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Protein-tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils

(signal transduction)

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ABSTRACT **Early signaling events that control the process of programed cell death are largely unknown. Tyrosine phosphorylation plays a major role in transmembrane signal transduction through most cell surface receptors. Granulocyte/macrophage colony-stimulating factor (GM-CSF), a cytokine released by activated T cells, has been shown to increase tyrosine phosphorylation in several cells and to inhibit granulocyte cell death *in vitro*. In this study, we demonstrate that the effect of GM-CSF on granulocyte cell death can be blocked by the tyrosine kinase inhibitor genistein, suggesting that increases in tyrosine phosphorylation are essential to inhibit cell death. To analyze the role of tyrosine phosphorylation for the regulation of granulocyte cell death more precisely, we increased levels of tyrosine phosphorylation using the protein-tyrosine phosphatase inhibitor phenylarsine oxide (PAO). Similar to GM-CSF, treatment of the cells with PAO was followed by high increases in tyrosine phosphorylation and inhibition of programed cell death in human eosinophils and neutrophils. Strikingly, at low concentrations of the inhibitor and low induction of tyrosine phosphorylation, acceleration of apoptosis was observed. Genistein and herbimycin A reversed the effects of PAO on tyrosine phosphorylation and granulocyte apoptosis. These results suggest that programed eosinophil and neutrophil death is regulated by early events of signal transduction pathways such as tyrosine phosphorylation.**

Apoptosis is a highly organized mechanism by which cells undergo programed cell death. Recently, there has been some progress regarding the genetic control of apoptosis (see ref. 1 for review). Individual genes have been identified that either promote or inhibit the process of apoptosis, although the mechanisms remain obscure. In contrast, the earliest events mediating programed cell death are largely unknown (2).

Phosphorylation and dephosphorylation of cellular proteins are implicated in many biologically important processes such as cell growth and differentiation. Less than 0.01% of total phosphorylation represents protein phosphorylation on tyrosine residues (3). Protein-tyrosine kinases (PTKs) and phosphatases (PTPases) jointly maintain the tyrosine phosphorylation of cellular proteins in homeostasis. Treatment of cells with receptor ligands changes this balance and consequently transduces the signal into the cell. There is a considerable interest regarding how PTKs and PTPases regulate signal transduction through the antigen or cytokine receptors in lymphocytes (see refs. 4 and 5 for reviews). Since the process of tyrosine phosphorylation and dephosphorylation is a major component of the intracellular response to external stimuli during signal transduction, such a protein modification may also play an important role in the mechanism of apoptosis.

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Recent studies using leukemia cell lines suggest a role of tyrosine phosphorylation in the regulation of programed cell death. For example, inhibition of tyrosine kinases leads to apoptosis in HL-60 and M07e cells (6). Tyrosine kinase inhibitors were further able to block the effect of growth factors on a growth factor-dependent cell line (7). On the other hand, tyrosine kinase activation was also implicated in the induction of apoptosis. Treatment of acute lymphoblastic leukemia cells with interferon α was accompanied with tyrosine phosphorylation of a 135-kDa protein and apoptosis of these cells (8). Furthermore, anti-immunoglobulin-induced apoptosis in murine CH31 lymphoma cells was dependent on the expression of the tyrosine kinase blk (9). These results raised the hypothesis that tyrosine phosphorylation determines whether a cell will survive or undergo apoptosis.

In this study, we provide evidence that changes in tyrosine phosphorylation regulate the process of granulocyte apoptosis *in vitro*.

MATERIALS AND METHODS

Purification of Cells. Eosinophils were isolated from human blood by negative selection of granulocytes, which do not express CD16, incorporating a magnetic cell separation system (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) as described (10). The purity was 98–99% as determined by light microscopy and flow cytometry. Neutrophils from the same donors were separated by Ficoll/Hypaque (Seromed, Fakola, Basel) centrifugation with no further purification, resulting in a 94–98% pure neutrophil preparation. Cells were washed and resuspended in RPMI 1640 medium plus 10% fetal calf serum to a density of 1×10^6 per ml.

