Nosocomial Pneumonia Elicits an Endothelial Proteinopathy: Evidence for a Source of Neurotoxic Amyloids in Critically Ill Patients

To the Editor:

Acute *Pseudomonas aeruginosa* infection elicits production of cytotoxic τ and $A\beta$ oligomers from pulmonary endothelium, and these amyloids accumulate in the BAL fluid of critically ill patients in the ICU (1–3). However, it remains unclear as to whether lung endothelial-derived amyloids access systemic tissues, and whether they contribute to end organ dysfunction. Accumulation of oligomeric τ and $A\beta$ within the brain contributes to various forms of neuronal dysfunction, leading to learning and memory deficits (4). Because *P. aeruginosa* is a common cause of nosocomial pneumonia, and as patients with nosocomial pneumonia are at high risk of developing memory loss within the Alzheimer's disease spectrum (5), we examined whether common gram-negative and gram-positive lung pathogens induce endothelial τ and $A\beta$ oligomers that are sufficient to impair neurological function. Part of this work was previously published in abstract form (6).

Lung endothelial cells were infected with *Klebsiella pneumoniae* and *Staphylococcus aureus* that had been isolated from the BAL fluid of intubated patients in the ICU. Supernatant was collected after bacterial infection (Figure 1A), prepared as described (1, 2), and incubated with brain endothelium. Lung endothelial supernatant generated in response to infection with both organisms induced intercellular gaps in brain endothelium (Figures 1B and 1C), and it contained transmissible and self-replicating τ and $A\beta$ oligomers, as previously described (data not shown) (1, 2). Neutralizing τ and $A\beta$ oligomers using T22 and A11 antibodies, respectively, abolished gap formation (Figures 1C and 1D). Amyloids were then eluted from the A11 antibody-bead complex, using a high-salt solution. Salt was removed by dialysis against Hanks' balanced salt solution, and the antibody bound to amyloid denatured by boiling. The resulting fraction was applied to endothelial cells. These heat-stable amyloids

disrupted the barrier (Figures 1C and 1D). Thus, three common pathogens responsible for nosocomial pneumonias induce lung endothelial production of cytotoxic τ and $A\beta$ oligomers that disrupt the brain endothelial barrier.

We determined whether the infection-induced lung endothelial amyloids are sufficient to impair long-term potentiation at the Schaffer collateral synapses, which is the best characterized *ex vivo* model for animal learning and memory (7). Endothelium was infected with *P. aeruginosa* that possessed either an intact (PA103) or deficient (Δ PcrV) type 3 secretion system, and supernatant was prepared. Acutely prepared rodent hippocampal brain slices were then incubated in artificial cerebrospinal fluid supplemented with vehicle control (Hanks' balanced salt solution) or with the endothelial cell supernatant at a 2:1 ratio, and changes in synaptic plasticity were assessed. High-frequency stimulation induced long-lasting synaptic strengthening, revealed as an increased field excitatory postsynaptic potential slope that lasted for more than 1 hour (Figures 1D, 1G, and 1H). Both the early (10 min after high-frequency stimulation) and late (60 min after high-frequency stimulation) phases of long-term potentiation were significantly impaired in brain slices bathed with the supernatant obtained from PA103-infected lung endothelial cells, whereas supernatant collected from Δ PcrV-infected lung endothelial cells was without effect. Amyloid neutralization rescued long-term potentiation (Figures 1E, 1G, and 1H), and amyloids eluted from the beads suppressed long-term potentiation (Figures 1F–1H). Early-phase long-term potentiation is attributable to AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor phosphorylation and insertion that acutely increases receptor conductance, whereas late-phase, long-term potentiation is caused by expansion of AMPA and NMDA (*N*-methyl-D-aspartate) receptor density and spine remodeling (8, 9). Thus, bacteria-induced lung endothelial amyloids impair long-term synaptic plasticity that is likely secondary to modifications in AMPA and/or NMDA receptor signaling.

