Apoptosis by pan-caspase inhibitors in lipopolysaccharide-activated macrophages

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Apoptosis by pan-caspase inhibitors in lipopolysaccharide-activated macrophages. Am J Physiol Lung Cell Mol Physiol 281: L1095–L1105, 2001.—Although apoptosis has been observed in macrophages during the course of infections, the mechanism of apoptosis in activated macrophages is not fully understood. This study shows that pan-caspase inhibitor benzylxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD) or t-butyloxycarbonyl-Asp-fluoromethylketone (Boc-D) caused the death of lipopolysaccharide (LPS)-activated macrophages and RAW 264.7 cells with apoptotic features. The apoptosis was also observed in lipoprotein-treated bacteria but not in CpG oligonucleotide- or flagellin-treated macrophages, indicating a difference of cellular responses downstream of different Toll-like receptors. Consistent with the induction of cell death by pan-caspase inhibitors, no activation of known caspases was detected in LPS-ZVAD-treated cells, suggesting an involvement of unknown proapoptotic caspases in the cell death. ZVAD inhibited the activation of extracellular signal-regulated kinase (ERK) and p38 but not of nuclear factor (NF)-κB induced by LPS, suggesting that the ZVAD-sensitive molecule lies upstream of the ERK and p38 pathways but downstream of the divergent site of NF-κB and mitogen-activated protein kinases. Our results demonstrate that apoptosis of macrophages induced by LPS+ZVAD is independent from the known proapoptotic caspases and suggest that activity of an unidentified ZVAD-sensitive molecule(s) is involved in the survival of LPS-activated macrophages.

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may also be important in immune responses. Indeed, apoptosis of macrophages has been observed during infection (55, 58). Death of macrophages induced by virulent bacteria such as Shigella flexneri may be important for the pathogen to successfully colonize the host (77). Macrophage death caused by S. flexneri results from the direct interaction of bacteria protein IpaB with caspase-1 (14, 27). Suppression of mitogen-activated protein (MAP) kinase (MAPK) and nuclear factor (NF)-κB pathways by ubiquitin-like protein proteases, such as YopJ from Yersinia pseudotuberculosis, was implicated as another mechanism used by bacteria to trigger macrophage cell death (52). Bacterial endotoxin lipopolysaccharide (LPS) is a complex glycolipid found in the outer membrane of all gram-negative bacteria and is believed to play a crucial role in gram-negative bacteria-induced cellular responses. The effect of LPS on macrophage cell death has been observed. LPS activates monocytes and macrophages and renders these cells to be more resistant to apoptosis (53, 70). In contrast, LPS can induce macrophage death in the presence of interferon (IFN)-γ (1, 59), and LPS can enhance Yersinia outer protein P-induced macrophage death (57). The present study showed that the death program of macrophages appears to be unique compared to other immune cells. As reported by others (53, 70), we found that activation of macrophages by LPS, which is a pathologically relevant stimulus, did not lead to cell death in vitro. Interestingly, the pan-caspase inhibitors, which prevent apoptosis in many different types of cells including T cells and B cells, caused apoptosis of LPS-activated macrophages. Induction of apoptosis by caspase inhibitors has not been reported, indicating that an unknown mechanism governs the apoptotic program in macrophages. Our data suggested the presence of a ZVAD- or Boc-D-sensitive molecule(s), probably an antiapoptotic caspase(s); the fate of LPS-activated macrophages is largely dependent on this antiapoptosis molecule(s).

**EXPERIMENTAL PROCEDURES**

**Cell culture.** The murine monocyte/macrophage cell line RAW 264.7 was maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, and 5 mM HEPES (complete DMEM). All experiments were performed in complete DMEM.

**Cell viability assays.** The extent of cell death was measured using either crystal violet uptake of live cells or propidium iodide (PI; Sigma, Irvine, CA) permeability analysis. The crystal violet uptake assays were performed as described previously (46). PI staining was assessed after incubation of the cells with 2 μg/ml of PI, followed by flow cytometric (fluorescence-activated cell sorter; FACS) analysis using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA) and CellQuest software. The extent of cell death was analyzed using either a dot plot or a histogram against the PI fluorescence.

