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**Pseudomonas aeruginosa**-Induced Human Mast Cell Apoptosis Is Associated with Up-Regulation of Endogenous Bcl-x\(_S\) and Down-Regulation of Bcl-x\(_L\)^1

Christopher E. Jenkins, Ania Swiatniowski, Melanie R. Power, and Tong-Jun Lin^2

Mast cells play a critical role in the host defense against bacterial infection. Recently, apoptosis has been demonstrated to be essential in the regulation of host response to *Pseudomonas aeruginosa*. In this study we show that human mast cell line HMC-1 and human cord blood-derived mast cells undergo apoptosis as determined by the ssDNA formation after infection with *P. aeruginosa*. *P. aeruginosa* induced activation of caspase-3 in mast cells as evidenced by the cleavage of D4-GDI, an endogenous caspase-3 substrate and the generation of an active form of caspase-3. Interestingly, *P. aeruginosa* treatment induced up-regulation of Bcl-x\(_S\) and down-regulation of Bcl-x\(_L\). Bcl-x\(_S\) and Bcl-x\(_L\) are alternative variants produced from the same Bcl-x pre-mRNA. The former is proapoptotic and the latter is antiapoptotic likely through regulating mitochondrial membrane integrity. Treatment of mast cells with *P. aeruginosa* induced release of cytochrome c from mitochondria and loss of mitochondrial membrane potentials. Moreover, *P. aeruginosa* treatment reduced levels of Fas-associated death domain protein-like II-1β-converting enzyme-inhibitory proteins (FLIPs) that are endogenous apoptosis inhibitors through counteraction with caspase-8. Thus, human mast cells undergo apoptosis after encountering *P. aeruginosa* through a mechanism that likely involves both the Bcl family protein mitochondrial-dependent and the FLIP-associated caspase-8 pathways. The Journal of Immunology, 2006, 177: 8000–8007.

Ast cells are abundant in the tissues adjacent to external surfaces such as lung, intestine, or skin. Mast cells have been repeatedly demonstrated to be critical in the host defense against bacterial infection (1–3). Direct evidence of a role for mast cells in host defense against bacterial infection comes from the study using mast cell-deficient W/Wv mice. In a model of cecal ligation and puncture-induced peritonitis and a model of *Klebsiella pneumonia* induced peritonitis or lung infection, animals with a normal number of mast cells survived bacterial challenge, whereas W/Wv mice did not (1, 2). To date, a role for mast cells in the host defense against bacterial infection has been attributed to their released products such as IL-1 and TNF, which in turn recruit other immune cells for the clearance of the pathogen (1, 2, 4). However, little is known about the fate of these mast cells after encountering live bacterial pathogens.

Apoptosis plays a central role in the balance between host defense and the invading pathogen (5). Depending upon the nature of the bacterial pathogen and the population of host cells, apoptosis of the host cells may be detrimental or beneficial to the survival of the host organism. *Pseudomonas aeruginosa* pneumonia-induced bronchial cell apoptosis is essential for survival, likely through shedding of infected apoptotic bronchial cells (6). In contrast, lymphocyte apoptosis during infection is detrimental, and prevention of lymphocyte apoptosis improves the chances of survival (7). Thus, it is important to differentiate and characterize the apoptotic response in a specific cell population during *P. aeruginosa* infection. Some cell types such as airway epithelium or endothelial cells are highly resistant to apoptosis in *P. aeruginosa* pneumonia (5, 8), whereas other cell types such as lymphocytes are highly susceptible to apoptosis during *P. aeruginosa* infection (5). Mast cells in the lung directly protrude into the airway space that allows the direct interaction of mast cells with bacterial pathogens (9). Although several purified bacterial products such as toxin A from *P. aeruginosa* (10) or *Clostridium difficile* (11), or LPS (12) modulate mast cell apoptosis, it is not known whether live *P. aeruginosa* infection induces mast cell apoptosis.

