Bacterial induction of pleural mesothelial monolayer barrier dysfunction

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Parapneumonic effusions develop in between 40 and 60% of patients with pneumonia (24). A complicated parapneumonic effusion or empyema is defined by the presence of grossly purulent pleural fluid or bacteria in the pleural space. Empyema is a serious complication of pulmonary parenchymal infection that rarely resolves without appropriate medical therapy and drainage procedures. In healthy individuals, the pleural fluid volume and turnover are finely controlled. Any imbalance in this homeostasis due to cardiac, renal, or hepatic disease leads to the development of transudative pleural effusion. An exudative pleural effusion may develop due to infection, toxic injury, malignancy, trauma, or other causes of inflammation (7, 23) of the pleura. Staphylococcus aureus accounts for a large number of the cases of pediatric empyema (16) and is a significant cause (19.7%) of bacterial pneumonia in patients with acquired immunodeficiency syndrome (1).

Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein that is secreted as a homodimer of 45 kDa (18). VEGF, also known as vascular permeability factor, was originally identified as a cause for permeability changes in peripheral vasculature and mitogenicity of endothelial cells (19, 21). There are several splice variants of VEGF. The major ones include 121, 165, 189, and 206 amino acids. Among these, the 165-amino acid VEGF variant is the predominant protein, but transcripts of the 121-amino acid VEGF variant may be abundant (32). VEGF is widely expressed in normal and diseased settings, and it is upregulated at the site of angiogenesis and inflammation (6, 14). VEGF serves as an endothelial cell-specific mitogen and a potent angiogenic factor. VEGF induces an increase in permeability of endothelial cell monolayers (10). However, the production of VEGF by activated mesothelial cells and the effect of VEGF on pleural permeability are unknown.

The pleural mesothelium is a monolayer of cells covering the lung and has multiple key functions. It has a pivotal role in inflammation of the pleura by releasing several cytokines (3). Pleural integrity has a central role in barrier maintenance. Histologically, the pleura has tight junctions with ZO-1 (zona occludens)-like connections (35). During the process of inflammation, pericellular permeability increases and mesothelial barrier dysfunction develops (29). Complicated parapneumonic effusions are typically exudative in nature, with a high protein content (30). However, the mechanism that mediates the protein exudation through an intact mesothelium is not known. In the current study, we have developed an in vitro model of the pleura to evaluate its responses during S. aureus infection. We report that S. aureus infection induces mesothelial barrier dysfunction, leading to protein exudation in pleural empyema.
MATERIALS AND METHODS

Reagents, bacteria, and cell cultures. Recombinant VEGF, VEGF antibody, and mouse isotype antibody were purchased from Peprotech (Rocky Hill, NJ), fetal bovine serum was from Atlanta Biologicals (Norcross, GA), and medium 199 was from Gibco BRL (Baltimore, MD). Human VEGF Quantikine ELISA kit was purchased from R&D Systems (Minneapolis, MN). Human pleural mesothelial cells (PMCs, Met-5A), human macrophage cell line U-937, and S. aureus strain Rose were purchased from American Type Culture Collection (Manassas, VA). The PMCs procured from ATCC were isolated from pleural fluids obtained from noncancerous individuals.

Human pleural fluids. Sixteen patients with pleural effusions were studied. Pleural fluids were obtained via thoracentesis from patients with empyema (n = 5), parapneumonic effusion (n = 5), and congestive heart failure (n = 6) after informed consent was obtained (26). The pleural fluids were collected within the first 6 h of the patient’s presentation to the hospital and recognition of a pleural effusion. Patients with a complicated parapneumonic effusion or empyema were defined as those who had pleural fluids that had a positive bacterial culture, loculations on chest radiograph, and a pleural fluid with a pH less than 7.20. An uncomplicated parapneumonic effusion was defined as one that had a negative bacterial culture, free-flowing fluid without loculation on chest radiograph, and a pleural fluid with a pH of more than 7.20. A congestive heart failure effusion was defined as a transudative effusion in a patient with clinical stigmas of congestive heart failure. After thoracentesis, the pleural fluid was centrifuged at 1,000 g and stored at −70°C.

