Extracellular Signal-Related Kinase 1/2 and p38 Mitogen-Activated Protein Kinase Pathways Serve Opposite Roles in Neutrophil Cytotoxicity

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Background: Inflammatory stimuli rapidly activate mitogen-activated protein kinases (MAPKs) in neutrophils (PMNs). However, their role in cytotoxic function remains unknown. Elucidating the signals involved in release of cytotoxic agents from PMNs may provide new avenues for therapy in diseases of diminished or excessive PMN function.

Hypothesis: The p38 MAPK and extracellular signal-related kinase 1/2 (ERK1/2) modulate superoxide generation and elastase release in activated human PMNs.

Study Design: Isolated human PMNs were incubated with specific inhibitors of MAPK pathways, or vehicle control solution, before activation with the bacterial peptide f-Met-Leu-Phe.

Main Outcome Measures: The rate of superoxide release from activated PMNs was measured by the superoxide dismutase–inhibitable reduction of cytochrome-c. Elastase release from PMNs was determined by cleavage of the substrate Ala-Ala-Pro-Val-pNA.

Results: Superoxide release from activated PMNs was inhibited by blockade of p38 MAPK activation but unaffected by blockade of ERK1/2. Conversely, elastase release was unaffected by p38 MAPK inhibition and increased by ERK1/2 inhibition.

Conclusions: Activation of p38 MAPK promotes superoxide release from PMNs activated by f-Met-Leu-Phe. The ERK1/2 pathway may serve as a negative feedback mechanism for granule exocytosis.

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THE NEUTROPHIL (PMN) is a critical effector of the response to invading microorganisms, as evidenced by disorders of diminished PMN microbicidal function such as chronic granulomatous disease.1 On the other hand, excessive PMN activity has also been implicated in disease, including the hyper-inflammatory states of adult respiratory distress syndrome and multiple-organ failure.2 Appropriate regulation of PMN cytotoxic mechanisms is thus crucial to the wellbeing of the host and may represent an emerging area for therapy in the critical care setting. Developing such strategies to modify inflammatory responsiveness, however, will hinge on our understanding of the cellular signaling that determines PMN cytotoxic function.

Mitogen-activated protein kinases (MAPKs) encompass a group of enzymes recruited in response to cellular stress, including heat stress, osmotic stress, cytokines, and UV irradiation.3 The accepted role of MAPKs is to produce cellular responses to those stresses by integrating multiple stimuli and activating transcription factors. In most cell lines, MAPKs have been implicated in regulating mitogenic responses and the synthesis of stress response proteins.4 To date, the following 3 major MAPK families have been characterized: p38 MAPK, extracellular signal-related kinase 1/2 (ERK1/2 or p42/44 MAPK), and stress-activated protein kinase (SAPK/JNK). Although they are often thought of and presented as parallel pathways, there is evidence of crosstalk between these families, and the net result of their activation is likely to be an integration of multiple effector mechanisms.

The PMN is a terminally differentiated cell with a limited cell cycle and synthetic repertoire. Nonetheless, recent work has demonstrated that 2 of the MAPK families (p38 MAPK and ERK1/2) are present in PMNs, and are activated within seconds by a variety of stimuli, including bacterial peptides, cytokines, lipopolysaccharides, and chemoattractants.5,6 However, the potential mechanistic role of this MAPK activation with respect to PMN cytotoxic function remains to be elucidated.
MATERIALS AND METHODS

MATERIALS

Reagents were obtained from Sigma-Aldrich Corporation, St Louis, Mo, unless otherwise specified. All solutions for cell separation were prepared in a sterile fashion using endotoxin-free water and filtered through 0.22-µm filters. The p38 MAPK inhibitor SB 203580 and MEK1/2 inhibitor PD 98059 were obtained from Calbiochem, La Jolla, Calif. Inhibitors were dissolved in dimethyl sulfoxide and diluted 1:100 in cell suspensions to produce final concentrations of 0.1 to 100 µmol/L.

ISOLATION OF PMNs

Blood drawn from healthy human volunteers was anticoagulated with 10 U/mL of heparin and mixed with an equal volume of 3% dextran in 0.9% isotonic sodium chloride solution. The whole blood-dextran admixture sedimented for 25 minutes, and the leukocyte-rich upper layer was removed and centrifuged for 10 minutes at 200 × g. The resultant pellet was resuspended in 8 mL of room-temperature phosphate-buffered isotonic sodium chloride solution and layered over 5 mL of Ficoll (Pharmacia Biotech, Upsalla, Sweden). The layered solutions were centrifuged at 400g for 30 minutes. All subsequent isolation steps were performed at 4°C. Residual erythrocytes were removed by hypotonic lysis, and the remaining cells were resuspended in Kreb-Ringer phosphodextrose (pH 7.35) at a final concentration of 2.5 × 10^6 cells/mL. Neutrophils prepared in this manner were more than 98% pure on results of Wright stain and more than 98% viable on results of trypan blue exclusion.

