

Cathepsin H and napsin A are active in the alveoli and increased in alveolar proteinosis

Markus Woischnik¹, Anette Bauer^{1*}, Rola Aboutaam², Asli Pamir¹, Franz Stanzel³, Jacques de Blic², Matthias Griese¹.

¹ Childrens' Hospital of the Ludwig-Maximilians-University, Lindwurmstr. 4, 80337 Munich, Germany

² Hôpital Necker Enfants Malades, Service de Pneumologie et d'Allergologie Pédiatriques, 149 rue de Sèvres, 75015 Paris, France

³ ASKLEPIOS Fachkliniken, Zentrum für Pneumologie und Thoraxchirurgie, Robert-Koch-Allee 2, 82131 Munich, Germany

Short title:

Cathepsin H and napsin A in alveolar proteinosis

Corresponding author:

Matthias Griese

Children's Hospital of the Ludwig-Maximilians-University

Lindwurmstr. 4

D-80337 Munich

Phone: +49-89-5160-7870

Fax: +49-89-5160-7872

E-mail: Matthias.griese@med.uni-muenchen.de

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Abstract

Pulmonary alveolar proteinosis (PAP) is a group of rare diseases with a disturbed homeostasis of alveolar surfactant. While 90% of the primary adult forms are caused by GM-CSF auto-antibodies, the underlying cause of the juvenile form remains unknown. In order to distinguish primary from secondary effects in the pathogenesis of these two forms, we studied the surfactant protein processing proteases napsin A and cathepsin H.

We enrolled sixteen controls, 20 patients with the juvenile form of PAP, and 13 adults with idiopathic PAP. Amounts and activities of the proteases in the bronchoalveolar lavage fluid were determined by immunoblotting and specific substrate cleavage.

Both proteases were present and active in lavage fluid from controls and increased in PAP patients of both forms. The amount of active cathepsin H in relation to total cathepsin H was increased in PAP patients compared to controls. Cystatin C, the physiological inhibitor of cathepsin H in the alveolar space, was not increased to the same degree as cathepsin H, resulting in an imbalance of inhibitor to protease in the alveolar space.

A general defect in napsin A and cathepsin H expression or activity as the specific cause for abnormal surfactant accumulation in juvenile PAP was excluded.

Keywords: Alveolar proteinosis, cathepsin H, napsin A, surfactant protein B, surfactant protein C

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Supplementary Online Material

Introduction

Normal composition, pool size and homeostasis of pulmonary surfactant are critical for appropriate function and gas exchange of the alveolar region of the lungs. Surfactant contains in addition to lipids, the hydrophobic surfactant proteins B (SP-B) and C (SP-C). SP-B is synthesized by alveolar type II cells as a 42 kDa proprotein (proSP-B), which is intracellularly processed to the mature 8 kDa protein [1]. Bronchoalveolar Clara cells also express proSP-B, but do not process it to the mature form [2]. Instead, a 24 kDa proSP-B form is secreted into the alveolar space. SP-C is exclusively synthesized by alveolar type II pneumocytes as a 21 kDa proprotein (proSP-C), which is then proteolytically processed inside type II cells to the 4.2 kDa mature protein [3]. Cathepsin H and napsin A are known to be necessary for this intracellular processing [4-7].

Cathepsin H is a lysosomal cysteine protease and, like other members of the family, is involved in lysosomal protein processing [8, 9]. It is synthesized as an inactive proenzyme (41 kDa) and then proteolytically processed within the lysosomes to an active form of size 28 kDa [10]. In the lung cathepsin H is localized to lamellar bodies, dense multivesicular bodies and composite bodies of type II pneumocytes [5], which are the sites of surfactant maturation. Ishii et al. identified active cathepsin H in BAL fluid and suggested type II cells and alveolar macrophages as the main sources of the enzyme [11, 12]. Others have found altered cathepsin H amounts and activities in BAL fluids of different patient groups [13, 14] or in animal models of lung diseases [15]; the amount and activity of cathepsin H in relation to its natural substrates, the hydrophobic surfactant proteins SP-B and SP-C, are unknown.

Napsin A, an aspartyl protease, is mainly expressed in lung and kidney [16, 17]. In the lung, it co-localizes with proSP-B and SP-B in multivesicular, composite, and lamellar bodies of alveolar type II cells [4]. Immune histological studies suggest its presence in the alveolar space [4, 6]. However, there are no reports on the amounts and activity of napsin A in BAL fluids and in relation to its substrates, proSP-B and proSP-C.

