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*J Immunol* 1999; 162:1692-1700; 
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Role of p38-Mitogen-Activated Protein Kinase in Spontaneous Apoptosis of Human Neutrophils

Kazutetsu Aoshiba, Shuji Yasui, Mitsutoshi Hayashi, Jun Tamaoki, and Atsushi Nagai

Neutrophils constitutively undergo apoptosis at both normal and inflamed sites: an important process that limits the toxic potential of the neutrophil. However, the signal pathway for neutrophil apoptosis is currently unknown. In this study, we evaluated the role of p38-mitogen-activated protein kinase (MAPK) in the spontaneous apoptosis of neutrophils in vitro. We found that p38-MAPK was constitutively tyrosine phosphorylated and activated during spontaneous apoptosis of neutrophils. Inhibition of p38-MAPK by SB203580 and an antisense oligonucleotide delayed apoptosis by approximately 24 h. The antioxidants catalase and N-acetylcysteine delayed neutrophil apoptosis, but failed to inhibit phosphorylation and activation of p38-MAPK. Granulocyte-macrophage CSF and anti-Fas Ab, which altered the rate of apoptosis, did not affect phosphorylation and activation of p38-MAPK. These results suggest that the constitutive phosphorylation and activation of p38-MAPK are involved in the program of spontaneous apoptosis in neutrophils. The Journal of Immunology, 1999, 162: 1692–1700.

Materials and Methods

Reagents

All reagents for cell culture were obtained from Life Technologies (Gaithersburg, MD). MTT, bisBenzimidze (Hoechst33258), thymus DNA, genistein, N-acetylcysteine, catalase, leupeptin, aprotinin, PMSF, β-glycerophosphate, sodium vanadate, DTT, and polymyxin B sulfate were purchased from Sigma (St. Louis, MO). Recombinant human GM-CSF, anti-Fas Ab (CH-11), protein A plus G agarose, and PD98059 were purchased from Pharma Biotechnologie (Hannover, Germany), Medical and Biological Laboratories (Nagoya, Japan), Oncogene Research Product (Cambridge, MA), and Biomol Research Laboratories (Plymouth Meeting, PA), respectively. SB203580 was a generous gift of SmithKline Beecham Pharmaceuticals (King of Prussia, PA). The phosphorylated forms of the proteins p38-MAPK, ERK, and JNK were obtained from New England Biolabs (Beverly, MA). MAPKAP kinase-2-GST was purchased from Upstate Biotechnology (Lake Placid, NY).
**Neutrophil preparation and cell culture**

Human neutrophils were isolated from EDTA-anticoagulated venous blood samples by dextran sedimentation and centrifugation on a Histopaque gradient (without endotoxin; Sigma), as previously described (17). Contaminating erythrocytes were removed by hypotonic water lysis. The isolated neutrophils were washed twice with PBS and resuspended in RPMI 1640 containing 5% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To avoid accidental neutrophil activation and clumping, neutrophil preparations were carefully washed by centrifugation at 200 × g and aspiration of the supernatant, followed by gentle resuspension with a pipet. Neutrophil preparations were carefully washed by centrifugation at 200 × g and aspiration of the supernatant, followed by gentle resuspension with a pipet. Neutrophil populations were stained with May-Grünwald-Giemsa stain, and neutrophil viability was >98%, as determined by trypan blue dye exclusion. Less than 5% of neutrophil preparations showed a polarized shape, a sensitive marker for neutrophil activation (18). Unless otherwise stated, to assay for apoptosis, cell survival, and ROS generation, 1 × 10^6 cells were incubated in 200 μl medium in 96-well round-bottom plates (Becton Dickinson, Lincoln Park, NJ) at 37°C in a humidified incubator containing 5% CO₂. For p38-MAPK assay and immunoblotting, 5 × 10^6 cells were suspended in 10 ml medium in a polycarbonate tube (Becton Dickinson) and cultured as above.

Normal human fetal lung fibroblasts (IMR-90; Clonetics, San Diego, CA) and human bronchial epithelial cells (16HBE) were cultured in DMEM or 1:1 Ham’s F-12/DMEM, respectively, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Antisense oligonucleotide treatment of cells**

Phosphorothioate-modified antisense oligonucleotides specific for p38-MAPK (5'-CGAGGAGCTGAACAAGAC-3') (19) as well as sense (5'-TGCTTGTGCTACCTTCGCTCGG-3') oligonucleotides were synthesized. Neutrophils were incubated with 1 μM of each oligonucleotide in serum-free RPMI 1640 for 6 h before addition of 5% heat-inactivated FCS. After 24 h of culture, the cells were prepared for apoptosis assay and immunoblot analysis, as described below.

