Monocytic cell necrosis is mediated by potassium depletion and caspase-like proteases

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Warny, Michel, and Ciarán P. Kelly. Monocytic cell necrosis is mediated by potassium depletion and caspase-like proteases. Am. J. Physiol. 276 (Cell Physiol. 45): C717–C724, 1999.—Apoptosis is a physiological cell death that culminates in mitochondrial permeability transition and the activation of caspases, a family of cysteine proteases. Necrosis, in contrast, is a pathological cell death characterized by swelling of the cytoplasm and mitochondria and rapid plasma membrane disruption. Necrotic cell death has long been opposed to apoptosis, but it now appears that both pathways involve mitochondrial permeability transition, raising the question of what mediates necrotic cell death. In this study, we investigated mechanisms that promote necrosis induced by various stimuli (Clostridium difficile toxins, Staphylococcus aureus alpha toxin, ouabain, nigericin) in THP-1 cells, a human monocytic cell line, and in monocytes. All stimuli induced typical features of necrosis and triggered protease-mediated release of interleukin-1β (IL-1β) and CD14 in both cell types. K⁺ depletion was actively implicated in necrosis because substituting K⁺ for Na⁺ in the extracellular medium prevented morphological features of necrosis and IL-1β release. N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone, a broad-spectrum caspase inhibitor, prevented morphological features of necrosis, plasma membrane destruction, loss of mitochondrial membrane potential, IL-1β release, and CD14 shedding induced by all stimuli. Thus, in monocytic cells, necrosis is a cell death pathway mediated by passive K⁺ efflux and activation of caspase-like proteases.

apoptosis; toxin; N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone; interleukin-1; CD14

Materials and Methods

Cells. Human peripheral blood monocytes were isolated by Ficol-Paque gradient centrifugation and Percoll discontinuous density gradient (Pharmacia, Piscataway, NJ) (26). Preparations contained 50–80% monocytes as determined by CD14 expression. THP-1 cells, a human monocytic cell line (American Type Culture Collection (ATCC), Manassas, VA), were cultured in RPMI 1640 supplemented with 5 mM HEPES, 5% FCS, and 50 µM β-mercaptoethanol (GiBCO, Grand Island, NY).

Toxin purification. Clostridium difficile toxin A (TcdA) and toxin B (TcdB) were purified from VPI 10463 strain (ATCC) culture supernatant as previously reported (24, 29).

Cell stimulation. Monocytes were kept in suspension by gentle agitation in glass tubes. THP-1 cells (500,000 cells/ml) were preincubated in 100 ng/ml lipopolysaccharide (LPS) from Escherichia coli 055:B5 (Sigma) for 1 h to trigger pro-IL-1 synthesis. Then cells were suspended in fresh medium and stimulated with TcdA, TcdB, Staphylococcus aureus alpha toxin (SAT) (Sigma, St. Louis, MO), ouabain, or nigericin (Sigma) for 2 h. Monocytes and THP-1 cells were stimulated in RPMI 1640, 5 mM HEPES, 50 U/ml penicillin G, and 50 µg/ml streptomycin. CD14 expression was induced in THP-1 cells with 1α, 25-dihydroxyvitamin D₃ (100 nM for 48 h; BioMol, Plymouth Meeting, PA) (21). The following protease inhibitors were added 15 min before stimulation: 100 µM Nα-p-tosyl-L-phenylalanine chloromethyl ketone (Sigma), 50 µM N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (Calbiochem, San Diego, CA), 50 µM N-acetyl-Tyr-Val-Ala-Asp-Aldehyde or 50 µM N-acetyl-Asp-Glu-Val-Ala-Ser-Aldehyde (DEVAD-cho; BioMol). Stock solutions (10 mM) of caspase inhibitors were prepared in DMSO. K⁺ buffer (10 mM) contained (in mM) 150 KCl, 5 NaH₂PO₄, 10 HEPES, 1 MgCl₂, 1 CaCl₂, and 5 glucose and 0.5% BSA.