**FACS analysis of mitochondrial membrane potential.** Cells (70–80% confluent) were treated with various agents and then detached from the plate using 0.5 mM EDTA in phosphate-buffered saline (PBS), washed with PBS, and incubated with a potential sensitive dye, 3,3′-dihexyloxycarbocyanine iodide (DiOC6(3) 40 nM) for 30 min at 37°C followed by washing with PBS. Just before FACS analysis, PI was added to each sample to a final concentration of 2 μg/ml. For each sample, 10,000 cells were examined and only PI-negative cells were gated to measure their membrane potential.

**FACS analysis of annexin and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining.** Apoptotic cells that are accompanied by phosphatidylserine exposure to the outer leaflet were analyzed by incubation of cells with FITC-conjugated annexin V (Roche Molecular Biochemicals, Indianapolis, IN). Labeling procedures followed those suggested by the manufacturer’s manual. Briefly, cells were resuspended in annexin labeling solution containing 10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM CaCl2, and fluorescein-conjugated annexin V for 15 min. After being washed twice with PBS, cell pellets were resuspended in PI (2 μg/ml) containing PBS and analyzed by flow cytometry.

**DNA strand breaks induced by apoptosis were identified using a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay kit following the manufacturer’s protocol (Roche Molecular Biochemicals).** Briefly, one to two million cells were fixed in 4% paraformaldehyde for 30 min at room temperature. After being washed twice with PBS, the cells were resuspended in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Cells were then washed twice with PBS followed by incubation for 60 min at 37°C in TUNEL reaction mixture containing 0.3 nM FITC-dUTP, 3 nmol dATP, 50 nM CoCl2, 5 U of terminal deoxynucleotidyltransferase, 0.2 M sodium cacodylate, 0.25 mg/ml of BSA, and 25 mM Tris·HCl, pH 6.6. After incubation, the cells were washed twice with PBS and analyzed by flow cytometry.

**Transmission electron microscopy.** Cells were grown in 35-mm petri dishes, fixed for 30 min on ice in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) plus 1 mM sodium cacodylate, and washed in 0.1 M cacodylate buffer. After treatment with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature and further washing in 0.1 M sodium cacodylate buffer, each sample was incubated in 0.5% tannic acid in 0.05 M cacodylate for 30 min, rinsed in 1% Na2SO4 in 0.1 M cacodylate for 10 min, and then rinsed in 0.1 M cacodylate buffer. The cells were dehydrated in 30% ethanol and en bloc stained in 1% uranyl acetate in 50% ethanol for 15 min followed by further dehydration in 50, 70, 90 and 100% ethanol. Because the cells were grown in regular plastic petri dishes, they were then cleared in 2-hydroxypropyl methacrylate two times for 15 min each and embedded in LX-112 (Ladd, Burlington, VT). After polymerization at 60% for 24 h, the plastic petri dish was broken away, and the thin resin block with cells was cut up into small pieces that were attached to blank blocks with Superglue. Thin sections were cut using a diamond knife, mounted on copper slot grids, stained with uranyl acetate and lead citrate, and examined on a Philips CM-100 electron microscope. Specific images were recorded photographically using Kodak SO-163 film.

**Colorimetric caspase assay.** Caspase activities were measured using a colorimetric caspase assay kit (Calbiochem, San Diego, CA). Assay protocols were followed as the product monograph indicated. Briefly, after induction of apoptosis, 1 × 106 cells were washed three times with PBS and lysed in 200 μl of cell lysis buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM 1,4-dithiothreitol (DTT), and 0.1 mM EDTA. The cell lysates were centrifuged at 10,000 g for 10 min at 4°C to collect cytosolic fractions.
Caspase activities were measured with the use of 200 μM caspase substrate and 10 μg of cell extracts at 37°C for 30, 60, 90, and 120 min. The substrates used were Ac-WEHD-pNA (detecting caspase-1, -4, and -5), Ac-DEVD-pNA (detecting caspase-3, -6, -7, -8, and -10), Ac-VEID-pNA (detecting caspase-6), and Ac-IETD-pNA for granzyme B. Caspase activities were expressed as optical density per hour after subtraction from control activities.