STIMULATION OF ISOLATED PMNs

Cells were warmed to 37°C for 5 minutes before any treatment. Cells were preincubated with the MEK1/2 inhibitor PD 98059 (10^−4 to 10^−6 mol/L), the p38 MAPK inhibitor SB 203580 (10^−7 to 10^−2 mol/L), or vehicle (dimethyl sulfoxide). The cells were warmed to 37°C for 5 minutes before any treatment. Cells were then activated with the bacterial formyl peptide f-Met-Leu-Phe (fMLP, 1 µmol/L) and assessed for activation of p38 MAPK and ERK1/2.

IMMUNOBLOTTING FOR ACTIVATED p38 MAPK AND ERK1/2

Aliquots of 1 million PMNs each were activated with fMLP (1 µmol/L) and assessed for activation of p38 MAPK and ERK1/2 using immunoblotting with antibodies specific to the active forms (dual phosphorylated) of these enzymes (New England Biolabs, Beverly, Mass). Briefly, activated cells were lysed at varying time points in sample buffer (Owl Scientific, Woburn, Calif) containing phenylmethylsulfonyl fluoride, leupeptin, sodium orthovanadate, and nitrophenylphosphate. Cell lysates were boiled for 5 minutes, and the proteins were separated on a polyacrylamide gel of 4% to 20% gradient under reducing conditions. Proteins were then transferred to a polyvinylidene difluoride membrane and detected using antibody labeling and chemiluminescence with a horseradish peroxidase-conjugated secondary antibody. In selected experiments, PMNs were preincubated with PD 98059, which selectively inhibits the phosphorylation of ERK1/2. Because SB 203580 does not block the phosphorylation of p38 MAPK (rather, it blocks enzymatic activity by binding the adenosine triphosphate site on the protein), this compound was not used in the immunoblot preparations.

MEASUREMENT OF ELASTASE RELEASE

Elastase release was measured after 10 minutes of activation by incubation of supernatants from PMNs with the substrate methoxyxuccinyl-Ala-Ala-Pro-Val-pNA (AAPV-pNA). Cells were pelleted using centrifugation, and the supernatant was incubated for 1 hour at 37°C in a buffer of combined HEPES and sodium chloride (pH 7.5) containing AAPV-pNA (final concentration, 0.33 mmol/L). Cleavage of the substrate with release of the colored product pNA was measured using optical density at 405 nm in duplicate wells, minus control wells containing the elastase inhibitor methoxyxuccinyl-AAPV-chloromethyl ketone. Data are expressed as a percentage of total cell elastase (cells lysed with 0.1% Triton X-100).

MEASUREMENT OF SUPEROXIDE RELEASE

The rate of O_2^- release was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome-c in 96-well microtiter plates. Cells were suspended at 2.5 × 10^6 cells/mL in a final volume of 150 µL of Kreb-Ringer phosphodextrose containing 75-µmol/L cytochrome-c. Duplicate-reaction wells were used and compared with a single control well containing 3 µg/mL superoxide dismutase. Immediately after the addition of fMLP, the plates were read at 550 – 450 nm every 20 seconds for 5 minutes. The maximal rate of production of O_2^- (V_max) was calculated using 5 points on the curve (100-second interval). Results are expressed as nanomoles per minute per 3.75 × 10^5 cells.

STATISTICS

All data are expressed as mean ± SEM and underwent analysis of variance with Dunnett correction for planned multiple comparisons.
Recognition of MAPK involvement in PMN cytotoxic function would represent a relatively novel role for MAPKs in rapid cellular responses. Furthermore, elucidation of these PMN-signaling pathways may provide new avenues for therapeutic manipulation of cytotoxic function in syndromes of excess PMN function (eg, multiple-organ failure) or syndromes of blunted PMN function (eg, postburn susceptibility to sepsis). We sought to characterize the role of the p38 MAPK and ERK1/2 pathways in the cytotoxic function of the PMN.

Two key PMN responses responsible for microbicidal and cytotoxic activity are the release of superoxide anion ($\text{O}_2^-$) and the release of granule contents (eg, the proteolytic enzyme elastase). We interrogated the role of MAPK pathways in these responses by incubating PMNs with specific inhibitors of these pathways before activating the cells. The results of our study indicate that these signaling cascades serve opposite roles in cytotoxicity: p38 MAPK activity promotes superoxide release, whereas ERK1/2 activity inhibits the release of granule contents.

