Surfactant protein A and D expression in the porcine Eustachian tube[1]

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Received 15 April 1999

Abstract Surfactant proteins A and D are collectins which are considered to play an important role in the innate immunity of lungs. Our aim was to investigate whether surfactant protein A or D is expressed in the porcine Eustachian tube originating from the upper airways. Both surfactant proteins A and D were present in the epithelial cells of the Eustachian tube, as shown by strong immunostaining. Using RT-PCR and Northern hybridization, these collectins were detected in the Eustachian tube. The present study is the first report demonstrating surfactant protein gene expression in the Eustachian tube. Surfactant proteins A and D may be important in the antibody-independent protection of the middle ear.

Key words: Surfactant protein A; Surfactant protein D; Eustachian tube; Collectin; Gene expression

1. Introduction

Surfactant proteins A and D (SP-A and SP-D) are C-type lectins (collectins) found in the epithelial lining of the lung [1]. They are multimeric proteins, which have a carbohydrate recognition domain (CRD) bound via the neck region to a collagenous domain [2]. The other collectins are serum mannose-binding protein [3], conglutinin [4] and collectin-43 (CL-43) [5]. According to current evidence, collectins mediate the innate immunity through binding to carbohydrates on micro-organisms and to specific receptors on phagocytic cells [6].

The main sites of SP-A and SP-D synthesis and secretion include alveolar type II and bronchiolar Clara cells [7]. They are also detected in the epithelium of the conducting airways and in the tracheal and bronchial glands of the lower airways [8,9]. SP-A expression has further been reported in rat intestinal epithelium [10] and in a phospholipid-rich layer from rat and human colon [11]. SP-D protein was also detected in the salivary and lacrimal gland [12] and gastric antrum [13]. SP-D mRNA was present in some tissues derived from the primitive gut [14].

SP-A has been shown to bind to pulmonary type II alveolar cells and immune cells, particularly to alveolar macrophages, and to regulate the functions of these cells [15]. The proposed functions of SP-A in vitro include the enhancement of surface activity [16,17], maintenance of homeostasis between the extra- and intracellular surfactant pools [18] and involvement in non-antibody-mediated defense against micro-organisms [15]. Unlike SP-A, SP-D does not bind to the surfactant complex or enhance the surface activity. However, it binds to carbohydrate structures on the surfaces of pathogens, resulting in agglutination of the target [6]. Deletion of SP-A [19] or SP-D [20,21] did not compromise the alveolar stability or the respiratory function in mice. Instead, SP-A knock-out mice showed a reduced intracellular killing and clearance of group B streptococci [22,23] and *Pseudomonas aeruginosa* [24]. Thus far, there are no reports on the host defense function of SP-D knock-out mice.

The Eustachian tube (ET) is important for the function of the middle ear, as it maintains the transient patency of the upper airways during swallowing. Evidence of a surface tension lowering substance in the canine ET has been found [25] and concentric lamellar bodies have been detected within the epithelial cells of the ET [26]. In 1992, Kobayashi et al. reported of a 80 kDa protein in the human middle ear, which cross-reacted with monoclonal antibodies to human SP-A [27]. However, the expression of surfactant protein genes has not been shown.

According to the present hypothesis, by maintaining the dynamic function and by enhancing the resistance of the middle ear against infection, surfactant components in the ET could prove to be instrumental in the protection of the middle ear. The aim was to investigate whether the C-type lectin genes associated with the surfactant system are expressed in the ET.

2. Materials and methods

2.1. Preparation of RNA

The pharyngeal opening of the porcine and ovine ET was prepared and a 20 G catheter was inserted into the tube. RNA was recovered from the ET lining by lavaging the tube with phenol-based RNA-STAT 60 (Tel-Test). Total RNA was isolated from the lavage return. The RNA pellet was dried, resuspended in diethyl pyrocarbonate-treated water and stored at −70°C before RT-PCR and Northern blot analysis. Total RNA from porcine lung was isolated as control. Approximately 15 mg was pulverized and homogenized in RNA-STAT 60 solution.

