

Acceleration of Neutrophil Apoptosis by Glucose-Containing Peritoneal Dialysis Solutions: Role of Caspases

MARINA PENÉLOPE CATALAN, ANA REYERO, JESÚS EGIDO, and ALBERTO ORTIZ

Unidad de Dialisis, Fundacion Jimenez Diaz, Universidad Autónoma, Madrid, Spain.

Abstract. Commercial, glucose-containing peritoneal dialysis (PD) solutions have deleterious effects on leukocytes and mesothelial cells that contribute to an impaired peritoneal defense. However, the molecular mechanisms of these deleterious effects are poorly understood. The effect of PD solutions on neutrophil viability, the molecular mechanisms of cell death, its functional consequences, and the possibilities for pharmacologic modulation have now been studied. The effect of newly available, bicarbonate-buffered PD solutions were further investigated. Lactate-buffered, glucose-containing PD solutions increased the apoptosis rate of cultured neutrophils (control media *versus* 4.25% glucose PD solution: $31 \pm 3\%$ *versus* $52 \pm 3\%$ apoptosis at 24 h, $P < 0.001$). Bicarbonate-buffered, 4.25% glucose-containing PD solutions with low concentration of glucose degradation products did not increase the rate of apoptosis. Apoptosis induced by lactate-buffered, 4.25% glucose PD solutions was not related to hyperosmolality or acidic pH and was not reproduced by increasing the glucose

concentration by the addition of glucose to a commercial, lactate-buffered fluid. Neutrophil apoptosis was associated with caspase-3 activation. Inhibition of caspase-3 by the use of the caspase-3 inhibitor acetyl-Asp-Glu-Val-Asp-fmk or the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) prevented features of apoptosis, such as morphologic changes, internucleosomal DNA degradation, and the appearance of hypodiploid cells and increased the number of viable, trypan blue-excluding neutrophils. Furthermore, zVAD-fmk increased neutrophil phagocytosis of bacteria. However, the caspase-1 inhibitor acetyl-Tyr-Val-Ala-Asp-aldehyde did not prevent cell death. These data suggest that unidentified components in commercial, lactate-buffered, high-glucose PD fluid accelerate the rate of neutrophil apoptosis. Glucose degradation products may be such unidentified components. Acceleration of neutrophil apoptosis may contribute to the impaired local defense system of patients undergoing PD.

Long-term peritoneal dialysis (PD) is marred by complications that may be favored by the poor biocompatibility of PD solutions. Peritonitis is one of the main complications of PD. PD solutions impair the peritoneal inflammatory response through actions on leukocytes. Adverse effects of PD solutions on leukocytes include inhibition of phagocytosis, oxidative burst, and enzyme release (1,2). However, the molecular mechanisms that lead to these abnormalities are poorly understood. More recently, glucose-containing PD solutions have been shown to promote death of monocytes and neutrophils (3,4). However, the cellular and molecular mechanisms of neutrophil death induced by PD solutions have not been explored.

Apoptosis is an active form of cell death that requires energy in the form of ATP to proceed (5). Apoptosis is under molecular control and offers the opportunity for a therapeutic intervention. The main intracellular effectors of apoptosis are a family of cysteine proteases known as caspases (6). Caspases

are activated by cleavage of procaspases in a sequential manner during apoptosis. Caspase-3 is an effector caspase that plays a role in cell death induced by a variety of stimuli (6). Data from knockout mice have identified, however, caspase-3-independent apoptosis pathways that involve the activation of additional caspases (7). Moreover, caspase-3 activation in the absence of cell death is also known to occur (8). More than 40 substrates for caspases have been identified whose cleavage can be either an activating or inactivating event for the function of the protein. Caspase actions include the inactivation of protective proteins, such as BclxL and Bcl2, dismantling of structural proteins, and activation of DNases (6). Certain caspases, such as caspase-1, have a minor role in apoptosis, and their main function is to regulate inflammation. The availability of specific inhibitors allows the therapeutic targeting of caspases (9).

Apoptosis contributes to normal tissue homeostasis, but changes in the physiologic rate of apoptosis may lead to disease (5). Neutrophils undergo spontaneous apoptosis in culture or in sites of inflammation (10). This is a physiologic mechanism to limit inflammation and tissue injury. However, the premature demise of neutrophils may compromise their defensive function. We have now explored the effect of PD solutions on apoptosis of neutrophils, the molecular mechanisms involved, and their functional consequences.

