B, H, K, L and S in human inflammatory bronchoalveolar lavage fluids from patients with silicosis. Conversely we failed to identify zymogens, suggesting the lack of a pool of activatable cysteine cathepsins. Weak amount of immunoreactive cystatin C was detected in BALF supernatants, and kininogens are degraded. As a possible result, the uncontrolled cathepsins could participate to the pathophysiological breakdown/remodeling of the extracellular matrix occurring during silicosis.

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