Streptococcal Pyrogenic Exotoxin B Induces Apoptosis and Reduces Phagocytic Activity in U937 Cells

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Treatment of U937 human monocyte-like cells with Streptococcus pyogenes led to an induction of apoptosis in these cells. A comparison between the wild-type strain and its isogenic protease-negative mutant indicated that the production of streptococcal pyrogenic exotoxin B (SPE B), a cysteine protease, caused a greater extent of apoptosis in U937 cells. Further study using purified SPE B showed that this protease alone could induce U937 cells to undergo apoptosis, which was characterized by morphologic changes, DNA fragmentation laddering on the gel, and an increase in the percentages of hypodiploid cells. The protease activity of SPE B was required for apoptosis to proceed, since treatment with cysteine protease inhibitor E64 or heat inactivation abrogated this death-inducing effect. The SPE B-induced apoptosis pathway was interleukin-1β converting enzyme (ICE) family protease dependent. Further experiments showed that the phagocytic activity of U937 cells was reduced by SPE B. Treatment with E64 and heat inactivation both abrogated this phagocytosis-inhibitory effect. Taken together, the present data show that SPE B not only possesses the ability to induce apoptosis in mononuclear cells but also helps bacteria to resist phagocytosis by host cells.

Streptococcus pyogenes can cause serious diseases in humans, including life-threatening streptococcal toxic shock syndrome and necrotizing fasciitis (21, 24, 25). Streptococcal pyrogenic exotoxins (SPE) and various M proteins have been implicated as the virulence factors involved in S. pyogenes infection. Several lines of evidence suggest that SPE B, which functions as a cysteine protease and whose gene is carried by every strain of S. pyogenes, may be a critical virulence factor in streptococcal infections (7, 8, 14, 17, 29). Using intraperitoneal inoculation, Lukomska et al. (17) showed that speB mutants lost the ability to secrete IL-1β or had a decreased ability to cause mouse death. We adopted an air pouch model to compare the virulence of protease-negative mutants to that of S. pyogenes wild-type strains. Results showed that rates of mortality and severe skin injury were lower in mice infected with speB mutants than in those infected with wild-type strains (14). Reconstitution of SPE B in the speB mutant inoculum caused increases in the mortality rate and tissue damage.

Apoptosis has been implicated in the mechanism of bacterial pathogenesis (33), which involves a variety of host-pathogen interactions. The bacterial pathogens that are known to induce apoptosis in macrophages include Shigella spp. (31, 32), Salmonella spp. (3, 16, 20), and Yersinia spp. (19, 23). Bacterial exotoxins such as diphtheria toxin, Pseudomonas exotoxin A, and anthrax toxin may kill macrophages before they ingest and destroy the bacteria (5, 13). In Shigella infections, apoptosis was shown to be mediated by the specific activation of interleukin-1β (IL-1β) converting enzyme (ICE). Shigella flexneri produces an invasin, invasion plasmid antigen B (IpaB), that binds to ICE and causes ICE activation by a yet-unknown mechanism (4). It was hypothesized that ICE-mediated apoptosis in macrophages allows the efficient release of IL-1β, which triggers the acute inflammation that is typical of shigellosis. The ICE-dependent apoptosis has been suggested to be relevant to other bacterial diseases. The likely candidates for ICE-activating proteins include Salmonella invasion protein B (SipB) and anthrax toxin (33). SPE B was shown in vitro to cleave IL-1β precursor to produce biologically active IL-1β (11). Using U937 mononuclear cells, we asked whether a streptococcal infection induces these cells to undergo apoptosis and, further, whether SPE B plays a role in the apoptosis.

It has previously been demonstrated in vivo that the disruption of the speB gene decreased the resistance of the mutant to phagocytosis by polymorphonuclear neutrophils (PMNs) and impaired its subsequent dissemination to organs (18). In this study, the effect of SPE B as a pathogenic factor was studied in vitro. Both apoptosis and phagocytic activity were investigated with U937 cells. The requirement of SPE B protease activity and the involvement of ICE family proteases in SPE B-mediated apoptotic pathway were investigated.

MATERIALS AND METHODS

Cell culture. Human monocyte-like U937 cells were grown in RPMI 1640 medium containing 50 μM 2-mercaptoethanol supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamate, and 50 μg of gentamicin per ml.

Bacteria. S. pyogenes NZ131 (type M49, T14) was a gift from D. R. Martin, New Zealand Communicable Disease Center, Porirua. This bacterial strain lacks speA, speC, and speF genes (2, 27). The speB gene as previously described and was designated SW510 (26). Cultivation and quantification of bacteria were carried out as previously described (14).