Caspase-3 activation plays a central role in the execution of apoptosis. Depending upon the specific cell type, two pathways have been reported to be involved in the activation of caspase-3. The death receptor-caspase-8 pathway is essential for apoptosis in type I cells such as lymphocytes (13). Fas-associated death domain-like IL-1-converting enzyme-inhibitory proteins (FLIPs)^3 are endogenous inhibitors that counteract caspase-8 pathway activation (14). Although a mitochondria-caspase-9 pathway is required for robust apoptosis in the type II cells such as hepatocytes (15, 16), the balance between the antiapoptotic Bcl family members such as Bcl-x\(_L\) and Bcl-2 and proapoptotic Bcl family members such as Bcl-x\(_S\) plays an essential role in maintaining the mitochondrial membrane integrity and regulates mitochondrial pathway-dependent apoptosis (17). Mast cells appear to have mechanisms involving both the FLIPs-associated receptor-caspase-8 pathway

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7 Abbreviations used in this paper used: FLIP, Fas-associated death domain protein-like II-1β-converting enzyme-inhibitory protein; DioC\(_6\), 3,3′-dihexyloxacarbocyanine iodide; CBMC, cord blood-derived mast cell; HMC; human mast cell; BMMC, bone marrow-derived mast cell; MOI, multiplicity of infection.

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Alternative splicing of the Bcl-x pre-mRNA gives rise to two transcripts, coding for either a long-form (Bcl-xL) or a short-form (Bcl-xS) of the protein (19). Bcl-xL inhibits apoptosis through heterodimerization with proapoptotic proteins (20). In contrast, Bcl-xS is proapoptotic through antagonizing survival proteins such as Bcl-xL or Bcl-2 (20). Due to the antagonistic functions of these two splice variants, a shift of the Bcl-xL to Bcl-xS ratio regulates the apoptotic process. The shift in splicing from Bcl-xL to Bcl-xS has been achieved by artificial antisense oligonucleotides and reverse transcriptase-PCR techniques (18) and the Bcl family protein-mitochondrial pathway to regulate apoptosis (12).

In this study, we demonstrated that human mast cells underwent apoptosis as determined by ssDNA formation in response to live, but not killed P. aeruginosa infection. Similarly, live but not killed P. aeruginosa induced caspase-3 activation in human mast cells. Interestingly, P. aeruginosa infection induced up-regulation of Bcl-xL and down-regulation of Bcl-xS, a shift from Bcl-xL to Bcl-xS expression in mast cells. In addition, P. aeruginosa reduced FLIP levels in mast cells. To our knowledge, this demonstration is the first to show that a shift from Bcl-xL to Bcl-xS is associated with bacteria-induced apoptosis in mammalian cells. Our results suggest that P. aeruginosa-induced human mast cell apoptosis likely involves Bcl family protein mitochondrial-dependent and FLIP-associated death receptor pathways.

Materials and Methods

Reagents

Mouse anti-ssDNA mAb (IgM), biotinylated mouse anti-Bcl-xL/Bcl-xS, rabbit anti-FLIPshort, and rabbit anti-FLIplong Abs were purchased from Chemicon International. Mouse anti-rat neutrophil mAb (RP-3, IgM) isotype control was a gift from F. Sendo (Yamagata University, Yamagata, Japan). Rabbit anti-active caspase-3 IgG was purchased from BD Biosciences. Mouse anti-D4-GDI (specific for the 23-kDa form) mAb was purchased from Imgenex. Mouse anti-human Bcl-2 (IgG1) was purchased from Upstate Biotechnology. Goat anti-actin IgG, donkey anti-goat IgG HRP, donkey anti-rabbit IgG HRP, and donkey anti-mouse IgG HRP Ab conjugates were purchased from Cell Signaling Technology. Rabbit FITC-conjugated anti-active caspase-3 IgG was purchased from BD Biosciences. Mouse anti-D4-GDI (specific for the 23-kDa form) mAb was purchased from Imgenex. Mouse anti-human Bcl-2 (IgG1) was purchased from Upstate Biotechnology. Goat anti-actin IgG, donkey anti-goat IgG HRP, donkey anti-rabbit IgG HRP, and donkey anti-mouse IgG HRP Ab conjugates were purchased from Santa Cruz Biotechnology. Goat PE-conjugated IgG to mouse IgM was purchased from Caltag Laboratories. 3,3′-Dihexyloxacarbocyanine iodide (DioC6) was from Molecular Probes. Purified P. aeruginosa exotoxin A was purchased from List Biological Laboratories. Camptothecin was obtained from Sigma-Aldrich. FBS, penicillin/streptomycin, IMDM, and RPMI 1640 medium were purchased from Biological Laboratories. Camptothecin was obtained from Sigma-Aldrich. FBS, penicillin/streptomycin, IMDM, and RPMI 1640 medium were purchased from Invitrogen Life Technologies. All other chemicals and reagents were of analytical grade.

