Surfactant protein D is expressed and modulates inflammatory responses in human coronary artery smooth muscle cells

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Surfactant protein D (SP-D) is a constituent of the innate immune system that plays a role in the host defense against lung pathogens and in modulating inflammatory responses. While SP-D has been detected in extrapulmonary tissues, little is known about its expression and function in the vasculature. Immunostaining of human coronary artery tissue sections revealed expression of SP-D in the vascular endothelium, media, and adventitia. Immunoblot analysis of human coronary artery smooth muscle cells (HCASMCs) by PCR and immunoblot analysis. Treatment of HCASMCs with endotoxin (LPS) stimulated the release of IL-8, a proinflammatory cytokine. This release was inhibited >70% by recombinant SP-D. Overexpression of SP-D by adenoviral-mediated gene transfer in HCASMCs inhibited both LPS- and TNF-α-induced IL-8 release. Overexpression of SP-D also enhanced uptake of Chlamydia pneumoniae elementary bodies into HCASMCs while attenuating IL-8 production induced by bacterial exposure. Both LPS and TNF-α increased SP-D mRNA levels by five- to eightfold in HCASMCs, suggesting that inflammatory mediators upregulate the expression of SP-D. In conclusion, SP-D is expressed in human coronary arteries and functions as an anti-inflammatory protein in HCASMCs. SP-D may also participate in the host defense against pathogens that invade the vascular wall.

MATERIALS AND METHODS

Preparation and culture of vascular cells. HCASMCs, human coronary artery endothelial cells (HCAECs), human aortic SMCs (HASMCs), and human aortic endothelial cells (HAECs) were isolated using a modification of a previously described method (41). Human aortas and coronary arteries removed at the time of heart transplantation surgery were obtained from the operating room at the University of Iowa Hospitals and Clinics according to a protocol approved by the University of Iowa Human Subjects Office. Fat and adventitia were thoroughly dissected from the vessel. The vessel was then cut longitudinally and incubated in ~40 ml PBS containing collagenase (type IV, 2 mg/ml, Worthington, Lakewood, NJ) for 20 min. The luminal surface was scraped gently, and the tissue was washed repeatedly in HBSS to remove endothelial cells. The arterial smooth muscle was then minced and incubated in 10 ml DMEM containing 2 mg/ml type IV collagenase, 0.4 mg/ml soybean trypsin inhibitor (Worthington), 0.5 mg/ml elastase (Worthington), and 1 mg/ml BSA (Sigma, St. Louis, MO) at 37°C with vigorous periodic mixing (every 15 min) for up to 2 h. Undigested tissue pieces were

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removed, and SMCs were collected by centrifugation. Cells were cultured in medium formulated for SMC [smooth muscle growth medium (SmGM), Cell Applications, San Diego, CA]. Cells from passages 4–9 were used in these experiments. Cells were treated with either 1–5 ng/ml TNF-α (Sigma) or 1–5 ng/ml rough LPS from *Esherichia coli* (List Biological Laboratories, Campbell, CA) for periods ranging up to 24 h, as previously described (35).

**Immunolocalization of SP-D protein.** Paraffin sections of human coronary arteries obtained from four individuals were immunostained for SP-D using a Vectastain Elite kit (Vector Laboratories, Burlingame, CA) and a rabbit polyclonal antibody directed against human SP-D (1:1,000, Chemicon, Temecula, CA). In addition, sections of formalin-fixed, paraffin-embedded cardiovascular tissues (7 μm thick) were obtained from the Department of Pathology at the University of Iowa and immunostained for SP-D. Nonspecific binding was blocked by incubating the sections with 2% normal goat serum for 30 min at room temperature. Sections were rinsed in PBS and then incubated for 1 h at 23°C with rabbit SP-D antiserum, nonimmune rabbit IgG, or PBS alone. Sections were washed twice in PBS followed by an incubation for 30 min with a biotinylated secondary antibody. After being rinsed in PBS, sections were incubated for 45 min in avidin-peroxidase reagent, rinsed again, and incubated in diaminobenzidine (0.7 mg/ml, Sigma) for 1–3 min. Sections were counterstained with hematoxylin, dehydrated, and mounted with coverslips.