PTPase Activity Assay. For PTPase assay, 2×10^6 cells were incubated with the indicated concentrations of phenylarsine oxide (PAO; Sigma) for 30 min at 37°C. The cell pellets were lysed on ice for 15 min using 20 μl of TKM buffer (50 mM Tris, pH 7.4/25 mM KCl/5 mM $MgCl_2$/1 mM EGTA) containing 0.5% Triton X-100. Phenylmethylsulfonyl fluoride (final concentration, 1 mM), aprotinin, and leupeptin (each at 10 μg/ml) were added to prevent proteolysis. After centrifugation at 14,000 rpm for 15 min, 4°C, the supernatant was diluted 10-fold by TKM buffer without Triton X-100. Aliquots of 100 μl were distributed in a 96-well plate (Nunc) and 10 mM of *p*-nitrophenyl phosphate was added. The plate was kept for 30 min at room temperature. The phosphatase activity was assayed by measuring released *p*-nitrophenol (absorbance at 405 nm).

Detection of Tyrosine Phosphorylation by Flow Cytometry. To analyze the amount of tyrosine-phosphorylated proteins

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; PAO, phenylarsine oxide; PTK, protein-tyrosine kinase; PTPase, protein-tyrosine phosphatase; P-Tyr, phosphotyrosine.

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by flow cytometry, 1×10^6 cells were incubated with 10 μM PAO for 30 min at 37°C, washed in phosphate-buffered saline (PBS) containing 2 mM vanadate, and fixed in 3% paraformaldehyde solution for 10 min at room temperature. After a washing step in PBS/vanadate, cells were resuspended in staining solution, which permeabilized the cell membrane for further intracellular staining [PBS containing 0.5 mg of saponin per ml, 1% bovine serum albumin (BSA), and 2 mM vanadate]. The monoclonal anti-phosphotyrosine (*P*-Tyr) antibody 4G10 (Biomol, Hamburg, Germany) was incubated for 60 min at room temperature. Unbound antibody was washed away with staining solution and cells were incubated with goat anti-mouse IgG fluorescein isothiocyanate conjugate (Tago-Inotech) for 30 min at room temperature. Following two washes, cells were resuspended in 300 μl of staining solution and immediately analyzed by flow cytometry in an EPICS Profile (Coulter).

Detection of Tyrosine Phosphorylation by Immunoblotting. For anti-*P*-Tyr immunoblotting, 2×10^6 cells were stimulated with 10 ng of granulocyte/macrophage colony-stimulating factor per ml (GM-CSF; GIBCO/BRL) for 5 min or with 10 ng of GM-CSF per ml after a 1-h preincubation with 50 μg of genistein per ml (Calbiochem). In additional experiments, cells were incubated at 37°C with the indicated concentrations of PAO for 30 min. In some experiments, cells were preincubated with 50 or 100 μg of genistein per ml for 1 h before addition of PAO. Cell pellets were lysed in SDS/PAGE sample buffer (10% SDS/100 mM Tris, pH 6.8/1% glycerol/125 μg of bromophenol blue per ml) and then sonicated, 1 mM dithiothreitol was added, and samples were boiled for 10 min. The lysates were centrifuged and the supernatants were separated by SDS/PAGE (8% gel) and transferred onto polyvinylidene difluoride membranes (Millipore). The filters were blocked in prewarmed blocking buffer (1% BSA/0.01 M Tris, pH 7.5/0.1 M NaCl/0.1% Tween 20). Immunoblots were carried out using the recombinant anti-*P*-Tyr antibody RC20 peroxidase conjugate (Affiniti, Nottingham, U.K.) at a 1:2500 dilution with prewarmed blocking buffer for 20 min at 37°C, and blots were washed extensively in blocking buffer without BSA. The blots were developed by an enhanced chemiluminescence technique (ECL kit, Amersham) according to the manufacturer's instructions.