**Apoptosis assay**

Aliquots of neutrophils were cytospun on glass slides and dried. Slides were stained with May-Grünwald-Giemsa. Apoptosis was assessed based on nuclear pyknosis or chromatin condensation together with cytoplasmic vacuolation on oil immersion microscopy (2). Three hundred cells were scored in each experiment to determine the percentage of apoptotic cells.

**Inhibition of p38-MAPK delays neutrophil apoptosis**

Apoptosis detection was also evaluated by detection of cytosolic histone-bound DNA fragments. Briefly, 2 × 10^6 cells were incubated in a 96-well round-bottom plate for 12 h. The plate was centrifuged and cell pellets were analyzed for histone-bound DNA fragments using the cell detection ELISA kit (Cell Death Detection ELISAPLUS; Boeringer Mannheim, Indianapolis, IN), according to the manufacturer’s instruction. The principle of this test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated antihistone- and peroxidase-coupled anti-DNA Abs. The enrichment of mono- and oligonucleosomes released into the cytoplasm is calculated as absorbance of incubated cells/absorbance of freshly isolated cells.

**Cell survival assay**

Cell survival was evaluated by both a colorimetric MTT assay and a fluorescence cellular DNA assay. For MTT assay, 0.5 mg/ml MTT was added to culture plates for the last 4 h of incubation. Then the plates were centrifuged and cell pellets were lysed in 200 μl DMSO and 25 μl Sorenson’s buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The OD was measured at 570 nm on a microplate immunoreader (Bio-Rad, Hercules, CA). For DNA assay, culture plates were centrifuged and cell pellets were lysed in 100 μl distilled water, followed by a cycle of freezing and thawing. Then, cell lysates were solubilized in 100 μl of TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) containing 10 μg/ml proteinase K, 1 mM PMSF, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate, and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant containing equivalent amounts of protein (250 μg) was precleared with protein A plus G agarose and immunoprecipitated with 4 μl of anti-p38-MAPK Ab for 1 h at 4°C. The immunocomplex was captured by protein plus G agarose for 4 h. Bead pellets were washed twice in lysis buffer and twice in kinase buffer (25 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM β-glycerophosphate, 2 mM DTT, 100 mM sodium orthovanadate). The beads were incubated in 30 μl of kinase buffer containing 12.5 μg/ml MAPKAP kinase-2-GST, 30 μM ATP, and 10 μCi [γ-32P]ATP for 20 min at 30°C. The reaction was terminated by the addition of 10 μl 5× SDS sample buffer and heating to 95°C for 5 min. Samples were resolved on a 12% acrylamide SDS-PAGE gel and subjected to autoradiography. The immunoprecipitated samples were also analyzed for p38-MAPK by immunoblotting to determine whether the same amount of p38-MAPK was immunoprecipitated. The OD of each band was determined, and the ratio of phosphorylated MAPKAP kinase-2-GST immunoprecipitated p38-MAPK was plotted.

**Immunocytochemistry for phosphorylation of p38-MAPK**

Venous blood was drawn and immediately smeared on a glass slide. Blood cells were fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with phospespecific Ab to p38-MAPK (1:50) or control rabbit IgG. Primary Ab was detected with FITC-conjugated Ab. Cells were counterstained with propidium iodide and observed under fluorescence microscopy.

**Statistics**

Results are presented as mean ± SEM. Comparisons were made by Student’s t test or ANOVA with Scheffe’s correction where appropriate.