2.2. Cloning of cDNA fragments for ET SP-A and SP-D

Altogether, 2 μg of total RNA was used for the RT-PCR reaction for SP-A with porcine-specific primers. The forward primer was 420 bp from the start site of porcine SP-A cDNA (L41350): (GGTGTCCTCAGTTTCCAGGAGT) and the reverse primer was 785 bp from the start of the cDNA: (CAGTCGGTACTGCTGGCAGTTC). RT-PCR reactions were performed using the Masteramp RT-PCR kit (Epicentre) with 1×enhancer in a one tube reaction. A 35 cycle RT-PCR was carried out (94°C 5 min, 94°C 30 s, 63°C 30 s, 72°C 45 s, 72°C 10 min). For SP-D, forward (TTCCCWRATGGCCRRAGTG) and reverse (AGATCTCCACACAGTYCTC) primers were selected from the carbohydrate-binding domain of human, bovine, rat and mouse lung SP-D cDNA. The primers were chosen from the area with the most conserved cDNA sequences. A 40 cycle RT-PCR for

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[1] The accession numbers of porcine and ovine surfactant D proteins are AJ133003 and AJ133002, respectively.

Abbreviations: SP, surfactant protein; ET, Eustachian tube; CRD, carbohydrate recognition domain

PII: S0014-5793(99)00602-X

porcine and ovine SP-D was carried out as for SP-A, except that the annealing temperature was 55°C. The resulting fragments were purified with the Qiaquick PCR purification kit (Qiagen) and the SP-A fragment was blunt-end-ligated into the *Eco*RV site of pBluescript KS (Stratagene). SP-D fragments from the porcine lung and ET and ovine ET were ligated to the pGEM-T Easy vector (Promega).

The clones obtained were identified by sequencing on both strands.

2.3. Northern analysis of SP-A and SP-D

For Northern analysis, total RNA was isolated as described above. Lung and ET mRNA were run on a formaldehyde gel. The samples were transferred with 20×SSC (1×SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7) overnight onto a Biodyne B membrane (Pall) and the filter was baked at 80°C for 2 h. For Northern hybridization, SP-A and SP-D antisense [^{32}P]CTP-labelled riboprobes were synthesized using T7 RNA polymerase (Promega) or Sp6 RNA polymerase (Promega) in a transcription reaction containing 1.2 μg linearized plasmid, 1×transcription buffer (Promega), 10 mM DTT and 0.5 mM ATP, GTP and UTP nucleotides (Amersham Pharmacia Biotech) and 13 U RNAse inhibitor (Amersham Pharmacia Biotech) for 1 h at 37°C. After DNAse I digestion, the labelled product was purified on a Sephadex G-50 column (Amersham Pharmacia Biotech). The hybridizations were performed at 42°C overnight in 50% formamide, 1 M NaCl, 1% sodium dodecylsulfate (SDS), 10% dextran sulfate, 5×Denhardt's, 100 μg/ml carrier DNA (calf thymus DNA) and the high-stringency washes at 60–65°C in 0.1×SSC, 0.1% SDS.

2.4. Immunohistochemistry

The ETs from pigs were isolated and fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The 5 μm cross-sections of ET obtained for immunostaining were deparaffinized and rehydrated. After that, they were treated by heating in citrate-phosphate buffer (0.01 M citric acid, pH 6) and incubated in 0.03% H_2O_2. After the inhibition of endogenous peroxidase, the sections were incubated in fetal calf serum in PBS to prevent non-specific staining.

The sections were incubated with primary antibodies. For SP-A, monoclonal human anti-SP-A antibody PE-10 was a kind gift from Prof. T. Akino (Sapporo, Japan) [28]. The dilution used was 1:1000. Porcine anti-SP-D polyclonal antibody, dilution 1:2500, was a kind gift from Dr M. van Eijk (Utrecht, The Netherlands). For SP-A, the secondary antibody was a biotinylated rabbit anti-mouse immunoglobulin antibody (DAKO) diluted 1 to 300. For SP-D, the secondary antibody was a biotinylated swine anti-rabbit immunoglobulin antibody (DAKO), dilution 1:200. After the addition of the secondary antibody, the sections were treated with ABC complex (DAKO) and stained with DAB substrate (ZYMED). Counterstaining was done with hematoxylin.