**Immunoblot analysis.** Cardiovascular tissues and cells were homogenized in sterile water containing 1 mM PMSE; the homogenate was centrifuged at 600 g for 10 min at 4°C. The supernatant was collected, and the protein concentration was measured using the Bradford method (6). Homogenate proteins (50 μg) were separated by one-dimensional gel electrophoresis on a 10% polyacrylamide-SDS minigel (Bio-Rad, Hercules, CA), and proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad) electrophoretically. Nonspecific binding sites were blocked by incubating the membranes in Blotto (7% nonfat dry milk in 20 mM Tris buffer). Nonspecific binding was blocked by incubating the membranes in Blotto (7% nonfat dry milk in 20 mM Tris buffer containing 0.1% Tween 20) overnight at 4°C. Membranes were then incubated with antibodies for SP-D (1:1,000 in Blotto) for 1 h at room temperature with gentle agitation. After three 15-min rinses in 0.1% Tris-NaCl with 0.1% Tween 20 buffer, membranes were incubated in a secondary antibody conjugated to horseradish peroxidase (Cappel, Irvine, CA, diluted 1:10,000 in Blotto) for 45 min at room temperature. After three 15-min washes in Tris-buffered saline, membranes were incubated in ECL solution (Amersham, Piscataway, NJ) and assayed for IL-8. After the cells had been washed and extracted with lysis buffer, immunoblot analysis was performed to determine the amount of *C. pneumoniae* major outer membrane protein (MOMP; antibody from Fitzgerald Industries, Cambridge, MA) as an index of bacterial content. Intracellular inclusions in HACSMC containing viable *C. pneumoniae* were quantified using a bioassay and the Pathfinder Chlamydia Culture Confirmation Test (Bio-Rad) as previously described (27). Fifteen photomicrographs (×40) of random fields from each treatment group were scored blindly by two investigators to determine the number of cells infected with *C. pneumoniae* per field in two separate experiments.

**Statistical analyses.** All data are expressed as means ± SE. Differences between mean values of multiple groups were analyzed by Student’s *t*-tests. Differences between mean values of multiple groups were analyzed by one-way ANOVA with a Newman-Keul post hoc analysis. Probability values of 0.05 or less were considered to be statistically significant.

**RESULTS**

**Detection of SP-D in coronary arteries and vascular cells.** SP-D was detected by immunostaining in tissue sections of coronary arteries obtained from four donor hearts. SP-D protein was present in the cytoplasm of both endothelial cells and vascular SMCs (Fig. 1, A and C). As a positive control, human fetal lung explants were immunostained for SP-D; as expected, staining was restricted to alveolar type II epithelial cells (Fig.
Negative controls included omission of the primary antibody (Fig. 1, B and D) and staining with nonimmune IgG (Fig. 1F).

RT-PCR analysis of total RNA isolated from primary cultures of HCAECs, HCASMCs, HASMCs, and HAECs revealed the presence of SP-D mRNA in each of these cell types (Fig. 2A). In addition, SP-A mRNA was detected in HCASMCs (data not shown). SP-D protein was detected in HCAECs and HCASMCs, migrating as a single immunoreactive band at ~43 kDa, the same molecular weight as a SP-D standard (Fig. 2B).

**Effect of SP-D on IL-8 release in HCASMCs.** The biological activity of SP-D in the vascular system has not been investigated. However, in the lung, SP-D binds to bacterial pathogens, facilitates their interaction with alveolar macrophages, and suppresses inflammatory responses (28, 32). Therefore, we investigated the ability of exogenous SP-D protein to suppress LPS-induced inflammation in HCASMCs. Treatment of HCASMCs with 1 ng/ml LPS induced a ~10-fold increase in the production of the proinflammatory cytokine IL-8 during a 24-h incubation (Fig. 3). Coincubation of HCASMCs with 0.36 μg/ml rat SP-D abolished the LPS-induced IL-8 release, consistent with a potent anti-inflammatory effect of SP-D on

**Fig. 2.** SP-D mRNA in ECs and vascular SMCs. Total RNA and protein from cultured vascular cells were analyzed for SP-D mRNA and protein using RT-PCR (A) and Western blot analysis (B), respectively. SP-D mRNA (431-bp RT-PCR product) was detected in HCAECs, human aortic ECs (HAECs), HCASMCs, and human aortic SMCs (HASMCs) (A). SP-D protein was detected in HCAECs and HCASMCs (B). Lane 1 contains a SP-D standard, which migrates at ~43 kDa.

