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

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ABSTRACT: Pulmonary alveolar proteinosis (PAP) is a group of rare diseases with disturbed homeostasis of alveolar surfactant. While 90% of the primary adult forms are caused by granulocyte-macrophage colony-stimulating factor autoantibodies, the underlying cause of the juvenile form remains unknown. In order to distinguish primary from secondary effects in the pathogenesis of these two forms, the present authors studied the surfactant protein processing proteases napsin A and cathepsin H.

In total, 16 controls, 20 patients with juvenile PAP and 13 adults with idiopathic PAP were enrolled. Amounts and activities of the proteases in the bronchoalveolar lavage fluid (BALF) were determined by immunoblotting and specific substrate cleavage.

Both proteases were present and active in BALF from controls and increased in juvenile and adult PAP patients. The amount of active cathepsin H in relation to total cathepsin H was increased in PAP patients compared with 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 or cathepsin H expression or activity was not the specific cause for abnormal surfactant accumulation in juvenile pulmonary alveolar proteinosis.

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

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 (SP)-B and SP-C. SP-B is synthesised 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 into the mature form [2]. Instead, a 24 kDa proSP-B form is secreted into the alveolar space. SP-C is exclusively synthesised 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 synthesised 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 localised to lamellar bodies (LBs), dense multivesicular bodies and composite bodies of type II pneumocytes [5], which are the sites of surfactant maturation. ISHII and co-workers [11, 12] identified active cathepsin H in bronchoalveolar lavage fluid (BALF) and suggested type II cells and alveolar macrophages as the main sources of the enzyme. Others have found altered amounts and activities of cathepsin H in BALF of different patient groups [13, 14] and 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 the lung and kidney [16, 17]. In the lung, it colocalises with proSP-B and SP-B in multivesicular bodies, composite bodies and LBs of alveolar type II cells [4]. Immunohistological studies suggest its presence in the alveolar space [4, 6]. However, there are no reports on the

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amounts and activity of napsin A in BALF and in relation to its substrates, proSP-B and proSP-C.

A major goal of the present study was to investigate the physiology of these two proteases in normal human BALF and to look for potential abnormalities in alveolar filling syndromes. Comparison of BALF samples allowed distinction of primary from secondary effects in the pathogenesis of these diseases.

MATERIALS AND METHODS

Study subjects

BALF from three groups of subjects was investigated: 20 juvenile pulmonary alveolar proteinosis (PAP) patients; 13 adult PAP patients; and 16 patients with chronic bronchitis (CB; mean age 3.5 yrs, 95% confidence interval (CI) 1.37–5.68 yrs) who served as the control group. These control subjects had a history of $\geqslant$3 months·yr^{-1} of chronic non-productive cough for at least 2 yrs, had no signs of atopy or asthma and were classified as the ''chronic cough'' group. Underlying immune deficiency, cystic fibrosis, primary ciliary dyskinesia, malformations, foreign bodies, micro-aspirations from gastro-oesophageal reflux disease, chemical exposures, tracheomalacia or nicotine abuse were excluded. Bronchoalveolar lavage (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 juvenile patients, mean (95% CI) age 2.01 (0.84–3.18) yrs, PAP was diagnosed by the characteristic histological pattern of alveolar filling with periodic acid-Schiff positive material in open or transbronchial lung biopsy in 12 children. All 20 patients had the milky lavage effluent, demonstrated a pathognomic cytological pattern and had the characteristic radiographical findings of PAP on high-resolution computed tomography (HRCT). Mutations of SP-B, SP-C or ATP-binding cassette, sub-family A, member 3 (ABCA3) were not detected in any of these children. Granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies were negative in the serum and lavage fluid of all these patients.

In total, 13 adult PAP patients were diagnosed with the idiopathic form of adult PAP (PAPad). Their mean (95% CI) age was 43.54 (37.45–49.62) yrs. Of these, five were diagnosed by open or transbronchial lung biopsy. A combination of typical clinical and radiographical findings on HRCT and a diagnostic BAL showing milky fluid and abundant extracellular periodic acid-Schiff positive material on cytopreparations was seen in all other patients. Of the 13 adult patients, eight had high titres of GM-CSF autoantibodies.

