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Treatment of Cells with Detergent Activates Caspases and Induces Apoptotic Cell Death

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Abstract. Due to their amphiphilic properties, detergents readily disrupt cellular membranes and cause rapid cytolysis. In this study we demonstrate that treatment of cells with sublytic concentrations of detergents such as Triton X-100, Nonidet P-40, n-octylglucoside and the bile salt sodium deoxycholate induce typical signs of apoptosis including DNA fragmentation and cleavage of poly(ADP-ribose) polymerase molecules. The detergent concentration required for apoptosis was below the critical micellar concentration. Induction of apoptosis was not restricted to human cells but similarly occurred in a variety of other vertebrate cell lines. Unstimulated peripheral blood mononuclear cells were susceptible to apoptosis induction by detergent suggesting that apoptosis in this circumstance is not mediated by CD95. Cell death was not due to influx of calcium from the medium. Apoptosis was blocked and cytolysis prevented by treatment with peptide inhibitors of caspases. These findings suggest a process of apoptosis that is initiated upon nonspecific alterations at the cell membrane level. Physiologic correlates of this process still have to be defined.

Key words: Detergent — Triton X-100 — Nonidet P-40 — n-Octylglucoside — Sodium deoxycholate — Apoptosis — Caspase — Jurkat T lymphoblast

Introduction

Apoptosis describes a distinctive actively executed process of cell death. It is characterized microscopically and biochemically by a variety of features including shrinkage of the cell, membrane blebbing, chromatin condensation and DNA fragmentation (Wyllie, Kerr & Currie, 1980), and degradation of intracellular proteins such as the poly(ADP-ribose) polymerase (PARP), lamin and others (Casciola-Rosen et al., 1994; Lazebnik et al., 1994; Casiano et al., 1996). Apoptosis is induced in susceptible cells by a broad spectrum of events and agents such as crosslinking of the CD95 molecule (Trauth et al., 1989; Yonehara, Ishii & Yonehara, 1989) and tumor necrosis factor receptor-1 (TNF-R1) (Schmid, Tite & Ruddle, 1986; Dealtry et al., 1987; Rubin et al., 1988), withdrawal of growth factors (Duke & Cohen, 1986; Kyprianou & Isaacs, 1988; Martin et al., 1988; Terada et al., 1989; Williams et al., 1990), viral infections (Wattre, Bert & Hober, 1996), cytotoxic T cell activity (Sanderson, 1976; Don et al., 1977; Russell et al., 1982), anticancer drugs such as etoposide, daunorubicin, adriamycin and several others (Kaufmann, 1989; Barry, Behnke & Eastman, 1990), ceramide (Obeid et al., 1993), ionizing radiation (Skalka, Matyasova & Cejkova, 1976; Sellins & Cohen, 1987; Yamada & Ohyama, 1988), calcium ionophores (Kizaki et al., 1989; McConkey et al., 1989), glucocorticoids (Burton, Storr & Dunn, 1967; Cohen & Duke, 1984), hyperthermia (Fairbairn et al., 1995) and other events (reviewed in: (Schwartzman & Cidlowski, 1993)). The first links in the chain of events stimulated by these factors are diverse and have only partially been elucidated. They include crosslinked cell membrane receptors, damage of the DNA and activated cytoplasmic or nuclear enzymes. However, a process central to the majority if not all apoptotic processes is activation of a series of caspases (reviewed in: (Nagata, 1997)).

Cell membrane integrity is essential for the cell to maintain intracellular homeostasis, viability and function. Small irritations of the membrane may alter the

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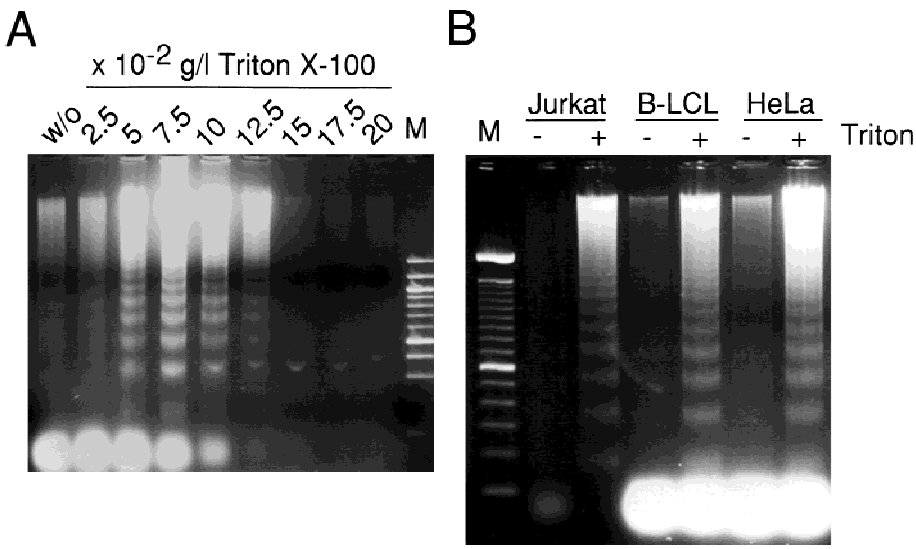

