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Amyloid fibril formation by pulmonary surfactant protein C

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Abstract Lung surfactant protein C (SP-C) is a lipopeptide that contains two fatty acyl (palmitoyl) chains bound via intrinsically labile thioester bonds. SP-C can transform from a monomeric α-helix into β-sheet aggregates, reminiscent of structural changes that are supposed to occur in amyloid fibril formation. SP-C is here shown to form amyloid upon incubation in solution. Furthermore, one patient with pulmonary alveolar proteinosis (PAP, a rare disease where lung surfactant proteins and lipids accumulate in the airspaces) and six healthy controls have been studied regarding presence and composition of amyloid fibrils in the cell-free fraction of bronchoalveolar lavage (BAL) fluid. Abundant amyloid fibrils were found in BAL fluid from the patient with PAP and, in low amounts, in three of the six healthy controls. SDS-insoluble fibrillar material associated with PAP mainly consists of SP-C, in contrast to the fibrils found in controls. Fibrillated SP-C has to a significant extent lost the palmitoyl groups, and removal of the palmitoyl groups in vitro increases the rate of fibril formation.

Key words: Amyloid fibril; Lipid-associated peptide; Protein palmitoylation; Pulmonary surfactant; Surfactant protein C

1. Introduction

Amyloid fibril formation is associated with neurodegenerative disease and systemic amyloidosis. The amyloid-related proteins are constitutively present in their soluble state, but form insoluble aggregates under some conditions [1]. In spite of close structural similarities of the fibrils found in different amyloid diseases, the proteins or peptides forming the amyloid lack structural similarities in their native states, which indicates that fibrils can be formed via different pathways. Some mutant amyloid-forming proteins are significantly destabilized compared to the corresponding non-amyloidogenic wild-type forms, indicating that intrinsic protein stability is one important factor in amyloid formation [2]. For most amyloid-associated proteins, however, no clear-cut reason for unfolding and amyloid formation is known [3].

Pulmonary surfactant is essential for normal respiration by lowering the alveolar surface tension and by contributing to pulmonary host-defence. Four surfactant-associated proteins are known, SP-A, SP-B, SP-C and SP-D, whereof only SP-C appears to be uniquely expressed in the lungs [4,5]. SP-C is a transmembrane 35-residue lipopeptide in which amino acid residues 9–34 form an α-helix [6]. SP-C is one of the most hydrophobic naturally occurring peptides known; the human molecule contains 28 residues with aliphatic side-chains, including two palmitoyl chains bound to Cys-5 and Cys-6 via thioester bonds, which are labile and are readily cleaved by nucleophilic agents [7]. SP-C in solution transforms from a monomeric α-helix into aggregates with β-sheet conformation. In contrast, SP-C is thermodynamically stable and remains helical in lipid membranes [8]. The α-helix to β-sheet conversion of SP-C resembles the structural transitions which have been reported to take place in many proteins and peptides associated with amyloid formation [9]. Initial studies confirmed that SP-C, dissolved in chloroform/methanol/0.1 M HCl or ethanol (about 100 μM) and incubated at 37°C, forms elongated fibrils with a diameter of 2–4 nm, thus exhibiting amyloid-like appearance (unpublished data). This prompted us to further investigate SP-C fibril formation.

PAP is an uncommon lung disease in which lipoproteinaceous material is accumulated within the airspaces of otherwise preserved alveolar lung tissue, causing impaired gas exchange and dyspnea [10]. PAP can occur as a secondary event to lung infections, hematologic malignancies, and after exposure to inhaled chemicals and minerals, but many cases of PAP are idiopathic [11]. Mice with severe combined immunodeficiency and mice deficient in the granulocyte macrophage colony-stimulating factor (GM-CSF), or its receptor, show alveolar accumulation of surfactant proteins and lipids similar to that of human PAP [12–14]. The PAP-like changes in GM-CSF deficient mice can be reversed by administration of aerosolized GM-CSF [15,16].

2. Materials and methods

2.1. Handling of BAL fluid

The PAP patient was diagnosed by lung histology and electron microscopy. He had no other diseases and was on no treatment. Lung lavage was performed because of progressive dyspnea and hypoxemia. BAL fluid from six healthy volunteers (three men and three women, age 18 to 49 years) were used as controls. Bronchoalveolar lavage fluid was obtained by instillation of sterile saline, and subsequent gentle aspiration (approved by ethical committee). The BAL fluid collected was first centrifuged at $150 \times g$ for 15 min to remove cells and debris. Subsequently the supernatant was centrifuged at $20\,000 \times g$ for 2 h at 4°C, and the resulting pellet was equilibrated in 5 mM Tris-HCl, 100 mM NaCl, pH 7.4, 1.64 M NaBr and centrifuged at $100\,000 \times g$ for 4 h at 4°C. The pellets obtained after 20 000

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Abbreviations: BAL, bronchoalveolar lavage; GM-CSF, granulocyte macrophage colony-stimulating factor; PAP, pulmonary alveolar proteinosis; SP, surfactant protein

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and 100 000×*g* centrifugation were washed with distilled water and used for further analyses.

