

Differential Expression of Fas (CD95) and Fas Ligand on Normal Human Phagocytes: Implications for the Regulation of Apoptosis in Neutrophils

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Summary

Human neutrophils, monocytes, and eosinophils are known to undergo apoptotic cell death. The Fas/Fas ligand pathway has been implicated as an important cellular pathway mediating apoptosis in diverse cell types. We conducted studies to examine the importance of the Fas/FasL system in normal human phagocytes. Although Fas expression was detected on neutrophils, monocytes, and eosinophils, constitutive expression of FasL was restricted to neutrophils. The three types of phagocytes demonstrated differential sensitivity to Fas-induced apoptosis. Only neutrophils were highly susceptible to rapid apoptosis *in vitro* after stimulation with activating anti-Fas IgM (mAb CH-11). Fas-mediated neutrophil apoptosis was suppressed by incubation with G-CSF, GM-CSF, IFN- γ , TNF- α , or dexamethasone, as well as the selective tyrosine kinase inhibitors, herbimycin A and genistein. Spontaneous neutrophil death *in vitro* was partially suppressed by Fas-Ig fusion protein or antagonistic anti-Fas IgG₁ (mAb ZB4). In coculture experiments, neutrophils released a soluble factor inducing death in Fas-susceptible Jurkat cells via a mechanism sensitive to the presence of Fas-Ig or anti-Fas IgG₁. Immunoblot analysis using specific anti-human FasL IgG₁ (mAb No. 33) identified a 37-kD protein in lysates of freshly isolated neutrophils and a 30-kD protein in the culture supernatant of neutrophils maintained *in vitro*. Our results suggest that mature neutrophils may be irrevocably committed to autocrine death by virtue of their constitutive coexpression of cell-surface Fas and FasL via a mechanism that is sensitive to proinflammatory cytokines, glucocorticoids, and inhibitors of tyrosine kinase activity. Furthermore, neutrophils can serve as a source of soluble FasL, which may function in a paracrine pathway to mediate cell death.

Apoptosis, or programmed cell death, plays a critical role in the regulation of inflammation and the host immune response (1). Distinct from necrosis, the apoptotic process induces a series of coordinated morphologic and biochemical events in the affected cell, resulting in its demise and removal by scavenger phagocytes (1). Characteristically, a cell undergoing apoptosis initially develops cytoplasmic shrinkage and membrane blebbing (zeiosis), which is subsequently followed by chromatin condensation (i.e., "apoptotic bodies") and DNA fragmentation. Although these changes generally occur sequentially in an affected cell, these events may occur separately, subject to the control of independent regulatory pathways (2–5).

The professional human phagocytes — neutrophils, monocytes, and eosinophils — die via apoptosis when maintained *in vitro* (6–15). Mature human neutrophils undergo spontaneous apoptosis most rapidly, resulting in the demise of >50% of a population within 48 h (6, 9–11). Spontaneous apoptosis occurs more slowly in eosinophils, where apoptotic morphologic features are generally delayed until 48–72 h (14, 15). The rate of spontaneous apoptosis in monocytes has been reported to vary between that of neutrophils and eosinophils, depending partly on the experimental culture conditions used (13, 16).

Although neutrophils appear to be committed to apoptotic death *in vitro* and *in vivo*, the life span and functional activity of mature neutrophils can be extended *in vitro* by incubation with either proinflammatory cytokines, including G-CSF, GM-CSF, IFN- γ , TNF- α , and IL-2 (6, 17), or glucocorticoids (9, 10). Similarly, spontaneous apoptosis is suppressed by TNF- α , IL-1 β , GM-CSF, and LPS in mono-

The data in this paper were presented in part in abstract form at the 36th Annual Meeting of the American Society of Hematology, 2–6 December 1994, Nashville, TN, and published in part in abstract form (*Blood*. 84(Suppl. 1):371a).

cytes (13) as well as IL-5, TGF β , and GM-CSF in eosinophils (14, 15).

The Fas (APO-1; CD95)/Fas ligand (FasL)¹ system has emerged as an important cellular pathway regulating the induction of apoptosis in a wide variety of tissues. Fas is a widely expressed 45-kD type I membrane protein member of the TNF/nerve growth factor family of cell surface molecules (18–20). Fas is activated to mediate apoptosis in susceptible tissues following interaction with agonistic anti-Fas IgM mAb or its natural ligand, FasL, a 37-kD type II protein (18–21). In contrast to the tissue distribution of Fas, constitutive expression of FasL is relatively limited (19, 20).

The biological importance of the Fas/FasL system has been extensively studied in T cells, where it plays a critical role in the clonal deletion of autoreactive T cells and activation-induced suicide of T cells (19, 20, 22–27). FasL can function in either autocrine or paracrine pathways to cell death. Although not constitutively expressed in mature T cells, FasL is induced after activation of mature T cells by a variety of stimuli, including PMA, ionomycin, anti-CD3, and bacterial superantigen (22–25). The subsequent interaction of FasL and Fas on the cell surface initiates a signal transduction pathway leading to autocrine T cell suicide (22–25). Cytotoxic T cells can deploy FasL as a death effector molecule in their strategies to induce killing of target cells (28, 29). Physiological activation induces FasL expression in distinct cytotoxic T cell clones, which can then use FasL to induce apoptosis upon interaction with Fas-bearing target cells (28–31). Furthermore, activated T cells can secrete a biologically active soluble form of FasL that may possibly contribute to systemic tissue injury during inflammation (32).

The role of the Fas/FasL system in the fate of phagocytes has been less well characterized. To assess the potential importance of this system in the regulation of spontaneous apoptosis of normal human phagocytes, we examined the relative surface expression of Fas and FasL on neutrophils, monocytes, and eosinophils. Experiments were performed to determine the relative sensitivity of these phagocyte populations to Fas-induced apoptosis. We also examined the effects of stimuli known to suppress spontaneous neutrophil apoptosis on Fas-mediated apoptosis in neutrophils. Additional experiments were conducted to determine whether neutrophils could serve as a source of soluble FasL and, thereby, induce death of heterologous cells that are susceptible to the Fas-mediated death pathway.

Materials and Methods

Special Reagents. The following reagents were obtained from commercial sources: FITC-conjugated annexin V (annexin V-FITC) (BRAND Applications b.v., Rotterdam, The Netherlands); dexamethasone, progesterone, H-7, acridine orange, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), Triton

X-100, human serum albumin (fraction V), and FITC-conjugated affinity-purified F(ab')₂ goat anti-human IgG Ab (Sigma Chemical Co., St. Louis, MO); genistein (ICN Biomedicals, Cleveland, OH); herbimycin A (GIBCO BRL, Gaithersburg, MD); murine mAb CH-11 (anti-human Fas IgM); and murine mAb ZB4 (anti-human Fas IgG₁; Medical & Biological Laboratories Co. Ltd., Nagoya, Japan, and Upstate Biotechnology Inc., Lake Placid, NY); murine mAb UB2-FITC (anti-human Fas IgG₁; Oncor, Inc., Gaithersburg, MD); murine mAb MMA (anti-CD15 IgM) and murine IgG₁-FITC control Ab (Becton Dickinson, San Jose, CA); and murine mAb No. 33 (anti-human FasL IgG₁; Transduction Laboratories, Lexington, KY). G-CSF and GM-CSF were purchased from Amgen, Inc. (Thousand Oaks, CA) and Immunex, Inc. (Seattle, WA), respectively. IFN- γ and TNF- α were provided as gifts from Genentech, Inc. (South San Francisco, CA).