A major goal of this study is to investigate the physiology of these two proteases in normal human BAL fluid, and to look for potential abnormalities in alveolar filling syndromes. Comparison of BAL fluid samples would allow to distinguish primary from secondary effects in the pathogenesis of these diseases.

Materials and Methods

Study objects

We investigated the bronchoalveolar lavages of three groups of subjects; 16 patients (age 3.5 y (1.37-5.68) confidence interval (CI)) with chronic bronchitis served as the control group. These subjects had a history of ≥ 3 months per year of chronic non-productive cough for at least two years, had no signs of atopy and asthma, and were classified as the “chronic cough” group. An underlying immune deficiency, cystic fibrosis, primary ciliary dyskinesia, malformations, foreign bodies, microaspirations from gastroesophageal reflux disease, chemical exposures, tracheomalacia or nicotine abuse were excluded. BAL was performed during a period free of exacerbation and pulmonary infiltrate as evaluated by C-reactive protein measurement and chest radiographs.

In the 20 pediatric patients (age 2.01 y, (0.84 – 3.18)) PAP was diagnosed by the characteristic histological pattern of alveolar filling with periodic acidic Schiff positive material in open or transbronchial lung biopsy in 12 children, and in addition all had the milky lavage effluent, showed the pathognomonic cytological pattern and all children had the characteristic radiological findings of PAP on HRCT. Mutations of SP-B, SP-C, or ABCA3 were not detected in any of these children. GM-CSF auto-antibodies were negative in all these patients in serum and lavage.

13 patients had the idiopathic form of adult PAP (age 43.54 y (37.45 – 49.62), diagnosed by open or transbronchial lung biopsy in 5 patients and by a combination of typical clinical and radiological findings on HRCT and a diagnostic BAL showing milky fluid and abundant extracellular periodic acidic Schiff positive material on cytopreps in all other patients. Eight of 13 of the adult patients had high titers of GM-CSF auto-antibodies.

BAL sample preparation, isolation of large and small aggregate surfactant

Bronchoscopy and BAL (4 x 1 ml of 0.9% NaCl per kg body weight) were performed as described previously [18]. For the isolation of large (LA) and small (SA) aggregate surfactant, aliquots of the BAL fluid were centrifuged at 40,000 xg for 30 minutes.

Immunoblot analysis of human BAL fluid, LAs, and SAs

Aliquots of human BAL fluid, LAs, and SAs were separated under reducing conditions using 10% NuPage Bis-Tris gels and transferred to PVDF membranes to detect

SP-B, SP-C, proSP-B, Napsin A and Cathepsin H. The protein bands were densitometrically quantified with QuantityOne. For details see the online supplement.

Cathepsin H and napsin A activity assay

Cathepsin H and napsin A activity were determined by measuring cleavage of a specific synthetic substrate. Details are in the online supplement.

Statistical methods

Non-parametric tests were used for comparison of two unpaired groups (Mann Whitney test). The results are given as means \pm standard error (SE) of the individual number of different subjects, each individual value representing the mean of 2–3 determinations. For correlations, Spearman's non-parametric test was used. A p value of less than 0.05 was considered statistically significant.

Results

Cathepsin H amounts and activity are elevated in BAL fluid from patients with alveolar proteinosis

Cathepsin H in BAL fluids was quantified by immunoblotting. The assay is linear and specific over a wide concentration range (Fig. 1A). The dominant immunoreactive forms of the protein have a size of 28 kDa, corresponding to the active form of cathepsin H (Fig. 1B). No larger proforms or smaller degradation products are detected. Concentrations of cathepsin H in BAL fluid are three times higher in patients with juvenile and adult PAP as compared to subjects in the control group (CB: 5.7 ± 0.6 $\mu\text{g/ml}$, PAPjuv: 15.9 ± 1.5 $\mu\text{g/ml}$, PAPad: 14.0 ± 0.9 $\mu\text{g/ml}$; means \pm SE, $P_{\text{PAPjuv-CB}} < 0.0001$, $P_{\text{PAPad-CB}} < 0.0001$) (Fig. 1C). As these results do not distinguish active cathepsin H from inactive, we determined the proteolytic activity of cathepsin H in BAL fluid (Fig. 2A). PAP patients have significantly higher levels than the control group (CB: 0.20 ± 0.06 $\mu\text{g/ml}$, PAPjuv: 6.8 ± 2.07 $\mu\text{g/ml}$, PAPad: 13.09 ± 3.79 $\mu\text{g/ml}$; means \pm SE, $P_{\text{PAPjuv-CB}} < 0.0001$, $P_{\text{PAPad-CB}} < 0.0001$). Activity and mass of cathepsin H correlate with each other ($r=0.64$; $P < 0.0001$). Interestingly, dissimilar percentages of active cathepsin H are observed for each group of subjects. In control patients only 3% of the cathepsin H is active, whereas 46% and 85% are active in juvenile and adult PAP patients (table 1).