2.2. Isolation of SDS-insoluble aggregates

Equal amounts (typically 2–5 mg) of the PAP and control pellets were suspended in 1% (w/v) SDS by vortexing, and centrifuged for 20 min at 100 000×*g* at 20°C [17]. After removal of the supernatant, the same procedure was repeated four times. The SDS-insoluble pellets were analyzed by SDS-PAGE, polarization microscopy after staining with Congo red, and electron microscopy. The SDS-soluble supernatants were lyophilized and analyzed by SDS-PAGE.

2.3. SDS-PAGE and amino acid sequence analysis

Samples were solubilized in 4% (w/v) SDS/45 mM Tris-HCl, pH 8.3, with or without 5% (v/v) β-mercaptoethanol, by incubation for 10 min at 110°C. Electrophoresis was performed in 10–20% gradient Tricine gels with 0.1% SDS in the running buffer. Gels were stained with Coomassie brilliant blue or silver. Alternatively, proteins were electro-transferred from the electrophoresis gels to polyvinyledinedifluoride (PVDF) membranes at 25 V for 100 min at room temperature. After staining of the PVDF membrane, bands were excised and analyzed for N-terminal amino acid sequence by Edman degradation in an Applied Biosystems 494 cLC protein sequencer with on-line HPLC for detection of phenylthiohydantoin derivatives. Western blotting was performed after protein transfer to PVDF membranes by using an alkaline phosphatase-labelled monoclonal anti-human IgG antibody.

2.4. Analysis of SP-C fibril formation

Chemical depalmitoylation of SP-C [7] was performed by treating the peptide, dissolved in chloroform/methanol, 1:2 (v/v), with trimethylamine for 1.5 h at room temperature, whereafter equimolar amounts, relative to trimethylamine, of trifluoroacetic acid were added. The removal of both palmitoyl chains was confirmed by mass spectrometry [18].

For fibrillation studies, 100 μM of native SP-C or depalmitoylated SP-C in chloroform/methanol/0.1 M HCl, 32:64:5 (by vol) or ethanol was incubated at 37°C. After different time intervals, aliquots were removed and centrifuged at 20 000×*g* for 20 min at 22°C [19]. The supernatants were analyzed by amino acid analysis for determination of peptide content, and the pellets were analyzed by electron microscopy.

2.5. Light microscopy after staining with Congo red

Staining was performed essentially as described [20]. Briefly, SDS-insoluble aggregates were suspended in 50 μl phosphate-buffered saline by sonication before addition of 10 μl Congo red (2%, w/v). Samples were incubated for 1 h at ambient temperature and aggregated proteins were collected by centrifugation at 13 000×*g*, for 5 min. The aggregates were washed twice with 100 μl of water before resuspension in 50 μl of water. Ten μl of the suspension was placed on a glass slide and allowed to dry. Samples were viewed by polarization microscopy using a Zeiss Axialfold microscope.

2.6. Electron microscopy

The pellets obtained after centrifugation of BAL, SDS extractions, or after incubation of SP-C or depalmitoylated SP-C in solution were suspended in a small volume of water by low-energy sonication for 5 s. Aliquots of 8 μl were placed on grids covered by a carbon-stabilized formvar film. Excess fluid was withdrawn after 30 s, and after air-drying the grids were negatively stained with 3% uranyl acetate in water. The stained grids were examined and photographed in a Philips CM120TWIN electron microscope operated at 80 kV.

3. Results

3.1. Amyloid fibrils in BAL fluid

Alveolar contents from one PAP patient and six healthy controls were recovered by bronchoalveolar lavage. After removal of cells and coarse debris, a particulate fraction was obtained after ultracentrifugation. Electron microscopy revealed the presence of significant amounts of amyloid fibrils in the pellet from the PAP patient (Fig. 1A). In three of the six control subjects, fibrils of similar appearance could be detected, but they were much less abundant than in the PAP case. The pellet from the PAP patient was then suspended in 1% SDS and centrifuged at 100 000×*g* for 20 min. The supernatant was removed and the resuspension and centrifugation procedure was repeated four times for the pellet. This procedure readily removes soluble proteins and has previously been used for isolation of the Alzheimer amyloid beta (Aβ) peptide from brain tissue [17]. The final pellet obtained was analyzed for ultrastructural properties,