Determination of Cell Death by Flow Cytometry. At time zero, cells were treated with PAO or GM-CSF. In some experiments, granulocytes were preincubated with genistein (50 μg/ml) or herbimycin A (5–10 μg/ml; GIBCO/BRL) for 1 h before addition of PAO or GM-CSF. The relative amounts of viable cells were determined at different time points by uptake of ethidium bromide and acridine orange (Becton Dickinson) in dead cells by fluorescence-activated cell sorter analysis. Granulocytes were separated from possible contaminating lymphocytes and monocytes by gating. Each experiment compared eosinophils and neutrophils derived from one individual. On average, 40% of untreated eosinophils and 25% of untreated neutrophils were dye-permeable after 18 h.

Determination of Cell Death by Fluorescence Microscopy. For fluorescence microscopy analysis, PAO-treated or nontreated cells (10^6 per 20 μl of PBS plus 10% glycerol) were exposed to a final concentration of 5 μg of propidium iodide per ml and immediately viewed under a fluorescence microscope (Zeiss). Many nuclei were fragmented in apoptotic cells. Other nuclei were homogeneously stained, also characteristic of apoptosis.

DNA Fragmentation Assay. For DNA analysis, PAO-treated or nontreated cells were harvested and washed with PBS at room temperature. Cell pellets were resuspended at 2×10^7 cells per ml in cell lysis buffer [10 mM EDTA/50 mM Tris, pH 8.0, containing 0.5% (wt/vol) *N*-lauroylsarcosine and 0.5 mg of proteinase K per ml] and incubated for 1 h at 50°C. RNase A (250 μg/ml) was added and incubated at 50°C for another hour. The solution was extracted two times with phenol, buffered with 0.1 M Tris·HCl (pH 7.4), followed by two chloroform/isoamyl alcohol (24:1) extractions. The DNA solution was brought to 2.5 volumes by the addition of Tris/EDTA (TE) buffer (pH 8) and centrifuged at 12,000 rpm for 20 min at room temperature to separate nucleosomal DNA. The supernatants, containing fragmented DNA, were precipitated for 24 h in 2 volumes of ethanol at −70°C. The DNA precipitates were recovered by centrifugation at 14,000 rpm for 1 h. After drying, DNA was dissolved in TE buffer and stored at 4°C. DNA was mixed with 10× loading buffer (0.25% bromophenol/0.1 M EDTA, pH 8/1% SDS/20% Ficoll 400) before loading into wells of a 1.5% agarose gel containing 0.5 μM ethidium bromide. Electrophoresis was carried out in 90 mM Tris base/90 mM boric acid/2 mM EDTA, pH 8. After electrophoresis, gels were visualized by ultraviolet light.

RESULTS AND DISCUSSION

GM-CSF-Induced Inhibition of Granulocyte Cell Death Is Associated with Increases in Tyrosine Phosphorylation. It is now generally accepted that defective regulation of programed cell death may play a role in the etiology of different diseases (11). In allergic inflammation, an accumulation of eosinophils but not neutrophils has been observed (12, 13). Inhibition of eosinophil apoptosis by growth factors was suggested to be one reasonable explanation for the eosinophilia associated with various diseases, including allergy and asthma (14, 15). To determine whether the effect of physiological growth factors, such as GM-CSF, on granulocyte viability depends on changes in tyrosine phosphorylation, we determined GM-CSF-induced increases in tyrosine phosphorylation and its effects on granulocyte life-span. As

