p38 Mitogen-activated Protein Kinase-dependent and -independent Intracellular Signal Transduction Pathways Leading to Apoptosis in Human Neutrophils*

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Human neutrophils undergo apoptosis spontaneously when cultured in vitro; however, the signal transduction pathways involved remain largely unknown. In some cell types, c-Jun NH₂-terminal kinase and p38 mitogen-activated protein kinase (MAPK) have been implicated in the pathways leading to stress-induced apoptosis. In this study, we begin to define two pathways leading to apoptosis in the neutrophil induced either by stress stimuli (UV, hyperosmolarity, sphingosine) or by anti-Fas antibody or overnight culture in vitro (spontaneous apoptosis). Apoptosis induced by stress stimuli activated p38 MAPK, and apoptosis was inhibited by the specific p38 MAPK inhibitor, 6-(4-Fluorophenyl)-2,3-dihydro-5-(4-puridinyl)imidazo[2,1-b]thiazole dihydrochloride. Furthermore, differentiation of HL-60 cells toward the neutrophil phenotype resulted in a loss in c-Jun NH₂-terminal kinase activation with concomitant acquisition of formylmethionylleucylphenylalanine-stimulable and stress-inducible p38 MAPK activity as well as apoptosis blockade by the p38 MAPK inhibitor. In contrast, anti-Fas-induced or spontaneous apoptosis occurred independent of p38 MAPK activation and was not blocked by the inhibitor. Both pathways appear to utilize member(s) of the caspase family, since pretreatment with either Val-Ala-Asp-fluoromethyl ketone or Asp-Glu-Val-Asp-fluoromethyl ketone inhibited apoptosis induced by each of the stimuli. We propose the presence of at least two pathways leading to apoptosis in human neutrophils, a stress-activated pathway that is dependent on p38 MAPK activation and an anti-FAS/spontaneous pathway that is p38 MAPK-independent.

Human neutrophils have a life span of only hours following release from bone marrow into systemic circulation. At sites of inflammation, neutrophils are short lived and, under normal circumstances, undergo apoptosis with subsequent recognition and removal by phagocytic cells such as macrophages (1–3). Cultured in vitro, neutrophils isolated from the blood undergo apoptosis spontaneously, with greater than 50% of the population becoming apoptotic within 16 h (1, 2, 4). It has been assumed that the neutrophil half-life in vitro is a reflection of their life span in vivo and that this cell possesses an “inbuilt” clock leading inexorably to apoptosis within a certain time range after maturation in, or release from, the bone marrow. In inflammatory lesions, the presence of neutrophil stimuli such as granulocyte-macrophage colony-stimulating factor or lipopolysaccharide could extend this life span by inhibiting apoptosis (5–8), while interleukin-10 might shorten it by enhancing the apoptotic process (9). Removal of apoptotic inflammatory cells by macrophages before they lyse and release their toxic contents may represent an important mechanism for limiting tissue injury and for resolution of inflammation (10–13). Thus, induction and control of neutrophil apoptosis appears to be central to resolution or persistence of an inflammatory state.

Recently, a number of gene products have been identified as important regulators involved in the pathways leading to apoptosis. However, the relative involvement of any one gene product may vary depending on the cell type. Although apoptosis appears to be a universal phenomenon observed in virtually all cells, the specific signal transduction pathways mediating the death program can be biochemically and functionally distinct in response to different stimuli as well as vary between cell types.

Neutrophil intracellular signal transduction in response to a wide variety of stimuli appears to utilize the MAPK kinase cascades. Each MAPK kinase cascade involves phosphorylation and activation of a MAP ERK kinase kinase, which in turn activates members of the MAP ERK kinases (MEK). MEKs then activate a specific member of the MAPK family by dual phosphorylation of a threonine and tyrosine residue. This system of parallel intracellular signaling pathways activated in response to a specific external stimulus and leading to a unique set of functional responses is well defined in yeast and is now recognized to exist in most mammalian cells including the human neutrophil. Three distinct MAPKs have been identified to date in mammalian cells: p42/p44 ERKs are activated by growth factors (14); JNK/stress-activated protein kinase is potentially activated by irradiation and other environmental stresses such as hyperosmolarity (15, 16); and p38 MAPK is activated by proinflammatory cytokines, osmotic stress, and UV irradiation (16). Irradiation and other stress stimuli are

