Surfactant protein A increases matrix metalloproteinase-9 production by THP-1 cells

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Vazquez de Lara, Luis G., Todd M. Umstead, Sara E. Davis, and David S. Phelps. Surfactant protein A increases matrix metalloproteinase-9 production by THP-1 cells. Am J Physiol Lung Cell Mol Physiol 285: L899–L906, 2003.—Matrix metalloproteinase (MMP)-9 from alveolar macrophages is a major source of elastolytic activity in the lung. It is increased in the bronchoalveolar lavage fluid of patients with emphysema. Although the importance of macrophage-derived elastolytic activity in the pathogenesis of emphysema is well established, questions remain about MMP-9 regulation. Because surfactant protein A (SP-A) is capable of modulating other functions of human mononuclear cells, we hypothesized that SP-A may regulate MMP-9 expression. Vitamin D3-differentiated THP-1 cells and peripheral blood mononuclear cells were stimulated in vitro with several concentrations of SP-A for different incubation times. MMP-9 mRNA expression was measured by dot-blot analysis, gelatinolytic activity in the medium was determined by gel zymography, protein expression was determined by ELISA, and a specific MMP-9 activity assay was used to measure the state of activation of this enzyme in the cell supernatants. SP-A induced the expression of MMP-9 in both cell types, the effect was time and dose dependent, and MMP-9 was released in its zymogen form. On the basis of the results of neutralizing antibody studies, we believe that SP-A action is mediated through Toll-like receptor-2. Even though the biological meaning of these findings remains to be elucidated, these observations suggest the presence of a novel, locally controlled mechanism by which MMP-9 levels may be regulated in alveolar macrophages. We speculate that SP-A may influence the protease/antiprotease balance in the lungs of patients with qualitative and/or qualitative changes in surfactant constituents favoring an abnormal breakdown of extracellular matrix components.

gelatinase; macrophage; chronic obstructive pulmonary disease

CIGARETTE SMOKING CAUSES a number of changes to occur in the lung, many of which are still poorly understood. Among these are changes in the number and character of immune cells in the alveolar spaces and in the function and composition of pulmonary surfactant. The latter is becoming an increasingly significant issue given the abundance of recent findings that surfactant regulates immune cell function in the lungs. The most accepted theory for the pathogenesis of emphysema involves an imbalance between proteases and antiproteases (9). Historically, neutrophils and neutrophil proteases have been thought to play a major part in the development of this disease; however, evidence is emerging that macrophages also play an important role in the pathogenesis of smoking-related emphysema (48). Cells of the monocyte/macrophage lineage have the ability to secrete several members of the matrix metalloproteinase (MMP) family, including MMP-1 (collagenase 1), MMP-3 (stromelysin), MMP-7 (matrilysin), MMP-9 (gelatinase B), MMP-12 (macrophage metalloelastase), and MMP-14 (42). It has been demonstrated that patients with emphysema and/or chronic obstructive pulmonary disorder (COPD) have elevated levels of MMP-9 and that alveolar macrophages were the major source of the MMP-9 (6, 28). Despite the fact that MMP-9 is also known as gelatinase B, it has been shown to have substantial elastolytic activity as well (30, 41). Although most attention has been paid to the matrix degrading and remodeling functions of the MMPs, recent evidence suggests that they are also involved in the regulation of the inflammatory response and other biological processes (33, 46).

Surfactant protein A (SP-A) is a member of a family of C-type lectins recently termed “collectins” because of the presence of a collagenous domain. This molecule is the most abundant surfactant protein in the alveolar space and plays roles in the structure, metabolism, and function of surfactant. It is also an important regulator of local host defense mechanisms that make up the innate immune system (49). SP-A levels in the alveolar spaces are altered by a variety of factors and circumstances (8, 11). Among these are increased oxygen, ozone, and nitrogen dioxide (38). Often, these changes in SP-A levels are accompanied by changes in the levels of various surfactant lipids.