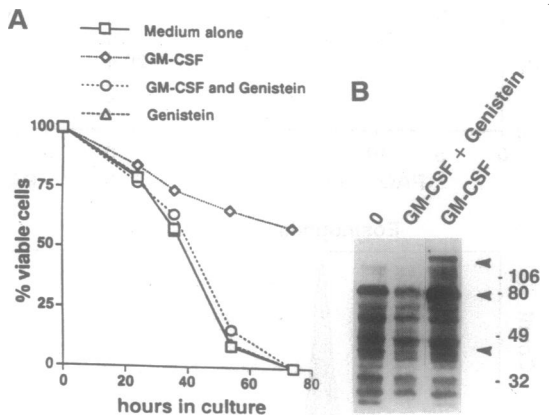

FIG. 1. Effects of GM-CSF and their reversibility by genistein on granulocyte death and tyrosine phosphorylation. (*A*) Eosinophil viability without and in the presence of 10 ng of GM-CSF per ml was assessed by uptake of ethidium bromide and flow cytometry. In addition, experiments were performed in the presence of 50 μg of genistein per ml. Each value is the mean of data from duplicate experiments. Error bars are within the symbols. GM-CSF increased the *in vitro* survival of human eosinophils. Pretreatment with genistein almost completely abolished the effect of GM-CSF. Genistein alone had no effect. (*B*) GM-CSF-induced increases in tyrosine phosphorylation in eosinophils were determined by anti-*P*-Tyr immunoblotting. Eosinophils were treated without or with 10 ng of GM-CSF per ml or with 50 μg of genistein per ml and 10 ng of GM-CSF per ml. Units are in kDa. GM-CSF induced tyrosine phosphorylation of several intracellular proteins. Pretreatment with genistein completely abolished the effect of GM-CSF on tyrosine phosphorylation. This figure is representative of two other experiments that yielded the same results.

shown in Fig. 1*B*, treatment of eosinophils with 10 ng of GM-CSF per ml for 5 min consistently resulted in increases of tyrosine phosphorylation of proteins with molecular masses of approximately 150, 74, and 40 kDa, confirming previously published results obtained with human neutrophils (16). In contrast, pretreatment of the eosinophils for 1 h at 37°C with the tyrosine kinase inhibitor genistein at 50 μg/ml completely inhibited tyrosine phosphorylation of the 150-, 74-, and 40-kDa bands (Fig. 1*B*). We next investigated whether blocking of increases in tyrosine phosphorylation by genistein also inhibits the prolonged eosinophil survival effect of GM-CSF. Indeed, pretreatment with 50 μg of genistein per ml for 1 h almost completely abolished the effect of GM-CSF on eosinophil viability *in vitro* (Fig. 1*A*). Genistein alone had no effect in this system. Similar results were seen using neutrophils (data not shown). These results suggest that GM-CSF induces tyrosine phosphorylation and that this signaling event is an obligatory step for the effect on granulocyte viability.

Inhibition of PTPase Activity Increases Tyrosine Phosphorylation in Human Eosinophils and Neutrophils. To further investigate the role of tyrosine phosphorylation for the inhibition of granulocyte death we wanted to mimic the GM-CSF signaling pathway. One possible event for sending a cytokine signal into a cell is the inactivation of a PTPase (17). PAO was previously shown to selectively inhibit PTPase activity and consequently to increase tyrosine phosphorylation in T cells (18, 19). As shown in Fig. 2*A*, PAO inhibited PTPase activity in human eosinophils and neutrophils. Inhibition of cellular PTPases resulted in a large increase in the level of tyrosine phosphorylation as assessed by flow cytometry using an anti-*P*-Tyr antibody (Fig. 2*B*). In addition, *P*-Tyr-containing proteins in human eosinophils and neutrophils were detected more precisely with an anti-*P*-Tyr immunoblotting technique. Incubation of human eosinophils and neutrophils with PAO increased tyrosine phosphorylation of several cellular proteins (Fig. 2*C*). In preliminary studies, we established that the degree of cellular protein tyrosine phosphorylation begins to increase after 5 min, is optimal after 10 min, and does not decline thereafter (data not shown). As shown in Fig. 2*C*, the minimal dose required to induce detectable increases in tyrosine phosphorylation is 0.1 μM PAO in both granulocyte types. Whereas 10-fold more PAO (1 μM) had no additional effect on tyrosine phosphorylation of eosinophil proteins, it markedly potentiated tyrosine phosphorylation of cellular proteins in neutrophils (Fig. 2*C*). A similar increase in tyrosine phosphorylation was produced in eosinophils treated with 5 μM PAO (Fig. 2*C*). Further increases of the PAO concentration did not lead to further increases of tyrosine phosphorylation (Fig. 2*C*). Thus, PAO inhibits PTPase activity and increases tyrosine phosphorylation in human eosinophils and neutrophils.