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The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; FasL, Fas ligand; ATF-2, activated transcription factor-2; MEK, MAP-ERK kinase; ERK, extracellular signal-regulated kinase; ICE, interleukin-1β-converting enzyme; TNF, tumor necrosis factor; NF-κB, nuclear factor-kappa B; PGE₂, polyarachidonic acid; ATP, ATP; IP3, inositol triphosphate; GTPase, GTPase activating protein; Tryptophan, tryptophan-specific serine/threonine protein kinase; RIPA, radioimmune precipitation; SK & P 86062, 6-(4-Fluorophenyl)-2,3-dihydro-5-(4-puridinyl)imidazo[2,1-b]thiazole dihydrochloride; VAD-fmk, Val-Ala-Asp-fluoromethyl ketone; DEVD-fmk, Asp-Glu-Val-Asp-fluoromethyl ketone.
known to induce apoptosis in a variety of cell types (17, 18). Accordingly, JNK and/or p38 have been implicated by some investigators in the process leading to apoptosis in response to these stimuli (17, 18). In Jurkat cells, JNK and p38 MAPK activation have been coupled to Fas-induced apoptosis, and it appears as though this coupling requires the activation of ICE-like proteases (caspases) (19).

Both Fas and the caspase superfamily have emerged as key players in the regulation of the apoptotic pathway. The Fas/FasL system has been extensively studied in T cells and has been shown to be intimately involved in the removal of autoreactive B cells (20) as well as in activation-induced T cell death (21–23). Recently, it has been reported that spontaneous apoptosis in neutrophils may be in part mediated by Fas/FasL (24, 25). However, the signal transduction pathway leading from Fas receptor cross-linking to apoptosis in the neutrophil is unknown.

The caspase superfamily contain a highly homologous group of proteins that can be subdivided into three categories including ICE-like (caspase 1), CPP32-like (caspase 3), and ICH-1-like (caspase 2) proteases. The involvement of caspases in apoptosis has been demonstrated in a number of ways including the use of tri- and tetrapeptide inhibitors that take advantage of the different substrate specificity of the enzymes or by the cowpox viral protein, CrmA, which is a natural inhibitor of caspase 1 (26, 27). Several lines of evidence have suggested an involvement of caspase 1 and caspase 3 in Fas-mediated apoptosis. For instance, treatment with either YVAD-cmk, an inhibitor of caspase 1, or DEVD-fmk, an inhibitor of caspase 3, prevents apoptosis induced by Fas cross-linking in Jurkat and U937 cells (28). Although there is detailed information regarding the involvement of caspases in apoptosis in other cell types, little is known about the role of these enzymes in neutrophil apoptosis.

In this report, we examine signal transduction pathways leading to apoptosis in human neutrophils induced by in vitro culture (spontaneous apoptosis), Fas cross-linking, or stress stimuli. At least two pathways leading to apoptosis have been identified, both requiring caspase involvement, a stress-activated pathway that is p38 MAPK-dependent and a spontaneous/Fas-mediated pathway that appears to be p38 MAPK-independent.

**EXPERIMENTAL PROCEDURES**

**Materials**—Endotoxin free reagents and plastics were used in all experiments. Neutrophils were resuspended in Krebs-Ringer phosphate buffer, pH 7.2, with 0.2% dextrose. fMLP, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, bovine serum albumin fraction V, propidium iodide, and tissue culture grade MeSO4 were purchased from Sigma. Protein A-Sepharose was purchased from Zymed (South San Francisco, CA). DEVD-fmk and VAD-fmk were purchased from Enzyme Systems (Livermore, CA). Anti-JNK1(C-17) rabbit and goat polyclonal antibody, anti-p38 phosphospecific polyclonal antibody, anti-ERK2(C-16) polyclonal antibody, and anti-ERK2 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-p38 phosphospecific polyclonal, horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody and PD98059 were purchased from Calbiochem. A MAP kinase assay kit and anti-Fas IgM (CH-11) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). 1,25-dihydroxy(2,4-puridinyl)imidazo(2,1-b)benzimidazol-3(2H)-one dihydrochloride (SK F 86002-A2, or SK & F 86002) was kindly provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

