Exogenous surfactant changes the phenotype of alveolar macrophages in mice

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Kramer, Boris W., Alan H. Jobe, and Machiko Ikegami. Exogenous surfactant changes the phenotype of alveolar macrophages in mice. Am J Physiol Lung Cell Mol Physiol 280: L689–L694, 2001.—Alveolar macrophages are essential for the maintenance of surfactant homeostasis. We asked whether surfactant treatment would change alveolar macrophage number and whether the alveolar macrophage phenotype would become activated or apoptotic when challenged in vivo with exogenous surfactant. Surfactant pool size in mice was increased by repetitive surfactant treatments containing 120 mg/kg (110 μmol/kg) saturated phosphatidylcholine. The number of alveolar macrophages recovered by alveolar lavage decreased after the first dose by 49% and slightly increased after the second and third doses. Up to 28.5% of the macrophages became large and foamy, and their appearance normalized within 12 h. Surfactant treatment did not increase the percent of apoptotic or necrotic cells. The alveolar macrophages were not activated as indicated by no change in expression of CD14, CD16, CD54, CD95, and scavenger receptor class A types I and II after surfactant treatment. Surfactant treatment in healthy mice transiently changed the phenotype of alveolar macrophages to large and foamy without indications of changes in the surface markers characteristic of activation.

Once surfactant is secreted to the airspaces of the lung, its clearance and catabolism are mediated primarily by alveolar type II cells and macrophages, with minimal amounts of surfactant cleared to the systemic circulation or up the airways (16, 21, 22, 26). The lipid and protein components of surfactant are taken up by both type II cells and macrophages in vivo and in vitro (26, 33). Type II cells can sort surfactant components that are taken up for either recycling or catabolism by lysosomal degradation (27). Alveolar macrophages only degrade surfactant components that are internalized (2–4). In adult rabbits, about 50% of the saturated phosphatidylcholine (Sat PC) is recycled by type II cells, with macrophages accounting for about 20% of the catabolism of Sat PC (26). In mice, Sat PC is also recycled, with a 50% efficiency, but the catabolic contribution appears to be lower than in rabbits based on the accumulation of the diether analog of dipalmitoylphosphatidylcholine (DPPC) in macrophages recovered by alveolar wash (8, 18).

Alveolar macrophages can be exposed to increased amounts of surfactant in vivo acutely by exogenous surfactant therapy or chronically in alveolar lipodosis or alveolar proteinosis syndromes. The macrophages recovered by alveolar lavage from patients and mice with alveolar proteinosis syndromes are referred to as foamy macrophages because they are large and filled with lipid vacuoles (30). In mice, foamy macrophages are present when granulocyte-macrophage colony-stimulating factor (GM-CSF) deficiency or GM-CSF signaling abnormalities cause abnormally slow catabolism (12, 25). Macrophages from these animals demonstrate slow catabolism of DPPC and surfactant protein A (SP-A) in vitro (33). Foamy alveolar macrophages are present in mice with the alveolar proteinosis phenotype resulting from interleukin-4 overexpression (13, 14). Macrophages are also large and foamy in the SP-D-deficient mouse that has a selective alveolar lipodosis (31). The interpretation has been that lipid-laden macrophages are abnormal and contribute to the lipid accumulation in the airspaces (9, 20).

Alveolar pool sizes of surfactant are acutely increased when infants with respiratory distress syndrome or adults with acute respiratory distress syndrome are treated with doses of 100–200 mg surfactant/kg body weight (7, 15). These doses will increase the alveolar pool size >10-fold (24). Mice and rabbits with injured lungs can increase the net catabolic rate of Sat PC when given large doses of surfactant by intratracheal instillation (1, 18, 23). The responses of alveolar macrophages to surfactant treatment in vivo have not been studied. We asked whether surfactant treatment would change alveolar macrophage number and whether the alveolar macrophage phenotype would be altered to become lipid laden, activated, or apoptotic when challenged in vivo with exogenous surfactant.

