Kidney International, Vol. 53 (1998), pp. 84–91

Interleukin-8 delays spontaneous and tumor necrosis factor-α-mediated apoptosis of human neutrophils

Ralph Kettritz, Marcia L. Gaido, Hermann Haller, Friedrich C. Luft, Charles J. Jennette, and Ronald J. Falk

Franz-Volhard Clinic and Max Delbrück Center for Molecular Medicine, Humboldt University of Berlin, Germany, and Departments of Medicine and Pathology, University of North Carolina, Chapel Hill, North Carolina, USA

Interleukin-8 delays spontaneous and tumor necrosis factor-α-mediated apoptosis of human neutrophils. During inflammation, polymorphonuclear neutrophils (PMN) are exposed to and influenced by various cytokines, including the chemoattractant interleukin-8 (IL-8). We tested the hypothesis that IL-8 affects apoptosis in PMN. We investigated which IL-8 receptor (RI or RII) might be involved, as well as the role of Bcl-2. Human PMN were isolated and cultured up to 30 hours. Apoptosis was detected by UV and light microscopy, as well as by DNA-fragmentation assay, and quantitated by flow cytometry. Interleukin-8 significantly delayed spontaneous apoptosis at 10, 20, and 30 hours in a dose-dependent fashion. Polymorphonuclear neutrophil treatment with the highest concentration of IL-8 (100 nM) decreased the percentage of apoptotic cells from 2.1 ± 1.5 to 0.8 ± 0.2 after 10 hours, from 31 ± 14 to 8 ± 5 after 20 hours, and from 47 ± 15 to 18 ± 8 after 30 hours of incubation ($P < 0.05$ for all time points, $N = 6$). Interleukin-8 also inhibited TNFα-mediated PMN apoptosis. Incubation with 20 ng/ml TNFα resulted in 23 ± 6% apoptotic cells at four hours, whereas pretreatment with IL-8 (50 nM) decreased this percentage to 11 ± 3 ($N = 5$, $P < 0.05$). We next studied the role of both types of IL-8 receptors, RI and RII, by comparing the effect of IL-8 and the product of growth-related oncogene alpha (Groα) on PMN cultured for 20 hours. Both IL-8 and Groα attenuated apoptosis, although IL-8 was more effective than Groα. Bcl-2 was detected by intracellular fluorescent antibody cell sorter analysis, Western blot, and reverse transcription-polymerase chain reaction (RT-PCR). Neither resting PMN nor IL-8-treated neutrophils expressed BCL-2 protein, which was readily detected in control cells. Furthermore, we could not detect BCL-2 gene expression by RT-PCR. We conclude that IL-8 prolongs the lifespan of human neutrophils *in vitro* by delaying apoptosis. This effect may be important for a controlled and effective inflammatory response. The delay in apoptosis can be mediated by the IL-8 RII, while RI may provide an added effect. The actions of IL-8 on apoptosis are Bcl-2 independent.

Polymorphonuclear neutrophils (PMN) play an important role in a variety of inflammatory renal diseases. Activated PMN respond with release of reactive oxygen species, degranulation of tissue degrading enzymes, and phagocytosis [1–4]. The acute process is self-limiting. Pathological conditions, such as impaired function or decreased PMN number, may result in life-threatening diseases. Apoptosis is an important regulator of cell turnover. Several diseases result from abnormalities in apoptosis [5]. Chemotaxis is a mechanism through which PMN are recruited to sites of inflammation, and it is mediated by several substances including chemokines [6–9]. The chemokine α-family targets mainly PMN. Members of this family are all about 70 to 80 amino acids in size and contain four conserved cysteines. The first two cysteines are separated by one variable amino acid (CXC). After the discovery of neutrophil-activating peptide 1, which is identical to interleukin-8 (IL-8), more than 12 other family members have been described including the growth-related oncogene alpha (Groα). Polymorphonuclear neutrophils express high numbers of two CXC cytokine receptor types, namely IL-8 RI and IL-8 RII. The latter is characterized by a high affinity for all members of the α-chemokine family including Groα, whereas IL-8 RI has high affinity for only IL-8 [10–12]. Interleukin-8 was initially classified according to its chemotactic effect on PMN; however, additional biological functions of IL-8 have been described. Whether or not IL-8 also modulates apoptosis is unclear. Apoptotic PMN have a decreased capability to respond to activating stimuli, such as FMLP or opsonized zymosan [13] and are swiftly removed by phagocytosis [14]. Thus, studies on regulation of PMN apoptosis are relevant and may provide information with therapeutic implications. We investigated the effect of IL-8 on spontaneous apoptosis in PMN and on rapidly-occurring tumor necrosis factor (TNFα)-mediated apoptosis. We studied the relative participation of IL-8 RI and IL-8 RII in this process, and investigated changes in the expression of Bcl-2. Our data suggest that IL-8 is indeed important in regulating PMN apoptosis.

Key words: apoptosis, human neutrophils, interleukin-8, Bcl-2.

Received for publication June 9, 1997
and in revised form August 20, 1997
Accepted for publication August 21, 1997

METHODS

Reagents

The following reagents were purchased from Sigma (St. Louis, MO, USA or Dreisenhofen, Germany): propidium iodide (PI), Ficoll-Hypaque, bovine serum albumin (BSA), DNase-free RNase, modified Wright Giemsa, 4′,6 diamidino-2-phenylindole (DAPI), and sulforhodamin 101 (SR 101). Recombinant IL-8 and recombinant TNFα were obtained from Genzyme (Rüsselsheim, Germany), and recombinant Groα from Laboserve (Giessen, Germany). All antibodies were purchased from Dako (Hamburg, Germany): murine monoclonal anti-human Bcl-2-antibody (clone 124), mouse IgG1 negative control as isotype control (generated

against *Aspergillus niger* glucose oxidase, an enzyme that is irrelevant for mammalian cells), and FITC-conjugated F(ab′)2 fragment of goat anti-mouse immunoglobulin as secondary antibody.

Human polymorphonuclear neutrophil isolation, cell count determination and cell viability

Polymorphonuclear neutrophils were prepared from heparinized whole blood of healthy donors. The isolation protocol included red blood cell sedimentation by Plasmagel (Cellular Products Inc., Buffalo, NY, USA) followed by Ficoll-Hypaque density gradient centrifugation, and hypotonic red cell lysis. Cells were resuspended at 10^7/ml in RPMI 1640 supplemented with 2 mM glutamine and penicillin/streptomycin. Trypan blue exclusion was used to determine cell viability. Ten microliters of PMN in suspension were incubated with 40 μl Trypan blue (Seromed, Berlin, Germany) for five minutes at room temperature. Cells were counted in duplicate using a hemocytometer and considered viable if able to exclude Trypan blue.

