

Alkaline Conditions Accelerate Polymorphonuclear Leukocyte Apoptosis In Vitro

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Apoptosis was monitored in polymorphonuclear leukocytes (PMNs) cultured under mildly acidic, neutral, and alkaline conditions. Within 3 h, 9.0% of the PMNs underwent apoptosis at pH 6.7, as did 12% at pH 7.2, 38% at pH 7.7, and 60% at pH 8.2. Inhibitors of serine proteases, caspase-1, or caspase-3 significantly inhibited PMN apoptosis at pH 8.2, suggesting an involvement by these enzymes.

Polymorphonuclear leukocytes (PMNs) are the first line of defense against subgingival bacterial plaque, the etiology of human inflammatory periodontal disease (14). These phagocytic cells have short life spans and are committed to death via apoptosis (programmed cell death). Recent studies suggest that apoptosis plays an integral role in resolving acute inflammation by down-regulating PMN activities after an infection has been contained (19). Apoptotic PMNs remain viable for hours but exhibit a diminished ability to degranulate, generate a respiratory burst, or undergo shape changes in response to external stimuli (20, 22). Apoptosis proceeds through cleavage of intracellular proteins, and caspases and serine proteases appear to be involved in modulating this process (15, 17, 24).

Previous investigations have shown that the human gingival crevice ranges from mildly acidic in the healthy sulcus to alkaline at sites affected by inflammatory periodontal disease (11). The alkaline environment associated with inflammation results from the catabolism of proteins and the release of ammonia and other basic metabolites from subgingival gram-negative bacteria (11). Little is known about the potential impact of these conditions on PMN behavior, but alkaline pH has the potential to alter the rate of PMN apoptosis. To assess this possibility, human PMNs were isolated from peripheral blood collected from healthy donors. The blood was subjected to Ficoll-Hypaque density gradient centrifugation and dextran sedimentation (4), and residual erythrocytes were eliminated by hypotonic lysis. The PMNs were washed three times in phosphate-buffered saline solution and resuspended at 5×10^6 /ml in HEPES-buffered RPMI 1640 medium adjusted to pH 6.7, 7.2, 7.7, and 8.2. PMNs were cultured at 37°C and maintained in suspension by gentle shaking (16). At the indicated times, small aliquots of cells were stained with acridine orange and ethidium bromide for assessment of apoptotic changes and cell viability by fluorescence microscopy (5). In some experiments, apoptosis was assessed by *in situ* labeling of cells containing fragmented DNA with a commercially available kit (FragEL-Klenow DNA fragmentation detection kit; Amersham Life Science, Inc.). After labeling, the percentage of PMNs containing fragmented DNA was determined by microscopic evaluation (9).

Only a small proportion ($\leq 12\%$) of PMNs incubated for 3 h at pH 6.7 or pH 7.2 underwent apoptotic changes (Fig. 1).

During the same period, approximately 38% of PMNs incubated at pH 7.7 underwent apoptosis, and 60% of those incubated at pH 8.2 underwent apoptosis (treatment effect significant at $P = 0.0005$; repeated-measures analysis of variance [ANOVA]). Apoptosis progressed linearly under alkaline conditions and approached 100% after approximately 7 h. After 3 h at pH 6.7 or pH 7.2, PMNs began to undergo apoptosis at a rate that paralleled that observed under alkaline conditions. Thus, exposure to alkaline conditions did not appear to alter the maximal rate at which PMNs eventually undergo apoptosis, but it induced PMNs to undergo apoptosis hours before they otherwise would. Interestingly, PMN viability (plasma membrane integrity) was not significantly affected by pH at any time during the course of the experiment (data not shown).

To confirm the effect of alkaline conditions on apoptosis, we examined their effect on DNA fragmentation, which accompanies the morphological changes associated with PMN apoptosis (12). After incubation for 4 h, the proportion of cells exhibiting DNA fragmentation was 25.6% at pH 6.7, 32.5% at pH 7.2, 43.8% at pH 7.7, and 55.4% at pH 8.2 (data not shown). The pH of the extracellular medium had a significant effect on DNA fragmentation ($P = 0.011$; repeated-measures ANOVA).

Tumor necrosis factor alpha (TNF- α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are found in the diseased periodontium, have been shown to inhibit apoptosis in PMNs (13). Their effects on apoptosis are most significant when incubated with PMNs for at least 6 h. As shown in Fig. 2 (upper panel), TNF- α (1 ng/ml for 6 h) maintained its ability to inhibit PMN apoptosis at pH 7.2 and pH 8.2 ($P < 0.05$), but its effects were not significant at pH 6.7. GM-CSF (1 ng/ml for 7 h) significantly inhibited PMN apoptosis at pH 6.7, 7.2, and 8.2 (Fig. 3, lower panel). Thus, these cytokines can influence PMN apoptosis under alkaline as well as neutral conditions.