Cystatin C in BAL fluid

Varying amounts of endogenous protease inhibitor in the BAL fluid may cause different fractions of active cathepsin H. Cystatin C is known to reversibly inhibit cathepsin H. About 5-fold higher concentrations of cystatin C are present in BAL fluid of PAP patients compared to controls (CB: 13.6 ± 1.9 ng/ml , PAPjuv: 69.6 ± 12.8 ng/ml , PAPad: 80.9 ± 14.6 ng/ml ; means \pm SE, $P_{\text{PAPjuv-CB}} = 0.0003$, $P_{\text{PAPad-CB}} < 0.0001$) (table 1).

Inhibition by cystatin C is a stoichiometrical event on a 1:1 molar basis [19]. With increasing concentrations of cathepsin H we observed an increasing imbalance of cathepsin H activity in relation to cystatin C concentration (table 1). In the control group there is a 10-fold excess of cathepsin H activity over inhibitor, which is about 42-fold for juvenile, and 77-fold for adult PAP patients. In contrast, the ratio of the concentration of cathepsin H to its inhibitor does not significantly vary among the groups. This clearly indicates a lack of cathepsin H inhibitory capacity in PAP.

Napsin A is present in human BAL fluid, and is increased in alveolar proteinosis patients

The other protease known to participate in the processing of the hydrophobic surfactant proteins is napsin A [6, 20]. A single dominant immunoreactive band of 38 kDa size, corresponding to the active form of napsin A [21], is detected by immunoblotting in all BAL fluid samples (fig. 4A). No larger proforms or smaller degradation products of napsin A are detectable. The concentration of napsin A is significantly larger in PAP patients than in the control group (CB: 504 ± 392 AU/ml, PAPjuv: 3038 ± 1571 AU/ml, PAPad: 3054 ± 1505 AU/ml; means \pm SE, $P_{\text{PAPjuv-CB}} < 0.0001$, $P_{\text{PAPad-CB}} < 0.0001$) (fig. 4B).

Napsin A in BAL fluid is proteolytically active, and is increased in juvenile, but not in adult PAP patients

The enzymatic activity of napsin A in BAL fluid is increased about 3 to 4-fold in juvenile PAP patients (CB: 58 ± 14 AU/ml, PAPjuv: 258 ± 32 AU/ml, means \pm SE, $P_{\text{PAPjuv-CB}} < 0.0001$), but not in adult PAP patients (PAPad: 119 ± 29 AU/ml; means \pm SE, $P_{\text{PAPad-CB}} = 0.083$) (fig. 5A). Napsin A activity correlates only weakly with napsin A mass (fig. 5B). Approximately 11% of total napsin A is active and there are no differences between the groups (CB: 11.5 ± 2.7 , PAPjuv: 10.6 ± 1.9 , PAPad: 6.9 ± 3.4 ; means \pm SE), consistent with an unchanged degree of napsin A activity in the three groups.

Increased protease levels are not due to increased cell damage or lysis

To exclude the possibility that the increased amounts of proteases in the BAL fluid are due to an increased damage or lysis of cells in PAP patients, we determined the activity of free LDH in BAL fluid. LDH activity does not differ between PAP patients and the control group; although 4 PAP patients show elevated LDH activity, there is no correlation between the amount of either of the proteases and the LDH activity (data not shown).

Napsin A is located in large aggregates, cathepsin H in the small aggregate fraction of BAL fluid

From previous studies it is known that the intracellular localization of napsin A and cathepsin H in type II pneumocytes is in the lamellar bodies (lbs) and in their precursor vesicles [4-6]. In contrast to napsin A, cathepsin H expression is not limited to type II pneumocytes, and increased cathepsin H amounts in BAL fluid of PAP patients may arise from several sources within the lung. In order to identify the origin of the proteases in BAL

fluid, cell-free BAL fluid was separated by ultracentrifugation at 40,000 xg into a large aggregate pellet, i.e. large surfactant particles primarily derived from lamellar bodies, and a small aggregate supernatant; the fractions were subjected to immunodetection with either cathepsin H or napsin A antibody.

