Exotoxin A induces human mast cell apoptosis by a caspase-8 and -3-dependent mechanism.

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Mast cells play an important role in both allergy and innate immunity. Recently, we demonstrated an active interaction between human mast cells and Pseudomonas aeruginosa leading to the production of multiple cytokines. Here, we show that both primary cultured human cord blood-derived mast cells and the human mast cell line HMC-1 undergo apoptosis as determined by single-stranded DNA (ssDNA) formation after stimulation with P. aeruginosa exotoxin A (ETA), a major toxin produced by this bacterium. ETA-induced ssDNA formation was completely inhibited by Z-VDAD (where Z is benzoyloxy carbonyl), which blocks multiple caspases, suggesting a role for caspases in this process. Active caspase-3 formation in mast cells after an ETA challenge was detected by both Western blotting and flow cytometry. ETA-induced caspase-3 activity in human mast cells was demonstrated by the detection of a characteristic 23 kDa product of D4-GDI (where GDI is guanine nucleotide dissociation inhibitor), an endogenous caspase-3 substrate. Interestingly, a specific caspase-8 inhibitor, Z-IETD-fmk (where fmk is fluoromethyl ketone), blocked ETA-induced cleavage of D4-GDI, but a caspase-9 inhibitor (Z-LEHD-fmk) did not. Treatment of mast cells with caspase-3 inhibitor Z-DEVAD-fmk or caspase-8 inhibitor Z-IETD-fmk reduced the generation of ssDNA induced by ETA, suggesting a role for caspase-8 and -3 in ETA-induced mast cell apoptosis. Furthermore, treatment of mast cells with ETA induced decreases of the short form and a long form (p43) of Fas-associated death domain protein (FADD)-like interleukin-1β-converting enzyme (FLICE) (caspase-8)-inhibitory proteins (FLIPs), which are endogenous caspase-8 inhibitors. Taken together, these results suggest that ETA-induced mast cell apoptosis involves down-regulation of antiapoptotic proteins, FLIPs, and activation of caspase-8 and -3 pathways.

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, is a leading cause of infections in people who have cystic fibrosis, burn victims, and immunocompromised individuals. P. aeruginosa synthesizes a number of extracellular toxic products believed to be involved in the pathogenesis of these infections. Exotoxin A (ETA), a 66-kDa protein, is considered to be the most toxic factor secreted by P. aeruginosa (2–6). Because of its potent cytotoxicity, ETA has been widely used to generate fusion proteins to kill target cells. For example, chimeric cytotoxins have been constructed by the fusion of growth factors or antibodies with the enzymatic region of ETA to specifically target and eliminate cancer cells, virally infected cells, or mast cells (7–12). It is generally accepted that ETA is internalized by the cell surface receptor CD91 (the α2-macroglobulin receptor/low density lipoprotein receptor-related protein) (13) and asserts its cellular toxicity by blocking protein synthesis through ADP ribosylation of translation elongation factor 2 (14–16). However, protein synthesis inhibition by toxins is not sufficient to mediate target cell lysis (17). In addition, decreased ADP ribosylation activity does not affect ETA-induced cytotoxicity, suggesting a dissociation between protein synthesis and cytotoxicity (18). Thus, additional mechanisms may be involved in ETA-induced cytotoxicity.

Apoptotic cell death has been implicated in ETA-induced cytotoxicity because ETA increases caspase-like activities in monocytic cell lines (19) and induces nuclear morphological changes and DNA fragmentation in these cells (19, 20). The role of apoptosis in ETA-induced cytotoxicity, however, seems controversial because DNA fragmentation and nuclear morphological changes are not specific to apoptosis. Moreover, some cell types such as epithelial respiratory cells and HUT-102 cells that are killed by ETA do not undergo apoptotic cell death (7, 21). Thus, a role for apoptosis in ETA-induced cytotoxicity requires further study.