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Correspondence to Dr. Alberto Ortiz, Unidad de Diálisis, Fundación Jiménez Díaz, Av Reyes Católicos 2, 28040 Madrid, Spain. Phone: 3491-5504940; Fax: 3491-5494764; E-mail: aortiz@fjd.es

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Materials and Methods

Isolation and Culture of Neutrophils

Neutrophils were isolated from peripheral blood of healthy volunteers by Ficoll gradient centrifugation (Rafer SL, Zaragoza, Spain) and hypotonic lysis of red blood cells (11). Briefly, each 10-ml sample of heparinized blood was diluted with 10 ml of 0.9% NaCl, underlayered with 10 ml of Ficoll, and centrifuged at 1800 rpm for 30 min at room temperature. The upper layer was discarded, and 2.5% gelatin in phosphate-buffered saline (PBS) was added to the buffy coat. After 15 min at 37°C, the supernatant was harvested and centrifuged at 2500 rpm for 15 min. Residual erythrocytes were lysed with hypotonic saline, and neutrophils were resuspended in serum-free RPMI 1640 (Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin. The percentage of apoptotic neutrophils right after isolation was <1%, as assessed by flow cytometry of permeabilized, propidium iodide-stained cells. Neutrophils were incubated in serum-free RPMI 1640, or lactate-buffered PD solutions that contained 1.5% glucose, pH 5.5 (CAPD-2, Fresenius Medical Care, Barcelona, Spain), or 4.25% glucose, pH 5.5 (CAPD-3, Fresenius Medical Care), or bicarbonate-buffered, 4.25% glucose, pH 7.4 (stay.safe BIC, Fresenius Medical Care), for 15 min. The culture media were then diluted 1:1 by the addition of serum-free RPMI 1640, to neutralize the acid pH and reproduce the physiologic conditions in PD.

The incubation was prolonged for 2, 4, 6, and 24 h. In some experiments, the pH of PD solutions was elevated to 7.4 through the addition of NaOH. In other experiments, the osmolarity of lactate-buffered, 1.5% glucose-containing solutions was elevated to that of 4.25% glucose-containing solutions by the addition of D-mannitol or D-glucose.

Caspase Inhibitors

The caspase inhibitory peptides acetyl-Tyr-Val-Ala-Asp-aldehyde and benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) were from Bachem (Bubendorf, Switzerland). Acetyl-Asp-Glu-Val-Asp-fmk (DEVD-fmk) was from Calbiochem (Darmstadt, Germany). DEVD-fmk and acetyl-Tyr-Val-Ala-Asp-aldehyde were dissolved in DMSO and zVAD-fmk in methanol. Final concentration of DMSO or methanol was <0.1%. This concentration did not modulate cell death. Peptides were added at the time of dilution of the PD solution.

Assessment of Cell Death and Apoptosis

For quantification of cell death, 150,000 cells were seeded in 12-well plates (Beckton-Dickinson, Franklin Lakes, NJ) under the experimental conditions. At defined time points, the cells were harvested by pooling nonadherent cells with adherent cells, which were detached by gentle trypsinization. The total number of cells were counted, and the viability was determined by trypan blue exclusion. For this purpose, at least 100 cells from triplicate wells were counted in a Fuch-Rosenthal chamber (12).

Apoptosis was also quantified by flow cytometry (13). Pooled attached and detached cells present in the supernatant were spun and resuspended in a cell permeabilization buffer that contained 100 µg/mL propidium iodide, 10 µg/mL RNase A, and 0.05% NP-40 in PBS, incubated at 4°C for 1 h, and analyzed on the FACScan by use of LYSIS II software (Becton Dickinson, Franklin Lakes, NJ). By permeabilizing the cells, we allowed propidium iodide access to both dead and live cells. The percentage of cells with decreased DNA staining, made up of apoptotic cells with fragmented nuclei, was counted. Apoptosis in the presence of PD solutions was considered to

be 100%, and apoptosis in the presence of inhibitors of caspases was expressed as a percentage of this (14).

To assess for the pyknotic nuclear changes seen in apoptosis, cells were fixed and stained with propidium iodide. After fixation, propidium iodide stained both live and dead cells. Neutrophils were plated onto Labtek slides (Nunc, Inc., Naperville, IL). The cells were stained with propidium iodide basically as described elsewhere (13). Briefly, the slides were fixed for 20 min in 1:1 methanol/acetone at –20°C, washed with PBS, and then stained for 30 min at 37°C in 0.1 µg/mL propidium iodide and 100 µg/mL RNase A in PBS (pH 7.2). Finally, the slides were washed with PBS and coverslips mounted by use of a 90% glycerol solution. The slides were examined with a fluorescence microscope with an ultraviolet light source filtered for propidium iodide. Images were photographed on Kodak TMAX 3200 film (Eastman Kodak, Rochester, NY) and printed at equivalent exposures. Cells were also stained with hematoxylin-eosin.

For assessment of internucleosomal genomic DNA fragmentation, a characteristic of apoptosis, 10⁶ neutrophils were cultured for 24 h under different experimental conditions, washed three times in PBS, lysed in 20 µl of lysis buffer (50 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid, and 0.5% sodium laurylsarkosine [pH 8.0]), and incubated for 30 min at 4°C. The samples were centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was incubated for 1 h with 5 µl of proteinase K (1 mg/ml) at 50°C and then for another hour at 50°C with 5 µl of RNase A (10 ng/ml). Finally, the temperature was increased to 70°C for 10 min, and the loading buffer was added. Samples were separated in a 1.5% agarose gel and stained with ethidium bromide.