Analysis for MAPK and NF-κB activation. MAPK activities were determined by immunoblotting analysis with phospho-specific antibodies for extracellular signal-regulated kinases (ERKs) and p38 as previously described (37). The activation of NF-κB was measured by analyzing degradation of inhibitor κB (IκB) (3) and by electrophoretic mobility shift assay (EMSA). Briefly, untreated RAW 264.7 cells or cells pretreated with ZVAD (50 μM) for 30 min were exposed to LPS (0.1 μg/ml) for 15 min, 30 min, or 1 h. The reactions were stopped by the addition of ice-cold cell lysis buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM Na₂EDTA, 1 mM Na₂VO₃, 1 mM DTT, 75 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, 10 μg/ml of pepstatin A, 1 μg/ml of leupeptin, and 1% Triton X-100, followed by sonication on ice. Cell extracts were obtained by centrifugation of the homogenate at 13,000 rpm for 10 min. The extracts (20 μg/lane) were electrophoretically resolved in 11% SDS-PAGE gel, followed by transfer onto nitrocellulose membranes. Membranes were subsequently blocked with 5% BSA for 30 min, immunoblotted with antibodies, and developed by using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) detection system. Activation of MAPKs was quantified by densitometric analysis of the bands. Antibodies for ERKs, p38, NF-κB, and IκB-α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and those for phospho-specific ERKs and p38 were obtained from New England Biolabs (Beverly, MA). For EMSA, nuclear extracts of RAW 264.7 cells were incubated with a double-strand, 32P-labeled oligonucleotide containing a NF-κB binding site as a probe as described elsewhere (39).

RESULTS

LPS-induced cell death in the presence of the pan-caspase inhibitor ZVAD. LPS is a well-known activator of macrophages. LPS treatment (100 ng/ml for 24 h) of primary murine macrophages and the murine macrophage cell line RAW 264.7 induced elongation and spreading of the cells but no obvious cell death (Fig. 1A). Unexpectedly, in the presence of a pan-caspase inhibitor, ZVAD (50 μM), LPS induced cell death in both primary macrophages and RAW 264.7 cells (Fig. 1A). ZVAD by itself had no effect on cell viability (Fig. 1A). Similar results were observed in J774 cells, another murine macrophage cell line (data not shown). Recent studies revealed that Toll-like receptors (TLRs) play an essential role in mediating innate immune responses initiated by bacterial components (31, 33). LPS, lipoprotein, CpG oligonucleotide, and flagellin were shown to activate cells through different TLRs (13, 28, 62, 63). Although common responses such as NF-κB activation were observed in LPS-, lipoprotein-, CpG-, or flagellin-stimulated cells (20, 24), we found that ZVAD only caused cell death of lipoprotein- and LPS-treated but not of CpG- or flagellin-stimulated RAW 264.7 cells (Fig. 1B). LPS-induced cell death in RAW 264.7 cells in the presence of ZVAD occurred as early as 6 h after the start of treatment and reached 65% by 24 h (Fig. 2A). Complete cell death can be observed by 72 h (Fig. 2A). The percentage of cell death was dependent on both the dose of drugs and the length of time of treatment. LPS alone or ZVAD alone had no effect on cell viability even over a long time period. To attain the killing effect within 24 h, the concentration of ZVAD needed to be above 1 μM (Fig. 2B). The extent of cell death also correlated with the dose of LPS, with maximal killing achieved at a concentration of 100 ng/ml of LPS in the presence of 50 μM ZVAD (Fig. 2C).

The quantitative analysis of cell death was measured by use of two different methods: TNF cytolysis analysis (based on crystal violet dye uptake by attached living cells) or flow cytometric analysis of PI-negative cells (based on impermeability to PI of surviving cells). Both of these analyses showed similar results.