**Induction of Apoptosis**—Apoptosis was induced by exposure of neutrophils or HL-60 cells reconstituted to either 5 × 10⁶ cells/ml or 20 × 10⁶ cells/ml in Krebs-Ringer phosphate buffer or RPMI 1640 plus 10% FBS and plated at 2–7 × 10⁵ cells/ml to UV irradiation (254 nm) on a UV transilluminator for 10 min, sphingosine, 450 mosm (0.25 mosmol/L) sucrose in Krebs-Ringer phosphate buffer), anti-Fas IgM, or 18–24 h culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS (spontaneous apoptosis) followed by incubation at 37 °C for the times indicated.

p38 MAPK Immunoprecipitation and Kinase Assay—p38 MAP ki- nase activity measurements were performed essentially as described (29, 30). Either HL-60 cells cultured in RPMI 1640 with 10% FBS or neutrophils isolated by the plasma Percoll method (31) were resuspended to 20 × 10⁶/ml in RPMI 1640 supplemented with 10% FBS, 20 × 10⁶ cells (1 ml) were plated with well of a 12-well tissue culture plate. Cells were stimulated with UV irradiation, 1,25-dihydroxy(2,4-puridinyl)imidazo(2,1-b)benzimidazol-3(2H)-one dihydrochloride (SK F 86002-A2, or SK & F 86002) was kindly provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

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FIG. 1. Induction of apoptosis in human neutrophils. Neutrophils were stimulated with UV irradiation, overnight culture in vitro, anti-Fas antibody, hyperosmolarity, or sphingosine. Apoptosis was quantitated by propidium iodide staining and flow cytometry (a–d) or morphologically by stained cytospin preparations (e, f). Panels a–d, representative histograms from four different determinations, demonstrating DNA fragmentation by the appearance of a sub-G₀ peak in cells treated with no stimulus (a), overnight culture in vitro (b), anti-Fas antibody (c), or UV irradiation (d). Panel e, apoptosis in cells cultured for 4 h at 37 °C with various stimuli. Panel f, representative photograph of a stained cytospin preparation demonstrating nuclear condensation and membrane blebbing in neutrophils treated with UV irradiation and incubated for 4 h at 37 °C. Open arrow, normal neutrophil; solid thick arrow, apoptotic neutrophil; solid thin arrow, membrane blebbing.

RESULTS

Selective Activation of p38 MAPK with Stimuli That Induce Apoptosis in Human Neutrophils—While the susceptibility of neutrophils to spontaneous apoptosis (1, 2, 4) or to Fas cross-linking (24) is well described, the sensitivity of neutrophils to other apoptosis-inducing stimuli, such as UV irradiation or hyperosmolarity, is less well understood. Propidium iodide staining and flow cytometric analysis or stained cytospin preparations were used to determine DNA fragmentation and nuclear condensation (respectively) characteristic of apoptosis in human neutrophils in response to a variety of stimuli (Fig. 1). Neutrophils exposed to overnight culture in vitro (spontaneous apoptosis (Fig. 1b)), anti-Fas (Fig. 1c), and UV irradiation (Fig. 1d) all show reduced PI staining, evident by the appearance of a sub-G₀ peak, characteristic of apoptosis. Likewise, these stimuli as well as treatment with sphingosine and culture in hyperosmolar conditions (Fig. 1e) showed an increase in nuclear condensation over time to levels comparable with the amount of DNA fragmentation analyzed by PI staining and flow cytometry. Morphological features of apoptosis, including nuclear condensation and membrane blebbing, were seen in neutrophils treated with UV irradiation followed by incubation for 4 h at 37 °C (Fig. 1f). Apoptosis was also confirmed by demonstrating DNA fragmentation with typical internucleosomal cleavage and laddering on agarose gels (data not shown). These data demonstrate that in addition to spontaneous and anti-Fas-induced apoptosis, neutrophils undergo apoptosis with stress stimuli in a rapid and relatively synchronous manner.

Studies in other cell types demonstrate that multiple pathways may lead to apoptosis, and some of these pathways are beginning to be elucidated. In particular, some studies have suggested that JNK and p38 MAPK, members of the MAP kinase superfamily, participate in the process leading to apoptosis induced by stress stimuli (i.e. UV irradiation, hyperosmolality) or by Fas ligation (19, 35). Therefore, the activation of JNK, p38 MAPK, and p42/p44 ERKs by stimuli that also induce apoptosis was investigated. JNK, p38, and p42/p44 ERK MAP kinase activities were measured in neutrophils exposed to UV irradiation, hyperosmolarity, sphingosine, or anti-Fas antibody (Fig. 2). Unlike other cell types (17, 36–38), p38 MAPK but not JNK was activated by stress stimuli within 30 min following stimulation. In contrast, Fas/receptor ligation failed to activate either p38 MAPK or JNK. Neither the stress stimuli
nor anti-Fas activated the p42/p44 ERKs. These results suggest that p38 MAPK, and not JNK or p42/p44 ERK, might be involved in the pathway leading to stress-induced apoptosis in human neutrophils.

