Glucocorticoids Inhibit Apoptosis of Human Neutrophils

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Human neutrophils rapidly undergo apoptotic cell death. Because glucocorticoids are known to modulate an array of neutrophil functional activities as well as induce rapid apoptosis in susceptible lymphocyte populations, we have examined the effects of glucocorticoids on apoptosis in mature human neutrophils. In cultures of neutrophils maintained in vitro, the glucocorticoids, dexamethasone, 6a-methylprednisolone, and hydrocortisone, inhibited the development of apoptotic morphology by 58% to 30% when assessed at 12, 24, and 48 hours. In contrast, corticosteroids lacking anti-inflammatory activity and progesterone failed to affect development of the morphologic features of apoptosis. The concentration of dexamethasone required to reduce apoptosis by 50% at 24 hours was approximately 5 x 10^{-8} mol/L, a concentration that is achievable in plasma after dexamethasone treatment. Dexamethasone (10^{-6} mol/L), but not progesterone, reduced the percentage of hypodiploid (apoptotic) nuclei by 40% to 90% over this time course. Similarly, dexamethasone reduced the DNA cleavage associated with apoptosis and prolonged the viability of neutrophils maintained in culture for 12 to 48 hours. Glucocorticoid-mediated modulation of neutrophil apoptosis was qualitatively similar, but lesser in magnitude, when compared with the effects of granulocyte colony-stimulating factor (100 ng/mL). Thus, glucocorticoids exert a protective effect on human neutrophil survival by delaying apoptosis.

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MATERIALS AND METHODS

Special reagents. Dexamethasone, 6a-methylprednisolone, hydrocortisone, 11-deoxycortisol, tetrahydrocortisol, progesterone, Triton X-100, acridine orange, and propidium iodide were purchased from Sigma Chemical Co (St Louis, MO). Stock solutions of the steroids were prepared at a concentration of 10 mmol/L in ethanol and stored at 4°C before use. G-CSF was purchased from Amgen, Inc (Thousand Oaks, CA). Culture media components, including RPMI 1640, endoxin-free fetal calf serum (FCS), HEPES buffer, penicillin-streptomycin solution, and L-glutamine were purchased from BioWhittaker, Inc (Walkersville, MD). FCS was heat-inactivated (56°C X 30 minutes) before use.

Preparation of purified neutrophils. Venous blood was collected from normal human volunteers using 0.2% dipotassium ethylenediaminetetraacetic acid (K2-EDTA) as anticoagulant. Neutrophils were isolated by sequential sedimentation in Dextran T-500 (Pharmacia LKB Biotechnology, Piscataway, NJ) in 0.9% sodium chloride, centrifugation in Histopaque-1077 (Sigma Chemical Co), and hypotonic lysis of erythrocytes, as described previously. The preparation contained greater than 97% polymorphonuclear leukocytes, of which greater than 95% were neutrophils. Cell viability was greater than 98% as determined by trypan blue exclusion.

Culture conditions. The neutrophil preparations were suspended in RPMI 1640-10% FCS (supplemented with 10 mmol/L HEPES, 0.2 mmol/L L-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin) at a concentration of 5 X 10^6 cells/mL and incubated in the presence or absence of steroids or G-CSF, as indicated in the figure presentation.

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Viability. Neutrophil viability was assessed by exclusion of propidium iodide, a dye excluded from cells with intact membrane integrity. Briefly, approximately $5 \times 10^6$ PBS-washed cells were gently suspended in PBS containing propidium iodide (5 $\mu$g/mL) in 12-× 75-mm polystyrene tubes and then analyzed (forward scatter versus FL-2 height) on a FACScan cytometer (Becton Dickinson, Mountain View, CA). A minimum of 20,000 events were recorded per sample. Data were analyzed using Consort 30 software (Becton Dickinson), and results are reported as the percentage of viable cells (ie, percentage excluding propidium iodide).