Caspase-3 Activity

Neutrophils were incubated with PD solutions as described and with or without 200 µM zVAD or 200 µM DEVD for 24 h. During the last 30 min of incubation, a DEVD substrate was added that, when cleaved by caspase-3 or similar caspases, emits green fluorescence (PhiPhiLux, Oncoimmunin, Gaithersburg, MD). The cells were washed, and the fluorescence was analyzed by flow cytometry, following the manufacturer's instructions.

Bacteria Phagocytosis Assay

Bacteria phagocytosis capacity was assayed by means of a commercially available kit (Vybrant phagocytosis assay kit; Molecular Probes, Eugene, OR). Neutrophils were cultured with PD solutions, with or without 200 µM zVAD, as described above, and cultured for 24 h at a cell density of 10⁶/ml, in quintuplicate wells, in 96-well plates. Then the media were removed, fluorescein-labeled *Escherichia coli* were added, and the procedure was performed following the manufacturer's instructions.

Statistical Analyses

Results are expressed as mean ± SEM. Significance at the 95% level was established by use of one-way ANOVA and the two-tailed *t* test. The presence of significant differences between groups was examined by a *post hoc* test (Bonferroni's method) by means of SigmaStat statistical software (Jandel, San Rafael, CA). At least four independent experiments with neutrophils from different donors were performed.

Results

Commercial, Lactate-Buffered, Glucose-Containing PD Solutions Induce Neutrophil Apoptosis

We have observed that exposure of peripheral blood neutrophils to commercial, lactate-buffered, 4.25% glucose-containing