Preparation of Fas-Ig and B7-Ig Fusion Proteins. The Fas-Ig and B7-Ig fusion proteins were constructed using the procedures essentially as described by Hollenbaugh and Aruffo (33). The extracellular domains of human Fas and human B7 were isolated by PCR using primers based on previously reported sequences (18, 34). The resulting fragments were then separately placed in frame with the constant region of human IgG₁. Each fusion gene was inserted into separate modified pCDM8 vectors, which were then transfected into COS cells using DEAE-dextran. After 6 d of culture, the COS cell supernatants were harvested, and the Fas-Ig and B7-Ig were purified on protein A affinity columns.

Preparation of Purified Populations of Normal Human Phagocytes. Venous blood was collected from normal human volunteers using 0.2% K₂EDTA as an 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 (35). The preparation contained >97% PMN, of which >95% were neutrophils. Platelet-depleted monocytes were isolated as described previously (35). Eosinophils were isolated by centrifugation on discontinuous gradients of metrizamide, as described previously (35). The preparations contained 95–100% eosinophils, as determined by Wright-Giemsa staining of cytocentrifuged samples. Cell viability of all three populations of phagocytes was >98%, as determined by trypan blue exclusion.

Culture Conditions for Normal Human Phagocytes. Preparations of isolated normal human phagocytes were maintained in RPMI 1640–10% FCS (supplemented with 10 mM HEPES, 0.2 mM L-glutamine, 25 U/ml penicillin, and 25 mg/ml streptomycin) at a concentration of 5×10^6 cells/ml in 48-well cell culture clusters (Costar 3548; Costar Co., Cambridge, MA) at 37°C in a humidified CO₂ incubator (5% CO₂–95% air). Aliquots were removed and washed once in PBS before use in assays of neutrophil apoptosis (see below).

Immunofluorescence Flow Cytometry for Detection of Fas Expression. Cell surface expression of Fas was assayed by direct immunofluorescence flow cytometry using saturating concentrations of mAb UB2-FITC. In brief, freshly isolated populations of normal human phagocytes (neutrophils, monocytes, and eosinophils) were suspended in ice-cold PBS at a concentration of 2×10^7 cells/ml. An aliquot of the respective phagocyte suspension (50 μ l, 10^6 cells) was added to 50 μ l of mAb UB2-FITC dissolved in PBS containing 0.1% BSA and 0.1% sodium azide in the wells of a 96-well vinyl microtiter assay plate (Costar 2596; Costar) kept on ice. Cells were stained for 45 min at 4°C, washed once with PBS containing 0.1% sodium azide, and then fixed with 1% paraformaldehyde in PBS. Simultaneous negative control staining reac-

¹ Abbreviations used in this paper: FasL, Fas ligand; MFI, mean fluorescence intensity; sFasL, soluble FasL.

tions were performed with a saturating concentration of irrelevant murine IgG₁-FITC in place of mAb UB2-FITC. The plates were kept at 4°C until the stained cells were analyzed by flow cytometry using a FACScan® and BDIS Consort software (Becton Dickinson & Co., Mountain View, CA). Mean fluorescence intensity (MFI) was calculated by subtraction of the mean fluorescence channel of the appropriate negative control.

Immunofluorescence Flow Cytometry for Detection of FasL Expression. Cell-surface expression of FasL on freshly isolated populations of normal human phagocytes was assayed by indirect immunofluorescence flow cytometry using Fas-Ig for primary staining and FITC-conjugated, affinity-purified F(ab')₂ goat anti-human IgG for secondary staining. In brief, primary staining was performed with 10⁶ cells incubated with Fas-Ig (20 µg/ml) in PBS containing 10% normal human serum in wells of a 96-well vinyl microtiter assay plate (Costar 2596; Costar) kept on ice. Cells were stained for 45 min at 4°C, then washed once with PBS containing 0.1% sodium azide and 0.1% BSA. Secondary staining was performed with FITC-conjugated, affinity-purified F(ab')₂ goat anti-human IgG (10 µg/ml) in PBS containing 10% goat serum. After a single PBS wash, the cells were fixed with 1% paraformaldehyde in PBS. Simultaneous negative control staining reactions were performed by omitting Fas-Ig in the primary staining step. In separate neutrophil assays, B7-Ig was also substituted for Fas-Ig as a negative control. The plates were kept at 4°C until the stained cells were analyzed by flow cytometry.

DNA Electrophoresis. DNA electrophoresis was performed to assess DNA fragmentation. Normal human neutrophils, monocytes, and eosinophils (10⁶ cells/sample) were removed from culture after incubation with mAb CH-11 for 12 h to induce Fas-mediated apoptosis, pelleted by centrifugation, washed once in PBS, and lysed by the addition of 0.5 ml lysis buffer (10 mM EDTA, 50 mM Tris, 1% SDS, 250 µg/ml proteinase K [Boehringer Mannheim, Mannheim GmbH, Mannheim, Germany], pH 8.0) at 37°C for 16 h. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 0.5 vol 7.5 M sodium acetate and 2 vol ethanol at -70°C, resuspended in Tris-EDTA buffer containing 250 µg/ml RNase (Boehringer Mannheim), and incubated at 65°C for 5 min before electrophoresis in 1.2% agarose at 50 V for 3 h. After staining with ethidium bromide, DNA was visualized by UV examination for photography.

Analysis of Apoptotic Cellular Morphology of Neutrophils. For the analysis of apoptotic morphology, ~10⁶ PBS-washed neutrophils were suspended in 1 ml of PBS containing acridine orange (5 µg/ml). An aliquot was then examined by fluorescence microscopy on a Leitz Ortholux microscope (Ernst Leitz, Wetzlar, Germany), and cells were scored as apoptotic vs. nonapoptotic. The morphologic changes of apoptosis in neutrophils are easily visualized by this technique, and they consist of diminution in cell volume and chromatin condensation yielding fragmented or bright, homogeneously stained nuclei (1, 10). 500 cells were counted per sample, and data were reported as the percentage of cells with apoptotic morphology.

Quantitation of DNA Fragmentation. Quantitation of DNA fragmentation was performed by determination of fractional solubilized DNA by diphenylamine assay (10, 36). In brief, 5 × 10⁶ PBS-washed neutrophils were lysed in 0.5 ml lysis buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0), and the lysates were centrifuged (15,000 g) 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.

Immunofluorescence Flow Cytometry of Annexin V-FITC Binding. The binding of annexin V-FITC was used as a sensitive measure of neutrophil apoptosis and performed by modification of a previously described method (11). Specific binding of annexin V-FITC was performed by incubation of neutrophils (5 × 10⁵ cells) in 50 µl of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing a saturating concentration of annexin V-FITC for 30 min at 4°C. Nonspecific binding was determined using calcium-free binding buffer (10 mM Hepes, 140 mM NaCl, 10 mM EDTA, pH 7.4). After incubation, neutrophils were pelleted at 400 g for 10 min at 4°C, then fixed in 1% paraformaldehyde in PBS and stored in the dark at 4°C before analysis. Neutrophils were analyzed within 24 h of fixation in a Coulter Epics Elite ESP flow cytometer (Coulter Corp., Hialeah, FL) while gating on physical parameters to exclude cell debris.