Culture conditions of human polymorphonuclear neutrophils

A total of 250 μl PMN at 10^7/ml RPMI without fetal calf serum (FCS) were pipetted into 12 $\times$ 75 mm polypropylene culture tubes (Fisher, Fair Lawn, NJ, USA). Then 210 μl of supplemented RPMI 1640 containing 20% heat-inactivated FCS together with IL-8, or Groα, or an equal volume of carrier protein containing PBS-solution without cytokines (0.5% BSA), was added giving a final concentration of 5×10^6 cells per ml. Cell suspensions were incubated at 37°C in 5% CO_2 for up to 30 hours. In another set of experiments we tested the effect of IL-8 on TNFα-mediated apoptosis. Cells were cultured as described above except that IL-8 (100 nM) or an equal volume of carrier was added before PMN were challenged with TNFα (20 ng/ml). We checked all solutions and reagents used in cell culture for the presence of endotoxin; the solutions contained less than 0.05 ng/ml endotoxin as demonstrated by a *Limulus* amoebocyte lysate assay (Biowhittaker, Walkersville, MD, USA). All experiments were done in duplicate.

Wright Giemsa, 4′,6 diamidino-2-phenylindole, and Sulforhodamin 101 cell staining

Cytocentrifuge cell preparations were either stained using modified Wright Giemsa stain or were fixed in 70% ethanol and stained with a solution containing 1 μg/ml 4′,6 diamidino-2-phenylindole (DAPI). Morphologic features of apoptosis, as described by Kerr, Whyte and Haslet [15] including pyknotic nuclei, nuclear and cytoplasmic condensation, and the formation of apoptotic bodies, were considered as evidence for apoptosis.

DNA fragmentation assay

DNA fragmentation was studied using isolated low molecular weight DNA. A total of 5×10^6 PMN were resuspended in lysis buffer (0.5 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) and the lysate was centrifuged at 20,000 $\times$ g for 20 minutes at 4°C to select the low molecular weight DNA. Supernatants were extracted twice with an equal volume of PCIA (Tris-saturated phenol:chloroform:isoamyl alcohol at 25:24:1) and twice with CIA (chloroform:isoamyl alcohol at 24:1). DNA was precipitated at −20°C overnight by adding 2.2 volumes of absolute ethanol in the presence of 0.3 M sodium acetate pH 5.2. DNA was centrifuged at 13,500 g for 20 minutes at 4°C, air dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing RNase Plus (5 Prime-3 Prime). After a 30 minute incubation at 37°C, DNA was quantitated and 7.5 μg were electrophoresed in an 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were visualized under UV light.

Apoptosis measurements with propidium iodide-stained polymorphonuclear neutrophils and flow cytometry

Flow cytometry was used to measure DNA content at the single cell level as described previously [16]. The method is based on the fact that endonuclease activation generates low molecular weight DNA fragments in apoptotic cells. After membrane permeabilization, these fragments leak out resulting in decreased DNA-content in apoptotic cells, while the DNA content in non-apoptotic cells remains unchanged. Briefly, freshly isolated or cultured cells were spun at 200 $\times$ g for five minutes at 4°C and carefully resuspended in PBS containing 0.5 mM EDTA. Chilled 95% ethanol was added to a final concentration of 70% and the cell mixtures were stored at −20°C for one to two days. The PMN were pelleted (200 g, 5 min, 4°C) and resuspended in 250 μl PBS/0.5 mM EDTA/1% BSA; 250 μl PBS containing 200 μg DNase-free RNase and 500 μl PBS containing 50 μg propidium iodide were added. Cells were held for 15 minutes in the dark at room temperature and then stored at 4°C for six to eight hours in the staining mixture allowing low molecular DNA fragments to leave permeabilized cells. PMN were analyzed using a fluorescent antibody cell sorter (FACscan; Becton Dickinson, Heidelberg, Germany) and 10,000 events per sample were collected in list-mode using Lysis II software for data acquisition and analysis.

Western blot analysis

Cell lysates were prepared by resuspending 5×10^6 cells in 500 μl of ice-cold lysing solution (40 mM Tris-HCl, pH 8.0, containing 276 mM NaCl, 2% NP 40, 20 μg/ml leupeptin, 2 mM PMSF, 20 μg/ml aprotinin, 4 mM EDTA, and 20% glycerol). Samples were stored for 30 minutes on ice and centrifuged at 12,000 $\times$ g for two minutes. Supernatant was recovered and protein concentration was estimated by BCA protein assay (Pierce, Rockford, IL, USA). Samples were incubated for five minutes at 95°C in loading buffer (250 mM Tris-HCl, pH 6.8 with 4% SDS, 20% glycerol, 0.01% bromphenol blue) and 20 μg protein per lane were loaded on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electropheresed, and blotted onto polyvinylidene difluoride membrane by a semi-dry technique. Bcl-2 was detected using a monoclonal antibody to human Bcl-2 oncoprotein (4 μg/ml) and an alkaline phosphatase-labeled goat anti-mouse IgG (1:10,000; Boehringer Mannheim, Germany). The blot was developed by incubation in a chemiluminescence substrate (Tropix, Bedford, MA, USA) and exposed to a x-ray film.

Flow cytometry analysis of intracellular Bcl-2 protein

Freshly isolated or cultured PMN or U937 were stained with Trypan blue. Cell count and viability was assessed using a hemocytometer. Then, 10^6 cells were pelleted (200 g, 5 min, 4°C) and resuspended in 250 μl of 4% paraformaldehyde in PBS. Cells were stored on ice for 20 minutes, washed once in PBS plus 1% BSA (buffer A) and resuspended in 100 μl permeabilization buffer containing PBS, 1% BSA, and 0.2% Saponin (buffer B). Monoclonal antibody to Bcl-2 (8 μg/ml), or an equal amount of

mouse IgG1 negative control (isotype control) or PBS only was added and samples were kept for 30 minutes on ice. Cells were washed and incubated with 10 μg FITC-conjugated F(ab′)2 fragment of goat anti-mouse immunoglobulin in 110 μl of buffer A. After washing, cells were resuspended in 500 μl of buffer A, and stored on ice in the dark until analyzed using a FACScan flow cytometer equipped with lysis II software. Antibody concentrations were based on results of preliminary titration experiments. Each experiment included a sample for measurement of autofluorescence and fluorescence caused by secondary antibody alone.