Cell death assays and flow cytometry. Necrosis and apoptosis were distinguished on the basis of four independent assays: 1) morphology, 2) plasma membrane permeability to propidium iodide, 3) DNA content, and 4) cell surface expression of phosphatidylinerse. Control apoptotic cells were ob-
tained by incubation in 100 µM Na-tosyl-L-lysyl chloromethyl ketone and 25 µM cycloheximide as previously described (31). Necrotic THP-1 cells and monocytes were identified by microscopy on the basis of cell swelling and cytoplasm translucidity. Loss of membrane integrity was determined by flow cytometry using propidium iodide. Propidium iodide (10 µg/ml; Sigma) was added just before analysis. Cell DNA content was quantified by flow cytometry using propidium iodide (18). After stimulation, cells were successively fixed in 70% ethanol for 30 min, incubated with RNase (1 mg/ml) for 1 h, and stained in 10 µg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Nonidet P-40. Phosphatidylserine expression was detected by flow cytometry using FITC-annexin V (Trevigen, Gaithersburg, MD). Mitochondrial membrane potential was measured using rhodamine 123 (Molecular Probes, Eugene, OR) as previously described (27). Cell surface CD14 was quantified by flow cytometry using FITC-MY4 monoclonal antibodies (Coulter, Miami, FL). Flow cytometry analysis was performed using a FACScan and the Cellquest software (Becton-Dickinson, San Jose, CA).
IL-1β and soluble CD14 concentrations. Mature IL-1β levels were measured by ELISA (Cistron, Pine Brook, NJ). Soluble CD14 was also quantified by ELISA (Medgenix Diagnostics, Fleurus, Belgium).

Intracellular K⁺. Intracellular K⁺ concentration ([K⁺]ᵢ) was measured using the fluorescent dye K⁺-binding benzofuran isophthalate (PBFI) (13). THP-1 cells (10⁶ cells/ml) were loaded with 5 µM PBFI for 90 min (Molecular Probes, Eugene, OR). After a washing, cells were resuspended (500,000 cells/ml) in (in mM) 150 NaCl, 5 NaH₂PO₄, 10 HEPES, 1 MgCl₂, 1 CaCl₂, and 5 glucose and 0.5% BSA. Cells were stimulated for 2 h at 37°C, and fluorescence was measured using a Perkin-Elmer LS-50B spectrofluorometer (Norwalk, CT). Excitation wavelength was set at 340 or 380 nm, the isosbestic point. Emission was measured at 505 nm (5-nm slit width). The ratio of the fluorescence intensities (340/380 nm) was used to determine K⁺ concentration. The PBFI assay was calibrated by equilibrating [K⁺]ₑ with known extracellular K⁺ concentration ([K⁺]ₑ). [K⁺]ₑ was increased from 0 to 150 mM in 50-mM increments by substituting K⁺ for Na⁺, and cells were permeabilized using 30 nM SAT. There was a close linear correlation between fluorescence values and [K⁺]ₑ (Pearson correlation coefficient ≥ 0.95).

Statistical analysis. Statistical analyses were performed using one-way ANOVA followed by the Student-Newman-Keuls test (SigmaStat, Jandel Scientific Software, San Rafael, CA).

RESULTS

Necrosis of THP-1 cells and monocytes is associated with release of CD14 and IL-1β. In preliminary experiments, we tested a series of agents for their ability to induce monocyte necrosis. We found that TcdA (100 nM) and TcdB (10 nM), SAT (10 µg/ml), nigericin (10 µM), and ouabain (200 µM) cause monocyte and THP-1 cell necrosis within 1.5–3 h. Morphological features of necrosis were identical in both cell types (Fig. 1, A-F). The cytoplasmic translucence associated with necrosis caused a drop in forward scatter height as measured by flow cytometry (Fig. 1, K-L). The toxins used in these experiments have distinct mechanisms of action. Briefly, TcdA and TcdB catalyze the monoglucosylation of Rho proteins, which disrupts the actin cytoskeleton (12). SAT forms plasma membrane pores permeable to monovalent cations (2, 3). Ouabain inhibits the Na⁺-K⁺ pump ATPase, whereas nigericin is a K⁺ ionophore. All stimuli caused cell swelling and permeability to prop-
idium iodide in <2 h (nigericin and ouabain not shown) (Figs. 1 and 2A). In contrast to apoptotic THP-1 cells, necrotic cells did not show a decrease in DNA content (Fig. 1, A-D) or phosphatidylserine exposure before loss of membrane integrity (data not shown). In addition to causing cell necrosis, all stimuli induced the release of mature IL-1β from LPS-stimulated THP-1 cells within 2 h (Fig. 2B). In normal monocytes TcdB (1 nM) also induced a 16.6 ± 2.0-fold increase in IL-1β release as 30 min (means ± SE, n = 3, P < 0.05). All stimuli also caused a marked reduction in cell surface expression of the LPS receptor CD14 in vitamin D3 differentiated THP-1 cells (undifferentiated THP-1 cells do not express CD14; Fig. 2C). In monocytes TcdA, TcdB, and SAT also reduced cell surface CD14 expression (by 91, 81, and 89%, respectively). This was associated with a corresponding release of soluble CD14 (Fig. 2, D-E). Thus, in normal monocytes as well as in THP-1 cells, necrosis induced by different toxins is associated with the rapid release of IL-1β and soluble CD14. Despite their distinct mechanisms of action, these agents stimulate a common pattern of events, suggesting activation of common mediators of necrosis.