Coculture Experiments with Neutrophils and Jurkat Cells. Freshly isolated neutrophils (2.5 × 10⁶/ml) and Jurkat cells (2.5 × 10⁶/ml) were cocultured in RPMI 1640-10% FCS at 37°C in a humidified CO₂ incubator (5% CO₂-95% air) in six-well Transwell tissue culture plates (Corning Costar Corp., Cambridge, MA). The two cell populations were physically separated by a polycarbonate membrane with a 0.4-µm pore size. Specific wells were supplemented with mAb CH-11 (anti-human Fas IgM, 100 ng/ml), mAb ZB4 (anti-human Fas IgG₁, 1 µg/ml), or Fas-Ig (50 µg/ml), as indicated in the figure legend. At designated times, aliquots of the Jurkat cells were removed, and viability was determined using a commercial fluorescence microscopy viability kit (LIVE/DEAD® Viability/Cytotoxicity Kit; Molecular Probes, Inc., Eugene, OR).

Identification of Membrane-associated and Soluble FasL from Neutrophils by Immunoblot. Lysate samples of freshly isolated neutrophils (10⁷) from two normal volunteers were prepared in 200 µl of CHAPS lysis buffer, as previously described (35). Neutrophil-conditioned culture supernatants were obtained by maintaining neutrophils in vitro in RPMI 1640 (supplemented with 10 mM Hepes, 0.2 mM L-glutamine, 25 U/ml penicillin, and 25 µg/ml streptomycin) plus 0.1% human serum albumin at a concentration of 20 × 10⁶ cells/ml in 48-well cell culture clusters. Culture supernatants were removed at designated time points, and were cleared of intact cells and cellular debris by centrifugation. Membrane-associated FasL from lysates of freshly isolated neutrophils and soluble FasL from supernatants of neutrophils maintained in vitro were detected by immunoblot analysis on Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) with murine mAb No. 33 (anti-human FasL IgG₁). Lysates were mixed 1:1 with 2× SDS sample buffer and boiled for 5 min immediately before electrophoretic analysis (40 µl loaded per well) on 8.5% SDS polyacrylamide gels. Culture supernatants were handled similarly, except that aliquots were mixed 2:1 with 3× SDS sample buffer before loading. Immunoblots were developed using an enhanced chemiluminescence detection kit (ECL; Amersham Life Science, Buckinghamshire, UK).

Statistical Analysis. Mean values were compared using Student's two-tailed *t* test for independent means; differences were not regarded as significant if *P* ≥ 0.05.

Results

Differential Cell-surface Expression of Fas and FasL on Normal Human Neutrophils, Monocytes, and Eosinophils. Cell-surface

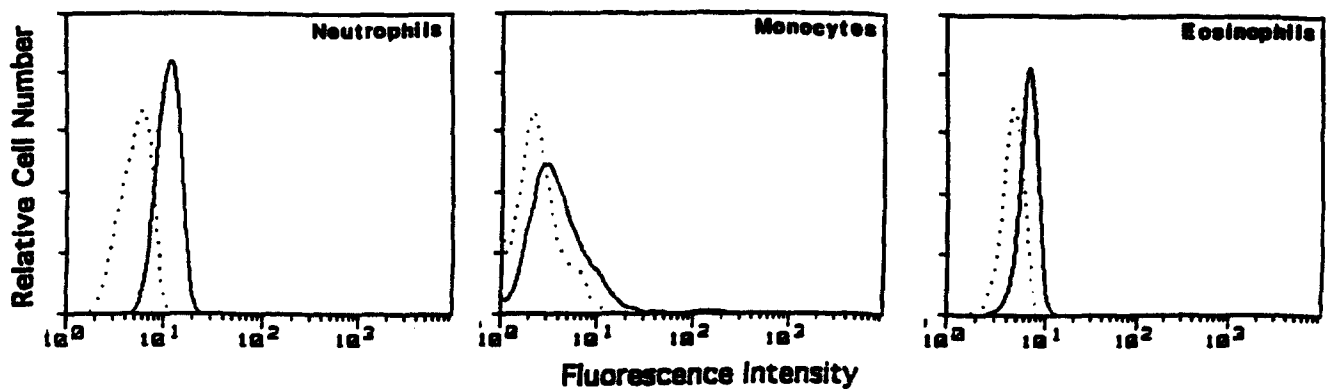


Figure 1. Cell-surface expression of Fas on normal human phagocytes. Direct immunofluorescence flow cytometry was performed to detect cell surface Fas expression on freshly isolated normal human neutrophils, monocytes, and eosinophils. Data are expressed in the form of a fluorescence histogram overlay depicting specific Fas staining (*solid line*) vs. negative control staining (*broken line*). Data are representative of five independent experiments with similar results. MFI values cited in the text represent mean values \pm SD from five independent donors.

expression of Fas was detected on normal human neutrophils, monocytes, and eosinophils by direct immunofluorescence flow cytometry using mAb UB2-FITC (Fig. 1). Fas expression was highest on neutrophils, where the average MFI was 19.5 ± 0.3 (mean \pm SD; log scale; $n = 5$ independent donors). The relative intensity of Fas expression on monocytes and eosinophils was $<50\%$ of that observed on neutrophils, with MFI values of 6.2 ± 1.6 and 9.0 ± 0.5 for monocytes and eosinophils, respectively ($n = 5$ independent donors).

Cell-surface expression of FasL was detected by indirect immunofluorescence flow cytometry using Fas-Ig fusion protein. In contrast to Fas, the FasL immunofluorescence histograms for monocytes and eosinophils were virtually superimposable on their respective negative control immunofluorescence histograms (Fig. 2), yielding MFI values for monocytes and eosinophils that were not significantly greater than 0 (2.0 ± 1.0 and 0.9 ± 0.4 , respectively [$n = 5$ independent donors]). Significant FasL expression, however, was detected on neutrophils with an MFI of 12.5 ± 0.9 ($n = 5$ independent donors). Substitution of B7-Ig for

Fas-Ig in the initial binding reaction yielded an immunofluorescence histogram superimposable on the negative control (data not shown).

Differential Sensitivity of Neutrophils, Monocytes, and Eosinophils to Fas-induced Apoptosis. To determine the relative sensitivity of normal human neutrophils, monocytes, and eosinophils to Fas-induced apoptosis, isolated populations of these cells were maintained in vitro in serum-containing medium and stimulated with agonistic anti-Fas IgM (mAb CH-11) for 12 h. Because DNA fragmentation is considered to be the biochemical hallmark of apoptosis (1), a quantitative assay of DNA fragmentation was performed (Fig. 3 A). Stimulation by mAb CH-11 induced a dramatic increase in the content of low molecular weight DNA in neutrophils from a control value of 7% to 48% ($P < 0.01$). In contrast, the proportion of soluble DNA in monocytes and eosinophils was relatively unaffected by mAb CH-11. The percentage of soluble DNA was unchanged in eosinophils (3% for both treated and control cells) and only modestly increased in monocytes from a control value of 1–6% ($P < 0.05$).

