Degradation of surfactant protein D by alveolar macrophages

QUN DONG AND JO RAE WRIGHT
Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Dong, Qun, and J O Rae Wright. Degradation of surfactant protein D by alveolar macrophages. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L97–L105, 1998.—Surfactant protein (SP) D is a pulmonary surfactant-associated protein that may function in lung host defense. SP-D is produced by alveolar type II cells and nonciliated bronchiolar epithelial (Clara) cells of the airway and is secreted into the air space. Here we investigated whether alveolar macrophages degraded SP-D in vitro. We also examined the effects of SP-A and lipids on SP-D metabolism. The results showed that alveolar macrophages bound and degraded SP-D in a time- and temperature-dependent fashion. After 100 min of incubation, the formation of trichloroacetic acid-soluble degradation products increased 4-fold in the medium and 30-fold in the cells. The degradation of SP-D was via a cell-associated process because SP-D was not degraded when incubated in medium previously conditioned by alveolar macrophages. Gel autoradiography of cell lysate samples after incubation with 125I-labeled SP-D demonstrated an increase in degradation products, further confirming the degradation of SP-D by alveolar macrophages. In addition, the degradation of SP-D was not affected by coincubation with SP-A or surfactant-like liposomes containing either phosphatidylinositol or phosphatidylglycerol. In conclusion, alveolar macrophages rapidly degrade SP-D and may play an important role in SP-D turnover and clearance.

alveolar surfactant; collectin; C-type lectin; alveolar type II cells

SURFACTANT PROTEIN (SP) D is one of the four surfactant-associated proteins. Although a role for SP-D in regulation of alveolar surface tension or regulation of surfactant metabolism has not been defined, several studies suggest that SP-D may function in pulmonary host defense. For example, SP-D binds and aggregates various gram-negative bacteria (11), interacts with Pneumocystis carinii and mediates organism adherence to alveolar macrophages (19), inhibits the hemagglutination activity of influenza virus (6), and enhances the production of superoxide by alveolar macrophages (23).

Because alveolar macrophages are the predominant phagocytic cell in the air space in healthy animals, they may play a role in SP-D metabolism. Alveolar macrophages have been shown to participate in various aspects of surfactant metabolism. For example, alveolar macrophages internalize and degrade surfactant lipids (5, 16, 28) and SP-A (1, 28) and contribute significantly to the clearance of SP-A and surfactant lipids (28). Immunocytochemistry studies showed that Clara cells, type II cells, and alveolar macrophages contained immunoreactive SP-D (24), but only type II cells and Clara cells contained SP-D mRNA by in situ hybridization (25). In addition, SP-D was shown to bind to alveolar macrophages specifically (10), which is consistent with the idea that SP-D binds to receptors on alveolar macrophage membranes. Furthermore, immunocytochemistry and electron microscopy studies showed that in addition to localization on the cell surface, immunoreactive SP-D was detected intracellularly, predominantly in the vacuolar or vesicular compartments (10) of alveolar macrophages, indicating that SP-D may be internalized and degraded in alveolar macrophages in vivo.

Although SP-A has been implicated in regulation of the metabolism of surfactant lipids, the role of SP-D in this process has not been reported. It is reasonable to speculate that SP-D may be involved in lipid metabolism because it has been reported that lipids coisolate with SP-D from the lavage of silica-treated rats (13). Furthermore, SP-D binds to phosphatidylinositol (PI) and aggregates PI-containing liposomes (20). Although PI is a minor component of surfactant lipids isolated from healthy animals (<5% of total surfactant lipids; see Ref. 27), there can be an increase in PI and a decrease in phosphatidylglycerol (PG) in surfactant lipids under certain pathological conditions (7, 8). Moreover, SP-D coexists with other SPs in the air space, and it has been shown that SP-D binds to SP-A (18) in vitro. Therefore, it would be interesting and important to examine the effects of surfactant-like lipids and SP-A on SP-D metabolism.

In the current study, we investigated whether SP-D was degraded by alveolar macrophages in vitro. In addition, we examined the effects of SP-A and surfactant-like lipids on SP-D metabolism by alveolar macrophages. These studies provide direct evidence of degradation of SP-D by alveolar macrophages for the first time.