We examined whether patients with nosocomial pneumonia harboring monomicrobial infections possess τ and $A\beta$ that can be captured using the A11 antibody within the BAL and cerebrospinal fluids. ICU patients with a primary *K. pneumoniae* or *S. aureus* infection were enrolled with uninfected patients. Co-immunoprecipitation experiments illustrate that A11 captures τ and $A\beta$ species that are recognized by the MOAB and TNT1 antibodies, respectively (Figure 2A), in the BAL fluid of the patients with pneumonia, indicating the presence of infection-induced amyloids. Uninfected control patients displayed no $A\beta$ immunoreactivity, whereas two of four patients showed minor τ immunoreactivity.

We then examined whether the cerebrospinal fluid from either infected or uninfected patients impairs synaptic plasticity dependent on τ and $A\beta$. Whereas long-term potentiation was prominent in slices incubated with cerebrospinal fluid from uninfected patients, it was abolished altogether by the cerebrospinal fluid from infected patients (Figures 2B–2E). Neutralizing amyloid proteins within the cerebrospinal fluid of infected patients using the A11 antibody partially rescued early long-term potentiation and fully rescued late long-term potentiation, whereas IgG neutralization was without effect. Amyloids eluted from the A11 antibody-bead complex and boiled abolished long-term potentiation, indicating amyloids within the cerebrospinal fluid of patients harboring bacterial pneumonia infection were acutely neurotoxic and sufficient to impair synaptic plasticity.

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Figure 1. *Klebsiella pneumoniae* and *Staphylococcus aureus* elicit lung endothelial amyloids that are cytotoxic to brain endothelial cells and inhibit long-term potentiation (LTP). (A) Bacterial strains isolated from the BAL fluid of ICU patients cause time-dependent lung endothelial cell injury. (B) Supernatant obtained from lung endothelial cells after *K. pneumoniae* and *S. aureus* infection are cytotoxic to brain endothelial cells. Images were taken 30 hours after supernatant application to the cells, and black/white cell masks generated to illustrate gap area for quantification. Supernatant neutralization using antibodies that capture τ and A β (i.e., A11) and τ (i.e., T22) prevented supernatant cytotoxicity, whereas cell treatment with eluate from the A11 antibody was sufficient to cause cytotoxicity. Supernatant treatments were performed in parallel experiments, with the same control conditions. Therefore, the control image (i.e., vehicle) is the same in both panels. (C) Averaged time course responses from *K. pneumoniae* (left) and *S. aureus* (right) supernatant treatments are shown with vehicle control, A11 and T22 neutralizations, and A11 eluate treatments. Because experimental treatments were conducted in parallel, the control data is the same in both panels (i.e., vehicle). Data are shown as means $\pm$ SEM, $n = 6-9$ experiments per group. $*P < 0.05$ vs. vehicle; data were analyzed using two-way ANOVA with Tukey's *post hoc* tests. (D) Time course of normalized field excitatory postsynaptic potential (fEPSP) slope (mean $\pm$ SEM) from control hippocampal slices bathed in Hanks' balanced salt solution (HBSS, black), or from slices bathed in PA103 (blue) or Δ PcrV (gray) bacterium-free supernatant for 1.5 hours. fEPSP responses were normalized to the mean of those before the high-frequency stimulation (HFS) delivered at time 0, which induced LTP. Representative average of 10 fEPSP traces taken from the indicated points before (a, black), 7–10 min after (b, blue), and 57–60 minutes after HFS (c, red) are shown as mean $\pm$ SEM (shaded area). (E) Normalized fEPSP slope from control slices (dashed line) and from slices bathed in PA103 supernatant that had been immunoneutralized with A11 and IgG control antibodies. (F) Endothelial-derived amyloid proteins bound to the respective antibodies were eluted, boiled, and applied to brain slices, and fEPSP was recorded (open symbols). Representative fEPSP traces are shown on the right (mean $\pm$ SEM). (G and H) Summary bar graphs showing the normalized amount of LTP with various treatments. Percentage LTP was calculated from fEPSP slopes at 7–12 minutes (G, early-phase LTP [E-LTP]) or 55–60 min (H, late-phase LTP [L-LTP]) after HFS versus 5 to 0 min before HFS. The number of recordings included in the summary data is shown in the bars. $*P < 0.05$ versus HBSS (ANOVA with Tukey's *post hoc* tests). Scale bars, 20 mm.

