Neutrophil Extracellular Traps and Fibrin in Otitis Media
Analysis of Human and Chinchilla Temporal Bones

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BACKGROUND  Bacterial resistance in acute otitis can result in bacterial persistence and biofilm formation, triggering chronic and recurrent infections.

OBJECTIVE  To investigate the middle ear inflammatory response to bacterial infection in human and chinchilla temporal bones.

DESIGN, SETTING, AND PARTICIPANTS  Six chinchillas underwent intrabullar inoculations with 0.5 mL of 10^6 colony-forming units (CFUs) of Streptococcus pneumoniae, serotype 2. Two days later, we counted bacteria in middle ear effusions postmortem. One ear from each chinchilla was processed in paraffin and sectioned at 5 μm. The opposite ear was embedded in epoxy resin, sectioned at a thickness of 1 μm, and stained with toluidine blue. In addition, we examined human temporal bones from 2 deceased donors with clinical histories of otitis media (1 with acute onset, 1 with recurrent infection). Temporal bones had been previously removed at autopsy, processed, embedded in celloidin, and cut at a thickness of 20 μm. Sections of temporal bones from both chinchillas and humans were stained with hematoxylin-eosin and immunolabeled with antifibrin and antihistone H4 antibodies.

MAIN OUTCOME MEASURES  Histopathological and immunohistochemical changes owing to otitis media.

RESULTS  Bacterial counts in chinchilla middle ear effusions 2 days after inoculation were approximately 2 logs above initial inoculum counts. Both human and chinchilla middle ear effusions contained bacteria embedded in a fibrous matrix. Some fibers in the matrix showed positive staining with antifibrin antibody, others with antihistone H4 antibody.

CONCLUSIONS AND RELEVANCE  In acute and recurrent otitis media, fibrin and neutrophil extracellular traps (NETs) are part of the host inflammatory response to bacterial infection. In the early stages of otitis media the host defense system uses fibrin to entrap bacteria, and NETs function to eliminate bacteria. In chronic otitis media, fibrin and NETs appear to persist.

The pathogenesis of otitis media involves bacterial factors and the host defense system. Activation of the host contact defense system plays a crucial role as the first line in host resistance against pathogens. It is part of the host inflammatory response that induces the entire coagulation cascade, intrinsic and extrinsic, both of which lead to the formation of fibrin. Furthermore, according to accumulating evidence, coagulation and innate immunity have coevolved—they are now believed to function as a highly integrated defense mechanism against infectious diseases.

Esmon et al found that infection can trigger responses that lead to coagulation and that coagulation (the fibrin network) can limit the invasiveness of the pathogen. The middle and inner ears present a challenge to the host defense system. The need to contain bacteria in such a large space filled with air and/or fluid requires some type of scaffolding to entrap bacteria to limit their dissemination and to facilitate clearance by host inflammatory cells. Neutrophils are the first line of the host inflammatory cell response to infection, eliminating bacteria by phagocytosis. A form of neutrophil death, NETosis, results in the release of extracellular nucleic acids decorated with histones and granular proteins that form neutrophil extracellular traps (NETs). These NETs stimulate both the extrinsic and intrinsic coagulation pathways as well as the formation and deposition of fibrin. Neutrophil extracellular traps are commonly formed.
Neutrophil Extracellular Traps and Fibrin in Otitis Media

Findings
In this laboratory-based case series study, fibrin and neutrophil extracellular traps (NETs) were part of the middle ear’s response to bacterial infection in chinchillas with experimentally induced acute otitis media and in middle ears from human temporal bones with acute onset and recurrent otitis media.

Meaning
The middle ear uses fibrin and NETs as part of the inflammatory response to contain bacteria and limit their dissemination, but it can also protect these organisms from clearance by the host defense.

Key Points

Question What is the inflammatory response of the middle ear to bacterial infection?

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Meaning The middle ear uses fibrin and NETs as part of the inflammatory response to contain bacteria and limit their dissemination, but it can also protect these organisms from clearance by the host defense.

In this study we examined the relationship between NETs, fibrin, and bacteria in experimentally induced otitis media in chinchillas and in human temporal bones with acute onset and recurrent otitis media.

Methods

Bacterial Culture
To induce otitis media in the 6 chinchillas we used in our study, we grew Streptococcus pneumoniae D39, serotype 215 in Todd Hewitt broth containing 0.5% yeast extract (BD Diagnostics), plated on sheep blood agar plates, and stored in 10% glycerin solution at −80°C. Optical densities were measured at 660 nm. Bacteria were diluted (10-fold) in phosphate-buffered saline (PBS), then plated, and viable colonies counted to confirm the concentration.

