Surface tension influences cell shape and phagocytosis in alveolar macrophages

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Akei, Hiroko, Jeffrey A. Whitsett, Michelle Buroker, Takafumi Ninomiya, Haruyuki Tatsumi, Timothy E. Weaver, and Machiko Ikegami. Surface tension influences cell shape and phagocytosis in alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 291: L572–L579, 2006. First published April 21, 2006; doi:10.1152/ajplung.00060.2006.—The effect of surface tension on alveolar macrophage shape and phagocytosis was assessed in vivo and in vitro. Surface tension was regulated in vivo by conditionally expressing surfactant protein (SP)-B in Sftpb−/− mice. Increased surface tension and respiratory distress were produced by depletion of SP-B and were readily reversed by repletion of SP-B in vivo. Electron microscopy was used to demonstrate that alveolar macrophages were usually located beneath the surfactant film on the alveolar surfaces. Reduction of SP-B increased surface tension and resulted in flattening of alveolar macrophages on epithelial surfaces in vivo. Phagocytosis of intratracheally injected fluorescent microbeads by alveolar macrophages was decreased during SP-B deficiency and was restored by repletion of SP-B in vivo. Incubation of MH-S cells, a mouse macrophage cell line, with inactive surfactant caused cell flattening and decreased phagocytosis in vitro, findings that were reversed by the addition of sheep surfactant or phospholipid containing SP-B. SP-B controls surface tension by forming a surfactant phospholipid film that regulates shape and nonspecific phagocytic activity of alveolar macrophages on the alveolar surface.

Surfactant protein-B; transgenic mice; MH-S cell; Sftpb−/− mice; pulmonary surfactant

PULMONARY SURFACANT is a complex mixture of lipids and surfactant proteins synthesized and secreted by alveolar type II epithelial cells. Secreted surfactant adsors to the air-liquid interface and spreads over the alveolar surface to reduce surface tension in a process greatly accelerated by surfactant protein (SP)-B (37). The phospholipid-rich surfactant film reduces the surface tension generated by the air-liquid interface (70 mN/m) to near 0 mN/m (40). SP-B is required for surfactant function and respiration after birth. Lack of SP-B caused by mutations in the human SFTPB gene (23), deletion of the SftpB gene in mice (6), and conditional reduction of SP-B in adult mice (17, 21, 22) causes respiratory failure.

Pulmonary surfactant plays an important role in innate defense of the lung. Both SP-A and SP-D are members of the collectin family of innate immune molecules and play critical roles in innate host defense and the regulation of macrophage function (14, 20, 39). Likewise, SP-B protects the lung during inflammation (36). Partial deficiency of SP-B in Sftpb−/− mice renders the adult lung more susceptible to oxygen- or intratracheal endotoxin-induced lung injury (8, 33, 34). Thus maintenance of SP-B homeostasis is critical for pulmonary homeostasis after lung injury (13).

Previous studies demonstrated that alveolar macrophages are usually closely associated with alveolar epithelial cells (12) and are located beneath the surfactant film (4). When surfactant was replaced by a detergent with low surface activity (high surface tension), flattened alveolar macrophages were observed in adult rabbit lung (4). Alveolar macrophages play an important role in innate host defense of the lung, internalizing and degrading microbial pathogens encountered on the respiratory surface. Because surface tension in the alveolus influences the shape of the alveolar macrophages, we hypothesized that surface tension might also influence phagocytic activity of alveolar macrophages. Transgenic mice were produced in which human SP-B cDNA was expressed in the alveolar epithelium of Sftpb−/− mice under conditional control of a doxycycline-inducible transgene (17, 22). The level of SP-B in the alveolus can be conditionally controlled by addition of doxycycline, which determines the surface tension in the alveolus. In the present study, the effect of altered SP-B levels and, therefore, surface tension on alveolar macrophage shape and nonspecific phagocytosis was determined in vivo and in vitro.