BAL sample preparation, isolation of large and small aggregate surfactant

Bronchoscopy and BAL (4 × 1 mL of 0.9% NaCl per kg body weight) were performed as described previously [18]. For the isolation of large and small aggregate (LA and SA, respectively) surfactant, aliquots of BALF were centrifuged at 40,000 × *g* for 30 min.

Immunoblot analysis of human BALF, LA and SA

Aliquots of human BALF, LA and SA were separated under reducing conditions using 10% NuPAGE® Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes (Millipore, Concord, MA, USA) to detect SP-B, SP-C, proSP-B, napsin A and cathepsin H. The protein bands were densitometrically quantified with QuantityOne (Biorad, Hercules, CA, USA). Cystatin C levels were determined by sandwich ELISA. (For full details see supplementary data.)

Cathepsin H and napsin A activity assay

Cathepsin H and napsin A activity were determined by measuring cleavage of a specific synthetic substrate. (See supplementary data for details.)

Statistical methods

Nonparametric tests were used for comparison of two unpaired groups (Mann–Whitney U-test). Data are presented as mean ± SE of the individual values of different subjects, with each individual value representing the mean of two to three experiments. For correlations, Spearman's nonparametric test was used. A p-value <0.05 was considered statistically significant.

RESULTS

Cathepsin H amounts and activity were elevated in BALF from PAP patients

Cathepsin H in BALF was quantified by immunoblotting. The assay was linear and specific over a wide concentration range (fig. 1a). The dominant immunoreactive forms of the protein had a size of 28 kDa, corresponding to the active form of cathepsin H (fig. 1c). No larger proforms or smaller degradation products were detected. The concentrations of cathepsin H in BALF were three times higher in patients with juvenile PAP (PAPjuv) and PAPad, compared with subjects in the control CB group (15.9 ± 1.5, 14.0 ± 0.9 and 5.7 ± 0.6 μg·mL^{-1}, respectively; $p<0.0001$ for comparisons of both PAPjuv and PAPad with CB; fig. 1d). As these results did not distinguish active cathepsin H from inactive, the proteolytic activity of cathepsin H in BALF was determined (fig. 2a). PAPjuv and PAPad patients had significantly higher levels than the CB group (6.8 ± 2.07, 13.09 ± 3.79 and 0.20 ± 0.06 μg·mL^{-1}, respectively; $p<0.0001$ for comparisons of both PAPjuv and PAPad with CB). The activity of cathepsin H correlated with its concentration ($r=0.646$, $p<0.0001$; fig. 2b). Interestingly, dissimilar percentages of active cathepsin H were observed for each group of subjects. In control patients, only 3% of the cathepsin H was active, whereas 46 and 85% were active in PAPjuv and PAPad patients, respectively (table 1).

Cystatin C in BALF

Varying amounts of endogenous protease inhibitor in the BALF may cause differences in the percentage of active cathepsin H. Cystatin C is known to reversibly inhibit cathepsin H. Approximately five-fold higher concentrations of cystatin C were present in BALF of PAPjuv and PAPad patients compared with the CB group (69.6 ± 12.8, 80.9 ± 14.6 and 13.6 ± 1.9 ng·mL^{-1}, respectively; $p=0.0003$ for PAPjuv *versus* CB, $p<0.0001$ for PAPad *versus* CB; table 1, fig. 3).