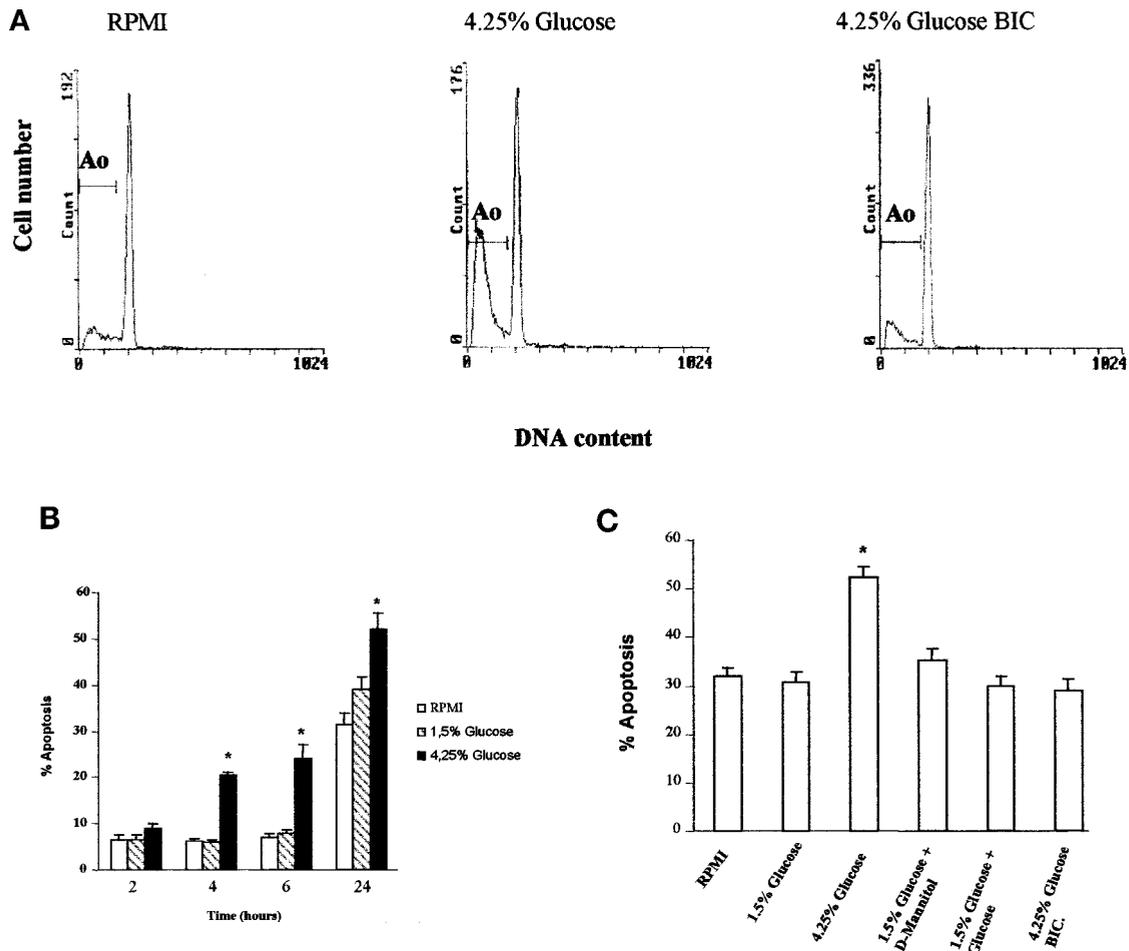


Figure 1. Peritoneal dialysis (PD) solutions induce apoptosis of neutrophils. Neutrophils were cultured in the presence of control culture medium (RPMI) or commercial PD solutions. (A) Flow cytometry diagrams of permeabilized, propidium iodide-stained cells after a 24-h incubation. Note the percentage of cells with decreased DNA staining (A_0) peak that corresponds to apoptotic, hypodiploid cells. (B) Time response of neutrophil apoptosis induced by commercial, lactate-buffered, glucose-containing PD solutions. Spontaneous neutrophil apoptosis in the presence of control culture medium is also observed. * $P < 0.001$ versus RPMI. Mean \pm SEM of six independent experiments. (C) The increase of culture media osmolarity to the levels of 4.25% PD solution by the addition of D-mannitol or glucose to lactate-buffered, 1.5% glucose PD solutions did not enhance the spontaneous rate of neutrophil apoptosis at 24 h. In addition, commercial, bicarbonate-buffered, 4.25% glucose PD solution (BIC) did not increase neutrophil apoptosis. Mean \pm SEM of four independent experiments. * $P < 0.001$ versus other groups.

ing PD solutions increases the spontaneous rate of apoptosis, as assessed by the percentage of cells with hypodiploid DNA content (Figure 1A). The spontaneous rate of neutrophil apoptosis observed in our studies is similar to that reported in the literature (10). The lethal effect of high-glucose (4.25%) PD solutions was noted as early as 4 h after exposure (Figure 1B). The microscopical examination of neutrophils exposed to PD solutions revealed typical morphologic features of apoptosis, such as decreased cell and nuclear size and nuclear condensation and fragmentation. Increasing the pH of lactate-buffered glucose-containing PD solution to 7.4 did not modify the rate of apoptosis at 24 h (apoptosis rate of cells exposed to 4.25% or 1.5% glucose [pH 7.4] PD solution was 100% of that observed in cells exposed to 4.25% or 1.5% glucose [pH 5.5] PD solution). By contrast, bicarbonate-buffered, 4.25% glucose (pH 7.4) PD solution did not increase the rate of sponta-

neous neutrophil apoptosis (Figure 1C). Increasing the osmolarity of lactate-buffered, 1.5% glucose PD solutions to the level of 4.25% glucose PD solutions by the addition of D-mannitol or glucose did not modify the rate of apoptosis (Figure 1C). This effectively excludes a role of either lactate or glucose itself in the phenomenon.

Caspases Are Mediators of Neutrophil Apoptosis Induced by Commercial, Lactate-Buffered, High-Glucose PD Solutions

We then addressed the molecular mechanisms of apoptosis induced by commercial, lactate-buffered, high-glucose PD solutions. We observed that both spontaneous neutrophil apoptosis and apoptosis induced by PD solutions was characterized by activation of caspase-3, the main effector caspase (Figure 2). Caspase-3 activation in intact neutrophils was prevented in