Tyrosine Phosphorylation Regulates the Life-Span of Human Eosinophils and Neutrophils. It was next investigated whether inhibition of PTPase activity and consequent changes in tyrosine phosphorylation may influence the lifespan of human granulocytes *in vitro*. Cell viability was assessed by flow cytometry. Human eosinophils (Fig. 3*A*) and neutrophils (Fig. 3*B*) were exposed to different concentrations of PAO. Higher PAO concentrations (3–10 μM for

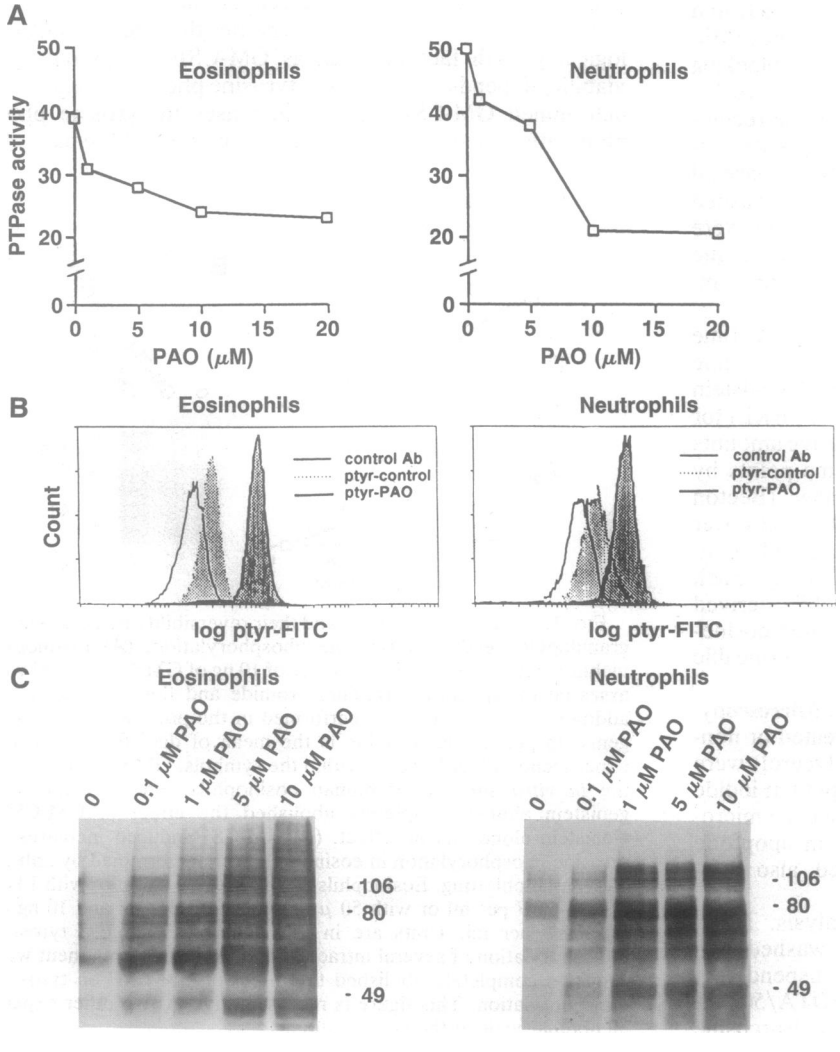

FIG. 2. Effects of the PTPase inhibitor PAO on tyrosine phosphorylation in human granulocytes. (*A*) PAO inhibited PTPase activity in human eosinophils and neutrophils in a dose-dependent manner. Scales at the left of each tracing indicate the amount of released *p*-nitrophenol (μM). Each value is the mean of data from duplicate experiments. (*B*) As a consequence of inhibition of PTPase activity, PAO induced tyrosine phosphorylation in human eosinophils and neutrophils as assessed by flow cytometry using an anti-*P*-Tyr antibody (Ab). FITC, fluorescein isothiocyanate. (*C*) Effects on tyrosine phosphorylation were also determined by immunoblotting. Granulocytes were treated with different concentrations of PAO for 30 min. The minimal dose required for induction of tyrosine phosphorylation in both cell types was 0.1 μM PAO. Potentiation of tyrosine phosphorylation was achieved with 1 μM PAO in neutrophils, whereas 5 μM PAO was needed in eosinophils. Units are in kDa. This figure is representative of two other experiments that yielded the same results.