Figure 2. Nosocomial pneumonia elicits τ and $A\beta$ oligomers that reduce long-term potentiation (LTP). (A) BAL fluid (BALF) was collected from patients with pneumonia and uninfected control patients. Patients 1, 3, and 4 had a *Klebsiella pneumoniae* infection, and patient 2 had a *Staphylococcus aureus* infection. BALF was immunoprecipitated (IP) using the A11 antibody and then immunoblotted (IB) for $A\beta$, using the MOAB-2 antibody, and for τ using the TNT1 antibody. All samples obtained from infected patients were immunoreactive for both $A\beta$ and τ . Two of the four uninfected patient samples showed minor immunoreactive τ bands. (B) Time course of normalized field excitatory postsynaptic potential (fEPSP) slope from hippocampal slices bathed in cerebrospinal fluid (CSF) from control (patients without nosocomial pneumonia bacterial infection) and *K. pneumoniae*-infected patients without immunoneutralization and after immunoneutralization (solid symbols) with A11 and control IgG antibodies. The lower panel shows brain slices treated with amyloid proteins eluted and boiled from A11 (A11-Eluate) and IgG (IgG-Eluate) antibodies. (C) Time course of normalized fEPSP slope from slices bathed in control human CSF (patient without nosocomial pneumonia bacterial infection, black), CSF from a patient with *S. aureus* (blue), and *S. aureus* CSF that had been immunoneutralized with the A11 antibody. Representative traces obtained from the indicated points are shown in corresponding colors (mean $\pm$ SEM). (D and E) Summary of CSF treatments and the amount of LTP. Percentage LTP was calculated from average fEPSP slope at 7–12 minutes (D, early LTP [E-LTP]) or 55–60 minutes after high-frequency stimulation (HFS) (E, late LTP [L-LTP]), normalized to that at 5 to 0 minutes before HFS. The number of recordings included in the summary data is shown in the bars. $*P < 0.05$ versus CSF control (ANOVA with Tukey's *post hoc* tests). MW = molecular weight.

It is noteworthy that this amyloid suppression of long-term potentiation appeared to be cytokine independent (data not shown).

Endothelial amyloids are heat stable, protease resistant, and RNase and DNase insensitive (1). They exhibit certain features of prion disease (10); for example, they are self-replicating and transmissible among cells (1). Here, we provide evidence that nosocomial pneumonia induces lung endothelial production of τ and A β oligomeric species that impair neurological information processing, supporting the hypothesis that these amyloids contribute to insidious end-organ dysfunction and suggesting the need for a larger cohort trial addressing this issue. In our studies, injurious amyloids were detected in the cerebrospinal fluid of infected, but not in uninfected, patients. However, our current sample size is small, and we have not determined whether other bacteria, viruses, fungi, or inflammatory conditions, such as the systemic inflammatory response syndrome, also elicit this endotheliopathy. Our studies focus on the acute consequences of infection-induced amyloids; it remains unknown as to whether the mechanisms tested here represent a cause of progressive and persistent memory loss in ICU patients. Indeed, in studies moving forward, it will be essential to determine the infection-induced τ and A β oligomer fate within the lung, blood, and other peripheral organs, including the brain. It will also be essential to determine whether infection-induced amyloid production and biodistribution contributes to cardiovascular disease, stroke, renal dysfunction, and pulmonary dysfunction in the aftermath of critical illness. ■

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Time of Day Affects Eosinophil Biomarkers in Asthma: Implications for Diagnosis and Treatment

To the Editor:

Asthma is characterized by strong time-of-day rhythms: symptoms worsen around 04:00 (1) along with increased airway narrowing, as

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