Mast cells and culture conditions

Human mast cells HMC-1 5C6 were maintained in IMDM in a 5% CO2-humidified atmosphere at 37°C. Culture medium was supplemented with 10% FBS and 50 U/ml each of penicillin and streptomycin.

Highly purified cord blood-derived mast cells (CBMC) (>95% purity) were obtained by long term culture of cord blood progenitor cells as previously described (23). The percentage of mast cells in the cultures was determined by toluidine blue staining (pH 1.0) of cytocentrifuged samples. Mature mast cells after more than 8 wk in culture were identified by their morphological features and the presence of metachromatic granules, at which time they were used for this study.
Murine primary cultured bone marrow-derived mast cells (BMMC) were harvested from the femurs and tibias of C57-black mice from Charles River Breeding Laboratories and maintained as previously described (24). Following 5 wk of culture, mast cell purity of >98% was achieved as assessed by toluidine blue staining. Mature mast cells were identified by their morphological features and granule prevalence.

Bacterial preparation and treatment with mast cells

*P. aeruginosa* strain 8821, a gift from Dr. A. Chakrabarty, University of Illinois (Chicago, IL), is a mucoid strain isolated from a cystic fibrosis patient (25). *P. aeruginosa* was cultured in Luria-Bertani broth and harvested when the culture reached an OD at 640 nm of 2 OD units (early stationary phase). Bacteria were washed in PBS and density adjusted to 1 OD unit before use. For killed *P. aeruginosa* experiments, bacteria were treated with gentamicin (100 μg/ml) for 2 h and exposed directly to UV light illumination for 20 min before experimental use. For live *P. aeruginosa* experiment, mast cells were treated with live *P. aeruginosa* for various multiplicity of infection (MOI) values, and then a mixture of antibiotics was added to kill *P. aeruginosa* (200 μg/ml gentamicin, 1% penicillin/streptomycin (v/v) each 50 U/ml, 100 μg/ml ceftazidime, and 100 μg/ml piperacillin). Death of bacteria was confirmed by plating mast cell culture on Luria-Bertani agar plates.

Detection of ssDNA by flow cytometry

*P. aeruginosa*-treated or sham-treated mast cells were fixed, permeabilized, and stained with a mAb specific for segments of ssDNA as previously described (26). Briefly, mast cells were fixed for 1–3 days in methanol at −20°C and subsequently heated in formamide at 70°C for 10 min. Non-specific binding was blocked with 1% nonfat dry milk (w/v) in PBS. Cells were stained with anti-ssDNA or IgM isotype control, followed by washing and incubation with a PE-conjugated anti-mouse IgM Ab. After washing, cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Preparation of total cell lysate

Treated cells (0.25 × 10^5–2.5 × 10^6) were homogenized in ice-cold radioimmune precipitation assay radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaHPO_4, 0.25% sodium...
deoxycholate (w/v), 0.1% Nonidet P-40 (v/v), 1 mM Na3VO4, and 1 mM NaF) containing freshly added protease and phosphatase inhibitors (2 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 5 mM EDTA, 5 mM EGTA, and 2 mM iodoacetamide). Lysates were typically incubated on ice for at least 20 min before centrifugation at 15,000 x g to remove cellular debris. Protein was quantified using a protein quantification reagent according to the manufacturer (Bio-Rad).