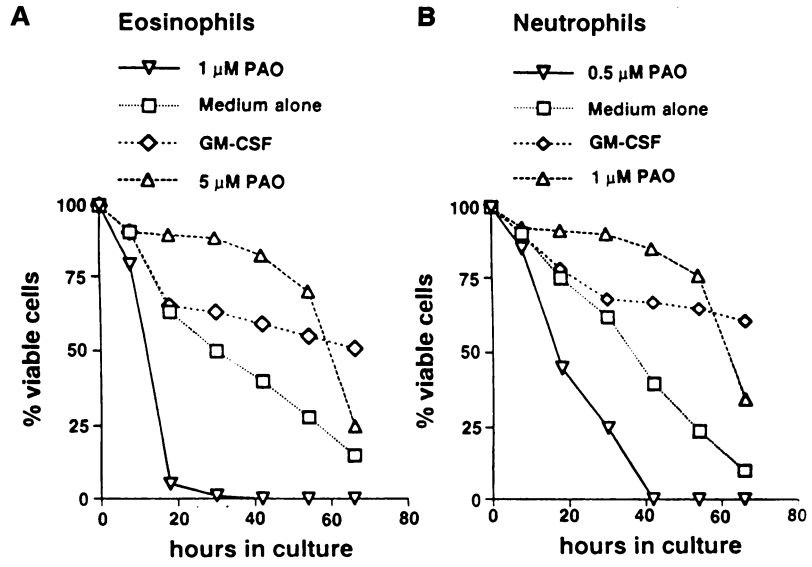

FIG. 3. Effects of PAO on granulocyte viability. (*A*) Cell death in eosinophils was accelerated by 1 μM PAO, while eosinophils treated with 5 μM PAO or 10 ng of GM-CSF per ml demonstrated inhibition of cell death. (*B*) Similar results were obtained using neutrophils. Acceleration of cell death occurred with 0.5 μM PAO, whereas 1 μM inhibited neutrophil death. Each value is the mean of data from duplicate experiments. Error bars are within the symbols. This figure is representative of >20 independent experiments that yielded the same results.

eosinophils, 1–5 μM for neutrophils) inhibited granulocyte death *in vitro*, at least within the first 48 h, and therefore mimicked the effect of GM-CSF. In contrast, low concentrations of PAO (0.1–1 μM for eosinophils, 0.1–0.5 μM for neutrophils) induced cell death.

Comparisons of the PAO concentrations used in our assays to determine cell death (Fig. 3) and to induce tyrosine phosphorylation (Fig. 2*C*) imply that low induction of tyrosine phosphorylation in human eosinophils and neutrophils is associated with induction of granulocyte death. However, maximal induction of tyrosine phosphorylation by inhibition of PTPases correlates with prolonged granulocyte survival.