MATERIALS AND METHODS

Materials. Lipids (L-α-PG, L-α-dipalmitoylphosphocholine, L-α-Pi, and cholesterol) were purchased from Avanti Polar Lipids (Birmingham, AL). L-[α-14C]dipalmitoylphosphatidylcholine (DPPC; 110 µCi/mmol) was purchased from NEN (Boston, MA). Dulbecco’s phosphate-buffered saline, F-12 medium, and lipofectamine were purchased from Gibco BRL (Gaithersburg, MD). α-Eagle’s minimum essential medium (MEM) medium and HB-CHO serum-free medium were from Irvine Scientific (Santa Ana, CA). The pE14 vector was purchased from Celltech Therapeutics (Berkshire, UK). The biotin-dependent protein assay reagent was purchased from Pierce Chemical (Rockford, IL). Ecolite liquid scintillation cocktail was from ICN (Costa Mesa, CA). Bolton-Hunter reagent and Amplify solution were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Other chemicals were from Sigma Chemical (St. Louis, MO). Polyclonal rabbit anti-rat SP-D antibody was kindly provided by...
Dr. Samuel Hawgood, University of California, San Francisco.

Expression of recombinant rat SP-D in Chinese hamster ovary cells. A full-length rat SP-D cDNA construct was kindly provided by Dr. James H. Fisher, Wayne State University. The cDNA was ligated into the pEE14 vector, and the orientation of the subclones was determined by restriction mapping. The Chinese hamster ovary (CHO-K1) cells in F-12 medium were transfected with pEE14-rat SP-D or were mock transfected with pEE14 using lipofectamine. The cells were incubated for 4 h, then 10% (vol/vol) dialyzed fetal calf serum was added, and the incubation continued overnight. The next day, the medium was changed to α-MEM containing 25 mM methionine sulfoximine (MSX; see Ref. 3) and 10% dialyzed fetal calf serum. Because SP-D was incorporated into the pEE14 proximal to the glutamine synthetase minigene driven by the SV40 promoter, incubation with MSX will select the colonies that express SP-D. Nicely formed individual colonies were generally seen after ~2 wk of culture, and 5–8 colonies were transferred from each plate into 6-well culture plates and were cultured in the same medium with MSX as before. After ~1 wk, the medium from each well was collected and screened for SP-D expression by Western blot. About three to five high producers were selected, and the cells were split into 100-mm culture plates and were further selected by exposing the cells to higher MSX concentrations (ranging from 0.1 to 1 mM). The highest producers were selected and further isolated by limiting dilution using 96-well cell culture plates in the presence of MSX.

For the purpose of collecting SP-D-containing medium, the selected cells were cultured in HB-CHO serum-free medium in the presence of MSX for 6–8 days. Freshly prepared ascorbate (50 µg/ml) was added to the medium every other day, presumably to facilitate the hydroxylation of proline residues (3).

Purification of recombinant SP-D. The serum-free HB-CHO medium was collected after ~8 days of culture and was dialyzed at room temperature against 25 mM tris(hydroxymethyl)aminomethane (Tris), 140 mM NaCl, and 2 mM CaCl2 with four changes. Subsequently, the medium was applied to a maltose column. After washing with dialysis buffer, SP-D was eluted from the column with 25 mM Tris, 140 mM NaCl, and 2 mM EDTA (pH 7.4). The purified SP-D was analyzed by Coomassie blue staining and Western blot, and the concentration of purified SP-D was measured by BCA assay.

3H labeling of recombinant rat SP-D. The rat SP-D CHO-K1 cells were cultured in serum-free medium as described previously except that 20 µCi/ml [3H]iodine were included in the medium. The medium was collected after ~1 wk, and SP-D was purified from the medium as described above. The [3H]SP-D was analyzed by Western blot, Coomassie blue staining, and gel autoradiography. The specific activity was ~6 × 104 counts·min⁻¹ (cpm)·µg protein⁻¹.

