Tumor Necrosis Factor-α Activation of the c-Jun N-terminal Kinase Pathway in Human Neutrophils

The intensity and duration of an inflammatory response depends on the balance of factors that favor perpetuation versus resolution. At sites of inflammation, neutrophils adherent to other cells or matrix components are exposed to tumor necrosis factor-α (TNFα). Although TNFα has been implicated in induction of pro-inflammatory responses, it may also inhibit the intensity of neutrophilic inflammation by promoting apoptosis. Since TNFα is not only an important activator of the stress-induced pathways leading to p38 MAPk and c-Jun N-terminal kinase (JNK) but also a potent effector of apoptosis, we investigated the effects of TNFα on the JNK pathway in adherent human neutrophils and the potential involvement of this pathway in neutrophil apoptosis. Stimulation with TNFα was found to result in β2 integrin-mediated activation of the cytoplasmic tyrosine kinases Pyk2 and Syk, and activation of a three-part MAPk module composed of MEKK1, MKK7, and/or MKK4 and JNK1. JNK activation was attenuated by blocking antibodies to β2 integrins, the tyrosine kinase inhibitors, genistein, and tyrphostin A9, a Pyk2-specific inhibitor, and piceatanol, a Syk-specific inhibitor. Exposure of adherent neutrophils to TNFα led to the rapid onset of apoptosis that was demonstrated by augmented annexin V binding and caspase-3 cleavage. TNFα-induced increases in annexin V binding to neutrophils were attenuated by blocking antibodies to β2 integrins, and the caspase-3 cleavage was attenuated by tyrphostin A9. Hence, exposure of adherent neutrophils to TNFα leads to utilization of the JNK-signaling pathways that may contribute to diverse functional responses including induction of apoptosis and subsequent resolution of the inflammatory response.

The rapid influx of polymorphonuclear leukocytes (neutrophils) into sites of injury is an important component of the acute inflammatory response in humans (1). The resulting adherence and chemotaxis of leukocytes in the appropriate cytokine milieu are not only necessary for efficient clearance of microorganisms (2) but may also participate in appropriate resolution of the inflammatory response through the subsequent induction of cell death by apoptosis.

1 The abbreviations used are: TNFα, tumor necrosis factor-α; MAP, mitogen-activated protein; MAPk, MAP kinase; ERK, extracellular signal-regulated kinase; pNPP, p-nitrophenyl phosphate; MKK, MAPk/ERK kinase (MEK); MEKK, MEK kinase (MAPk kinase); JNK, c-Jun N-terminal kinase; JNKind, recombinant wild type JNK1; JNKinac, recombinant kinase-inactive JNK1; SEK, SAPK/ERK kinase; P65 kinase, phosphorytidylinositol 3-kinase; PAF, platelet-activating factor; Pyk2, Proline-rich tyrosine kinase 2; TNFR, TNF receptor; PMSP, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; ANOVA, analysis of variance; NBD,12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)).

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plasmic tyrosine kinase Pyk2 (proline-rich tyrosine kinase 2 (24)), also known as CAKβ (cell adhesion kinase β (25)), RAFTK-related adhesion focal tyrosine kinase (26)), CADTK-calcium-dependent tyrosine kinase CADTK (27)), as a member of the FAK family. Pyk2 has been identified in brain cells, cell lines and hematopoietic cells (24, 25, 28, 29). Studies in adherent neutrophils exposed to TNFα demonstrate that tyrosine phosphorylation of Pyk2 is β2 integrin-dependent and that Pyk2 appears to lie downstream of Lyn, Syk, protein kinase C, and reorganization of the cytoskeleton (30). Furthermore, over-expression of Pyk2 in PC12 cells, which have been stimulated with TNFα, induces JNK activation in a calcium-independent manner (31). Thus the altered response of the adherent neutrophil to TNFα may reflect in part differences in tyrosine kinase signaling through β2 integrin-mediated mechanisms.