**Fig. 3.** SP-D inhibits LPS-induced IL-8 release in cultured HCASMCs. Cells were treated for 24 h in medium containing either vehicle (control) or 1 ng/mL LPS with or without 0.36 μg/ml recombinant rat SP-D. Media were removed, and IL-8 was quantified by ELISA. Responses were normalized to control values (i.e., cells not treated with LPS). Data are representative of 2 experiments with 3 samples/data point. *P < 0.05 from control.
SP-D added alone had no effect on IL-8 release by cells (Fig. 3). Preliminary experiments indicated that the application of SP-A also inhibited LPS-induced IL-8 release by HCASMCs (data not shown).

Overexpression of SP-D. Purified SP-D can contain contaminants such as LPS (26). Therefore, an adenovirus containing the human SP-D gene (AdSP-D) was constructed to overexpress SP-D protein. Infection of HCASMCs with AdSP-D resulted in a MOI-dependent increase in SP-D protein detected in the culture medium within 2 days after adenoviral infection (Fig. 4, A and B). Infection with 50-100 MOI resulted in as much as a fivefold increase in the levels of SP-D protein released into the medium compared with control levels (Fig. 4B). In addition, the amount of SP-D protein present within HCASMCs increased approximately fourfold after infection with AdSP-D (Fig. 4B). To examine the cellular distribution of SP-D protein, immunofluorescence confocal microscopy was performed. As shown in Fig. 4C, SP-D protein was detected throughout the cytoplasm in HCASMCs after adenoviral-mediated overexpression of SP-D.

Effects of overexpression of SP-D on LPS-induced IL-8 release. The biological activity of SP-D protein produced by AdSP-D-infected cells was evaluated by treating transfected HCASMCs with LPS and then measuring IL-8 release into the medium. Transfection with 100 MOI of AdSP-D, the highest titer employed, did not affect basal IL-8 release from HCASMCs during the 24-h MOI-dependent fashion in cells infected with AdSP-D (Fig. 5A). Treatment with LPS (2 ng/ml) produced an increase in IL-8 release that was significantly inhibited in a MOI-dependent manner in cells infected with AdSP-D (Fig. 5A). Incubation of HCASMCs with another inflammatory mediator, 2 ng/ml TNF-α, increased the release of IL-8 by approximately fivefold over control levels (Fig. 5B). In cell cultures infected with increasing MOI of AdSP-D for 2 days before a 24-h challenge with TNF-α, IL-8 secretion was significantly diminished at all levels of AdSP-D infection (Fig. 5B).

Inflammatory stimuli affect SP-D mRNA. Having shown that SP-D is expressed in vascular cells and modulates LPS- and TNF-α-induced cytokine production in HCASMCs, we next investigated whether the level of SP-D mRNA in cells is regulated by proinflammatory stimuli. HCASMCs were exposed to either 2 ng/ml LPS or 2 ng/ml TNF-α for 2-24 h, and levels of SP-D mRNA were quantified by real-time PCR. LPS treatment rapidly increased SP-D mRNA levels in HCASMCs, with a maximum increase (−10-fold) observed by 12 h (Fig. 6). By 24 h after LPS application, SP-D levels had declined substantially. TNF-α likewise increased SP-D mRNA levels by nearly 10-fold. However, the stimulation of SP-D mRNA by TNF-α was more gradual in onset than was observed with LPS and continued to rise throughout the course of the experiment (Fig. 6).

