Increased Expression of Surfactant Protein A and D in Rheumatoid Arthritic Synovial Fluid

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Aim To determine the concentrations of surfactant protein (SP)-A and SP-D in synovial fluid samples from patients with rheumatoid arthritis and healthy controls and correlate them with rheumatoid factor, C-reactive protein (CRP), immunoglobulin (Ig) A, IgM, and IgG, total protein, and total lipid content.

Methods The concentrations of SP-A, SP-D, CRP, IgA, IgM, IgG, and rheumatoid factor were measured in the synovial fluid of 7 patients with rheumatoid arthritis and 3 samples of healthy synovial fluid obtained at autopsy. The bands obtained by specific antibodies in Western blotting to determine the surfactant protein concentration were analyzed densitometrically and synovial fluid total phospholipids and protein content were analyzed spectrophotometrically.

Results Patients with rheumatoid arthritis had increased concentrations of proteins and lipids in the synovial fluid, which correlated with 3.5-fold increase in SP-A and 6.1-fold increase in SP-D concentrations. Total protein content in synovial fluid samples from patients with rheumatoid arthritis showed a 2.1-fold increase and phospholipid content showed 7.0-fold increase in comparison with samples of healthy synovial fluid. Rheumatoid factor, CRP, IgA, IgM, and IgG concentrations in synovial fluid samples from patients with rheumatoid arthritis were 40-2660 KIU/L, 4-35 mg/L, 0.10-2.70 g/L, 0.50-1.90 g/L, and 5.3-15.4 g/L respectively.

Conclusion SP-A and SP-D were present and expressed in various degrees in the synovial fluid of patients with rheumatoid arthritis. SP-A and SP-D may play role in the initiation of immune system and joint inflammation, and may be an integral component of synovial fluid and provide insights for in innate and adaptive immunity within the joint.

Pulmonary surfactant is mainly a mixture of lipids and proteins, which covers the peripheral airway. Surfactant in the lungs has two distinct functions. It reduces the surface tension at the air-liquid interface of the lung (1) and plays a role in host defense against infection and inflammation (2). About 10% of surfactant consists of proteins. So far, 4 surfactant proteins (SP) have been defined – SP-A, SP-D, SP-B, and SP-C. SP-B and SP-C are small and extremely hydrophobic proteins. SP-A and SP-D are members of the collectin family of proteins containing collagen regions (3); they play important roles in host defense mechanisms and mediation of immune cell functions of the lung (4). The main site of surfactant biosynthesis is the lung, although surfactant protein mRNA and, in some cases, proteins have been found at non-pulmonary sites (5). SP-A and SP-D have been localized to non-pulmonary sites including the small intestines, colon and intestinal lumen mesenteric cells (6), lung, heart, stomach, kidney (7), gastric mucosa, Eustachian tube (8,9), brain, testis, pancreas, salivary gland, heart, prostate, small intestine, placenta (10), human skin (11), female genital tract, the placenta and amniotic fluid (12), vaginal mucosa, vaginal lavage fluid (13), and synovial fluid (14-17).

There may be a correlation between the presence of lamellar bodies and surfactant protein secretion in the synovium (18). Lamellar bodies are specialized intracellular organelles of epithelial cells for packaging and secretion of surfactant (14). Dobbie et al (19) and Schwarz and Hills (20) reported the presence of lamellar bodies in type B synoviocytes, and synovial B cells seem to be the source and secretion site of synovial surfactant within the joint. Dobbie et al (19) also found an SP-A-like protein in human synovium, which provided a new physiological and pathological insight into these tissues and suggested that the lamellar bodies present in synovium type B cells were the major targeted cytoplasmic organelle in rheumatoid diseases. Additionally, it was reported that SP-A, as expressed by immunoglobulin G (IgG) and autoantibodies, was present in the synovial fluid isolated from patients with rheumatoid arthritis (16).