Fig. 1. Treatment of Jurkat T lymphoblasts with Triton X-100 causes apoptosis. Agarose gel electrophoresis of the intracellular LMW DNA of 1–2 × 10^6 cells treated with Triton X-100 for 4 hr. (*A*) Jurkat cells incubated in cell culture medium without (w/o) treatment or with 2.5–20 × 10^{-2} g/l Triton X-100. (*B*) Jurkat T cells, 7.5 × 10^{-2} g/l Triton; B-LCL, 10 × 10^{-2} g/l Triton; HeLa cells, 10 × 10^{-2} g/l Triton X-100. (*C*) C32 chicken fibroblasts, 10 × 10^{-2} g/l Triton; Vero African green monkey fibroblasts, 12.5 × 10^{-2} g/l Triton; P815 mouse mastocytoma cells, 7.5 × 10^{-2} g/l Triton X-100. (*D*) Unstimulated human PBMCs, 7.5 × 10^{-2} g/l Triton X-100. M: molecular weight marker. (*Continued on next page.*)

permeability for water, small neutral solutes and ions. If the membrane defect is more profound, lysis occurs accompanied by efflux of proteins and other macromolecules (Helenius & Simons, 1975). Since a balanced intracellular milieu is critical for the function of the enzymes both in the cytosol and in the organelles, circumstances that lead to altered cell membrane permeability may cause the cell to counteract by regulatory mechanisms. Alternatively, it could be hypothesized that the affected cell may execute its own death to avoid overt cytolysis and damage due to this process. It has recently been reported that Triton X-100 induces signs of apoptotic cell death in prostate, colon, and hepatic tumor cells by a mechanism not requiring protein synthesis (Borner et al., 1994; Ahn et al., 1997). The goal of our study was to investigate whether induction of apoptosis represents a more general aspect of the interaction of low concentrations of detergent with a cell and to determine the role of caspases in this apoptotic process.

Materials and Methods

Cells and Media

The CD4+ human T lymphoblastoid cell line Jurkat E6-1 (Weiss, Wiskocil & Stobo, 1984) and the mouse mastocytoma cell line P815 were obtained from the American Tissue Culture Collection (ATCC). B lymphoblastoid cell lines (B-LCL) were generated by transformation of B lymphocytes with Epstein-Barr virus (EBV) in vitro as described previously (Walker et al., 1988). Jurkat cells, B-LCL, and P815 cells were cultured in RPMI-1640 medium supplemented with 10 or 15% FCS, antibiotics and Hepes buffer. HeLa carcinoma cells and Vero African green monkey fibroblasts were obtained from the ATCC and cultured in minimal essential medium containing 10% FCS. The chicken fibroblast cell line C32 was cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 8% FCS and 2% chicken serum. Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy laboratory workers by Ficoll-Hypaque (Life Technologies, Karlsruhe, Germany) density gradient centrifugation. PBMCs were maintained in RPMI-1640 medium with 10% FCS.

Treatment of Cells with Detergents

Triton X-100, Nonidet P-40, n-octylglucoside and sodium deoxycholate (Sigma Biochemicals, Deissenhofen, Germany) were used as detergents. Cells were incubated in medium with or without 0.1–20 × 10^{-2} g/l detergent at 37°C for 4–8 hr. Cell suspensions were centrifuged and cells processed for subsequent analysis. As positive control for apoptosis, Jurkat cells were treated for 4 hr with the anti-CD95 mAbs CH-11 (200–500 ng/ml) or 7C11 (200 ng/ml, Coulter-Immunotech, Hamburg, Germany).