3. Results

3.1. Isolation of porcine SP-A cDNA

RT-PCR amplification of the porcine ET was carried out using specific primers for porcine lung SP-A cDNA. The resulting 365 bp fragment was amplified and cloned. Sequencing of this fragment revealed 100% homology compared to the fragment obtained from porcine lung.

3.2. Isolation of porcine and ovine SP-D cDNA

Primers chosen from the conserved regions of the carbohydrate-binding domain of SP-D cDNA were used for RT-PCR amplification. The 313 bp PCR product was obtained from porcine lung and ET RNA. Ovine lung and ET RNA revealed a 304 bp product with the same primers. The fragments were cloned and sequenced. These sequences were highly conserved, the similarity with the SP-D cDNA of the known species ranging between 77 and 86%. The sequences were identical to those in the lung. The porcine sequence showed a three amino acid insertion, compared to the other species. The sequences of rat, mouse, human, bovine, ovine and porcine cDNAs and the deduced porcine SP-D amino acid sequence are shown in Fig. 1.

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human    GCTTTCTCTC AGTATAAGAA AGTTGAGCTC -----AA--- ---AA-----
rat      GCCTTTTCTC GCTATAAGAA AGCCGCGCTC -----TG--- ---AA-----
mouse    GCCTTCTCCC ACTATCAGAA AGCTGCATTG -----TG--- ---GA-----
bovine   GCCTTCTCTC AGTATAAGAA AGCGATGCTC -----TA--- ---GG-----
ovine    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ -----AG--- ---GG-----
porcine  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ TTCCCTGATG GCCGGAGTGT

porcine amino acid sequence                F  P  D   G  R  S  V

human    C--G--G--G --T--C-A-A -A-C-GG--T T-T-A-AC-A ---AC---G-
rat      T--A--C--A --C--C-G-G -G-C-AA--C G-A-G-GC-T ---GA---T-
mouse    T--A--C--G --C--C-G-A -A-C-GA--C T-A-A-GC-T ---GA---T-
bovine   C--G--G--G --C--T-A-A -G-T-GG--C T-A-A-AA-G ---CA---T-
ovine    C--G--G--G --C--T-A-A -G-C-GG--C T-A-A-AA-G ---CA---T-
porcine  CGGGGAGAAG ATCTTCAAGA CGGGAGGCTT TGAAAAGACT TTTCAGGATG

porcine    G  E  K   I  F  K   T  G  G  F   E  K  T   F  Q  D

human    -AC--CT-C- G----CA--- --T--T--A- --T--G---- T-----C---
rat      -CA--GA-A- G----GG--- --C--A--G- --C--G---- C-----T---
mouse    -CC--GA-A- G----AA--- --T--A--A- --C--G---- C-----T---
bovine   -CC--CA-A- C----CA--- --T--A--A- --T--C---- C-----T---
ovine    -CC--CA-G- C----CA--- --T--A--A- --C--C---- C-----T---
porcine  CTCAGCAGGT ATGCACACAG GCCGGGGGAC AGATGGCCTC CCCACGCTCT

porcine  A  Q  Q  V   C  T  Q   A  G  G   Q  M  A  S   P  R  S

human    -CCG----G- -T-CC--CT- GCAA--G--G GT-GT---TA -G-ACG-G--
rat      -CTA----G- -T-CT--AG- ACAG--G--C GT-AC---CC -C-GCA-A--
mouse    -CTA----G- -T-CT--CA- ACAG--A--C AT-AC---CC -C-ACA-G--
bovine   -GAG----A- -C-AG--CT- GACT--G--G GC-AC---CC -G-ACA-G--
ovine    -CAG----G- -T-AG--TT- GACT--G--G GC-AC---CC -G-ACA-G--
porcine  GAGACTGAGA ACGAGGCCTT GAGCCAGCTG GTCACAGCTC AGAATAAGGC