To determine whether the observed effects on cell viability were specifically due to PAO-induced changes in tyrosine phosphorylation and not due to other possible pharmacological activities of PAO, cells were pretreated with the tyrosine kinase inhibitor genistein. Indeed, incubation with 50 μg of genistein per ml for 1 h completely prevented PAO-induced cell death (Fig. 4*A*). In addition, genistein partly converted PAO-induced inhibition into acceleration of cell death (Fig. 4*A*). Similar results were observed using the tyrosine kinase inhibitor herbimycin A (data not shown). We also performed experiments and determined the effects of genistein on PAO-induced increases in tyrosine phosphorylation. Genistein completely abolished increases in tyrosine phosphorylation usually induced by low PAO concentrations in eosinophils (Fig. 4*B*). Under these conditions, PAO-induced granulocyte apoptosis was abolished, and no effect on cell viability was observed (Fig. 4*A*). Genistein only partially blocked increases in tyrosine phosphorylation induced by higher PAO concentrations (Fig. 4*B*). The result of such a treatment is a low induction of tyrosine phosphorylation that activates cell death (Fig. 4*A*). These results suggest that PAO-induced changes on the granulocyte life-span are mediated by tyrosine phosphorylation and not the consequence of a nonspecific effect.

Alterations of Granulocyte Life-Span by Changes in Tyrosine Phosphorylation Are Due to Activation and Inhibition of Apoptosis. We next investigated whether the observed cell death was apoptosis. Using fluorescence microscopy and a DNA fragmentation assay, we demonstrated that the type of cell death induced by low PAO concentrations is apoptotic and that PAO at higher concentrations inhibits apoptosis in both human eosinophils and neutrophils. As shown in Fig. 5*A*, eosinophils exposed to 1 μM PAO for 8 h demonstrated evidence for apoptosis as assessed by fluorescence microscopy. At higher magnification, we also observed nuclear fragmentation, chromatin condensation, and homogeneous nuclear staining that are characteristic of apoptosis but not necrosis (20) (data not shown). Exposure to the same PAO concentration for 30 h led to virtually complete cell death. In contrast, no evidence for apoptosis was observed after 30 h in eosinophils exposed to 5 μM PAO (Fig. 5*A*). Similar results were observed in neutrophils using appropriate PAO con-