MATERIALS AND METHODS

Surfactant. The surfactant used for intratracheal instillation was a mixture of lipid-extracted sheep surfactant and DPPC (Sigma, St. Louis, MO). The DPPC was added to increase the concentration of DPPC and to minimize the
volume of the injection solution (18). Sheep surfactant was recovered from alveolar lavage fluid of adult sheep by centrifugation and extracted with chloroform-methanol (2:1) (21). Of the total amount of Sat PC, two-thirds was from sheep surfactant and one-third was from supplemental DPPC. Animals were given 120 mg/kg (110 µmol/kg) Sat PC as a treatment dose. The normal alveolar pool size in C57BL/6 mice is 22 µmol/kg (18). Specificity of the responses to the treatment dose of surfactant was verified by also giving a trace dose of about one-tenth of the alveolar pool size (11). The surfactant preparations were negative for endotoxin when tested with the Limulus amebocyte lysate assay (Sigma).

Mice. Female C57BL/6 mice at 8–10 wk of age were anesthetized with methoxyflurane and orally intubated with a 25-gauge animal-feeding needle. A volume of 60 µl was instilled, delivering the trace dose or the treatment dose of surfactant in saline. A total of three treatment doses of surfactant, with each dose separated by 12 h, was given to groups of 4–10 mice who were evaluated 1, 3, 6, and 12 h after each instillation. Groups treated with the trace dose were used for comparison. Normal values were measured in untreated animals.

As a control for changes in expression of cell surface markers, we instilled 0.5 mg of *Escherichia coli* endotoxin, serotype 055:B5, 1 h after a treatment dose of surfactant. After an additional 1 h, the lungs were lavaged and the cells were stained. Four animals were studied.

Alveolar lavage. Mice were deeply anesthetized with pentobarbital sodium (ip) and exsanguinated by cutting the distal aorta (11). The thorax was opened, and a 20-gauge blunt needle was tied into the proximal trachea for alveolar lavage. Lungs were washed sequentially with five aliquots of 1 ml of 0.9% saline. Each aliquot was infused and withdrawn by syringe three times. The aliquots from each animal were pooled for analysis. Lavage fluids were immediately centrifuged for 15 min at 500 g. Total cells were stained with trypan blue and counted. Differential cell counts were performed on cytospin preparations after staining with Diff-Quik (Scientific Products, McGaw Park, IL). Cells on slide after cytopsin were stained with oil red O (Poly Scientific R & D, Bay Shore, NY) to detect lipids. Cell numbers are given per kilogram of body weight.

Flow cytometry. Aliquots of alveolar cells were incubated in the dark on ice with phycoerythrin-labeled monoclonal anti-mouse antibodies against CD14 (receptor for complex of lipopolysaccharide and lipopolysaccharide binding protein), CD16 (component of low-affinity Fc receptor; FCγRIII), CD54 (intercellular adhesion molecule (ICAM)-1), and CD95 (Fas). Isotype antibodies were used to detect nonspecific binding. Antibodies were purchased from PharMingen (Mountain View, CA). A monoclonal antibody against murine scavenger receptor class A type I (SR-AI) and type II (SR-AII; old nomenclature SR 1 and 2) (19) was obtained from SEROTEC (Raleigh, NC). After incubation with the primary antibody, cells were washed and incubated with phycoerythrin-labeled F(ab′)2 anti-IgG fragments (secondary antibody) in the dark on ice. The cell pellet was washed twice with PBS to remove unbound antibody. Control staining was performed with isotype antibodies and with secondary antibody alone to obtain background fluorescence. Cells were washed twice, resuspended in PBS, kept on ice, and immediately analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Apoptotic cells and necrotic cells were detected by annexin V and propidium iodide staining (29) (PharMingen). Apoptotic and necrotic cells expose the inner cell membrane to the outside, allowing annexin V to bind to phosphatidylserine. Cell aliquots were stained with fluorescent-labeled annexin V. Counterstaining with propidium iodide detected necrotic cells. Cell aliquots were incubated with camptothecin, a topoisomerase I inhibitor that induces apoptosis, for 3 h at 37°C as a positive control (17).

