Role of Apoptosis in Pseudomonas aeruginosa Pneumonia

Apoptosis plays a central role in the complex balance between invading pathogen and host defense. Depending on the pathogen, apoptosis of host cells may be beneficial or detrimental to survival. Studies using a clinically relevant intra-abdominal model of sepsis (peritonitis) have indicated that two types of cells, lymphocytes and gastrointestinal epithelial cells, undergo accelerated apoptosis (1, 2). Apoptosis appears to be confined predominantly to these two cell types, possibly because these cells normally die by apoptotic mechanisms. Furthermore, blocking lymphocyte apoptosis in peritonitis has been shown to improve survival (3–5). Consequently, some investigators have speculated that prevention of apoptosis may be efficacious in sepsis by preventing immune suppression (6, 7).

In striking contrast to the concept that apoptosis is detrimental in sepsis, Grassmé et al. (8) reported that Pseudomonas aeruginosa pneumonia resulted in bronchial cell apoptosis that was essential for survival. The survival benefit of apoptosis was demonstrated by showing that bronchial cell apoptosis did not occur in mice deficient in the cell death receptor Fas (CD95) and that mortality was greatly increased in Fas-deficient mice as well. Grassmé et al. (8) speculated that shedding of infected apoptotic bronchial cells in control mice (mice with normal Fas receptors) prevented bacterial dissemination and improved survival.

Although Grassmé et al. employed several methods to detect cell apoptosis in vitro, only the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) method was employed for in vivo determination of bronchial cell apoptosis. Because TUNEL may not reliably discriminate between apoptosis and necrosis and may yield false positives (9–11), however, their conclusions regarding in vivo effects of pneumonia must be interpreted cautiously.

Using the identical model of Grassmé et al. (8), we employed three methods in addition to TUNEL to determine if P. aeruginosa induced bronchial apoptosis (12). No bronchial apoptosis was detected by conventional light microscopy, electron microscopy (Fig. 1), or immunohistochemical staining for active caspase 3 (Fig. 2). Lymphocytes, occasional polymorphonuclear neutrophils, and, to a much lesser degree, capillary endothelial and alveolar cells demonstrated characteristic apoptotic changes of condensed and fragmented nuclei (Fig. 2). The incidence of apoptotic capillary endothelial and alveolar epithelial cell apoptosis was rare (less than one cell per five high powered fields; magnification, ×400). Although TUNEL was positive for bronchial cell apoptosis in mice with pneumonia, it was also positive in saline-treated (control) mice (Fig. 3). That TUNEL positive bronchial cells in both control and P. aeruginosa–treated mice were negative for active caspase 3 and had no apoptotic nuclear morphologic changes by conventional or electron microscopy strongly supports the contention that the TUNEL positive bronchial cells were falsely positive.

The present study agrees with the work of Rajan et al. (13), who reported that airway epithelium is highly resistant to apoptosis in P. aeruginosa pneumonia and is also consistent with clinical studies in which no bronchial apoptosis was detected in lungs of patients who died of pneumonia (14, 15). Interestingly, the TUNEL method did accurately demonstrate apoptosis in lymphocytes in mice with pneumonia (apoptosis confirmed by four methods), but not in controls. Recent studies have indicated that the accuracy of the TUNEL method may be related to cell-specific endogenous endonucleases and that TUNEL is falsely positive in cells with high endonuclease activity (11).

The most remarkable finding in the present study was the extensive lymphocyte apoptosis that occurred in lung, spleen, and thymus during pneumonia (Fig. 4). The percentage apoptosis increased from 3.3 ± 0.4% to 6.0 ± 0.8% (p < 0.05) in splenocytes and from 4.6 ± 1.0 to 20.0 ± 4.2 (p < 0.05) in thymocytes. Fas receptor–deficient mice (MRL/MPJ = Fas<sup>−/−</sup>) had no protection from lymphocyte apoptosis; percentage apoptosis was 3.7 ± 0.5% in control splenocytes versus 6.9 ± 0.4% in septic splenocytes and 2.8 ± 1.0% in control thymocytes versus 24.0 ± 4.3% in septic thymocytes (p < 0.05). Although there was a trend toward improved survival in Fas receptor–deficient mice compared with control mice, the difference was not statistically significant (15). Lymphocyte apoptosis was totally inhibited in transgenic mice that overexpressed Bel-2 in T cells;
Fig. 3. Lung tissue obtained 24 hours after intratracheal injection and evaluated by TUNEL method. TUNEL-positive bronchial epithelial cells have brown staining nuclei and are present in both (A) saline-treated (sham) lung and (B) bacterial-treated (pneumonia) lung. Magnification, ×400. It should be noted that the nuclei of the bronchial epithelial cells have normal morphology, without evidence of contraction or fragmentation.

percent apoptosis was 2.5 ± 0.7% in control splenocytes versus 1.9 ± 0.4% in septic splenocytes and 2.62 ± 0.2% in control thymocytes versus 3.3 ± 0.4 in septic thymocytes in such mice. The results of the Fas and Bcl-2 transgenic mice are consistent with previous studies in sepsis that indicate that lymphocyte apoptosis occurs by a mitochondrial rather than a cell receptor death pathway (4, 16).