**RESULTS**

**ACTIVATION OF p38 MAPK AND ERK1/2 BY fMLP**

Activated forms of these enzymes were not detected in unstimulated PMNs. Figure 2 shows that, after stimulation with 1-µmol/L fMLP, both MAPKs rapidly phosphorylated, peaking at 60 to 90 seconds. Consistent with its reported activity, PD 98059 inhibited the activation of ERK1/2 in a dose-dependent fashion, with nearly complete inhibition at 10 µmol/L (Figure 3).

**$\text{O}_2^-$ RELEASE**

Incubation of PMNs with the p38 MAPK inhibitor SB 203580 produced a dose-dependent decrease in the rate of superoxide production in response to fMLP (Figure 4). Control cells (preincubated with dimethyl sulfoxide) produced superoxide at a $V_{\text{max}}$ of $0.35 \pm 0.05$ nmol/min per $3.75 \times 10^5$ cells, whereas cells treated with 1 and 10 µmol/L SB 203580 produced $0.15 \pm 0.04$ and $0.21 \pm 0.06$ nmol/min per $3.75 \times 10^5$ cells, respectively. Although there was a trend toward less inhibition with the 10-µmol/L dose, this did not reach statistical significance. The MEK1/2 inhibitor PD 98059 had no effect on superoxide produced in response to fMLP when compared with vehicle control, even at concentrations well above the median effective concentration ($EC_{50}$) for the compound (about 10 µmol/L) (data not shown).

**ELASTASE RELEASE**

Although inhibition of p38 MAPK decreased oxidant production by fMLP-stimulated PMNs, there was no effect on release of granule contents (data not shown), even at doses well above the $EC_{50}$ of the SB 203580 compound (about 1 µmol/L). In contrast, inhibition of MEK1/2 had an effect on degranulation, as measured by release of elastase. The PD 98059 produced a dose-dependent increase in release of elastase (Figure 5). Control cells released $11.39\% \pm 1.36\%$ of their elastase after fMLP stimulation, which was increased to as much as $24.31\% \pm 3.21\%$ with MEK1/2 inhibition.
Our study supports the hypothesis that MAPK activation is important in the regulation of PMN cytotoxic function. Furthermore, it underscores the concept that parallel MAPK pathways are not functionally redundant but, rather, act to integrate responses to stimuli. In the PMN, our results suggest that, although p38 MAPK promotes cytotoxic function, simultaneous activation of the ERK1/2 pathway may serve as a negative feedback mechanism for granule release.

Previous investigators have characterized a role for the activation of MAPK pathways in response to stress using multiple cell types. In general, MAPK activation is thought to be integral to mitogenic responses and mediated primarily through transcription factors and the modification of protein synthesis. However, the rapid (within seconds) activation of 2 such MAPK pathways in the PMN, a terminally differentiated cell, suggested that MAPKs can serve other roles. The stimuli shown to activate the p38 MAPK and ERK1/2 pathways in the PMN are the same stimuli that provoke its cytotoxic function. For instance, p38 MAPK activation has been described in the PMN in response to bacterial products (eg, fMLP, lipopolysaccharide) and cytokines (tumor necrosis factor and granulocyte colony-stimulating factor).5-7,14-17 The fMLP results in the rapid activation of p38 MAPK and ERK1/2 in human PMNs.10 Our results show that activation of p38 MAPK is necessary for normal release of \( \cdot O_2^- \) in response to fMLP. Inhibition of p38 MAPK decreased the fMLP-stimulated production of superoxide by 57% ± 14%. In contrast to our findings with the p38 MAPK pathway, the ERK1/2 pathway did not appear to stimulate cytotoxic function, but rather attenuate it, as ERK1/2 inhibition increased elastase release in response to fMLP by as much as 113% ± 61%.

Other investigators have recently examined the role of MAPKs in oxidant production by PMNs. Our results agree with those of Zu et al,17 who used an end point assay for superoxide to show that total oxidant production in 3 minutes was reduced by 92% after treatment with SB 203580. The more pronounced effect they observed may be due to the fact that they preincubated with the inhibitor for 40 minutes before rewarming the cells, which may not be an ideal model for therapeutic application of p38 MAPK inhibitors. These authors, as well as Coffer et al,14 also found no effect of ERK1/2 inhibition on oxidant production.