Iodination of recombinant SP-D. Iodination was performed using the Bolton-Hunter reagent according to the method described by Kuan et al. (10). Approximately 20 µg of recombinant rat SP-D were incubated with dried Bolton-Hunter reagent in a final volume of 0.2 ml of 25 mM N-2-hydroxymethylpiperazine-N’-2-ethanesulfonic acid-150 mM NaCl (HBS; pH 7.4) containing 2 mM CaCl2, 0.1% Triton X-100, and 30 mM maltose at 4°C for 15 min. The iodinated SP-D was separated from free iodine by a P-2 column (Bio-Rad) in HBS containing 0.1% Triton X-100 at room temperature. The specific activity of the protein was ~4 × 10⁹ cpm/µg protein. The protein was subsequently dialyzed against HBS containing 2 mM EDTA at room temperature to decrease further the free iodine associated with the protein. Iodinated SP-D was used within 24 h after the labeling.

Preparation of liposomes. Small unilamellar liposomes were prepared by extrusion from a French pressure cell as described previously (28). The PG-containing liposomes consisted of (by weight) 54% DPPC, 27% egg PC, 11% egg PG, and 8% cholesterol, and the PI-containing liposomes were the same as PG-containing liposomes except that PI (13% by weight) was substituted for PG.

Isolation of cells. Alveolar macrophages were isolated by lung lavage of 250- to 300-g male Sprague-Dawley rats (Charles River Laboratory, Raleigh, NC). Briefly, rats were killed by injection of Nembutal and exsanguination. The lungs were removed from the chest and were lavaged eight times with phosphate-buffered saline (PBS) containing 0.2 mM EDTA. The alveolar macrophages were collected by centrifugation at 200 g for 10 min. The purity and viability of the cells were routinely >95%.

Degradation of [3H]SP-D by alveolar macrophages. The isolated alveolar macrophages (2.5 × 10⁴) were resuspended in 0.5 ml of incubation buffer (PBS with 0.9 mM CaCl2 and 0.1% bovine serum albumin) and various concentrations of [3H]SP-D. In some experiments, primary cultured rat lung fibroblasts (15) were used in the degradation assay. To study the effects of lipids and SP-A on SP-D metabolism, human SP-A (purified from proteinosis patient lavage) or PI- or PG-containing liposomes were coincubated with [3H]SP-D and cells in the experiments. In gel autoradiography experiments, 125I-labeled SP-D was used instead of [3H]SP-D to increase the sensitivity. The cells were incubated at 37 or 4°C with gentle shaking for various amounts of time. After incubation, the cells were collected by centrifugation at 200 g for 10 min (or 500 g for 5 min), and the medium was saved for further analysis. The cells were washed one time and then transferred to a new tube to minimize nonspecific binding of radioactivity to the tubes, followed by two washes. For binding experiments, the cells were lysed in 0.2 ml of cell lysis buffer (150 mM NaCl, 50 mM phosphate buffer, 0.5% Nonidet P-40, and 2 mM EDTA), of which 0.15 ml was analyzed for radioactivity in an LS 1800 scintillation counter (Beckman, Fullerton, CA), and the remaining 0.05 ml was assayed for protein using BCA assay. For protein degradation studies, the cells were resuspended into 0.5 ml of the incubation buffer containing 0.1% BSA, and trichloroacetic acid (TCA) was added to a final concentration of 10% to both the cells and medium. The samples were then incubated on ice for 25 min and were centrifuged at 9,000 g for 10 min at 4°C. The supernatants were transferred to scintillation vials, and the pellets were resuspended in PBS containing 0.1% BSA and were transferred to scintillation vials. Four milliliters of scintillation cocktail were added to each sample.

In some experiments, alveolar macrophages were first preincubated with 2 µg/ml [3H]SP-D at 4°C for 2 h. Then after being washed and transferred to new tubes, the cells were warmed to 37°C and were incubated at 37°C for another 100 min, followed by TCA precipitation of medium and cell samples. Control experiments were performed by preincubation of cells without SP-D at 4°C for 2 h, addition of 2 µg/ml [3H]SP-D to the cells after they were warmed to 37°C, and continuation of the incubation for another 100 min.

Gel autoradiography. Samples with 125I-Sp-D were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and were stained with Coomassie blue. After being destained, the gels were dried and exposed to X-OMAT film (Sigma Chemical) for various amounts of
time at −80°C depending on the radioactivity of the samples loaded on the gels.

Gels containing [3H]SP-D samples were first incubated in Amplify solution for 30 min to enhance the radioactive signal before they were dried and exposed to the films.