Purification of SPE B. SPE B was purified from S. pyogenes NZ131 according to the methods of Kapur et al. (12) and Ohara-Nemoto et al. (22), with modifications as described previously (14, 26). Analysis by sodium dodecyl sulfate-polycrylamide gel electrophoresis and Coomassie blue staining showed a single band with an apparent molecular mass of 28 kDa. The N-terminal sequence of SPE B was confirmed by an Applied Biosystems 477A autosequencer. Purified SPE B was stored at –20°C in aliquots for use in the experiments.

Bacterial infection in U937 cells. U937 cells were suspended in serum-free, antibiotic-free RPMI 1640 medium, and 2 × 10⁵ cells were seeded into each well of a 96-well plate. The cells were infected with S. pyogenes NZ131 or its speB mutant SW510 at multiplicities of infection (MOI) of 40:1, 20:1, and 10:1. After a 2-h incubation, culture supernatant was removed and an equal volume of fresh
RPMI 1640 medium consisting of 5% fetal calf serum, 2 mM L-glutamate, 50 μg of gentamicin per ml, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml was added to kill extracellular bacteria. After an additional 22 h, U937 cells were harvested and fixed with 70% ethanol. Resuspended cells were washed once with phosphate-buffered saline and resuspended in 800 μl of phosphate-buffered saline. Next, 100 μl of l-mercapto-5 nucleoside and 100 μl of 400-μg/ml propidium iodide were added and the mixture was incubated at room temperature for 30 min as previously described (26). The stained cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, Calif.) with excitation set at 488 nm.

SPE B treatment of U937 cells. U937 cells were cultured at a density of 2 × 10^5/ml in a volume of 100 μl at 37°C, and various doses of purified SPE B in a 100-μl volume were added at the beginning of incubation. After various time intervals, the percentages of apoptotic cell death were determined by propidium iodide staining and flow cytometric analysis. Results of time course studies indicated that the percentage of apoptotic (hypodiploid) cells determined by propidium iodide staining as described above. Cells were also stained with Liu’s solution for the characterization of morphologic changes. In some experiments, cells were treated with 4 μg AcYVAD-CMK or zVAD-FMK (Clontech Laboratories, Inc., Palo Alto, Calif.) or 16 μM E64 (Sigma Chemical Co., St. Louis, Mo.) immediately before the addition of SPE B.

Detection of apoptosis by DNA extraction and agarose gel electrophoresis. The procedure described previously was followed (15). A total of 2 × 10^6 cells were lysed in 500 μl of cold lysis buffer containing 20 mM Tris-Cl (pH 7.4), 10 mM EDTA, and 0.2% Triton X-100 for 10 min. The lysates were centrifuged at 12,000 rpm in a microcentrifuge tube for 10 min, and the supernatants which contained fragmented DNA were collected. The fragmented DNA was incubated with 200 μg of protease K per ml at 60°C for 1 h and then digested with 100 μg of RNase per ml for an additional 2 h at 37°C. The DNA solution was extracted twice with phenol and once with chloroform-isooamyl alcohol (24:1) and then precipitated overnight with 50% isopropanol and 20°C. After centrifugation, the presence of 50% isopropanol and 20 μg of glycogen per ml at −20°C. After centrifugation, the pellet was washed once with 70% ethanol and resuspended in TE buffer containing 10 mM Tris-Cl at 4°C. SPE B, heat-inactivated SPE B (56°C for 20 to 30 min), or SPE B plus E64 were added to the different cultures. The unbound beads were separated from U937 cells by density gradient centrifugation in 2% bovine serum albumin. The binding of fluorescent beads to U937 cells was determined by flow cytometric analysis. The background fluorescence of U937 cells alone was used as the threshold level. Cells with fluorescence intensity higher than the threshold level were considered to have significant incorporation. The percent incorporation was calculated as the number of positive cells relative to the total count.

Statistical analysis was performed by using Student’s t test. Differences were considered significant at P of <0.05.