Effects of overexpression of SP-D on responses to C. pneumoniae in HCASMCs. To determine whether SP-D can modulate the inflammatory response produced by an LPS-bearing pathogen, C. pneumoniae, HCASMCs were infected with 0–100 MOI AdSP-D to produce elevated levels of SP-D in cells and culture medium. Two days later, HCASMCs were exposed to C. pneumoniae elementary bodies (2 MOI). In the presence of increasing amounts of SP-D, there was a concentration-dependent increase in the levels of intracellular C. pneumoniae MOMP in HCASMCs as detected by Western blot analysis (Fig. 7A). We also evaluated cell cultures for the presence of viable Chlamydia intracellular inclusions (an index of bacterial infection). Infection in the presence of AdSP-D resulted in an ∼40% reduction in the number of viable Chlamydia intracellular inclusions compared with noninfected control cells or infection with AdLacZ (average number of infected cells in 15 photomicrographs/treatment: control, 15.9 ± 0.9 cells/field; 100 MOI AdLacZ, 15.2 ± 1.2 cells/field; and 100 MOI AdSP-D, 9.2 ± 0.6 cells/field). Moreover, the inflammatory response to C. pneumoniae by HCASMCs (indicated by IL-8 release into the culture medium) was progressively reduced with increasing SP-D levels (Fig. 7B). Together, these results suggest that SP-D in SMC participates both in the host defense and modulation of the inflammatory response to invading pathogens.
DISCUSSION

Innate immune system proteins, such as Toll-like receptors and CD14, have been detected in both endothelial cells and vascular SMCs (35). We now report that human vascular SMCs express SP-D, a member of the collectin family of proteins that modulates innate inflammation in the lung. SP-D inhibited IL-8 released from HCASMCs in response to LPS and TNF-α (9). In addition, LPS and TNF-α upregulated the expression of SP-D mRNA in HCASMCs. SP-D also facilitated the uptake of C. pneumoniae elementary bodies by HCASMCs while modulating the host cell inflammatory response. Collectively, these data suggest that SP-D may play a role in regulating inflammatory processes and innate host defense in vascular SMCs.

SPs are synthesized as glycoprotein monomers that initially form trimers covalently linked by disulfide bonds in their collagen domains; the trimers then aggregate in groups of six (SP-A) or four (SP-D) to form the mature, functional collectin molecules (9). All of the collectins are involved in innate host defense (16). Although first described in lung surfactant (29), SPs (particularly SP-D) are widely distributed in other mammalian tissues (2, 22, 34), including human vascular endothelial cells (20). However, to our knowledge, SP-D expression has not been examined in atherosclerosis-prone blood vessels such as coronary arteries. It is conceivable that some of the SP-D protein detected in arteries was taken up from serum. However, SP-D mRNA and protein were also detected in cultured human vascular SMCs and endothelial cells, indicating that SP-D is endogenously produced by these cells. Likewise, SP-A mRNA was detected in HCASMCs. Moreover, we found that HCASMCs respond to an inflammatory challenge with LPS or TNF-α by increasing the expression of SP-D. These results suggest that SP-D gene expression in HCASMCs may be regulated via proinflammatory signaling pathways, as has been demonstrated in the lung in a variety of injury models, including LPS administration (9).

In addition to demonstrating that HCASMCs express SP-D, we found that the application of exogenous SP-D, or the overexpression of endogenous SP-D, significantly decreased the IL-8 release induced by either LPS or TNF-α. IL-8 is an 8.4-kDa cytokine with potent proinflammatory activity that is believed to play an important role in monocyte chemotaxis and atherosclerotic lesion formation (12). TNF-α is an inflammatory cytokine that stimulates IL-8 production through a different family of receptors than LPS; however, both LPS and TNF-α share some common signaling intermediates and act via a common transcription factor, NF-κB (25, 36). Thus, our results suggest that SP-D can modulate inflammatory responses to mediators that may contribute to the initiation and/or progression of atherosclerosis.

The precise mechanisms whereby SP-D exerts its anti-inflammatory effects in SMCs remain to be determined. Protection against LPS-mediated inflammatory signaling could potentially be mediated by SP-D binding to LPS and/or CD14. However, such a mechanism could not explain the inhibitory effects of SP-D on TNF-α signaling, which does...
Cultures were assayed. The experiment described above was determined by ELISA. Media from 3 replicate cultures were combined for each condition. The protein (MOMP; quantified by densitometry and expressed as relative OD; estimated by immunoblot analysis for other inflammatory mediators such as heat shock proteins and other inflammatory mediators such as heat shock proteins, including glycoprotein 340 (15), signal inhibitory regulatory peptide-α, and/or a calreticulin/CD91 complex (11). The latter two receptors have been postulated to initiate a signal transduction cascade that inhibits inflammation. Thus, SP-D may act as part of a feedback mechanism to repress inflammatory signaling pathways in vascular cells.