The relative sensitivity of neutrophils to Fas-induced DNA

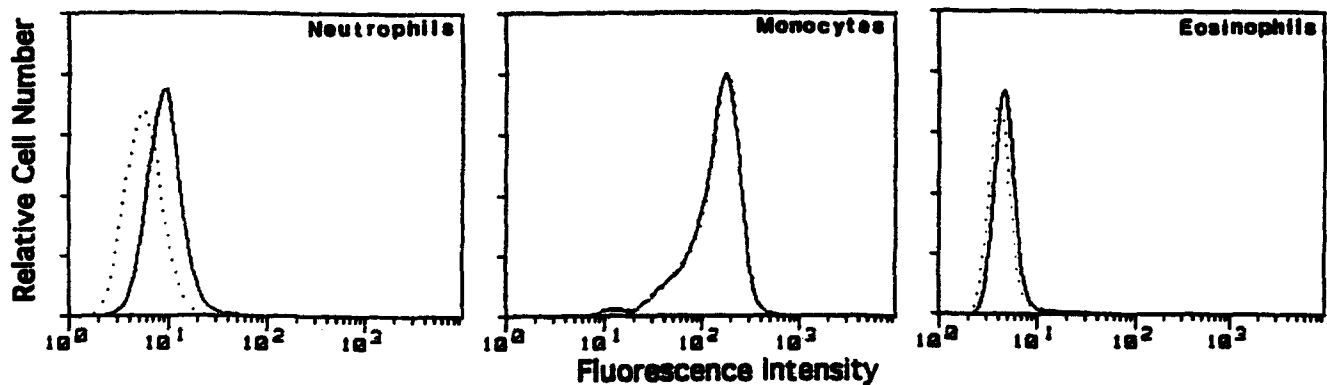


Figure 2. Cell-surface expression of FasL on normal human phagocytes. Indirect immunofluorescence flow cytometry was performed to detect cell-surface FasL expression on freshly isolated normal human neutrophils, monocytes, and eosinophils. Data are expressed in the form of a fluorescence histogram overlay depicting specific FasL staining (*solid line*) vs. negative control staining (*dotted line*). Data are representative of five independent experiments with similar results. MFI values cited in the text represent mean values \pm SD from five independent donors.

fragmentation was confirmed by conventional "DNA ladder" analysis (Fig. 3 B). Whereas negligible or modest DNA cleavage was present in freshly isolated (0 h, lane 1) neutrophils and neutrophils maintained in vitro for 12 h (lane 2), a substantial level of DNA cleavage products was readily apparent in neutrophils after stimulation with mAb CH-11 for 12 h (lane 3).

When undergoing apoptosis, cells develop characteristic morphologic changes, such as chromatin condensation and cytoplasmic shrinkage, before apparent DNA fragmentation (1, 2, 5). Stimulation with mAb CH-11 rapidly accelerated the development of apoptotic morphology in neutrophils maintained in vitro (Fig. 3 C). Greater than 30% of the mAb CH-11-stimulated population developed apop-

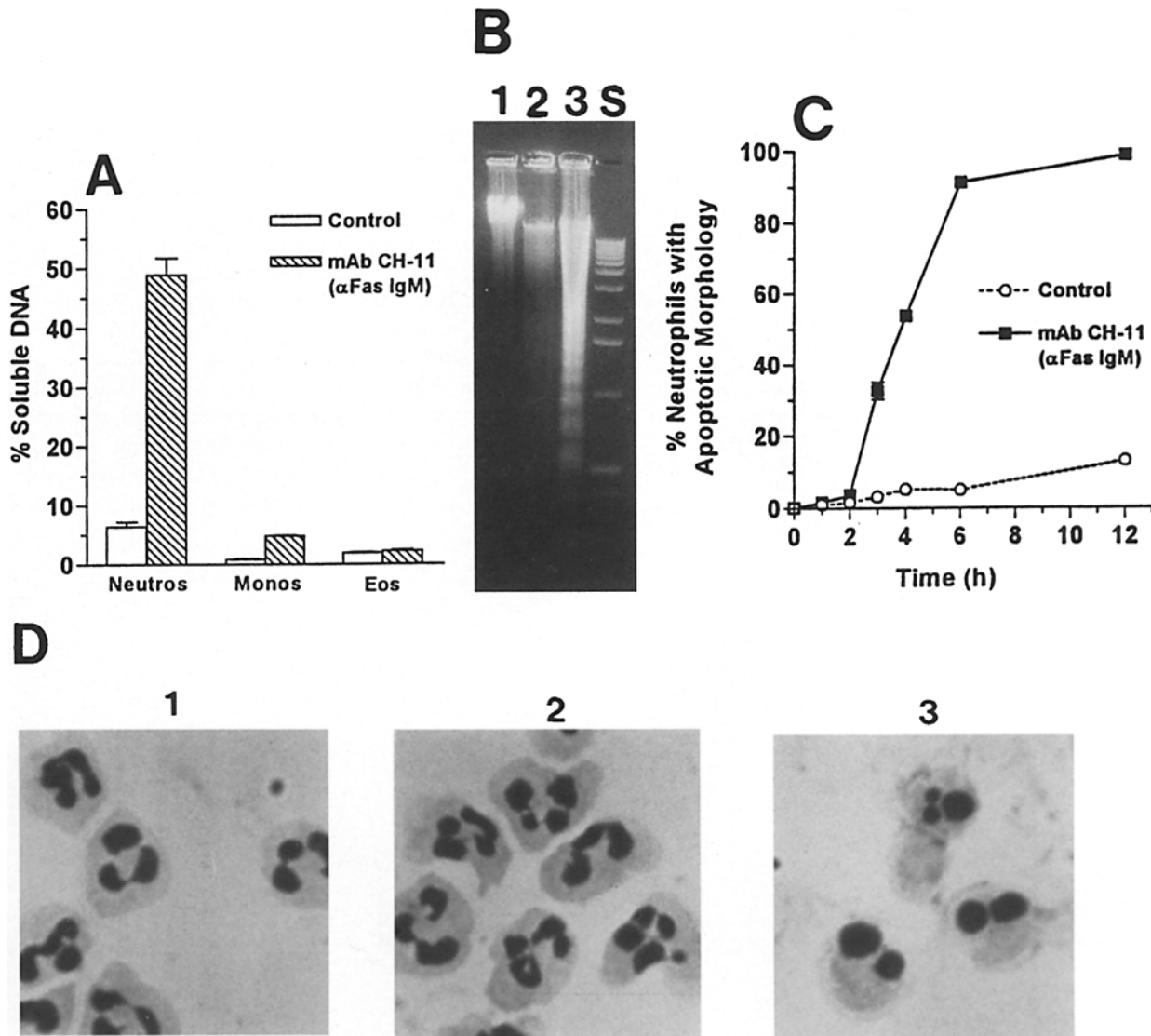


Figure 3. Differential sensitivity of normal human phagocytes to Fas-induced apoptosis. (A) Fas-induced DNA fragmentation in normal human neutrophils, monocytes, and eosinophils. Freshly isolated populations of normal human phagocytes were maintained in vitro in the absence (control, open bars) and presence of mAb CH-11 (100 ng/ml) (diagonally striped bars), as indicated. After incubation for 12 h, aliquots of phagocytes were processed for quantitation of DNA fragmentation by the diphenylamine assay method. Data are reported as the ratio of DNA cleavage products to high molecular weight DNA expressed as a percentage. Results represent the mean \pm SE of five separate experiments performed with phagocyte populations isolated from independent donors. (B) Gel electrophoresis demonstrating Fas-induced DNA fragmentation in neutrophils maintained in vitro: (lane 1) 0 h (freshly isolated); (lane 2) control \times 12 h; (lane 3) mAb CH-11 (100 ng/ml) \times 12 h. Lane S represents DNA 1-kb size marker standards (GIBCO BRL). (C) Time course of Fas-induced apoptosis in neutrophils maintained in vitro. At the designated time points, aliquots of neutrophils were prepared and scored for apoptotic morphology by fluorescence microscopy after staining with acridine orange: (a) control ($\cdots\circ\cdots$), and (b) mAb CH-11 (100 ng/ml) ($\text{---}\blacksquare\text{---}$). Data are reported as the percentage of cells demonstrating morphologic features of apoptosis, and they represent the mean \pm SD of five separate experiments performed with neutrophils isolated from independent donors. Substitution of mAb MMA (anti-CD15 IgM; 1 μ g/ml) for mAb CH-11 during incubation resulted in a rate of apoptosis identical to control (data not shown). (D) Morphologic features of Fas-induced apoptosis in neutrophils. Cytospin preparations of neutrophils maintained in vitro were stained by the Wright-Giemsa method: (1) control \times 0 h; (2) control \times 12 h; and (3) mAb CH-11 (100 ng/ml) \times 12 h.