The extensive apoptotic loss of lymphocytes in P. aeruginosa pneumonia is comparable to that occurring in sepsis due to peritonitis (3, 4). Importantly, prevention of lymphocyte apoptosis by overexpression of Bcl-2 or administration of caspase inhibitors has been shown to improve survival in animal models of sepsis (4, 15, 17) and caused a non–statistically significant trend toward improved survival in pneumonia as well (15).

Thus, extensive lymphocyte apoptosis may be a fundamental abnormality in bacterial sepsis irrespective of site of infection and may contribute to the accompanying immune suppression and mortality that characterize this highly lethal disorder. A recent clinical study in patients dying of sepsis showed profound loss of B and CD4+ T cells via apoptosis (18), a finding that highlights the potential importance of the current study.

Richard S. Hotchkiss
Departments of Anesthesiology, Medicine, and Surgery
Washington University School of Medicine
660 South Euclid
St. Louis, MO 63110, USA
E-mail: hotchk@morpheus.wustl.edu

W. Michael Dunne
Paul E. Swanson
Department of Pathology
Washington University School of Medicine

Christopher G. Davis
Kevin W. Tinsley
Katherine C. Chang
Department of Anesthesiology
Washington University School of Medicine

TECHNICAL COMMENTS

Timothy G. Buchman
Department of Surgery
Washington University School of Medicine

Irene E. Karl
Department of Medicine
Washington University School of Medicine

References and Notes
12. Methods used in these experiments are briefly discussed in (19–21), below, a complete description of methods and results appears at http://elysium.wustl.edu/rhlab/.
15. R. S. Hotchkiss et al., unpublished data.
19. C3H HeN mice were from Harlan (Omaha, NE). Fas receptor–deficient mice (MRL/MpJ-Fas lpr) and transgenic mice heterozygous for overexpression of human Bcl-2 in T cells were from Jackson Laboratory (Bar Harbor, ME).
20. Although Grassmé et al. (8) used nasal application of bacteria, our studies found that intratracheal injection gave a more consistent and reproducible method of assuring bacterial delivery. For intratracheal injection, mice were anesthetized with halothane and the trachea exposed by a midline incision. A tuberculin syringe was used to inject 40 to 50 μl of solution.
21. P. aeruginosa (ATCC 27853) were grown overnight in trypticase soy broth. A 10-ml volume of the culture medium was placed in a 50-ml conical tube and bacteria were harvested by centrifugation. The pellet was resuspended, centrifuged, and density of inoculum adjusted to 0.3 A595, corresponding to a density between 5 x 107 and 1 x 108 CFU/ml as determined by serial dilution and colony counts. Survival studies demonstrated an ~20% survival at 7 days in mice injected with bacteria versus a 100% survival in saline-treated mice. Livers and spleens obtained from mice ~24 hours after bacterial injection were positive for P. aeruginosa. Lungs from saline treated and P. aeruginosa treated mice were obtained at 6 and 24 hours after injection. Detection of active caspase 3, TUNEL, flow cytometry, and electron microscopy were performed as described in previous studies (4, 5, 14, 18).
22. This work was supported by the National Institutes of Health (Grants GM44118 and GM55194) and by the Alan A. and Edith L. Wolf Foundation.

29 June 2001; accepted 18 September 2001
Response: TUNEL has been employed by many investigators to show apoptosis in the lung induced by a variety of stimuli [see, e.g., (1–11)]. In our study (12), using TUNEL, we showed that several P. aeruginosa strains induce apoptosis of lung epithelial cells by an up-regulation of the CD95/CD95 ligand system. Epithelial cells from lpr or gld mice lacking either CD95 or CD95 ligand were resistant to P. aeruginosa–triggered apoptosis. These mice, which were too young to suffer from lymph-adenoproliferative symptoms or reduction of body weight, were unable to control the infection and died by sepsis, while normal mice rapidly cleared pulmonary P. aeruginosa infections. Apoptosis as part of the host defense was recently also shown for Salmonella typhimurium infections of Caenorhabditis elegans (13). In this response to the comments of Hotchkiss et al., we here confirm induction of apoptosis in lung epithelial cells by P. aeruginosa.

Transmission electron microscopy [TEM (14)] revealed marked chromatin condensation and fragmentation in nuclei of lung epithelial cells from infected mice, while the chromatin in nuclei from uninfected mice was homogenous (Fig. 1, A and B). The presence of cilia in apoptotic cells identifies them as lung epithelial cells (Fig. 1C). Apoptosis in lung epithelial cells was also confirmed by detection of single-stranded DNA (15), which was present only in nuclei of infected mice (Fig. 2). Single-stranded DNA is a typical marker of apoptosis and is absent in necrosis (16).