Chinchilla Temporal Bones
Care and use of chinchillas were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. For our current study we selected the method of inoculation and the amount of inoculum that resulted in otitis media in most of the chinchilla ears based on our previous studies.16 All 6 chinchillas were anesthetized with 0.25 mL of a combination of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (10 mg/kg). Then they underwent intrabullar inoculation with 0.5 mL of 10^6 colony forming units (CFU) per mL of S pneumoniae.

Two days later, we administered an overdose of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (10 mg/kg), followed by cervical dislocation and decapitation, and took middle ear effusions for CFU counts. We removed the bullae and fixed 1 ear from each chinchilla in 4% paraformaldehyde in 0.1 M phosphate buffer, decalcified it in 10% EDTA, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections were cut at a thickness of 5 μm and mounted on glass slides, some sections were stained with hematoxylin-eosin. Other sections were immunostained with antifibrin antibody for assessing the presence of fibrin or with antihistone H4 antibody for assessing the presence of NETs.

We fixed the opposite ears from the 6 chinchillas in 2% glutaraldehyde in 0.1 M phosphate buffer, decalcified in 10% EDTA, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Sections were cut at a thickness of 1 μm and stained with toluidine blue for light microscopic examination.

Human Temporal Bones
We selected 2 temporal bone specimens from deceased donors from our archived human temporal bone collection at the University of Minnesota for histopathologic and immunohistochemical analyses and for comparisons with our chinchilla experimental model. The first specimen was from an 18-month-old girl who before her death had appeared ill and lethargic.

She was believed to have influenza; 2 days later, she was unresponsive and transported to the hospital. The following day, an ear, neck, and throat physician, after an otoscopic examination, described bulging of the left tympanic membrane; in the right ear the tympanic membrane was red and retracted. From her cerebral spinal fluid, S pneumoniae was cultured. She was treated with extensive antibiotic therapy, but died the following day. The autopsy report listed the cause of death as pneumococcal meningitis.

The second specimen was from a 64-year-old woman who had had biliary cirrhosis since age 24. April and December of the year of her death, she experienced gastrointestinal bleeding. After surgery for her last bleed, she developed profound hepatic encephalopathy and did not regain consciousness. She was on and off a respirator until the time of her death. Her clinical history during the period of her last hospitalization included recurrent infections in her lungs, ears, and urine; culture results were positive for both gram-negative and gram-positive bacteria. She was given antibiotic treatment during that period. The autopsy report listed cause of death as primary biliary cirrhosis with liver failure and organized pneumonia.

The temporal bones from both these donors had been removed at the time of autopsy, fixed in 10% formalin, decalcified in EDTA, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections were cut at a thickness of 1 μm and stained with hematoxylin-eosin for histological examination. Other sections were immunolabeled with antifibrin (for localization of fibrin fibers) and antihistone H4 antibody (for localization of NET fibers).

Immunohistochemical Analyses
For immunohistochemical analysis, we first removed paraffin (from chinchilla sections) and celloidin (from human temporal bone sections). Sections were then rehydrated in a graded series of ethanol and double deionized water; permeabilized with proteinase K (20 μg/mL; Sigma-Aldrich Corporation) for 30 minutes at 37°C; washed in PBS; and immersed in PBS containing 3% hydrogen peroxide for 30 minutes at room temperature to block endogenous peroxidase activity; washed again in PBS and incubated overnight at 4°C in a humidified chamber with the appropriate primary antibody. We used antifibrin β-chain immunoglobulin (IgG1, mouse antihuman antibody (Sekisui Diagnostics, LLC) at a dilution of 1:100 and...
antihistone H4, mouse antihuman monoclonal antibody (Cell Signaling Technology) at a dilution of 1:50.

Next, sections were again washed in PBS and incubated with secondary antibody, goat antimouse IgG1-horseradish peroxidase (HRP) (Abcam) for 1 hour at 37°C. To visualize immunolabeling we used a 3,3-diaminobenzidine peroxidase substrate kit (Vector Laboratories Inc). Coverslips were mounted with VectaMount (Vector Laboratories Inc).

Results

Chinchilla Temporal Bones
Bacterial counts in chinchilla middle ear effusions 2 days after inoculation were approximately 2 logs above the initial inoculum levels. Hematoxylin-eosin staining showed middle ear effusions (Figure 1A). Effusions contained bacteria, inflammatory cells, and an extensive fibrous network that was composed of both thick and thin fibers (Figure 1B). Immunohistochemical analysis of the fibrous network demonstrated fibers that immunostained positively with antifibrin antibody (Figure 1C). Bacteria were frequently seen surrounded by fibrin fibers. Other fibers associated with bacteria extended from neutrophils. Immunostain findings were positive for antibody against histone H4 (Figure 1D), indicating the presence of NETs. Epoxy-embedded sections stained with toluidine blue revealed polymorphonuclear neutrophils with long projections entrapping bacteria (Figure 2).