Inhibition by cystatin C occurs stoichiometrically on a 1:1 molar basis [19]. With increasing concentrations of cathepsin H, an increasing imbalance of cathepsin H activity in relation to cystatin C concentration was observed (table 1). In the control

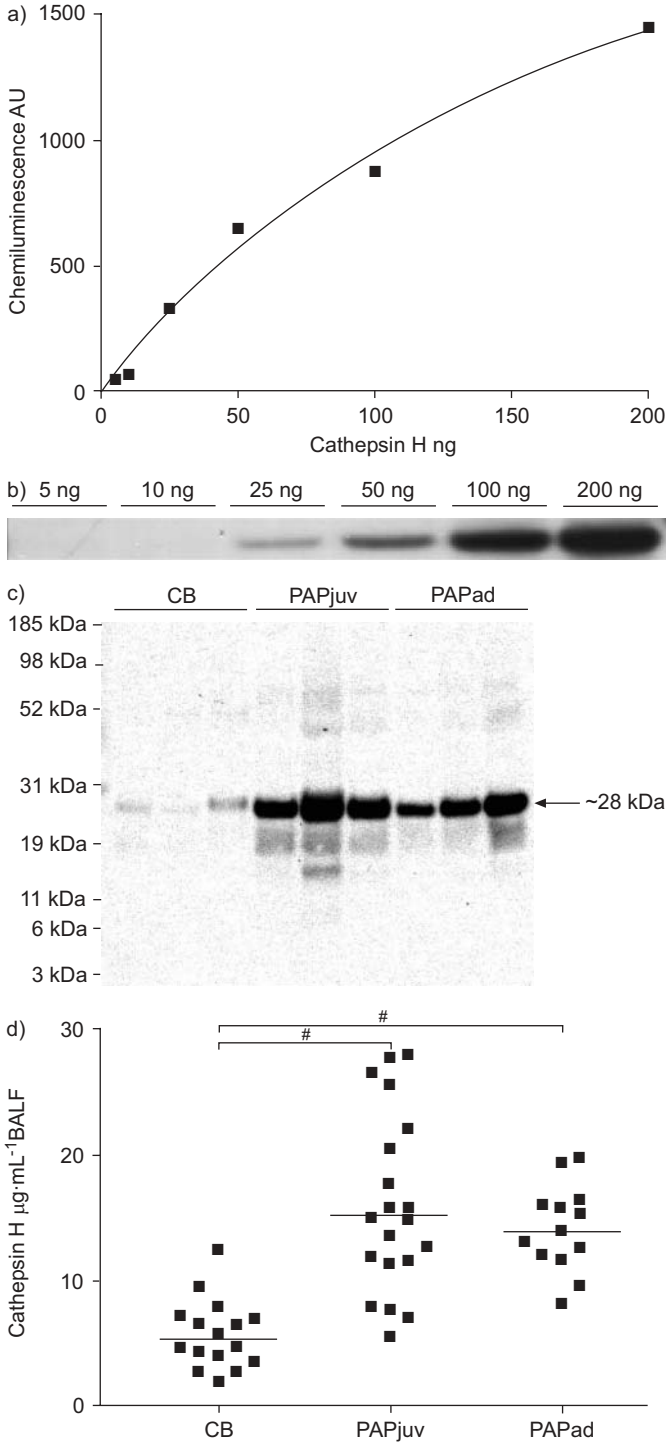

FIGURE 1. Cathepsin H in human bronchoalveolar lavage fluid (BALF). To quantify cathepsin H, a standard curve (a) was created by measuring the chemiluminescence signal of varying amounts of cathepsin H (5–200 ng) on a Western blot (b). Aliquots (50 μL) of cell-free human BALF samples were separated on NuPAGE® gels, followed by immunodetection with cathepsin H antibody. c) A representative immunoblot, showing three 50 μL BALF samples for each of the patient groups, with the signal for mature cathepsin H at ~28 kDa. d) The distribution of cathepsin H concentrations in BALF in the patient groups. The horizontal bars represent the median. AU: arbitrary units; CB: chronic bronchitis control patients; PAPjuv: juvenile pulmonary alveolar proteinosis (PAP) patients; PAPad: adult PAP patients. #: $p<0.0001$.

group there was a 10-fold excess of cathepsin H activity over its inhibitor cystatin C, which was ~42-fold for PAPjuv and 77-fold for PAPad. In contrast, the ratio of the concentration of total cathepsin H to its inhibitor did not vary significantly between the groups. This clearly indicates a lack of cathepsin H inhibitory capacity in PAP.