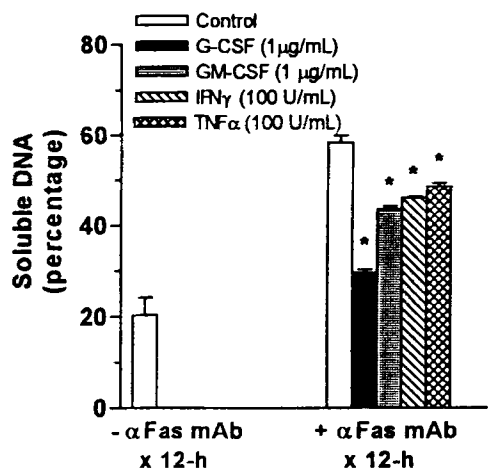
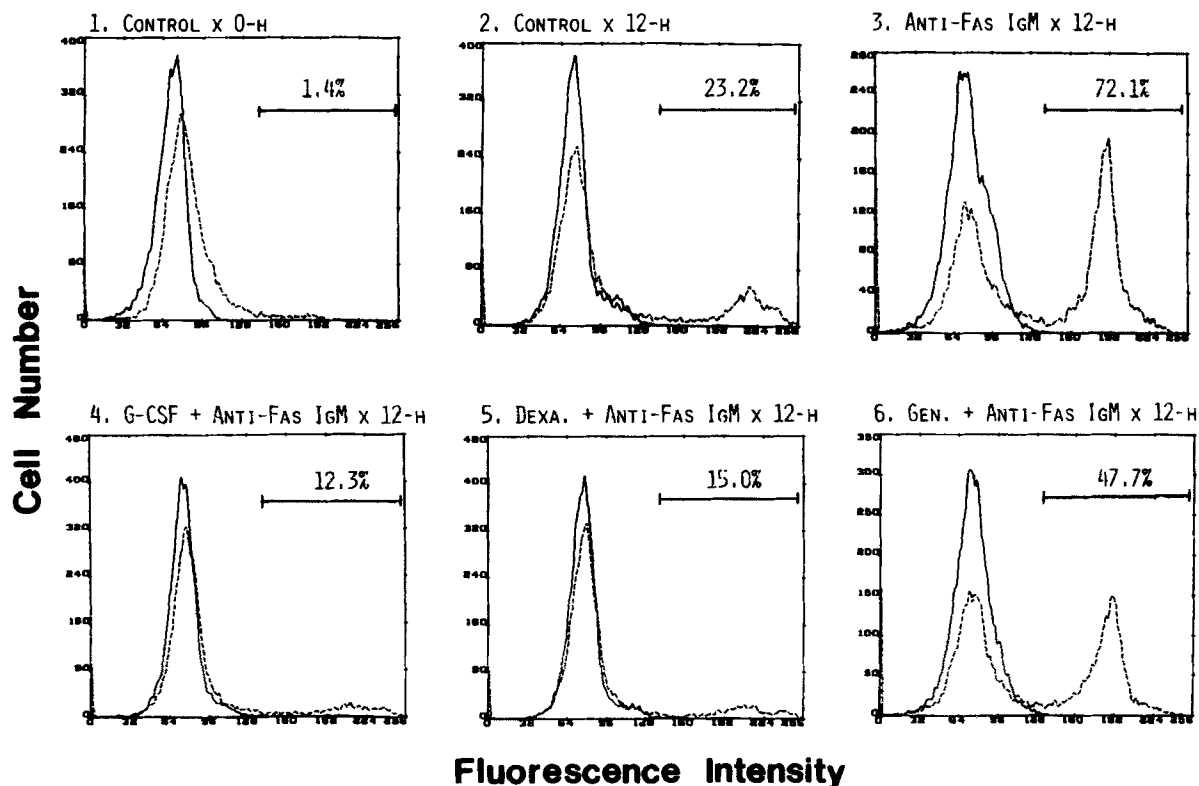


Figure 4. Proinflammatory cytokines reduce Fas-induced DNA fragmentation in normal human neutrophils. Neutrophils were maintained in vitro in the absence and presence of anti-Fas IgM (mAb CH-11, 100 ng/ml), as indicated. Designated samples of neutrophils were preincubated with G-CSF (1 μg/ml), GM-CSF (1 μg/ml), IFN-γ (100 U/ml), or TNF-α (100 U/ml) for 1 h before the addition of mAb CH-11. After incubation for 12 h, aliquots of neutrophils were processed for quantitation of DNA fragmentation by the diphenylamine assay method. Data are reported as the ratio of DNA cleavage products to high molecular weight DNA expressed as percentage. Results represent the mean ± SE of four separate experiments performed with neutrophils isolated from independent donors. Among the neutrophils stimulated with anti-Fas IgM (mAb CH-11), the asterisk denotes a statistically significant difference in DNA fragmentation in cytokine-treated cells as compared to control cells ($P < 0.05$).

otic morphology within 3 h, compared to <5% of neutrophils maintained without mAb CH-11. By 6 h, apoptotic morphology was apparent in 90% of the neutrophils stimulated with mAb CH-11. Neutrophils maintained in vitro for 12 h without mAb CH-11 (Fig. 3 D, panel 2) retained morphologic features essentially similar to those of freshly isolated neutrophils (panel 1). In contrast, typical apoptotic morphologic features, consisting of chromatin condensation ("apoptotic bodies") and reduced cytoplasmic volume, were readily apparent in neutrophils maintained in vitro with mAb CH-11 for 12 h (Fig. 3 D, panel 3).

Effect of Proinflammatory Cytokines on Fas-induced Apoptosis of Neutrophils. Proinflammatory cytokines have been shown to suppress or delay spontaneous apoptosis of neutrophils maintained in vitro (6). To determine whether proinflammatory cytokines could exert a similar suppressive effect on Fas-mediated apoptosis of neutrophils, quantitative DNA fragmentation assays were performed on neutrophils pretreated in vitro for 1 h with G-CSF (1 μg/ml), GM-CSF (1 μg/ml), IFN-γ (100 U/ml), or TNF-α (100 U/ml) before the addition of mAb CH-11 (Fig. 4). All four cytokines significantly reduced the development of low molecular weight DNA induced by activation of Fas ($P < 0.05$). G-CSF exerted the strongest effect on Fas-mediated apoptosis, reducing the percentage of soluble DNA from a control value of 58% to 29%. Less pronounced, but significant, suppression of Fas-mediated apoptosis was observed when



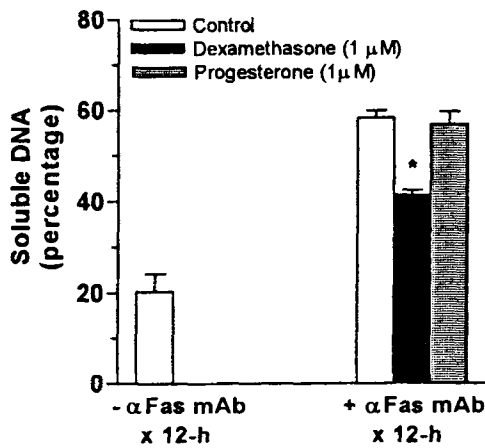


Figure 6. Dexamethasone reduces Fas-induced DNA fragmentation in normal human neutrophils. Neutrophils were maintained in vitro in the absence and presence of anti-Fas IgM (mAb CH-11, 100 ng/ml), as indicated. Designated samples of neutrophils were preincubated with dexamethasone (1 μM) or progesterone (1 μM) for 1 h before the addition of mAb CH-11. After incubation for 12 h, aliquots of the neutrophil suspensions were processed for quantitation of DNA fragmentation by the diphenylamine assay method. Data are reported as the ratio of DNA cleavage products to high molecular weight DNA expressed as percentage. Results represent the mean ± SE of four separate experiments performed with neutrophils isolated from independent donors. Among the neutrophils stimulated with anti-Fas IgM (mAb CH-11), the asterisk denotes a statistically significant difference in DNA fragmentation in steroid-treated cells as compared to control cells ($P < 0.05$).