Preparation of S. aureus culture and PMC stimulation. S. aureus (subspecies Rosenbach, strain Rose) was cultured as reported earlier (27). The bacteria were stored on agar slants at 4°C. A loopful of S. aureus was inoculated into nutrient broth and incubated overnight at 37°C. The bacterial concentrations were determined by colony counts, plating known volumes of serial dilutions over nutrient agar. The bacteria were harvested by centrifugation, washed twice in PBS, and resuspended in medium 199. PMCs were incubated in the presence of varying concentrations of live S. aureus (PMCs to bacteria in ratios of 1:0.1, 1:1, 1:10, and 1:100) in serum-free medium and incubated 12 h at 37°C in 5% CO2. PMC viability was checked by trypan blue dye exclusion test at 24 h. The cell viability was >99% in all of these selected concentrations. Based on a concentration-response curve, the optimal concentration of S. aureus (1:1) was selected. To evaluate the response over time, PMCs were incubated in the presence of live S. aureus (1:1) in serum-free medium at 37°C in 5% CO2. The cultures were terminated at different time points (3, 6, 12, and 24 h), and supernatants were collected and stored at −70°C until estimation of VEGF levels by ELISA. The cells were subjected to total RNA extraction for the analysis of VEGF-specific mRNA by reverse transcriptase (RT)-polymerase chain reaction (PCR).

Measurement of VEGF by ELISA. VEGF levels in pleural fluids and S. aureus-induced PMC culture supernatants were measured by “sandwich” enzyme immunoassay (Quantikine ELISA, R&D Systems) as previously described (28). Briefly, the samples were added to 96-well microtiter plates that were coated with murine monoclonal antibody to VEGF. The unbound protein was washed three times, and an enzyme-linked polyclonal antibody specific to VEGF was added. The plate was washed again three times, and substrate solution was added to the wells. After 30 min of incubation, stop solution was added to each well. The amount of VEGF was determined by optical density of the samples by comparing to the standards at 450 nm using the ELISA reader. The detection limit of the kit was 2.0 pg/ml for cell culture supernatant and 3.0 pg/ml for serum or plasma samples.

Isolation of RNA and RT-PCR. Total cellular RNA was isolated from PMCs as reported earlier using TRI reagent (9). Total cellular RNA also was isolated from the macrophage cell line U-937. Because the resting U-937 cells were found to express VEGF splice variants 567 bp (VEGF206) 516 bp (VEGF165), and 444 bp (VEGF165), these cells served as positive controls for RT-PCR. One microgram of total RNA was reverse transcribed into cDNA. The first strand of cDNA was synthesized in a total volume of 20 μl in the presence of 5 mmol/l MgCl2, 50 mmol/l KCl, 10 mmol/l Tris·HCl, pH 8.3, 1 mmol/l dNTPs, 1 μg/μl RNase inhibitor, primer (15 μM) and 2.5 U/μl of M-MuLV RT (PerkinElmer Cetus, Norwalk, CT). The reverse transcription was conducted at 42°C for 15 min, and the reaction was stopped by incubation at 99°C for 5 min.

The cDNA was then amplified using specific primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Primers for human VEGF were synthesized by the phosphoramidite method with a Beckman 200A synthesizer. The primers used were 5’-GCTGCGGATCCAGGTTGGTGGT-3’ (sense) and 5’-TGTTGCGCATGAGGTTCCACCAC-3’ (antisense) for GAPDH and 5’-AGGGCAAGATCATACGGAG-3’ (sense) and 5’-TTTAACTCAAGGCTTGGCCTGCC-3’ (antisense) for VEGF (GenBank accession X62568); PCR was performed with 5 μl of RT product in a reaction mixture containing 2 mmol/l MgCl2, 50 mmol/l KCl, 10 mmol/l Tris·HCl, pH 8.3, specific oligonucleotide primers (15 μM), and 2.5 U Taq DNA polymerase (PerkinElmer Cetus). The samples were amplified in a thermal cycler (GeneAmp PCR System 9600 PerkinElmer Cetus), preheated for 1.30 min at 95°C, followed by 30 cycles. Each cycle was composed of 95°C for 15 s of denaturation, 55°C for 45 s of primer annealing for VEGF, and extension at 72°C for 45 s. The amplification products were analyzed by agarose gel electrophoresis, and their identities were confirmed after sequence determination.