Time courses of p38 MAPK, JNK, and ERK activation using antibodies specific to their activated forms, revealed that p38 MAPK activation with UV began as early as 10 min and remained persistent 1.5–2 h following stimulation (Fig. 3a), unlike p38 MAPK activation with fMLP where peak activity was observed at 2 min and was down to background levels by 10 min (30). On the other hand, p38 MAPK activation with anti-Fas antibody was not evident at any time during the time course, consistent with data presented in Fig. 2. Also consistent with data presented in Fig. 2 was the lack of JNK and ERK activation following UV irradiation or treatment with anti-Fas (Fig. 3, b and c). Western blots of both JNK and ERK show that both proteins were present in the cell but were not activated with stimuli that induced apoptosis. These results support further the notion that p38 MAPK, but not JNK or ERK, are involved in stress-induced apoptosis. A time course of p38 MAPK with sphingosine and hyperosmolarity showed activation kinetics similar to that of UV (data not shown), suggesting that this response is similar for all stress stimuli. Likewise, time courses of JNK and ERK activation with sphingosine and hyperosmolarity were similar to that with UV irradiation (data not shown).

p38 MAPK Involvement—To determine more directly whether p38 MAPK activation was necessary for apoptosis to occur, a specific p38 MAP kinase inhibitor, SK & F 86002, was used (30, 39, 40). Neutrophils pretreated with the inhibitor were allowed to undergo apoptosis either spontaneously in overnight culture or by stimulation with UV irradiation, hyperosmolarity, sphingosine, or anti-Fas, and the degree of apoptosis was determined (Table I). Consistent with the activation of p38 MAPK by the different stimuli, treatment with SK & F 86002 (10 μM) protected the cells from UV irradiation, hyperosmolarity, and sphingosine-induced apoptosis but had no effect on anti-Fas or spontaneous apoptosis, suggesting that p38 MAPK activation is required for signal transduction leading to stress-induced apoptosis in human neutrophils. A dose-response of the SK & F 86002 on p38 activity shows that concentrations of 10 μM and lower were efficient at completely inhibiting p38 activity (data not shown and Ref. 30). Pretreatment of neutrophils with PD98059, an inhibitor of MEK1 kinase that is responsible for the phosphorylation and activation of p42/p44 ERKs (41) had no effect on UV-induced apoptosis, thus supporting the lack of p42/p44 ERK involvement (Fig. 4).

Switch in MAP Kinase Usage during Differentiation—In contrast to neutrophils, the HL-60 cell line, a cell that can be terminally differentiated to the neutrophil phenotype by treatment with Me2SO, demonstrates activation of both JNK and p38 MAPK in response to UV irradiation (Fig. 6). JNK has been implicated in ceramide-induced apoptosis in undifferentiated HL-60 cells (42). As both JNK and p38 MAPK were activated by UV irradiation in the undifferentiated HL-60 cell, SK & F

**FIG. 2.** Specific activation of p38 MAPK by stress stimuli in neutrophils. Neutrophils were treated with UV irradiation, hyperosmolarity, sphingosine, and anti-Fas antibody. p38 MAPK, JNK1, and ERK2 kinase activities were measured in immune complex protein kinase assays using [γ-32P]ATP and ATF-2, c-Jun, and myelin basic protein as substrates for p38 MAPK, JNK1, and ERK2, respectively. The phosphorylated substrates (ATF-2 and c-Jun) were detected after SDS-PAGE by autoradiography (representative of three experiments) or by scintillation counting after binding to P81 phosphopaper (myelin basic protein) (means ± S.D.; n = 3). Western blots (representative of three experiments) comparing the amount of p38 MAPK, JNK1, or ERK2 are shown. Each stimulus demonstrates equivalent amounts of protein present in each sample.