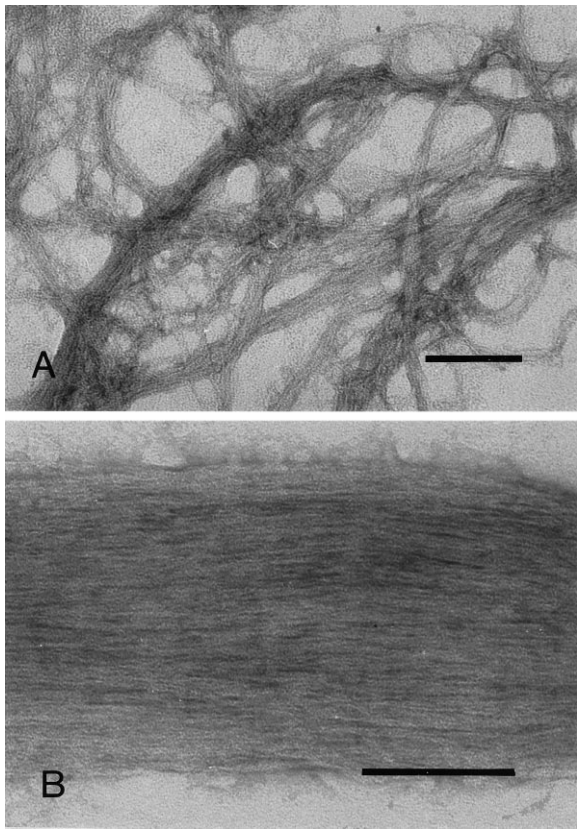

Fig. 1. Amyloid fibrils are present in pulmonary alveolar proteinosis. Cell-free BAL fluid from a PAP patient was pelleted and analyzed by electron microscopy (A), and further extracted five times with 1% SDS, whereafter the insoluble pellet was analyzed by electron microscopy (B). Bar = 100 nm.

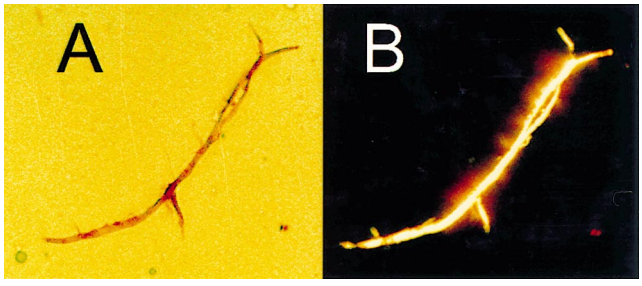

Fig. 2. Tinctorial properties of isolated fibrils. Congo red birefringence of SDS-insoluble pellet from PAP BAL fluid. Protein aggregates were stained with Congo red, washed and applied to microscope slides and observed by bright field microscopy (A) or by polarization microscopy (B). Magnification 360×.

which revealed fibrillar structures with amyloid appearance (Fig. 1B), that stain with Congo-red and exhibit green birefringence (Fig. 2). This confirms that amyloid fibrils are abundant in PAP. In contrast, SDS extraction of the control samples yielded only a very faint pellet.

3.2. Amyloid fibril protein composition

In order to define the constituent proteins of the PAP alveolar amyloid, the pellet obtained by 1% SDS extraction was resolved by SDS-PAGE under reducing conditions, followed by sequence analysis and Western blotting. This showed one strong band at about 4 kDa and one band at about 8 kDa, corresponding to monomeric and dimeric SP-C, respectively, as well as one weaker band at 50 kDa, corresponding to IgG heavy chain (Fig. 3, lane 1). The same analysis of the SDS-soluble supernatant obtained from PAP alveolar material identified several abundant proteins (Fig. 3, lane 2), i.e. albumin (65 kDa), SP-A (35 kDa), IgG light chain (25 kDa), and SP-B (10 kDa), and weak bands corresponding to SP-C are seen. In the healthy control, in sharp contrast, the SDS-insoluble pellet contains no detectable SP-C (Fig. 3, lane 3), but SP-C and the other surfactant proteins are found exclusively in the SDS-soluble supernatant (Fig. 3, lanes 4 and 5). SDS-PAGE of the SDS-insoluble pellets from all healthy controls confirmed that significant amounts of aggregated SP-C are specific for PAP (data not shown).