Determination of PMC permeability by measuring 125I-albumin transfer. Human mesothelial cells were cultured to confluence on the abluminal surface of polycarbonate membranes in Transwell plates as reported earlier (29). The PMC confluence was confirmed by microscopic analysis and by electrical resistance. The Transwell plates have an inner and an outer chamber separated by a polycarbonate membrane. This membrane contained 3-μm pores.

Bovine serum albumin (BSA) was labeled with 125I in IODO-GEN precoated iodination tubes obtained from Pierce (Rockford, IL). To evaluate the effect of S. aureus on PMC protein permeability, confluent PMC monolayers in Transwell chambers were preincubated with S. aureus. After an 8-h incubation, 0.05 ml of 125I-labeled albumin (150,000 counts/min) was added into the inner chambers and reincubated for 2 h. The 2-h time point after adding 125I-BSA was selected based on the time-response curve. Some of the chambers received either VEGF antibody or isotype antibody. The lower chamber of the Transwells were filled with 1.5 ml of 199 medium. Protein leak across the membrane was quantitated by measuring radiolabeled 125I in the lower chamber as mean corrected counts per minute.

Measuring PMC electrical resistance. PMC electrical resistance was measured by an electrical cell substrate impedance-sensing system (Applied Biophysics, Troy, NY) as described earlier (31). Briefly, PMCs were cultured on a small gold electrode (10−2 cm2), and culture medium was used as the electrolyte. The total electrical resistance was measured...
dynamically across the PMC monolayer and was determined by the combined resistance between the ventral surface of the cell and the electrode reflective to the resistance between the cells. The small gold electrode and the larger counterelectrode (1 cm²) were connected to a phase-sensitive lock-in amplifier (model 5301A, EG and G Instruments, Princeton, NJ) with a built-in differential preamplifier (model 5316A, EG and G Instruments). A 1-V 4,000-Hz alternating-current signal was supplied through a 1-MΩ resistor to approximate a constant current source. Voltage and phase data were stored and processed with a Pentium 400-MHz computer that controlled the output of the amplifier and relay switches to different electrodes. Experiments were conducted on wells that achieved 5,000 Ω of steady-state resistance. Resistance is expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized value.

Statistical analysis. The significance of differences between experimental and control groups was tested by ANOVA using SigmaStat statistical software. The significance of difference between the two groups was tested by all pairwise multiple comparison procedure (Student-Newman-Keuls method), and \( P < 0.05 \) was considered significant.

RESULTS

Empyema pleural effusions have higher VEGF levels than uncomplicated parapneumonic and congestive heart failure effusions. Parapneumonic pleural fluids contain severalfold higher VEGF levels compared with fluids from patients with congestive heart failure (Fig. 1). Among the parapneumonic effusions, the VEGF levels were significantly higher in empyema pleural fluids (4,048 ± 499 pg/ml) than in uncomplicated parapneumonic (775 ± 284 pg/ml) pleural fluids (\( P < 0.001 \)). The VEGF concentrations were 14.31-fold higher in empyema effusions and 2.74-fold higher in uncomplicated parapneumonic pleural fluids than in fluids from patients with congestive heart failure.

S. aureus induces VEGF production in PMCs in vitro. S. aureus-activated PMCs released VEGF in a concentration-dependent manner (Fig. 2A). A 1:1 (S. aureus-to-PMCs) concentration effectively induced VEGF release in PMCs. Using the optimal (1:1) concentration of S. aureus, we also evaluated the time course of VEGF release until 24 h after S. aureus activation. S. aureus induced PMCs to release VEGF in a time-dependent manner (Fig. 2B). At all time points (3–24 h), S. aureus-stimulated mesothelial cells released significantly higher (\( P < 0.001 \)) amounts of VEGF compared with unstimulated control cultures.