Cathepsin H is found mainly in the SA fraction; only in a 5-fold concentrated sample from PAP patients it becomes detectable in the LAs. Thus, the majority of cathepsin H is localized in the SAs (fig. 6A). In contrast to the distribution of cathepsin H, a noticeably higher amount of napsin A protein is localized in the LA fraction (fig. 6B).

Napsin A and cathepsin H amounts and activities correlate with the intra-alveolar amounts of mature surfactant proteins SP-B and SP-C, and to the physiological processing intermediates of pro-SP-B

Napsin A and cathepsin H are involved in the intracellular processing of surfactant protein B and C to their mature forms. Because this study demonstrates that these proteases are also present and active in the extracellular space, we investigated their potential role in relation to the hydrophobic surfactant proteins. Both, the concentrations of cathepsin H (Supplementary fig. 1A and 1B), and of napsin A (Supplementary fig. 1C and 1D) correlate significantly with the concentrations of the mature forms of SP-B and SP-C. In agreement, the activities of the two proteases also correlate with these surfactant proteins (for SP-B, cathepsin H ($r=0.511$, $P<0.0002$), napsin A ($r=0.489$, $P<0.0004$) and for SP-C, cathepsin H ($r=0.645$, $P<0.0001$), napsin A ($r=0.518$, $P<0.0001$)).

The majority of all subjects has the physiologic proSP-B processing form of 24-26 kDa [22-24] in their BAL, as assessed by immunoblotting (table 2). As for mature SP-B, the abundance of this common precursor is proportional to the concentrations and activities of cathepsin H (concentration $r=0.638$, $P<0.0001$, activity $r=0.636$, $P<0.0001$) and napsin A (concentration $r=0.407$, $P=0.0082$, activity $r=0.738$, $P<0.0001$).

The fraction of mature SP-B, calculated as a percentage of SP-B and proSP-B forms, does not differ between the patient groups. About 80% of all SP-B is in its mature form (table 2). This indicates that the processing of the final product SP-B is not altered in PAP patients. Of interest, in about 50% of the patients with juvenile PAP additional aberrant 30-32 kDa proSP-B forms are present in significant amounts (table 2).

Discussion

Pulmonary alveolar proteinosis (PAP) is a group of rare diseases with a disturbed surfactant homeostasis, resulting in altered composition and pool size of alveolar surfactant. The adult form can be divided into so-called idiopathic, primary forms, and secondary forms associated with other diseases. Primary forms are caused by a defect in reuptake of the surfactant due to auto-antibodies against granulocyte–macrophage colony-stimulating factor (GM-CSF) in blood and tissues, including pulmonary alveoli [25-27]. These auto-antibodies neutralize the biologic activity of GM-CSF. Pediatric PAP is a rare disease, very few patients have been described [28, 29]. Causally GM-CSF autoantibodies play no role (this study and [29, 30]). Our hypothesis was that napsin A or cathepsin H, crucial proteases for the intracellular synthesis of SP-B and SP-C, are causally involved in the pathogenesis of juvenile PAP.

We found that napsin A was present and active in the alveolar space under physiological conditions. In patients with PAP napsin A concentration was increased 6-fold compared to controls. We made similar observations for cathepsin H. The results exclude a general deficiency of napsin A and cathepsin H as the specific cause for abnormal surfactant accumulation in juvenile PAP, because essentially the same results were found in adult PAP, which is caused by GM-CSF auto-antibodies. Both enzymes were active, abundantly present and proportional to the alveolar amounts of the mature surfactant proteins.

There are limitations of this study. First, protease amounts and activities were not determined inside alveolar type II cells, where surfactant is synthesized. The minor differences between the PAP groups, and the large differences to the control group are unlikely to stem from dissimilar rates of secretion of proteases into the alveolar space. Cell damage or lysis was excluded. Moreover, this limitation is offset by the interesting finding that these proteases were active in the alveolar space and potentially have yet unexplored functions. Another limitation is the lack of an age matched comparison group for adult PAP. However, given the large differences in protease amounts and activities between the juvenile PAP and the control group, compared to the small differences among the PAP groups, even with somewhat higher baseline levels in an adult comparison group, the same conclusions would have been derived. Furthermore, there was no indication of age dependency over the available age-range, and human and animal data show that surfactant pools are stable after the neonatal period [31].