Apoptotic characteristics of LPS+ZVAD-induced cell death: nuclear and extranuclear events. To investigate whether the cell death described above was mediated by an apoptotic pathway, three nuclear changes were analyzed in LPS+ZVAD-treated RAW 264.7 cells. First, nuclear labeling with HOE-33258 indicated that LPS+ZVAD-treated cells had the typical feature of chromatin condensation by 12 h, whereas no such con-
The later stages of chromatin condensation, respectively. 

Panels of Fig. 3 ZVAD (Fig. 3 1). The B LPS mission electron microscope also conspicuously shows A (Fig. 3). Second, morphological study using a trans-densation was detected in LPS- or ZVAD-treated cells A ZVAD. 

DNA fragment in LPS+ZVAD-treated cells appears to be large, since we did not detect the 0.5- to 1.5-kb fragment ladder using agarose gel (data not shown). A similar pattern of DNA fragmentation has been observed in a number of apoptosis studies using induction by apoptosis-inducing factor (AIF), serum deprivation, transforming growth factor (TGF)-β1, or etoposide in different cell lines (40, 51, 61, 65).

In addition to nuclear changes, several extranuclear changes are often associated with the process of apoptosis (43). For example, shrinkage or condensation of cell volume is a major characteristic of apoptotic cells (10, 43, 48, 76). The population of condensed cells can be distinguished from nonapoptotic cells by FACS analysis on the basis of patterns of the forward scatter signal (indication of cell size) and intensity of PI staining (indication of the integrity of plasma membrane) (30). As shown in Fig. 4A, the population of shrunken cells (region R2) increased from 2% in untreated cells to 20% after the cells were treated with LPS+ZVAD for 12 h. Cell populations in regions R1 and R3 were dead (PI positive) and live (PI negative) cells, respectively. Another marker for the early stage of apoptosis is perturbation of the plasma membrane, which is manifested by translocation of phosphatidylserine (PS) from the inner layer of membrane to the outer leaflet (68). Double labeling with annexin V-FITC and PI was used to detect PS flipping in intact cells. As shown in Fig. 4B, PS staining was significantly increased in the PI-negative cells after LPS+ZVAD treatment. Last, it is well established that disruption of the mitochondrial inner transmembrane potential (ΔΨm) precedes nuclear changes and cell death in many cell types (29, 66).

Accordingly, ~79% of the RAW 264.7 cells lost ΔΨm 9 h after incubation with LPS+ZVAD (Fig. 4C).

LPS+ZVAD-induced apoptosis was independent of known proapoptotic caspases. To further investigate what isoform(s) of caspase was involved in this apoptotic process, we tested a battery of caspase inhibitors on the LPS-activated cells. None of the isoform-specific caspase inhibitors tested induced cell death, except for another pan-caspase inhibitor, Boc-D (Fig. 5A). This result suggested that the proapoptotic effect of ZVAD in LPS-treated cells was not mediated by inhibition of known caspases. We also measured caspase activities when the cells were treated with a nitric oxide (NO) producer sodium nitroprusside (SNP) and an anti-tumor drug etoposide that can induce RAW cell apoptosis (32, 47, 71). Caspase activity toward Ac-WEHD-pNA, which is a substrate for caspase-1, -4, and -5, was only observed in SNP-treated cells but not in cells after treatment with other stimuli including LPS+ZVAD (Fig. 5B). Activities toward Ac-DEVDP-pNA (substrate for caspase-3, -6, -7, -8, and -10) and Ac-VEID-pNA (substrate for caspase-6) were detected after 6- or 9-h treatments with SNP or etoposide, respectively, whereas no activation was induced by LPS or LPS+ZVAD treatment (Fig. 5, C and D). Activation of granzyme B (based on activity toward Ac-IETD-pNA) was observed in none of the above treatments (data not shown).