porcine  E  T  E   N  E  A  L   S  Q  L   V  T  A   Q  N  K  A

human    ---------- --C--G-CT- -TTCCAAG-C A-----C-AG ----CC----
rat      ---------- --T--G-CA- -TGTGGGC-C G-----C-AG ----CT----
mouse    ---------- --T--G-CA- -TGTGGGC-C A-----C-AG ----CT----
bovine   ---------- --C--G-GC- -CACCAGG-A G-----T-CT ----TC----
ovine    ---------- --C--A-CC- -TACCAGG-A G-----T-CT ----TC----
porcine  TGCTTTCCTG AGCATGACTG ACATCAAGAC GGAGGGCAAT TTCACCTACC

porcine  A  F  L   S  M  T  D   I  K  T   E  G  N   F  T  Y  P

human    ----A--A-- -T-------- ---T-C--C- ----C--AGG ----------
rat      ----A--A-- -G-------- ---T-T--C- ----T--AGG ----------
mouse    ----A--A-- -C-------- ---T-T--T- ----T--AGG ----------
bovine   ----G--G-- -C-------- ---T-C--C- ----C--CCA ----------
ovine    ----G--G-- -C-------- ---T-C--C- ----C--CCA ----------
porcine  CCACGGGGGA GCCCCTGGTC TATGCCAACT GGGCCCCTGG GGAGCCCAAC

porcine    T  G  E   P  L  V   Y  A  N  W   A  P  G   E  P  N

human    G-TG----C- -GTCA..... ....---G-- ---------- ---TCACCAA
rat      A-CA----A- -GGCA..... ....---A-- ---------- ---TCACCAA
mouse    A-CA----T- -AGCA..... ....---A-- ---------- ---TCACCAA
bovine   A-TG----C- -CTCA..... ....---A-- ---------- ---TTCCCAA
ovine    A-TG----C- -CTCA..... ....---G-- ---------- ----------
porcine  AACAATGGTG GCAGCAGCGG AGCAGAGAAC TGTGTGGAGA TCT~~~~~~~

porcine  N  N  G  G   S  S  G   A  E  N   C  V  E  I
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Fig. 1. Mammalian SP-D cDNA sequences. Alignment of the ovine and porcine SP-D cDNA sequences to human (X65018), rat (M81231), mouse (L40156) and bovine (X75911) cDNAs from SP-D CRD and the deduced porcine amino acid sequence. Similarities in nucleotide sequences are shown with dashes (-) and gaps are shown with points (.).

3.3. Northern analysis of porcine SP-A and SP-D

Northern blotting revealed hybridization of the porcine SP-A probe to a single band corresponding to a mRNA of approximately 2.0 kb present in porcine lung and 1.8 kb in the ET (Fig. 2A). With the porcine SP-D probe, the bands hybridized were approximately 1.4 kb in both the ET and lung (Fig. 2B).

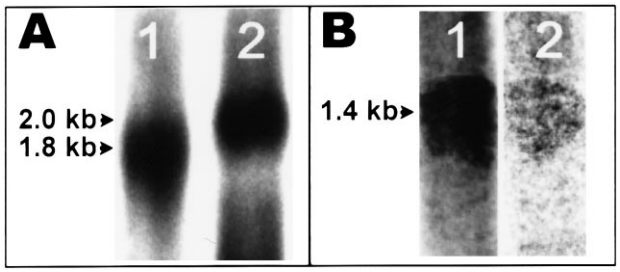

Fig. 2. Northern blot of SP-A (A) and SP-D (B) mRNA from the porcine ET. The gel contained 10 μg of total RNA from the ET (lanes 1) or 2 μg of total RNA from porcine lung (lanes 2). The arrows show deductive sizes of the mRNAs. Exposure times were 15 h for SP-A and either 3 (lane 1) or 12 h (lane 2) for SP-D.

3.4. SP-A and SP-D immunoreactivity in the porcine ET

Many epithelial cells in the porcine ET were immunopositive when human SP-A antibody was used (Fig. 3A). To demonstrate SP-D in the porcine ET, immunohistochemistry studies were performed with sections of the porcine ET using SP-D polyclonal antibody. Intense reactivity was found in the epithelial cell lining of the tubal roof (Fig. 3B).