Napsin A

To our knowledge, this is the first report to show that active napsin A is present in human BAL fluid of normal and diseased humans. Napsin A is required for the synthesis of surfactant; the proforms of SP-B and SP-C are the physiological substrates of napsin A [5, 6, 20]. The close correlations observed between the processing enzyme napsin A and SP-B, proSP-B and SP-C in the alveolar space suggest that increased protease amounts and activities are indeed involved in the increase of mature surfactant proteins in patients with expanded alveolar surfactant pool sizes. LDH activity levels in BALF were not different between the three groups and exclude the release of napsin A (or cathepsin H) by increased cell damage or lysis in patients with PAP. By fractionating BAL fluid, we identified large aggregates (LA) as the primary location of napsin A; LAs are derived from the lamellar bodies of pulmonary type II cells. This is in agreement with the localization of napsin A in lamellar bodies, shown by immunoelectron microscopy [4, 6], and its association with cellular membranes [32].

Napsin A is an intracellular aspartic acid protease with maximum activity at an acidic pH. Of note, at a pH of 7 and a temperature of 37° C napsin A activity has a half-life of 4 hours [33]. Thus we expected to find active enzyme in the BALF, if secreted. Napsin A may be involved in many extracellular enzymatic processes. In line with extracellular functions of napsin A is its secretion from the kidney into the urine of normal human subjects [16, 17, 34]. All this fits well into the more general paradigm that enzymatic activities, previously believed to be responsible for entirely intracellular processes, may also have specific functions after secretion or release into the extracellular environment. Caspases, for example, known to function as intracellular executioners of apoptosis, retain enzymatic activity in various extracellular fluids [35, 36]. This extracellular caspase activity is relevant for tissue remodeling during development and disease, as it provides coordinated cell and extracellular matrix loss [37].

Cathepsin H

Similar to napsin A, we found cathepsin H present and active in all BAL samples analyzed. No precursor forms of cathepsin H were detected by immunoblotting, which is in agreement with studies of BAL fluids from patients with inflammatory lung diseases and silicosis [13, 14]. In PAP patients amount and activity were elevated in proportion to increased SP-B and SP-C levels. At least two cell types, alveolar macrophages and type II cells, express cathepsin H in the lung [12, 38]. Cathepsin H amounts did neither correlate with the number of alveolar macrophages recovered from BAL fluid, nor with the degree of

inflammation, as represented by neutrophils in BAL fluid (results not shown). Support for type II cells as the origin of cathepsin H comes from immunoelectron microscopy, which shows localization of cathepsin H in lamellar bodies of alveolar type II cells [5]. After secretion, partitioning into the small aggregate fraction may occur. Due to its pH optimum at neutral pH, it is expected to be active in the epithelial lining fluid, the pH of which has been estimated at 6.92 [39].

Taken together, the increased cathepsin H level in PAP is most likely related to the expanded alveolar pool of the hydrophobic surfactant proteins and not to inflammation or cell damage.

Under physiological conditions endogenous broad-range inhibitors of the cystatin family strictly control cathepsin H activity [40]. The levels of cystatin C, the major cathepsin H inhibitor [19], were 2-fold higher in PAP patients compared to controls; however, the percentage of active cathepsin H increased from 3% in control subjects to 85% in juvenile PAP patients. Obviously, the capacity to cope with excessive proteolytic activity in the human lung is limited, which is supported by animal data [15]. Cystatin C is synthesized by all nucleated cells [41, 42]. A cell specific defect in cystatin C expression in the lung is therefore unlikely.

We can only speculate on the physiologic functions of intra-alveolar cathepsin H. Clara cells secrete proSP-B as a 24 kDa proform [2], and this might be processed in the alveoli, thereby increasing SP-B levels from a latent reservoir. This concept is supported by *in vitro* studies with recombinant proSP-B and isolated cathepsin H, which have shown the generation of the COOH terminus of mature SP-B [6].

In summary, the results reported here exclude a general deficiency of napsin A and cathepsin H expression or activity as the specific cause for abnormal surfactant accumulation in juvenile PAP. Both primarily intracellular proteases napsin A and cathepsin H are abundant and active in the alveolar space and correlate with SP-B, proSP-B forms and SP-C, supporting their role in intra- and extracellular surfactant metabolism.

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Table legends

Table 1: Cathepsin H amounts, activities and inhibitor concentrations and their ratios

Table 2: Surfactant protein B proforms in BAL fluid from the patient groups investigated

Figure legends

Figure 1: Cathepsin H in human BAL fluid. To quantify cathepsin H, a standard curve was created by measuring the chemiluminescence signal of varying amounts of cathepsin H (5 ng – 200 ng) on a Western immunoblot (A). Aliquots (50 μ l) of cell-free human BAL fluid samples were separated on NuPage gels, followed by immunodetection with cathepsin H antibody. Panel B shows a representative immunoblot (3 samples of each of the patient groups) with the signal for mature cathepsin H at ~28 kDa. Cathepsin H signals from all patients were densitometrically quantified, and absolute amounts were calculated based on a calibration curve (see panel A). Panel C shows the distribution of cathepsin H amounts in [μ g/ml] BAL fluid in the patient groups. The horizontal bar represents the median.