Caspase activation plays a central role in the execution of apoptosis (22, 23). Depending on the nature of the stimuli and the cell types, two caspase activation pathways have been described, including the receptor-initiated caspase-8-dependent pathway and the mitochondria-initiated caspase-9-mediated pathway (22–24). Activated caspase-8 or -9 initiates a downstream cascade of effector caspases, such as caspase-3, which cleaves various substrates such as D4-GDI, and leads to the execution of cell death (22, 23). The specific roles of caspase-8 and caspase-9 pathways as well as caspase-3 in ETA-induced apoptosis are unclear.

Death receptor caspase 8-induced apoptosis is counteracted by Fas-associated death domain (FADD)-like interleukin-1-
converting enzyme (FLICE)-inhibitory proteins (FLIPs) (25). FLIPs structurally resemble caspases but lack proteolytic activity. There are two isoforms of cellular FLIPs, FLIP short (FLIPshort, 26 kDa) and FLIP long (FLIPlong, 55 kDa), of which the latter can be cleaved into FLIP(p43) and FLIP(p12). Both FLIPlong and FLIPshort can be recruited into the death-inducing signaling complex and associate with caspase-8 (26). Accordingly, FLIPs have been proposed as antiapoptotic proteins because activation of caspase-8 is blocked. Modulation of the FLIPs expression level regulates the apoptotic process in various cell types including mast cells (27).

Among granulocytes, mast cells are exceptionally long-lived (up to months), suggesting that they are not normally programmed for spontaneous apoptosis (28). Because mast cells play an essential role in allergy, several approaches have been used to deplete mast cells, including ETA (9, 10). A chimeric protein composed of an Fc fragment of mouse IgE and a truncated form of ETA demonstrates potent mast cell cytotoxicity in vitro and prevents mast cell-dependent passive cutaneous anaphylaxis in mice in vivo (9, 10). Mechanisms of ETA-induced mast cell cytotoxicity have not been reported.

In this study, using an apoptosis-specific marker, the generation of single strand DNA, we demonstrated that human mast cells undergo apoptosis after exposure to pathologically relevant levels of ETA. ETA-induced human mast cell apoptosis is shown to be mediated by caspase-3 activation through a caspase-8-dependent but not a caspase-9-dependent pathway. Furthermore, ETA down-regulates FLIPshort and FLIP(p43) levels in human mast cells. Thus, ETA-induced human mast cell apoptosis involves down-regulation of antiapoptotic FLIPs and activation of caspase-8 or -3 pathway.

**MATERIALS AND METHODS**

**Reagents**—Z-VAD-fmk (multiple caspases inhibitor), Z-DEVD-fmk (caspase-3 inhibitor), Z-IETD-fmk (caspase-8 inhibitor), and Z-LEHD-fmk (caspase-9 inhibitor) were purchased from R&D Systems (Minneapolis, MN). Purified *P. aeruginosa* ETA was purchased from List Biologicals (Campbell, CA). Fetal bovine serum, penicillin/streptomycin, Iscove’s modified Dulbecco’s medium, and RPMI 1640 medium were purchased from Invitrogen. Mouse anti-single-stranded DNA (ssDNA) monoclonal antibody (IgM), rabbit anti-FLIPshort and rabbit anti-FLIPlong antibodies were purchased from Chemicon International (Temecula, CA). Mouse anti-rat neutrophil mAb (RP-3, IgM) isotype control was a gift from F. Sendo (Yamagata University, Japan). Rabbit anti-active caspase-3 IgG was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit FITC-conjugated anti-active-caspase-3