**FIG. 3.** Time course of p38 MAPK, JNK, and ERK2 activation. Neutrophils were untreated or treated with UV irradiation or anti-Fas antibody and harvested at the times indicated. p38 MAPK, JNK1, or ERK2 was immunoprecipitated with specific antibodies. Equal amounts of protein were separated on 10% SDS-PAGE, transferred to nitrocellulose, and probed for total p38 MAPK, JNK, and ERK2 as well as for their activated forms by a Western blot (representative of three experiments).
86002 was used to determine whether p38 MAPK or JNK was the dominant kinase involved in the pathway leading to apoptosis. HL-60 cells pretreated with and without SK & F 86002 prior to UV irradiation were incubated at 37 °C over a range of times. The p38 MAPK inhibitor failed to prevent UV-induced apoptosis in undifferentiated HL-60 cells (Fig. 5a). It has been suggested by Whitmarsh et al. (43) that some p38 MAPK inhibitors can inhibit JNK activation. A dose-response analysis of JNK activation with increasing concentrations of SK & F 86002 demonstrates that at 10 μM, the concentrations used in this study, there was no inhibition of JNK activity (Fig. 5b). These results suggest that, in HL-60 cells, UV-induced apoptosis does not require p38 MAPK and are consistent with the possibility that signaling occurs through the JNK MAP kinase cascade in this cell.

Since HL-60 cells can be differentiated to the neutrophil phenotype, direct determination of the pattern of MAP kinase usage during maturation was studied. HL-60 cells were induced to differentiate with Me2SO and harvested at day 3. The percentage of differentiated cells was determined by the ability of cells to reduce nitro blue tetrazolium, and p38 MAPK and JNK kinase activities were measured. We have shown previously that p38 MAPK is activated by fMLP in neutrophils (30); therefore, fMLP stimulation of p38 MAPK was used as an additional marker of the neutrophil phenotype in differentiated HL-60 cells. As HL-60 cells became more neutrophil-like, there was a decrease of JNK activation and a simultaneous acquisition of fMLP-inducible p38 MAPK activity (Fig. 6a). Western blot analysis of p38 MAPK and JNK show that the level of protein is the same in differentiated HL-60 cells as undifferentiated HL-60 cells, suggesting the decrease in JNK activation was not due to decreased JNK expression. At day 3 following the addition of Me2SO, 70% of HL-60 cells were differentiated (as determined by nitro blue tetrazolium reducing activity (33)). Since HL-60 cells are capable of robust JNK activation in response to UV irradiation, the residual JNK activity observed is probably due to the remaining undifferentiated HL-60 cells. Furthermore, the differentiated HL-60 cells, unlike the undifferentiated HL-60 cells, were protected from UV-induced apoptosis by 10 μM SK & F 86002 (Fig. 6b). These results suggest that during maturation of HL-60 cells toward the neutrophil phenotype, there is a decrease in JNK activation, which may lead to a more physiologically prominent role for p38 MAPK in the signaling pathway leading to stress-induced apoptosis in neutrophils.

Effects of fMLP on UV-induced Apoptosis—Although p38 MAPK is activated under conditions that induce apoptosis, it is also activated by the chemotaxtract fMLP and utilized in pathways leading to cell activation for superoxide anion release, actin assembly, adherence, calcium influx, and chemotaxis (44, 45). Therefore, we tested the effects of fMLP directly and also on UV-induced apoptosis. Neutrophils were pre-treated with fMLP for 10 min prior to UV irradiation and allowed to incubate at 37 °C for 4 h. fMLP treatment did not directly induce apoptosis and, in fact, inhibited DNA fragmentation induced by UV irradiation although p38 MAPK was activated under these conditions (Fig. 7, a and b). Furthermore, fMLP did not have an effect under conditions where p38 MAPK was not activated, such as anti-Fas-induced or spontaneous apoptosis (Fig. 7, c and d).

p42/p44 ERKs are also activated by fMLP in neutrophils (30,
cytospin preparations in undifferentiated cells (M. Iments).

HL-60 cells. Phosphorylated ATF-2 and c-Jun were detected after SDS-UVT
neutrophils, undifferentiated HL-60 cells, and differentiated (C), or UV-stimulated C

described in unstimulated ( ), differentiated HL-60 cells, and differentiated HL-60 cells. Phosphorylated ATF-2 and c-Jun were detected after SDS-PAGE by autoradiography (representative of three independent experiments).