The role of MAPK pathways in degranulation is less well defined. A single report10 suggested a role for ERK1/2; however, the study used neutrophils pretreated with cytochalasin B, which perturbs the cytoskeleton and makes results concerning mobilization of granules difficult to interpret. The apparent inhibitory role of ERK1/2 in PMN degranulation may prove to be via the induction of cellular phosphatase activity and, therefore, termination of the activity of enzymes activated by phosphorylation. Recently, ERK1/2 has been shown to induce phosphatases specific for MAPKs.18,19 Although p38 MAPK has now been implicated in several PMN cytotoxic functions, including oxidant production, chemotaxis, and cytokine production,14,16 it appears to have no role in the release of granule contents. This argues that specific elements of PMN cytotoxic mechanisms are regulated independently, and not in an all-or-none fashion. Indeed, we have noted disparate changes in oxidant production and elastase release in patients after injuries (J.L.J. and E.E.M., unpublished observations, October 1998). This has important implications for the potential therapeutic application of agents that alter PMN function.

As our understanding of the cellular control of PMN cytotoxic function develops, we may be able to develop novel strategies to modify the inflammatory response. The present finding that p38 MAPK serves a proinflammatory role in the PMN may provide a target for therapy in hyperinflammatory states. Indeed, our work in pulmonary endothelial cells20 corroborates a proinflammatory role for p38 MAPK in adhesion molecule expression and, thus, the potential for regulating PMN-endothelial interactions. Inhibition of p38 MAPK activity may provide an avenue for controlling the cytotoxic potential of primed PMNs, which have been implicated in the patho-
The neutrophil (PMN) has been implicated as a principal effector of organ injury in adult respiratory distress syndrome and multiple organ failure.21 The finding that ERK1/2 may serve a negative feedback role in granule release may have similar implications for the diagnosis and treatment of disease. For instance, because elastase release is a central mechanism of cell death after CD18–intercellular adhesion molecule-1 interactions,22 augmentation of ERK1/2 may serve a negative feedback role in granule release may have similar implications for the diagnosis and treatment of disease. For instance, because elastase release is a central mechanism of cell death after CD18–intercellular adhesion molecule-1 interactions,22 augmentation of ERK1/2 activity may prove to be a useful tool in preventing damage to the host during syndromes characterized by neutrophil sequestration. Of course, because MAPK pathways are found in many cells, the clinical efficacy of manipulating these signaling events remains to be proven.

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REFERENCES

Having been in the practice of “rural” general surgery for the last decade, plus working in a US Air Force small hospital practice, I must echo Bill Rainer’s comments regarding caution in designing a track for rural-based surgeons. In fact, a separate track would likely exacerbate the already often contentious relationships between small town and big city surgeons. It is difficult enough to deal with the growing need for the training centers to maintain a patient base, often at our expense, but to add the question of adequacy or intent of training credentials would certainly add to the wedge that practice economics drives between us these days.

The better answer is to provide better exposure to rural medicine during residency—not just watching cases as a preceptee third or fourth year medical student, but being in the pit with them for 2 to 3 months, living the life. The 3 months I spent away from the University of Colorado, Boulder, in postgraduate year 3 with William Miller, MD, and Zef Arroyo, MD, in Garden City, Kan, molded my future practice aims and led me out of the myth that bigger is better and that only in the big city surgeons. It is difficult enough to deal with the contentious relationships between small town and big city surgeons. The overall success rate, measured by fistula closure, was 56%; this was considered unsatisfactory by the scientific and ethical committee at our institution, and the trial was stopped. When analyzing our results, we came to the conclusion that inadequate removal of the infective source of the fistula could be the cause of our failure. Most fistulae derive from sepses originating in the glands of the anus at the dentate line, and the failure of traditional surgical treatment worldwide is related to residual infection. According to the criteria of Parks et al, fistulae in our series were classified as intersphincteric in 16 patients (10 with a low and 6 with a high internal orifice), transphincteric in 10 (6 with a low and 4 with a high internal orifice), and extrasphincteric in 4, all with a high internal orifice.

While an effective cleansing of the fistulous tract was attempted in all cases, it is conceivable that in complex fistulae and in those with a high internal orifice, the procedure might have been inadequate. Fistulae with a low internal orifice healed in 100% (10 patients) of intersphincteric tract cases, in 83% (5 patients) of transphincteric cases, and in no extrasphincteric cases. The height of the internal orifice is the determinant factor affecting recurrence (χ² = 21.2; P < .001). We have recently resumed fibrin glue treatment exclusively in fistulae with a low internal orifice. Follow-up data are being collected, and present results reveal a recovery rate of more than 90%.

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Correction

Error in Byline. In the article titled “Extracellular Signal-Related Kinase 1/2 and p38 Mitogen-Activated Protein Kinase Pathways Serve Opposite Roles in Neutrophil Cytotoxicity” (Arch Surg. 1999;134:1074-1078), there was an error in the spelling of an author’s name. The author should have been listed as David A. Partrick, MD. The ARCHIVES regrets the error.