**Results**

**Inhibition of p38-MAPK delays neutrophil apoptosis**

When cultured in DMEM containing 5% FCS, neutrophils underwent apoptosis constitutively, with 25.1% of cells apoptotic at 16 h and 94.3% apoptotic by 72 h, as assessed by light microscopy (Fig. 1). The constitutive rate of apoptosis in neutrophils shown in this work is within the range reported previously (2–5). Incubation of neutrophils with 50 μM of SB203580, a selective p38-MAPK inhibitor that has no inhibitory action on ERK and JNK (20), significantly inhibited apoptosis at each time point, with delayed onset of apoptosis by approximately 24 h (Fig. 1A). Genistein, a broad spectrum tyrosine kinase inhibitor, modestly inhibited apoptosis. In contrast, PD98059, an inhibitor of ERK kinase, had no effect on the rate of neutrophil apoptosis. Inhibition of apoptosis by SB203580 was dose dependent and was observed at a concentration as low as 5 μM, which is comparable with effective concentrations (1–25 μM) reported previously (12, 20–25) (Fig. 1B). SB203580 also inhibited nucleosomal DNA fragmentation, as assessed by a cell death ELISA assay (Fig. 2), which detects cytosolic histone-bound DNA fragments formed in cells undergoing apoptosis. The inhibition of neutrophil apoptosis by SB203580 was supported by the fact that this compound prolonged survival of neutrophils, as assessed by an MTT assay (Fig. 3A), and by measurement of residual DNA in culture wells (Fig. 3B). In contrast to SB203580, the ERK inhibitor PD98059 did not affect neutrophil survival, consistent with the apoptosis data. These results...
suggest that the pharmacologic inhibition of p38-MAPK, but not ERK, delays spontaneous apoptosis with resultant extension of survival in neutrophils.

Although SB203580 has been shown to date to be specific for p38-MAPK (12, 20–25), the rather high doses used for the long incubation time may result in nonspecific effects. Thus, to confirm the results described above, phosphorothioate-modified antisense oligonucleotides were used to specifically deplete p38-MAPK. Treatment of neutrophils with the antisense oligonucleotides for 24 h reduced phosphorylated and total p38-MAPK in cell lysates by approximately 50% as compared with treatment with sense and scrambled oligonucleotides (Fig. 4A). When cells were treated with the antisense oligonucleotides, neutrophils exhibited a decreased level of apoptosis in comparison with cells treated with sense or scrambled oligonucleotides (Fig. 4B). These experiments suggest that p38-MAPK activity may play a role in spontaneous apoptosis in neutrophils.

**p38-MAPK is continuously phosphorylated and activated in neutrophils**

To assess p38-MAPK activity in neutrophils, we evaluated the phosphorylation and activation of p38-MAPK. Phosphorylation of p38-MAPK, ERK, and JNK was assessed by SDS-PAGE fractionation of equivalent amounts (50 μg) of protein from each sample, followed by immunoblotting with phosphospecific Abs. In freshly isolated neutrophils (time 0), a significant level of p38-MAPK phosphorylation was detected (Fig. 5A). This basal phosphorylation level was sustained during a 24-h period, while neutrophils in parallel cultures increasingly underwent apoptosis with time (data not shown). In contrast to p38-MAPK, ERK and JNK showed no significant phosphorylation during the same period of culture (Fig. 5A). This was not due to the absence of ERK and JNK proteins because these proteins were detectable by immunoblotting with Abs to ERK and JNK. To determine whether phosphorylation of p38-MAPK is also seen in quiescent cultures of other cell types, we evaluated fibroblasts (IMR-90) and bronchial epithelial cells (16HBE). In contrast to neutrophils, fibroblasts and bronchial epithelial cells cultured in quiescent conditions showed no apparent phosphorylation of p38-MAPK (Fig. 5B).

There was a possibility that the p38-MAPK phosphorylation seen in freshly isolated neutrophils was the result of accidental activation of neutrophils or endotoxin contamination during and after cell preparation because p38-MAPK has been shown to be phosphorylated following stimulation of neutrophils (12–14). However, this was unlikely for six reasons. First, less than 5% of neutrophil preparations showed polarization that reflects activation of neutrophils (18). Second, very few cells isolated exhibited spontaneous migration in a chemotaxis chamber (data not shown). Third, ERK, also known to be activated following stimulation of neutrophils (15, 26), was not significantly phosphorylated unless neutrophils were stimulated with PMA, an activator of protein kinase C (Fig. 5C). Stimulation of neutrophils with PMA modestly potentiated phosphorylation of p38-MAPK (Fig. 5C). Fourth, endotoxin was not detected (<5 pg/ml) in cell preparation reagents or culture medium by a Limulus lysate test. Fifth, inclusion of the endotoxin inhibitor polymyxin B (100 U/ml) in culture medium

**FIGURE 1.** Effects of SB203580, PD98059, and genistein on neutrophil apoptosis. Neutrophils were cultured for varying times (A) or 24 h (B) in RPMI 1640 containing 5% FCS with or without SB203580, PD98059, or genistein. Apoptotic cells were identified by morphology on light microscopy, and percentage of apoptosis was obtained from 300 counts per experiment. Data represent mean ± SEM of six experiments. ***, p < 0.01 versus control cells in medium alone.