MATERIALS AND METHODS

Transgenic mice. The Animal Care and Use Committee of the Cincinnati Children’s Hospital Research Foundation (Cincinnati, OH) approved all animal procedures and protocols. The conditional transgenic mice [CCSP-rtTA, (tetO)7, SFTPBSftpb−/−] were generated from two transgenic mouse lines (25, 32): 1) “responder” transgenic mice [CCSP-rtTA, SFTPBSftpb−/−] expressing reverse tetracycline transactivator transcription factor (rtTA) under the control of a 2.3-kb element from the rat Clara cell secretory protein (CCSP) gene promoter that confers expression in both conducting and alveolar respiratory epithelial cells and 2) “responder” transgenic mice [(tetO)7, SFTPBSftpb−/−] in which the human SP-B cDNA was expressed under the control of the tetracycline response elements. CCSP-rtTA/(tetO)7, SFTPBSftpb−/− mice were bred with Sftpb−/− mice to generate CCSP-rtTA/(tetO)7, SFTPBSftpb−/− mice (17, 22), here termed “conditional SP-B mice.” Because Sftpb−/− mice die perinatally unless the SP-B transgene is expressed, conditional transgenic mice were maintained on doxycycline-containing food from embryonic day 1 until 7 wk of age. Doxycycline was removed for 4 days and resumed before study on days 7 and 13. In the conditional SP-B mouse model, SP-B deficiency was induced by removal of doxycycline, resulting in increased equilibrium and minimum surface tensions. SP-B was significantly decreased on day 1 of removal from doxycycline. Equilibrium surface tension of surfactant was increased when doxycycline was removed for 2 days, and minimum surface tension of CCSP-rtTA/(tetO)7, SFTPBSftpb−/− mice was increased 2 days before doxycycline was removed.

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tension was significantly higher on day 1 after removal from doxycycline (17). Slow respiratory failure developed within 4 days after removal from doxycycline. Surface activity was restored when SP-B was reexpressed under control of doxycycline. Four days of SP-B deficiency did not alter SP-C or surfactant phospholipid content or composition (17).

Surface tension measurement. Surface activity was measured with a captive bubble surfactometer as previously described (17, 29). The concentration of each sample was adjusted to 7 nmol phospholipid/μl, and 3 μl of the sample was applied to the air-water interface of a 25-μl-volume bubble by microsyringe (n = 3). Equilibrium surface tension was measured at 300 s, and then bubble pulsation was started. The minimum surface tension was measured after 80% bubble volume reduction at a rate of 12 cycle/min. Surface tension did not change significantly after the third pulsation. Minimum surface tension was measured at the fifth pulsation.

Electron microscopic analysis. To preserve lung structure and cell morphology, fixatives were perfused through the vasculature (2, 9). Briefly, intravascular blood coagulation was inhibited by an intraperitoneal injection of heparin (250 U/mouse) 30 min before sedation with pentobarbital sodium. The chest cavity was opened, and an incision was made on the auricle of the left atrium. A 30-gauge needle was introduced into the pulmonary artery through the right ventricle. Inflation pressure was then reduced to 5 cmH2O (60% TLC) and maintained during fixation. The fixative solution, 2.5% glutaraldehyde (glutaraldehyde, EM grade; Electron Microscopy Sciences, Washington, PA) in phosphate-buffered saline (PBS), was perfused from the needle in the right ventricle at the rate of 2.5 ml/min for 12 min. The minimum (or absent) effects of this fixative on surfactant surface activity were shown previously (3). After perfusion, lung tissues were postfixed in Karnovsky fixative solution overnight, cut into 1- to 2-mm pieces, fixed overnight in Karnovsky fixative, and stained with osmium tetroxide and uranyl acetate, followed by embedding in resin (Embed 812; Electron Microscopy Sciences).

Four unit square areas of lung tissue on 200-mesh grid (total 0.04 mm2) were randomly selected in a blinded fashion. All macrophages in these areas were evaluated with image analysis software (Image-Pro Plus, version 5.0; MediaCybernetics, Silver Spring, MD). Macrophages at the corner of alveolar walls were excluded from the percent area (% area) analysis. A straight line was drawn between the two edge points of the pseudomorphs of macrophages attached to the alveolar epithelial cell, and % area over the line relative to total surface area of the macrophage was measured. The percentage of macrophages associated with epithelial cells relative to the total number of macrophages in the same four unit square areas of lung tissue on 200-mesh grid was determined.