Apoptosis. Apoptosis of neutrophils was assessed by four distinct methods: (1) analysis of apoptotic cellular morphology; (2) analysis of apoptotic (hypodiploid) nuclei by flow cytometry; (3) quantitation of DNA fragmentation; and (4) DNA electrophoresis. For the analysis of apoptotic morphology, approximately $1 \times 10^6$ PBS-washed neutrophils were suspended in 1 mL of PBS containing acridine orange (5 $\mu$g/mL). An aliquot was then examined by fluorescence microscopy on a Leitz Ortholux microscope (Ernst Leitz, Wetzlar, Germany), and cells were scored as apoptotic versus non-apoptotic. The morphologic changes of apoptosis in neutrophils are easily visualized by this technique and consist of diminution in cell volume and chromatin condensation yielding fragmented or bright, homogeneously stained nuclei.\textsuperscript{11,12} Five hundred cells were counted per sample, and data are reported as the percentage of cells with apoptotic morphology. Cytospin (Shandon Southern Cytospin; Shandon Inc, Pittsburgh, PA) preparations of neutrophils were also prepared and stained using the Wright-Giemsa technique for black-and-white photographic demonstration of apoptotic morphologic features.

For analysis of apoptotic nuclei, $5 \times 10^6$ PBS-washed neutrophils were gently resuspended in 0.5 mL of hypotonic fluorochrome solution (50 $\mu$g/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) in 12-× 75-mm polystyrene tubes and immediately analyzed by flow cytometry. Propidium iodide fluorescence of individual nuclei was filtered through a 585/42-nm band pass filter and measured on a logarithmic scale using a FACScan cytometer (Becton Dickinson) while gating on physical parameters to exclude cell debris. Data were analyzed using CellFIT Cell-Cycle Analysis Version 2.0 software (Becton Dickinson). A minimum of 10,000 events were counted per sample. Results are reported as the percentage of hypodiploid nuclei reflecting the relative proportion of apoptotic cells.\textsuperscript{3,34}

Quantitation of DNA fragmentation was performed by determination of fractional solubilized DNA by diphenylamine assay.\textsuperscript{3,36} In brief, $5 \times 10^6$ PBS-washed neutrophils were lysed in 0.5 mL of lysis buffer (5 mMol/L Tris- HCl, 20 mMol/L EDTA, 0.5% Triton X-100, pH 8.0), and the lysates were centrifuged (15,000g) to separate high molecular weight DNA (pellet) and cleaved, low molecular weight DNA (supernatant). After precipitation with 0.5 N perchloric acid, DNA was quantitated by spectrophotometry after the addition of diphenylamine reagent. Data are reported as the relative proportion (percentage) of soluble, low molecular weight DNA.

DNA electrophoresis was performed to assess DNA fragmentation. Neutrophils ($1 \times 10^6$) were removed from culture at varying time points, pelleted by centrifugation, washed once in PBS, and lysed by the addition of 0.5 mL of lysis buffer (10 mMol/L EDTA, 50 mMol/L Tris, 1% sodium dodeyl sulfate, 250 $\mu$g/mL proteinase K [Boehringer Mannheim, Mannheim, Germany], pH 8.0) at 37°C for 16 hours. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 0.5 vol 7.5 mol/L sodium acetate and 2 mol ethanol at −70°C, resuspended in Tris-EDTA buffer containing 250 $\mu$g/mL RNAse (Boehringer), and incubated at 65°C for 5 minutes before electrophoresis in 1% agarose at 50 V for 3 hours. After staining with ethidium bromide, DNA was visualized by UV examination for photography.

Statistical analysis. Mean values were compared using the Student's two-tailed t-test for independent means; differences were not regarded as significant if $P > .05$.

RESULTS

Effects of steroids on the development of apoptotic morphology in neutrophils maintained in vitro. All three of the glucocorticoids examined (dexamethasone, 6a-methylprednisolone, and hydrocortisone) inhibited the development of apoptotic morphology in neutrophils at each of the time points assessed (Figs 1 and 2). In control neutrophils obtained from 4 separate healthy donors, the mean percentage of cells showing apoptotic morphology increased steadily from 15% at 12 hours to 44% at 24 hours to 86% at 48 hours. Incubation with glucocorticoid effectively reduced the mean percentage of apoptotic cells by 59% to 90% at each of the time points. The differences in degree of apoptosis in glucocorticoid-treated versus control cells was statistically significant at each time point ($P < .05$). In cells incubated with dexamethasone (10 to 60 mol/L), the mean percentage of neutrophils with apoptotic morphology was 2%, 13%, and 35% at 12, 24, and 48 hours, respectively. Similar values were observed after incubation with either 6a-methylpred-
Fig 2. Morphologic features of neutrophils maintained in suspension culture in the absence and presence of dexamethasone. Cytospin preparations of neutrophils were prepared and stained using the Wright-Giemsa method after incubation in vitro for 0, 24, 48, and 72 hours. Samples 1 through 4 represent control neutrophils: (1) 0 hours, (2) 24 hours, (3) 48 hours, and (4) 72 hours. Samples 5 through 7 represent neutrophils maintained in the presence of dexamethasone (10^{-6} mol/L): (5) 24 hours, (6) 48 hours, and (7) 72 hours.