Fig. 2. Time- and dose-dependent RAW cell death induced by LPS+ZVAD. A: RAW 264.7 cells were treated with LPS (0.1 μg/ml), ZVAD (50 μM), or both for indicated periods, and the extent of cell death was measured by flow cytometric analysis of plasma membrane permeability to propidium iodide (PI). The results shown are the means ± SE (n = 3). B and C: RAW 264.7 cells were treated with different concentrations of ZVAD (B) in the presence of 0.1 μg/ml of LPS or various doses of LPS in the presence of 50 μM ZVAD. The extent of cell death was measured by use of crystal violet staining of attached live cells as indicated in EXPERIMENTAL PROCEDURES. Results represent the means ± SE (n = 3).
shown). Therefore, the known proapoptotic caspases are not involved in the LPS+ZVAD-induced cell death.

LPS+ZVAD-induced apoptosis of RAW 264.7 cells is mediated by a mechanism not yet described. Because LPS+ZVAD-induced apoptosis appears to be unique with regard to the role of caspases, we have compared this cell death with other cell death processes that are either caspase independent or caspase inhibitor enhanced. TNF-induced necrotic death of L929 cells can be significantly enhanced by ZVAD (67). This enhancement correlates with the production of oxygen radicals and can be blocked by butylated hydroxyanisole (BHA), a free radical scavenger. BHA in the present study had only a modest effect (~10% inhibition of cell death) on LPS+ZVAD-induced cell death (data not shown), suggesting a different mechanism of the cell death. Watson et al. (73) reported that inhibition of caspase-1 promoted neutrophil apoptosis during inflammation. They found that production of IL-1β is required to delay apoptosis in neutrophils and that inhibition of caspase-1 by blocking processing of proIL-1β to IL-1β accelerates apoptosis. In contrast to neutrophil death, LPS+ZVAD-induced RAW 264.7 cell apoptosis appears to be independent of the inhibition of caspase-1 (Fig. 4, A and B). Moreover, addition of IL-1β into the medium had no effect on the viability of LPS- or LPS+ZVAD-stimulated RAW 264.7 cells (data not shown). Cell death induced by LPS in IFN-γ-primed macrophages was observed, and this cell death was shown to have resulted from NO production (1, 59). We analyzed NO production in LPS-stimulated RAW 264.7 cells in the presence or absence of ZVAD and found no difference in NO production between these samples (data not shown), suggesting LPS+ZVAD-induced cell death is not a NO-dependent secondary effect. Because LPS stimulation leads to a massive secretion of TNF-α, which has been implicated in the induction of death in many different types of cells, it is possible that the cell death induced by LPS+ZVAD was a secondary event of TNF production. However, addition of TNF into culture medium had no influence on the cell viability in LPS- or LPS+ZVAD-treated RAW 264.7 cells. Thus LPS+ZVAD-induced apoptosis of macrophages is governed by an unknown mechanism. Although we cannot formally exclude the possibility that some unknown secondary events led to cell death in LPS+ZVAD-treated cells, the above data together with the kinetics of cell death shown in Fig. 2A suggest that LPS+ZVAD-induced cell death is likely to be a direct consequence of interplay between intracellular events induced by LPS and ZVAD.

A ZVAD-sensitive molecule(s) functions upstream of ERK and p38 MAPKs and downstream of the divergent point of NF-κB and MAPKs. It has been shown that LPS activates MAPKs and NF-κB, the activation of which is a key intermediary step in the production of cytokines (22, 64). We investigated whether LPS-induced activation of these molecules was influenced by ZVAD.