Western blotting for active caspase-3, D4-GDI, Bcl-xL/Bcl-xS, and FLIPs

Sample lysates containing 75 μg of protein (for caspase-3), 15 μg (for D4-GDI), 5 μg (for FLIP<sub>short</sub>), and 30 μg (for FLIP<sub>long</sub>, Bcl-x<sub>L</sub>/Bcl-x<sub>S</sub>, Bcl-2, or cytochrome c) were boiled for 5 min and subjected to SDS-10% PAGE. Gels were transferred to polyvinylidene difluoride membrane, and nonspecific binding was blocked using 10% nonfat dry milk. Membranes were then incubated overnight at 4°C with Abs to active caspase-3, D4-GDI, FLIP<sub>short</sub>, FLIP<sub>long</sub>, Bcl-x<sub>L</sub>/Bcl-x<sub>S</sub>, Bcl-2, or cytochrome c and detected by ECL detection reagent (Amersham Biosciences). Membranes were subsequently stripped (62.5 mM Tris-HCl (pH 6.8), 20% SDS (w/v), 100 mM 2-ME) and reprobed for actin.

Detection of active caspase-3 by flow cytometry

Treated mast cells (0.5–1 x 10⁶) were fixed in 4% paraformaldehyde and subsequently stored in 10% DMSO in PBS at −80°C until staining. Cells were thawed and permeabilized with 0.1% saponin in PBS for 1 h followed by incubation in 3% BSA/PBS for 1 h to block nonspecific binding. Cells were then stained with FITC-conjugated rabbit mAb to active caspase-3, washed, and analyzed by flow cytometry.

Detection of mitochondrial membrane potential using DioC₆

Changes of mitochondrial membrane potential were measured using DioC₆ as described elsewhere with minor modifications (27). Mast cells were suspended in 40 nM DioC₆ in medium for 30 min at 37°C. Cells were then transferred to flow cytometry polystyrene tubes and kept on ice. Cells were then analyzed by flow cytometry for green fluorescence.

Statistical analysis

Data were analyzed by one way ANOVA followed by Tukey’s posttest, using Instat GraphPad software (version 3.0) to determine the statistical difference between individual treatments. Statistical significance was defined as p < 0.05.

Results

P. aeruginosa induces mast cell apoptosis

To determine whether P. aeruginosa induces mast cell apoptosis, an Ab specific for ssDNA was used because the generation of ssDNA is a specific indicator of apoptosis (26). HMC-1 cells were treated with various concentrations of live or killed P. aeruginosa strain 8821. Treatment of HMC-1 cells with live P. aeruginosa induced ssDNA formation in a concentration-dependent manner (Fig. 1, a and b). Interestingly, no ssDNA formation was detectable when HMC-1 cells were treated with killed P. aeruginosa strain 8821 (Fig. 1, a and b).

P. aeruginosa induces caspase-3 activation in mast cells

Because caspase-3 plays a central role in the execution of apoptosis, several approaches were taken to examine the activation of caspase-3

FIGURE 4. P. aeruginosa up-regulates Bcl-x<sub>S</sub> and down-regulates Bcl-x<sub>L</sub> in human mast cells. HMC-1 cells were treated with live P. aeruginosa (strain 8821, MOI = 25, 50, or 100) for 24 h (Antibiotics were added at 3 h and throughout the rest of the incubation period as in Fig. 1). Cells without P. aeruginosa treatment served as controls (NT). Cell lysates were subjected to SDS-PAGE and Western blotting with an Ab that recognizes both Bcl-x<sub>S</sub> and Bcl-x<sub>L</sub>. Blots were subsequently stripped and reprobed for Bcl-2 or actin (a). Densitometry analysis of Bcl-x<sub>L</sub> (b) or Bcl-x<sub>S</sub> (c) was performed based on three separate experiments. The increase of Bcl-x<sub>L</sub> to Bcl-x<sub>S</sub> ratio induced by P. aeruginosa treatment was shown (d).
in mast cells following *P. aeruginosa* infection. To directly examine caspase-3 activation in human mast cells, an Ab that specifically recognizes the activated form of caspase-3 was used. HMC-1 cells were treated with live or killed *P. aeruginosa* with various MOI for 24 h. Cell lysates were used to determine caspase-3 activation by Western blotting. Treatment of mast cells with live *P. aeruginosa* induced an increase of active caspase-3 (Fig. 2a). Similar to ssDNA formation and D4-GDI cleavage, killed *P. aeruginosa* has little effect on caspase-3 activation (Fig. 2a).

