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Physiological concentration of calcium inhibits elastase-induced cleavage of a functional recombinant fragment of surfactant protein D

Annelyse Duvoix[a], Rose-Marie Mackay[b], Neil Henderson[b], Eamon McGreal[c], Anthony Postle[b], Kenneth Reid[a], Howard Clark[b,*]

[a] MRC Immunochemistry Unit, Biochemistry Dept, University of Oxford, South Parks Road, OX1 3QU Oxford, United Kingdom
[b] IIR, School of Medicine, University of Southampton, MP 803 Level F, South Block, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom
[c] Department of Child Health, Cardiff University, Wales College of Medicine, 5th Floor University Hospital, Heath Park, Cardiff CF14 4XN, United Kingdom

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ABSTRACT

Surfactant protein D (SP-D) plays an important role in lung host defence. SP-D levels have been shown to be depleted in cystic fibrosis (CF) patients. A recombinant fragment of the human SP-D (rfhSP-D) which consist of a hydrophobic neck and a CRD has been shown to be active *in vivo* and partially reverses the symptoms of the SP-D deficiency in the lungs when administered to SP-D knock-out mice. In this paper we studied the *in vitro* effect of different proteolytic enzymes commonly found in CF patients lungs, such as neutrophil elastase, cathepsin G and protease 3 as well as *Pseudomonas* elastase, on rfhSP-D. It was also shown that cleavage was inhibited by physiological concentration of calcium. When Western blot was compared with ELISA, we show that an anti-SP-D ELISA is a not a reliable assay of functional SP-D levels since non-functional fragments of SP-D are also detected. Thus, ELISA cannot be used as a reliable "diagnostic" tool for SP-D deficiency. Finally, we observe that SP-D is not cleaved in control patients but is degraded in about half the samples from cystic fibrosis patients, indicating that degradation of endogenous SP-D, by enzymes present in CF bronchioalveolar lavage fluid (BALF), may lead to deficiency of the protein as seen in CF and therefore rfhSP-D may be a useful future therapy.

1. Introduction

Surfactant protein D (SP-D) is a calcium-dependent carbohydrate molecule of the innate immune system involved in first-line defences of the mucosal surfaces against microorganisms, virus, fungi or allergens.

SP-D is a member of the collectin family and consists of a short N-terminal domain, a collagen domain, a hydrophobic neck and a calcium-dependant carbohydrate recognition domain (CRD). The association of 3 SP-D molecules, through the formation of a triple helical collagen region and stabilising interchain disulfide bonds, forms basic subunits that then associate in dodecamers linked by their N-terminal regions (cruciform structure). SP-D is secreted by alveolar type II cells in the lungs (Crouch et al., 1991) and participates in the pulmonary host defence as well as surfactant homeostasis. In particular, it has been shown that SP-D can lead to microbacterial agglutination (Hartshorn et al., 1996; Ferguson et al., 1999), plays a role in the clearance of pathogens (Hartshorn et al., 1998) and apoptotic cells (Vandivier et al., 2002), can modulate the cellular immune response (Borron et al., 1998), has a direct effect on microbacterial growth (Wu et al., 2003), modulate the allergic response (Strong et al., 2002), plays a role in the regulation of inflammation (Crouch and Wright, 2001; Gardai et al., 2003) and binds and helps to clear DNA from the lungs (Palaniyar et al., 2004).

Our team has cloned and purified a fragment of the human SP-D (rfhSP-D), consisting of the neck and CRD regions, which, when used in SP-D KO mice, has been shown to reduce lung inflammation, decrease the excessive number of apoptotic and necrotic alveolar macrophages and inflammatory cells as well as reduce the level of inflammatory cytokines (Clark et al., 2003).

The level of SP-D has been shown to be reduced in the BALF of healthy smokers (Honda et al., 1996) and in cystic fibrosis (CF) patients (Postle et al., 1999).

CF is a lethal hereditary disease caused by mutations of the CF transductance regulator gene leading to abnormal exocrine gland secretion and chronic inflammation of the airways. The disease is characterised by a massive influx of neutrophils and as a consequence by high levels of proteolytic enzymes that play a major role in the progression of CF lung disease. The low levels of SP-D could be explained at least in part by the increased proteolytic activity. Indeed, it has been shown that neutrophil serine proteases (NSPs),

Abbreviations: SP-D, surfactant protein D; CF, cystic fibrosis; BALF, bronchoalveolar lavage fluid; CRD, carbohydrate recognition domain; NSP, neutrophil serine protease; NE, elastase; CG, cathepsin G; P3, protease 3; PE, *Pseudomonas* elastase.

* Corresponding author.
E-mail address: H.W.Clark@soton.ac.uk (H. Clark).

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such as elastase (NE), cathepsin G (CG) and protease 3 (P3), which are released from degranulating or necrotic neutrophils, can cleave SP-D *in vitro*, inhibiting its ability to agglutinate microorganisms (Hirche et al., 2004).

The presence of the microorganism *Pseudomonas aeruginosa*, the predominant infectious factor in CF patients, could also be a cause of SP-D degradation. In fact, *Pseudomonas* elastase (PE) has been shown to cleave SP-D *in vitro* (Alcorn and Wright, 2004). However, controversy arose when it was described that CF patients presenting infection show lower levels of SP-D and SP-A whereas show an increased level of SP-A during infections (Postle et al., 1999; Hull et al., 1997). Yet, both these teams use the technique ELISA in order to measure the surfactant proteins level. Moreover, there is little evidence on the effect of these proteolytic enzymes *in vivo* and the effect of those enzymes on the fragment of SP-D has yet to be studied.