S. aureus induces VEGF mRNA expression in PMCs. VEGF-specific mRNA expression was tested by RT-PCR. The primers designed in this study amplified three VEGF isoforms: a 444-bp (165 amino acids), a 516-bp (189 amino acids), and a 567-bp variant (206 amino acids). S. aureus-activated PMCs expressed VEGF-specific mRNA in a time-dependent manner (Fig. 3). S. aureus induced VEGF mRNA as early as 3 h, with expression similar at 12 and 24 h after S. aureus stimulation. Among the three variants, the 444-bp variant is highly expressed, and the other two variants

Fig. 1. Vascular endothelial growth factor (VEGF) levels in pleural fluids. The results are means ± SE of triplicate determinations from number of patients studied: parapneumonic effusion (Uncomp Parapneu, \( n = 5 \)), empyema (\( n = 5 \)), congestive heart failure (CHF, \( n = 6 \)). *Statistical significance (\( P < 0.001 \)) compared with CHF.

Fig. 2. Staphylococcus aureus activated mesothelial cell release of antigenic VEGF in vitro. Pleural mesothelial cells (PMCs) were incubated in serum-free medium (SFM) or stimulated with S. aureus. A: concentration-dependent response at 12 h. The ratios (1:0.1, 1:1, 1:10, and 1:100) indicate PMCs to bacteria. B: time-dependent response with 1:1 (PMCs-to-bacteria) concentration. The values are means ± SE of 3 separate experiments. *Statistical significance (\( P < 0.001 \)) compared with control.
were comparatively less expressed. However, in U-937 histiocyte lymphoma, all three VEGF variants were constitutively expressed.

*S. aureus causes a decrease in electrical resistance of mesothelial monolayers.* Confluent PMC monolayers exhibited a mean electrical resistance of 200 Ω cm². A significant decrease in electrical resistance was noticed across PMC monolayers that were incubated with *S. aureus* (Fig. 4). *S. aureus* caused barrier dysfunction as early as 2 h, which decreased further until 24 h. Recombinant VEGF decreased the electrical resistance in mesothelial monolayers. Antibodies to VEGF significantly inhibited PMC barrier dysfunction across *S. aureus*-induced PMC monolayers, whereas inclusion of an isotype antibody did not recover *S. aureus*-mediated PMC barrier dysfunction.

*S. aureus enhances protein permeability across PMC monolayers.* Confluent PMC monolayers were preincubated with live *S. aureus* for 8 h. ¹²⁵I-albumin clearance increased when *S. aureus* was incubated with confluent PMC monolayers (Fig. 5). Recombinant VEGF enhanced the protein exudation across the mesothelial monolayer. Inclusion of antibodies to VEGF significantly (59.25 ± 10.31%) decreased the *S. aureus*-induced ¹²⁵I-albumin clearance, whereas inclusion of an isotype antibody had no significant effect on *S. aureus*-induced albumin clearance. Although VEGF antibody did not completely neutralize *S. aureus*-induced permeability, there was no significant difference between control and VEGF antibody-treated cultures.

**DISCUSSION**

Parapneumonic effusions are frequent complications of bacterial pneumonia. Despite extensive use of antibiotics for respiratory infections, pleural empyema is still seen as a complication of pneumonia. Empyema is the result of a pathophysiological process that occurs commonly but is still not well understood. We have attempted to elucidate this process by developing an in
vitro model of the pleura as reported earlier (29). The findings in this study demonstrate that parapneumonic effusions contain higher levels of VEGF than effusions due to congestive heart failure. Among the parapneumonic effusions, empyema fluid contains fivefold higher VEGF levels than uncomplicated parapneumonic effusions. In vitro, \textit{S. aureus} induces VEGF expression in PMCs and causes mesothelial barrier dysfunction, resulting in protein leakage across mesothelial monolayers. Neutralizing antibodies to VEGF inhibit the \textit{S. aureus}-induced permeability across mesothelial monolayers. Although the \textit{S. aureus}-mediated VEGF production constantly increased in PMCs in vitro, experiments were designed for 24 h because no significant decrease in electrical resistance was noticed thereafter.

In an healthy individual, pleural fluid volume is relatively constant because the parietal pleural membranes participate in the reabsorption of fluid (36, 37). Parapneumonic effusions account for about one-third of all pleural effusions, and \~40% of patients with pneumonia develop a concomitant effusion (17). When the inflammatory process is anatomically close to the pleura, a parapneumonic effusion develops within hours. This effusion contains a higher level of protein and a significant increase in phagocytic cells. Even before any pathogens reach the pleural space, neutrophil transmigration across the pleura stimulated by the release of cytokines and other inflammatory mediators is initiated.