Statistical analysis. Most of the results are shown as means ± SE. Student’s t-test was performed for the comparison of different values with the corresponding controls for significance, which was accepted at P < 0.05.

RESULTS

3H-labeled recombinant rat SP-D. Recombinant rat SP-D was expressed in CHO-K1 cells as described in MATERIALS AND METHODS. To radiolabel SP-D, CHO cells were incubated with [3H]proline-supplemented serum-free medium. The medium was collected after 7–8 days of culture and was applied to a maltose-affinity column to purify the [3H]SP-D. The results (Fig. 1, lanes 1 and 2) of immunoblotting demonstrated the comparison of recombinant [3H]SP-D and wild-type SP-D. The major form of SP-D is the high-molecular-mass form of ∼43 kDa; the low-molecular-mass form is probably the deglycosylated form of SP-D because the 43-kDa form shifted to the low-molecular-mass form after treatment with N-glycosidase. On nonreducing gels, the major form of both recombinant and wild-type SP-D was ∼130 kDa (data not shown). Coomassie blue staining (Fig. 1, lane 3) showed that SP-D was the only detectable protein that eluted from the column. This was confirmed by gel autoradiography (Fig. 1, lane 4), demonstrating that [3H]SP-D was the only tritiated protein purified from the column. The specific activity of the [3H]SP-D used in the experiments was ∼6 × 10^4 cpm/µg SP-D. The [3H]SP-D was stable for at least 1–2 mo after the labeling when tested by TCA precipitation and gel autoradiography.

Degradation of [3H]SP-D by alveolar macrophages. At 4°C (Fig. 2A), the recombinant [3H]SP-D bound to isolated alveolar macrophages in a Ca2+- and concentration-dependent manner. The binding was saturated at an ∼1 µg/ml SP-D concentration. Binding of [3H]SP-D to isolated alveolar macrophages was also observed at 37°C (Fig. 2B), although it did not reach saturation. The reason for the lack of saturation at 37°C may be due to the simultaneous uptake and degradation of SP-D by alveolar macrophages at 37°C.

The degradation of [3H]SP-D was measured by the formation of TCA-soluble radioactivity in the cells and in the medium (28). The results were expressed as the percentage of radioactivity that was TCA soluble as shown in Fig. 3, A and B. The TCA-soluble radioactivity increased both in the cells (Fig. 3A) and in the medium (Fig. 3B) as a function of incubation time at 37°C. In the cells, the percentage of TCA-soluble radioactivity increased rapidly from a starting value of ∼0.50% at the beginning of the incubation to ∼1.97 ± 1.85% at 25 min and remained near this level over 100 min of incubation. In the medium, the TCA-soluble radioactivity increased from an initial value of 0.50 ± 0.07% to 2.06 ± 0.16% at 100 min of incubation, which was approximately a fourfold increase. These data were consistent with a rapid degradation of SP-D by alveolar macrophages. At 4°C there was no increase in the
was analyzed. As shown in Table 1, 13.77 \( \pm 4.18\% \) (\( n = 3 \)) of the total radioactivity associated with the cells was precipitated by trichloroacetic acid (TCA), indicating that this process is temperature dependent. Experiments were also performed under the same conditions in the absence of SP-D, and there was no detectable degradation (data not shown).

To characterize further the degradation of SP-D by alveolar macrophages, macrophages were preincubated with \([3H]SP-D (1 \mu g/ml)\) for 2 h (Fig. 3), and the degradation products were mostly released to the medium. This indicated that SP-D was most likely degraded via a cell-associated process.

To determine whether SP-D could be degraded in macrophage-conditioned medium, cells were incubated in the absence of \([3H]SP-D\) for 75 min, the conditioned medium was then transferred to a new tube to which \([3H]SP-D\) was added, and the incubation was continued for another 75 min. As shown in Fig. 4, \([3H]SP-D\) was not degraded when incubated with alveolar macrophage-conditioned media, whereas the cells from the same source degraded \([3H]SP-D\) and released the TCA-soluble radioactivity into the medium. This indicated that SP-D was most likely degraded via a cell-associated process.