Alveolar macrophage phagocytosis assays in vivo. Albumin-coated fluorescent latex beads were prepared with 2-μm-diameter fluorescein isothiocyanate (FITC)-labeled latex microbeads (Molecular Probes, Eugene, OR) by incubation with bovine serum albumin (fraction V; Sigma, St Louis, MO; 10 mg/ml, 37°C, 60 min), followed by washing three times and resuspension in PBS at a concentration of 5 × 108 microbeads/ml. The suspension of microbeads was sonicated for 10 s in a bath sonicator before use (5). Study groups included conditional SP-B mice that were 1) continuously treated with doxycycline (control), 2) removed from doxycycline for 4 days, and 3) removed from doxycycline for 4 days and then retreated with doxycycline for 7 and 13 days. Mice were anesthetized by isoflurane inhalation in the box and then by face mask. After the neck was disinfected with iodine and ethyl alcohol, the trachea was exposed through a small incision and a 100-μl microbead suspension was injected, resulting in its relatively homogenous distribution in the airways (41). After the skin wound was closed with Nexabrand liquid topical tissue adhesive (Abbott Laboratories, North Chicago, IL), mice were recovered from the anesthesia instantly. One hour after intratracheal injection, mice were deeply anesthetized with intraperitoneal heparin and tolbutamide, the trachea was opened by a small incision and a 100-μl microbead suspension was injected, resulting in its relatively homogenous distribution in the airways (41). After the skin wound was closed with Nexabrand liquid topical tissue adhesive (Abbott Laboratories, North Chicago, IL), mice were recovered from the anesthesia instantly. One hour after intratracheal injection, mice were deeply anesthetized with intraperitoneal pentobarbital sodium, and the distal aorta was cut (24). A 20-gauge blunt needle was tied into the trachea, the chest was opened, and 0.9% NaCl was flushed into the airways until the lungs were fully expanded (∼1 ml). The fluid was then withdrawn three times with syringes for each aliquot. Bronchoalveolar lavage (BAL) was repeated three times and pooled [BAL fluid (BALF)]. Cells (1 × 106) were immediately analyzed on a FACSCaliber.
Fig. 2. Alveolar macrophage shape is influenced by surface tension in vivo. Alveolar macrophage shape was evaluated by electron microscopy after perfusion fixation of lungs from adult mice. Alveolar macrophages were located beneath the surfactant film (arrows). Controls were conditional SP-B mice with continuous treatment with doxycycline (A). After discontinuation of doxycycline for 4 days, alveolar macrophage were flattened and located deep in the alveolar septae (B). After resumption of doxycycline (7d (C) and 13d (D) On Dox), alveolar macrophage were not different from controls (A). Scale bars, 2 μm.

Fig. 3. The shape of the alveolar macrophage was assessed in SP-B-replete and depleted mice by estimation of the % of cell area above the line drawn from 2 edge points of the pseudopodium of macrophages attached to the alveolar epithelial cell, as seen in (A) control and (B) 4 days after withdrawal from doxycycline. Scale bars, 2 μm. C: after 4 days of deletion of SP-B (Off Dox), macrophage area over the line was significantly decreased. Increased surface tension by SP-B deficiency caused flattening of the alveolar macrophage. *P < 0.01 vs. others by ANOVA; 3 mice/group. No. of cells analyzed: control = 23, 4d Off Dox = 45, 7d On Dox = 23, 13d On Dox = 20.
macrophages. MH-S cells (10^5/well) were plated on six-well plates 1 day before phagocytosis analysis in DMEM (Invitrogen, Grand Island, NY) containing 1% fetal bovine serum, 1% L-glutamine, and 1% antibiotic-antimycotic (Invitrogen). After removal of culture medium, 25 or 50 μl of 10 μg lipid/μl testing suspension (described above) was carefully added by application of 10 drops/well on the surface of cultured cells. This concentration of surfactant lipid is similar to physiological levels (15). After wells were gently shaken horizontally five times to spread suspension, 20 μl of 0.9% NaCl containing 1 × 10^7 albumin- or SP-B-coated FITC-labeled microspheres was applied to each well and incubated at 37°C for 10 min. FITC-labeled 2-μm microbeads were coated with human SP-B in the same way as the albumin-coated microbeads described above. Cells were removed with 0.25% trypsin at 37°C for 5 min, followed by scraping to remove adherent cells from the wells. Cell suspensions were analyzed by FACSscan flow cytometer, and the phagocytic index was calculated as described above.