RESULTS

Induction of apoptosis by S. pyogenes NZ131 and its speB mutant in U937 cells. Adherence of S. pyogenes NZ131 and its speB mutant SW510 to U937 cells was first determined by the methods described previously (26). Results showed that the adherence abilities of NZ131 and SW510 represented as means ± standard deviations (SD) from three experiments were (4.84 ± 0.65) × 10^4 and (3.94 ± 0.70) × 10^4 CFU/ml, respectively. The ability of NZ131 to adhere to U937 cells was slightly greater than that of SW510, but the difference was not statistically significant (P = 0.33). We next investigated whether group A streptococcal infection could induce apoptosis in U937 cells and also whether the presence or absence of SPE B would cause any difference in the magnitude of apoptosis. The U937 cells were infected with NZ131 and SW510 for 2 h, and the apoptotic cell death was determined after an additional 22 h by propidium iodide staining followed by flow cytometric analysis. The percentages of hypodiploid cells were found to be 39.8 and 15.1% in NZ131- and SW510-treated groups, respectively, with an MOI of 40:1 (Fig. 1A). The percentage of apoptotic cells in the nontreated group was 5%. Figure 1B shows that the apoptosis in U937 cells was dependent on the bacterial dose. These results therefore indicated that group A streptococcal infection could induce apoptosis in U937 cells. Furthermore, the protease-positive wild-type strain caused a significantly greater extent of apoptosis than did the protease-negative mutant at MOI of 40:1 and 20:1 (Fig. 1B), suggesting an important role played by SPE B protease in augmentation of U937 cell apoptosis.

Induction of apoptosis by SPE B in U937 cells. As the wild-type strain led to a greater extent of apoptosis than did its speB mutant, we then asked whether SPE B by itself would induce apoptosis in U937 cells or whether it merely acted as an enhancing factor. Purified SPE B was added at the beginning of cultivation. The percentage of apoptotic (hypodiploid) cells was determined by propidium iodide staining and flow cytometric analysis. Results of time course studies indicated that after 24 h, there was an increase in the percentages of apoptosis when SPE B was present compared with the level for the medium-only control (Fig. 2A), and the percentages of apoptosis were more apparent after 48 h (Fig. 2B). The cell viabilities at 24 h as determined by trypan blue staining were 95% in the nontreated group and 90, 88, and 83% in the groups treated with 5, 10, and 20 μg of SPE B, respectively; at 48 h the results were 92% in the nontreated group and 79, 78, and 75% in the groups treated with 5, 10, and 20 μg of SPE B, respectively. The SPE B-induced apoptotic cell death was further
confirmed by the characteristic morphological pattern showing chromatin condensation and apoptotic-body formation (compare Fig. 3B and A) as well as by the presence of fragmented DNA bands on the gel (Fig. 3C).

**Effect of cysteine protease inhibitors on SPE B-induced U937 cell apoptosis.** The cysteine protease activity was diminished when SPE B was treated with cysteine protease inhibitor E64 or subjected to heat inactivation (14). Both treatments blocked the SPE B-induced apoptosis in U937 cells (Fig. 4A), indicating that SPE B protease activity was required for its capacity to induce apoptotic cell death. The involvement of the caspase (for cysteine-containing aspartate-specific proteases) cascade in the process of apoptosis was next assessed. Selective inhibitors of caspases were used to investigate the role of ICE family proteases in SPE B-induced U937 cell apoptosis. The caspase inhibitors were added to the cultures immediately before the addition of SPE B. Compared to the SPE B-treated group, there was a reduction in the level of apoptosis after addition of AcYVAD-CMK, an irreversible synthetic peptide inhibitor of ICE (caspase 1). The percentage of apoptotic cells reverted to a level similar to that for the medium control (Fig. 4B). Treatment with zVAD-FMK, a synthetic peptide that irreversibly inhibits the activity of a broad spectrum of ICE family proteases, also blocked SPE B-induced apoptosis completely. In an attempt to examine whether the caspase inhibitors would directly affect the activity of SPE B, the protease activity was determined in vitro by the methods described previously (22), with modifications (14). Results showed a 38% inhibition of SPE B protease activity by AcYVAD-CMK, i.e., absorbance (at 450 nm) of 0.589 ± 0.006 in the presence of 4 mM AcYVAD-CMK compared to 0.955 ± 0.015 in the control group, and no effect by zVAD-FMK, i.e., absorbance of 0.924 ± 0.040 in the presence of 4 mM zVAD-FMK compared to 0.955 ± 0.015 in the control group. Although the ICE inhibitor AcYVAD-CMK partially inhibits SPE B activity, studies showing the complete blockage of apoptosis by both AcYVAD-CMK and zVAD-FMK suggest that SPE B-induced apoptosis is mediated via the caspase pathway.

**Reduction of U937 phagocytic activity by SPE B.** A further study was performed to evaluate the effect of SPE B on phagocytosis. The phagocytic activity of U937 cells was assayed by
the cell or whether it acts through its surface receptor to initiate the apoptotic pathway.