To further determine the percentage of mast cell population positive for activated caspase-3, flow cytometry analysis was conducted using intracellular staining with a FITC-labeled mAb specific for the activated caspase-3. The population of mast cells positive for activated caspase-3 after *P. aeruginosa* treatment is dependent on the MOI (Fig. 2, b and c). Approximately 56% of HMC-1 cells were stained positive for activated caspase-3 when mast cells were infected with *P. aeruginosa* at the MOI of 1:100. Consistent with previous results, killed *P. aeruginosa* had little effect on caspase-3 activation (Fig. 2, b and c).

To confirm *P. aeruginosa*-induced caspase-3 activation in primary cultured human mast cells, CBMC were treated with live *P. aeruginosa* at the MOI of 1:100 for 24 h. CBMC were permeabilized and stained with anti-active caspase-3. Similar to HMC-1 cells, CBMC were stained positive for active caspase-3 after treatment with live, but not killed *P. aeruginosa* (Fig. 2, d and e).

**P. aeruginosa** induces D4-GDI cleavage in mast cells

We further confirmed *P. aeruginosa*-induced caspase-3 activation in mast cells by measuring D4-GDI cleavage. D4-GDI is one of the endogenous substrate for caspase-3 (28). Accordingly, cleavage of D4-GDI has been used as an indicator of caspase-3 activity (28). HMC-1 cells were treated with various concentrations of live or killed *P. aeruginosa* (strain 8821) for 24 h, and D4-GDI cleavage was determined by Western blotting. Treatment of *P. aeruginosa* induced significant D4-GDI cleavage leading to the generation of a 23-kDa product, which is specific to caspase-3 activity (Fig. 3).

It is noteworthy that only live *P. aeruginosa*, but not killed *P. aeruginosa*, induced enhanced D4-GDI cleavage in mast cells, a pattern consistent with that of ssDNA formation.

**P. aeruginosa** increases Bcl-x<sub>S</sub> and decreases Bcl-x<sub>L</sub> levels in mast cells and decreases mitochondrial membrane potential

Mitochondria play an essential role in the initiation of the apoptotic process by release of proapoptotic substances into the cytosol to activate caspase-3. The balance between the antiapoptotic Bcl family members such as Bcl-x<sub>L</sub> and Bcl-2 and the proapoptotic Bcl family members such as Bcl-x<sub>S</sub> controls apoptosis through several mechanisms including maintaining the integrity of the mitochondria membrane by preventing the release of proapoptotic substances from the mitochondria (29). To examine whether *P. aeruginosa* regulates the levels of Bcl family members in mast cells, HMC-1 cells were treated with live *P. aeruginosa* with various MOI for 24 h. Cell lysates were used to examine the levels of Bcl-x<sub>S</sub>, Bcl-x<sub>L</sub>, and Bcl-2. Treatment of mast cells with *P. aeruginosa* induced decrease of the antiapoptotic Bcl family member Bcl-x<sub>L</sub> and increased the proapoptotic member Bcl-x<sub>S</sub> (Fig. 4). Interestingly, the level of Bcl-2 was unaffected by *P. aeruginosa* treatment (Fig. 4). Because Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub> are splice variants, we determined the ratio of Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub>. Treatment of mast cells with *P. aeruginosa* induced a consistent increase in the Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub> ratio.

To examine whether Bcl-x<sub>S</sub> expression is a specific effect induced by *P. aeruginosa* treatment, we examined the effect of two apoptosis-inducing agents (camptothecin and exotoxin A) on Bcl-x<sub>S</sub> expression in HMC-1 cells. Camptothecin is an anticancer chemical that induces cell apoptosis by inhibiting topoisomerase I (30). Treatment of HMC-1 with camptothecin (1 μM) induced...
FIGURE 6. *P. aeruginosa* treatment induces loss of mitochondrial potential and release of cytochrome c from mitochondria to cytosol. a and b, HMC-1 cells were treated with various concentrations of live *P. aeruginosa* for 3 h (MOI = 25, 50, or 100). Then antibiotics (see Fig. 1) were added to kill bacteria and were present throughout the rest of 21 h incubation time (total 24 h incubation). Cells treated with killed *P. aeruginosa* (MOI = 100) or camptothecin (1 μM) served as controls. Cells were then used to stain with DioC6 and analyzed by flow cytometry. Representative flow cytometry histogram showed that *P. aeruginosa* treatment induces loss of mitochondrial potential in mast cells (a). Results are expressed as mean percentage of positive staining cells ± SEM of three independent experiments. *, p < 0.01 compared with no treatment group (b). c, HMC-1 cells were treated with live *P. aeruginosa* (Psa Live), killed *P. aeruginosa* (Psa Killed) (MOI = 100), or camptothecin (Camp, 1 μM) for 24 h. Cells were then subjected to mitochondria/cytosol fractionation to obtain mitochondria-free cytosol fraction. Cytosol proteins were probed for cytochrome c, D4-GDI, or actin by Western blotting. Cells without *P. aeruginosa* treatment (NT) were used as a control.