Three distinct MAPK families have been described to date that may act downstream of tyrosine kinases. The prototypical p42/p44 ERK MAPKs are activated in response to growth factors and chemoattractants, whereas the p38 MAPK and c-Jun N-terminal kinase (JNK) families modulate stress-activated responses (32, 33). Each MAPK cascade requires sequential activation of a MAPK kinase kinase (or equivalent) which can then activate a MAPK kinase, resulting in turn in the phosphorylation and activation of a MAPK (34, 35). In the neutrophil, only the p38 MAPK cascade has been clearly shown to be utilized in response to TNFα stimulation (36). Involvement of the p42/p44 ERK MAPK pathway in response to TNFα is controversial. Activation of a JNK pathway in the neutrophil has not as yet been shown in any context. Furthermore, regulation of apoptosis may represent a functional consequence of JNK, and this has yet to be delineated in the context of the neutrophil inflammatory response.

Apoptosis is an important mechanism for regulating the extent of an inflammatory response, making it self-limiting and thus preventing massive tissue damage. TNFα is a potent inducer of apoptosis in neutrophils, but its mechanisms of action are unknown. Recent reports have suggested that TNFα in conjunction with β2 integrins induces apoptosis in a tyrosine kinase-dependent manner (37), whereas other studies have shown activation of the cysteine proteases, caspase-3 and -8 (8). The role of MAPK members, in particular JNK pathway activation, in the TNFα induction of apoptosis remains controversial (38, 39).

The present study was undertaken to determine the conditions under which TNFα might activate the JNK pathway in human neutrophils, to define components of a putative MAPK module, and to delineate key upstream components, as well as downstream functional consequences (in particular apoptosis). We report that in adherent neutrophils, activation of JNK1 in response to TNFα results from the mobilization of an integrin-mediated cascade involving activation of tyrosine kinases, in particular Syk and Pyk2, and activation of a MAPK module in which MEKK1 activates either or both of the MAPK kinases, MKK7 and MKK4. Among possible functional consequences of this pathway, we show that TNFα-elicited JNK activation is accompanied by a dramatic acceleration of neutrophil apoptosis which is modulated both by integrins and tyrosine kinases. Together, these results provide the first description to date of the activation of the JNK MAPK cascade in the human neutrophil, while providing a potential mechanism for TNFα-induced apoptosis in the human neutrophil.

**EXPERIMENTAL PROCEDURES**

**Materials**—Endotoxin-free reagents and plasticware were used throughout the experimental process. Neutrophils, prepared by previously described methods (40), were resuspended in Krebs-Ringer phosphate buffer, pH 7.2, with 0.2% dextrose (5% dextrose in 0.2% NaCl, injectable, Abbott) KRPD. KRPD salts were purchased from Mallinckrodt Chemical Works, and all components were diluted in endotoxin-free saline (0.9% saline for irrigation, Abbott). Aprotinin, leupeptin, PMSF, bovine serum albumin fraction V, sodium orthovanadate (Na3VO4), p-nitrophenyl phosphate (pNPP), Brij 97, protein A-Sepharose, workman, and propidium iodide were purchased from Sigma. Recombinant human TNFα from Pharmingen (San Diego, CA). Genistein was purchased from Life Technologies, Inc., and piceatannol, tyrphostin A9, and tyrphostin A63 were from Calbiochem. Human serum albumin was from Intergen (Purchase, NY). NBD-phallacidin was from Molecular Probes (Eugene, OR). ECL reagents and [γ-32P]ATP were from Amersham Pharmacia Biotech. Anti-Receptors—Anti-CD11b and Anti-CD18 antibodies were from Immunotech (Marseille, France). Anti-CD11b receptor was from Becton Dickinson, and the phosphospecific anti-SEK1 was from New England Biolabs (Beverly, MA). Anti-CR3 receptor (anti-CD11b) was purchased from Dako (Denmark). Mouse IgG1 was from Upstate Biotechnology Inc. (Lake Placid, CA). Anti-CD11b (Fab')2 Preparation kit, using immobilized ficin (Pierce).

**Histidine-tagged Recombinant Proteins**—Wild type SEK1 (SEK1 wt), wild type JNK1 (JNK1 wt), and kinase-inactive JNK1 (JNK1 km) were expressed in Escherichia coli. Recombinant SEK1 (40), recombinant JNK1 for 2 ha t4° C., activated in vitro.