Blocking K+ efflux prevents monocyte necrosis and IL-1β release. In many cell types, both SAT and ouabain cause K+ depletion as a direct consequence of their mechanism of action. We tested the hypothesis that K+ depletion is involved in THP-1 cell and monocyte necrosis. In THP-1 cells all stimuli caused a decrease in [K+] in controls (140 ± 5 mM) and in cells stimulated with SAT (42 ± 5 mM), TcdB (38 ± 4 mM), nigericin (5 ± 20), and ouabain (80 ± 4; all values are means ± SE). We next examined the effect of blocking K+ efflux by substituting K+ for Na+ in the extracellular medium. In control THP-1 cells, the high K+ buffer inhibited morphological features of necrosis (B, E) and IL-1β release (G) induced by TcdB or SAT. TcdB-exposed monocytes show shape modification (elongation and teardrop morphology) associated with toxin-mediated Rho glucosylation (B, arrows). High-K+ buffer did not prevent toxin binding or internalization, inasmuch as its subsequent replacement by normal buffer was followed quickly by necrosis (C, F) and IL-1β release (G), which occurred within 20 min. Mean IL-1β ± SE are shown; n = 3, *P < 0.05 vs. control in that buffer.

Fig. 3. Substitution of K+ for Na+ in extracellular medium prevents necrosis of THP-1 cells and IL-1β release. THP-1 cells were stimulated with 100 ng/mL LPS for 1 h to trigger pro-IL-1 synthesis and then incubated for 2 h with 10 nM TcdB or 10 nM SAT in physiological buffer containing 150 mM NaCl and 5 mM KH2PO4 (A, D) or in buffer containing 150 mM KCl, 5 mM NaH2PO4 (B, E). In normal buffer TcdB and SAT induced necrosis in 50 and 90% of cells, respectively (A, D, arrows show examples of necrotic cells). High-K+ buffer inhibited morphological features of necrosis (B, E) and IL-1β release (G) induced by TcdB or SAT. TcdB-exposed monocytes show shape modification (elongation and teardrop morphology) associated with toxin-mediated Rho glucosylation (B, arrows). High-K+ buffer did not prevent toxin binding or internalization, inasmuch as its subsequent replacement by normal buffer was followed quickly by necrosis (C, F) and IL-1β release (G), which occurred within 20 min. Mean IL-1β ± SE are shown; n = 3, *P < 0.05 vs. control in that buffer.
by normal medium triggered necrosis in up to 40% of
the control monocytes. Taken together, these data
support the hypothesis that K⁺ efflux regulates mono-
cyte necrosis induced by various stimuli.