D4-GDI cleavage, active caspase-3 production, and ssDNA formation (Fig. 5a) and reduced FLIPshort level (see Fig. 7a below). However, camptothecin at all concentrations tested (0.1, 1, and 5 μM) did not induce Bcl-xL expression, although at higher concentrations (1 and 5 μM) it reduced Bcl-xL level (Fig. 5b). This result is in contrast to live *P. aeruginosa* infection, which consistently induces Bcl-xL expression. Killed *P. aeruginosa* appears to have no effect on Bcl-xL expression (Fig. 5c).

Effects of *P. aeruginosa* exotoxin A on Bcl-xL formation were examined because we showed previously that exotoxin A induced HMC-1 cell apoptosis (10). HMC-1 cells were treated with exotoxin A (300 ng/ml) for 24 h. Interestingly, no effect of exotoxin A on Bcl-xL was observed (Fig. 5c).

To examine whether *P. aeruginosa* induces Bcl-xL formation in primary cultured human CBMC, mature CBMC were treated with live or killed *P. aeruginosa* for 24 h. Cell lysates were examined for Bcl-xL. As seen in HMC-1, Bcl-xL expression was induced by treatment with live but not killed *P. aeruginosa* (Fig. 5d). Camptothecin was used as a control and showed no effect on Bcl-xL expression.

To examine whether primary cultured mouse BMMC respond to *P. aeruginosa*, mature mouse BMMC were treated with live *P. aeruginosa*, killed *P. aeruginosa*, or camptothecin. Live *P. aeruginosa* induced increase of Bcl-xL, caspase-3 activation, and D4-GDI cleavage in mouse BMMC (Fig. 5, e and f). In contrast, killed *P. aeruginosa* had minor or no effects on Bcl-xL, active caspase-3, or D4-GDI levels, a pattern similar to that seen in HMC-1 cells. Similarly, although camptothecin induced caspase-3 activation and D4-GDI cleavage, it did not induce Bcl-xL formation (Fig. 5, e and f). Thus, mouse mast cells and human mast cells respond to *P. aeruginosa* and undergo apoptosis likely through similar mechanisms.

To determine whether *P. aeruginosa* treatment affects mast cell mitochondrial membrane permeability, DioC6 was used. HMC-1 cells were treated with live *P. aeruginosa* with various MOI for 24 h or treated with killed *P. aeruginosa* (MOI = 100) for 24 h. HMC-1 cells were also treated with camptothecin (1 μM) for 24 h as a control. After treatment, cells were stained with DioC6. Treatment with camptothecin or live, but not killed *P. aeruginosa* induced loss of mast cell mitochondrial membrane potential (Fig. 6, a and b), suggesting an increase of mitochondrial membrane permeability.

To further determine a role of mitochondrial pathway in *P. aeruginosa*-induced mast cell apoptosis, we examined whether *P. aeruginosa* treatment induces cytochrome c release into cytosol compartment. HMC-1 cells were treated with camptothecin (1 μM), live or killed *P. aeruginosa* (MOI = 100) for 24 h. Cells were then subjected to mitochondria-cytosol separation using a mitochondria-cytosol fractionation kit (BioVision). The release of cytochrome c in the cytosol fraction was examined by Western blotting. Treatment of mast cells with live *P. aeruginosa* induced release of cytochrome c into cytosol (Fig. 6c). In contrast, killed *P. aeruginosa* only induced a low level of cytochrome c release. Treatment with camptothecin also induced a significant release of cytochrome c. Similarly, camptothecin or live but not killed *P. aeruginosa* induced D4-GDI cleavage when cytosol fractions were probed for cleaved D4-GDI product by Western blotting (Fig. 6c).