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*Fig. 1. Induction of human mast cell apoptosis by *P. aeruginosa* ETA.* A and B, HMC-1 cells were treated with increasing concentrations of ETA for 24 h prior to flow cytometric analysis for ssDNA. ETA induced the dose-dependent generation of ssDNA in HMC-1 5C6. Results are expressed as the mean percentage of ssDNA-positive cells ± S.E. of five independent experiments (*, p < 0.01 compared with HMC-1 cells treated with medium alone). Cells without ETA treatment served as controls (NT). C–E, CBMC were treated with medium or increasing concentrations of ETA for 24 h and then fixed and stained for ssDNA. A representative flow cytometry histogram from one donor’s CBMC is shown (C). Results from two individual donors are expressed as the mean percentage of ssDNA-positive cells ± S.E. (D and E)
IgG was purchased from BD Biosciences. Mouse anti-D4-GDI (specific for the 23-kDa form) mAb was purchased from Imgenex (San Diego, CA). Mouse anti-human Bcl-2 (IgG1) and rabbit anti-human Bax were purchased from Upstate Biotechnology (Lake Placid, NY). Goat anti-actin IgG, donkey anti-actin IgG horseradish peroxidase, donkey anti-rabbit IgG horseradish peroxidase, and donkey anti-mouse IgG horse-

radish peroxidase antibody conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat phycoerythrin-conjugated IgG to mouse IgM was purchased from Caltag (Burlingame, CA). All other chemicals and reagents were of analytical grade.

**Mast Cells and Culture Conditions**—HMC-1 5C6 human mast cells were maintained in Iscove’s modified Dulbecco’s medium in a 5% CO2-humidified atmosphere at 37 °C. Culture medium was supplemented with 10% fetal bovine serum and 50 units/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 described previously (29). The percentage of mast cells in the cultures was determined by toluidine blue staining (pH 1.0) of cytocentrifuged samples. After >8 weeks in culture, mature mast cells were identified by their morphological features and the presence of metachromatic granules, at which time they were used for this study.

**Detection of Single-stranded DNA by Flow Cytometry**—Exotoxin-treated mast cells were fixed, permeabilized, and stained with a mAb specific for segments of ssDNA as described previously (30). 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. Nonspecific binding was blocked with 1% nonfat dry milk (w/v) in phosphate-buffered saline.

Cells were stained with anti-ssDNA or IgM isotype control, followed by washing and incubation with a phycoerythrin-conjugated anti-mouse IgM antibody. After washing, cells were analyzed with a FACScaliber flow cytometer (BD Biosciences).

**Preparation of Total Cell Lysate**—Treated cells (0.25 × 106–5 × 106) were homogenized in ice-cold radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaH2PO4, 0.25% sodium deoxycholate (w/v), 0.1% Nonidet P-40 (w/v), 1 mM Na3VO4, and 1 mM NaF) containing freshly added protease and phosphatase inhibi-
tors, 2 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM EDTA, 5 mM EGTA, and 2 mM iodoacetamide. Lysates were typically incubated on ice for at least 20 min prior to centrifugation at 15,000 × 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, and FLIPs**—Sample lysates containing 75 μg of protein for caspase-3, 15 μg for D4-GDI, 5 μg (for FLIPshort), and 30 μg (for FLIPnort, XIAP, Bax, and Bcl-2) 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 antibodies to active caspase-3, D4-GDI, FLIPshort, FLIPnort, XIAP, Bax, or Bcl-2 and detected by enhanced chemiluminescence detection reagent (Amersham Biosciences). Membranes were subsequently stripped (62.5 mM Tris-HCl, pH 6.8, 20% SDS (w/v), 100 mM β-mercaptoethanol) and re-probed for actin.

**Detection of Active Caspase-3 by Flow Cytometry**—Treated mast cells (0.5–1 × 106) were fixed in 4% paraformaldehyde and subsequently stored in 10% dimethyl sulfoxide in phosphate-buffered saline at −80 °C until staining. Cells were thawed and permeabilized with 0.1% saponin in phosphate-buffered saline for 1 h followed by incubation in 3% bovine serum albumin/phosphate-buffered saline for 1 h to block nonspecific binding. Cells were then stained with FITC-conjugated rabbit polyclonal antibody to active caspase-3, washed, and analyzed by flow cytometry.