**FIGURE 2.** Effect of SB203580 on nucleosomal DNA fragmentation in neutrophils. Neutrophils were cultured with or without 10 μM SB203580 for 12 h and analyzed for nucleosomal DNA fragmentation using a cell death ELISA kit. The enrichment of mono- and oligonucleosomes released into the cytoplasm is calculated as absorbance of cultured cells/absorbance of freshly isolated cells. Data represent mean ± SEM of three experiments. ***, p < 0.01 versus control cells in medium alone.
did not affect the phosphorylation of p38-MAPK (Fig. 5D). Sixth, to avoid endotoxin contamination and accidental cell activation during cell preparation, fresh blood smears made on glass slides were immunostained with a phosphospecific p38-MAPK Ab. A low but significant level of p38-MAPK phosphorylation was detectable in neutrophils in blood smears (Fig. 6).

Activation of p38-MAPK was assessed by its ability to phosphorylate MAPKAP kinase-2-GST (27, 28). Consistent with the phosphorylation data, a basal activity of p38-MAPK that could be inhibited by SB203580 was detectable in freshly isolated neutrophils, and this activity lasted at 24 h of culture (Fig. 7). These experiments indicate that p38-MAPK in neutrophils is continuously phosphorylated and activated during the program of spontaneous apoptosis. Furthermore, a lack of phosphorylation in ERK and JNK confirms that p38-MAPK is selectively phosphorylated during spontaneous apoptosis.

**FIGURE 3.** Effect of SB203580, PD98059, and genistein on neutrophil survival. Neutrophils were cultured for 24 h in RPMI 1640 containing 5% FCS with or without SB203580, PD98059, or genistein. Neutrophil survival was evaluated by MTT assay (A) or by measurements of DNA content per well (B). Data represent mean ± SEM of six experiments. **p, p < 0.01 versus control cells in medium alone.

**FIGURE 4.** Effect of p38-MAPK antisense (AS), sense (SS1), and scrambled (SS2) oligonucleotides on p38-MAPK expression and apoptosis in neutrophils. Neutrophils were incubated with 1 μM of each oligonucleotide in serum-free RPMI 1640 for 6 h before addition of 5% heat-inactivated FCS. After 24 h, the cells were assessed for p38-MAPK expression by immunoblot analysis (A) and apoptosis by morphology on light microscopy (B). A, Immunoblotting with control and phosphospecific Abs to p38-MAPK was done as described in Fig. 5. A representative experiment of two performed is shown. The OD of each band was determined and plotted. Data represent mean ± SEM. B, Data represent mean ± SEM of three experiments. **p, p < 0.01 versus control cells in medium alone.

*Phosphorylation and activation of p38-MAPK are not affected by a Fas agonist or GM-CSF*

We examined whether p38-MAPK activity was affected by Fas ligand, GM-CSF, and intracellular ROS, which are all known to affect the rate of neutrophil apoptosis (4–8, 29). The agonistic Fas
Ab CH-11 (100 ng/ml) shortened neutrophil survival and promoted apoptosis (Fig. 8). In contrast, GM-CSF (100 ng/ml) and the antioxidants catalase (500 U/ml) and N-acetylcysteine (500 μg/ml) extended survival and inhibited apoptosis (Fig. 8). However, none of these reagents or H2 O2 significantly affected p38-MAPK phosphorylation and activation in neutrophils (Fig. 9, A and B). These results indicate that p38-MAPK function in neutrophils is independent of signaling pathways triggered by Fas ligand, GM-CSF, and ROS.

**Discussion**

It is now clear that neutrophil apoptosis is a novel part of homeostasis and the inflammatory response (1). Unlike many other cell types, neutrophils undergo spontaneous apoptosis without any need for apparent external inductive stimuli. However, no signal-transduction pathways have been shown to mediate spontaneous neutrophil apoptosis to date. The present study demonstrates that the p38-MAPK pathway is involved in spontaneous apoptosis in
neutrophils. To our knowledge, this is the first study to demonstrate that a specific kinase is involved in signaling pathways that lead to spontaneous apoptosis in neutrophils.