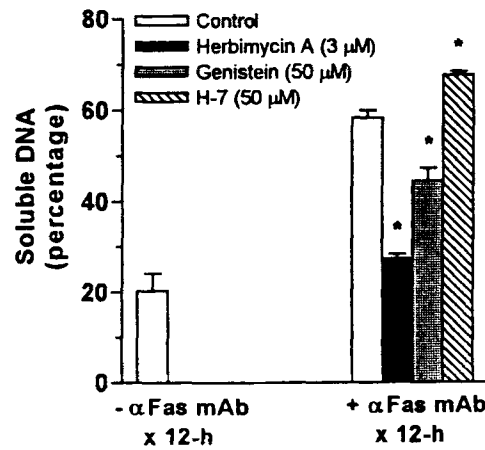


Figure 7. Genistein and herbimycin A reduce Fas-induced DNA fragmentation in normal human neutrophils. Neutrophils were maintained in vitro in the absence and presence of anti-Fas IgM (mAb CH-11, 100 ng/ml), as indicated. Designated samples of neutrophils were preincubated with herbimycin A (3 μM), genistein (50 μM), or H-7 (50 μM) for 1 h before the addition of mAb CH-11. After incubation for 12 h, aliquots of the neutrophil suspensions were processed for quantitation of DNA fragmentation by the diphenylamine assay method. Data are reported as the ratio of DNA cleavage products to high molecular weight DNA expressed as percentage. Results represent the mean ± SE of four separate experiments performed with neutrophils isolated from independent donors. Among the neutrophils stimulated with anti-Fas IgM (mAb CH-11), the asterisk denotes a statistically significant difference in DNA fragmentation in cells incubated with protein kinase inhibitor as compared to control cells ($P < 0.05$).

neutrophils were preincubated with GM-CSF, IFN-γ, or TNF-α.

The ability of G-CSF to suppress Fas-mediated apoptosis in neutrophils was confirmed by annexin V-FITC flow cytometric experiments (Fig. 5). Apoptosis is associated with alterations in plasma membrane structure before the development of DNA fragmentation (5). These structural changes lead to the exposure of phosphatidylserine on the cell surface early in the apoptotic process (11, 37). Thus, apoptotic cells can be detected and quantified by their ability to bind annexin V, a Ca^{2+} -dependent phospholipid-binding protein with high affinity for phosphatidylserine (11, 38). High specific binding of annexin V was detected in <2% of freshly isolated neutrophils, compared to 14% of neutrophils maintained in vitro for 12 h. Activation of Fas by mAb CH-11 increased the percentage of annexin V-positive cells to 78% at 12 h. Incubation with G-CSF reduced the proportion of annexin V-positive cells in Fas-activated neutrophil populations to 28% (mean; $n = 5$ independent donors).

Effect of Dexamethasone on Fas-induced Apoptosis of Neutrophils. In contrast to their induction of apoptosis in immature T cells, glucocorticoids suppress the rate of spontaneous apoptosis in neutrophils maintained in vitro (9, 10). To determine whether glucocorticoids could similarly inhibit Fas-mediated neutrophil apoptosis, we compared the relative effects of dexamethasone (1 μM) and progesterone (1 μM) on the development of DNA fragmentation induced by mAb CH-11 (100 ng/ml × 12 h) (Fig. 6). Dexamethasone, but not progesterone, significantly reduced the percentage of soluble, low molecular weight DNA in mAb CH-11-stimulated neutrophil populations from 58% to 42% ($P < 0.05$). To confirm this finding, flow cytometric analysis of annexin V binding was performed (Fig. 5). The percentage of specific annexin V-positive cells induced by mAb CH-11 was reduced from 78% to 30% (mean; $n = 5$ independent donors).

Effects of Genistein and Herbimycin A on Fas-induced Apoptosis of Neutrophils. Tyrosine kinase activity has been implicated as an essential component of the Fas-mediated signal

Figure 5. Proinflammatory cytokines, dexamethasone, and genistein reduce Fas-induced apoptosis in normal human neutrophils as detected by annexin V binding. Immunofluorescence flow cytometry was performed to detect binding of annexin V-FITC to neutrophils maintained in vitro. Experimental conditions: (1) control × 0 h; (2) control × 12 h; (3) mAb CH-11 (100 ng/ml) × 12 h; (4) G-CSF (1 μg/ml) × 1 h followed by mAb CH-11 (100 ng/ml) × 12 h; (5) dexamethasone (1 μM) × 1 h followed by mAb CH-11 (100 ng/ml) × 12 h; and (6) genistein (50 μM) × 1 h followed by mAb CH-11 (100 ng/ml) × 12 h. Data are expressed in the form of a fluorescence histogram overlay depicting specific annexin V-FITC (broken line) vs. nonspecific background binding (solid line). Data are representative of five independent experiments with similar results. Percentages represent the proportion of neutrophils demonstrating high specific binding of annexin V-FITC (i.e., apoptotic cells). The percentage values cited in the text represent mean values from five independent donors.

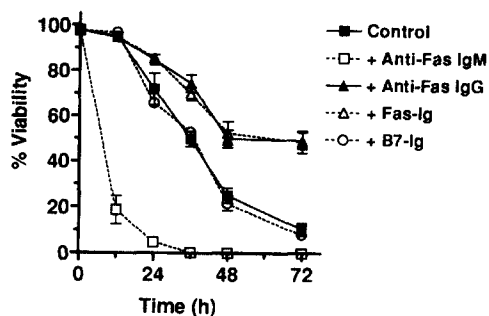


Figure 8. Spontaneous neutrophil death in vitro is suppressed by antagonistic anti-Fas IgG₁ and Fas Ig. Normal neutrophils were maintained in vitro under the following conditions: control (—■—); + anti-Fas IgM (mAb CH-11; 100 ng/ml) (—□—); + anti-Fas IgG (mAb ZB4; 1 μg/ml) (—▲—); + Fas-Ig (50 μg/ml) (—△—); and + B7-Ig (50 μg/ml) (—○—). At the designated time points, aliquots of the neutrophils were removed, and cell viability was determined. The data represent the mean ± SE for four independent experiments performed with neutrophils isolated from independent donors.

transduction pathway leading to apoptosis in T cells (39). To determine whether tyrosine kinase activity is involved in Fas-mediated apoptosis in neutrophils, we examined the effects of two selective tyrosine kinase inhibitors with different mechanisms of action, genistein (40), and herbimycin A (41) on Fas-induced DNA fragmentation in neutrophils (Fig. 7). Both herbimycin A (3 μM) and genistein (50 μM) significantly reduced the percentage of soluble, low molecular weight DNA in populations of Fas-stimulated neutrophils from 58% to 28% and 43%, respectively ($P < 0.05$). In contrast, the protein kinase C inhibitor, H-7, failed to suppress Fas-induced DNA fragmentation. Confirmatory flow cytometric experiments demonstrated that genistein reduced the percentage of annexin V-positive cells in Fas-stimulated populations of neutrophils from 78% to 40% (mean; $n = 5$ independent donors) (Fig. 5).