Apoptosis has been associated with bacterial pathogenesis (33). For example, activation of apoptosis in the phagocytes, like neutrophils and macrophages, would be beneficial to the bacteria. Utilization of apoptosis to trigger inflammation, which involves ICE activation and IL-1 release, has also been considered one of the pathogenic strategies in bacterial infections. In Shigella infections, apoptosis was shown to be mediated by ICE activation. S. flexneri produces an invasin, IpaB, that binds directly to ICE and causes ICE activation (4). It was proposed that ICE activation serves the dual purposes of clearing IL-1β and initiating apoptosis. The release of IL-1β triggers inflammation, which is typical for shigellosis. Salmonella SipB, which has homology to IpaB (6, 9, 10), is a likely candidate for an ICE-activating protein (33). In this study, we showed that SPE B-induced apoptosis in U937 cells was completely abrogated by treatment with zVAD-FMK, indicating the involvement of an ICE family protease(s) in this pathway. zVAD-FMK has also been shown to block completion of the Yersinia-induced apoptotic program (23). We further showed that SPE B-induced apoptosis was blocked by AcYVAD-CMK, an irreversible ICE inhibitor. Although AcYVAD-CMK by itself partially inhibits SPE B protease activity, the same dose causes a complete inhibition of SPE B-induced U937 cell apoptosis. SPE B, which is a cysteine protease, possesses a function similar to that of ICE in that it can be cleaving IL-1β precursor to produce biologically active IL-1β (11). It is intriguing how SPE B is incorporated into the mechanism of ICE activation, which causes cleavage and release of IL-1β and triggers apoptosis, and meanwhile itself induces IL-1β production. SPE B thus seems to play redundant and multiple roles in group A streptococcal pathogenesis.

Our previous study showed that numbers of infiltrated cells in the exudates from air pouches of mice infected with SPE B-producing S. pyogenes were higher than those in exudates from mice infected with protease-negative mutants at 12 h (14). Studies by Lukomski et al. (18) showed that for 4 h following intraperitoneal injection, there were approximately equivalent amounts of PMN influx in the animals receiving the wild-type and speB mutant strains. By 22 h, animals receiving the speB mutant actually had higher peritoneal PMN counts than those injected with the wild-type strain. These studies showed that inactivation of SPE B resulted in loss of toxicity to PMNs. The reason that at 22 h animals receiving the mutant had higher PMN counts was because the mutant fails to kill PMNs. Consistent with the findings by Lukomski et al. (18), we show in this study, using U937 cells as a model, that SPE B has the capacity to augment cell death. In the animal model established previously (14), it was unclear whether SPE B would cause a higher level of infiltrated PMN apoptosis to result in a lower PMN count at a later time. An in vivo study of S. flexneri infection showed massive numbers of apoptotic cells in rabbit Peyer's patches. Macrophages, T cells, and B cells all underwent apoptosis (34). Our preliminary results show that in addition to causing apoptosis in the phagocytic cells described in this study, SPE B may play a role in the induction of non-phagocytic-cell apoptosis, such as in epithelial cell lines and T-cell lines. The binding and mode of action of SPE B on these nonphagocytic cells are subjects for future interest. It should be noted that the clinical features of S. pyogenes infection are largely characterized by tissue necrosis. Whether the increase in apoptosis caused by SPE B observed in vitro may also occur in vivo remains to be determined in further studies. The role that apoptotic cell death may play in group A streptococcal infection is not known.
Lukomski et al. (18) showed that in vivo, speB gene disruption decreased the resistance of the mutant to phagocytosis. We also found in this study that the phagocytic activity of U937 cells was reduced in the presence of purified SPE B. Results obtained from experiments in which SPE B was treated with E64 and subjected to heat inactivation revealed that SPE B protease activity was required for this inhibitory effect. The mechanism responsible for SPE B-mediated inhibition of phagocytosis remains to be elucidated. We have preliminary results showing the downregulation of cell surface molecules after SPE B treatment; its causal relationship with phagocytic activity needs further investigation.

Previous studies with insertion mutants provided evidence that SPE B may serve as an important virulence factor in group A streptococcal infection (14, 17). The roles played by SPE B as previously suggested include the enhancement of bacterial invasion (26), degradation of the extracellular matrix proteins fibronectin and vitronectin (12), inhibition of monocyctic cell activity by cleavage of urokinase plasminogen activator receptor (30), activation of matrix metalloproteinase (1), and cleavage of IL-1β precursor to biologically active IL-1β (11). We show here that in addition to these functions which contribute to endothelial cell damage, tissue destruction, and bacterial invasion and dissemination, SPE B also causes an increase in apoptotic cell death and resistance to phagocytic activity. Bacteria may therefore benefit, through the release of SPE B, by escaping from being ingested and moreover may kill phagocytes.

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REFERENCES