To characterize further the degradation of SP-D, recombinant SP-D was iodinated as described in MATERIALS AND METHODS. \(^{125}\)I-SP-D was dialyzed immediately after the labeling to remove the remaining free iodine. Because we found that \(^{125}\)I-SP-D was unstable and rapidly formed TCA-soluble products, the dialysis was continued until the protein was utilized, which was within 24 h of the labeling. The stability of the protein was monitored by TCA precipitation and gel autoradiography. At least 90% of the counts were TCA precipitable at the time the experiments were initiated, and no degradation products were detected by autoradiography (Fig. 5, lane 1). After the iodinated SP-D was incubated with the cells, the medium and the cell lysate were analyzed by SDS-PAGE and gel autoradiography. The results demonstrated that, when cells were incubated at 37°C for 2 h, there was a significant increase in small degradation products at approximately the position of the dye front of the gel in the cell lysate (Fig. 5, lane 2). No small degradation products were detectable after incubation at 4°C (Fig. 5, lane 4). The inability to detect any increase in degradation products in the sample of medium at 37°C (Fig. 5, lane 3) is probably due to the very low percentage of total TCA-soluble radioactivity in the medium. Based on the results from Fig. 3, A and B, \(>97\%\) of the total radioactivity in the medium (including TCA soluble and precipitable) is TCA-precipitable radioactivity, the majority of which is from the input SP-D added at the beginning of the experiment. In the cells, \(>20\%\) of the total radioactivity is TCA-soluble. Therefore, when equal amounts of radioactivity were loaded in each lane of the gels, the amount of TCA-soluble radioactivity in the medium sample would be much lower than that in the cell lysate samples.

In addition, when the cells were incubated at 37°C, there was a change in the intensity of the high (\(~45\ kDa\) ) and low (\(~38\ kDa\)) molecular-mass forms of SP-D in the cell lysate compared with the control \(^{125}\)I-SP-D (Fig. 5, lane 1) that was not incubated with...
cells. In contrast, at 4°C, there was no significant change in intensity of the high and low forms of SP-D (Fig. 5, lanes 4 and 5). This suggests that the changes in distribution of the high and low forms of SP-D were not a result of preferential binding of the low-molecular-mass form of SP-D to alveolar macrophages, which could still presumably occur at 4°C. The change in the intensity of the high- and low-molecular-mass forms of SP-D was also detected when similar experiments were performed using [3H]SP-D instead of 125I-SP-D (data not shown).

To test if [3H]SP-D can be degraded by other cell types, the degradation assay was also performed using primary cultured rat lung fibroblasts. As shown in Table 2, there was no significant increase in TCA-

![Image](https://example.com/image.png)

Fig. 4. Effect of macrophage-conditioned medium on degradation of [3H]SP-D. Alveolar macrophages (AM) were incubated (5 × 10^6/ml) in the incubation buffer at 37°C for 75 min. Conditioned medium was then collected by centrifugation, added to [3H]SP-D (1 µg/ml), and continuously incubated at 37°C for another 75 min. TCA precipitation was performed as described in MATERIALS AND METHODS. For comparison, degradation experiments were also performed with alveolar macrophages from the same source as the conditioned medium ([3H]SP-D with AM). SP-D was incubated for 75 min in the incubation buffer ([3H]SP-D in phosphate-buffered saline (PBS)), and TCA precipitation with [3H]SP-D (control) was analyzed. Values are expressed as the degree of increase of TCA-soluble radioactivity in the medium or in the cells with fibroblasts after incubation at 37°C for 100 min.

![Image](https://example.com/image.png)

Table 1. Degradation of [3H]SP-D by SP-D prebound alveolar macrophages

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>%TCA-Soluble Radioactivity With Cells</th>
<th>%TCA-Soluble Radioactivity in Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-D prebound to cells</td>
<td>SP-D present during incubation</td>
<td>SP-D prebound to cells</td>
</tr>
<tr>
<td>5–15 s</td>
<td>13.77 ± 3.54 (3)</td>
<td>18.77 ± 0.76% (3)</td>
</tr>
<tr>
<td>100 min</td>
<td>ND</td>
<td>15.57 ± 4.18 (3)</td>
</tr>
</tbody>
</table>