Therefore, the aim of this study was to determine if the 4 proteolytic enzymes, NE, CG, P3 and PE, also cleave rfhSP-D *in vitro* in the absence or presence of calcium. We also wanted to investigate the best method of investigating levels of cleaved protein by comparing ELISA to SDS-PAGE or Western blot. Finally, we want to study the fate of rfhSP-D when incubated with CF patient and control BALF. N-terminal sequencing of the cleavage product was used both *in vitro* and *in vivo* in order to determine which enzyme is implicated in the cleavage of rfhSP-D.

2. Materials and methods

2.1. rhfSP-D

The recombinant fragment of SP-D (20 kDa) was purified from *E. coli* as previously described (Strong et al., 2002). The anti-SP-D antibody used was raised against rfhSP-D.

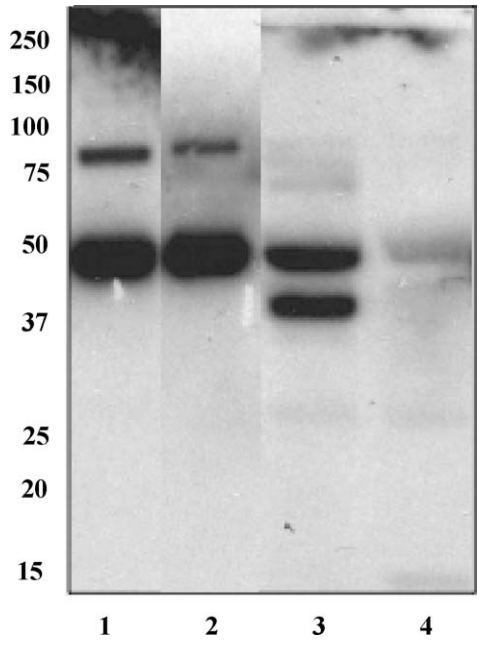

Fig. 1. State of NhSP-D in CF BALF. Individual BALF samples from 3 control patients and 24 CF patients were used. 200 μl of BALF was incubated with 20 μl of startaclean for 30 min at room temperature. The samples were spun and the strataclean pellet was used for Western blot using anti-SP-D antibody: (1) control BALF and (2–4) CF BALF showing increased NhSP-D degradation.

2.2. Proteases

Pseudomonas elastase, neutrophil elastase, protease 3 and cathepsin G were purchased from Elastin Product Company

Table 1
N-terminal sequencing of cleavage products of rfhSP-D. rfhSP-D was incubated with P3, CG, NE and PE and the cleavage products were sequenced. The band numbers are shown in Fig. 2. ⇓ shows cleavage site.

Enzyme	Band number	Fragment size (kDa)	N-terminal sequence
No enzyme		20	ASPGLKG...
Protease 3	1	11	GEKIFK...
		11	ASPGLK...
	2	10	GFVKPF
		10	ASPGLK...
		10	QLL-TQ...
	3	9	QLL-TQ...
		9	ASPGLK...
	4	7	ASPGLK...
		7	ALQQLV...
	5	6	ASPGLK...
Cathepsin G	6	17	ASPGLK...
	7	14	ASPGLK...
	8	10	ASPGLK...
	9	6	ASPGLK...
Neutrophil elastase	10	12	SPGLK...
		12	ASPGLKG...
	11	7	FLSMTDS...
		7	ASPGLKG...
Pseudomonas elastase	12	15	ASPGLK...
	13	14	ASPGLK...
	14	10	FVKPFT...
		10	ASPGLK...
	15	6	FVKPFT...
		6	ASPGLK...
BALF	Fig. 6	12	ASPGLK...

rfhSP-D sequence: ASPGLKGDKGIPGDKGAKGESGLPDVASLRQQVEALQGQVQHLQAAFSQYKKVELFPNGQSVGEKIFKTAGFVKPFTEAQLLCTQAGGQLASPRSAAENAALQQLVVAKNEAAFLSMTDSKTEGKFTYPTGESLVYSNWAPGEPNDDGGSEDCVEIFTNGKWNDRACGEKRLVVCEF-.
Protease 3 cleavage pattern: ASPGLKGDKGIPGDKGAKGESGLPDVASLRQQVEALQGQVQHLQAAFSQYKKVELFPNGQSV⇓GEKIFKTA⇓GFVKPFTEA⇓QLLCTQAGGQLASPRSAAENA⇓ALQQLVVAKNEAAFLSMTDSKTEGKFTYPTGESLVYSNWAPGEPNDDGGSEDCVEIFTNGKWNDRACGEKRLVVCEF-.
Neutrophil elastase cleavage pattern: A⇓SPGLKGDKGIPGDKGAKGESGLPDVASLRQQVEALQGQVQHLQAAFSQYKKVELFPNGQSVGEKIFKTAGFVKPFTEAQLLCTQAGGQLASPRSAAENAALQQLVVAKNEAA⇓FLSMTDSKTEGKFTYPTGESLVYSNWAPGEPNDDGGSEDCVEIFTNGKWNDRACGEKRLVVCEF-.
Pseudomonas elastase cleavage pattern: ASPGLKGDKGIPGDKGAKGESGLPDVASLRQQVEALQGQVQHLQAAFSQYKKVELFPNGQSVGEKIFKTAG⇓FVKPFTEAQLLCTQAGGQLASPRSAAENAALQQLVVAKNEAAFLSMTDSKTEGKFTYPTGESLVYSNWAPGEPNDDGGSEDCVEIFTNGKWNDRACGEKRLVVCEF-.