**Preincubation of Neutrophils to Promote Adherence**—Neutrophils were resuspended at 20 × 10^6/ml in complete KRPD containing 0.25% human serum albumin, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Following this, 20 × 10^6 cells were either preincubated in a tube under static conditions for 35 min at 37°C (cell-cell/homotypic adherence; hereafter referred to as adherent neutrophils) or preincubated in a tube under static conditions for 30 min at 37°C and then aliquoted into 1 well of a 12-well plate (cell-substratum adherence) for an additional 25 min prior to stimulation with 10 ng/ml TNFα for the intervals indicated hereafter. Cells were either harvested from wells into a microcentrifuge tube and pelleted at 15,000 rpm for 20 s or those stimulated in microcentrifuge tubes were directly pelleted.

**JNK Immunoprecipitation**—Cell lysates were prepared using cold JNK lysis buffer, JLB (50 mM Tris, pH 7.5, 10% glycerol, 1% Nonidet P-40, 137 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 2 mM NaF, 1 mM Na3VO4) and centrifuged at 15,000 rpm for 10 min at 4°C. Triton-soluble cell lysates were preincubated with protein A-Sepharose for 15 min, 4°C, prior to immunoprecipitation with anti-JNK1 for 2 h at 4°C.

**MEKK1-coupled in Vitro Kinase Assay**—Adherent neutrophils were washed once in JNK lysis buffer and twice in JNK reaction mix (20 mM β-glycerophosphate, pH 7.2, 50 mM Hepes, pH 7.6, 50 μM Na3VO4, 1 mM dithiothreito1, 10 mM MgCl2, 12 mM pNPP). 40 μl of in vitro kinase assay mix containing 10 μCi of [γ-32P]ATP and 500 ng of c-Jun-(1–79) in JNK reaction mix were added to samples that were incubated for 30 min at 30°C. Reactions were terminated with addition of 5% Laemmli sample buffer, subjected to 15% PAGE, and proteins transferred to nitrocellulose. c-Jun-(1–79) phosphorylation was quantified by PhosphorImager (Molecular Dynamics) analysis and visualized by autoradiography.

**JNK activity was expressed as a ratio percentage of unstimulated control (% BL). Baseline-BL.**

**MKK4/MKK7 in vitro Kinase Assays**—Adherent neutrophils were exposed to TNFα, lysed in JNK lysis buffer, and immunoprecipitated with either anti-MKK4 or anti-MKK7. MKK4-bound beads or MKK7-bound beads were then washed once in JNK lysis buffer and twice in JNK reaction mix. For direct in vitro kinase assays, samples were incubated with 40 μl of JNK reaction mix containing 10 μCi of [γ-32P]ATP and 500 ng of JNK1 km for 30 min at 30°C. For coupled in vitro kinase assays, samples, immunoprecipitated and washed as above, were incubated with 40 μl of JNK reaction mix containing 10 μCi of [γ-32P]ATP, 250 μCi α32P ATP, 50 ng of JNK1 km, and 500 ng of c-Jun-(1–79) for 30 min at 30°C. All reactions were terminated with the addition of 5× Laemmli sample buffer, subjected to SDS-PAGE, and proteins transferred to nitrocellulose. Phosphorylation of substrates (JNK1 km, c-Jun-(1–79), and JNK1 wt) was quantified by PhosphorImager (Molecular Dynamics) analysis and visualized by autoradiography.

**MEKK1-coupled in vitro Kinase Assay**—Adherent neutrophils were stimulated with TNFα, pelleted, lysed in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM Na3VO4, and 2.1 μg/ml aprotinin), and immunoprecipitated with anti-MEKK1. MEKK1-bound protein A-Sepharose beads were washed once in RIPA, once in PAN (10 mM Pipes pH 7.0, 100 mM NaCl, 21 μg/ml
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apotinin, and then incubated with 45 μl of the MEKK in vitro kinase mix (25 μM β-glycerophosphate, pH 7.2, 20 mM Hepes, pH 7.6, 100 μM Na3VO4, 2 μM dithiothreitol, 20 μM MgCl2, and 100 μM ATP) containing 10 ng of SEK1 and 100 ng of JNK1, for 10 min at 30 °C. 2 μl of a mix containing 15 μCi of [γ-32P]ATP and 500 ng of c-Jun-(1–79) were added to the samples which were further incubated for 30 min at 30 °C. Reactions were terminated with the addition of 5 × Laemmli sample buffer, and proteins were separated by SDS-PAGE. Phosphorylation of JNK1 and c-Jun-(1–79) was quantified by PhosphorImager analysis and visualized by autoradiography.