Data analysis. Results are given as means ± SE. Comparisons between groups of animals at individual time points were made with two-tailed t-tests. Significance was accepted at *P < 0.05.*

RESULTS

Number of alveolar macrophages. In untreated animals, 1.5 ± 0.3 × 10⁷ macrophages/kg body wt were recovered by alveolar lavage (Fig. 1, top). One hour after the first instillation of 120 mg/kg of surfactant, the number of alveolar macrophages decreased by 49% compared with that in untreated animals (*P < 0.05*). The number of alveolar macrophages was unchanged 3 h after the first instillation. After 6 h, the number of alveolar macrophages increased to the value seen in untreated animals. A second instillation of surfactant was administered 12 h after the first instillation. The number of alveolar macrophages increased after the second instillation by 42%, a nonsignificant increase compared with that in untreated animals. After a third
surfactant instillation, the number of alveolar macrophages increased significantly by 82% compared with those in untreated animals. Within 6 h after the third instillation, the number of alveolar macrophages returned to normal. Instillation of a trace dose of surfactant did not change the number of alveolar macrophages (Fig. 1, bottom). More than 96% of the cells were alveolar macrophages at all the time points for both treatment and trace doses. Surfactant treatment made small changes in alveolar macrophage number and did not result in recruitment of granulocytes into the lungs.

**Phenotype.** The appearance of the macrophages changed after exogenous surfactant treatment. The cells appeared larger, with vacuoles characteristic of lipid-laden or foamy macrophages, as seen by light microscopy 1 h after surfactant instillation (Fig. 2A). These vacuoles stained positively for lipids with oil red O (Fig. 2B). Alveolar macrophages from animals given the trace dose of surfactant did not have these changes (Fig. 2, C and D) and were indistinguishable from alveolar macrophages from untreated animals (Fig. 2, E and F).

The proportion of large alveolar macrophages in the population was quantified by forward-scatter flow cytometry. Constant numbers of alveolar macrophages were sized at different times after surfactant treatment (Fig. 3). In untreated animals, the normal-size cells were defined by an arbitrary cutoff (dashed line), which included 98.7% of the cells. This cutoff was used to estimate the population shift to large alveolar macrophages after surfactant treatment. For each time point, four animals were analyzed, and representative measurements are shown. Large macrophages increased to 28.5% (mean) of the population, a 22-fold increase, after instillation of the first treatment dose of surfactant. The population of large cells decreased to 18.1% (14-fold increase) and to 7.7% (6-fold increase) by 6 h. After 12 h, the size of the macrophages had returned to normal. After the second treatment dose of surfactant, the proportion of large alveolar macrophages increased again.

**Apoptosis.** The number of alveolar macrophages decreased after the first treatment dose of surfactant. Therefore, we hypothesized that necrosis or apoptosis might be causing the decrease in macrophage number. Alveolar macrophages from alveolar washes were analyzed for externalized phosphatidylserine, and propidium iodide was used to identify necrotic cells. In Fig. 4, representative measurements for every time point are shown. Four independent experiments were
done for each time point. The number of necrotic and late apoptotic cells from untreated animals was <3% of the population, and there were no changes 1 and 3 h after the treatment dose of surfactant. The positive control using camptothecin demonstrated that 28% of the macrophages could become apoptotic.

Flow cytometry. Because the alveolar macrophages became lipid laden, we wanted to know whether they also became activated. The expression of CD14, CD16, CD54, CD95, SR-AI, and SR-AII was analyzed by flow cytometry (Fig. 5). There were no significant changes for any of the cell-surface antigens either 1 or 3 h after surfactant treatment in four independent experiments. However, after endotoxin exposure, the expression of CD14 increased from 51 ± 17 to 121 ± 25 mean fluorescence units on large alveolar macrophages. This experiment showed that changes in the expression of cell surface markers can increase on lipid-laden alveolar macrophages and that the change can be detected by flow cytometry.

DISCUSSION

Exogenous surfactant acutely changes the phenotype of alveolar macrophages, causing them to become large and foamy. The phenotype of the foamy macrophages returned to normal within 6 h, even after re-
petitive doses of surfactant. The number of alveolar macrophages decreased 1 and 3 h after a first treatment dose of surfactant. A second and third dose of exogenous surfactant increased the number of alveolar macrophages. The initial decrease of alveolar macrophages was not due to apoptosis or necrosis. The alveolar macrophages did not change expression of CD14, CD16, CD54, CD95, SR-AI, or SR-AII after exposure to exogenous surfactant, indicating no generalized activation. Normal alveolar macrophages can respond to increased surfactant in their environment by becoming transiently large and foamy.