Specific and reproducible proteolytic cleavage of cellular proteins has been identified as an early step in apoptosis (15, 17, 21). Serine proteases (e.g., elastase) and cysteine proteases (e.g., caspase-1 and caspase-3) have been implicated in apoptosis of PMNs and other types of cells (15, 22, 24). We used inhibitors of these enzymes to study their role in the acceleration of PMN apoptosis under alkaline conditions. Figure 3 portrays the effects of the serine protease inhibitor DFP (diisopropyl fluorophosphate; Sigma), the selective caspase-3 inhibitor DEVD-CHO (*N*-acetyl-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Glu-Val-Asp-CHO; Biomol), and the caspase-1 inhibitor Z-VAD-FMK [Z-Val-Ala-Asp(OMe)-CH₂F; Calbiochem] on apoptosis in

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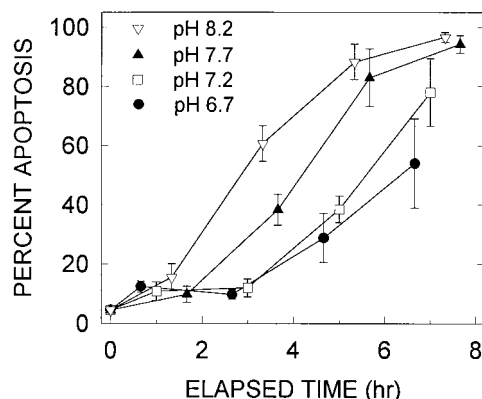


FIG. 1. Acceleration of PMN apoptosis under alkaline conditions. PMNs were suspended at 5×10^6 /mL in HEPES-buffered RPMI 1640 medium adjusted to the indicated pHs and incubated at 37°C. At the indicated time points, a small volume of cell suspension was withdrawn, stained, and evaluated for apoptotic changes by fluorescence microscopy. The proportion of apoptotic cells was calculated from the number of cells with apoptotic nuclei divided by the total number of cells counted. Data are expressed as the means \pm standard errors of the means (error bars) of four experiments.

PMNs cultured for 4 h at pH 7.2 and pH 8.2. DFP (1 mM) significantly inhibited apoptosis at pH 8.2 but had no significant effect at pH 7.2 ($P < 0.05$; paired t test). Similar results were obtained with PMNs treated with 1 μ M DEVD-CHO. Treatment with 100 μ M Z-VAD-FMK produced significant inhibition of apoptosis at pH 7.2 as well as at pH 8.2. Although caspase-1 activity is difficult to recover from PMN lysates, the presence of cellular caspase-3 can be readily determined with the fluorescent caspase-3 substrate DEVD-AFC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; Bi-mol) (10). In our experiments, caspase-3 activity in lysates from cells incubated for 4 h at pH 8.2 was 2.5-fold higher than that recovered from cells incubated at pH 7.2 ($P < 0.05$ [data

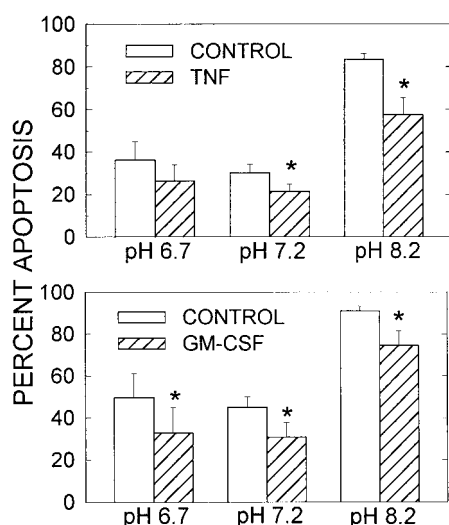


FIG. 2. Effect of TNF- α and GM-CSF on apoptotic changes in PMNs incubated under neutral and alkaline conditions. PMNs were suspended in media adjusted to the indicated pHs and then were treated with TNF or GM-CSF (1 ng/ml) for 6 or 7 h, respectively. The results are based on three experiments and are expressed as means \pm standard errors of the means (error bars). Asterisks indicate a significant difference between treatment and control groups ($P < 0.05$).