Human Temporal Bones
In the specimen from the donor with acute onset otitis media, the middle ear contained purulent effusion (Figure 3A). Within the effusion there were bacteria in a fibrous network (Figure 3B). Fine fibers showed positive immunostaining with antibody to fibrin (Figure 3C), others were immunolabeled with antihistone H4 antibodies (Figure 3D).

In the specimen from the donor with recurrent otitis media, the middle ear was filled with effusion (Figure 4A)
containing bacteria embedded in a fibrous matrix (Figure 4B). Some fibers immunostained positively with antifibrin (Figure 4C) and others with antihistone H4 (Figure 4D) antibodies.

Discussion

Neutrophil extracellular traps are part of the innate response that binds microorganisms, prevents them from spreading, and ensures a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria. They have been described using scanning electron microscopy in middle ear effusions of chinchillas with S pneumoniae otitis media, and a recent study using proteomic analysis in children with chronic otitis media showed that NETs are macromolecular constituents of middle ear effusions. Routine histopathologic techniques cannot specifically identify these fibers as NETs; however, because neutrophil DNA containing fibers in the extracellular space are specific to NETs, they can be identified with antibodies to histones. To our knowledge, our study is the first immunohistochemical identification of NETs, using specific antibody to NET fibers in middle ears of chinchillas with pneumococcal otitis media and in human temporal bones from patients with acute onset otitis media and chronic recurrent otitis media.

In addition, our immunohistochemical analysis further revealed an extensive network of fibrin fibers in the middle ear effusion using an antibody specific for fibers composed of fibrin monomers. Fibrin matrices have likewise been shown to physically entrap or encapsulate bacteria, thereby limiting their growth and dissemination and supporting the recruitment and activation of host immune cells, which in turn mediate the elimination of invading microbes. Similar to the middle ear, the peritoneal cavity is another large space in which fibrin has been reported to promote clearance of bacteria either by
helping to activate the microbicidal properties of phagocytes or by physically trapping bacteria, directly limiting their dissemination. 18

Although the host defense system uses fibrin to entrap bacteria for containment and elimination, it may also protect these organisms from clearance by inflammatory cells and antibiotics, providing a mechanism for bacterial aggregation and growth of biofilms, and the potential for reinfec-

tion and chronicity of disease. Hau et al19 showed that entrapment of bacteria by fibrin in the peritoneum abolished systemic sepsis, but also protected bacteria against the action of antibiotics.

Bacterial resistance to antibiotics is one of the characteristics of biofilms. Biofilms have been demonstrated in recurrent acute otitis media,20 chronic otitis media with effusion,21 chronic suppurative otitis media,21,22 and cholesteatoma.23 The extracellular matrix in biofilms has been described as being derived from bacteria24; however, host-derived components are also associated with biofilm formation.24 Hong et al25 showed that a substantial portion of the nucleic acid in biofilms in vivo is derived from host cells. Neutrophil extracellular traps have been demonstrated to be associated with biofilm formations in middle ear effusions of chinchillas with nontypeable Haemophilus influenzae,25 and Thornton et al20 showed that host DNA in middle ear effusions of children was predominately from neutrophils, derived from NET formations. The conversion of fibrinogen into fibrin has also been reported to be an essential factor for biofilm formation.26

This study shows that both NETs and fibrin are part of the early host response to middle ear infection that can persist in chronic forms of the disease. These 2 entities can be potential therapeutic targets to reduce the progression of otitis media.

Figure 4. Temporal Bone Section From a 64-Year-Old Woman Who Had Recurrent Ear Infections

A. The yellow arrowhead indicates the malleus. Stained with hematoxylin-eosin (original magnification ×20). The black arrowhead indicates the area of higher magnification seen in B.

B. A fibrous matrix containing bacteria (arrowheads) and inflammatory cells (original magnification ×20). C. Some fibers stained with antibodies to fibrin (original magnification ×600).

D. Other fibers immunostained with antihistone H4 antibody (original magnification ×600).
Conclusions

We observed bacteria entrapped in a network of fibrin and NETs in animal temporal bones with experimentally induced acute otitis media and in human temporal bones from deceased donors with acute onset and recurrent otitis media. Our findings support an important role of fibrin fibers and NETs in the host inflammatory response to bacterial infection of the middle ear.