Recent studies suggest the importance of tyrosine phosphorylation and dephosphorylation events in signaling pathways that result in neutrophil apoptosis. For example, a study showed that increased tyrosine-phosphorylated proteins were detected in GM-CSF-treated neutrophils that showed increased survival, while genistein, a broad tyrosine kinase inhibitor, abrogated the survival-promoting effect of GM-CSF (9). Another study indicated that the tyrosine kinase lyn is specifically involved in GM-CSF-mediated signaling that promotes neutrophil survival (10). These studies indicate that tyrosine phosphorylation of certain proteins is necessary for the inhibition of apoptosis by GM-CSF. On the other hand, the present study indicates that tyrosine phosphorylation events are also important for the induction of apoptosis that occurs spontaneously. Our data suggest that continuous activation of p38-MAPK is involved in spontaneous apoptosis in neutrophils.

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Endotoxin contamination during and after cell preparation is a constant problem that may prime neutrophils and confuse results. Although endotoxin has been shown to activate p38-MAPK in
neutrophils (13, 14), we could not obtain any evidence favoring the possibility that p38-MAPK phosphorylation and activation seen in freshly isolated neutrophils were due to endotoxin contamination or accidental neutrophil activation. However, although a relationship between apoptosis and p38-MAPK is clearly demonstrated, our results may neither exclude contamination by undetectable levels of endotoxin, nor rule out the necessity of a certain degree of neutrophil activation for constitutive activation of p38-MAPK. It should also be emphasized that because cell preparation procedures induce some degree of neutrophil activation (18), extrapolation of in vitro data to in vivo situations requires caution.

Nevertheless, additional evidence for a close relationship between neutrophil apoptosis and p38-MAPK is provided by a recent paper published after our original submission of this manuscript (30). Frasch et al. have reported that SK & F 86002, a specific p38-MAPK inhibitor, suppressed neutrophil apoptosis induced by stress stimuli such as UV, hyperosmolarity, and sphingosine (30). In contrast to us, they observed no inhibition of spontaneous apoptosis by the p38-MAPK inhibitor. However, there are several methodologic differences between their study and our own. First, they used a different p38-MAPK inhibitor (SK & F 86002) than we used (SB203580). Second, they assessed the effect of the p38-MAPK inhibitor on spontaneous apoptosis only at one time point (24 h). Third, they assessed apoptosis by fluorometric measurement of DNA content in propidium iodide-stained cells by flow cytometry. It should be noted that in our experiments, when compared with morphologic (Fig. 1B) and MTT-based assays (Fig. 3A), the fluorometric measurement of DNA content (Fig. 3B) detected a lower degree of inhibition of apoptosis by the same dose (10 μM) of p38-MAPK inhibitor. It is likely that the different methods of assessing apoptosis detect various stages of apoptosis. Fourth, neutrophils isolated in their study may not have exhibited basal phosphorylation and activation of p38-MAPK. However, in accordance with our own, their figures show low but significant levels of basal phosphorylation and activation of p38-MAPK at time 0 and 90 min of incubation.

Recent studies suggest that spontaneous neutrophil apoptosis is elicited by the interaction between the constitutively expressed Fas and Fas ligand molecules in neutrophils (7, 8, 31). In the present study, we found that an agonistic Fas Ab, which promoted neutrophil apoptosis, did not affect the phosphorylation and activation of p38-MAPK. This finding is consistent with a recent report that the incubation of neutrophils with anti-Fas Ab did not affect the tyrosine phosphorylation or tyrosine phosphatase activity of the cells (31) nor the phosphorylation and activation of p38-MAPK (30). In Jurkat T lymphocytes, however, Fas has been shown to activate p38-MAPK (32, 33). Although we cannot explain these differences, our data suggest that p38-MAPK works independent of or in parallel with Fas-Fas ligand systems in neutrophils.