Panel a, apoptosis was quantitated morphologically by stained

HL-60 cells decreased JNK activation and acquired p38 activity while having no effect on the activation of p38 MAPK (Fig. 8b).

Since PD98059 can reduce ERK2 activity without affecting the activation of p38 MAPK, its effect on fMLP inhibition of stress-induced apoptosis was examined. Pretreatment of neutrophils with PD98059 prior to fMLP stimulation and UV irradiation resulted in reversal of the protective effects of fMLP on UV-induced apoptosis (Fig. 8c), supporting the hypothesis that fMLP protection may be due, in part, to the activation of p42/p44 ERKs and that activation of p38 MAPK in the absence of p42/p44 ERK activity results in apoptosis.

These results suggest that although p38 MAPK is activated under conditions that lead to cell activation (in the case of fMLP stimulation) and apoptosis (in the case of UV irradiation), the context by which p38 MAPK is activated determines the fate of the cell.

Involvement of CED3/ICE-like Proteases in Apoptosis—As mentioned above, p38 MAPK seemed to be involved in stress-induced apoptosis and did not appear to participate in anti-Fas-induced or spontaneous apoptosis, suggesting an alternative pathway leading to DNA fragmentation. Another class of enzymes implicated in apoptosis are Ced-3/ICE-like proteases, or caspasases. We sought to determine whether member(s) of the caspase family were involved in stress-induced, anti-Fas-induced, or spontaneous apoptosis. In these studies, two inhibitors were used; VAD-fmk is an irreversible caspase 1-like protease inhibitor, and DEVD-fmk inhibits the caspase 3-like proteases (50, 51). Both inhibited apoptosis induced by any of the stimuli (Fig. 9).

Since stress-induced apoptosis involves both p38 MAPK and at least one member of the caspase family, the possible regulatory relationship between p38 MAPK and either caspase 1-like or caspase 3-like proteases during UV-induced apoptosis was determined. Neutrophils were pretreated with VAD-fmk or DEVD-fmk for 1 h prior to UV irradiation. Following UV irradiation, p38 MAPK activity was measured as before. Treatment of neutrophils with VAD-fmk or DEVD-fmk had no effect on either fMLP or UV-induced p38 MAPK activity, suggesting that p38 MAPK activation is independent of caspase activity (data not shown).

**DISCUSSION**

The requirement for JNK and/or p38 MAPK in stress-induced or Fas-mediated apoptosis has been a matter of controversy. In some cell types, JNK activation has been uncoupled from apoptosis, although it is activated with stimuli that also induce apoptosis. For instance, in MCF7 cells, JNK activation has been dissociated from TNF receptor-mediated apoptosis (52). In contrast, in PC-12 cells JNK activation is required for apoptosis induced by nerve growth factor withdrawal (17). Likewise, there appears to be a requirement for JNK activation in ceramide-mediated apoptosis in U937 cells, suggesting that there is coordinated regulation of the apoptotic pathway by both the sphingomyelinase and the JNK pathways (18). UV irradiation has recently been reported to accelerate apoptosis in human neutrophils, although the mechanism by which apoptosis was mediated remains unclear (34). We have shown that in neutrophils, there is selective activation of the p38 MAPK, and not JNK, in response to stress stimuli that induce apoptosis providing a possible mechanism of stress-induced apoptosis in these cells. Several lines of evidence support this conclusion. In addition to activation of p38 MAPK, but not JNK or p42/p44 ERKs, with stress stimuli (Fig. 2), we have shown that inhibition of p38 MAPK with SK & F 86002 protects from apoptosis induced by UV irradiation, hyperosmolarity, and sphingosine (Table 1).