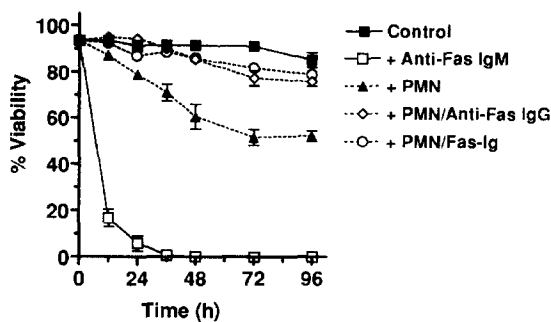


Figure 9. Neutrophils release a soluble factor that induces Jurkat cell death. Jurkat cells were cocultured with normal neutrophils in tissue culture wells providing physical separation of the two cell populations by a porous polycarbonate membrane. Conditions: control, Jurkat cells alone (—■—); Jurkat cells + anti-Fas IgM (mAb CH-11; 100 ng/ml) (—□—); Jurkat cells + normal neutrophils (—▲—); Jurkat cells + normal neutrophils + anti-Fas IgG₁ (mAb ZB4; 1 μg/ml) (—△—); and Jurkat cells + normal neutrophils + Fas-Ig (50 μg/ml) (—○—). At the designated time points, aliquots of the Jurkat cells were removed, and cell viability was determined. The data represent the mean ± SE for four independent experiments performed with neutrophils isolated from independent donors.

Spontaneous Neutrophil Apoptotic Death Is Partially Inhibited by Fas-Ig or Inhibitory anti-Fas IgG. To test whether the Fas system plays a critical role in the control of spontaneous neutrophil apoptotic death, neutrophils were maintained in vitro with antagonistic anti-Fas IgG₁ (mAb No. 33, 1 μg/ml) or Fas-Ig (50 μg/ml) deployed as a soluble receptor to block the interaction of FasL with cell-associated Fas (Fig. 8). Both anti-Fas IgG₁ and Fas-Ig significantly reduced spontaneous neutrophil death at 24–72 h ($P < 0.05$). At 48 h, viability in control neutrophils declined to 25%, but remained >50% in neutrophils treated with either anti-Fas IgG₁ or Fas-Ig. B7-Ig, used as a negative control fusion protein, did not significantly affect neutrophil viability.

Neutrophils Release a Soluble Factor That Induces Jurkat Death. It has been recently recognized that FasL can be secreted as a soluble, functionally active ligand (23, 25, 32, 42, 43). Thus, cells capable of expressing FasL can induce death of heterologous Fas-susceptible cells via a paracrine pathway that does not require direct cell–cell contact (23–25, 32). Furthermore, Fas-Ig fusion protein can be deployed as a competitive inhibitor of Fas/FasL interactions to block the physiological effects of soluble FasL in culture supernatants (23–25).

Stimulated neutrophils are known to secrete a soluble factor (or factors) that suppresses the biologic activity of certain populations of lymphocytes (44). This neutrophil-derived suppressive activity has been attributed, at least in part, to released myeloperoxidase and H₂O₂ (44). Because FasL is constitutively expressed on normal neutrophils, we hypothesized another mechanism for the immunosuppressive activity of neutrophils. To test whether neutrophils could serve as a source of biologically active soluble FasL (sFasL), we performed experiments in which Jurkat cells, a cloned human T cell line is highly susceptible to Fas-mediated death (Fig. 9, —□—), were cocultured with normal neutrophils in a system that allows diffusion of soluble factors, but prevents physical contact between the two cell populations. Neutrophil coculture reduced Jurkat viability at 72 h to 51%, compared to 91% viability observed in control Jurkat cells maintained in normal medium (Fig. 9). Jurkat death induced by coculture with neutrophils was significantly reduced at 24–72 h by either antagonistic anti-Fas-IgG₁ (mAb No.33, 1 μg/ml) or Fas-Ig (50 μg/ml) ($P < 0.05$). Specifically, Jurkat viability at 72 h was increased to 77% and 82% by anti-Fas-IgG₁ and Fas-Ig, respectively. These results are consistent with the release of biologically active sFasL by normal human neutrophils maintained in vitro, suggesting a possible role for FasL in neutrophil-mediated cytotoxicity.

Immunoblot Detection of Intact, Membrane-associated FasL and sFasL from Normal Neutrophils. Whole-cell lysates of freshly isolated neutrophils from two normal donors were prepared, and immunoblot analysis was performed using an mAb specific for human FasL. A prominent 37-kD protein (Fig. 10, upper arrow) was identified by this technique in the neutrophil lysates from both individuals (Fig. 10, lanes 1 and 2). Samples of supernatant were also obtained from cultures of these neutrophils after maintenance in vitro for

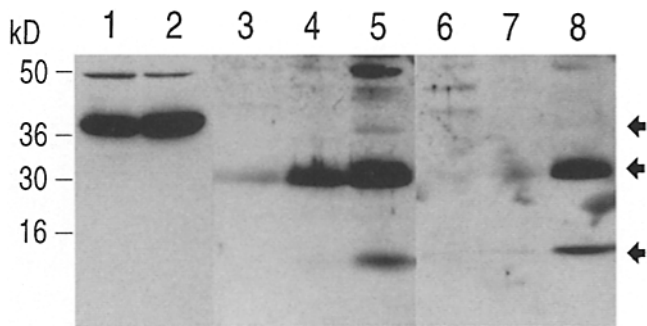


Figure 10. Detection of intact FasL and soluble FasL from normal neutrophils by immunoblot. Immunoblot analysis for FasL was performed using human FasL-specific mAb No. 33. Lysates and culture supernatants from two normal donors are shown. Lanes 1 and 2 represent whole-cell lysates of freshly isolated neutrophils from the two donors, respectively. Lanes 3–5 and 6–8 represent culture supernatants of neutrophils maintained in vitro from the two donors, respectively. Culture supernatants were obtained at 12 h (lanes 3 and 6), 24 h (lanes 4 and 7), and 48 h (lanes 5 and 8). Migration positions of prestained molecular mass markers are indicated in the left margin. Arrows in the right margin indicate the migration positions of proteins recognized by the anti-human FasL mAb used in the immunoblot.

12–48 h. A 30-kD protein (Fig. 10, *middle arrow*) was detected by FasL immunoblot in the neutrophil supernatants from both individuals (Fig. 10: donor 1, lanes 3–5, donor 2, lanes 6–8). In the supernatant from donor 1, the 30-kD protein signal was faintly apparent at 12 h (lane 3) and increased in intensity at 24 (lane 4) and 48 h (lane 5). In the supernatant from donor 2, the 30-kD protein signal was absent at 12 h (lane 6), but readily apparent by 48 h (lane 8). By 48 h, a 14-kD protein (*lower arrow*), presumably representing an sFasL degradation product, was detected in the neutrophil culture supernatants from both individuals (lanes 5 and 8).

Discussion

The Fas/FasL system is acknowledged to play a critical role in the regulation of both T cell and B cell development (19, 20, 22–27, 45). Whereas quiescent T cells lack FasL expression, physiologic activation of T cells in response to a number of stimuli induces FasL expression and upregulation of Fas, whose ensuing engagement on the cell surface initiates an autocrine cell death pathway (22–25). In this manner, T cells appear not to be committed but, rather, must be induced by activation signals to undergo apoptosis.