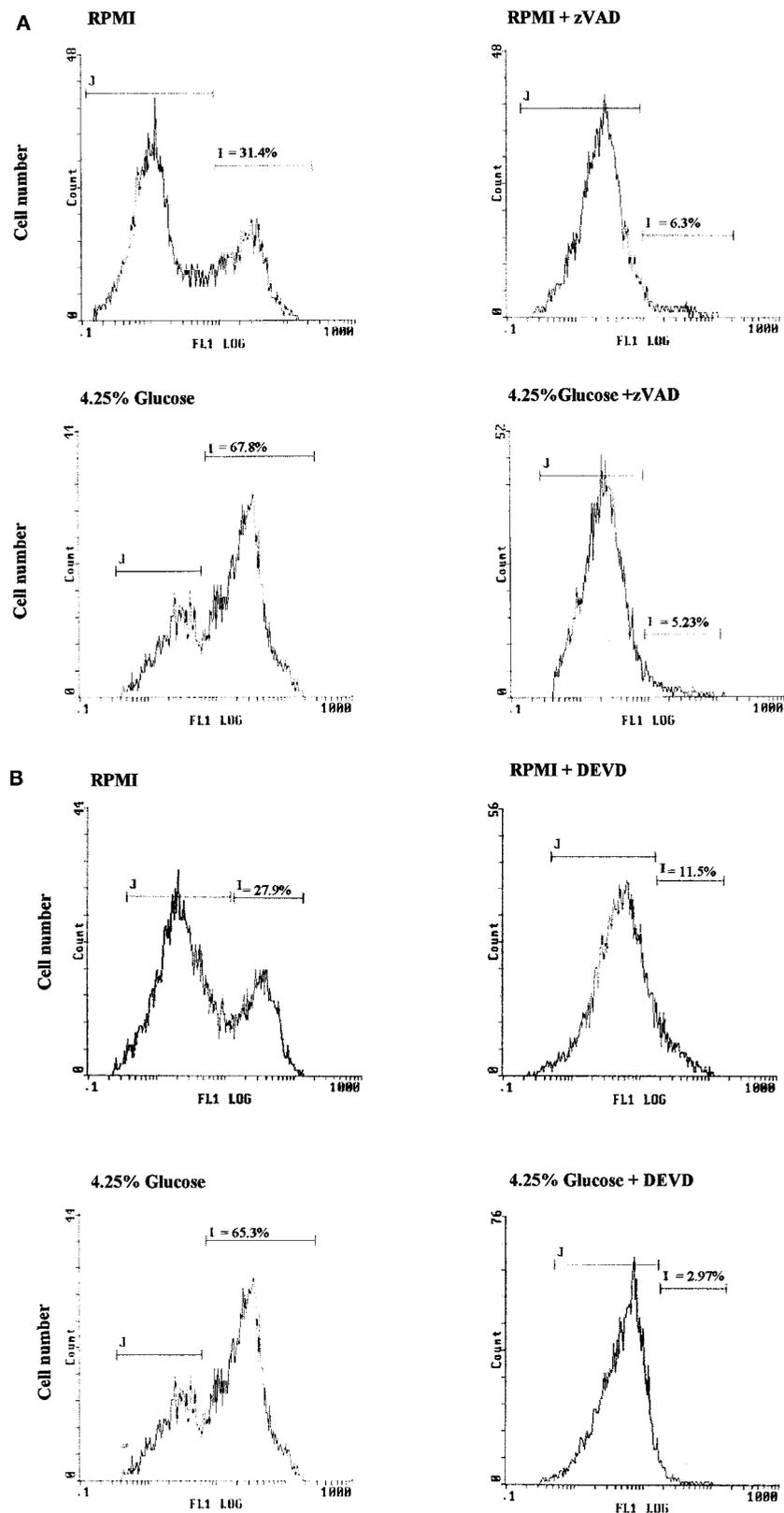


Figure 2. Neutrophil apoptosis is associated with caspase-3 activation. Caspase-3 activity was quantified as green fluorescence generated by cleavage of a peptide target. (A) Flow cytometry diagrams of cells cultured for 24 h in the presence of control culture medium (RPMI) or commercial, lactate-buffered PD solutions and 200 μ M benzylloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) or vehicle. zVAD-fmk prevents caspase-3 activation. (B) Cells cultured for 24 h in the presence of control culture medium (RPMI) or commercial PD solutions and 200 μ M Acetyl-Asp-Glu-Val-Asp-fmk (DEVD-fmk) or vehicle. DEVD-fmk prevents caspase-3 activation. The I bar encompasses cells with activated caspase-3.