Syk/Lyn in Vitro Kinase Autophosphorylation Studies—Adherent neutrophils were stimulated with TNFα and lysed in Syk lysate buffer (50 μM Tris, pH 7.5, 150 mM NaCl, 1% Brij 97, 1 mM Na3VO4, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Brij-soluble lysates were protein A-Sepharose immunoprecipitated with either anti-Syk or anti-Lyn. Syk-bound beads or Lyn-bound beads were washed twice in wash buffer A (25 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Brij 97, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and twice in wash buffer B (25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM Na3VO4, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Beads and kinase mix (25 mM Hepes, pH 7.5, 2 mM MnCl2, 20 mM MnPP, and 25 μCi of [γ-32P]ATP) were preincubated separately for 5 min at 30 °C and then 40 μl of kinase mix added to beads for 1 min at 30 °C. The reaction was terminated with 5 × Laemmli sample buffer. Samples were boiled for 5 min at 100 °C, subjected to SDS-PAGE, and transferred to nitrocellulose by immunoblotting. Autoradiography was detected and quantified by PhosphorImager Analysis.

Inhibitor Studies—Neutrophils were preincubated with the PI3-kinase inhibitor wortmannin and tyrosine kinase inhibitor genistein as described (40, 41). 10 and 30 μM picatannol were used for Syk and Lyn in vitro kinase autophosphorylation studies. Neutrophils were preincubated with tyrophostin A9 (5 μM) or tyrophostin A65 (50 μM) a negative control, for 1 h at 37 °C prior to stimulation with TNFα.

Apoptosis Assessment—For annexin V binding, externalized phosphatidylserine was determined by annexin V binding. Adherent neutrophils were exposed to TNFα for 1, 1.5, and 2 h. Aliquots containing 2.5 × 105 cells were resuspended in 100 μl of NBD buffer, pH 7.5 (157 mM NaCl, 2.7 mM KCl, 2 mM MgCl2, 5 mM glucose, 10 mM Hepes, and 2.5 mM CaCl2), incubated with 400 ng of annexin V fluorescein (Caltag Laboratories, Burligame, CA) and 500 ng of propidium iodide for 15 min at room temperature and placed on ice with the addition of 400 μl of cold NBD buffer. FACs analysis was performed by flow cytometry on a FACScan (Becton Dickinson), and annexin V binding was determined from the non-necrotic cell population, which stains negatively for propidium iodide. 5000 cells were analyzed in each sample. For caspase-3 cleavage, 20 × 106 adherent neutrophils were exposed to TNFα, 10 ng/ml for 0, 1, 1.5, and 2 h. Cells were lysed in RIPA, and whole cell lysates were prepared from the Triton-soluble fraction. Samples were subjected to SDS-PAGE using a 14% gel, and proteins were transferred to nitrocellulose membrane that was then probed with antibodies to caspase-3 by Western blot analysis.

RESULTS

TNFα Activates JNK in Adherent Neutrophils—Adherent human neutrophils were stimulated with TNFα, and JNK1 was immunoprecipitated from Triton-soluble cell lysates, and JNK activation was measured by the phosphorylation of an N-terminal fragment of c-Jun (c-Jun(1–79)) in the presence of [γ-32P]ATP. Phosphorylation of c-Jun(1–79) by JNK1 immunoprecipitates was augmented following TNFα stimulation of neutrophils (Fig. 1A). Activation was detected at 5 min and was maximal between 10 and 15 min. Immunoblots, probed with anti-JNK1 antibody, confirmed that equivalent amounts of JNK were immunoprecipitated and that in neutrophils the predominant immunoreactive JNK1 species is a 55-kDa protein (Fig. 1C). A change in the electrophoretic mobility of JNK1, consistent with phosphorylation, was detected as early as 1 min after TNFα exposure. JNK1 activation was further supported by immunodetection with a phosphospecific anti-JNK1 antibody, indicating increased phosphorylation of JNK in the Triton-soluble fraction of JNK1 immunoprecipitates 5 min after TNFα stimulation of neutrophils (Fig. 1B). The time course of JNK1 phosphorylation mirrored the phosphorylation of c-Jun(1–79).