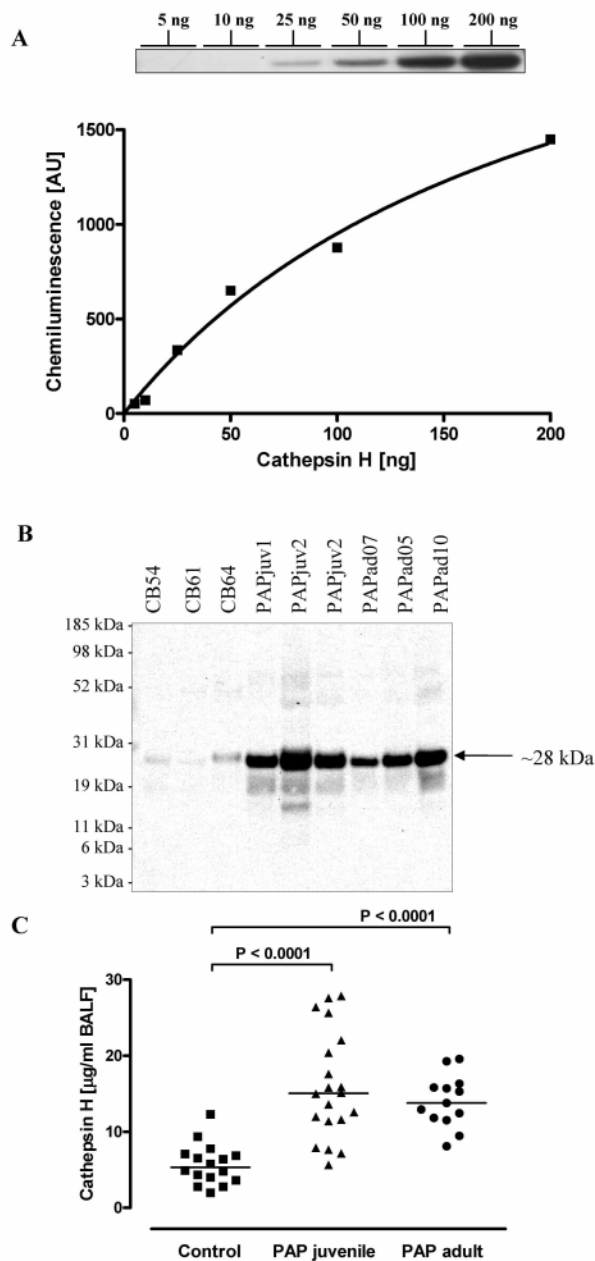


Figure 2: Cathepsin H activity in human BAL fluid. The enzymatic activity of cathepsin H in human BAL fluid was determined by measuring the turnover of a specific substrate. Panel A shows the amount of proteolytically active cathepsin H in human BAL fluid in the patient groups. The horizontal bar represents the median. Panel B shows the correlation of cathepsin H amount with cathepsin H activity over all patients.