RNA extraction and RT-PCR

RNA was purified by resuspending 5×10^6 to 1×10^7 PMN or control HL-60 cells in 1 ml RNA-STAT 60 (Tel-Test "B", Inc., Friendswood, TX, USA) following the Tel-Test "B" procedure, wherein the RNA/RNA-STAT mixture is extracted with chloroform, the RNA precipitated with isopropanol, and dissolved in 1 mM EDTA, pH 8.0. Gene-specific RNA was analyzed by RT-PCR using 1 μg of RNA per reaction. First strand cDNA for two independent RNA samples and PCR (performed in triplicate) were prepared as described in the Clontech (Palo Alto, CA, USA) 1 st-Strand cDNA Synthesis and RT-PCR kits. Five microliters of cDNA were used per 50 μl PCR reaction and cycling conditions were 94°C for 45 seconds, 58°C for one minute and 72°C for two minutes for 30 cycles using a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Clontech) were used as an internal control.

Primers for Bcl-2 were selected using the MacVector (Kodak) primer pair program and included 5′-GCCTTCTTTGAGTTCGGTGGG-3′ and 5′-GAGCAGAGTCTTCAGAGACAGCCAG-3′. The primers were synthesized using an Applied Biosystems Model 394 DNA synthesizer by the University of North Carolina Pathology Department Oligonucleotide Synthesis Facility. PCR products were electrophoresed in 1.8% Metaphor (FMC BioProducts, Rockland, ME, USA) agarose gels in TBE (Tris pH 8.0, 89 mM borate and 2 mM EDTA) buffer, stained in 0.5 μg/ml ethidium bromide and photographed. A control reaction was run on at least one sample from each RNA preparation with PCR of samples without reverse transcriptase to assess contamination by genomic DNA. No bands were seen in these lanes (data not shown).

Statistical analysis

Statistical analysis was performed on a Macintosh computer (Apple Inc., Cupertino, CA, USA) equipped with commercially available program (Statview, Cricket Software Inc., Philadelphia, PA, USA). The nonparametric signed Wilcoxon rank test was used to compare results between paired groups to test if the differences were unequal to 0. Differences were considered to be significant when the P value was < 0.05.

RESULTS

To determine the involvement of IL-8 in the regulation of neutrophil apoptosis, the effect of IL-8 on cultured PMN was studied in six independent experiments, each using cells from a single donor preparation. Cells were cultured with or without IL-8 at concentrations ranging from 1 to 100 nM. Samples were harvested at 10, 20, and 30 hours and the percentage of apoptotic PMN was assessed by flow cytometry. The FACs results showed

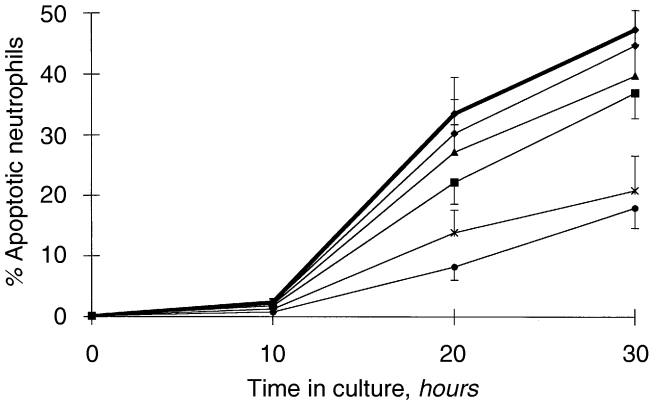

Fig. 1. Apoptosis was measured in freshly isolated polymorphonuclear neutrophils (PMN) and in samples cultured for 10 hours, 20 hours, and 30 hours in the absence (bold line) or in the presence of interleukin-8 (IL-8). Cells were treated with IL-8 at 1 nM (♦), 5 nM (▲), 10 nM (■), 50 nM (*), or 100 nM (●). Neutrophils (PMN) from the same donor preparation was used in each experiment to assure compatible conditions ($N = 6$). Data are depicted as mean ± SEM. Significant inhibition ($P < 0.05$) was found for incubation with the following IL-8 concentrations: at 10 hours ≥ 50 nM, at 20 hours for all concentrations, at 30 hours ≥ 5 nM.

that IL-8 treatment caused a dose- and time-dependent delay of spontaneous apoptosis (Fig. 1). A significant reduction in the percentage of apoptotic PMN was observed as early as 10 hours after cells were cultured with the highest cytokine concentration of 100 nM (mean of the difference of the pairs: −1.6%). All IL-8 concentrations were significant after 20 hours (100 nM, −24.2%; 50 nM, −19.6%; 10 nM, −11.3%; 5 nM, −6.3%; 1 nM, −3.2%). At 30 hours all concentrations save the lowest (1 nM) remained significant (100 nM, −29.3%; 50 nM, −26.5%; 10 nM, −10.4%; 5 nM, −7.6%). No significant cell loss was observed up to 30 hours and cell viability exceeded 92%, as determined by Trypan blue exclusion (data not shown). No significant cell loss was observed up to 30 hours and cell viability exceeded 92%, as determined by Trypan blue exclusion (data not shown).

Polymorphonuclear neutrophils cultured for 20 hours developed morphological signs of apoptosis, including pyknotic nuclei, nuclear condensation, and formation of apoptotic bodies, as identified by staining with DAPI (Fig. 2) or with modified Wright Giemsa (data not shown). Morphological features of apoptosis were clearly inhibited in samples treated with 100 nM IL-8 for 20 hours (Fig. 2). Also, IL-8 diminished DNA-fragmentation, a hallmark of apoptosis. Low molecular weight DNA isolated from 5×10^6 PMN was electrophoresed on agarose gels. Extensive DNA "laddering" was observed, when cells were cultured for 20 hours without IL-8. In contrast, IL-8 treatment diminished the occurrence of typically fragmented low molecular DNA (Fig. 3).

Since our earlier work had shown that TNFα treatment accelerated PMN apoptosis [16], it was of particular interest to determine if IL-8 specific regulation of apoptosis could, in fact, override TNFα-mediated pathways. We stimulated PMNs to apoptosis with TNFα (20 ng/ml). For this set of experiments we concentrated on the four-hour time point, since we knew from earlier studies that the TNF effect plateaus at that time [16]. Interleukin-8 (50 nM) pretreatment significantly diminished apoptosis (23 ± 6 to 11 ± 23, $N = 5$; Fig. 4).