Napsin A was present in human BALF and was increased in PAP

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, corresponding to the active form of napsin A [21], was detected by immunoblotting in all BALF samples (fig 4a). No larger proforms or smaller degradation products of napsin A were detectable. The concentration of napsin A was significantly larger in PAPjuv and PAPad than in the CB group (3038 ± 1571, 3054 ± 1505 and 504 ± 392 arbitrary units (AU)·mL^{-1}, respectively; $p<0.0001$ for comparisons of both PAPjuv and PAPad with CB; fig. 4b).

Napsin A in BALF was proteolytically active and increased in PAPjuv but not in PAPad

The enzymatic activity of napsin A in BALF was increased approximately three- to four-fold in PAPjuv compared with CB controls (258 ± 32 and 58 ± 14 AU·mL^{-1}, respectively; $p<0.0001$), but not in PAPad (119 ± 29 AU·mL^{-1}; $p=0.083$; fig. 5a). Napsin A activity correlated only weakly with napsin A concentration (fig. 5b). Of the total napsin A, ~11% was active and there were no differences between the groups (10.6 ± 1.9, 6.9 ± 3.4 and 11.5 ± 2.7%; PAPjuv, PAPad and CB, respectively), consistent with an unchanged fraction of active napsin A in the three groups.

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

To exclude the possibility that the increased amounts of cathepsin H and napsin A proteases in the BALF were due to an increased damage or lysis of cells in PAP patients, the activity of free lactate dehydrogenase (LDH) in BALF was determined. LDH activity did not differ between PAP patients and the CB group; although four PAP patients showed elevated LDH activity, there was no correlation between the amount of either of the proteases and the LDH activity (data not shown).

Napsin A was located in the LA and cathepsin H in the SA fraction of BALF

From previous studies it is known that napsin A and cathepsin H are intracellularly localised in type II pneumocytes to the LBs and their precursor vesicles [4–6]. In contrast to napsin A, cathepsin H expression is not limited to type II pneumocytes, and increased cathepsin H levels in BALF of PAP patients may arise from several sources within the lung. In order to identify the origin of the proteases in BALF, cell-free BALF was separated by ultracentrifugation at $40,000\times g$ into an LA pellet, *i.e.* large surfactant particles primarily derived from LBs, and an SA supernatant. The fractions were subjected to immunodetection with either cathepsin H or napsin A antibody.

Cathepsin H was mainly found in the SA fraction; it only became detectable in the LA in a five-fold concentrated sample