Determination of Intracellular DNA Fragmentation

Low molecular weight (LMW) DNA was prepared as described previously (McConkey et al., 1991; Heinkelein, Pilz & Jassoy, 1996). Briefly, after incubation, cells (1.5–2 × 10^6) were lysed for 10 min on ice in a 20 mM Tris/EDTA solution containing 0.5% Triton X-100. High molecular weight DNA was separated from LMW DNA by high speed centrifugation. LMW DNA in the supernatant was obtained after phenol/chloroform extraction and isopropanol precipitation. DNA electrophoresis was performed using 1.8% agarose gels and a 100 base pair ladder molecular weight marker.

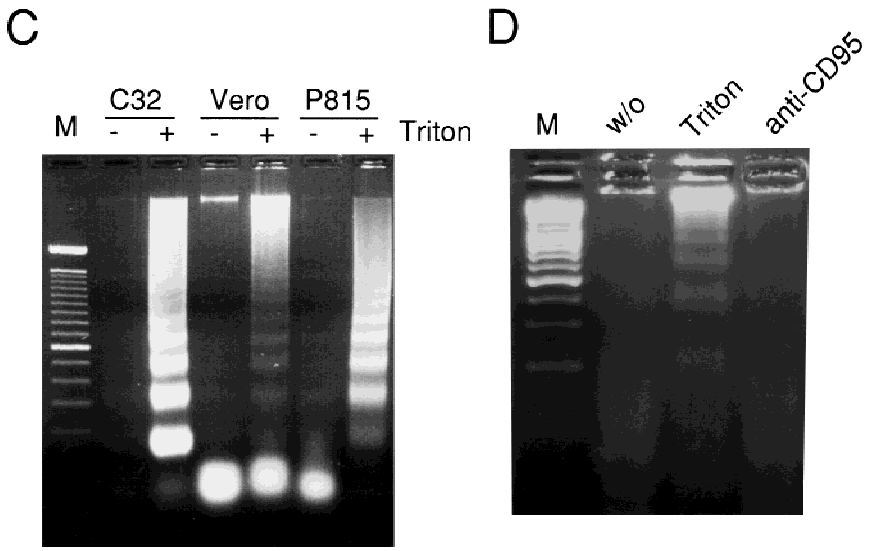

Fig. 1. *Continued.*

Analysis of Poly(ADP-Ribose) Polymerase (PARP) Cleavage

Jurkat lymphoblasts ($2–2.5 \times 10^6$) were treated with detergent for 4 hr. Cells were centrifuged, resuspended and lysed in 30 μl Tris/EDTA solution (20 mM) containing 0.5% Triton X-100. Proteins were separated by SDS polyacrylamide gel electrophoresis and analyzed by Western blotting using the mouse anti-PARP mAb C2-10 (diluted 1:2000; PharMingen, San Diego, CA), which recognizes both the 116 kD intact and 85 kD cleaved forms of the molecule. A peroxidase-conjugated rabbit anti-mouse antiserum (diluted 1:500; Dako, Hamburg, Germany) was used as a secondary antibody, and the PARP protein visualized using as substrate freshly prepared 0.02% 3-amino-9-ethyl-carbazole in 5% dimethylformamide, 0.015% H_2O_2 in 50 mM sodium acetate (pH 5.0).

Inhibitors of Caspases

Cells were treated with detergents in the presence and absence of agents that interfere with the activity of caspases including the peptides ZVAD-FMK (Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, 50 μM; Enzyme Systems Products, Sierra Lane, CA) and YVAD-CMK (Acetyl-Tyr-Val-Ala-Asp-chloromethylketone, 300 μM; Bachem Biochemica, Heidelberg, Germany) which are broadly active against several caspases, and DEVD-CHO (Acetyl-Asp-Glu-Val-Asp-aldehyde, 300 μM; Bachem Biochemica) which has a predilection for caspase-3. As control, the thrombin inhibitor FPR-CMK (H-D-Phe-Pro-Arg-chloromethylketone, 300 μM; Bachem Biochemica) was used. The peptides were not cytotoxic at the concentrations indicated (H. Ohnimus and C. Jassoy, *data not shown*).

Cytotoxicity Assay

For the determination of detergent cytotoxicity, the standard chromium release assay was performed (Heinkelein et al., 1996). Briefly, Jurkat cells (2×10^4) were labeled with 100 μCi $Na_2^{51}CrO_4$ for 60 min and treated with detergent in the absence or presence of the caspase inhibitor ZVAD (50 μM) or the control peptide FPR (50 μM) for 8 hr. After incubation, supernatants were harvested and counted in a gamma counter. Percent specific lysis was calculated by the formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum chromium release was determined by lysis of the cells in 1.5% Triton X-100.