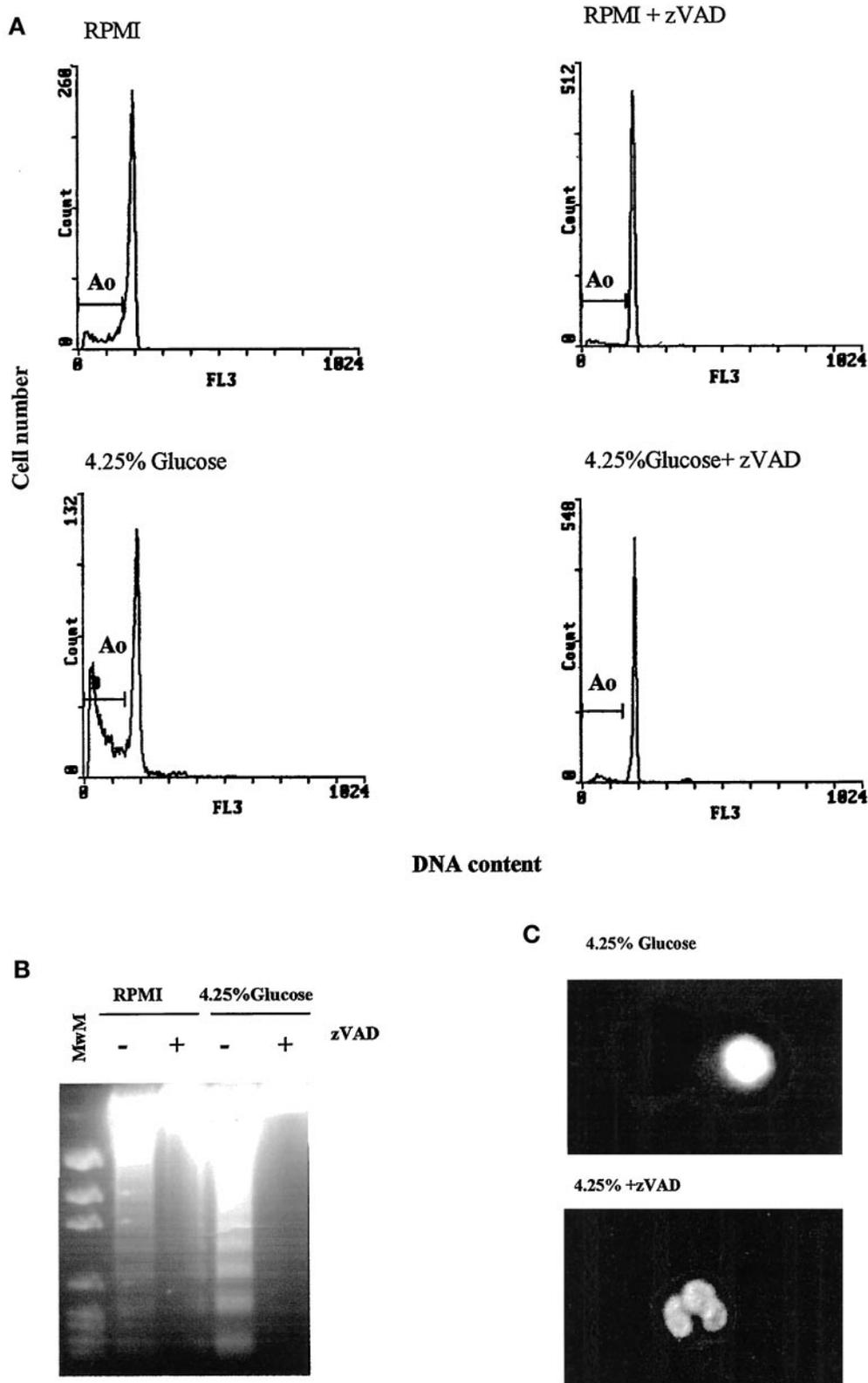


Figure 3. Caspase inhibition prevents both spontaneous and PD solution–induced apoptosis in neutrophils. Cells were cultured for 24 h in the presence of control culture medium (RPMI) or commercial, lactate-buffered PD solutions and 200 μ M zVAD-fmk or vehicle, and apoptosis was studied by different approaches. (A) Flow cytometry diagrams of permeabilized, propidium iodide–stained cells. Note the disappearance of the A₀ peak corresponding to apoptotic, hypodiploid cells in the presence of zVAD-fmk. (B) zVAD-fmk inhibits the internucleosomal DNA degradation characteristic of apoptosis. (C) zVAD-fmk prevents the occurrence of the nuclear apoptotic morphology (propidium iodide staining of permeabilized cells. Magnification, $\times 1000$).

both situations by zVAD-fmk, a broad-spectrum, irreversible caspase inhibitor (Figure 2A). Caspase-3 activation was also prevented by DEVD-fmk, an irreversible caspase inhibitor specific for effector caspase-3, -6, and -7 (Figure 2B).

Under certain circumstances, caspase-3 is activated, but its activation does not lead to cell death (8). We thus evaluated the hypothesis that caspase-3 activation was required for neutrophil apoptosis to proceed. zVAD-fmk decreased dramatically the spontaneous and PD solution-induced apoptosis of neutrophils (Figure 3). Decreased percentage of hypodiploid cells (Figure 3A), prevention of internucleosomal DNA degradation (Figure 3B), and absence of morphologic features of apoptosis were noted (Figure 3C). Indeed, zVAD reduced apoptosis to $35 \pm 6\%$, $12 \pm 1\%$, and $24 \pm 1\%$ of that observed in the presence of its vehicle in cells cultured in RPMI, 1.5% glucose, and 4.25% glucose PD solution, respectively ($P < 0.001$). DEVD-fmk had a lesser effect than zVAD-fmk on PD solution-induced apoptosis (apoptosis induced by 4.25% glucose PD solution + zVAD was $61 \pm 6\%$ of that observed in 4.25% glucose PD solution + vehicle, $P = 0.003$). However, DEVD-fmk had no effect on spontaneous neutrophil apoptosis. The caspase-1 inhibitor acetyl-Tyr-Val-Ala-Asp-aldehyde did not prevent features of apoptosis in neutrophils exposed to PD solutions (data not shown).

Caspase Inhibition Also Prevents Nonapoptotic Cell Death and Preserves Function

In some cell systems, caspase inhibitors prevent features of apoptosis, such as DNA degradation and the appearance of hypodiploid cells, but cells die a nonapoptotic cell death (15). We have thus addressed whether inhibition of caspases prevents nonapoptotic cell death. Nonapoptotic cell death is characterized by an early loss of cell membrane integrity, so we chose to use trypan blue staining, an assay that is not specific for any type of cell death and that picks both necrotic and late apoptotic cells. In that assay, zVAD-fmk increased the proportion of viable cells after a 24-h exposure to high-glucose PD solutions (Figure 4).