In the lung, surfactant proteins assist in the formation of a monolayer of surface-active phospholipid at the liquid-air interface of the alveolar lining, reducing the surface tension (21). In contrast, surface-active phospholipid adsorbed to articular surfaces has been identified as the load-bearing boundary lubricant of the joint (22). This raises the question whether or not surfactant proteins in the synovial fluid are an integral part of normal joints.

The hypothesis in this study was that surfactant proteins may play a significant role in the functioning of joints. The aim was to determine the presence of SP-A and SP-D in the synovial fluid from patients with rheumatoid arthritis and healthy controls and to correlate the changes in synovial fluid SP-A and SP-D concentration with rheumatoid factor, C-reactive protein (CRP), IgA, IgM, IgG, total protein, and total lipid content.

Material and methods

The experimental protocol for this study was approved by the Human Subjects Review Committee at the University of Queensland, Brisbane, Queensland, Australia.

Preparation of human lung bronchoalveolar lavage fluid

Three bronchoalveolar lavage (~ 0.5 mL) samples were collected from patients undergoing lung lavage at Princess Alexandra Hospital (Brisbane, QLD, Australia) and diluted in 5 mL of BN buffer (0.01 mol/L sodium borate at pH 7.4, 0.15 mol/L NaCl, and 3 mmol/L $CaCl_2$). The lavage was centrifuged at 250 × g for 10 minutes at 4°C to remove cellular debris. The supernatant was then centrifuged at 27 000 × g for 2 hours at 4°C, and the resulting pellet was resuspended in 100 μL BN buffer and kept at -20°C (23).

Preparation of human synovial fluid samples

Synovial samples collected from 7 patients with rheumatoid arthritis (4 men and 3 women, aged between 60 and 78 years). Three control samples of healthy synovial fluid were obtained at autopsy by knee-aspiration 1-5 hours after death. Within this time window, the degradation in synovial fluid constituents was minimal. Rheumatoid arthritis in patients was diagnosed on the basis of clinical symptoms, physical examination, and laboratory testing on rheumatoid factor and CRP. Synovial fluid samples from patients with rheumatoid arthritis were obtained at synovectomy or before intra-articular corticosteroid injection. All samples were stored at -20°C until used. Six patients with rheumatoid arthritis were in the active state of disease. Synovial fluid was centrifuged at 1200 × g for 10 minutes to remove cellular debris and treated with hyaluronidase (1 μg/mL, Type I-S, bovine testes, 320 U/mg; Sigma Laboratories, St. Louis, MO, USA) for 1 hour at 37°C to reduce sample viscosity.

Protein assay

Micro assay with bicinchoninic acid (BCA) described by Smith et al (23) uses BCA to detect the copper ions generated from cupric ions by reaction with protein under alkaline conditions. The protein concentration range of this assay is 0.1-1 mg/mL. Total protein estimation was performed with microbicinchoninic protein assay in 96-well Microplate as described previously (24). Absorbance at 562 nm was measured with Spectra MAX250 microtiter plate reader (Molecular Devices Corporation, Sunnydale, CA, USA) controlled by SoftMax PRO software, version P 1.12 (Molecular Devices Corporation).

Synovial fluid lipid estimation

Surfactant lipid was extracted from the samples of synovial fluid by the standard method of Folch et al (25). A volume of 0.4 mL of sample (20 μL of synovial fluid diluted in 380 μL distilled water or up to 50 mg dry weight) was placed in a glass or polyethylene centrifuge tube. A volume of 1.5 mL of methanol-chloroform (2:1, v/v) was added, gently shaken for 1 hour, and centrifuged to extract lipids into methanol-chloroform phase and suppress any biochemical degradation that could otherwise occur. Analysis for phospholipid followed the standard method of Rouser et al (26). Essentially, the inorganic phosphate was left behind in the aqueous phase during the chloroform-methanol extraction. All elemental phosphorus in the chloroform-methanol extract was then oxidized to phosphate by using perchloric acid and estimated colorimetrically in a spectrophotometer set at 660 nm, with ammonium molybdate as the indicator. Calibration was effected using dipalmitoyl phosphatidylcholine (P6267, Sigma Chemicals). Dipalmitoyl phosphatidylcholine was used as the standard because phosphatidylcholine is the major phospholipid component of synovial fluid, while synovial phosphatidylcholine is predominantly saturated (27). Each test was repeated and the mean value recorded.