Flow Cytometric Analysis

Jurkat E6-1 cells (10^5) were seeded in the wells of a 96-well flat-bottomed plate and incubated in the presence of detergent for 5 hr. Some of the wells contained the caspase inhibitor ZVAD (50 μM). Propidium iodide was added and cells were analyzed by flow cytometry using a BD Facscan and Cell Quest software.

Results

Treatment with Triton X-100 Causes Apoptosis

Jurkat T lymphoblasts were treated with Triton X-100 at concentrations ranging from 2.5 to 20×10^{-2} g/l. After incubation for 4 hr, intracellular LMW DNA was prepared and analyzed by agarose gel electrophoresis. Figure 1*A* shows that treatment of the cells with Triton X-100 at $5–12.5 \times 10^{-2}$ g/l caused fragmentation of the DNA into multiples of approximately 180 base pairs. No DNA fragmentation occurred in untreated cells. At higher detergent concentrations, cells were completely lysed and neither LMW DNA nor cellular RNA were detectable. Additional time-course experiments with Jurkat cells demonstrated that detectable DNA fragmentation occurred in less than 90 min (*data not shown*).

To examine whether induction of apoptosis by Triton X-100 similarly occurs in other cells and at which concentration of the detergent, a set of human cell lines

of different tissue origin was tested. Figure 1*B* shows that DNA fragmentation was observed with additional cell lines such as Epstein Barr virus-transformed B lymphocytes and adenocarcinoma cells. Apoptotic cell death was similarly induced by Triton X-100 in other B and T lymphoblastoid cell lines, including A3.01 and CEM-T2, as well as in HL-60 promyeloblasts and in melanoma cell lines that do not develop spontaneous metastases when growing in nude mice such as IF6 and 530 (*data not shown*).

To examine whether induction of apoptosis by Triton is limited to human cells or requires a human factor, cells from other vertebrate species were examined. Figure 1*C* illustrates DNA fragmentation upon treatment of African green monkey and chicken fibroblasts, as well as mouse mastocytoma cells. The concentration range at which apoptosis was induced in these diverse cell lines was similar to that observed with Jurkat cells.

Unstimulated human PBMCs and PBMCs activated for several hours with PHA are not susceptible to apoptosis induction through the CD95 molecule despite expression of this molecule on the cell surface (Peter et al., 1997). To examine whether the mechanism that prevents apoptosis induced by CD95 is similarly operative in detergent-induced apoptosis, freshly prepared PBMCs were treated with Triton. Figure 1*D* illustrates that unstimulated PBMCs underwent apoptosis when treated with Triton indicating that critical prerequisites for the execution of the apoptotic program are present in unstimulated PBMCs and demonstrating that detergents induce apoptosis by a different mechanism.

Initiation of Apoptosis by Detergent Does not Depend on the Presence of Calcium-ions in the Medium

The interaction of detergent with biomembranes may result in increased permeability for water and electrolytes. Several electrolytes have been attributed to the development of apoptosis including calcium and magnesium (Patel, Bronk & Gores, 1994; McConkey & Orrenius, 1997). To test whether the initiation of apoptosis is due to influx of calcium ions from the extracellular medium, cell death induction by detergent was tested in Jurkat cells treated with Triton X-100 in culture medium containing 1.5 mM of the calcium chelating agent EGTA. The concentration of EGTA used is sufficient to bind all calcium ions in the medium. Figure 2 shows that detergent-induced apoptosis was not affected by depletion of extracellular calcium ions indicating that influx of calcium from the extracellular space does not underly initiation of apoptosis.

Detergent Activates Caspases

Apoptotic cell death is generally mediated and executed by a series of caspases. To test whether induction of apoptosis by detergents similarly involves caspase activity, cleavage of the PARP molecule which is a substrate of several of the caspases was analyzed in Jurkat cells treated with Triton X-100. As shown in Fig. 3, treatment with detergent caused cleavage of PARP and peptide inhibitors of caspases prevented PARP cleavage and subsequent DNA fragmentation. To examine whether treatment with detergent stimulates additional cytoplasmic enzymes, we performed in vitro protein kinase assays to investigate whether kinases of the mitogen and stress pathway are stimulated. As positive controls, we used phorbolester- and anisomycin-stimulated cells, which demonstrate the phosphorylation of extracellular regulated protein kinase (ERK) and stress activating protein kinase (SAPK), respectively. In contrast to its effect on caspases, Triton X-100 did not activate the protein kinases ERK or SAPK during the 4 hr time period examined. This indicates that in contrast to the caspase pathway, the mitogen- and stress-activated signaling pathways were not induced by the detergent (*data not shown*).