Data are means ± SE; no. of experiments (n) is shown in parentheses. Cells were first preincubated with [3H]surfactant protein (SP)-D (2 µg/ml) at 4°C for 2 h. After being washed and transferred to new tubes, cells were warmed to 37°C and were incubated for another 100 min. Control experiments were performed by preincubation of cells without SP-D at 4°C for 2 h, and addition of 2 µg/ml [3H]SP-D to the cells after warming the cells to 37°C, and continuation of the incubation for another 100 min. Cells were then separated from medium by centrifugation, washed one time, transferred to new tubes, washed two more times, and resuspended into the incubation buffer. Cells and medium were trichloroacetic acid (TCA) precipitated as in MATERIALS AND METHODS. Results are expressed as percentage of counts in the supernatant after TCA precipitation. ND, not detectably greater than background, e.g., percentage of TCA-soluble radioactivity in [3H]SP-D at the beginning of the experiment, the value of which is 0.28 ± 0.02% (n = 3).

Fig. 5. Detection of degradation of 125I-labeled SP-D by gel autoradiography. 125I-SP-D (1 µg/ml) was incubated with alveolar macrophages (5 × 10^6/ml) at 37 or 4°C for 2 h. Cells were separated from medium by centrifugation, washed 1 time, transferred to new tubes, and washed 2 more times. Cells were then lysed, and both cell lysate and medium samples with equal amounts of radioactivity were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were Coomassie blue stained, dried, and exposed to film. Lane 1, 125I-SP-D; lane 2, cell lysate sample after incubation at 37°C; lane 3, medium sample after incubation at 37°C; lane 4, cell lysate sample after incubation at 4°C; lane 5, medium sample after incubation at 4°C. Molecular-mass markers are shown on left.
of both types of liposomes under the same conditions (data not shown).

**DISCUSSION**

The major findings of this study are summarized as follows. First, SP-D is degraded by isolated alveolar macrophages by a relatively rapid process. Second, the degradation is via a cell-associated process. Third, neither SP-A nor surfactant lipids affect SP-D degradation by alveolar macrophages under current conditions.

Initially, both wild-type and recombinant rat SP-D were radiolabeled with $^{125}$I using either Iodo beads (26) or the Bolton-Hunter method (see Ref. 10). However, the $^{125}$I-SP-D was not stable for routine metabolism studies because it underwent rapid and spontaneous degradation as assessed by the appearance of TCA-soluble iodinated products. Therefore, we labeled the newly synthesized SP-D in CHO cells. The soluble iodinated products. Therefore, we labeled the degradation as assessed by the appearance of TCA-soluble radioactivity. ND, not detectably greater than background, e.g., percentage of TCA-soluble radioactivity in the [3H]SP-D at the beginning of the experiments, the value of which is 0.50 ± 0.07% ($n = 12$).

**Table 2. Comparison of degradation of [3H]SP-D by alveolar macrophages and fibroblasts**

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>%TCA-Soluble Radioactivity With Cells</th>
<th>%TCA-Soluble Radioactivity in Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alveolar macrophages</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>5–15 s</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>100 min</td>
<td>22.31 ± 2.55 (8)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE; $n$ is shown in parentheses. Cells were incubated with [3H]SP-D (1 µg/ml) at 37°C for 100 min. Cells were separated from medium by centrifugation, washed one time, transferred to new tubes, washed two more times, and resuspended in the incubation buffer. Results are expressed as percentage of counts in the supernatant after TCA precipitation. ND, not detectably greater than background, e.g., percentage of TCA-soluble radioactivity in the [3H]SP-D at the beginning of the experiments, the value of which is 0.50 ± 0.07% ($n = 12$).
products are found in the medium (4.25 ng in medium + 2.5 ng in cells). This indicated that the cells degraded SP-D and released most of the degradation products into the medium. The comparison of the degradation of SP-D by alveolar macrophages with that by primary cultured lung fibroblasts (Table 2) demonstrates that, in contrast to alveolar macrophages, there was little increase in TCA-soluble radioactivity in the medium or in the cells with fibroblasts after incubation. This result indicates that the rapid degradation of SP-D by alveolar macrophages is not a general process carried out by all types of cells.