Our findings invoke a different scenario for the Fas/FasL pathway in neutrophils. Our results not only confirm that Fas is constitutively expressed on human neutrophils, monocytes, and eosinophils (16, 46), but they also demonstrate that, among normal mature phagocytes, FasL expression is restricted to neutrophils (Figs. 1 and 2). This constitutive coexpression of Fas and FasL suggests a plausible mechanism responsible for the rapid rate of spontaneous apoptosis observed in mature human neutrophils. Unlike mature T cells, which must be activated to undergo Fas-mediated

apoptosis, neutrophils may be irrevocably committed to autocrine death resulting from Fas/FasL engagement on the cell surface. Alternatively, neutrophil apoptosis could occur via a “fratricide” pathway in which membrane-bound Fas interacts with FasL expressed on an adjacent cell (47, 48).

To test the potential importance of the Fas/FasL pathway in regulation of spontaneous neutrophil apoptotic death, we performed experiments examining the effects of antagonistic anti-Fas IgG₁ and Fas-Ig (deployed as a soluble receptor for FasL) on the viability of neutrophils maintained in vitro (Fig. 8). Both agents significantly reduced spontaneous neutrophil death by ~50% during the 72-h time course. This partial, but not complete, inhibition of neutrophil death suggests that the Fas/FasL pathway is an important, but not exclusive, mechanism involved in the regulation of spontaneous neutrophil apoptosis. We interpret these results to indicate that redundant mechanisms exist to insure that neutrophils undergo apoptosis in the event that the Fas pathway is interrupted or fails. Thus, we propose that the Fas/FasL pathway may represent a key mechanism regulating the rapid spontaneous turnover of neutrophils after their release from the marrow.

Our results demonstrate differential sensitivity among neutrophils, monocytes, and eosinophils to Fas-induced apoptosis (Fig. 3). Although all three types of phagocytes were shown to express cell-surface Fas, only neutrophils were found to undergo rapid apoptosis after stimulation with mAb CH-11. In monocytes, mAb CH-11 induced a modest, albeit statistically significant, degree of DNA fragmentation. Thus, the observed magnitude of apoptosis in our study differs from a previous report, suggesting that monocytes exhibit relatively high sensitivity to the Fas apoptotic pathway (49). Moreover, the relative sensitivity of the phagocyte populations to Fas-induced apoptosis correlated with the relative rate of spontaneous apoptosis observed in vitro (i.e., neutrophils > monocytes > eosinophils) (6–16, 46). Both proinflammatory cytokines and dexamethasone, an antiinflammatory glucocorticoid, suppressed Fas-induced apoptosis in normal neutrophils (Figs. 4–6), in a manner similar to their effects on spontaneous apoptosis and viability of neutrophils maintained in vitro (6, 9, 10). Of the proinflammatory cytokines investigated in our study, G-CSF exerted the strongest effect on reducing spontaneous (58) and Fas-induced neutrophil apoptosis (Figs. 4 and 5). Lesser but significant reductions in Fas-induced apoptosis were observed with GM-CSF, IFN- γ , and TNF- α (Fig. 4). Together, these findings are consistent with the hypothesis that proinflammatory cytokines and glucocorticoids suppress neutrophil apoptosis via inhibition of a component of the Fas death program.

The mechanism by which proinflammatory cytokines and glucocorticoids mediate their antiapoptotic effects has yet to be determined. We doubt that simple downregulation of cell-surface Fas expression is involved, based on our observation that incubation with G-CSF in vitro fails to alter the MFI signal for Fas in flow cytometry experiments (data not shown). Ceramide, generated by activation of an acidic sphingomyelinase, has also been implicated as an im-

portant intracellular messenger of the Fas death pathway in several cell types, including lymphocytes (51, 52). Although we cannot exclude the possibility that ceramide is an Fas-induced intracellular messenger of apoptosis in neutrophils, we have failed to observe accelerated apoptosis in neutrophils treated with C₂ ceramide (Liles, W.C., and S.J. Klebanoff, unpublished data), a cell permeable, synthetic analogue of endogenous free ceramide capable of inducing apoptosis in other cell types (51, 52). Fas activation has also been found to stimulate an elevation in the concentration of cytosolic Ca²⁺ in certain cell types (53). Although a potential role for a Fas-mediated Ca²⁺ signal has yet to be described in phagocytes, it is interesting to note that small, transient elevations in intracellular Ca²⁺ have been reported to paradoxically inhibit apoptosis in normal human neutrophils (54).

Induction of antiapoptotic oncogene expression represents another potential mechanism for inhibition of apoptosis. *Bcl-2* and related oncogenes such as *bcl-x* have received substantial attention because of their ability to inhibit apoptosis in a variety of systems (55–57), including hematopoietic development (58, 59). The relative resistance of monocytes to Fas-induced apoptosis has been attributed to expression of the antiapoptotic gene *bcl-2* (16). Furthermore, mice deficient in *bcl-2* expression suffer massive death of hematopoietic precursors, and spontaneous apoptosis is significantly impaired in mature neutrophils from transgenic mice that express *bcl-2* (4). However, neither *bcl-2* nor *bcl-x* is expressed in mature neutrophils, nor are they induced by treatment with G-CSF in vitro (Liles, W.C., J.R. Park, D.M. Hockenbery, and S.J. Klebanoff, unpublished data). Our findings suggest that proinflammatory cytokines and glucocorticoids may modulate Fas-mediated signal transduction, thereby suppressing both Fas-induced and spontaneous apoptosis of normal neutrophils. The demonstration that both genistein and herbimycin A suppress apoptosis in neutrophils stimulated with mAb CH-11 is consistent with this hypothesis (Figs. 5 and 7), especially in light of the recent report implicating an essential role for

tyrosine kinase activity in Fas-mediated signalling in lymphocytes (39). Furthermore, we have recently found that spontaneous neutrophil apoptosis in vitro is partially suppressed by both genistein and herbimycin A (50).

The neutrophil is recognized as an important cellular mediator of tissue damage during inflammation (60). It has been hypothesized that release of sFasL from lymphocytes represents a pathologic pathway to promote injury in Fas-susceptible tissues (32, 43). The finding that neutrophils release a soluble factor that induces Jurkat cell death via a mechanism sensitive to either antagonistic anti-Fas IgG₁ or Fas-Ig (Fig. 9) suggested that the neutrophil also may serve as a source of sFasL. Normally, senescent neutrophils are removed from an inflammatory site by macrophages via phagocytosis (1). It is believed that this process minimizes the potential risk of tissue injury related to the release of neutrophil-derived toxic mediators (1). Conceivably, failure of this system to recognize and remove senescent neutrophils could lead to increased FasL release and exacerbation of tissue injury at sites of inflammation.

Immunoblot analysis confirmed that a soluble 30-kD protein recognized by an mAb specific for human FasL is released into the culture supernatant of neutrophils maintained in vitro. Thus, we propose that neutrophils may not only facilitate their own suicide, but also induce apoptosis in heterologous cells via the Fas death program. The soluble 30-kD protein detected in our FasL immunoblot experiments is slightly larger than the 26-kD sFasL released from activated T cells (32). Recently, a matrix metalloproteinase was implicated in the mechanism responsible for the release of sFasL from lymphocytes (43). The difference in molecular mass of the neutrophil-derived product suggests that the proteolytic mechanisms regulating the release of sFasL from neutrophils and lymphocytes may differ. Thus, it will be of considerable interest to examine whether a specific neutrophil-derived metalloproteinase mediates shedding of biologically active sFasL from normal human neutrophils.

W.C. Liles is a Pfizer Postdoctoral Fellow. The study was supported by U.S. Public Health Service grants AI-07763 and HL-53515 Grant info from the National Institutes of Health.

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Received for publication 9 November 1995 and in revised form 14 May 1996.

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