In PMN, stimulation of the IL-8 RI and RII generates a greater

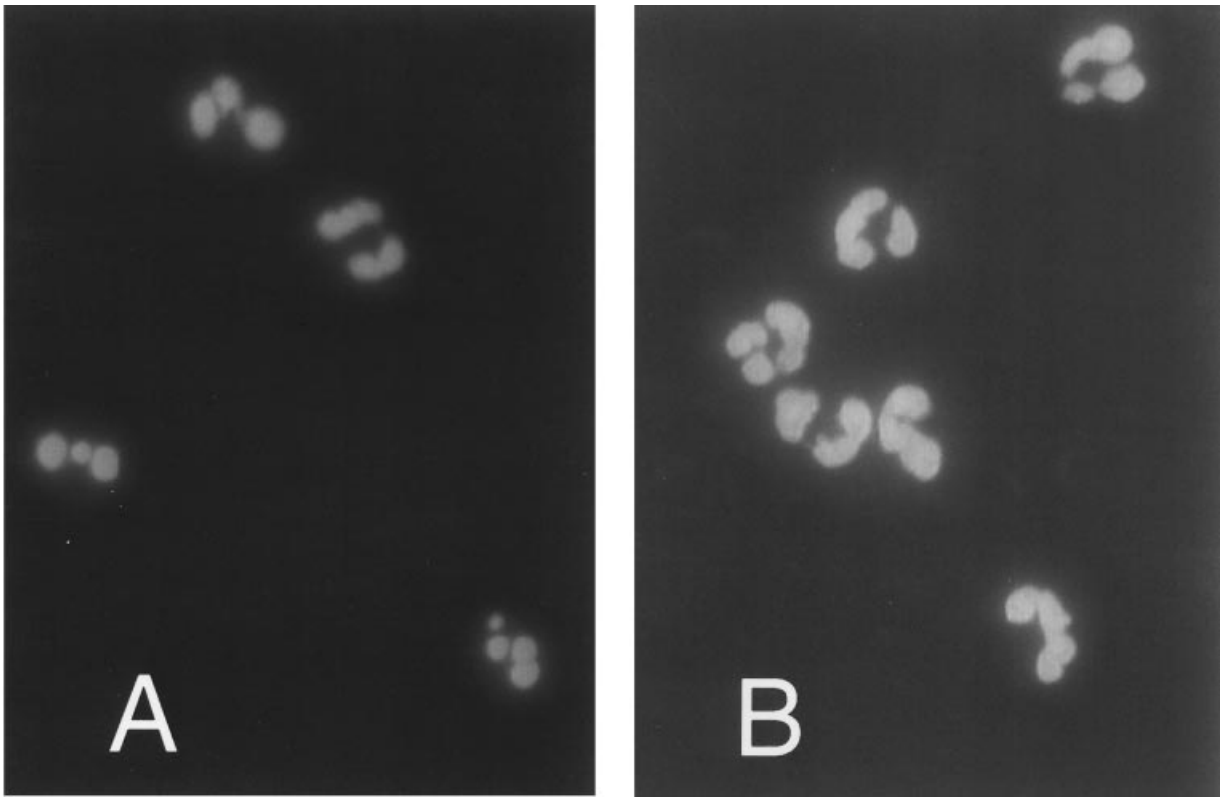

Fig. 2. Polymorphonuclear neutrophils (PMN) were cultured for 20 hours in the absence (*A*) and in the presence of 100 nM interleukin-8 (IL-8) (*B*). Cells were stained in DAPI and photographs were taken using an UV-filter. A representative image shows that IL-8 treatment prevented typical findings of apoptosis, such as pyknotic nuclei, chromatin condensation, and nuclear fragmentation.

biochemical response than binding to IL-8 RII only [17, 18]. To determine the relative effects of the two IL-8 receptors on PMN apoptosis we compared the effect of IL-8 and Groα in parallel. Based on the experiments shown in Figure 1, the 20-hour time point was selected. Using flow cytometry, five different concentrations of each chemokine were tested in seven independent experiments. The results demonstrated that both agents were capable of mediating a delay in PMN apoptosis compared to controls (Fig. 5). The difference compared to untreated control cells was significant for IL-8 at all concentrations and for Groα at $\geq$ 5 nM. At 50 nM and 100 nM, IL-8 inhibited apoptosis significantly more than Groα (mean of the difference of the pairs: -3.9% for 50 nM; -6.4% for 100 nM). We tested for the possibility of an additive effect of both cytokines in two separate experiments. The percentage of apoptotic PMN was 30% for untreated samples, 21% for 10 nM IL-8, 21% for 10 nM Groα, and 20% for the combination of 10 nM IL-8 and 10 nM Groα. The results demonstrate no additive effect of IL-8 and Groα on PMN apoptosis. We chose this cytokine concentration, since it gave half of the maximal inhibitory effect without causing a stronger IL-8 effect.

To investigate possible mechanisms involved in IL-8-mediated cell rescue, we next tested whether or not IL-8 delayed PMN apoptosis by inducing Bcl-2 production. Using FACs analysis Bcl-2 protein was not detectable in freshly isolated PMN, as well as in PMN that were cultured for 24 hours in the presence or in the absence of IL-8 (100 nM; $N = 3$), whereas Bcl-2 was readily detectable in the U 937 cells, which were used as a positive control (data not shown). Neither freshly isolated PMN nor PMN that were cultured for 24 hours with or without IL-8 (100 nM) expressed detectable Bcl-2 protein levels as determined by Western blotting (data not shown). In contrast, Bcl-2 was clearly measurable in the monocytic cell line U937. To test for gene expression, we assayed for Bcl-2 mRNA by RT-PCR. Two independent experiments were performed. No measurable up-regulation of Bcl-2 mRNA by IL-8 treatment was observed (Fig. 6). The promyelocytic cell line HL-60 served as a positive control.