Fig. 3. Nuclear features of the cell death induced by LPS and ZVAD in RAW 264.7 cells. A: after treatment with LPS (0.1 μg/ml), ZVAD (50 μM), or both for 12 h, cells were fixed and stained with Hoechst 33258. The results shown are representative photomicrographs (fluorescence). Arrows indicate condensed and fragmented nuclei. B: nontreated cells (control; top left) or cells treated with LPS+ZVAD for 12 h were fixed as described in EXPERIMENTAL PROCEDURES, and their chromatins were analyzed by transmission electron microscope. The bottom right panel shows a shrunken dense cytosol of chromatin-condensed apoptotic cell (left) compared with that in normal cell (right). C: cells treated with LPS+ZVAD were analyzed for DNA strand breaks (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; TUNEL) at 0 h (shaded area), 6 h (thick solid line), and 9 h (broken line). The graph shown is representative of 3 separate experiments.
**Fig. 4.** Extranuclear features of the cell death induced by LPS and ZVAD in RAW 264.7 cells. A and B: flow cytometric density plots showing PI staining vs. forward scatter (FSC; A) or annexin (B) of cells treated for 14 and 9 h, respectively. The different cell populations were grouped as R1 (dead cells), R2 (apoptotic), and R3 (viable) in A or in quadrants in B. The results shown are representative of 3 separate experiments. C: flow cytometric histogram of cells undergoing reduction of mitochondrial transmembrane potential (ΔΨₘ). Cells were double stained with PI and 3,3′-dihexyloxacarbocyanine iodide (DiOC₆) as indicated in EXPERIMENTAL PROCEDURES. PI-negative cells were gated to analyze the ΔΨₘ of viable cells. Events under the bar were cells with dissipated ΔΨₘ. The results shown are representative of 2 separate experiments.

**Fig. 5.** Analysis of caspases in LPS-treated and ZVAD + LPS-treated RAW 264.7 cells. A: RAW 264.7 cells were incubated for 24 h with various caspase inhibitors (50 μM) in the presence of LPS (0.1 μg/ml). The extent of cell death was measured by flow cytometric analysis for PI permeability of cells as described in EXPERIMENTAL PROCEDURES. Results show the means of ± SE (n = 3). Boc-D, t-butylxycarbonyl-Asp-fluoromethylketone; zYVAD, benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethylketone; zVDVAD, benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone; zDEVD, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; zVEID, benzyloxycarbonyl-Val-Glu-Ile-Asp-fluoromethylketone; zWEHD, benzyloxycarbonyl-Trp-Glu-His-Asp-fluoromethylketone; zLEHD, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone. B–D: caspase activities were measured colorimetrically as described in EXPERIMENTAL PROCEDURES with the use of Ac-WEHD-pNA as a substrate for caspase-1, -4, and -5; Ac-DEVD-pNA for caspase-3, -6, -7, -8, and -10; and Ac-VEID-pNA for caspase-6. Cells were treated with either LPS (0.1 μg/ml), LPS (0.1 μg/ml) + ZVAD (50 μM), sodium nitroprusside (SNP; 1 mM), or etoposide (200 μM) for the periods indicated. Results show the means of 2 separate experiments, each performed in duplicate. OD₄₀₅, optical density at 405 nm.

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**L1100** ZVAD INDUCED APOPTOSIS IN ACTIVATED MACROPHAGES

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ZVAD. It is known that IκB-α mediates NF-κB activation in LPS-stimulated RAW 264.7 cells (9). A Western blotting analysis revealed that IκB-α was degraded by LPS treatment within 30 min (Fig. 6A). The presence of ZVAD did not influence LPS-induced IκB-α degradation. No influence on LPS-induced NF-κB activation by ZVAD was confirmed by mobility shift assay (Fig. 6B). LPS activated ERK1, ERK2, and p38 with similar kinetics (Fig. 6, C and D). When cells were preincubated for 30 min with ZVAD before LPS treatment, activation of the MAPKs was reduced by 50% for ERK1, 70% for ERK2, and 80% for p38 (Fig. 5, B and C). These results indicate that the ZVAD-sensitive molecule(s) may lie upstream of MAPK cascades in LPS-activated cells and downstream of the divergent point of NF-κB and MAPKs.

DISCUSSION

Although the role of caspases in apoptosis has been well established, ZVAD and other caspase inhibitors do not always prevent cell death. Although these inhibitors clearly prevent the nuclear events of apoptosis, cell death occurred at the same rate (12, 15, 18, 35, 41, 45, 61). Nevertheless, these data still support the role of caspases in mediating the nuclear events of apoptosis. The present study shows that the pan-caspase inhibitors, ZVAD and Boc-D, can induce cell death in LPS-activated macrophages (Figs. 1 and 2). The cell death manifested both nuclear and cytosolic characters of apoptosis, including shrinkage of cell size, chromatin condensation and positive TUNEL label, loss of ΔΨm, and exposure of PS (Figs. 3 and 4). The observation that a pan-caspase inhibitor is required for apoptotic cell death is unique. This extreme case supports the notion that caspases may not be indispensable for apoptosis and further indicates a possible existence of a caspase-like molecule(s) required for cell survival.