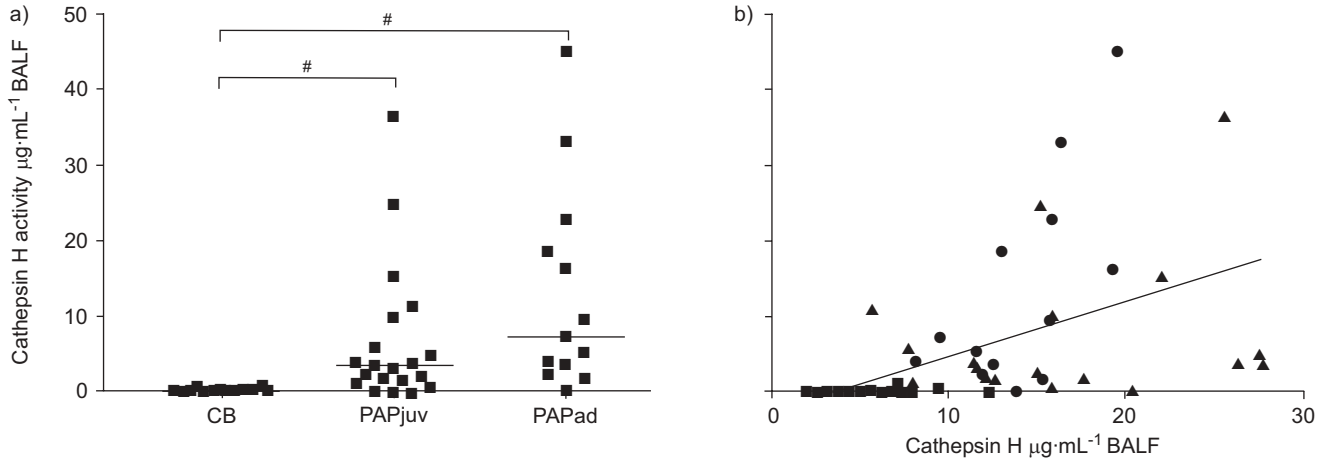

FIGURE 2. Cathepsin H activity in human bronchoalveolar lavage fluid (BALF). The enzymatic activity of cathepsin H in human BALF was determined by measuring the turnover of a specific substrate. a) The amount of proteolytically active cathepsin H in human BALF. The horizontal bars represent the median. b) Correlation of cathepsin H concentration with cathepsin H activity for all patients. CB: chronic bronchitis control patients (■); PAPjuv: juvenile pulmonary alveolar proteinosis (PAP) patients (▲); PAPad: adult PAP patients (●). #: $p<0.0001$. b) $r=0.646$, $p<0.0001$.

from PAP patients. Thus, the majority of cathepsin H was localised in the SA (fig. 6a). In contrast to the distribution of cathepsin H, a noticeably higher amount of napsin A protein was localised in the LA fraction (fig. 6b).

Napsin A and cathepsin H amounts and activities correlated with the intra-alveolar amounts of mature SP-B and SP-C, and with the physiological processing intermediates of proSP-B

Napsin A and cathepsin H are involved in the intracellular processing of SP-B and SP-C to their mature forms. The present study demonstrates that these proteases are also present and active in the extracellular space, so their potential role in relation to the hydrophobic surfactant proteins was investigated. Both the concentrations of cathepsin H (see figs 1a and b in the supplementary data) and napsin A (see figs 1c and d in the supplementary data) correlated significantly with the concentrations of the mature forms of SP-B and SP-C. In addition, the activities of the two proteases also correlated with the concentrations of these SPs (SP-B: cathepsin H $r=0.511$ and $p<0.0002$, napsin A $r=0.489$ and $p<0.0004$; SP-C: cathepsin H $r=0.645$ and $p<0.0001$, napsin A $r=0.518$ and $p<0.0001$).

The majority of subjects had the physiological proSP-B form of 24–26 kDa [22–24] in their BALF, as assessed by immunoblotting (table 2). As for mature SP-B, the abundance of this common precursor was 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, did not differ between the patient groups. Of all SP-B, ~80% was in the mature form (table 2). This indicates that the processing of the final product SP-B was not

TABLE 1 Cathepsin H concentrations, activities and inhibitor (cystatin C) concentrations, and ratios expressed on a molar basis

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

Data are presented as mean (95% confidence interval). PAP: pulmonary alveolar proteinosis; CatH: cathepsin H; CysC: cystatin C.

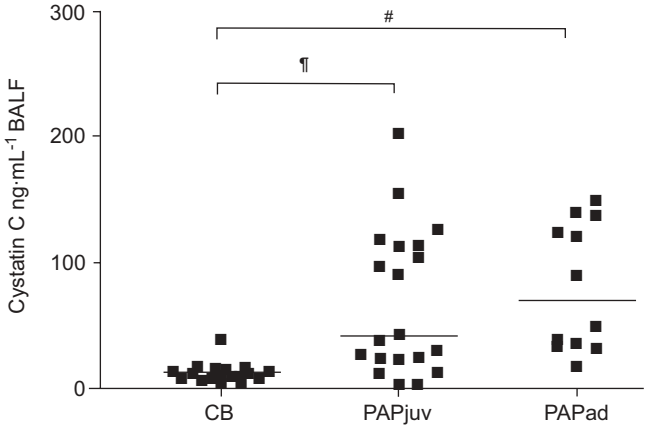

FIGURE 3. Cystatin C in human bronchoalveolar lavage fluid (BALF). The horizontal bars represent the median. CB: chronic bronchitis control patients; PAPjuv: juvenile pulmonary alveolar proteinosis (PAP) patients; PAPad: adult PAP patients. #: $p<0.0001$; ¶: $p=0.0003$.