DISCUSSION

Our results indicate that the prototype chemokine IL-8 affects programmed cell death of human PMN *in vitro*, resulting in a delay of spontaneous as well as TNFα-mediated apoptosis. We used the fact that PMN undergo apoptosis when placed in culture to demonstrate that the inhibitory effect of IL-8 on spontaneous PMN apoptosis occurs in a dose- and time-dependent fashion. We also provide evidence that both types of IL-8 receptors play a role in regulating apoptosis. Groα, which binds only RII, was effective compared to controls, while IL-8, which binds both RI and RII, was more effective than Groα. Thus, the delay in apoptosis can be mediated by RII in any event, while additional binding to RI may provide an added effect. Several genes have been characterized

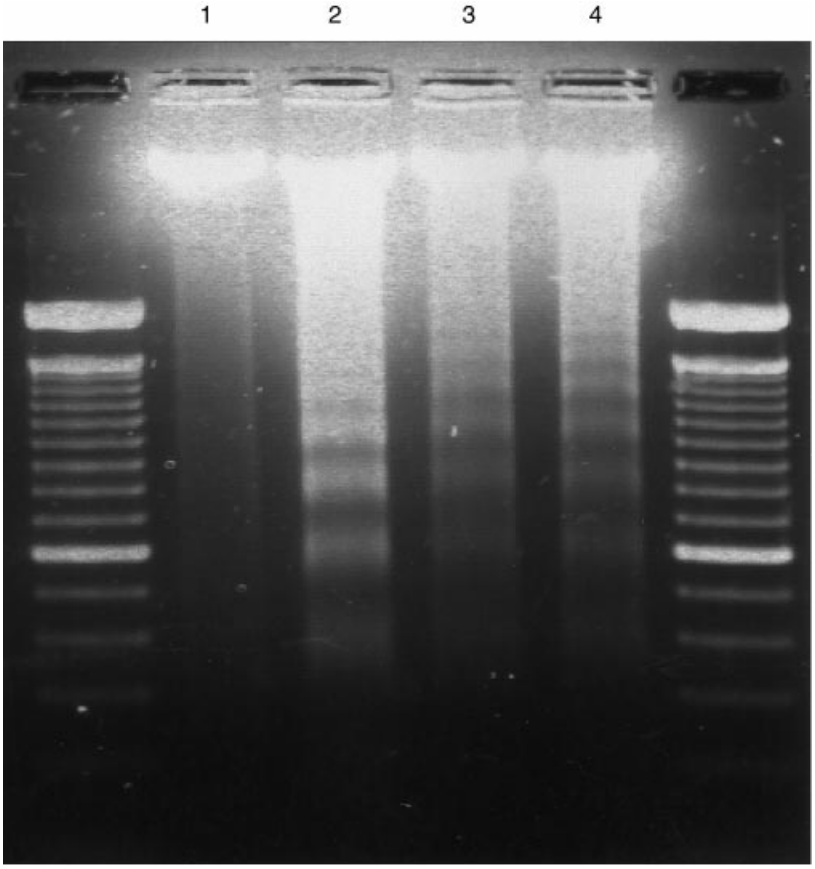

Fig. 3. Effect of interleukin-8 (IL-8) treatment on spontaneous apoptosis was studied employing DNA-fragmentation assay. Cells were cultured in the absence and in the presence of IL-8, or Groα (100 nM). Samples were harvested after 20 hours, DNA was extracted and 7.5 μg were electropheresed on an 1.5% agarose gel. No DNA-fragmentation was observed in freshly isolated PMN (lane 1), whereas extensive fragmentation occurred in untreated cells after 20 hours (lane 2). IL-8 (lane 3) and Groα (lane 4) inhibited DNA-ladders. A typical example of two distinct experiments is shown.

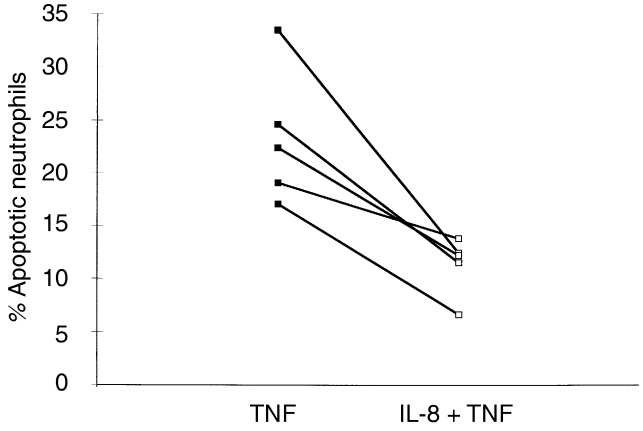

Fig. 4. Effect of interleukin-8 (IL-8) pretreatment on tumor necrosis factor alpha (TNFα)-mediated apoptosis was studied. Polymorphonuclear neutrophils (PMN) of the same preparation were first treated with either vehicle (closed symbols) or with 50 nM IL-8 (open symbols) and challenged with 20 ng/ml TNFα ($N = 5$). Samples were harvested after four hours and the percentage of apoptotic PMN was measured by flow cytometry.

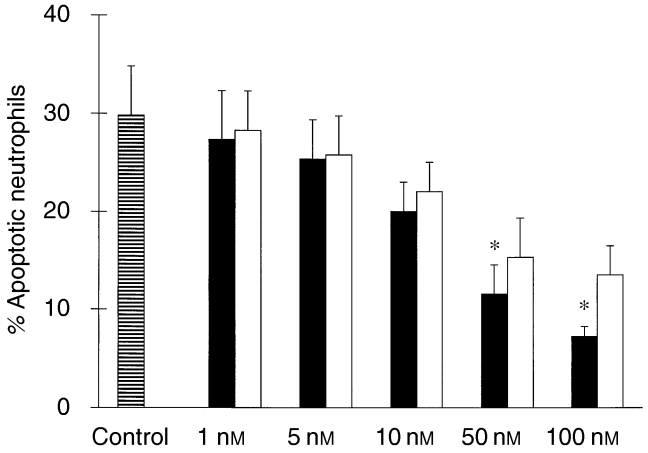

Fig. 5. Effect of interleukin-8 (IL-8) and Groα on polymorphonuclear neutrophil (PMN) apoptosis cultured for 20 hours was compared in parallel. Cells of the same preparation were exposed to either IL-8 (■) or Groα (□) at increasing concentrations: 1, 5, 10, 50, and 100 nM respectively. Data are given as mean ± SEM ($N = 7$). Differences between IL-8 and Groα were significant at 50 nM and at 100 nM (*).

that regulate apoptosis, including the Bcl family [19]. Polymorphonuclear neutrophils do not generally express the apoptosis-delaying Bcl-2 gene. We wondered whether or not IL-8 could inhibit apoptosis by inducing expression of Bcl-2. Our findings indicate that no such up-regulation occurs, as shown both at the protein and mRNA levels.