**Statistical Analysis**—Data were analyzed by one way analysis of variance followed by Tukey’s post-test, using InstatGraphPad software (version 3.0) to determine the statistical difference between individual treatments. Statistical significance was defined as p < 0.05.

**RESULTS**

**Exotoxin A Induces Mast Cell Apoptosis**—To determine whether ETA induces mast cell apoptosis, an antibody specific for ssDNA was used. The generation of ssDNA is a specific indicator of apoptosis (30). Human mast cell line HMC-1 cells were treated with various concentrations of ETA (50, 100, 300, and 1000 ng/ml) for 24 h and subjected to formamide-induced DNA denaturation and ssDNA staining with mAb against ssDNA. ETA induced concentration-dependent mast cell apo-

**P. aeruginosa Exotoxin A-induced Mast Cell Apoptosis**

**Fig. 2. ETA-induced mast cell apoptosis is dependent on caspase activation.** HMC-1 cells were treated with 100 μg Z-VAD-fmk for 1 h prior to a challenge with 900 ng/ml ETA for 18 h. Cells were analyzed for ssDNA using flow cytometry. A, representative flow cytometry histograms are shown. B, results are expressed as the mean percentage of cells staining positive for ssDNA ± S.E. of four independent experiments (*, p < 0.001 compared with medium-treated cells).

**ETA-induced Caspase-3 Activation in Mast Cells**—There are several pathways involved in apoptosis, including caspase-dependent and caspase-independent mechanisms (31, 32). To ex-
amine the role of caspases in ETA-induced mast cell apoptosis, HMC-1 cells were pretreated with Z-VAD-fmk for 2 h before ETA treatment (300 ng/ml, 18 h). Treatment with Z-VAD-fmk completely blocked ETA-induced mast cell apoptosis (Fig. 2). The complete inhibition of ETA-induced ssDNA formation by Z-VAD-fmk, which specifically blocks multiple caspases, sug-
gests an essential role of caspases in ETA-induced mast cell apoptosis.

Caspase-3 is one of the effector caspases that is central in executing the apoptotic process. To determine whether caspase-3 is involved in ETA-induced apoptosis of mast cells, several approaches were taken. Western blotting analysis using a mAb that specifically recognizes the activated form of caspase-3 demonstrated that ETA treatment induced activation of caspase-3 in mast cells in a dose-dependent manner.
Fig. 3. ETA induces dose-dependent activation of caspase-3 in mast cells. A, HMC-1 cells were treated with increasing concentrations of ETA for 24 h and then lysed in radioimmune precipitation assay buffer. Sample lysates were subjected to SDS-PAGE and analyzed by Western blotting with monoclonal antibodies specific for active caspase-3 or actin. B and C, HMC-1 cells were treated with medium or increasing concentrations of ETA for 24 h and then fixed and permeabilized for staining with FITC-conjugated anti-active caspase-3 antibody for flow cytometric analysis. Representative histograms show ETA-treated HMC-1 5C6 (B) but not medium-treated cells stain for active caspase-3. Results are expressed as the mean percentage of positive staining cells ± S.E. of five independent experiments (*, p < 0.05) (C).

(Fig. 3A). To further examine the population of caspase-3-positive cells after ETA treatment, flow cytometry analysis was carried out using intracellular staining with a FITC-labeled mAb specific for the activated caspase-3. ETA treatment of HMC-1 cells induced a dose-dependent increase of active caspase-3-positive cells (Fig. 3, B and C). Approximately 22% of the HMC-1 cells were stained positive for the active caspase-3.