Proapoptotic effect of pan-caspase inhibitors does not appear to occur solely in activated macrophages. Recently, Lüscher et al. (42) reported an apoptosis-sensitizing effect by ZVAD in TNF-treated NIH/3T3 cells (42). In this study, ZVAD aggravated TNF-induced cell death with apoptotic features including oligonucleosomal DNA fragmentation. Accumulation of cells in the G0/G1 phase was observed in TNF+ZVAD-treated NIH-3T3 cells. Blocking the cells in G0/M phase by nocodazole had no effects on LPS+ZVAD-induced cell death (data not shown). Preblockage of cell cycle at G0/M phase by nocodazole therefore, is unknown whether ZVAD targets the same molecule(s) to execute its proapoptotic function in both systems.

LPS, after forming a complex with LPS-binding protein and CD14, interacts with TLR4 and then initiates intracellular signaling. We showed here that signaling through TLR2 (receptor for lipoprotein) but not TLR9 (receptor for CpG) or TLR5 (receptor for flagellin) also caused macrophage death in the presence of ZVAD. Thus, although there are common downstream events of different TLRs mediating signaling, the different biological consequence of different TLR activation was detected in the present study. The biochemical basis of the similar downstream reaction shared by TLR2 and -4 may be due to the high amino acid sequence homology in their intracellular domains (48%), whereas TLR5 and -9 have only 26–28% sequence identity with TLR2 or -4.

It is interesting to note the different effects of ZVAD on activation of MAPK and NF-κB. These results suggest that the location of the ZVAD-sensitive molecule(s) is upstream of MAPK but downstream of the divergent point of NF-κB and MAPK pathways. Rapid activation of MAPK and NF-κB after LPS receptor ligation in macrophages has been subjected to intensive studies in recent years.
NF-κB activation is believed to be mediated by recruitment of a death domain-containing adaptor molecule, myeloid differentiation factor-88 (MyD88), to TLRs and sequential activation of MyD88-associated IL-1R-associated kinase-1, TNF-α receptor-associated factor-6, evolutionarily conserved signaling intermediate in Toll pathways, and IkB kinase complex (IKK-α and -β). IKK complex phosphorylates IkB, which will result in the degradation of IkB and nuclear translocation of NF-κB (4). NF-κB activation is known to have an antiapoptotic effect in RAW 264.7 cells (2, 6, 26, 38, 70). Because the present study showed that ZVAD had no effects on LPS-induced NF-κB activation (Fig. 6, A and B), ZVAD is unlikely to exert its proapoptotic effect through a component of the NF-κB pathway. LPS+ZVAD-induced cell death appeared to have no relationship with another survival pathway, the phophatidylinositol 3-kinase (PI3K)-Akt pathway because the PI3K inhibitor LY-294002 had no effect on LPS+ZVAD-induced cell death.

Although activation of MAPK is a hallmark of LPS activation in macrophages, the signaling cascades between LPS receptor and MAPK are largely unknown. The activation of MAPK was impaired in TLR4 mutant cells (5), suggesting the same signaling via TLR4 to activate MAPK and NF-κB. However, the normal activation of MAPK in MyD88-deficient cells (36) suggested that the signaling already diverged before MyD88. Activation of MAPK has been shown to be involved in both apoptosis and cell survival depending on the apoptotic stimuli. For example, in serum deprivation-, satratoxin G-, or vometoxin-induced apoptosis, activation of ERK5 prevented cell death (16, 75), whereas in NO- or hyperoxia-induced apoptosis, ERKs served as proapoptotic signaling pathways (49, 54). We have tested whether inhibition of ERK and p38 had any effect on LPS+ZVAD-induced macrophage cell death. Unfortunately, the commercially available MAPK or ERK kinase and p38 inhibitors do not work well in murine macrophages. There was not a significant inhibitory effect of U-0126 or SB-203580 on ERK or p38 activity when a dose below 10 μM was used (data not shown). High doses of U-0126 (50 μM; Calbiochem) and SB-203580 (50 μM; Calbiochem) enhanced LPS+ZVAD-induced cell death two- to three-fold (data not shown), which may imply that suppression of ERK and p38 contributed to LPS+ZVAD-induced cell death. However, because of the nonspecific effect of these inhibitors at high doses (8, 25), no conclusion can be made at present.