**FIGURE 9.** Effect of agonistic anti-Fas Ab, GM-CSF, and antioxidants, and H<sub>2</sub>O<sub>2</sub> on p38-MAPK phosphorylation and activation. Neutrophils were cultured for 6 h with or without anti-Fas Ab (CH-11; 100 ng/ml; Fas), GM-CSF (50 ng/ml; GM-CSF), catalase (500 U/ml; Cat), N-acetylcysteine (500 μg/ml; NAC), and H<sub>2</sub>O<sub>2</sub> (1, 10, and 100 mM). Cell lysates were analyzed for p38-MAPK phosphorylation by immunoblotting (A, B) and for p38-MAPK activation by immune complex protein kinase assay (C). The results are representative of three experiments. The OD of each band was determined, and the ratio of phosphorylated MAPKAP kinase-2-GST/immunoprecipitated p38-MAPK was plotted. Data represent mean ± SEM. We also found that catalase up to 1600 U/ml and N-acetylcysteine up to 5 mM did not affect p38-MAPK phosphorylation (data not shown).
Our finding that p38-MAPK phosphorylation and activation were not affected by stimulation of neutrophils with the Fas agonist or GM-CSF raises the question of how p38-MAPK in neutrophils is continuously activated without any need for apparent external stimuli. We initially hypothesized that ROS generated intracellularly may contribute to continuous phosphorylation and activation of p38-MAPK. This hypothesis was based on the following evidence. First, previous investigators showed that ERK, another MAPK family member, is activated following treatment of neutrophils with the oxidizing agent diamide and H₂O₂ (34). Second, we have found recently that ROS act as an activator of p38-MAPK in fibroblasts depleted of thiols. Third, like other aerobic cells, neutrophils constitutively generate ROS within cells and undergo apoptosis in response to ROS accumulation (4, 5, 35). In support of this, we observed that peroxides, as detected by CDDCF (6-carboxy-2’7’-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)) fluorescence, are accumulated within neutrophils cultured in quiescent conditions (unpublished data). Fourth, we have found in the present study that the antioxidants catalase and N-acetylcysteine inhibited spontaneous apoptosis and extended survival of neutrophils. Taken together, these lines of evidence confirm that ROS play an important role in spontaneous apoptosis in neutrophils. However, the lack of effect of these antioxidants and exogenous H₂O₂ on P38-MAPK phosphorylation and activation we observed suggests that ROS generation does not lie upstream of p38-MAPK activation.

In other mammalian cell types such as COS-1, COS-7, and HeLa cells, the small GTP-binding proteins Rac and Cdc42 (36, 37) and different MAPK kinases (MKK-3, MKK-4, MKK-6) (38, 39) have been implicated as upstream regulators of p38-MAPK. In neutrophils, however, the upstream regulators of p38-MAPK signaling remain ill defined. In this respect, a recent report suggested that activation of p38-MAPK by LPS in neutrophils uses MKK-3, but not Raf, mitogen-activated protein/ERK-1, or mitogen-activated protein/ERK-2 (14). Other reports showed that activation of p38-MAPK by chemoattractants is mediated through a pathway involving phosphatidylinositol 3-kinase, protein kinase C, and p38-MAPK by chemoattractants is mediated through a pathway involving phosphatidylinositol 3-kinase, protein kinase C, and p38-MAPK (12–16). However, many of these agonists activate ERK as well (15, 26, 42), indicating multiple MAPK activation by inflammatory stimuli. Our findings suggest that the activation of p38-MAPK may serve specifically to limit the longevity of neutrophil survival following activation.

Indeed, it seems clear that the constitutive rate of apoptosis in neutrophils is the major determinant of their survival and functional longevity at inflamed sites (1). Although elevation of intracellular Ca²⁺ (3) or cAMP (43) by agonistic stimuli has been shown to inhibit neutrophil apoptosis, the effect of inflammatory mediators such as FMLP, C5a, IL-1, and IL-8 on neutrophil apoptosis remains controversial (34, 44, 45). Based on our findings, activation of p38-MAPK by inflammatory mediators may activate an apoptotic program to counteract the antiapoptotic effects of inflammatory signals. This may be important to limit the toxic potential of neutrophils. The validity of this hypothesis remains to be determined.

In summary, our data suggest that p38-MAPK plays a role in driving spontaneous apoptosis of neutrophils. Clarification of this role perhaps awaits further investigations of its regulators and targets. Definition of the p38-MAPK-mediated pathway will provide clues to the complex mechanism underlying neutrophil apoptosis.

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