*P. aeruginosa* down-regulates FLIPshort and FLIPlong in mast cells FLIPs are endogenous proteins that regulate caspase-3 activation through interaction with caspase-8 (14, 31). To determine whether FLIPs are involved in live *P. aeruginosa*-induced mast cell apoptosis, HMC-1 cells were treated with live or killed *P. aeruginosa*...
We determined whether \textit{P. aeruginosa}\(^{\text{cytometry}}\) (data not shown). \textit{P. aeruginosa} (mast cell to bacteria ratio of 1:50) for various times of the incubation period. Cell lysates were examined by Western blotting (Antibiotics as described in Fig. 1 were added at 3 h and throughout the rest of the incubation period). Cell lysates were examined by Western blotting with Abs to FLIP\(_{\text{short}}\) (a), FLIP\(_{\text{long}}\) (b), or actin. Cells without \textit{P. aeruginosa} treatment (NT) were used as controls.

\textbf{Discussion}

Mast cells play a critical role in the host defense against bacterial infection (1–3). To date, this important role of mast cells has been attributed to the release of mast cell mediators that in turn recruit other immune effector cells such as neutrophil to clear bacterial pathogen (1–3). Recently, we demonstrated an active mast cell-pathogen (1–3). To date, this important role of mast cells has been shown about the fate of mast cells after encountering bacterial pathogen. Because apoptosis has been shown to be one of the critical mechanisms in the host defense against \textit{P. aeruginosa} infection, we investigated whether mast cells undergo apoptosis after encountering \textit{P. aeruginosa} and the mechanisms involved in \textit{P. aeruginosa}-induced mast cell apoptosis. We used a recently developed technique based on formamide-induced DNA denaturation combined with detection of denatured DNA with a mAb against ssDNA that allows specific detection of apoptotic cells (26). We provide compelling evidence that human mast cells undergo apoptosis after incubation with \textit{P. aeruginosa}.

\textit{P. aeruginosa}-induced apoptosis was confirmed by the detection of caspase-3 activation. An active form of caspase-3 in \textit{P. aeruginosa}-treated mast cells was detected by Western blotting and flow cytometry. \textit{P. aeruginosa}-induced activation of caspase-3 was further verified by the cleavage of D4-GDI, an endogenous caspase-3 substrate. A central role for caspase-3 in the process of apoptosis has been well recognized. Depending on a specific cell type, two major pathways have been well detailed in the initiation of caspase-3 activation, the FLIPs-associated death receptor-caspase-8 pathway and the Bcl family-regulated mitochondrial pathway. Both pathways exist in mast cells (12, 18). Bcl family members consist of proapoptotic proteins (Bcl-x\(_a\), Bax, Bad, and others) and the antiapoptotic proteins (Bcl-2, Bcl-x\(_b\), and Bcl-w). The balance between proapoptotic and antiapoptotic members determines the fate of many types of cells. Bcl-x\(_a\) and Bcl-x\(_b\) are splice variants produced by alternative splicing of Bcl-x pre-mRNA. The antagonistic functions of Bcl-x\(_a\) (antiapoptotic) and Bcl-x\(_b\) (proapoptotic) have prompted several studies in an attempt to shift the alternative splicing in an effort to control the apoptotic process (21, 22). We found that a shift from Bcl-x\(_a\) to Bcl-x\(_b\) in human mast cells was induced by treatment with \textit{P. aeruginosa}. To our knowledge, this demonstration represents the first that shows that alternative splicing of Bcl-x\(_a\) and Bcl-x\(_b\) is involved in bacterial pathogen-induced apoptosis in mammalian cells. One major advantage of using the mechanism of splicing shift producing variants with opposing effect is that one molecule of antiapoptotic Bcl-x\(_a\) is replaced with one molecule of proapoptotic Bcl-x\(_b\), leading to an increased net effect in the control of apoptotic process.