nisolone (10^{-6} mol/L) or hydrocortisone (10^{-6} mol/L). For dexamethasone, the concentration required to inhibit neutrophil apoptosis by 50% (EC_{50}) was approximately 5 \times 10^{-8} mol/L (Fig 3). Apoptosis was clearly delayed but not prevented by dexamethasone, as shown in Fig 2. Microscopic examination showed that control neutrophils developed prominent morphologic features characteristic of apoptosis, including diminution in cell volume, nuclear condensation eventually resulting in apoptotic bodies, and cytoplasmic vacuolation, within 24 hours in culture. Although dexamethasone-treated cells generally retained a nonapoptotic morphologic appearance after 24 to 48 hours in culture, nuclear condensation and decreased cell volume were evident by 72 hours.

To assess the specificity of glucocorticoids in their ability to inhibit neutrophil apoptosis, we examined the effects of 11-deoxycortisone and tetrahydrocortisone, two corticosteroids that lack anti-inflammatory activity, and progesterone on the development of apoptotic morphology in human neutrophils maintained in vitro. In contrast to glucocorticoids, neither 11-deoxycortisone, tetrahydrocortisone, nor progesterone significantly affected the development of apoptotic morphology (P > .05; Fig 1). Thus, among the steroids tested, only the anti-inflammatory corticosteroids appear to inhibit apoptosis in neutrophils.

The effects of dexamethasone, G-CSF, and progesterone on the development of apoptotic nuclei and DNA fragmentation in human neutrophils maintained in vitro. Recent evidence indicates that apoptosis occurs via a sequential process. The initial stage is characterized by a decrease in cytoplasmic volume and chromatin condensation, followed later by internucleosomal cleavage of DNA that results in hypodiploid nuclei that can be identified by flow cytometry. Because internucleosomal cleavage of DNA, albeit a late manifestation of apoptosis, is considered to be its biochemical hallmark, we quantitated the development of hypodiploid nuclei (Fig 4) and DNA fragmentation (Fig 5) in neutrophils maintained in vitro in the presence and absence of dexamethasone (10^{-6} mol/L), progesterone.
Fig 4. Kinetic analysis of the effects of dexamethasone, progesterone, and G-CSF on the development of apoptotic nuclei in human neutrophils maintained in suspension culture. (a) Neutrophils were maintained in suspension culture at 37°C with and without dexamethasone (10^{-8} mol/L), progesterone (10^{-8} mol/L), or G-CSF (100 ng/mL), as designated above. After incubation for 0, 12, 24, and 48 hours, aliquots of the neutrophil suspensions were processed for DNA content analysis using propidium iodide staining as described in the Materials and Methods. Nuclei were analyzed using flow cytometry, data were plotted on log histograms as red fluorescence intensity versus relative cell number, and the percentage of hypodiploid nuclei was determined for each condition. Results are reported as the mean ± SE of four separate experiments performed with neutrophils isolated from independent donors. *A statistically significant difference in apoptosis as compared with control cells maintained in culture for the specific time period indicated (P < .05). (b) Representative log histograms of DNA content in neutrophils from a single donor are displayed. Neutrophils were maintained in suspension culture for 24 hours with and without dexamethasone, progesterone, or G-CSF, as outlined above. The percentage of hypodiploid nuclei (between dashed line markers) present in each condition is reported. Similar results were observed in neutrophils isolated from 3 other independent donors.
(10⁻⁶ mol/L), and G-CSF (100 ng/mL). The mean percentage of apoptotic nuclei steadily increased in control neutrophils when measured after 12 (24%), 24 (50%), and 48 hours (92%) in culture (Fig 4). The development of apoptotic nuclei was not affected by incubation with progesterone. In contrast, both dexamethasone and G-CSF significantly reduced the development of hypodiploid nuclei in cultures of neutrophils at each of the time points (P < .05). However, the magnitude of inhibition of apoptosis was greater for G-CSF at each determination. G-CSF reduced the mean percentage of apoptotic nuclei to 4%, 6%, and 55% at 12, 24, and 48 hours, respectively. By comparison, analysis of dexamethasone-treated neutrophils yielded mean apoptosis percentages of 6%, 16%, and 76% at the respective time points.