SDS/PAGE and Western blotting

Each sample was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide (12%) gel electrophoresis and Western blotting. Blots were probed with monoclonal antibody PE-10 (21 mg/mL) against human SP-A (DAKO Co. Ltd, Kyoto, Japan), polyclonal antibody PO-B (8 μg/mL) against SP-B (Dr Ian Doyle, Flinders University, Australia) and with a polyclonal goat antibody against SP-D (CHO SP-D, 190 μg/mL) (Dr Dennis R Voelker, National Jewish Medical and Research Center, Denver, CO, USA). Gels were prepared in a Bio-Rad Protean or Mini-Protean electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). Samples containing 15-150 mg of protein were subjected to one-dimensional SDS/PAGE (12%, 15%, mass/v, 1.5 mm-thick polyacrylamide gel) by the method of Laemli (28) with a Mini Protean II apparatus

(Bio-Rad) in the presence of 5% β-mercaptoethanol. Samples were added to the sample buffer (20 μL total) and boiled at 95°C for 5 minutes. After cooling, they were added by pipette to each well containing running buffer and run for 1 hour at room temperature at constant voltage of 200 V. After electrophoresis, gels were used in Western blotting of antigens by transferring separated proteins onto nitrocellulose membrane. Transfer was performed at 100 V, 350 mA for 1 hour using 200/2.0 power supply model (Bio-Rad). After blocking for 1 hour with 5% (mass/vol.) nonfat milk in 0.05 mol/L tris-buffered saline containing 0.05% Tween 20 (TBS-T), the nitrocellulose sheets were incubated overnight at 4°C with SP-A antibody (Dako), diluted to 1:500 with 0.05% TBS-T. After 3 × 10 minutes wash, the blot was incubated for 1 hour at room temperature with antimouse IgG antibody (2 mg/mL) diluted to 1:1000 with 0.05% TBS-T (developed in goat, M-8642, Sigma Chemical Company). After further 3 × 10 minutes wash, the blot was incubated with the secondary antibody, ie, antigoat IgG-peroxidase conjugate (1 mg/mL) diluted to 1:2000 with 0.05% TBS-T (A-4174, Sigma Chemical Company). For SP-B and SP-D detection, rabbit polyclonal antisera to human lung SP-B and SP-D antibodies were used that were diluted to 1:500 with 0.05% TBS-T. After further 3 × 10 minutes wash, the blots were incubated for 1 hour at room temperature with anti-rabbit IgG-peroxidase conjugate (1 mg/mL) diluted to 1:1000 with 0.05% TBS-T (A-0545, Sigma Chemical Company). After the final wash with TBS, the blots were developed with chemiluminescent detection system (ECL, Amersham Life Sciences, Arlington Heights, IL, USA).

SP-A and SP-D signal intensities in healthy and rheumatoid arthritis synovial fluids on autoradiographs were quantified using densitometry (Bio-Rad GS-670 Imaging Densitometer).

Measurement of C-reactive protein, immunoglobulins, and rheumatoid factor

The concentrations of C-reactive protein (CRP), immunoglobulins, and rheumatoid factor were measured in the samples of synovial fluid from patients with rheumatoid arthritis by specific assays using Beckman Systems at the Department of Pathology, Princess Alexandra Hospital, Brisbane-QLD-Australia.