Indolent bacterial infections may be a local source of LPS and other inflammatory mediators such as heat shock proteins that stimulate smooth muscle inflammatory mechanisms in atherosclerotic lesions. Although a causative association between atherosclerosis and *C. pneumoniae* has not been established, infection of atherosclerotic lesions by *C. pneumoniae* has been reported (18). When *C. pneumoniae* were incubated with HCASMCs in culture, we detected a robust increase in IL-8 production that corresponded to the level of bacterial exposure. Overexpression of SP-D in HCASMCs reduced this inflammatory response to *Chlamydia* infection in the same way that SP-D reduced LPS- and TNF-α-stimulated IL-8 release. Interestingly, with increasing levels of SP-D in the culture medium, there was an increase in intracellular *C. pneumoniae* MOMP (Fig. 7A), suggesting an increase in the uptake of *Chlamydia* by HCASMCs. While overexpression of SP-D increased *C. pneumoniae* MOMP within HCASMCs, the number of viable intracellular *C. pneumoniae* inclusions (an index of active bacterial infection) was reduced, suggesting that SP-D not only facilitates the uptake of *C. pneumoniae* elementary bodies by HCASMCs but may also direct the elementary bodies to a phagocytic cellular pathway that results in killing of the *Chlamydia*. Thus, our data are suggestive of two mechanisms whereby overexpression of SP-D may modulate responses to *C. pneumoniae* in HCASMCs: 1) repression of the inflammatory response and 2) promotion of the phagocytosis of *C. pneumoniae* by HCASMCs.

The involvement of SP-D in the pathogenesis of atherosclerosis may be complex. For example, deletion of SP-D has been reported to increase the levels of reactive oxygen species in lung macrophages, thereby increasing NF-kB activity and matrix metalloproteinase production (40). This observation suggests that SP-D may play a direct role in regulating macrophage activation, perhaps independently of innate immune signaling. Conversely, binding of SP-D to vascular cells can potentially augment proinflammatory responses, consistent with a dual-function mechanism that has been proposed in macrophages (38). In the latter study, the authors suggested that the physical presentation of the SP-D molecule to the cell may determine which of the cell surface receptors interact with the SP-D and, thus, whether the signal to the NF-kB pathway is either enhanced or repressed.

SP-D knockout mice have been generated and exhibit chronic pulmonary inflammation leading to emphysema and other lung maladies (38). These mice were also used to evaluate the effects of SP-D gene deletion on atherosclerosis in vivo (33). In this study, deletion of SP-D was reported to reduce atherosclerotic lesion formation in mice fed a high-fat diet, in conjunction with an improvement in plasma lipid profile and a reduction of plasma TNF-α levels. To our knowledge, the effects of overexpression of SP-D on atherosclerosis in mice have not been reported. Our data in human vascular cells suggest that overexpression of SP-D could potentially modulate innate immune responses elicited by LPS, a proatherogenic factor (14, 37). However, it is important to point out the species differences between mice and humans with regard to the innate immune system (for a review, see Ref. 24). For example, humans are extremely responsive to endotoxin, whereas mice are far less sensitive, requiring much higher doses of endotoxin to elicit a response (5). These differences have been attributed to structural differences in both Toll-like receptor 4 and an adaptor protein, MD-2, in the two species (1, 13, 17). Thus, it is difficult to predict whether overexpression of SP-D would sufficiently suppress innate immune activation in mice to attenuate atherosclerosis. In addition, it is not known whether endogenous SP-D expression is modulated in the cardiovascular system during the development of atherosclerosis in mice or humans. Further studies are needed to ascertain the role of SP-D in regulating atherosclerosis as well as other aspects of
cardiovascular pathophysiology, such as ischemic myocardial injury and repair.

In summary, the data presented in this study provide the first evidence that SP-D is expressed in human vascular SMCs. Moreover, we propose that SP-D may function to modulate inflammation and host defense in the vasculature, which may impact the development or progression of cardiovascular disease.

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