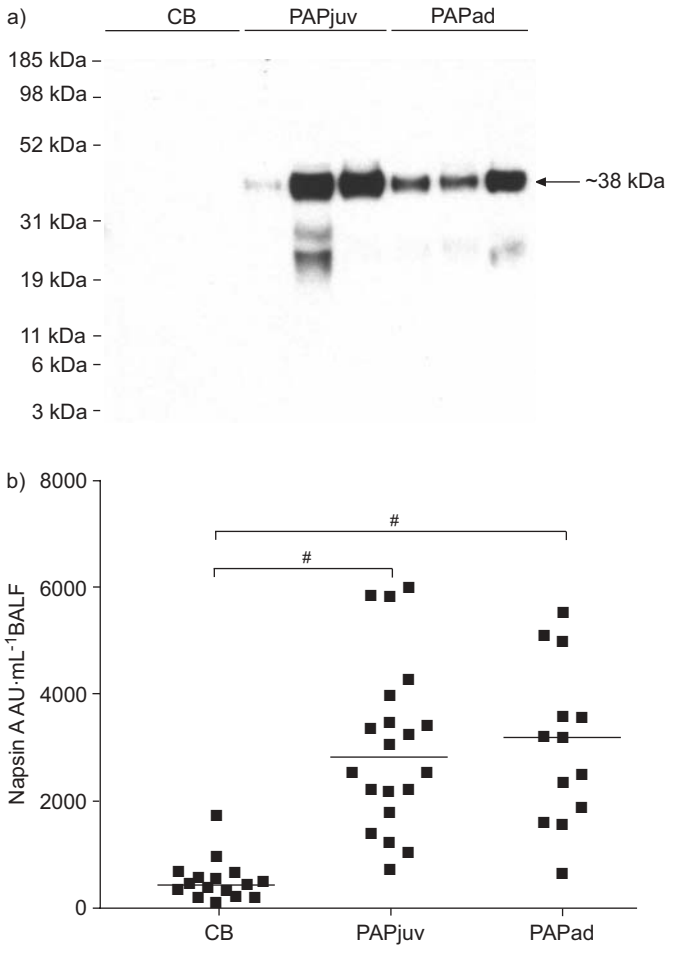

FIGURE 4. Napsin A in human bronchoalveolar lavage fluid (BALF). a) Aliquots (50 μL) of cell-free human BALF samples were separated on NuPAGE® gels, followed by immunodetection with napsin A antibody. A representative immunoblot, showing three 50 μL samples for each of the patient groups, with the signal for mature napsin A at ~38 kDa. Napsin A signals from all patients were densitometrically quantified, and calculated in relation to an internal standard. b) Distribution of relative napsin A concentrations in BALF. The horizontal bars represent the median. AU: arbitrary units; CB: chronic bronchitis control patients; PAPjuv: juvenile pulmonary alveolar proteinosis (PAP) patients; PAPad: adult PAP patients. #: $p<0.0001$.

altered in PAP patients. Interestingly, in ~50% of the patients with PAPjuv, additional aberrant 30–32 kDa proSP-B forms were present in significant amounts (table 2).

DISCUSSION

PAP is a group of rare diseases with disturbed surfactant homeostasis, resulting in altered composition and pool size of alveolar surfactant. PAPad can be subdivided into primary (so-called idiopathic) forms, and secondary forms associated with other diseases. Primary forms are caused by a defect in the re-uptake of the surfactant, due to autoantibodies against GM-CSF in blood and tissues, including pulmonary alveoli [25–27]. These autoantibodies neutralise the biological activity of GM-CSF. PAPjuv is a rare disease, and very few patients have been described [28, 29]. Causally, GM-CSF autoantibodies play no role, according to the present study and previous studies [29, 30].

The hypothesis of the present study 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 PAPjuv.