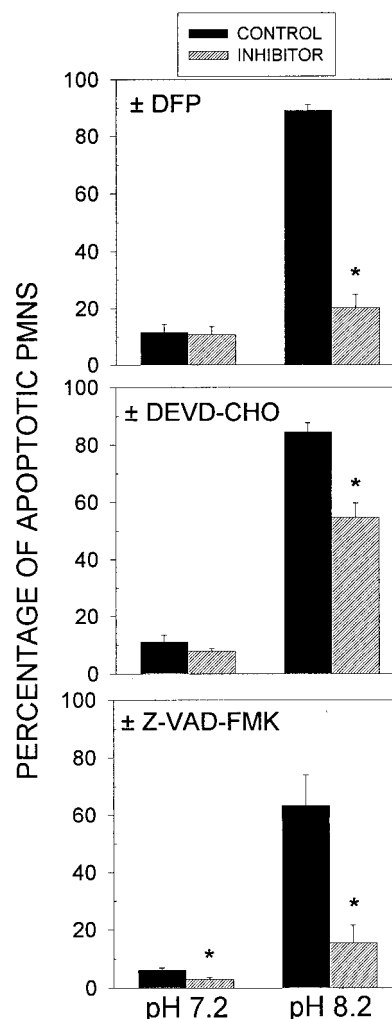


FIG. 3. Effect of protease inhibitors on acceleration of PMN apoptosis by alkaline conditions. PMNs were suspended in RPMI 1640 medium adjusted to the indicated pHs and then were incubated for 4 h in the presence and absence of inhibitors of serine proteases (DFP), caspase-1 (Z-VAD-FMK), and caspase-3 (DEVD-CHO). Morphological assessment of apoptosis was performed as previously described. The results are expressed as the means \pm standard errors of the means (error bars) of four experiments. Asterisks indicate a significant difference between treatment and control groups (as assessed by the paired t test).

not shown)). This increase in activity was almost completely inhibited by DEVD-CHO. Together, these data suggest that serine proteases, caspase-1, and caspase-3 could be involved in the acceleration of PMN apoptosis under alkaline conditions.

Like other enzymes, the activities of the proteases and endonucleases associated with apoptosis are pH dependent (6). Intracellular alkalinization has been shown to induce apoptosis in HL-60 cells (25). To assess the impact of the extracellular environment on intracellular pH, PMNs were loaded for 20 min with 5 μ M 2',7'-bis-(2-carboxyethyl)-5 (and 6)-carboxy-fluorescein (BCECF) acetoxymethyl ester (Molecular Probes) at 37°C and resuspended in HEPES-buffered balanced salts solutions adjusted to the appropriate pH. BCECF fluorescence was monitored ratiometrically (490- and 440-nm excitation; 530-nm emission), and a calibration curve was obtained by treating cells with 50 μ M nigericin in Ringer's solution as previously described (8). PMNs incubated for 1 h at pH 7.2 in

HEPES-buffered balanced salts solution had an intracellular pH of 7.11 ± 0.053 , but the intracellular pH decreased to 6.91 ± 0.046 in cells incubated for 1 h at pH 6.7. In PMNs incubated at pH 7.7 and 8.2, the intracellular pH increased to 7.30 ± 0.057 and 7.39 ± 0.081 , respectively. Although the magnitude of these changes was small, the pH of the extracellular medium had a statistically significant impact on intracellular pH ($P < 0.001$; repeated-measures ANOVA [$n = 4$]).

Several studies have reported an association between alkaline conditions and inflammation in the human gingival crevice (2, 3, 7, 11). In the present study, PMN apoptosis was assessed under conditions that ranged from the mildly acidic environment typical of healthy gingival crevices (pH 6.7) to the moderately alkaline environment commonly found in moderately inflamed crevices (pH 8.2). Our results show that exposure to alkaline conditions induces cytoplasmic alkalization and accelerates the onset of PMN apoptosis without significantly affecting viability or the maximal rate of apoptotic changes. This effect can be mitigated by TNF- α and GM-CSF and, to an even greater extent, by inhibitors of serine proteases, caspase-1, and caspase-3. Consistent with the last finding, PMN caspase-3 activity appears to increase when these cells are incubated at pH 8.2. Thus, proteases appear to play a role in the acceleration of PMN apoptosis under alkaline conditions. These findings complement previous reports that acidic conditions inhibit PMN apoptosis and impair the recognition of apoptotic PMNs by macrophages (1, 18).

Aside from its alkaline pH, the diseased gingival crevice is a unique infection site in several respects. It is inhabited by a complex bacterial flora, flushed by the flow of gingival crevicular fluid, and infiltrated by numerous PMNs. Gingival crevicular fluid flow plays a role in clearing PMNs from this site. Although apoptosis is not the sole mechanism for down-regulating the inflammatory response in the periodontium, it presumably plays an important role. Previous studies suggest that apoptosis is a major determinant of macrophage persistence in the inflamed periodontium (23). Interleukin-4, which induces apoptosis of gingival macrophages, is not found at diseased periodontal sites. The absence of this cytokine may contribute to chronic inflammation because it allows macrophages to loiter at inflamed gingival tissues. It has been suggested that topical application of interleukin-4 could be a useful approach for reducing gingival inflammation. As previously mentioned, apoptotic PMNs are less capable of responding to a bacterial challenge (20, 22). By accelerating the onset of PMN apoptosis, the alkaline environment of the diseased gingival crevice could limit the functional life span of PMNs at that site. However, accelerated apoptosis could benefit the host by minimizing tissue damage that might otherwise occur if aged PMNs underwent necrosis and spilled noxious, histotoxic products into the periodontium.

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