3.3. Importance of SP-C palmitoyl groups for fibril formation

SDS-PAGE of the PAP SDS-insoluble pellet without disulphide reduction revealed no SP-C bands, since the aggregates did not enter the gel. However, native SDS-soluble SP-C migrates as a monomer without disulphide reduction (Fig. 3, lane 5). Consequently, SP-C aggregates in the 1% SDS-insoluble pellet are disulphide-dependent. Since SP-C contains only two cysteines which both normally are palmitoylated, the SP-C population that forms SDS-insoluble disulphide-dependent aggregates must contain depalmitoylated peptides. The exact ratio of SP-C and depalmitoylated SP-C in the fibrils cannot

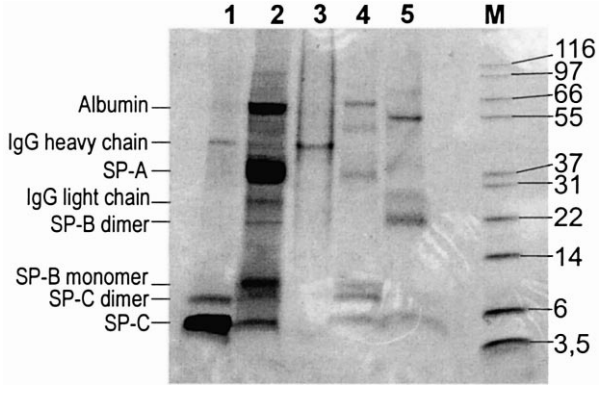

Fig. 3. Composition of PAP-associated insoluble fibrils. SDS-PAGE of proteins in BAL fluid which are insoluble (lanes 1 and 3) or soluble (lanes 2, 4, 5) after five extractions with 1% SDS. The samples were derived from a PAP patient (lanes 1 and 2) and a healthy control (lanes 3–5). The sample in lane 1 is the same material as analyzed in Figs. 1B and 2. The sample in lane 5 was not reduced, while samples in lanes 1–4 were reduced. The identity of the different bands, as deduced from amino acid sequence analysis or Western blotting, are given to the left. The lane marked M shows the migration of molecular size markers with indicated masses in kDa.

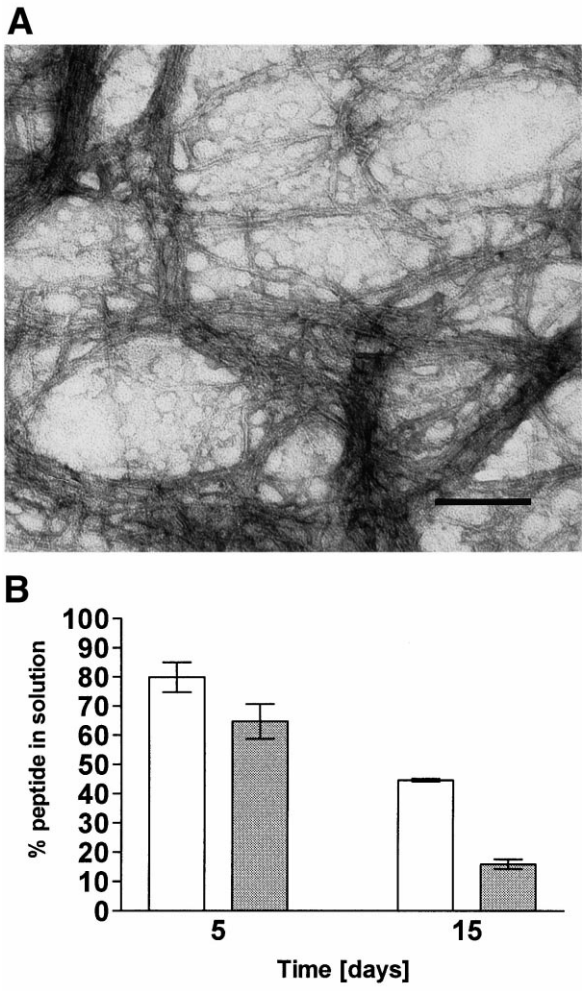

Fig. 4. Removal of SP-C palmitoyl groups increases fibril formation. A: Electron micrograph of fibrils formed after 1.5 h incubation of depalmitoylated SP-C in chloroform/methanol/0.1 M HCl, 32:64:5 (by vol). Bar = 100 nm. B: Peptide amounts in solution after 5 and 15 days of incubation of native SP-C (open bars) and depalmitoylated SP-C (filled bars).

be determined from the present data. However, a major part of the fibrillated material likely consists of depalmitoylated SP-C, since no electrophoretic bands corresponding to SP-C can be detected without disulphide reduction, and no detectable protein remains in the gel loading pocket with reduction. Consistent with the assignment of depalmitoylated SP-C to alveolar fibrils, chemical depalmitoylation of SP-C increases the rate of SP-C fibril formation in vitro (Fig. 4). Intriguingly, electron microscopy indicates that fibrils are formed from the depalmitoylated peptide as early as 1.5 h after starting the incubation (Fig. 4A). Moreover, determination of the amount peptide that remains in solution after 5 and 15 days of incubation confirms an increased rate of peptide aggregation after removal of the palmitoyl groups (Fig. 4B).