z-VAD-fmk, a broad-spectrum caspase inhibitor, pre-
vents THP-1 cell and monocyte necrosis. In monocytes
not undergoing necrosis, inducing a net K⁺ efflux
activates caspase-1 (formerly IL-1 converting enzyme),
Fig. 4. Broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) but
not caspase-3 inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-cho), prevents necrosis of THP-1 cells and
monocytes. THP-1 cells (A, B) and normal monocytes (C, D) were preincubated for 30 min in culture medium
supplemented with 0.5% DMSO (used as solvent for caspase inhibitor; A, C) or with 50 µM z-VAD-fmk (B, D). Then
they were exposed to TcdB. z-VAD-fmk delayed morphological features of necrosis induced by TcdB. z-VAD-fmk also
prevented necrosis induced by the other stimuli, whereas DEVD-cho had no effect (data not shown). E: THP-1 cells
were preincubated for 30 min with 50 µM z-VAD-fmk and then stimulated with TcdB (10 nM), SAT (10 nM), ouabain
(200 µM), or nigericin (5 µM). Necrotic cell number was determined by measuring cell permeability to propidium
iodide using flow cytometry. z-VAD-fmk caused significant reduction in percentage of necrotic cells following
exposure to all stimuli (mean ± SE, n = 3, P < 0.05). Caspase-3 inhibitor DEVD-cho does not prevent necrosis.
THP-1 cells were preincubated for 30 min in culture medium with 0.5% DMSO (F, G), 50 µM DEVD-cho (H), or 50
µM z-VAD-fmk, a broad-spectrum caspase inhibitor (I). Subsequently, cells were incubated for 90 min with 10 nM
SAT and then analyzed by flow cytometry for propidium iodide uptake. z-VAD-fmk but not DEVD-cho prevented
propidium iodide uptake (data representative of 3 experiments).
leading to IL-1β release (22, 30). In THP-1 cells undergoing necrosis, we found that N-acetyl-Tyr-Val-Ala-Asp-aldehyde, a specific caspase-1 inhibitor, abrogated IL-1β release by all stimuli, but did not prevent necrosis (data not shown). We tested whether other caspases, in addition to caspase-1, might be implicated in THP-1 cell necrosis. Cell preincubation with z-VAD-fmk (50 µM), a broad-spectrum caspase inhibitor, completely prevented the morphological features of necrosis in THP-1 cells and in monocytes (Fig. 4, A-E; in separate control experiments 0.5% DMSO, the solvent for caspase inhibitors, did not affect necrotic cell number in control or stimulated cells). z-VAD-fmk also prevented THP-1 cell membrane leakage in contrast to the caspase-3 inhibitor DEVD-cho (Fig. 4, F-I). In addition, z-VAD-fmk inhibited IL-1β release and CD14 shedding in THP-1 cells stimulated by all stimuli (>95% inhibition; Fig. 5). These findings indicate that key events in monocyte cell necrosis are mediated by activation of caspase-like proteases.

K+ depletion may precede or follow protease activation during monocyte necrosis. To determine whether K+ efflux was upstream or downstream of protease activation, we measured [K+]i in THP-1 cells protected from necrosis by preincubation with the caspase inhibitor z-VAD-fmk. The pore-forming toxin SAT and the Na+-K+ pump ATPase inhibitor ouabain both caused marked [K+]i depletion in z-VAD-fmk-treated cells, after 1 h of incubation. No changes in [K+]i were observed in z-VAD-fmk-treated cells challenged with TcdB or nigericin (Fig. 6). Thus agents that induce [K+]i depletion (e.g., SAT and ouabain) can trigger protease activation and subsequent necrosis. However, for other stimuli (e.g., TcdB or nigericin) [K+]i depletion is the result, not the cause, of protease activation.

z-VAD-fmk prevents loss of mitochondrial membrane potential. In the effector phase of apoptosis as well as in necrosis, mitochondria lose osmotic homeostasis and membrane potential. We tested whether activation of caspase-like proteases occurs upstream or downstream to mitochondrial membrane depolarization. THP-1 cells were stained with rhodamine 123, a fluorescent probe leading to IL-1β release (22, 30). In THP-1 cells undergoing necrosis, we found that N-acetyl-Tyr-Val-Ala-Asp-aldehyde, a specific caspase-1 inhibitor, abrogated IL-1β release by all stimuli, but did not prevent necrosis (data not shown). We tested whether other caspases, in addition to caspase-1, might be implicated in THP-1 cell necrosis. Cell preincubation with z-VAD-fmk (50 µM), a broad-spectrum caspase inhibitor, completely prevented the morphological features of necrosis in
uptaken by mitochondria that maintain a membrane potential. The distribution of rhodamine 123 fluorescence was bimodal in control cells, 70% of them showing a 10-fold higher signal than the remaining 30%. z-VAD-fmk preincubation prevented the drop in rhodamine 123 fluorescence caused by all stimuli (Fig. 7; TcDB and ouabain not shown). These findings suggest that activation of caspase-like proteases precedes mitochondrial membrane depolarization in THP-1 cells undergoing necrosis.

**DISCUSSION**

The present study shows that necrosis of monocytes and THP-1 cells induced by various stimuli is a cell death pathway associated with IL-1β release and CD14 shedding. Moreover, this pathway involves caspase-like proteases and K^+^ depletion.