Influence of surface tension on height of MH-S cells in culture. MH-S cells were cultured in a glass-bottom chamber slide glass system (Tissue-Tek II chambered coverglass; Nalge Nunc International, Rochester, NY) overnight. Cells were stained with a cell-labeling dye (DiD, V-22887, Vybrant cell-labeling solutions; Molecular Probes), for 25 min at 37°C. Cell surfaces were covered with experimental mixtures as described for the in vitro phagocytosis studies. The height of randomly selected cells (16 cells/group) was analyzed from serial z-axis at 0.57-μm intervals (LSM510; Carl Zeiss Microimaging, Thornwood, NY) with image-analyzing software (Zeiss LSM Image Browser, version 3.5.0, 276).

Statistics. Data are expressed as means ± SE. Comparisons between two groups were made by Student’s t-test or Mann-Whitney U-test, and comparisons among groups were made by ANOVA with post hoc comparison by Newman-Keuls test. Significance was accepted at the 5% level.

RESULTS

Surface tension influences macrophage shape in vivo. SP-B is a critical determinant of the surface activity of pulmonary surfactant in vitro and in vivo (17, 21, 22). The equilibrium surface tension and minimum surface tension of isolated surfactant from conditional SP-B mice correlated well with the levels of SP-B in BALF (Fig. 1, A and B), consistent with previous studies (17). Studies conducted in the lung in situ (30)
suggest that similar surface activity is present in vivo. The structure of surfactant film was assessed by electron microscopy of mouse lung tissue (Fig. 2). Alveolar macrophages were usually observed beneath the surfactant film. The shape of alveolar macrophages was strongly influenced by SP-B and surface tension. After discontinuation of doxycycline for 4 days, a time at which surface tension was increased because of SP-B deficiency, alveolar macrophages were flattened against the alveolar septa (Fig. 2B). In normal lung and lungs of Sftpβ−/− mice expressing SP-B under control of doxycycline (Fig. 2A), alveolar macrophages were less flattened. The changes in alveolar macrophage shape caused by SP-B deficiency were readily reversible by resuming doxycycline (Fig. 2, C and D). Changes in alveolar macrophage shape were quantitated by drawing a line between the distal edges of macrophage at sites where it adheres to alveolar epithelium. A representative line is shown in Fig. 3, A (control) and B (4 days off doxycycline). In the presence of SP-B, the % area of the macrophage above the line was twofold higher than that for cells in the SP-B-deficient lung (Fig. 3C). Thus the increased surface tension caused by the reduction of SP-B induced a pronounced flattening of the alveolar macrophages that was restored when surface tension was lowered by expression of SP-B.

The percentage of macrophages associated with epithelial cells relative to the total number of macrophages was determined by electron microscopy. An increased number of macrophages were associated with lung parenchyma after withdrawal of doxycycline (89 ± 6%, n = 38) compared with control (58 ± 1%, n = 44; P < 0.01). Thus SP-B and surface tension influence the distribution and possibly migration of alveolar macrophages in the lung.

Surface tension influences phagocytosis by alveolar macrophages in vivo. Phagocytosis was assessed after intratracheal injection of 2-μm fluorescent microbeads. Internalized microbeads were quantitated in alveolar macrophages 1 h after injection (Fig. 4A). After FACS, the percentage of cells containing microbeads in the gated macrophage population was significantly lower in the absence of SP-B (4 days off doxycycline) than in SP-B-sufficient groups (Fig. 4B). Representative fluorescence intensity measurements for each group are shown in Fig. 4C. Fluorescence peaks indicating the number of beads associated with the cells were fewer after removal from doxycycline (4 days off doxycycline group) than in the other groups. Phagocytic index was significantly decreased in alveolar macrophages isolated from BALF (Fig. 4D) or in tissue during SP-B deficiency (Fig. 4E). Phagocytic index was readily reversed when SP-B expression was corrected by doxycycline treatment. Thus increased surface tension caused by SP-B deficiency inhibited phagocytic activity of alveolar macrophages in vivo.