It was found that napsin A was present and active in the alveolar space under physiological conditions. In patients with PAP, the napsin A concentration was increased six-fold compared with controls. Similar observations were made for cathepsin H. The results excluded general deficiency of napsin A and cathepsin H from being the specific cause for abnormal surfactant accumulation in PAPjuv, because essentially the same results were found in PAPad, which is caused by GM-CSF autoantibodies. Both enzymes were active, and present abundantly and in proportion to the alveolar amounts of the mature SPs.

There are limitations to this study. First, protease amounts and activities were not determined inside alveolar type II cells, where surfactant is synthesised. The minor differences between the PAP groups, and the large differences between the PAP and control groups, 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 further unexplored functions. Secondly, an age-matched comparison group for PAPad was lacking. However, given the large differences in protease amounts and activities between the PAPjuv and the control CB group, compared with the small differences among the PAP groups, even with 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

The present study is the first to show that active napsin A is present in the BALF 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 amounts of 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 SPs 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 fractioning BALF, LAs were identified as the primary location of napsin A; LAs are derived from the LBs of pulmonary type II cells. This is in agreement with the localisation of napsin A in LBs, shown by immunoelectron microscopy [4, 6], and its association with cellular membranes [32].

Napsin A is an intracellular aspartyl protease with maximum activity at an acidic pH. Of note, at pH 7 and 37°C, napsin A activity has a half-life of 4 h [33]. Thus it was expected that active enzyme would be found in the BALF, if secreted. Napsin A may be involved in many extracellular enzymatic processes. In line with the extracellular functions of napsin A is

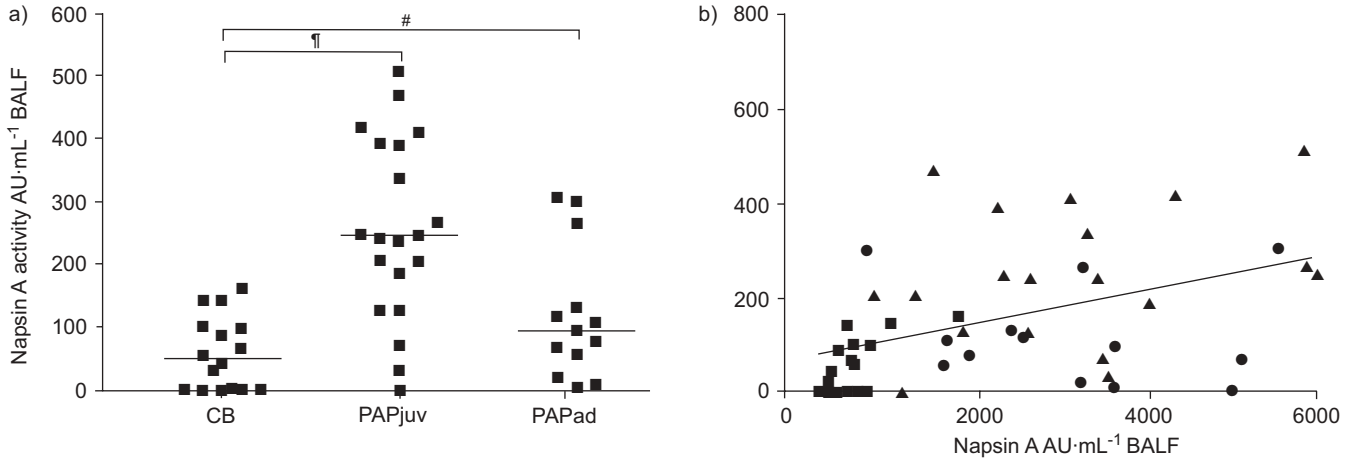

FIGURE 5. Napsin A activity in human bronchoalveolar lavage fluid (BALF). The enzymatic activity of napsin A in human BALF was determined by measuring the turnover of a specific substrate. The activity was based on an internal standard. a) The amount of proteolytically active napsin A in human BALF. The horizontal bars represent the median. b) Correlation of napsin A concentration with napsin A activity for all patients. CB: chronic bronchitis control patients (■); PAPjuv: juvenile pulmonary alveolar proteinosis (PAP) patients (▲); PAPad: adult PAP patients (●). #: p=0.0833; ¶: p<0.0001. b) r=0.475, p=0.0006.