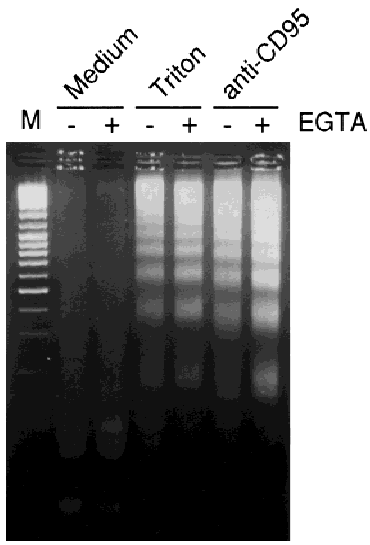

Fig. 2. Apoptosis induction by detergent does not require extracellular calcium ions. Agarose gel electrophoresis of LMW DNA from Jurkat cells cultured in the absence or presence of 1.5 mM EGTA. DNA from untreated cells and from cells treated with 7.5×10^{-2} g/l Triton X-100 or the anti-CD95 mAb 7C11 was prepared as described in Materials and Methods. M: molecular weight marker.

Detergent-Induced Apoptotic Cytotoxicity is Prevented by Caspase-Inhibitors

Apoptotic death of cells in culture ultimately leads to cytolysis. Similarly, higher concentrations of detergents disrupt the plasma membrane. To dissect cytoplasm lysis as the consequence of apoptotic cell death from membrane destruction due to dissolution by the detergent,

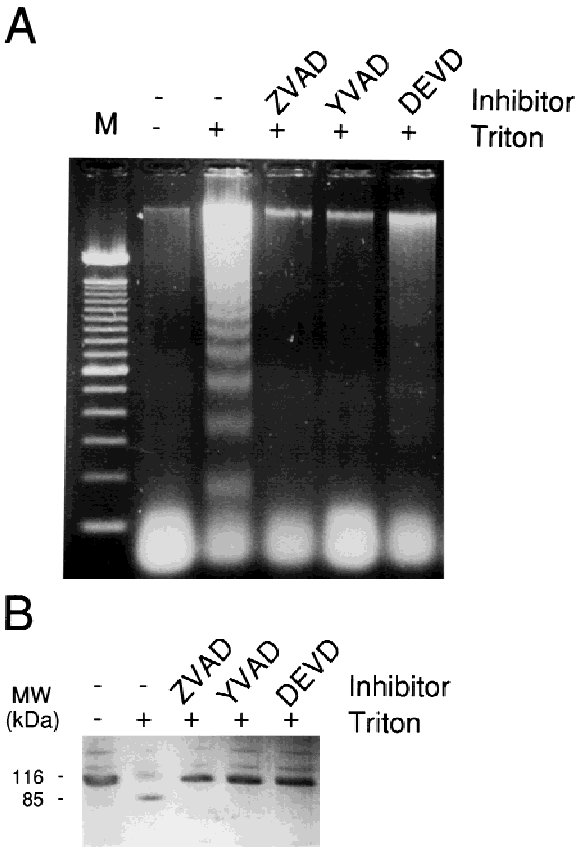

Fig. 3. Treatment with Triton X-100 activates caspases. Jurkat cells were treated with 7.5×10^{-2} g/l Triton X-100 for 4 hr in the absence or presence of the caspase inhibitors ZVAD (50 μM), YVAD (300 μM) or DEVD (300 μM). (*A*) Agarose gel electrophoresis of LMW DNA. (*B*) Western blot analysis of the poly(ADP-ribose) polymerase.

chromium release assays were performed with Triton-treated cells in the presence and absence of an apoptosis inhibitor. The results depicted in Fig. 4 show that lysis of Jurkat cells started at a Triton X-100 concentration of 5×10^{-2} g/l. The level of cytolysis correlated with an increasing Triton concentration and reached about maximum levels at 15×10^{-2} g/l. The cytolytic effect observed at concentrations between 5×10^{-2} and 10×10^{-2} g/l was completely or partially inhibited by an inhibitor of caspases indicating that at these concentrations lysis was secondary to the activity of these enzymes. In contrast, cytolysis at higher detergent concentrations was not inhibited by ZVAD indicating that cell permeability alterations at these concentrations were not of apoptotic nature.