Furthermore, as HL-60 cells differentiated toward the neutrophil phenotype, there was a loss in JNK activation in response to UV irradiation, with concomitant acquisition of fMLP-activable p38 MAPK activity. The notion that p38 MAPK is involved in neutrophil apoptosis is supported further by the observation that differentiated HL-60 cells were protected from UV-induced apoptosis by the p38 inhibitor, whereas undifferentiated HL-60 cells were unaffected by that treatment. Of interest, no stimulus has been identified that leads to activation of JNK in neutrophils using the current assay conditions, although the JNK protein is present in the cell (Fig. 2). These results strongly suggest that the requirement for either JNK or p38 MAPK in stress-induced apoptosis varies depending on the cell type as well as on the context in which the kinase is activated. For instance, in addition to stimuli that induce apoptosis, such as γ-irradiation (37, 38, 53), UV irradiation (15), and anti-Fas antibody (19, 35), JNK activation has been
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**Fig. 7.** fMLP inhibited UV-induced apoptosis. Panel a, neutrophils were treated in the absence (solid line) or presence (dashed line) of 100 nM fMLP for 10 min prior to UV irradiation. Apoptosis was detected by propidium iodide staining and flow cytometry (representative histogram of three experiments). Panel b, p38 MAPK activity in neutrophils treated with fMLP or UV irradiation was determined as described. Phosphorylated ATF-2 was detected after SDS-PAGE by autoradiography (representative of three experiments). Panels c and d, neutrophils were treated in the absence (solid line) or presence (dashed line) of 100 nM fMLP for 10 min prior to stimulation with anti-Fas antibody (c) or overnight culture (d). Apoptosis was detected by propidium iodide staining and flow cytometry (representative histograms of three experiments).

Associated with activation stimuli, including T cell activation signals (37, 38, 54), CD40 ligation (55, 56), and growth factors (57). Chen et al. (37, 38, 57) have shown that although JNK is activated by these various stimuli, resulting in different biological responses (i.e. proliferation versus apoptosis), the kinetics of activation determines the fate of the cell, such that a rapid and transient activation is associated with proliferation, while persistent and sustained JNK activation results in apoptosis. A similar situation is observed in the neutrophil where p38 MAPK, although it appears to be involved in the pathway leading to stress-induced apoptosis, is also activated under conditions that promote cell survival, such as treatment with chemoattractants such as fMLP. The kinetics of p38 MAPK activation induced by fMLP is rapid and transient (30), where peak activity is observed within 2 min following fMLP stimulation and has returned to background levels by 10 min. In contrast, in response to UV irradiation and other stress stimuli, activation is delayed and sustained, reaching maximal activity after 30 min of stimulation and maintaining activity for 1–2 h (Fig. 3). It is possible that the differences in activation kinetics of p38 MAPK by fMLP and UV irradiation in the neutrophil could contribute to the distinct biological responses elicited by each stimulus. Even when the potentially inhibitory ERK activation was blocked by treatment of neutrophils with PD98059 prior to fMLP stimulation, the transient p38 MAPK activation was apparently not enough to induce apoptosis (data not shown), supporting the hypothesis that the duration of p38 MAPK activation contributes to whether the cell will survive or undergo apoptosis.

There appear to be two distinct outcomes associated with p38 MAPK activation in the neutrophil: cell activation and apoptosis. This suggests that the microenvironment in which p38 MAPK is activated determines the ultimate biological response to be mediated by p38 MAPK. We and others (30, 46) have shown that in neutrophils, fMLP induces robust p42/p44 ERK activity. p42/p44 ERKs are activated by growth factors and are hypothesized to be involved in cell survival (17, 41, 48). The relative balance between JNK and p42/p44 ERK activity in PC-12 cells has been reported to determine whether the cell will survive or undergo apoptosis (17). Similarly, protection from TNF-α-induced apoptosis by fibroblast growth factor-2 requires the activation of p42/p44 ERK (48). Based on the results presented in this study, we suggest a similar situation where, although p38 MAPK activation is involved in the signal transduction pathway leading to stress-induced apoptosis, the “death” signal mediated by p38 MAPK can be overridden by the survival signal generated by activation of p42/p44 ERK. However, since fMLP stimulation evokes a variety of cellular responses, it is possible that there may be additional, as of yet unidentified mechanisms of cell survival. For instance, activation of NF-κB is critical in the regulation of cytokine-induced gene expression and has been associated with protection of TNF-induced apoptosis in 3T3 and Jurkat cells (58, 59) as well as in radiation-induced apoptosis in transfected HT1080 cells (60). In the neutrophil, NF-κB activation occurs in response to TNF as well as fMLP (61). It is possible, therefore, that protection of UV-induced apoptosis by fMLP occurs at the level of NF-κB activation. The observation that inhibition of UV-induced apoptosis by fMLP can be reversed by the MEK1 kinase inhibitor PD98059 strongly suggests that fMLP protection requires p42/p44 ERK activation. Preliminary evidence (data not shown) suggests that NF-κB activation by fMLP was not reduced in the presence of the MEK1 kinase inhibitor, PD98059, suggesting that NF-κB was not involved in the protection of UV-induced apoptosis by fMLP.