We are able to estimate the percentage of SP-D that is degraded by alveolar macrophages and the overall turnover and clearance of SP-D in vivo by making a few assumptions. If we assume that the total number of alveolar macrophages is \(27 \times 10^6\) per rat lung (2), the total volume of hypophase fluid is \(0.5\) ml/rat lung (22), and the concentration of SP-D in the air space of rats is \(3\) mg/ml (4), then using the results we obtained in this study, \(~105\) ng of SP-D would be degraded in 100 min, which is \(~7\)% of the total SP-D pool in the lung. Based on this calculation, if we assume that SP-D is degraded predominantly by alveolar macrophages in the lung, the turnover time of SP-D would be \(~24\) h. Because free amino acids could be released from SP-D during the degradation, some of the \([3H]\)proline might be released and reutilized within the incubation period. Therefore, the degradation of SP-D could be underestimated. In addition, these results are based on in vitro analyses and some perhaps oversimplified assumptions. Nevertheless, this is a general estimate of the capability of alveolar macrophages to degrade SP-D. Electron microscopy studies demonstrated that alveolar macrophages were one of the major sites for SP-D localization in the alveolar space, and no obvious staining was detectable in the alveolar lining (10). Taken together, our results, as well as the above observations, support the possibility that alveolar macrophages play a major role in SP-D degradation in the lung. Further studies with other pulmonary cells such as type II cells and in vivo turnover studies with SP-D should help to understand further the metabolic process of SP-D in the lung.

Based on the calculation from actual counts and the specific activities of \([3H]\)SP-D, \(~10\) ng of SP-D were degraded by \(2.5 \times 10^6\) alveolar macrophages after a 100-min incubation. This accounts for 2% of SP-D added to the medium (Table 4). In contrast, \(~15\)% of added SP-A was degraded after a 100-min incubation (28). The difference in the degradation of SP-A and SP-D may be due to the distinct metabolic pathway of these two proteins. It may also depend on the different binding properties of SP-A and SP-D to alveolar macrophages. It has been shown that SP-D binds to alveolar macrophages with a dissociation constant \((K_d)\) of \(1.4 \times 10^{-6}\) M (10), whereas SP-A binds to alveolar macrophages with a \(K_d\) of \(4 \times 10^{-9}\) M (21). Therefore, the binding affinity of SP-D to alveolar macrophages is much lower than that of SP-A. In addition, cell-associated partial degradation products were detected (Fig. 5), and there was a rapid increase in TCA-soluble radioactivity associated with the cells after incubation.

### Table 4. Effect of lipids on \([3H]\)SP-D degradation in medium

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>([3H])SP-D only</th>
<th>([3H])SP-D + PG liposomes</th>
<th>([3H])SP-D + PI liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–15 s</td>
<td>0.50 ± 0.07 (12)</td>
<td>0.64 ± 0.06 (4)</td>
<td>0.67 ± 0.06 (4)</td>
</tr>
<tr>
<td>25 min</td>
<td>0.82 ± 0.08 (8)</td>
<td>0.92 ± 0.03 (5)</td>
<td>0.79 ± 0.02 (4)</td>
</tr>
<tr>
<td>75 min</td>
<td>1.35 ± 0.21 (7)</td>
<td>1.47 ± 0.15 (4)</td>
<td>1.55 ± 0.05 (3)</td>
</tr>
<tr>
<td>100 min</td>
<td>2.06 ± 0.16 (12)</td>
<td>1.74 ± 0.11 (3)</td>
<td>1.92 ± 0.26 (4)</td>
</tr>
</tbody>
</table>

Data are means ± SE; n is shown in parentheses. Cells were incubated at 37°C for various lengths of time with \([3H]\)SP-D (1 µg/ml) alone or \([3H]\)SP-D and phosphatidylglycerol (PG)-containing liposomes (100 µg/ml phospholipids) or \([3H]\)SP-D and phosphatidylinositol (PI)-containing liposomes (100 µg/ml phospholipids). Medium was separated from cells by centrifugation and TCA precipitated as in MATERIALS AND METHODS. Results are expressed as percentage of counts in supernatant after TCA precipitation.