D4-GDI has been reported as an endogenous caspase-3 substrate (33). To further confirm ETA-induced caspase-3 activation in HMC-1 cells, D4-GDI cleavage was determined by Western blotting analysis. Treatment of these mast cells with ETA induced cleavage of D4-GDI in a dose- and time-dependent manner to yield a characteristic 23-kDa product (Fig. 4). Pretreatment of the cells with either Z-VAD-fmk or a specific inhibitor of caspase-3, Z-DEVD-fmk, strongly blocked ETA-induced cleavage of D4-GDI (Fig. 5), suggesting the cleavage of D4-GDI results exclusively from caspase-3 activity. Together, these results suggest a functionally active caspase-3 in mast cells after ETA stimulation.

To examine whether ETA has similar effects on other cell types, HL-60 cells (a promyelocytic cell line) were treated with ETA (500 ng/ml) for 18 h, and D4-GDI cleavage was examined by Western blot. Treatment of ETA also induced cleavage of D4-GDI to yield a 23-kDa product (data not shown), suggesting that ETA may also induce caspase 3 activation in additional cell types.

Caspase-8 but Not Caspase-9 Is Responsible for ETA-induced Caspase-3 Activation—Both caspase-8 and caspase-9 have been implicated in caspase-3 activation in different cell types (22–24). To determine whether these two caspases are involved in ETA-induced caspase-3 activation, HMC-1 cells were pretreated individually with inhibitors specific for caspase-8 or -9 before stimulation with ETA. Interestingly, a specific caspase-8 inhibitor (Z-IETD-fmk) but not a caspase-9 inhibitor (Z-LEHD-fmk) markedly blocked ETA-induced cleavage of D4-GDI (Fig. 6), suggesting an essential role of caspase-8 but not caspase-9 in ETA-induced caspase-3 activation.

ETA-induced Mast Cell Apoptosis Is Dependent on Caspase-3 and -8—To determine the importance of caspase-3 and caspase-8 in ETA-induced apoptosis of mast cells, HMC-1 cells were pretreated for 2 h with specific inhibitors for caspase-3 (Z-DEVD-fmk, 100 μM), or -8 (Z-IETD-fmk) before ETA treatment (300 ng/ml, 18 h). Mast cell apoptosis was determined by flow cytometry for the generation of ssDNA. Treatment of mast cells with Z-DEVD or Z-IETD significantly reduced the generation of ssDNA induced by ETA (Fig. 7), suggesting significant
roles for caspase-3 and caspase-8 in ETA-induced mast cell apoptosis.

ETA Down-regulates FLIPshort and FLIP(p43) but Not XIAP, Bax, and Bcl-2—Given that caspase-8 activity can be regulated by its natural inhibitor FLIPs, we determined whether ETA modulates FLIP levels in mast cells. HMC-1 5C6 cells were treated with ETA (50, 100, 300, and 1000 ng/ml) for 24 h. Cell lysates were used to determine the protein level of FLIPs by Western blotting. FLIPshort and FLIP(p43) were down-regulated by ETA treatment (Fig. 8, A and B), but other apoptosis-related molecules including Bcl-2, Bax, and XIAP were not affected by ETA treatment (Fig. 8C).

Because FLIPs are involved in regulating Fas-induced apoptosis, we determined whether ETA modulates Fas expression in mast cells. Flow cytometry analysis of ETA-treated HMC-1 cells revealed that there was no change in Fas expression on the cell surface after treatment with ETA for 3 or 24 h (data not shown). Tumor necrosis factor-mediated apoptosis, which shares common signaling pathways with Fas to caspase activation, also does not seem to be involved in the ETA-induced apoptosis because no tumor necrosis factor production can be detected in ETA-treated mast cells. Furthermore, the inclusion of anti-tumor necrosis factor neutralizing antibody (20 μg/ml, neutralization dose 50 = 0.04–0.08 μg/ml, according to the manufacturer, R & D Systems) during culture with ETA did not affect ETA-induced mast cell apoptosis (data not shown).