The behavior of alveolar macrophages after surfactant treatment has not been studied extensively. In vitro macrophages take up and catabolize the lipid and protein components of surfactant (33). Macrophages have been implicated as being major contributors to surfactant catabolism in vivo because mice that lack GM-CSF have alveolar proteinosis and foamy macrophages that degrade DPPC and SP-A very slowly (33). In the rabbit, about 20% of the phospholipase A-resistant diether analog of DPPC accumulates in the macrophages recovered by alveolar lavage after intratracheal injection of the analog (28). This result indicates that at least 20% of the major lipid of surfactant is catabolized by macrophages in the rabbit. In contrast, the recovery of this analog in macrophages after airway instillation in mice was only about 5% and did not accumulate after 3 consecutive instillations (18). However, in recent unpublished experiments, large numbers of macrophages containing the ether analog that were not recovered by saline lavage could be recovered from the lungs, contributing to the catabolism (unpublished observation). The acute change in phenotype of the macrophages to large and foamy after surfactant treatment, followed by a return to normal appearance within about 6 h, suggests that the macrophage takes up more lipid than it can catabolize after surfactant treatment. The net rate of catabolism increases after surfactant treatment (18). The factors that regulate uptake and catabolic rate of macrophages are not known.

We thought that the change in phenotype might be associated with activation of the macrophages. Endotoxin receptor CD14 is minimally expressed on resting alveolar macrophages despite its function for phagocytosis (35). In our endotoxin control experiment, the expression of CD14 increased, showing that cell surface markers can be detected by flow cytometry on lipid-laden alveolar macrophages. Surfactant treatment did not increase expression of CD14. Recently, a central function for CD14 in apoptosis and modulation of inflammation was shown (6). The expression of Fas (CD95), which also mediates apoptosis, was not changed with surfactant treatment. CD16, a component of low-affinity Fc receptor FeγRII, is expressed on a subset of monocytes and macrophages (34), and the expression of CD16 was not changed after exposure to exogenous surfactant. ICAM-1 (CD54), SR-AI, and SR-AII are involved in cell adhesion (10). SR-AI and SR-AII are highly expressed on alveolar macrophages (10) and are involved in bacterial phagocytosis and binding of endotoxin (5). The expression of ICAM-1, SR-AI, and SR-AII did not change significantly after surfactant treatment. This is of particular interest because an increased adhesion of macrophages might affect the efficiency of the alveolar lavage to recover alveolar macrophages. The administration of large doses of sheep surfactant did not change the expression of surface markers characteristic of activation in the alveolar macrophages in mice. The lack of neutrophil recruitment suggests that no important activation of macrophages occurred.

The unexplained finding is the decrease in macrophages after the first dose of surfactant and the small increase in macrophages after the subsequent doses of surfactant. Recovery of macrophages by alveolar lavage is incomplete, and distension of the lungs with saline that contains chelators at 37°C will strikingly increase the number of macrophages recovered by lavage (8). The surfactant treatments did not induce apoptosis or necrosis, and no indicators of inflammation such as granulocyte accumulation were apparent. We did not measure cytokine expression by the macrophages because there was no indication of activation. We assume that the changes in the number of macrophages recovered by alveolar lavage resulted from the subtle effect of the surfactant on the efficiency of recovery of the macrophages by alveolar wash. The expression of adhesion molecules ICAM-1 and SR-AI and SR-AII, however, were not changed. However, it is conceivable that the lungs are primed after the initial surfactant treatment, which might result in changes in the interstitial macrophages.

In vitro surfactant components have been shown to be both proinflammatory and anti-inflammatory (32). The surfactant given to the mice was an organic solvent extract of sheep surfactant supplemented with DPPC, which contained ovine SP-B and SP-C. This surfactant given repetitively and in large doses did not activate macrophages or change the number of macrophages or granulocytes in the lungs very much. The only response was a change in the phenotype of the macrophages indicating increased uptake of the surfactant. These studies demonstrate no adverse effect of surfactant instillation on the resident alveolar macrophage population. Alveolar macrophages can increase lipid uptake and catabolism without changing surface markers characteristic of activation.

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