Among the various apoptotic features, normotonic cell shrinkage is a hallmark of extranuclear apoptotic events (10, 23, 43, 48, 76). The apoptotic volume decrease occurs before cell fragmentation and is a prerequisite for apoptosis (43, 76). This early apoptotic event involves the volume regulatory K+- and Cl− channels (11, 43) and is independent of caspase activities (43). The presence of cell volume shrinkage was clearly observed only in LPS+ZVAD-treated RAW 264.7 cells (Fig. 3B and 4A), which could cue the apoptotic nuclear events. As for a mechanism in apoptotic nuclear events, AIF may be the only known factor so far that could play a role in LPS+ZVAD-induced nuclear condensation. This protein appears to be released from mitochondrial intermembrane space and cause chromatin condensation, ΔΨm disruption, and exposure of PS on the outer leaflet of the plasma membrane. More importantly, AIF executed its function without caspase activation. The involvement of AIF in LPS+ZVAD-induced cell death remains to be investigated.

The role of ZVAD in LPS+ZVAD-induced cell death may be to block an anti-apoptotic caspase. Tissue-specific expression of a truncated form of caspase-2, caspase-2s, was shown to be involved in an antiapoptotic process. Motor neurons and sympathetic neurons of caspase-2-deficient mice undergo apoptosis more effectively during development and nerve growth factor deprivation (7). Overexpression of caspase-2 also suppresses apoptosis induced by serum deprivation (72). RAW 264.7 cells expressed both the full-length and truncated forms of caspase-2 (data not shown), and thus caspase-2 is a candidate for the antiapoptotic caspase in RAW 264.7 cells.

Non-specific effects of ZVAD other than inhibition of caspases should also be considered. ZVAD was reported to inhibit calpain and cathepsin B (60). To test for the possible involvement of these proteases, the cells were exposed to pepstatin A, a calpain and cathepsin inhibitor. Pepstatin A by itself had no effect on cell viability and does not cause cell death of LPS-treated cells (data not shown). It also had no influence on LPS+ZVAD-induced cell death (data not shown). These results suggest that ZVAD does not cause cell death of LPS-activated cells by interfering with calpain or cathepsin. Although the known nonspecific effect of ZVAD is not responsible for ZVAD-mediated cell death, the possibility that ZVAD targets a caspase-unrelated molecule(s) to cause apoptosis in LPS-stimulated macrophages still cannot be excluded. Because another pan-caspase inhibitor, Boc-D, which has different structure compared with ZVAD, also has the same effect in causing cell death of LPS-activated macrophages, it is more likely that inhibition of a caspase family member(s) triggers death.

Infiltration and accumulation of macrophages often cause adverse effects in pathological conditions such as allergic and inflammatory diseases and in tumor growth. Regulation of the life span of macrophages could, therefore, play an important role in physiological and pathological processes. Under normal physiological conditions, homeostatic control of monocytes/macrophages occurs through proliferation of stem cells and apoptosis of differentiated cells. Caspase-3 has been shown to be involved in spontaneous apoptosis of monocytes (21). Activation of monocytes by LPS prolonged the life span of the cells (21). Here we showed that a pan-caspase inhibitor could induce apoptosis of LPS-activated macrophages. Our results suggested that the cell death pathway in macrophages may be altered after activation. Death of activated macrophages had a unique pattern distinct from death of other leukocytes. Selectively promoting macrophage...
death using ZVAD may be useful in developing novel therapeutic strategies for treating chronic inflammatory conditions as well as myeloid leukemia.

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