In an experimental model of \textit{Pseudomonas aeruginosa} pneumonia, it was demonstrated that pleural empyema fluid contained the alveolar protein tracer instilled at the same time as \textit{P. aeruginosa} into the lungs of anesthetized rabbits. This study also demonstrated that specific virulent products of the organism worsen the epithelial leak (38). In another study, the authors demonstrated that when the lung interstitium filled with interstitial fluid, the fluid crossed the visceral pleural barrier into the pleural space within 2 h after the development of lung injury (23). The authors hypothesized that it took 2 h for fluid to cross into the pleural space because subpleural fluid pressure took time to develop. We believe it is also possible that pleural mesothelial cell activation by bacteria or their products and release of permeability factors such as VEGF on stimulation take at least 2 h to occur, depending on the injurious stimulus.

The pleura was originally thought of as a fairly passive membrane in the process of development of a parapneumonic effusion. The pleural mesothelium is now emerging as the principal coordinator of a concerted response to a microbial invasion. Parapneumonic pleural fluids contain several cytokines (2, 26). We found fivefold higher VEGF levels in empyema effusions than in uncomplicated parapneumonic effusions. We speculate that this increase in VEGF levels in empyema may be due to bacterial and cytokine activation of the pleural mesothelium. Thus increased VEGF may alter the mesothelial permeability, resulting in a protein leak into the pleural space.

\textit{S. aureus} produces several toxins, including staphylococcal enterotoxins (SE) A–E, toxic shock syndrome toxin-1 (TSST-1), and protein A, that contribute to pathogenesis of a variety of diseases (5). Protein A elicits hypersensitivity and inflammation (5). In the current study, staphylococcus was used as a paradigm of bacterial infection. Staphylococcus may induce pleural permeability change directly or indirectly. TSST-1, SEA, and SEB have each been implicated as mediators of toxic shock syndrome (12, 13). When SEB was systemically infused in primates, endothelial cell injury was noticed, with cell fragmentation and plasma membrane interruption (15). TSST-1 had direct cytotoxic effects on porcine aortic endothelial cells (20). SEA directly altered bovine pulmonary artery endothelial cell monolayer permeability in vitro (25). SEB directly caused pulmonary endothelial cell barrier dysfunction,
protein leak, and intercellular gap formation in vitro (8). Our study demonstrated that S. aureus may cause focal breakdown of the mesothelial barrier and thereby result in a protein-rich exudative pleural effusion.

VEGF, initially described as a product of tumor cells (34), is also expressed and released by normal cells such as lung epithelium (4). VEGF is widely expressed in several disease conditions and is upregulated at the site of inflammation (14). VEGF causes barrier abnormality and enhances protein permeability in endothelial cells (10, 33). A drop in electrical resistance with VEGF in mesothelial monolayers and a decrease in mesothelial barrier dysfunction with neutralizing antibodies to VEGF suggest that VEGF can also cause mesothelial barrier dysfunction. Lichtenbeld et al. (22), in similar studies, noticed that neutralizing antibodies to VEGF reduced tumor microvessel permeability. VEGF mediates protein vascular permeability in vivo (11). S. aureus infection causes VEGF expression in PMCs in vitro. Besides, S. aureus infection caused a protein leak across confluent PMC monolayers, and neutralizing antibodies to VEGF decreased the protein leak. Parapneumonic effusions have high VEGF levels, and these may be responsible in part for the mesothelial barrier dysfunction. Parapneumonic effusions have high VEGF levels, and these may be responsible in part for the mesothelial barrier dysfunction and the development of effusions. However, the mechanism by which VEGF induces mesothelial permeability remains unclear. The change in mesothelial permeability in S. aureus infection was not cell death related as evidenced by the trypan blue viability test.

Pleural fluid is most often sterile during the early stage of pathogenesis of a parapneumonic effusion. Substances that create or mimic inflammatory conditions appear to cause increased permeability in the mesothelium. In our mesothelial model, bacterial infection increased protein permeability across the confluent mesothelial monolayer, suggesting that changes in pleural permeability lead to the formation of an exudative pleural effusion.

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