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 remodelling during development and disease, as it provides coordinated cell and extracellular matrix loss [37].

Cathepsin H

Similar to napsin A, cathepsin H was found to be present and active in all BALF samples analysed. No precursor forms of cathepsin H were detected by immunoblotting, which is in agreement with studies of BALF from patients with inflammatory lung diseases and silicosis [13, 14]. In PAP patients, the amount and activity of cathepsin H 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 neither correlated with the number of alveolar macrophages recovered from BALF, nor with the degree of inflammation as represented by neutrophils in BALF (results not shown). Support for type II cells as the origin of cathepsin H comes from immunoelectron microscopy, which shows localisation of cathepsin H to LBs of alveolar type II cells [5]. After secretion, partitioning into the SA fraction may occur. Neutral pH levels are optimal for cathepsin H activity, so 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 SPs and not to inflammation or cell damage. Under physiological conditions, endogenous broad-range inhibitors of the cystatin family control cathepsin H activity strictly [40]. The levels of cystatin C, the major cathepsin H inhibitor [19], were two-fold higher in PAP patients compared with controls; however, the percentage of active cathepsin H increased from 3% in control subjects to 85% in PAPjuv 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 synthesised by all nucleated cells [41, 42]. A cell-specific defect in cystatin C expression in the lung is therefore unlikely.

The physiological functions of intra-alveolar cathepsin H can only be speculated. Clara cells secrete proSP-B as a 24 kDa proform [2], and this might be processed in the alveoli, thereby

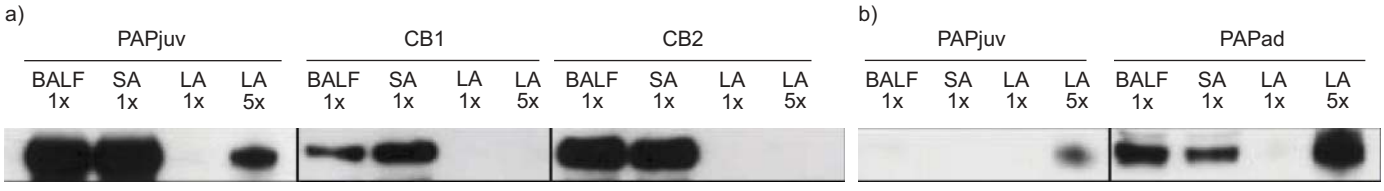

FIGURE 6. Localisation of cathepsin H and napsin A in human bronchoalveolar lavage fluid (BALF) subfractions. Human BALF, small aggregate (SA) and large aggregate (LA) surfactant were separated on NuPAGE® gels (Invitrogen), followed by immunodetection with a) cathepsin H or b) napsin A antibodies. Lanes were loaded with total BALF, SA or LA from a sample size of 100 μL BALF (1×), or LA from a sample size of 500 μL BALF (5×). Samples were from a) one juvenile pulmonary alveolar proteinosis (PAPjuv) patient and two control chronic bronchitis patients (CB; cathepsin H band size 28 kDa) or b) one PAPjuv patient and one adult PAP (PAPad) patient (napsin A band size 38 kDa).

TABLE 2 Surfactant protein (SP)-B proforms in bronchoalveolar lavage fluid (BALF) from the patient groups investigated

	Control	PAP juvenile	PAP adult
Subjects	16	20	13
SP-B proforms kDa			
9–15	0	2	0
19–21	3	3	0
24–26	13	17	13
30–32	0	13	0
proSP-B ng·mL^{-1}			
Total	29 (21–38)	662 (303–1021)	729 (234–1225)
24–26 kDa	30 (19–40)	531 (201–862)	729 (234–1225)
Mature SP-B %	71 (53–88)	88 (83–93)	82 (76–87)

Data are presented as n or mean (95% confidence interval). PAP: pulmonary alveolar proteinosis.

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 carboxy terminus of mature SP-B [6].

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

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