Statistical analysis

Given the relatively small number of samples, normal distributions of the samples could not be assumed. The Spearman's rank correlation coefficient was used to test bivariate associations between the relative densities of surfactant proteins A and D, and concentrations of rheumatoid factor, CRP and immunoglobulins G (IgG), M (IgM) and A (IgA) in disease affected joints. Statistical Package for Social Sciences, version 9 (SPSS Inc, Chicago, IL, USA) was used to analyze the data. *P* values <0.05 were considered statistically significant.

Results

Western blots of synovial fluid probed with antibodies against surfactant proteins SP-A and SP-D each contained a single major band that corresponded to proteins with apparent molecular weights of 34 000 and 43 000 kDa, respectively (Figure 1A). Human bronchoalveolar lavage samples were used as a positive control in all experiments (results not shown). Precaution was paid to all samples obtained at autopsy, so that samples with abnormal protein, lipid, and physical characters were omitted and not used as control. When the same amounts of synovial fluid protein were compared by Western blot analysis, SP-A and SP-D bands were more intense in patients with rheumatoid arthritis than healthy subjects ($P<0.001$; Figure 1A). Densitometric analysis of the blots showed that the SP-A and SP-D content in synovial fluid of patients with

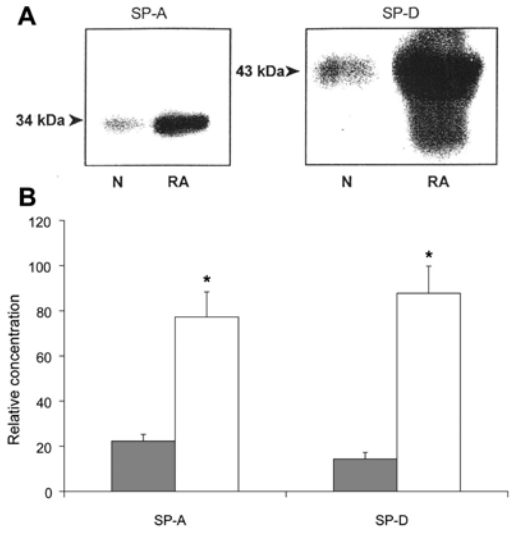

Figure 1. Western blot analysis of surfactant protein (SP)-A and SP-D in synovial fluid from normal and rheumatoid joints. **A.** Western blots of synovial fluid from normal (N) and rheumatoid arthritis (RA) joints were probed with antibodies against SP-A (left panel, 15 μg of protein per lane) and SP-D (right panel, 150 μg of protein per lane). **B.** Surfactant proteins were densitometrically quantified using one of the rheumatoid arthritis samples as a standard on each blot (intensity of 100%). Mean intensities (±standard error of the mean) are plotted for SP-A and SP-D in synovial fluid from 3 normal (closed bars) and 7 rheumatoid arthritis joints (open bars). The large amount of protein required for analysis of SP-D limited the number of samples of synovial fluid analyzed from normal joints. Asterisk indicates $P \leq 0.005$.

rheumatoid arthritis was 3.5 and 6.1 times higher than that in normal synovial fluid, respectively (Figure 1B). The increase in surfactant proteins was accompanied by a 2.1-fold increase in the total protein concentration and a 7.0-fold increase in the phospholipid concentration in synovial fluid of patients with rheumatoid arthritis. Total proteins in synovial fluid of patients with rheumatoid arthritis were significantly higher than those in healthy synovial fluid samples (5.32 ± 0.543 mg/mL vs 2.67 ± 0.62 mg/mL, respectively; $P<0.001$). Synovial fluid from rheumatoid arthritis contained significantly higher concentration of total synovial fluid lipid than the normal synovial fluid (0.938 ± 0.034 mg/mL vs 0.134 ± 0.026 mg/mL, respectively; $P<0.001$). The ratio of surfactant protein to phospholipid concentration in synovial fluid remained relatively unchanged for SP-A and SP-D. The value of rheumatoid factor in synovial fluid of patients with rheumatoid arthritis was 40-2660 KIU/L. CRP concentration in synovial fluid of patients with rheumatoid arthritis ranged from 4 to 35 mg/L, IgG concentration varied from 5.3 to 15.4 g/L, IgA concentration from 0.10 to 2.70 g/L, and IgM from 0.50 to 1.90 g/L.