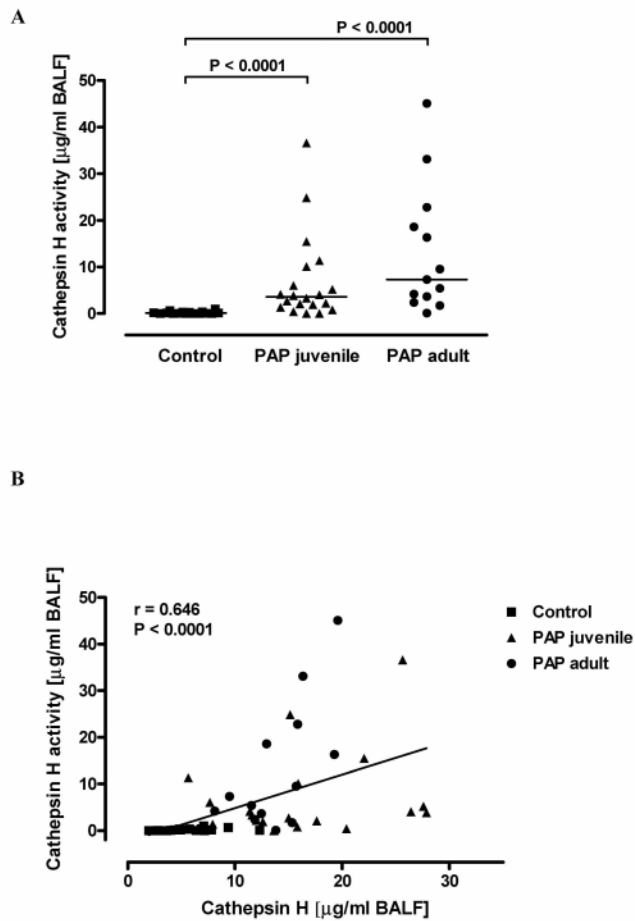


Figure 3: Cystatin C in human BAL fluid. Cystatin C was quantified in human BAL fluid samples by sandwich ELISA. The diagram shows the amount of cystatin C in human BAL fluid for the three groups control, PAP juvenile, and PAP adult. The horizontal bar represents the median.

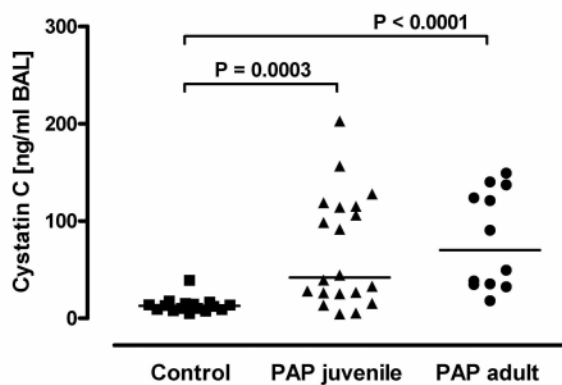


Figure 4: Napsin A in human BAL fluid. Aliquots (50 μ l) of cell-free human BAL fluid samples were separated on NuPage gels, followed by immunodetection with napsin A antibody. Panel A shows a representative immunoblot (3 samples of each of the patient groups) with the signal for mature napsin A at \sim 38 kDa. Napsin A signals from all patients were densitometrically quantified, and calculated in relation to an internal standard. Panel B shows the distribution of relative napsin A amounts per milliliter BAL fluid for the patient groups. The horizontal bar represents the median.

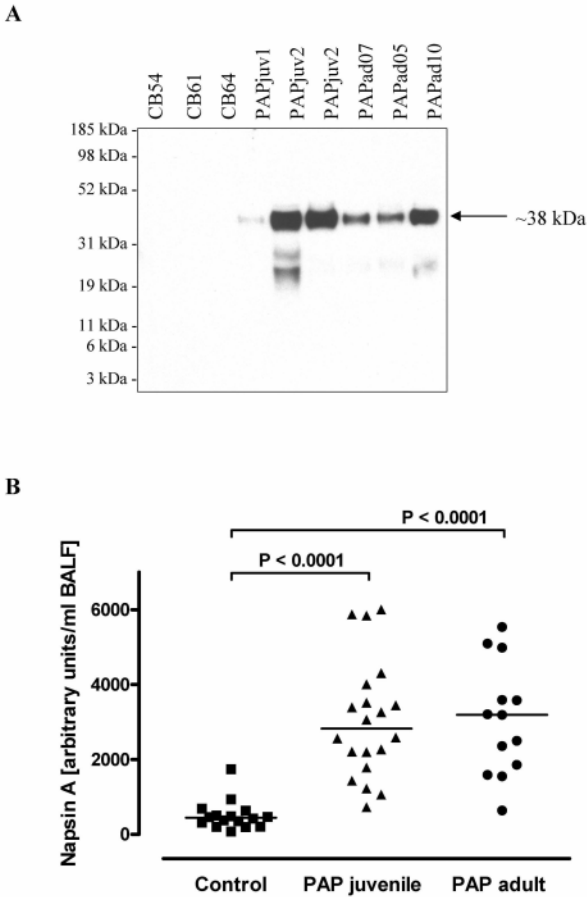
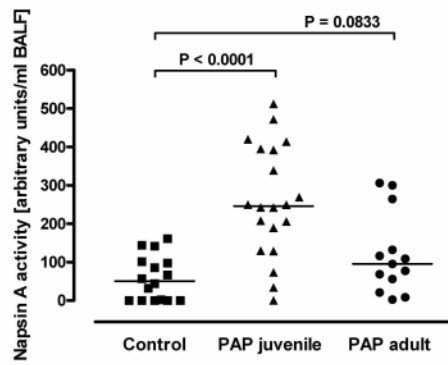


Figure 5: Napsin A activity in human BAL fluid. The enzymatic activity of napsin A in human BAL fluid was determined by measuring the turnover of a specific substrate. The activity was based on an internal standard. Panel A shows the amount of proteolytically active napsin A in human BAL fluid in the patient groups. The horizontal bar represents the median. Panel B shows the correlation of napsin A amount with napsin A activity over all patients.