Influence of surface tension on shape of cultured MH-S cells. Two suspensions were used in these studies: 1) a preparation with high surface tension of a mixture of phospholipids (DPC+PG; Fig. 1, C and D), and 2) a low-surface-tension surfactant isolated from normal adult sheep. The addition of sheep surfactant or phospholipid mixture to MH-S cells in culture influenced the height of the cells (Fig. 5, A and B). The height of the MH-S cells was decreased when cells were covered by phospholipid mixture with poor surface activity (Fig. 5C). Thus increased surface tension of the phospholipid mixture caused flattening of the MH-S cells in culture, consistent with the in vivo observations.

Influence of surface tension on MH-S cell phagocytosis in vitro. For both phospholipid mixture (DPC+PG) and sheep surfactant groups, the larger the microbeads the higher the phagocytic index (Fig. 6; P < 0.05). Phagocytic index was increased by lower volume (25 μl) of sheep surfactant (P < 0.05), which is relevant to the physiological surfactant amount. Phagocytic index was significantly increased when MH-S cells were incubated with natural sheep surfactant compared with phospholipid mixture (P < 0.05). To identify whether specific components in natural surfactant increased phagocytic index or, alternatively, whether low surface tension per se enhanced phagocytosis, the phagocytic index was determined in phospholipid mixtures in the presence and absence of SP-B (Fig. 7). Phagocytic index was increased by the addition of 2% SP-B to the phospholipid mixture, conditions in which the equilibrium surface tension and the minimum surface tension were low (Fig. 1, C and D). Phagocytic index for a mixture consisting of DPC+PG + SP-B was similar to that for natural sheep surfactant, suggesting that other surfactant components did not influence MH-S cell function under these study conditions. Phagocytic index observed with SP-B alone or SP-B-coated microbeads was similar to that with a lipid mixture lacking SP-B, indicating that SP-B does not directly influence phagocytic activity by alveolar macrophages but exerts its effect by reducing surface tension.

**DISCUSSION**

The present study demonstrated that changes in surface tension controlled by SP-B influence alveolar macrophage shape and phagocytosis in vitro and in vivo. Electron microscopic findings demonstrated that alveolar macrophages were usually located beneath the surfactant film on alveolar surfaces.
The shape of the alveolar macrophage was influenced by the surface tension of the surfactant film. Reduction of SP-B, which increases surface tension in vivo, caused flattening of alveolar macrophages and was associated with decreased macrophage phagocytic activity of alveolar macrophages. These findings suggest that normal surface-active surfactant is critical both for maintenance of pulmonary mechanics and for regulation of alveolar macrophage shape and phagocytic function.

The precise location of alveolar macrophages in the lung has been difficult to assess in vivo. The lung is usually fixed for microscopy by introduction of fixative via the airways, resulting in removal of the surfactant film and alveolar macrophages from the alveolar surface. The majority of alveolar macrophages are associated with lung tissue and are difficult to recover by BAL. By extensive BAL, only 30% of the total alveolar macrophages were removed from the mouse lung (19). In our previous study (12), ~20-fold more macrophages were isolated from enzymatically digested mouse lung tissue than from BALF. In the present study the lungs were fixed by perfusion through the vasculature to preserve lung structure from BALF. In the present study the lungs were fixed by perfusion through the vasculature to preserve lung structure from BALF. In the present study the lungs were fixed by perfusion through the vasculature to preserve lung structure from BALF.

Alveolar macrophages provide a first line of host defense by internalizing and killing microbial pathogens on the respiratory surface. The mechanisms by which macrophages internalize materials are influenced by the size and nature of the particles and pathogens. Alveolar macrophages are highly motile and are able to respond to a wide variety of particles and substances. Ligand-induced endocytosis is associated with rapid changes in cell shape that are controlled by the actin cytoskeleton (14, 39), which leads to the internalization of the particle. In contrast, internalization of large (>0.5 μm) inert particles like microbeads occurs by nonspecific phagocytosis (1). The large microbeads (0.5–2 μm) used in the present study are taken up by nonspecific phagocytic pathways. The uptake of large particles is initiated by extension of filopodia from the cell surface. The particle is surrounded and engulfed without the involvement of specific receptors. Phagocytosis of large particles occurs by an actin-dependent mechanism and is usually independent of clathrin (1). In the present study, the phagocytic index of the alveolar macrophages was altered by the lack of SP-B and lack of surface tension-lowering ability in vivo. Although increased proinflammatory cytokines and soluble L-selectin would activate macrophage migration and phagocytosis in the absence of SP-B (17), alveolar macrophage phagocytic index was decreased, demonstrating the critical influence of surface tension on phagocytosis.