The only significant bivariate correlation in the synovial fluid of patients with rheumatoid arthritis was the inverse correlation between SP-D and IgG (Spearman's correlation coefficient = -0.857; $P<0.02$). None of the disease markers for rheumatoid arthritis significantly correlated with SP-A.

Discussion

Our study showed that SP-A and SP-D were present in synovial fluid of patients with rheumatic arthritis. The monomeric forms of SP-A and SP-D present in the synovial fluid corresponded to the glycosylated monomeric 32 and 38 kDa form of pulmonary SP-A and 43 kDa form of pulmonary SP-D. SP-B, a functionally crucial component of lung surfactant (29), seems to be absent from synovial fluid. This may suggest that the properties of synovial surfactant were somewhat different from those of pulmonary surfactant and do not require the presence of SP-B. The presence of SP-A and SP-D might improve our understanding of the mechanism of defense in organs containing few immunocytes. Taking into account the properties of the collagenous C-type lectins as protein interacting with a variety of microorganisms (30) and with phagocytic cells (31), SP-A and SP-D might have functions in the protection of joints against pathogens.

The concentration of SP-A may be changed in some diseases. For example, SP-A concentration was decreased in the amniotic fluid of diabetic mothers (32,33). SP-A levels in bronchoalveolar lavage of patients with cystic fibrosis and lower airway infections were higher than in pa-

tients with cystic fibrosis without infection (34). Also, maximum SP-A immunoreactivity was detected in the synovium of patients with rheumatoid arthritis (16,19).

SP-A and SP-D are water-soluble surfactant proteins that have distinct functions in the lung. Whereas SP-A plays a role in the transit of surface-active phospholipid to the liquid-air interface, studies in SP-D knockout mice suggest that SP-D regulates phospholipid homeostasis (35). We postulated that SP-A and SP-D may have an equally important role in the lubrication of joints. It can be suggested that the SP-A facilitates the adsorption of surface-active phospholipid to the articular surface and that SP-D regulates phospholipid homeostasis in the joint. SP-B is a small hydrophobic protein in the lung that is important for the formation of a monolayer at the liquid-air interface of the lung. Its absence in synovial fluid suggests that it may not be required for the adsorption of phospholipid at the articular liquid-solid interface and this raises the possibility that its role may be specific for liquid-air interfaces. The presence of SP-A and SP-D in normal and their elevated levels in rheumatoid arthritis, however, indicates that they are normal components of synovium. The immunosupressive properties of lung surfactant have long been recognized, and its phospholipid components have been shown to inhibit the production of interleukin 2, an inflammation-promoting cytokine, by mononuclear cells in a dose-dependent manner (36). Antiinflammatory and immunosuppressive functions of joint surfactant, which contains both SP-A and SP-D, are therefore possible. The lipid concentration in the synovial fluid is normally low, 0.130-0.140 mg/mL (37), and very similar to plasma concentration. The protein concentration of synovial fluid varies between 1.2-3.0 mg/mL (38). The increase in total protein (39) and lipid content (40) has been reported in various joint diseases, including rheumatoid arthritis. Normally, the free entry of lipids into synovial fluid can be controlled. However, the levels of lipid and protein in synovial fluid increases within the synovium that are likely derived from the blood.

Further studies will be required to characterize the functions of SP-A and SP-D in synovium and to determine whether the particular human SP-A protein in synovium, is the product of the SP-A1 or SP-A2 gene, or both.

In conclusion, the present study showed that SP-A and SP-D were present and expressed in various degrees in the synovial fluid of patients with rheumatoid arthritis. SP-A and SP-D may play role in the initiation of immune system and joint inflammation, and may be an integral component of synovial fluid and provide insights for in innate and adaptive immunity within the joint.

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