Similar results were obtained when DNA fragmentation was quantitated (Fig 5). The mean percentage of soluble DNA (ie, low molecular weight DNA representing cleavage products) in neutrophils increased steadily with increasing time in culture (8% at 12 hours, 48% at 24 hours, and 59% at 48 hours). The amount of soluble DNA in progesterone-treated cells was not statistically different than that of the control at each of the time points (P > .05). However, both dexamethasone and G-CSF significantly reduced the development of soluble DNA in cultures of neutrophils over time (P < .05). As observed with inhibition of the development of hypodiploid nuclei, G-CSF exerted the greater effect. Electrophoretic analysis confirmed the ability of dexamethasone to delay DNA fragmentation in neutrophils maintained in vitro for 0 to 48 hours (Fig 6).

The effects of dexamethasone, G-CSF, and progesterone on viability of neutrophils maintained in vitro. To confirm that the observed reduction in apoptosis induced by glucocorticoids correlates with enhanced survival of neutrophils, we determined the viability of neutrophils maintained in the presence and absence of dexamethasone (10⁻⁶ mol/L), G-CSF (100 ng/mL), or progesterone (10⁻⁶ mol/L) for 24 and 48 hours (Fig 7). Viability was assessed using flow cytometry to quantitate the percentage of cells capable of excluding propidium iodide. After 24 hours in vitro, the mean proportion of viable cells declined to similar levels in control (81%) and progesterone-treated (82%) neutrophils. In contrast, viability was significantly increased in neutrophils treated with either dexamethasone (92%) or G-CSF (94%). The ability of dexamethasone and G-CSF to prolong neutrophil survival was even more apparent at 48 hours. Whereas the mean percentage of viable cells had declined to 35% to 40% in control and progesterone-treated neutrophils, treatment with either dexamethasone (61% viable) or G-CSF (80%) significantly enhanced neutrophil survival. Thus, similar to the effects reported for G-CSF, glucocorticoid inhibition of neutrophil apoptosis results in prolonged viability of human neutrophils in vitro.

**DISCUSSION**

Apoptosis is considered to be an active process dependent on specific RNA and protein synthesis. However, it is now clear that certain cell populations must be induced to undergo apoptosis by specific hormones or stimuli, whereas other cell populations appear to be committed to apopto-
Immature thymocytes represent a cell population that must be induced to undergo apoptosis. In these cells, glucocorticoids induce rapid apoptosis via a pathway that is independent, at least in part, of p53 expression. Mature neutrophils, on the other hand, represent a cell population that appears to be committed to a pathway of apoptosis. Recent evidence from several laboratories indicates that neutrophils can be partially rescued, or inhibited, from undergoing apoptosis by a variety of proinflammatory mediators and cytokines. Of the cytokines influencing apoptosis in human neutrophils, G-CSF exerts the greatest antiapoptotic activity.

In the present study, we examined whether glucocorticoids (ie, anti-inflammatory corticosteroids) could influence the development of apoptosis in human neutrophils. Our results show that glucocorticoids exert a protective effect on neutrophil survival in vitro via inhibition of apoptosis at concentrations that correlate with both the plasma level achieved after the administration of pharmacologic doses of dexamethasone in vivo and the concentration required to induce apoptosis of immature thymocytes.

Consistent with the commitment of neutrophils to apoptotic death, glucocorticoids delayed, rather than blocked, apoptosis, resulting in prolonged neutrophil survival in vitro. Thus, it is clear that glucocorticoids play divergent roles in the regulation of the apoptotic pathway in thymocytes and neutrophils.