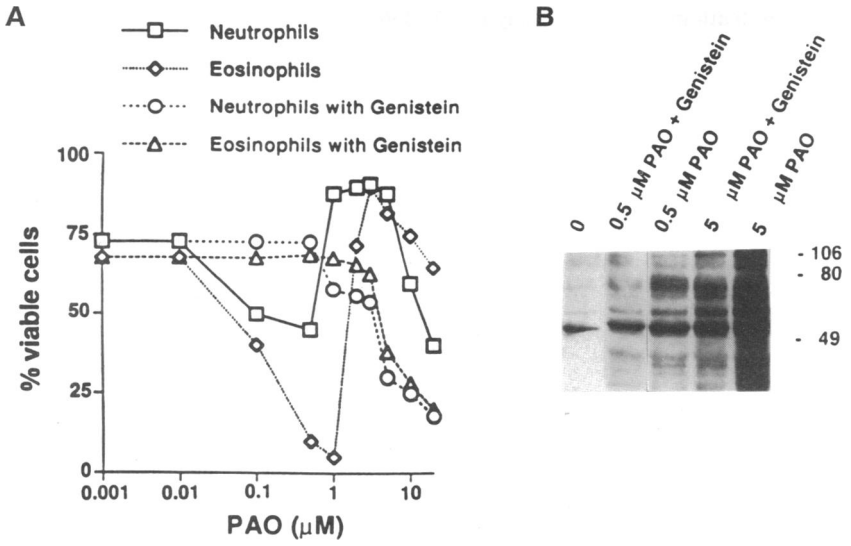

FIG. 4. Effects of PAO and their reversibility by genistein on eosinophil death and tyrosine phosphorylation. (*A*) Relatively small changes of the PAO concentrations led to opposite effects on 18-h granulocyte survival. PAO-induced cell death and prolonged survival, respectively, were abolished by genistein. Each value is the mean of data from duplicate experiments. Error bars are within the symbols. (*B*) Preincubation with 50 μg of genistein per ml prevented increases of tyrosine phosphorylation after 0.5 μM PAO treatment and markedly decreased tyrosine phosphorylation induced by 5 μM PAO in human eosinophils. Units are in kDa. This figure is representative of two other experiments that yielded the same results.

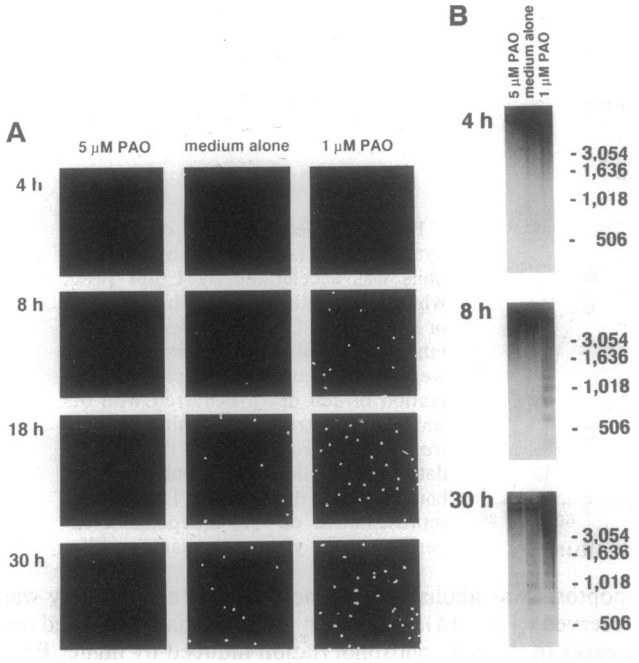

FIG. 5. Cell death in human granulocytes and its alteration by PAO is due to acceleration and inhibition of apoptosis—acceleration of eosinophil apoptosis by 1 μM PAO, but inhibition by 5 μM PAO. Apoptotic cells were analyzed by fluorescence microscopy (*A*) and by agarose gel DNA electrophoresis (*B*). Units are in bp. This figure is representative of two other experiments that yielded the same results.

centrations (data not shown). Apoptosis is often associated with a characteristic oligonucleosomal DNA fragmentation (21), and such DNA fragmentation in untreated eosinophils became evident after 18 h in culture. Again, as demonstrated in Fig. 5*B*, 1 μM PAO induced apoptotic cell death within 8 h, whereas the higher concentration of 5 μM PAO prevented apoptosis in eosinophils even after 30 h in culture.

Since relatively small differences in the concentrations of the PTPase inhibitor PAO caused opposing effects on cell death of human granulocytes, we hypothesize that tyrosine phosphorylation controls a "balancing act" between activation and inhibition of apoptosis in these cells. The results further suggest that PTPase activity may be a control point in the regulation of tyrosine phosphorylation and apoptosis. This view is supported by recent observations that the CD45 PTPase regulates apoptosis in thymocytes (22).

In summary, we provide evidence for a role of tyrosine phosphorylation in the regulation of programed cell death in freshly isolated human granulocytes. It should now be possible to dissect the early signaling pathways leading to activation of and protection from apoptosis. Furthermore, our observations provide an avenue to identify genes that control the life-span of human granulocytes and perhaps other cell types. It should be also possible to develop therapeutic agents that induce specific eosinophil apoptosis in patients with eosinophilic disorders by manipulating their PTPase activity. Apoptotic eosinophils may then easily be removed by macrophages without induction of further inflammation (23).

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