Apoptosis Induction by Other Detergents

To examine whether apoptosis is induced upon treatment with detergents other than Triton X-100, additional non-

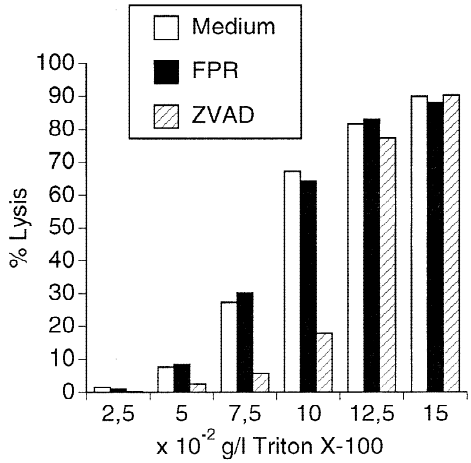

Fig. 4. Prevention of cytotoxicity by caspase inhibitors. Radioactive chromate-labeled Jurkat cells were treated for 8 hr with different Triton X-100 concentrations in the absence or presence of the control peptide FPR or the caspase inhibitor ZVAD.

ionic synthetic detergents such as Nonidet P-40 and n-octylglucoside, and the ionic detergent and bile salt sodium deoxycholate were tested. The observation that caspase inhibitors prevented detergent-induced cell death was utilized for these analyses. Thus, cells were treated with detergent in the absence or presence of ZVAD and cell death analyzed by propidium iodide staining. Figure 5 shows that an increase of the detergent concentration correlated with growth of the fraction of dead cells. The caspase inhibitor that specifically interferes with the apoptotic process shifted the deadly detergent concentration to higher levels. The area enclosed by the two curves represents the fraction of cells that was protected from caspase activation and subsequent cell death induced by the detergents. Similar results were obtained when cells were stained with annexin V or examined in the chromium release assay. In addition, DNA fragmentation identical to that with Triton X-100 and with the anti-CD95 mAb was observed upon treatment of cells with NP-40 and sodium deoxycholate (*data not shown*).

Discussion

Detergents are a chemically heterogeneous group of agents that contain both lipophilic and hydrophilic structures. It was recently reported that Triton X-100 induces apoptotic DNA fragmentation in tumor cell lines by a mechanism independent of protein synthesis (Borner et al., 1994; Ahn et al., 1997). We demonstrate that in addition to Triton X-100 other detergents similarly induce apoptosis indicating that apoptosis induction in this situation is not a feature peculiar to Triton X-100 but a more general effect of the interaction of detergents with cells. Moreover, detergent induced apoptosis in cell lines from