SP-A modulates a number of immune cell functions, including cell proliferation, cytokine production, the expression of cell surface markers, and the generation of oxidative activity (35). SP-A may also participate in adaptive immune responses (52). The mechanism of action is still unclear, but it is possible that many of its functions may be mediated via cell surface receptors (43). Recently, some evidence has been published showing that SP-A is a ligand of both Toll-like recep-

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tors 2 and 4 (TLR-2 and TLR-4) (12, 31). Given the likely importance of MMP-9 in immune processes and disease pathogenesis, we examined the role of SP-A in the regulation of MMP-9 production and function. Evidence is presented, for the first time, that SP-A increases the expression of MMP-9 in monocytic cells. We also present evidence that this effect may be mediated by TLR-2.

MATERIALS AND METHODS

Preparation of SP-A. SP-A was purified by butanol extraction from the bronchoalveolar lavage (BAL) fluid of alveolar proteinosis patients (13) as described previously (20). The purified protein was examined by two-dimensional gel electrophoresis and silver staining and was found to be >99% pure. Endotoxin content was determined with the QCL-1000 Limulus amebocyte lysate assay (BioWhittaker; Walkersville, MD). This test indicated an average endotoxin level in our samples of <3 pg of LPS/mg of SP-A.

Cell culture. The THP-1 cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were grown in suspension in complete RPMI 1640 (Sigma, St. Louis, MO) culture medium with 0.05 mM 2-mercaptoethanol and 10% fetal calf serum (FCS; BioWhittaker) at 37°C in a humidified incubator with a 5% CO2 atmosphere. Cells were only used for 15 passages. For all experiments, THP-1 cells were differentiated for 72 h with 10^{-8} M 1,25-dihydroxycholecalciferol (vitamin D3; Biomol Research Laboratories, Plymouth Meeting, PA) at a starting density of 5 × 10^5 cells/ml. After they were differentiated, cells were pelleted at 150 g for 10 min at 4°C, and the cell pellet was washed once with 50 ml of cold PBS and pelleted as before. The pellet was then resuspended in complete RPMI 1640 medium.

Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as described elsewhere (25). Briefly, blood was obtained by venipuncture and collected in Vacutainer blood collection tubes containing 0.1 ml of 15% EDTA (Becton Dickinson, Rutherford, NJ) according to a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine. The blood was layered over Ficoll-Hypaque (Histopaque-1077; Sigma Chemical). The gradients were centrifuged at 400 g for 30 min at room temperature. The mononuclear cells were then collected from the interface, washed twice, and resuspended in the same culture medium used for THP-1 cells.

The density of both cell types (THP-1 or PBMC) was adjusted to 2 × 10^6 cells/ml, SP-A was added at different concentrations, and cells were incubated in 24-well culture plates (BD Biosciences, Bedford, MA) for various intervals. To assess the specificity of the effect seen with SP-A, in some experiments, mouse IgG was used as a control at the same concentrations used for SP-A. After each incubation period, cells were counted and viability was assessed by trypan blue exclusion. Thereafter, cells were pelleted at 250 g for 2 min at 4°C, supernatants were aliquoted, and both were stored at −80°C until used. Under the conditions employed in this study, SP-A did not appear to have any effect on the viability of THP-1 cells.

Zymography. Proteins with gelatinolytic activity from culture medium were identified using sodium dodecyl sulfate (SDS) 10% polyacrylamide gels containing gelatin (0.5 mg/ml) (16). Thirty microliters of sample were diluted with 15 µl of 3× nonreducing sample buffer (30% wt/vol glycerol, 187.5 mM Tris-base, 6.9% SDS, 0.15% bromphenol blue, pH 6.8), incubated at 37°C for 15 min, and each lane was loaded with 30 µl of sample. After electrophoresis, gels were washed two times in 200 ml of 2.5% Triton X-100 at room temperature for 15 min and incubated in development buffer (0.05 M Tris-HCl, 5 mM CaCl2, 0.03% Triton X-100, pH 8.8) for another 15 min at room temperature, followed by an overnight incubation at 37°C in the same buffer to allow digestion of the gelatin substrate. After digestion, gels were rinsed briefly with water, fixed with 200 ml of 40% ethanol and 10% glacial acetic acid for 30 min at room temperature, stained for 2 h with 0.116% Coomassie blue in 25% ethanol and 8% acetic acid, and destained in a solution of 25% ethanol and 8% acetic acid. Gels were photographed, and the photog...
THP-1 cells were incubated with the antibodies for 30 min at room temperature before the addition of SP-A. The effectiveness of the antibodies was assessed by evaluating their blocking activity against known TLR-2 and TLR-4 agonists. Peptidoglycan (PG; Staphylococcus aureus, Sigma Chemical), a TLR-2 agonist, at 1 μg/ml, or lipopolysaccharide (LPS; Escherichia coli 055:B5, Sigma Chemical), a TLR-4 agonist, at 100 pg/ml, were used. These concentrations of LPS or PG increased gelatinolytic activity in THP-1 cell culture supernatants after a 24-h treatment. The LPS content of the PG was 1.2 pg of LPS/mg of PG. After the addition of SP-A, cells were incubated for 24 h in complete RPMI 1640 medium with 0.5% FCS. Supernatants were assayed for MMP-9 by either zymography or ELISA.