The production of specific Bcl isoforms with opposite effects on the apoptotic response is likely controlled by the promoter usage (33). Several \textit{bcl-x} gene promoters have been identified (33, 34). The usage of different promoters leads to the generation of different \textit{bcl-x} gene products, with promoter 1 primarily producing Bcl-x\(_a\) and promoter 2 producing Bcl-x\(_b\), and Bcl-x\(_c\) (33). Accordingly, it is possible that upon \textit{P. aeruginosa} infection human mast cells change the usage of \textit{bcl-x} gene promoter leading to the decrease of Bcl-x\(_a\) and increase of Bcl-x\(_b\). However, the promoter usage appears to be tissue specific (33). Thus, it remains to be determined whether \textit{P. aeruginosa}-induced Bcl-x isoform shift also occurs in other cell types or other pathogen-induced apoptosis because different cell types possess distinct mechanisms in response to different bacterial pathogen. In addition, because mechanisms involved in live \textit{P. aeruginosa}-induced mast cell apoptosis are likely multifactorial, the causative relationship between Bcl-x\(_a\)/Bcl-x\(_b\) levels and mast cell apoptosis requires further study.

It has been suggested that members of the Bcl-x\(_a\) family are likely multifactorial, the causative relationship between Bcl-x\(_a\)/Bcl-x\(_b\) levels and mast cell apoptosis requires further study.

An additional mechanism in the initiation of caspase-3 activation is the FLIPs-associated death receptor-caspase-8 pathway. Cellular FLIPs structurally resemble caspase-8 except that they lack proteolytic activity (14). Thus, FLIPs function as intrinsic inhibitors of caspase-8 pathway activation. Treatment of mast cells with live \textit{P. aeruginosa} reduced the protein levels of both FLIP\(_{\text{long}}\) and FLIP\(_{\text{short}}\), suggesting a potential role of death receptor-caspase-8 pathway in \textit{P. aeruginosa}-induced mast cell apoptosis. We also attempted to determine whether levels of cell surface Fas (CD95) on mast cells were altered by \textit{P. aeruginosa} treatment because Fas was up-regulated by \textit{P. aeruginosa} on epithelial cells (32). Little changes of surface Fas were observed when human mast cells were treated with \textit{P. aeruginosa} for various times...
(3–48 h) (data not shown), suggesting that mast cells and epithelial cells likely respond differently to \textit{P. aeruginosa}.

Interestingly, both ssDNA formation and activation of caspase-3 were induced by live, but not killed \textit{P. aeruginosa}. Further studies are needed to determine the specific bacterial components that are responsible for inducing mast cell apoptosis. It is possible that live \textit{P. aeruginosa} releases bacterial toxins that induce mast cell apoptosis. Our recent study showed that \textit{P. aeruginosa} exotoxin A induces human mast cell apoptosis that is associated with reduced levels of FLIPs, a feature similar to that induced by live \textit{P. aeruginosa} (10). However, we noticed that unlike live \textit{P. aeruginosa}, exotoxin A did not induce increased expression of Bcl-Xs. In addition, we have demonstrated that mitochondrial pathway is activated by live \textit{P. aeruginosa}. This result is in contrast to exotoxin A, which induces mast cell apoptosis through a mitochondrial-independent pathway (10). Thus, exotoxin A may not be a major factor responsible for \textit{P. aeruginosa}-induced mitochondrial pathway-mediated mast cell apoptosis. \textit{P. aeruginosa}-derived azurin and cytchrome c have been implicated in macrophage and mast cell apoptosis (36). Alternatively, \textit{P. aeruginosa} invasion into mast cells may initiate an intracellular event that directly targets the upstream of initiator caspases.

In summary, we reported for the first time that human mast cells undergo apoptosis after encountering live bacteria, \textit{P. aeruginosa}, through a mechanism that is associated with a shift of alternative splicing from Bcl-xL to Bcl-xS. \textit{P. aeruginosa}-induced caspase-3 activation in mast cell is accompanied by the loss of mitochondrial potential and reduced FLIPs levels, suggesting a potential role for the Bcl mitochondrial pathway and FLIPs-caspase-8 pathway in \textit{P. aeruginosa}-induced mast cell apoptosis.

\textbf{Disclosures}

The authors have no financial conflict of interest.

\textbf{References}