Polymorphonuclear neutrophil-mediated inflammatory responses are critically important for maintaining host integrity. To respond to tissue inflammation, PMN leave the circulation and migrate into the inflamed site. By so doing they are exposed to several external conditions known to affect their physical and

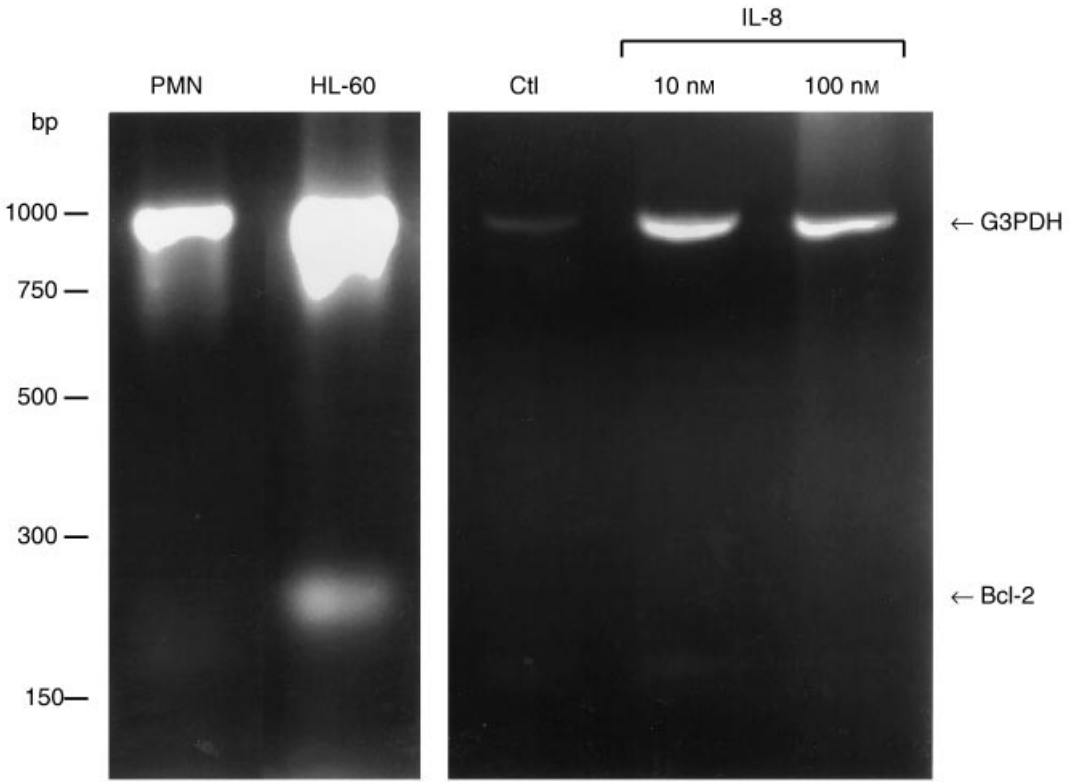

Fig. 6. Detection of Bcl-2 proto-oncogene by RT-PCR. RNA was purified from HL-60 cells or from PMN. First, strand cDNA was generated by reverse transcription, amplified by PCR, and electropheresed on 1.8% Metaphor gel. The upper band corresponds to G3PDH and the lower band to Bcl-2. Bcl-2 mRNA was not expressed in freshly isolated PMN (lane 1) but was detected in HL-60 (lane 2). Incubation of PMN in the absence or in the presence of IL-8 (10 nM, and 100 nM) for 20 hours did not result in up-regulation of Bcl-2 (lanes 3, 4, 5).

functional states. These environmental signals include cell-cell and cell-matrix interactions, as well as exposure to soluble substances such as cytokines. A variety of different cytokine effects on PMN have been characterized. Recently, attention has been directed towards cytokine-mediated effects on apoptosis. For instance, GM-CSF, G-CSF, IL-1, IL-15, and interferon-γ increase PMN survival by delaying programmed cell death [20–22]. In contrast, IL-6 and TNFα have been reported to accelerate apoptosis [16, 23–25]. Polymorphonuclear neutrophil apoptosis is important because it provides a signal for PMN removal [14], and because it results in the loss of functional PMN responsiveness [13]. Thus, modulation of apoptosis, either its delay or acceleration, may have a major effect on the inflammatory process.

Several studies have demonstrated an association of various diseases with the chemokine IL-8, such as rheumatoid arthritis [26], adult respiratory distress syndrome [27, 28], psoriasis [29, 30], proliferative glomerulonephritis, [31] and primary systemic vasculitis [32]. Interleukin-8 is produced by numerous cell types, including endothelial cells, epithelial cells, fibroblasts, hepatocytes, mesangial cells, T-cells, monocytes, and even PMN [9, 33]. Moreover, PMN stimulated *in vivo* show increased IL-8 production. Harvested from skin lesions on the forearm of volunteers, PMN expressed 100-fold more and released 50-fold more IL-8 than freshly isolated cells from the same donor [34]. Interestingly, PMN activation also induces release of proteolytic enzymes that can enhance IL-8 production by endothelial cells [35]. The same proteases cleave a longer form of IL-8, thereby generating a more active shorter form [36]. These examples demonstrate that inflammation creates an IL-8 rich milieu and that even PMN, once they have arrived at inflammatory sites, can promote their own recruitment.

Polymorphonuclear neutrophils have various response patterns to IL-8 including shape change [37], degranulation [38], actin polymerization [39], and up-regulation of adhesion molecules [40]. Critically important for the inflammatory response is the chemotactic effect of IL-8. The cytokine attracts PMN from the circulation into sites of inflammation. Our findings characterize an additional biological property of IL-8, namely, a delay of PMN apoptosis. We demonstrated a clear dose- and time-dependent inhibition of spontaneous apoptosis in culture. In contrast to our study, two other studies employing IL-8 doses similar to ours reported no modulation of PMN apoptosis. However, both studies used longer incubation periods: 72 hours [21] and 48 to 96 hours [20], respectively. The investigators did not study the early time points, that is, the first 30 hours. We believe that these early time points represent the greatest PMN activity in terms of killing and the recruitment of other inflammatory cells. After 48 hours, PMN activity may be less relevant than that of monocytes and other invading cells.

Stimulated PMN release cytokines, including both TNFα and IL-8 [9, 41]. The release process is interactive since endogenous synthesis of TNFα in lipopolysaccharide-treated PMN can enhance IL-8 generation in an autocrine/paracrine manner [41]. We and others have shown that TNFα can rapidly accelerate PMN

apoptosis within two hours of treatment [16, 24, 25]. In this study, we demonstrated that IL-8 not only inhibits spontaneous apoptosis but also counteracts TNFα-mediated apoptosis. Because of the fact that apoptotic PMN have a decreased capability to respond to activating stimuli, as shown for FMLP and opsonized zymosan [13], delaying apoptosis would lead to accumulation of cells still capable of responding properly. In that way, IL-8 may prolong and amplify PMN-related effects.