Statistical analysis. Data were analyzed with SigmaStat statistical software (SPSS, Chicago, IL). For comparison of two means, unpaired two-tailed t-test was used. Differences were judged to be significantly different at P < 0.05.

RESULTS

Gelatinolytic activity in THP-1 cell culture medium. To identify the effect of SP-A on the release of gelatinolytic activity in the culture medium by THP-1 cells, aliquots of 30 μl were analyzed by gelatin substrate gel zymography. With this technique, MMP proenzymes are activated in situ, presumably by the denaturation-renaturation process and autocatalytic cleavage (23). Accordingly, both the proenzyme and the active form can be detected and distinguished on the basis of molecular mass (~92 and 82 kDa, respectively). SP-A produced a time- and dose-dependent increase of the 92-kDa activity corresponding to pro-MMP-9 (Fig. 1). At 24 h of incubation in several experiments, average increases of 2.1- and 3.3-fold were seen in response to treatment with concentrations of SP-A of 25 and 50 μg/ml, respectively, and slight additional increases occurred at 48 h. The results of a representative experiment are shown in Fig. 1. The identification of this band as MMP-9 was further confirmed by Western blotting (not shown). When the experiments were done in the absence of 10% FCS, the results were the same. To avoid interassay variability caused by the addition of serum, further experiments were done in medium without FCS. With these conditions, there was no detectable 82-kDa activity. Cultures incubated with either albumin or human or mouse IgG were indistinguishable from the cells with no proteins added, confirming the specificity of the effect.

MMP-9 protein release by THP-1 cells. ELISA was used to measure the amount of pro-MMP-9 released by THP-1 cells. The kit used for this purpose also detects pro-MMP-9/tissue inhibitor of metalloproteinases-1 complex but not the active form of MMP-9. Figure 2 shows the results of three experiments, each done in triplicate. A seven- to eightfold increase in the concentration of MMP-9 was observed at 24 h of incubation with 25 or 50 μg/ml (P < 0.05). Results are in good agreement with those found with zymograms. However, although the gelatinolytic activity approaches a plateau after 24 h, the concentration of immunoreactive MMP-9 still markedly increases beyond this time point.

Activity assay of MMP-9 in THP-1 cell cultures. The absence of the 82-kDa band corresponding to mature activated MMP-9 in the zymograms suggested that MMP-9 is primarily released in its pro form. To further investigate this issue, a commercially available MMP-9 activity assay system was used. With this system, active gelatinase B is measured, but total enzyme can be detected as well by activating the samples with APMA. Results are presented in Fig. 3. In the absence
of APMA, no detectable MMP-9 activity was seen (Fig. 3A), supporting the notion that nearly all of the MMP-9 was secreted and remained in its inactive form during the course of our experiments with THP-1 cells. Conversely, when APMA was used, the activity assay followed the same kinetics seen with ELISA (Fig. 3B).