DISCUSSION

Mast cells not only play an essential role in allergy, they also have an important role in host defense against bacterial infection (34, 35). Mast cells are abundant in the lung, occupying 1.6–2.1% of the area of the alveolar wall (36). Importantly, many human lung mast cells directly protrude through the alveolar wall into air space (36), which allows direct interaction between mast cells and pathogens. Recently, we demonstrated active interactions between human mast cells and P. aeruginosa, resulting in the secretion of mast cell-derived cytokines and chemokines that are important in innate immunity (37–
Cells without ETA treatment (H9262NT) or treated with 5 ng/ml Campin (Camp) were used as controls. Apoptosis has been described as an essential host defense mechanism against P. aeruginosa lung infection, because a deficiency of apoptosis leads to the rapid development of polymicrobial sepsis (40). ETA is considered the most dominant and lethal of the virulent factors produced by the P. aeruginosa-induced sepsis (40). ETA down-regulates FLIP short and FLIP(p43) in mast cells. ETA-mediated responses likely involve both the surface receptor that mediates its internalization and the toxin’s intracellular targets. CD91 (the α2-macroglobulin receptor/low density lipoprotein receptor-related protein) has been identified as a receptor for ETA in mouse fibroblasts (13). In an attempt to determine whether CD91 is a receptor for ETA on mast cells, we used flow cytometry to examine CD91 expression on human mast cells. No CD91 was detected on HMC-1 or primary cultured CBMC (data not shown), but confocal microscopic analysis of ETA-treated mast cells showed intense intracellular localization (data not shown), suggesting that additional mechanisms may be involved in ETA internalization.

In conclusion, we demonstrated direct evidence of human mast cell apoptosis induced by ETA through detection of ssDNA formation. Caspase-3 activation through the caspase-8-dependent and mitochondria caspase-9-dependent pathways (22–24). Specific pathways involved in apoptosis are likely cell type-specific (24). In type I cells, such as lymphocytes, the caspase-8 pathway is sufficient to initiate apoptosis and cell death (24). Overexpression of mitochondria pathway-related antiapoptotic factors such as Bcl-2 cannot protect type I cells from death receptor-induced cytotoxicity (44). By contrast, type II cells, such as hepatocytes, require a mitochondria caspase-9 pathway for robust apoptosis (45, 46). Specific apoptotic pathways used in mast cells are unclear, although caspase-3 activity has been detected in mast cells (27, 47–50). We demonstrated that ETA-induced ssDNA formation and D4-GDI cleavage were inhibited by a caspase-8 inhibitor (Z-IETD-fmk) but not by a caspase-9 inhibitor (Z-LEHD-fmk), suggesting a role for a caspase-8 pathway in ETA-induced mast cell apoptosis.

Activation of caspase-8 is counteracted by its natural endogenous inhibitors, FLIPs. Regulation of FLIP’s expression modulates the sensitivity of mast cells to apoptosis (27). Accordingly, we examined whether ETA modulates FLIP’s expression levels in human mast cells. Our results indicated that FLIP short and FLIP(p43) were reduced by ETA treatment. FLIP short is widely recognized as a “dedicated” caspase-8 inhibitor. Although the full length of FLIP long (55 kDa) may have dual effects on pro-caspase-8 activation, its truncated form FLIP(p43) is considered to be a major inhibitor for caspase-8 activation (51). The down-regulation of FLIP short and FLIP(p43) may contribute to the ETA-induced caspase-8 or -3 pathway-dependent apoptosis in mast cells.
pathway appears to play a major role in ETA-induced human mast cell apoptosis. ETA-induced down-regulation of FLIPs likely contributes to ETA-induced activation of the caspase-8 or -3 pathway. Given that mast cells are now known to be multifunctional effector cells that have the capacity to mediate both innate and T helper type 2 cell-induced immune responses (28), these data not only provide direct evidence of the roles of caspases in ETA-mediated apoptosis but also have implications for developing strategies for manipulating mast cell homeostasis in allergy or infection.

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