In addition to stress-induced apoptosis, neutrophils undergo apoptosis spontaneously when cultured in vitro as well as by Fas receptor cross-linking; however, it appears as though apoptosis by these pathways is independent of p38 MAPK, since treatment of neutrophils with anti-Fas antibody induces apoptosis in the absence of p38 MAPK activation. Similarly, the p38 MAPK inhibitor had no effect on Fas-mediated or spontaneous apoptosis, suggesting further an alternative signal transduction pathway leading to apoptosis. However, one cannot rule out the possibility of different isoforms of p38 MAPK involved in these pathways, the activities of which may not be detected by the available methods. Since Fas-induced and spontaneous apoptosis appear to follow the same pathway, it is possible that
spontaneous apoptosis involves the Fas/FasL system. It has been shown previously (24) and confirmed by us (data not shown) that apoptosis induced by overnight culture in vitro could be partially inhibited (50%) by an antagonistic anti-Fas IgG antibody. This raises the question of whether neutrophils have an inbuilt clock that determines when the cell will die, or whether the life span is in part dependent on whether the neutrophil encounters FasL as it is filtered through the liver and spleen. It is also possible that the in vivo life span is a combination of both Fas/FasL expression on the neutrophil as well as the presence of an inbuilt clock such that the neutrophil ages, Fas and/or FasL expression is up-regulated, therefore increasing the likelihood of Fas/FasL ligation either by another neutrophil or another cell expressing FasL, the interaction of which would subsequently induce apoptosis. Both Fas and FasL have been detected on human neutrophils (24); however, whether Fas and/or FasL expression increases as neutrophils are cultured in vitro remains to be determined.

The Ced-3/ICE protease superfamily has been shown to be required for multiple pathways leading to apoptosis; however, it appears as though different apoptosis-inducing stimuli may utilize different members of the protease family, and the relevant protease(s) for these different apoptotic pathways have not been defined. In an attempt to distinguish stress-induced from Fas-mediated apoptosis pathways, we used tetrapeptide inhibitors of the Ced-3/ICE protease superfamily. We observed that treatment with either VAD-fmk or DEVD-fmk virtually blocked apoptosis induced by any stimuli used, whether it was stress-stimuli, anti-Fas, or spontaneous. Furthermore, we have shown that p38 MAPK activation was independent of caspase activity, suggesting that either p38 MAPK and caspase activation are distinct from each other or that p38 MAPK activation in upstream of caspase activation. The implication of these results is that although both p38 MAPK and member(s) of the caspase family appear to be involved in the pathway leading to stress-induced apoptosis, it is possible that their activities may follow parallel pathways in response to the same stimuli. In addition, these results suggest further that the anti-Fas-induced and spontaneous apoptosis pathways, both of which are independent of p38 MAPK activation, may converge with the stress-induced pathway at the level of the caspase(s).

REFERENCES

1. Newman, S. L., Henson, J. E., and Henson, P. M. (1982) J. Exp. Med. 156, 430–442
2. Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., and Haslett, C. (1989) J. Clin. Invest. 83, 865–875
3. Savill, J. S., Henson, P. M., and Haslett, C. (1989) J. Clin. Invest. 84, 1518–1527
4. Steinhäubl, K. H., Schick, P., and Trepel, F. (1979) Blut 39, 27–38
5. Lee, A., White, M. K. B., and Haslett, C. (1993) J. Leukocyte Biol. 54, 283–288
6. Coletta, F., Re, P., Polesaratturi, N., Sozzani, S., and Mantovani, A. (1992) J. Leukocyte Biol. 54, 283–288
7. Cox, G., Gauldie, J., and Jordana, M. (1992) J. Respir. Cell Mol. Biol. 7, 507–513
8. Brach, M. A., deVos, S., Gruss, H-J., and Herrmann, F. (1992) Blood 80, 2012–2020
9. Cox, G. (1996) Am. J. Physiol. 271, L566–L571
10. Henson, P. M., and Johnston, B. B. (1987) J. Clin. Invest. 86, 669–674
11. Cox, G., Crossley, J., and Zing, Z. (1995) Am. J. Respir. Cell Mol. Biol. 12, 232–237
12. Haslett, C., Savill, J. S., Whyte, B. K. B., Stern, M., Dransfield, I., and
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