4. Discussion

We have shown the presence of both SP-D and SP-A in the ET. Strong immunopositivity of both SP-A and SP-D was detected in the transitional epithelium present in the cartilaginous roof area of the porcine ET. Foci of intense intracellular immunostaining of SP-D were evident in some cells.

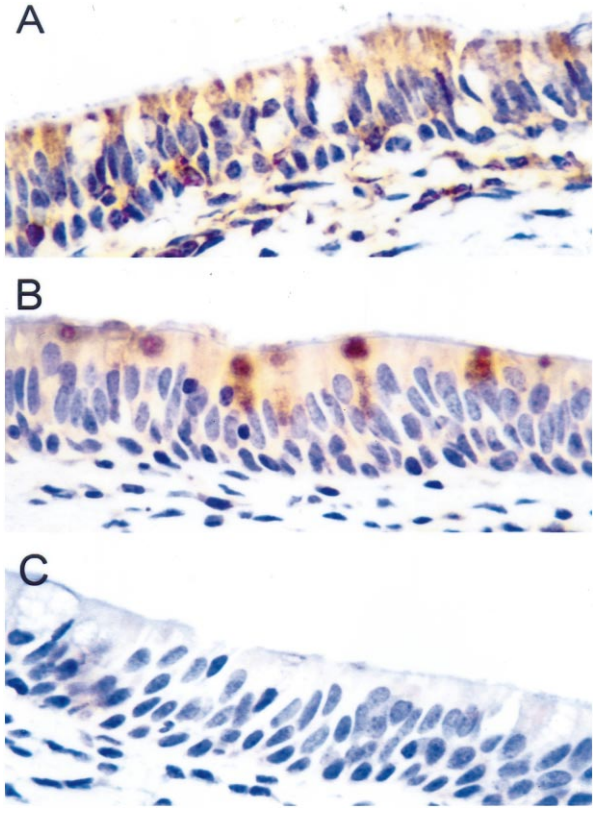

Fig. 3. Sections from the porcine ET immunostained with a human monoclonal antibody against SP-A (A) and a porcine polyclonal antibody against SP-D (B). A negative control is shown in C.

Using RT-PCR and Northern hybridization, the expression of SP-A and SP-D genes in the porcine ET was established. A 313 bp fragment of the porcine SP-D cDNA sequence from the CRD was identified. It showed a strong homology to the other species, with the exception of a three amino acid insertion in porcine SP-D. The SP-D cDNA sequence from sheep also showed a strong homology with the other species in both the amino acid and the nucleic acid sequences. The SP-A and SP-D sequences from the porcine ET revealed 100% homology with those in the lung.

According to morphologic [26] and functional [25] evidence, the epithelial lining of the ET may contain surface active material with possible functions of protecting the epithelial lining or allowing the patency of the ET during swallowing. The lung surfactant required for alveolar stability consists of specific phospholipids with prominent dipalmitoyl phosphatidylcholine and hydrophobic proteins, SP-B and SP-C. Although lamellar bodies are present in some epithelial cells of the ET, it is unclear whether they contain any hydrophobic peptides. SP-A isolated from lung improves the surface activity and serves as an essential component of alveolar tubular myelin [29]. However, SP-A is neither concentrated in lamellar bodies [30] nor required for pulmonary alveolar stability [22]. The structural and functional association of SP-D with alveolar surfactant aggregates is even less evident [20,21]. The surfactant proteins thus far identified in ET may not be essential for surface activity.

The evidence that SP-A and SP-D genes are expressed in the ET suggests that these proteins were involved in the immune defense, enhancing the host resistance against middle ear infections. According to experiments in vitro, SP-D enhances the clearance and phagocytosis of pathogens [6]. SP-A knock-out mice show a decreased resistance against pulmonary bacterial infections and an apparently normal respiratory function [22–24].

Despite the identity in the mRNA sequence between ET and alveolar SP-As, there are differences in the molecular mass [27], sedimentability (Paananen et al., unpublished) and mRNA size (Fig. 2), suggesting differences in transcription and processing. Further studies are required to define the characteristics and functions of SPs in the ET.

Acknowledgements: This research was supported by grants from the Biocenter Oulu and the Academy of Finland.

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