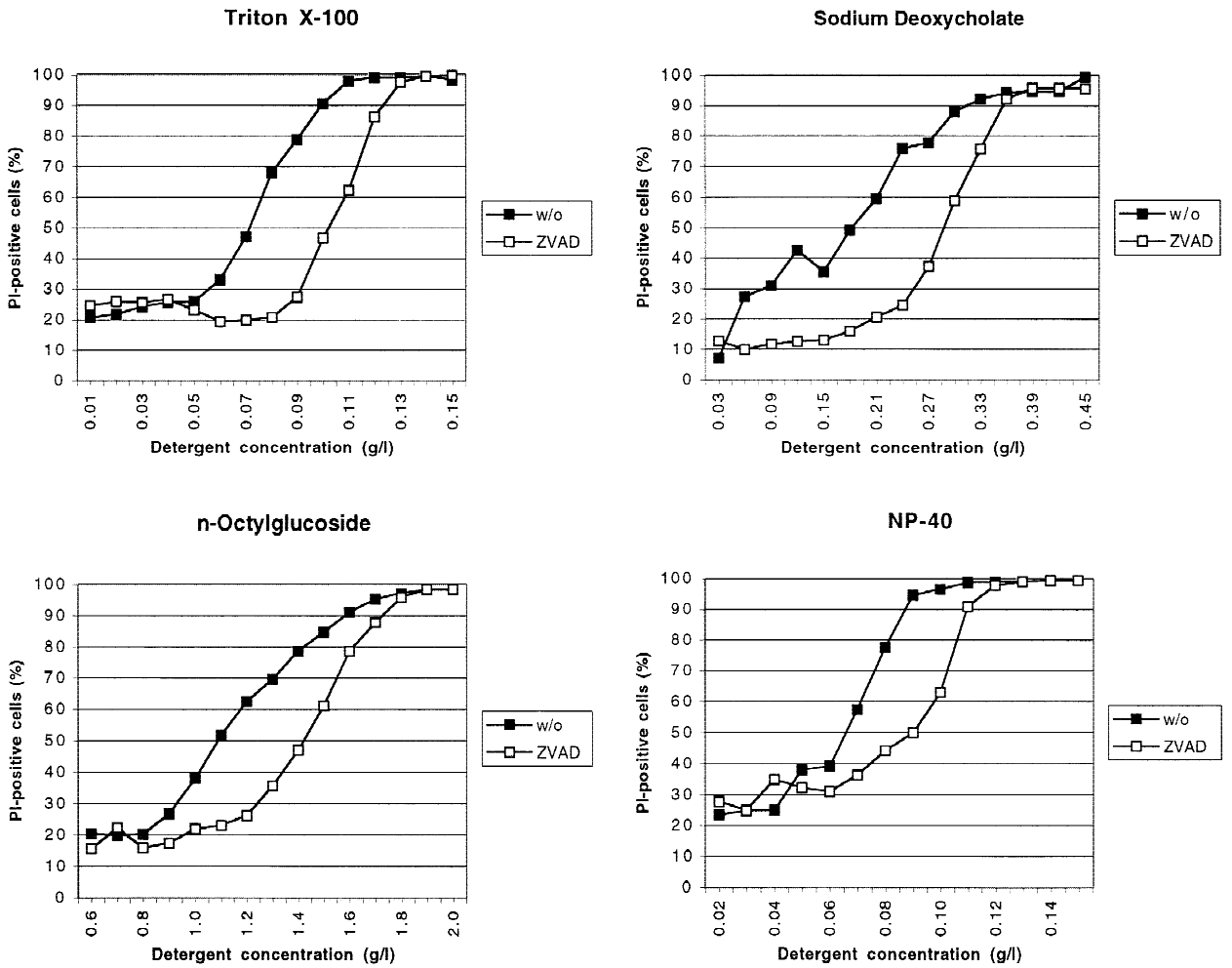

Fig. 5. Apoptosis induction by sodium deoxycholate, n-octylglucoside, and Nonidet P-40. Jurkat cells were treated for 5 hr with detergents in the absence (w/o) or presence of 50 μM ZVAD and the proportion of living and dead cells determined by staining with propidium iodide and flow cytometric analysis.

humans and a variety of nonhuman vertebrate animal species. This makes it unlikely that apoptosis induction is mediated by interaction of the detergent with a particular cell membrane receptor such as CD95, TRAIL or the TNF receptor 1.

Apoptosis was similarly induced in unstimulated PBMCs. It was recently demonstrated that T lymphocytes stimulated with PHA for 1 day are resistant to CD95-mediated apoptosis despite expression of the CD95 molecule. It was suggested that this might be due to Bcl-X_L overexpression (Peter et al., 1997). This block is not operative in the mononuclear cells treated with detergent because cells were rendered apoptotic without prior stimulation. Furthermore, this observation supports the notion that apoptosis induction by detergent is different from that mediated by CD95.

The concentration range at which apoptosis occurred was narrow and comprised changes by a factor of approximately 2 (non-ionic detergents) to 10 (deoxycholate) (Figs. 1 and 5). The concentration at which detergents induced apoptosis was below the critical micellar concentration (CMC). The effective detergent concentration in the cell culture was not determined and may be lower than that calculated due to binding of the agents to proteins such as those present in the FCS and to cells and, with respect to sodium deoxycholate, because of potential precipitations by divalent cations. In regard to the non-ionic detergents there was a correlation between the CMC and the apoptosis-inducing concentrations. For instance, Triton X-100 and NP-40 that have similar CMCs (0.2 and 0.25 × 10^{-3} mol/l at 25° in water, respectively) induced apoptosis at similar concentrations. N-octylglucoside that has a CMC of 14.5 × 10^{-3} mol/l induced apoptosis at more than ten times higher concentrations. Sodium deoxycholate which has the highest CMC induced apoptosis at a relatively broad concentra-

tion range below that of n-octylglucoside (Fig. 5). The difference between the potency of deoxycholate and the non-ionic detergents may be related to deoxycholate's sensitivity to low pH and divalent cations, to the ionic strength of the medium. Still, with this and the other detergents, the concentrations at which apoptosis was observed were contiguous to the concentration that causes nonapoptotic cytotoxicity (Fig. 5).