We believe that our results demonstrating a delay of PMN apoptosis by IL-8 could be relevant to inflammatory renal diseases. For instance, in a recently described rat model of immune complex-mediated glomerulonephritis, PMN-apoptosis was identified as one of the mechanisms involved in restoring PMN to basal activity [42]. Interestingly, IL-8 is present both in urine and in renal tissue in patients with glomerulonephritis and could be a modulator of PMN apoptosis in this setting [31]. Finally, recent *in vitro* data indicate that PMN apoptosis is important in the presentation of target antigens for ANCA [43]. Since increased levels of IL-8 are found in active ANCA-vasculitis [44, 45], an effect of IL-8 on PMN apoptosis could be of importance.

We examined the two IL-8 receptors, RI and RII, in terms of modulating apoptosis. Both receptor types bind to IL-8 with high affinity. Interleukin-8 RII, but not RI, binds to other members of the CXC-cytokine family, such as Groα. The fact that IL-8 treatment resulted in a stronger effect on apoptosis than Groα should not have been surprising. Interleukin-8 exposure also results in increased CD11b expression, enhanced phospholipase D activation, intracellular calcium surge, and accelerated chemotaxis, compared to Groα [17, 18]. The results show that the IL-8 RII is capable of delaying apoptosis in any event. The additional occupation of IL-8 RI, as is the case with IL-8 administration, may provide an additive effect.

The Bcl-2 protooncogene encodes a protein shown to block programmed cell death [46, 47]. While Bcl-2 has been detected in a variety of cell types, it is not expressed in freshly isolated resting human PMN [48, 49]. This lack of expression may be responsible for the very short lifespan of PMN. We looked for Bcl-2 protein and for Bcl-2 gene expression in IL-8 treated cells and found neither. Thus, we conclude that IL-8 must inhibit apoptosis by Bcl-2-independent mechanisms. The nature of these mechanism remains to be elucidated. Numerous possibilities exist; inhibition of interleukin I converting enzyme (ICE) and ICE-like proteases is one example.

In summary, human PMN undergo spontaneous apoptosis *in vitro*. Treatment with the proinflammatory cytokine TNFα results in rapid acceleration of programmed cell death. The chemoattractant cytokine, IL-8, delays spontaneous as well as TNFα-mediated apoptosis *in vitro*. The IL-8 receptor II is capable of mediating the signal, although the IL-8 receptor I may provide an added response. The effect on apoptosis is independent of Bcl-2. Our data suggest that IL-8 delays the removal of PMN by inhibiting programmed cell death. The number of functionally intact PMN at sites of inflammation could thereby be enhanced. This mechanism may be of importance for a controlled and effective inflammatory response.

ACKNOWLEDGMENTS

This work was supported by DK 40208-09. Ralph Kettritz was supported by a grant-in-aid from the Deutsche Forschungsgemeinschaft.

Reprint requests to Ralph Kettritz, M.D., Division of Nephrology, Franz-Volhard Clinic, Humboldt Universität Berlin, Wiltbergstrasse 50, 13122 Berlin, Germany.

REFERENCES

1. Babior BM: Oxygen-dependent microbial killing by phagocytes (first of two parts). *N Engl J Med* 298:659–668, 1978
2. Root RK, Cohen MS: The microbicidal mechanisms of human neutrophils and eosinophils. *Rev Infect Dis* 3:565–592, 1981
3. Wade BH, Mandel GL: Polymorphonuclear leukocytes: Dedicated professional phagocytes. *Am J Med* 74:686–693, 1983
4. Lehrer RI (moderator): Neutrophils and host defense. *Ann Intern Med* 109:127–142, 1988
5. Bellamy COC, Malcomson RDG, Harrison DJ, Wyllie AH: Cell death in health and disease: The biology and regulation of apoptosis. *Cancer Biol* 6:3–16, 1995
6. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K: Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol* 9:617–648, 1991
7. Miller MD, Krangel MS: Biology and biochemistry of the chemokines: A family of chemotactic and inflammatory cytokines. *Crit Rev immunol* 12:17–29, 1992
8. Ahuja SK, Gao JL, Murphy PM: Chemokine receptors and molecular mimicry. *Immunol Today* 15:320–326, 1994
9. Baggiolini M, Dewals D, Moser B: Interleukin-8 and related chemotactic cytokines–CXC and CC chemokines. *Adv Immunol* 55: 97–179, 1994
10. Moser B, Schumacher C, Von Tscharner V, Clark-Lewis I, Baggiolini M: Neutrophil-activating peptide 2 and gro/melanoma growth-stimulatory activity interact with neutrophil-activationg peptide 1/interleukin 8 receptors on human neutrophils. *J Biol Chem* 266:10666–10671, 1991
11. Schumacher C, Clark-Lewis I, Baggiolini M, Moser B: High- and low-affinity binding of Groα and neutrophil-activating pepetide 2 to interleukin 8 receptors on human neutrophils. *Proc Natl Acad Sci USA* 89:10542–10546, 1992
12. Lee J, Horuk R, Rice GC, Bennett L, Camerato T, Wood WI: Characterization of two high affinity human interleukin-8 receptors. *J Biol Chem* 267:16283–16287, 1992
13. Lee A, Whyte MKB, Haslet C: Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* 54:283–288, 1993
14. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslet C: Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 83:865–875, 1989
15. Kerr JFR, Wyllie RH, Currie AR: Apoptosis: A basic biological phenomenon with wideranging implications in tissue kinetics. *Br J Cancer* 26:239–257, 1997
16. Kettritz R, Gaido ML, Jennette JC, Falk RJ: Neutrophil superoxide release is required for spontaneous and FMLP-mediated but not for TNFα-mediated apoptosis. *J Am Soc Nephrol* 8:1091–1100, 1997
17. L'Heureux GP, Bourgoin S, Lean N, McColl SR, Naccache PH: Diverging signal transduction pathways activated by interleukin-8 and related chemokines in human neutrophils: Interleukin-8, but not NAP-2or Groα, stimulates phospholipase D activity. *Blood* 85:522–531, 1995
18. Geiser T, Dewald B, Ehrengruber MU, Clark-Lewis I, Baggiolini M: The interleukin-8-related chemotactic cytokines Groα, Groβ, and Groγ activate neutrophil and basophil leukocytes. *J Biol Chem* 268:15419–15424, 1993
19. Yang E, Korsmeyer SJ: Molecular thanatopsis: A discourse on the Bcl-2 family and cell death. *Blood* 88:386–401, 1996
20. Colotta F, Re F, Polentarutti N, Sozzani S, Montovani A: Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 80:2012–2020, 1992
21. Cox G, Gauldie J, Jordana M: Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *Am J Respir Cell Mol Biol* 7:507–513, 1992
22. Girard D, Paquet ME, Paquin R, Beaulieu AD: Differential effects of interleukin-15 (IL-15) and IL-2 on human neutrophils:

Modulation of phagocytosis, cytoskeleton rearrangement, gene expression, and apoptosis by IL-15. *Blood* 88:3176–3184, 1996
23. Afford SC, Pongracz J, Stockley RA, Crocker J, Burnett D: The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. *J Biol Chem* 267:21612–21616, 1992
24. Takeda Y, Watanabe H, Yonehara S, Yamashita T, Saito S, Sendo F: Rapid acceleration of neutophil apoptosis by tumor necrosis factor-α. *Int Immunol* 5:691–694, 1993
25. Ohta H, Yatomi Y, Sweeney EA, Hakomori S, Igarashi Y: A possible role of sphingosine in induction of apoptosis by tumor necrosis factor-α in human neutrophils. *FEBS Lett* 355:267–270, 1994
26. Seitz M, Dewald B, Gerber N, Baggiolini M: Enhanced production of neutrophil-activating peptide-1/interleukin-8 in rheumatoid arthritis. *J Clin Invest* 87:463–469, 1991
27. Brennan FM, Zachariae COC, Chantry D, Larsen CG, Turner M, Maini RN, Matsushima K, Feldmann M: Detection of interleukin 8 biological activity in synovial fluids from patients with rheumatoid arthritis and production of interleukin 8 mRNA by isolated synovial cells. *Eur J Immunol* 20:2141–2144, 1990
28. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC: Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 341:643–647, 1993
29. Sticherling M, Bornscheuer E, Schröder JM: Localization of neutrophil-activating peptide-1/interleukin-8 immunoreactivity in normal and psoriatic skin. *J Invest Derm* 96:26–30, 1991
30. Nickoloff BJ, Karabin GD, Barker JNWN, Griffith CEM, Sarma V, Mitra RS, Elder JT, Kunkel SL, Dixit VM: Cellular localization of interleukin-8 and its inducer, tumor necrosis factor-α in psoriasis. *Am J Pathol* 138:129–140, 1991
31. Wada T, Yokoyamma H, Tomosugi T, Hisada Y, Ohta S, Naito T, Kobayashi KI, Mukaida N, Matsushima K: Detection of urinary interleukin-8 in glomerular diseases. *Kidney Int* 46:455–460, 1994
32. Kekow J, Szymkowiak CH, Sticherling M, Schröder JM, Christophers E, Gross WL: Pro- and anti-inflammatory cytokines in primary systemic vasculitis, in *ANCA-Associated Vasculitidis: Immunological and Clinical Aspects*, edited by Gross WL, New York, Plenum Press, 1993, p 341
33. Hoch RC, Schraufstätter IU, Cochrane CG: In vivo, in vitro, and molecular aspects of interleukin-8 and the interleukin-8 receptors. *J Lab Clin Med* 128:134–145, 1996
34. Kuhns DB, Gallin JI: Increased cell-associated IL-8 in human exsudative and A23187-treated peripheral blood neutrophils. *J Immunol* 154:6556–6562, 1996
35. Berger SP, Seelen MAJ, Hiemstra PS, Gerritsma JSJ, Van Den Woude FJ, Daha MR: Proteinase 3, the major autoantigen of Wegener's granulomatosis enhances IL-8 production by endothelial cells in vitro. *J Am Soc Nephrol* 7:694–701, 1996
36. Padrines M, Wolf M, Walz A, Baggiolini M: Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-2. *FEBS Lett* 352:231–235, 1994
37. Wymann MP, Kernen P, Deranleau DA, Dewald B, Von Tscharner V, Baggiolini M: Oscillatory motion in human neutrophils resonding to chemotactic stimuli. *Biophys Res Commun* 147:361–368, 1987
38. Masure S, Proost P, Van Damme J, Opdenakker G: Purification and identification of 91-kDa neutrophil gelatinase–Release by the activating peptide interleukin-8. *Eur J Biochem* 198:391–398, 1991
39. Westlin WF, Kiely JM, Gimbrone MAJ: Interleukin-8 induces changes in human neutrophil actin conformation and distribution: Relationship to inhibition of adhesion to cytokine-activated endothelium. *J Leukocyte Biol* 52:43–51, 1992
40. Detmers PA, Lo SK, Olsen-Egbert E, Walz A, Baggiolini M, Cohn ZA: Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J Exp Med* 171:1155–1162, 1990
41. Cassatella MA: The production of cytokines by polymorphonuclear neutrophils. *Immunol Today* 16:21–26, 1995
42. Hughes J, Johnson RJ, Mooney A, Hugo C, Gordon K, Savill J: Neutrophil fate in experimental glomerular capillary injury in the rat. *Am J Pathol* 150:223–234, 1997
43. Gilligan HM, Bredy B, Brady HR, Hebert MJ, Slayter HS, Xu Y, Rauch J, Shia MA, Koh JS, Levine JS: Antineutrophil cytoplasmic autoantibodies interact with primary granule constituents on the surface of apoptotic neutrophils in the absence of neutrophil priming. *J Exp Med* 184:2231–2241, 1996
44. Tesar V, Masek Z, Rychlik I, Bartunkova J, Stejskalova A, Zabka J, Dostal C, Becvar R, Merta M, Tomasova Z, Tepla L, Zima T: Cytokines and adhesion molecules in ANCA-positive vasculitis. (abstract) *Clin Exp Immunol* 101:58, 1995
45. Arimura Y, Minoshima S, Nagasawa T, Suzuki K: Serum IL-8, soluble ICAM-1, glomerular expression of cellular adhesion molecules and myeloperoxidase (MPO) release from the neutrophils in MPO-ANCA associated crescentic glomerulonephritis (CRGN). (abstract) *Clin Exp Immunol* 101:27, 1995
46. Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2 (immunoglobulin transcript resulting from the t/14; 18) translocation. *Cell* 47:19–28, 1986
47. Tsujimoto Y, Croce CM: Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci USA* 83:5214–5218, 1986
48. Iwai K, Miyawaki T, Takizawa T, Konno A, Ohta K, Yachie A, Seki H, Taniguchi N: Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood* 84:1201–1208, 1994
49. Delia D, Aiello A, Soligo D, Fontanella E, Melani C, Pezella F, Pierotti MA: Bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. *Blood* 79:1291–1298, 1992