Acute lung injury is a common cause of mortality and morbidity in adults and children that is associated with surfactant deficiency and dysfunction (11, 26). Altered content, composition, or structure of surfactant, presence of inhibitory proteins, and loss of large-aggregate surfactant result in decrease in surface activity of surfactant during acute lung injury. SP-B plays a critical role in surfactant function, and SP-B concentrations in BAL from patients with established acute lung injury are significantly reduced compared to healthy controls. These findings suggest that SP-B may play a role in the pathogenesis of acute lung injury and support the development of SP-B as a potential therapeutic target for the treatment of acute lung injury.

**Fig. 6.** Surfactant influences phagocytic index of MH-S cells. Twenty-five or fifty microliters of phospholipid mixture (DPC+PG) or surfactant isolated from sheep BALF was applied to MH-S cells in culture. Phagocytic index was assessed with 0.5-, 1-, and 2-μm microbeads by FACS analysis. Phagocytosis index was significantly increased by surfactant (low surface tension) compared with DPC+PG suspension (high surface tension). Phagocytosis in the surfactant group was inhibited by a higher volume of surfactant suspension (P < 0.05, 25 μl vs. 50 μl); n = 3/group. *P < 0.01 vs. phospholipid mixture by ANOVA.

**Fig. 7.** Addition of SP-B to lipid mixture enhances phagocytic activity of MH-S cells. DPC+PG with and without 2% human SP-B was added to cultured MH-S cells. Phagocytosis of 2-μm microbeads was studied. To determine whether SP-B directly affects phagocytosis, 2% suspensions of SP-B or SP-B-coated microbeads (SP-B-beads) were also studied. Addition of SP-B to the phospholipid mixture significantly increased phagocytic index. Addition of DPC+PG, SP-B alone, or DPC+PG with SP-B-coated microbeads did not improve phagocytic index; n = 3/group. *P < 0.01 vs. others by ANOVA.
lung injury range from 25% to 55% of normal (11). Decreased SP-B was observed after pulmonary infection with a variety of pathogens (18). Because marked changes in surface tension may accompany lung injury, inhibition of phagocytic activity may render the lung susceptible to secondary infection or inflammation.

Surfactant in BALF consists of large (lamellar body and tubular myelin)- and small (lipid vesicle)-aggregate forms that can be separated by centrifugation (31). In the present study, large-aggregate surfactant isolated from sheep BALF, which contains surfactant phospholipids and SP-A, -B, and -C and is highly surface active, was used for the in vitro studies (38). Small-aggregate surfactant is the catabolic form, contains surfactant phospholipids and SP-D (26), and is not as surface active. SP-A is a member of the collectin family of innate immune molecules, known to regulate macrophage function (20, 28), but by itself does not play a major role in surface activity (16). Both SP-C and SP-B are hydrophobic peptides that dramatically enhance surface activity of surfactant phospholipids in vitro (27). The survival of SftpC−/− mice indicates that SP-C is not required for respiratory function in vivo (7, 10). In adult conditional SP-B mice, neither SP-C nor phospholipid content and composition were perturbed by the loss of SP-B. The period of 4 days of SP-B deficiency may not have been sufficient to perturb SP-C processing or phospholipid content. Despite maintenance of SP-C and surfactant phospholipid, the loss of SP-B in this mouse model resulted in surfactant dysfunction and decrease in phagocytic index, demonstrating the critical role of SP-B in surfactant function in vivo compared with SP-C.

Phagocytosis of pathogens, particles, and apoptotic cells by alveolar macrophages plays an important role in their clearance from the lung and initiates inflammation and innate immune responses that, in turn, modulate the adaptive responses in the lung. Although surfactant is known to play a critical role in reduction of surface tension required for ventilation, surface tension controls alveolar macrophage shape and phagocytic activity.

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