**MMP-9 mRNA expression.** To study the mechanisms of action of SP-A on THP-1 cells, mRNA levels of MMP-9 were assessed by dot-blot analysis of total RNA. Results are shown in Fig. 4. The values represent the means ± SE of three different experiments. At a concentration of 50 μg/ml of SP-A in the culture medium, a significant increase in MMP-9 mRNA level was seen at 6 h, peaking at 24 h of incubation (P < 0.05). At 48 h, although above control values, the difference was not statistically significant. It was concluded that enzyme expression is controlled at the pretranslational level.

**Effect of TLR-2 and TLR-4 blocking antibodies on the SP-A effect on MMP-9 expression.** To determine whether the observed effect of SP-A on the expression of MMP-9 is mediated by Toll-like receptors, THP-1 cells were coincubated with monoclonal antibodies against TLR-2 and TLR-4, using an antibody of the same isotype as control. The specificity of the action of the two antibodies was first determined using known TLR-2 and TLR-4 agonists (PG and LPS). In Fig. 5A, LPS treatment caused an increase in MMP-9 activity by zymography. This enhancement was not affected by the antibody against TLR-2 or the isotype control but was markedly reduced by the anti-TLR-4 antibody. In Fig. 5B, a similar set of experiments is shown with the TLR-2 agonist, PG. In these studies, the PG effect was not altered by the TLR-4 antibody or the isotype control but was reduced to untreated control levels by the anti-TLR-2 antibody. The effects of these antibodies were then examined on the ability of SP-A to increase MMP-9 activity. Figure 6 depicts the pooled data of four independent experiments done in duplicate. In the presence of anti-TLR-2 antibody, the effect of SP-A was inhibited by 83% (P < 0.05) compared with the isotype control, whereas the effect of the antibody against TLR-4 was not found to be statistically significant. These results strongly suggest that SP-A may be increasing the expression of MMP-9, at least in part, through TLR-2.

**Gelatinolytic activity in the culture medium of PBMC.** To assess whether the observed effect of SP-A on MMP-9 was also seen in other mononuclear cells, freshly drawn PBMC were incubated for 24 h either in the presence of 50 μg/ml of SP-A or in its absence. SP-A produced an increase in the release of gelatinolytic activity by these cells. Medium treated with APMA showed a shift of the 92-kDa band to ~82 kDa. The SP-A-induced increase in MMP-9 activity, both before and after APMA treatment, was apparent (Fig. 7A). Densitometric evaluation of three experiments (Fig. 7B) done in duplicate finds a 2.1-fold difference in MMP-9 for SP-A-treated cells compared with control (P < 0.05).
DISCUSSION

Theories on the pathogenesis of emphysema in cigarette smokers were extrapolated from studies of patients with α1-antitrypsin deficiencies (4). However, continuing studies based on several lines of investigation have spawned new theories. Key observations involved in these theories are the findings that the numbers of macrophages, rather than neutrophils, are markedly increased in the lungs of smokers (18), thereby raising questions about the involvement of neutrophil elastase, as has been previously postulated. Another pivotal observation originated from studies of neutrophil-depleted rats where emphysema developed even in the absence of neutrophils (32). As a result of these studies, the focus shifted to macrophage-derived enzymes with elastolytic activity, such as MMP-9. Subsequent studies have confirmed its importance in animal models of emphysema. MMP-9 is a major source of elastolytic activity in the lung and is increased in the BAL fluid of patients with emphysema (6, 7, 40). Together with MMP-12, MMP-9 accounts for most of the macrophage-derived elastase activity in smokers (30). However, numerous questions remain about the regulation of this enzyme and its activity.
Here, we present evidence that SP-A potently stimulates the expression of pro-MMP-9 in a vitamin D₃-differentiated human monocytic cell line and in PBMC as well. SP-A-treated cells transcribed significantly more MMP-9 mRNA than untreated cells. This increase in mRNA levels was followed by a corresponding increase in immunoactive MMP-9 concentration in the culture medium. By zymography, only a major band at ~92 kDa was seen, suggesting that THP-1-conditioned media lacks the active form of the enzyme. The results using the activity assay further supported this idea. Mechanisms for the activation in vivo of MMPs are not clear, but some serine proteases have been implicated in this process (5, 45), and activation of pro-MMP-9 in vitro by MMP-3 has been reported in a tumor cell model (15). Recently, an association between trypsin-2 and the activation of MMP-9 was found in BAL fluid of patients with inflammatory lung disorders (37). In the simple system utilized in the present study (THP-1 cells alone), these activating enzymes are not present. However, in the more complex environment of the alveolar space, it is likely that these and other potential activating enzymes would be produced by other cell types or by the macrophage under the influence of other regulatory molecules in the alveolar spaces. Although we did not examine alveolar macrophages in this study, the presence of SP-A responses in their precursor cells, peripheral blood monocytes, and the THP-1 cells (a commonly used surrogate), is relevant. Cells obtained from the alveolar spaces of individuals with diseased lungs often exhibit properties more similar to those of peripheral blood monocytes than alveolar macrophages from the lungs of normal subjects (26, 39). We speculate that the alterations in the SP-A levels (8, 11) found in many of these diseases may result in the observed changes in MMP-9 (1, 40).