The broad-spectrum caspase inhibitor z-VAD-fmk has been shown to inhibit various pathways of apoptosis in many cell types. In THP-1 cells, z-VAD-fmk (50 µM) prevented apoptosis (31) and inhibited the cleavage of caspase-2, -3, -6, and -7 (15). z-VAD-fmk also prevented z-DEVD-AFC (benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin) cleavage (a caspase-3 substrate; IC_{50} 1.2 µM) and activation of caspase-3 (15). To our knowledge, z-VAD-fmk has not been reported to inhibit necrosis. In contrast, it was shown to promote necrotic cell death in some other cell types (9, 27), suggesting important cell type-related differences in the control of necrosis. Caspase-3, a central effector of apoptosis, does not appear to be involved in THP-1 cell necrosis because DEVD-cho did not prevent necrosis. However, we cannot exclude the possibility that DEVD-cho did not achieve sufficient intracellular concentrations to block caspase-3 activity in our experiments.

Although the target(s) of z-VAD-fmk was not identified in this study, the data strongly suggest that monocyte necrosis involves the activation of caspases or caspase-like proteases. In monocytes not undergoing necrosis, IL-1β release involves caspase-1 activation, is enhanced by K^+^-efflux, and is blocked by increasing K^+^ concentration in the extracellular medium (22, 30). In this study, we show that IL-1β release and necrosis are associated and that both are inhibited by intracellular K^+^ and by a broad-spectrum caspase inhibitor. These data support the hypothesis that monocyte necrosis is mediated by activation of caspases or other z-VAD-fmk sensitive proteases.

Early studies reported that necrotic cell death is associated with rapid loss of intracellular K^+^ (9). To our knowledge, this is the first report that maintaining [K^+]_i can inhibit cell necrosis. A role for K^+^ in pro-apoptotic caspase regulation was recently reported. In HeLa cells and cerebellar granules, K^+^-deprivation caused caspase-3 activation and apoptosis (4, 5). Moreover, K^+, at a physiological intracellular concentration (150 mM), prevented recombinant caspase-1 activation in vitro (5). Although this effect was not specific to K^+^ (Na^+^, Ca^{2+} and Mg^{2+} at 150 mM were as potent as K^+^), these results support the hypothesis that caspase activation is prevented in healthy cells by K^+, the main intracellular cation. In support of this hypothesis, apoptosis induced by various stimuli in lymphocytes was associated with progressive K^+^ loss. Replacement of Na^+^ by K^+^ in the extracellular medium prevented lymphocyte apoptosis and caspase-3 activation (11). The time course of K^+^ depletion in these lymphocytes was much slower (56 mM after 8 h) than in THP-1 cells undergoing necrosis (median value 40 mM after 2 h). The rapid loss of plasma membrane integrity that characterizes necrosis is likely to allow rapid intracellular K^+^ depletion and may promote necrosis versus apoptosis. Thus intracellular K^+^ appears to be a key inhibitor of cell death through its ability to prevent caspase activation.

The findings that monocyte necrosis involves intracellular K^+^ and caspase-like proteases is compatible with the emerging concept that apoptosis and necrosis may be functionally linked cell death pathways (reviewed in Ref. 14). Both modes of cell death involve loss of mitochondrial membrane potential and osmotic homeostasis, under the control of Bcl-2 proteins. It is interesting that Bcl-xL, an anti-apoptotic protein of this family,
forms cation-selective membrane channels and is capable of conducting a K⁺ current (18). Recently, Bcl-xL was found to inhibit necrosis caused by inhibitors of oxidative phosphorylations (27). These studies provide further evidence that the regulation of intracellular cations plays a key role in both apoptosis and necrosis. Elucidating the mechanism by which intracellular K⁺ prevents caspase activation will represent a key step in understanding the regulation of cell death.

Based on the findings of this study and the current literature, we speculate that monocyte necrosis results from activation of K⁺-regulated caspases (or caspase-like proteases), which in turn cause loss of mitochondrial membrane potential and permeability transition. This pathway of cell death can be induced by marked K⁺ depletion or by other mechanisms independent of [K⁺]e. Permeability transition and loss of mitochondrial osmotic homeostasis further promote K⁺ loss and caspase activation by a self-amplifying mechanism involving ATP depletion (inhibition of K⁺ uptake) and plasma membrane disruption, two classical features of necrosis. Further studies are needed to verify this hypothesis and to identify these "pro-necrotic" caspases.

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