The administration of glucocorticoids in vivo induces a moderate increase in the number of blood neutrophils. This neutrophilia has been attributed to an increase in the release of neutrophils from bone marrow reserves and/or a decrease in neutrophil margination. The results from the present study suggest an alternative or additional mechanism that may play a role in glucocorticoid-induced neutrophilia. The apoptotic pathway is considered to be the primary means by which neutrophils undergo senescence. Macrophages recognize and phagocytose senescent, apoptotic neutrophils before their loss of viability in vitro and, apparently, in vivo. Thus, a decrease in the rate of development of apoptotic features in neutrophils would be expected to reduce the rate of their removal by macrophages in vivo, resulting in an apparent increase in neutrophil survival and lengthening of the blood neutrophil half-life. Thus, inhibition of neutrophil apoptosis may contribute to the neutrophilia that occurs after glucocorticoid administration in vivo.

Given the fact that the precise cellular and molecular events involved in the regulation of the apoptotic pathway in mature neutrophils have yet to be elucidated, the mechanism by which glucocorticoids inhibit apoptosis in human neutrophils remains unclear. Unlike the situation with lymphocytes, transient, but not sustained, increases in the level of cytosolic free calcium induced by low concentrations of ionophore have been reported to inhibit, rather than promote, apoptosis of mature human neutrophils.

Thus, modulation of intracellular free calcium could conceivably account for the inhibitory effects of glucocorticoids on neutrophil apoptosis. Alternatively, glucocorticoids could influence neutrophil apoptosis by modulating expression of p53 or bcl-2. Wild-type p53, a product of a tumor-suppressor gene, has been implicated in the induction of apoptosis in a variety of cells and tissues. Given that mature human neutrophils express wild-type p53, a possible mechanism by which glucocorticoids delay neutrophil apoptosis might involve a reduction in the level of p53 protein, resulting from either increased degradation or repression of p53 gene expression. However, we have been unable to detect alterations in p53 protein levels in either dexamethasone- or G-CSF-treated neutrophils by immunoblot (data not shown). Reinduction of bcl-2 gene expression is another potential mechanism by which neutrophil apoptosis could be delayed. Bcl-2 is an oncogene product that functions to prevent apoptosis in a variety of cell types and is endogenously expressed in early myeloid cells of the bone marrow but not in mature neutrophils. Although enforced expression of bcl-2 has been shown to inhibit apoptosis in transgenic mice and HL-60 myeloid leukemia cells during granulocytic differentiation, neither proinflammatory cytokines nor glucocorticoids induce bcl-2 expression in mature human neutrophils (Liles et al, unpublished data). However, these results do not exclude the possibility that glucocorticoids may modulate the expression of recently described bcl-2 homologs in neutrophils.

The Fas antigen (Fas; Apo-1; CD95), a member of the tumor necrosis factor receptor family, is capable of inducing rapid apoptosis in susceptible cell populations. The Fas-mediated apoptotic signal transduction pathway appears to involve the generation of ceramide, produced after activation of specific sphingomyelinase(s). The recent findings that Fas is expressed on human neutrophils and capable of inducing rapid apoptosis suggest that the Fas pathway may represent an important mechanism regulating spontaneous neutrophil apoptosis. Furthermore, dexamethasone has been shown to increase...
the sphingomyelin content of human neutrophils in vitro,26 invoking the possibility that glucocorticoids could depress the rate of spontaneous apoptosis via modulation of steady-state levels of ceramide. Further studies are underway to delineate which of these factors are important for the regulation of apoptosis in neutrophils.16,63

In summary, our results show that glucocorticoids prolong neutrophil survival in vitro via inhibition of apoptosis. These effects on neutrophil viability are qualitatively similar, but of lesser magnitude, to those exerted by G-CSF, a proinflammatory cytokine. On the basis of our findings, we postulate that the neutrophilia induced by glucocorticoid administration in vivo may be due, in part, to inhibition of apoptosis and prolonged survival of circulating neutrophils.

NOTE ADDED IN PROOF

We acknowledge the manuscript published by Cox et al64 after the submission of our report demonstrating similar effects of glucocorticoids on neutrophil apoptosis.

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Glucocorticoids inhibit apoptosis of human neutrophils

WC Liles, DC Dale and SJ Klebanoff