The concentration of SP-A in BAL fluid from healthy individuals has been reported to range from 1 μg/ml (36) to ~5 μg/ml (10). The lavage procedure probably dilutes the alveolar lining material by 10–100 times, thus the range of SP-A concentrations that increased pro-MMP-9 expression in our experiments is likely to be within physiological limits for levels of SP-A in the alveolar lining layer. Furthermore, in the normal lung, as much as 95% of the SP-A is thought to be complexed with surfactant lipid (2, 47) and may, therefore, be inactive with respect to many of its activities related to host defense function. It is believed that SP-A exerts many of its functions via cell surface receptors (43). A specific receptor for SP-A (SP-R210) on alveolar macrophages has been isolated and characterized (3). Recently, some evidence has been published showing that SP-A may act through the Toll-like receptors (12, 31). The transcription regulator nuclear factor (NF)-κB, which has a binding site in the promoter region of the MMP-9 gene (17), is activated by SP-A (24). Furthermore, it has been shown that SP-A requires a functional TLR-4 to induce the activation of the NF-κB signaling pathway in mouse bone marrow-derived macrophages (12). On the other hand, in rat alveolar macrophages and U-937 cells, SP-A attenuates the PG-induced expression of TNF-α by binding to the extracellular domain of TLR-2 (31). In our experiments, treatment of THP-1 cells with anti-TLR-2 antibody strongly inhibited the SP-A-induced expression of MMP-9. This was not seen when the antibody against TLR-4 was used, strongly suggesting that the effect of SP-A on MMP-9 expression is mediated, at least in part, by TLR-2, and further supporting the notion that SP-A is a ligand of this receptor.

The pathophysiological role of SP-A in patients with emphysema or COPD has not been established. However, alterations in surfactant composition in the BAL fluid from healthy smokers have been reported (11, 19, 29). In these studies, SP-A has been reported to be decreased in BAL fluid but increased in serum (22), a finding that may be associated with lung damage. There are also reports that there are alterations in the amounts and structure of surfactant lipids, with selective increases in components and morphological forms that may promote inflammation (21, 34, 53). It is also likely that there are some chemical alterations of surfactant components by some of the constituents of cigarette smoke, particularly reactive oxidants. We and others have previously demonstrated that oxidation of SP-A by ozone or other oxidants can alter its activity (14, 50). These changes are likely to affect the delicate balance between the immunostimulatory and immunosuppressive actions of surfactant (35, 51) and may, in turn, affect protease balance in the lung.

Even though the biological meaning of our findings remains to be elucidated, the observations reported here suggest the presence of a novel, locally controlled mechanism by which the lung can regulate MMP-9 levels. We speculate that SP-A may influence the protease/antiprotease balance in the lungs of patients with qualitative and/or qualitative changes in surfactant constituents favoring an abnormal breakdown of extracellular matrix components.

In summary, SP-A induces the expression of MMP-9 in THP-1 cells and in human PBMC. The effect was time and dose dependent, and most of the MMP-9 was released in its zymogen form. This finding raises the possibility that SP-A could have a role in the pathogenesis of diseases such as emphysema where MMP-9 has been implicated and a protease/antiprotease imbalance occurs.

DISCLOSURES

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REFERENCES

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