### Table 5. Effect of lipids on \([3H]\)SP-D degradation in alveolar macrophages

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>([3H])SP-D only</th>
<th>([3H])SP-D + PG liposomes</th>
<th>([3H])SP-D + PI liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–15 s</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>25 min</td>
<td>14.97 ± 1.85 (4)</td>
<td>19.41 ± 2.22 (4)</td>
<td>20.49 ± 1.72 (3)</td>
</tr>
<tr>
<td>75 min</td>
<td>19.88 ± 1.14 (4)</td>
<td>20.29 ± 0.95 (3)</td>
<td>23.76 ± 1.21 (3)</td>
</tr>
<tr>
<td>100 min</td>
<td>22.31 ± 2.55 (8)</td>
<td>16.23 ± 1.96 (3)</td>
<td>19.70 ± 0.66 (3)</td>
</tr>
</tbody>
</table>

Data are means ± SE; n is shown in parentheses. Cells were incubated at 37°C for various lengths of time with \([3H]\)SP-D (1 µg/ml) alone or \([3H]\)SP-D and PG-containing liposomes (50 µg phospholipids) or \([3H]\)SP-D and PI-containing liposomes (50 µg phospholipids). Cells were separated from medium by centrifugation, washed one time, transferred to new tubes, and washed two more times. Cells were resuspended in incubation buffer and TCA precipitated as in MATERIALS AND METHODS. Results are expressed as percentage of counts in supernatant after TCA precipitation. ND, not detectably greater than background, e.g., percentage of TCA-soluble radioactivity in the \([3H]\)SP-D at the beginning of the experiments, the value of which is 0.50 ± 0.07% (n = 12).
with SP-D (Fig. 3A). In contrast, there were no detectable partial degradation products (data not shown) and very little TCA-soluble radioactivity in the cells (28) after incubation with SP-A.

The effects of SP-A on SP-D metabolism were also studied. SP-D has been shown to interact with SP-A and to counteract the inhibition of type II cell lipid secretion by SP-A (12). However, our results indicated that under the current experimental conditions SP-A did not affect SP-D metabolism. It would be interesting to test if the incubation of SP-D and SP-A would affect SP-A degradation.

Although the majority of SP-D does not seem to be associated with lipids in the lavage fluid, some of the SP-D may associate with surfactant lipids and other surfactant components (13). SP-D has been shown to bind to PI and to aggregate PI-containing liposomes in vitro (20). Therefore, SP-D may interact with surfactant lipids via PI, especially under circumstances in which PI levels are elevated, such as in alveolar proteinosis and silicosis. We speculated, therefore, that the metabolism of SP-D might be affected by PI-containing liposomes to a greater extent than by PG-containing liposomes. However, neither PG- nor PI-containing liposomes affected the degradation process of SP-D by alveolar macrophages (Tables 4 and 5). The lack of any effect of lipids on SP-D metabolism could be due to the lack of aggregation of lipids by SP-D. Our results with the liposomes used in these studies showed that although SP-D bound to PI-containing liposomes, it did not aggregate either PG- or PI-containing liposomes. The disparity in the lipid aggregation results may be due to the different experimental conditions. In our experiments, we used PBS containing a lower Ca²⁺ concentration (1 mM), which is more physiologically relevant, and used liposomes with a lipid composition similar to that of native surfactant. Under the same conditions, SP-A dramatically enhanced lipid aggregation (data not shown). Therefore, the finding that the degradation of SP-A by alveolar macrophages is reduced by the presence of lipids (28) may be a result of the aggregation of lipids by SP-A.

It is important to understand the metabolic pathways of surfactant components because the maintenance of appropriate, functional pools of surfactant is required for normal lung function and possibly maintenance of host defense in the lung. Under certain pathological conditions such as silicosis and proteinosis, there is an accumulation of surfactant components, including SP-D (4). Although in both cases there is significantly more surfactant in the lung, the function of the lung is impaired, indicating that maintaining homeostasis is critical for the normal function of the lung. Whether alveolar macrophages are dysfunctional in the above pathological conditions, thereby causing the accumulation of surfactant, including SP-D, is not clear. The mechanism by which alveolar macrophages degrade SP-D under normal and pathological conditions requires further investigation.

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Address for reprint requests: J. R. Wright, Box 3709, Dept. of Cell Biology, Duke Univ. Medical Center, Durham, NC 27710.

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