We further explored whether neutrophils that were rescued through interference with caspase activation could be useful for the peritoneal defenses. To test this hypothesis, we performed a bacteria phagocytosis assay. Exposure to commercial, lactate-buffered, 4.25% glucose PD solutions for 24 h decreased the phagocytosis capacity of neutrophils ($66 \pm 10\%$ of RPMI control, $P < 0.01$). zVAD-fmk increased the phagocytosis capacity of both neutrophils cultured in control media and those cultured in PD solution ($158 \pm 12\%$ and $122 \pm 10\%$ of controls cultured in RPMI, respectively, $P < 0.001$ versus their respective vehicle-treated control).

Discussion

The presence of PD solutions is a disrupting event for peritoneal defense mechanisms. A variety of adverse effects of PD solutions on leukocyte function have been described elsewhere (1,2). More recently, it was observed that, in short-term experiments (4 h), neutrophils exposed to undiluted glucose-containing PD solutions undergo cell death (4). However, the

methodology used by those authors to quantify cell death does not differentiate necrotic cells from cells in the late stages of apoptotic cell death (16). Moreover, in the course of the PD dwell, the composition of intraperitoneal PD fluid changes, and the glucose concentration decreases over time. In an attempt to more closely reproduce the intraperitoneal changes in glucose concentration, the molecular and cellular mechanisms of death in neutrophils exposed initially to undiluted PD solution and for a prolonged follow-up to diluted PD solutions was explored. Under those conditions, commercial, lactate-buffered, high-glucose PD solutions promote neutrophil apoptosis.

The well-described phenomenon of spontaneous neutrophil apoptosis in culture has been observed elsewhere (10). Commercial, lactate-buffered PD solutions that contained 4.25% glucose accelerated the rate of spontaneous neutrophil apoptosis. Neutrophil apoptosis is a physiologic process that limits inflammation. However, premature neutrophil apoptosis may compromise the antibacterial potential of these leukocytes.

The lethal effect of lactate-buffered, high-glucose PD solutions was not accounted for by the low pH or by the high osmolality and should be ascribed to the high glucose concentration or, alternatively, to additional factors related to the high glucose levels in commercial PD solutions. Hyperglycemia *per se* induces apoptosis in cultured tubular epithelial cells, endothelial cells, and the blastocyst (17–19) through changes in the expression or activity in Bcl2-related molecules that, in turn, regulate caspase activation (5). However, addition of glucose to commercial, lactate-buffered, 1.5% glucose-containing PD solutions to achieve a 4.25% glucose concentration failed to reproduce the increased neutrophil apoptosis rate observed in commercial, lactate-buffered, 4.25% glucose PD solutions. Thus, the cell death cannot be ascribed to the lactate-glucose association. Furthermore, this supports the notion that it is not glucose *per se* but additional factors linked to the presence of glucose in commercial PD solutions that promotes neutrophil apoptosis. Glucose degradation products are produced in the course of heat sterilization of commercial, lactate-buffered PD solutions, especially in high-glucose solutions, and may be responsible for acceleration of neutrophil cell death. In this regard, the presence of increased concentrations of glucose degradation products in commercial, lactate-buffered, high-glucose PD fluids has been linked to cytotoxicity (20). In our experiments, a commercially available, bicarbonate-buffered, high-glucose PD solution, which contains a lower concentration of glucose degradation products (21), induced less apoptosis than conventional high-glucose PD fluids.

The molecular mechanisms responsible for PD solution-induced cell death had not been previously addressed. Bcl2 family members are difficult to target therapeutically. The availability of inhibitors makes caspases more attractive therapeutic targets (9). Our data indicate that caspases are indispensable effectors of glucose-containing PD solution-induced apoptosis in neutrophils. This conclusion is based on four lines of evidence: (1) glucose-containing PD solutions activate caspase-3 in neutrophils; (2) two different inhibitors of caspases block caspase-3 activation; (3) inhibitors of caspases prevent both features of apoptosis (morphology, internucleo-