4. Discussion

SP-C is here shown to form amyloid fibrils. This could be anticipated from its tendency to undergo $\alpha \rightarrow \beta$ transitions [8]. The present data further indicate that amyloid formation can occur in PAP (Figs. 1 and 2). The fibrils associated with PAP

are composed of SP-C, depalmitoylated SP-C and IgG heavy chain. The relative amounts of these proteins suggest that SP-C peptides are the main constituents (Fig. 3). Previously it has been shown that SP-A forms oligomeric aggregates in PAP [21], but no SP-A could now be detected in the insoluble fibrils (Fig. 3). We conclude that SP-C is the only surfactant protein present in PAP amyloid. The etiology of PAP is largely unknown, but decreased removal of the alveolar contents by alveolar macrophages appears to be a common denominator [22,23]. In GM-CSF knockout mice, as well as in SP-D knockout mice, the alveolar macrophages have been reported to be loaded with lipoproteinaceous material [13,24,25]. The clearance of alveolar SP-C fibrils is expected to be significantly impaired compared to that of native SP-C, since already dimeric/depalmitoylated SP-C is cleared from mouse lungs with a half-life which is more than twice that for monomeric/native SP-C [26]. Clearance of surfactant phospholipids and SP-A is, however, also impaired in PAP [27,28]. Alveolar accumulation of fibrils may be toxic; Aβ fibrils associated with Alzheimer's disease induce oxidative stress in microglial cells via binding to class A scavenger receptors [29]. It remains to be established whether alveolar macrophages could be affected by fibrils in an analogous manner. Notably, it has recently been shown that dimeric SP-C is toxic to alveolar macrophages through increased formation of superoxide anions [30].

Recent data indicate that amyloid formation in vitro is not restricted to a limited set of proteins. This implies that the development of amyloid diseases is governed by factors that influence the equilibria between different conformational states of amyloidogenic proteins [1,31]. The nature of these factors is to a large extent unknown, but protein concentration affects amyloid development [32]. Increased alveolar levels of surfactant proteins (including SP-C) [33,34], likely contribute to the formation of amyloid fibrils in PAP. In addition, removal of SP-C palmitoyl groups may influence amyloid formation. Fibrils formed in PAP contain disulphide-linked depalmitoylated SP-C (Fig. 3), and depalmitoylated SP-C is more prone to form fibrils (Fig. 4). Previous studies have revealed elevated levels of depalmitoylated forms of SP-C in PAP compared to healthy controls [26,35]. Protein palmitoylation often occurs in integral membrane proteins close to their transmembrane helices [36], but the biological function of this modification is not known. Depalmitoylation apparently destabilizes the SP-C α-helix [6]. Removal of the palmitoyl groups could therefore promote fibrillation directly by facilitating α-helix to β-sheet conversion. Moreover, depalmitoylation may induce fibrillation indirectly, by favoring the removal of SP-C from a lipid membrane where it is structurally stable [8], in contrast to the situation in solution (Fig. 4). In this context it is interesting to note that the Aβ peptide associated with Alzheimer's disease transforms into amyloid fibrils if it is removed from its membrane-associated precursor state [37]. No explanation for the observed loss of SP-C palmitoyl groups in PAP is presently available. Cleavage of thioester-linked acyl groups could be mediated by reactive oxygen species, such as hydrogen peroxide, superoxide anions and hypochlorite released from inflammatory cells [38]. Release of agents that cleave SP-C-linked palmitoyl chains, combined with disrupted removal of surfactant by alveolar phagocytes, could thus cause an exponential growth of amyloid fibrils. It should be pointed out, however, that the production of reactive oxygen species is decreased in GM-CSF null mice [39]. Further studies are motivated in order to assess the role of amyloid fibrils, and the importance of inflammatory cells and surfactant clearance in the pathogenesis of alveolar proteinosis.

The α-helix of SP-C is metastable in solution [8]. As pointed out previously [8,40] the long poly-Val segment of SP-C statistically favors β-sheet conformation over helix formation. It is reasonable to believe that this α/β discordance underlies structural transition and fibril formation by SP-C, and we suggest that also other amyloid-forming proteins may contain α-helices with high statistical propensity for β-sheet formation.

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