Hotchkiss et al. used P. aeruginosa in the plateau growth phase, whereas we employed bacteria cultured until the early mid-logarithmic growth phase (17). Because growth and infection conditions have been shown to be crucial for biological effects of many bacteria, such as Salmonella typhimurium, Yersinia enterocolitica, Yersinia pseudotuberculosis, Escherichia coli, and P. aeruginosa (18, 19), we tested the effect of different P. aeruginosa growth conditions on apoptosis. Our experiments revealed that P. aeruginosa cultured until early mid-logarithmic growth phase, but not bacteria in plateau growth phase or taken directly from the agar plate, induced apoptosis in lung epithelial cells in vivo (Fig. 2) or in vitro (Fig. 3 [20]). The very efficient induction of apoptosis by P. aeruginosa ATCC 27853 is consistent with our previous data (12).

Likewise, only early mid-logarithmic P. aeruginosa were internalized by mammalian cells in vitro and in vivo [Fig. 4 (21)]. P. aeruginosa grown to plateau phase or directly taken from agar plates were poorly internalized. Plateau phase P. aeruginosa, but not early mid-logarithmic grown P. aeruginosa, seemed to develop capsules (Fig. 4, A to C), which might interfere with the infection of mammalian cells.

Our TEM, single-stranded DNA, and FITC-Annexin/PI labeling studies confirm the previously described TUNEL assays (12) and demonstrate apoptosis of lung epithelial cells upon P. aeruginosa ATCC 27853 infection. In our previous studies, we have observed positive TUNEL exclusively in epithelial cells from infected normal mice. No signal was detected in lungs from uninfected normal or infected lpr or gld mice, respectively, which indicates the specificity of TUNEL, consistent with many previous studies (1–11) that have employed TUNEL to detect apoptosis in the lung under different conditions. None of those studies have reported unspecific TUNEL staining of naive lungs (1–11), in contrast to the data presented by Hotchkiss et al. In addition, because apoptosis may involve a variety of different caspases and even caspase-independent apoptosis has been observed, absence of active caspase 3 immunoreactivity does not rule out apoptosis.

Our data indicate the importance of P. aeruginosa growth conditions for triggering apoptosis and invasion of epithelial cells. However, early mid-logarithmic growth conditions of P. aeruginosa upon intranasal infections might be most appropriate for mimicking the clinical situation of an early pulmonary P. aeruginosa infection. Although CD95 stimulation and apoptosis of lung epithelial cells seem to be
beneficial in acute pulmonary *P. aeruginosa* infections, apoptosis of lymphocytes in *P. aeruginosa* peritonitis or sepsis might be detrimental. This suggests that induction of apoptosis has specific roles depending on the conditions of the bacterial infection.

Heike Grassmé
Susanne Kirschnek
Department of Immunology
St. Jude Children’s Research Hospital
Memphis, TN 38105, USA

Joachim Riethmüller
Department of Pediatrics
University of Tübingen
72076 Tübingen, Germany

Andrea Riehle
Department of Immunology
St. Jude Children’s Research Hospital

Gabriele von Kürthy
Department of Neurology
University of Tübingen

Florian Lang
Department of Physiology
University of Tübingen

Michael Weller
Department of Neurology
University of Tübingen
References and Notes
14. Electron microscopy was performed as described in (22). C57/Bl6 mice were intranasally infected with 1.4 x 10^9 CFUs of P. aeruginosa ATCC 27853.
15. Single-stranded DNA was detected with an alkaline phosphatase-labeled monoclonal antibody and fast red chromogen following the instructions of the vendor (Alexis).
17. Early mid-logarithmic growth phase bacteria were obtained after 1 hour subculture of overnight-grown P. aeruginosa ATCC 27853. Plateau-phase grown bacteria were prepared according to the methods of Hotchkiss et al. Plate-grown P. aeruginosa were directly resuspended in RPMI-1640, 10 mM HEPES.
20. In the in vitro tests, cells were simultaneously stained with FITC-labeled Annexin V (Roche, 1:50 dilution) and 5 μg/ml propidium iodide (PI) and analyzed by fluorescence-activated cell sorting (FACS). Bacteria were excluded by gating. WI-38 cells were infected with P. aeruginosa ATCC 27853 at a cell-to-bacteria ratio of 1:500, as in (23).
21. In vitro internalization was determined by crystal violet staining and polymyxin assays exactly as described in (24). In vivo internalization was determined by digestion of lungs in 0.5% trypsin, 1 mM EDTA, 0.2% NaN₃, and 100 μg/ml polymyxin, homogenization, and subjecting of the single-cell suspension to a polymyxin assay.
Role of Apoptosis in *Pseudomonas aeruginosa* Pneumonia
Richard S. Hotchkiss, W. Michael Dunne, Paul E. Swanson, Christopher G. Davis, Kevin W. Tinsley, Katherine C. Chang, Timothy G. Buchman and Irene E. Karl

*Science* **294** (5548), 1783.
DOI: 10.1126/science.294.5548.1783a