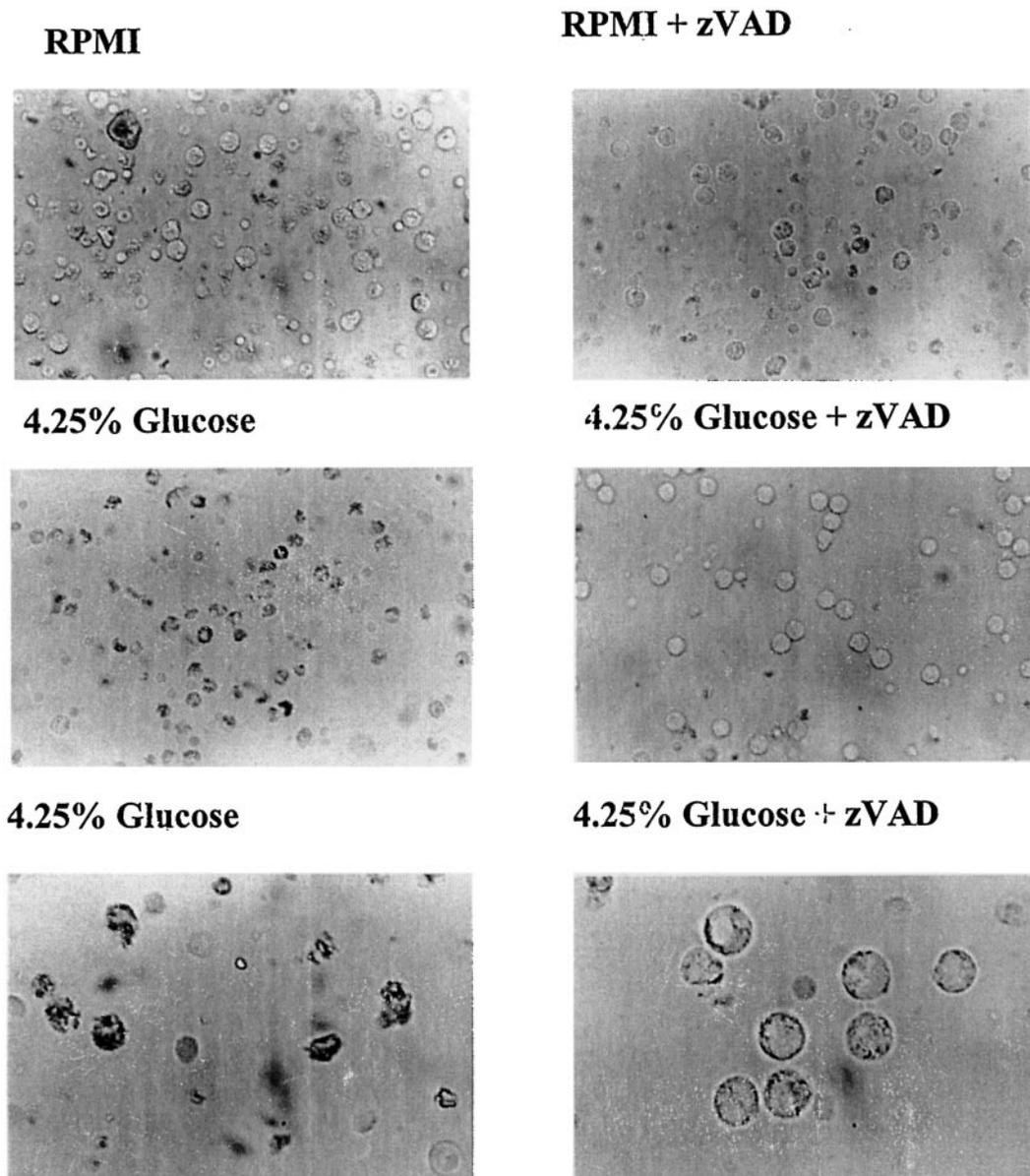


Figure 4. Caspase inhibition prevents both apoptotic and nonapoptotic cell death in neutrophils exposed to commercial, lactate-buffered, high-glucose PD solutions. In some cell death systems, zVAD prevents apoptotic cell death, but cells that are rescued from apoptosis do die by necrosis. To exclude this possibility, cell viability was evaluated by trypan blue staining after 24 h of incubation. Blue-stained cells are dead cells (both necrotic and late apoptotic cells), and this method does not provide information on the mode of cell death. Note stained, dark, dead cells. Magnifications: $\times 200$ in A; $\times 1000$ in B.

somal DNA degradation, and presence of hypodiploid cells) and nonapoptotic cell death (trypan blue exclusion); and (4) caspase inhibitors preserve the phagocytic function of neutrophils. The two latter aspects are important, because, in some models of cell death, caspase inhibition is unable to prevent disruption of the cell membrane and cell death even in conditions in which it completely blocks nuclear apoptosis (chromatin condensation and DNA fragmentation) (15). DEVD-fmk, an inhibitor of caspase-3 and related caspases, had a lesser effect than zVAD-fmk on PD solution-induced apoptosis, despite the fact that similar inhibition of caspase-3 activity was achieved. This suggests that additional caspases, not inhibited by DEVD-fmk, contribute to neutrophil

apoptosis in these circumstances. Moreover, the differential effect of DEVD-fmk observed under our culture conditions on spontaneous and PD solution-induced apoptosis implies that there may be different caspase pathways for neutrophil apoptosis that are stimulus dependent.

In summary, our findings suggest that factors present in commercial, lactate-buffered, high-glucose PD solutions but not in commercial, bicarbonate-buffered, high-glucose PD solutions accelerate neutrophil apoptosis. They further suggest that these factors may be glucose degradation products. Accelerated caspase-dependent neutrophil apoptosis may contribute to an impaired peritoneal defense. Indeed, preliminary reports

suggest a beneficial effect of acute, short-term administration of caspase inhibitors in experimental models of sepsis (22).

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