The fact that the concentration of detergent required for apoptosis induction is below the level required for overt lysis of the cells was specifically demonstrated by chromium release experiments. These assays indirectly measure the release of macromolecules. Two mechanisms of cell lysis could be discriminated: Cytolysis at lower detergent concentrations that was blocked by inhibitors of caspases and cell death at higher detergent levels that was not prevented by the enzyme inhibitors. Since caspases are involved in the execution of apoptotic cell death, the cytolysis at lower detergent concentrations reflects apoptosis. In contrast, cytolysis at higher detergent concentrations mirrors primary cell membrane rupture (Fig. 4). This fact is further supported by the data presented in Fig. 1*A*: At Triton concentrations between 5×10^{-2} and 12.5×10^{-2} g/l both fragmented DNA and RNA were visible upon electrophoresis of the LMW nucleic acids of the cells. In contrast, at higher concentrations, neither DNA fragments nor RNA could be retrieved due to complete cell membrane permeabilization.

The biochemical properties of detergents and biomembranes suggest that the initial event of apoptosis induction may occur at the cell membrane. Although the Triton concentration required for apoptosis induction did not immediately disrupt the cell membrane, detergent molecules may be incorporated into the lipid bilayer. This may result in subtle alterations of the physical properties of the membrane including an increased permeability for small charged and neutral solutes (Helenius & Simons, 1975). To investigate whether the influx of calcium is involved in the apoptotic process, experiments were made in media containing the calcium chelating agent EGTA. The data obtained demonstrate that calcium in the extracellular medium was not necessary for initiation of the apoptotic process (Fig. 2).

Treatment with detergents specifically targets the activity of caspases and does not, for instance, induce protein kinases of the stress- and mitogen-activated pathway. This further underlines the specificity of the events initiated by the detergent. It was suggested that the apoptotic process consists of a "common" pathway which represents enzymatic processes and morphological changes occurring in all apoptotic cells independent of the stimulus such as the involvement of the caspase cascade and a "private" pathway which depends upon the inducing event (Zhu, Fearnhead & Cohen, 1995). This concept can also be applied to treatment with detergent. The "private" signaling events of apoptosis induction by detergent i.e., the initiating step and the events that precede activation of the caspases have not yet been elucidated in this study. Clearly, the fact that apoptosis is induced by detergent in cells from different species points to a well preserved "private" mechanism of apoptotic signaling in this situation. The molecular nature of apoptosis induction by detergents still has to be elucidated. In particular, it will be important to determine the role of the plasma membrane phospholipid sphingomyelin. The product of hydrolysis of this molecule, ceramide, has been shown to mediate apoptosis (Obeid et al. 1993).

Induction of apoptosis by detergent may be relevant in pathophysiological situations. For instance, it was reported that low concentrations of bile salts induce apoptosis in rat hepatocytes possibly after uptake of the bile salt into the cell. It was suggested that this event may contribute to liver damage upon cholestasis (Patel et al., 1994). Our observations suggest that a mechanism different to uptake of bile salts may contribute to cell death by this agent, although this still has to be examined in hepatocytes. Moreover, apoptosis has been regarded as a physiological type of cell death (Vaux, Haecker & Strasser, 1994). If this view is to be applied to apoptosis induction by detergent, the physiologic function of apoptosis would be to prevent necrosis and concomitant tissue damage upon disturbances at the cell membrane. Factors that cause disarrangement of the plasma membrane in vivo still have to be defined.

In conclusion, treatment of human and nonhuman cells with detergent at concentrations below the level that causes cytolysis induced apoptotic death. The apoptotic signal generated by treatment with detergent is propagated by caspases and apoptosis is prevented by inhibitors of these enzymes. Influx of calcium from the extracellular medium is not required for the apoptotic signaling process. Initiation of the programmed cell death by agents that act primarily nonspecifically on cell membranes represents a peculiar type of apoptosis induction. It remains to be determined which physiologic relevant mechanism of apoptosis induction is imitated by detergent. Elucidation of the early "private" events of apoptosis in this circumstance will shed new light on both the apoptotic signaling pathway and the physiologic role of programmed cell death in mature cells.

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