A



B

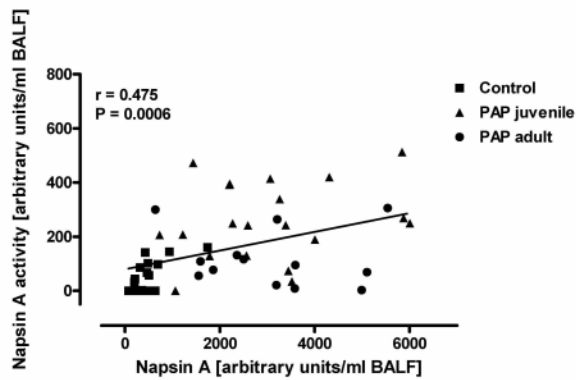
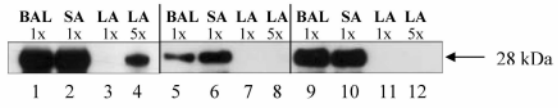


Figure 6: Localization of cathepsin H and napsin A in human BAL subfractions. Human BAL fluid, small (SAs) and large (LAs) aggregate surfactant were separated on NuPage gels, followed by immunodetection with cathepsin H (Panel A) or napsin A (Panel B) antibodies. Panel A: Lanes were loaded with total BAL fluid (1, 5, 9), SAs (2, 6, 10), or LAs (3, 7, 11) from a sample size of 100 μ l BAL fluid, or LAs from a sample size of 500 μ l BAL fluid (4, 8, 12); samples are from 1 juvenile PAP patient (1-4), and 2 control patients (5-8, 9-12). Panel B: Lanes were loaded with total BAL fluid (1, 5), SAs (2, 6), or LAs (3, 7) from a sample size of 100 μ l BAL fluid, or LAs (4, 8) from a sample size of 500 μ l BAL fluid; samples are from 1 juvenile (1-4) and 1 adult (5-10) PAP patient.

A



B

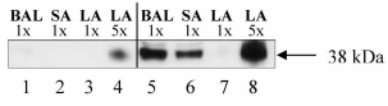


Table 1. Cathepsin H amounts, activities and inhibitor concentrations, and their ratios expressed on a molar basis

Patient Group	Cathepsin H amount [nM]	Cathepsin H activity [nM]	Cystatin C amount [nM]	Ratio Cat.H – Cat.H activity	% active cathepsin H	Ratio Cat.H activity – Cystatin C	Ratio Cat.H – Cystatin C
Control	203 (153 – 254)	7 (2 - 12)	1.0 (0.7 – 1.3)	85 (24 – 146)	3 (1 – 5)	10.4 (1.3 – 19.6)	237 (163 - 310)
PAP juvenile	569 (452 - 686)	243 (88 - 399)	5.2 (3.2 – 7.2)	7.5 (1.6 – 13.3)	46 (19 – 73)	41.6 (24.5 – 58.7)	276 (132 - 420)
PAP adult	500 (426 - 574)	467 (172 - 762)	5.6 (3.2 – 8.0)	15.2 (-13 – 43.3)	85 (40 - 129)	77.5 (48.2 - 107)	116 (70 - 163)

Data are means with upper and lower 95% confidence interval in brackets; PAP: pulmonary alveolar proteinosis

Table 2. Surfactant protein B proforms in BAL fluid from the patient groups investigated

Patient Group	9 - 15	19 - 21	24 - 26	30 - 32	proSP-B total	proSP-B 24 - 26	SP-B in % of
	[kDa]	[kDa]	[kDa]	[kDa]	[ng/ml]	[ng/ml]	proSP-B + SP-B
Control	0/16	3/16	13/16	0/16	29 (21 - 38)	30 (19 - 40)	71 (53 - 88)
PAP juvenile	2/20	3/20	17/20	13/20	662 (303 - 1021)	531 (201 - 862)	88 (83 - 93)
PAP adult	0/13	0/13	13/13	0/13	729 (234 - 1225)	729 (234 - 1225)	82 (76 - 87)

Data are number of subjects with the respective molecular weight form present in relation to the total number of subjects in a group or means with upper and lower 95% confidence interval in brackets; PAP: pulmonary alveolar proteinosis