The Role of Adherence in JNK Activation—JNK activation was studied in neutrophils preincubated under different conditions that promote adherence and then exposed to TNFα. Cells were maintained either stationary in a tube under conditions that favor cell-cell interactions or stimulated on a surface, favoring cell-substratum interactions (Fig. 2A). Neutrophil exposure to TNFα induced comparable amounts of JNK1 activation under both sets of conditions (Fig. 2B). Since integrins, in particular β2 and β2, are important components of neutrophil-adhesive interactions, we questioned whether β2 integrins were up-regulated by TNFα on the neutrophil surface. Neutrophils, exposed to TNFα for various time intervals, were stained with anti-CD11b, an antibody directed against β2 integrins, and their fluorescence was analyzed by flow cytometry. We observed a time-dependent up-regulation of β2 integrins on the neutrophil surface in those cells that had been exposed to TNFα (Fig. 2C).

β2 Integrin Dependence of JNK Activation—Previous studies have indicated a role for integrins in mediating a variety of neutrophil responses, including the activity of the Na+/H+ antiport and oxygen radical release (12, 42). To determine whether the TNFα-induced JNK activation we had observed was β2 integrin-mediated, neutrophils were pretreated with a blocking antibody directed against CD11b, prior to TNFα stimulation, and JNK activity was determined on JNK immunoprecipitates. The resultant JNK activation was significantly diminished compared with controls (Fig. 3, A and B).

To validate the specificity of blocking by anti-CD11b, two types of control experiments were performed. The first, using mouse IgG as an isotype-matched antibody, ruled out the possibility that the inhibition observed was due to anti-CD11b competition for protein A-Sepharose (preventing anti-JNK1 binding), whereas it also tested the blocking capabilities of anti-CD11b per se (Fig. 3, A and B). The second, carried out to determine whether anti-CD11b inhibited the TNFR and thus its downstream signaling, examined a TNFα-induced function, actin assembly. We have previously shown in neutrophils and HL-60 promyelocytic cells, stimulated with either chemoattractants or lipopolysaccharide, that actin assembly is differentiable from integrin-mediated adherence (43–45). Neutrophils, pretreated in the absence and presence of anti-CD11b and stimulated with TNFα, demonstrated identical actin assembly in both cases (Fig. 3C), indicating that anti-CD11b does not
modulate the TNFR or its immediate downstream signaling.

**M KK7 and M KK4 Activate JNK1**— Whereas JNK activation has been reported as a consequence of MKK4/SEK1 activation in many cell systems (46), the recent description of MKK7 (homologous to *hemipterous*, the *Drosophila* JNK kinase) (47) as a downstream effector of TNF-α suggests its potential role (48). Activation of MKK4 and MKK7 following TNF-α exposure was determined in MKK4 and MKK7 immunoprecipitates by direct phosphorylation of a kinase-inactive recombinant human JNK1 (JNK1<sub>km</sub>). A time-dependent increase in JNK1<sub>km</sub> phosphorylation by both MKK4 and MKK7 immunoprecipitates from TNF-α-stimulated neutrophils was detected (Fig. 4A). To determine whether the JNK1<sub>km</sub> phosphorylation induced by MKK4 and MKK7 resulted in enhanced activation of JNK1, we determined whether these MKK homologues could initiate a coupled reaction leading to c-Jun-(1–79) via kinase-active JNK1. Both MKK4 and MKK7 immunoprecipitates from TNF-α-exposed neutrophils induced phosphorylation of the JNK1<sub>km</sub> and c-Jun-(1–79) substrates with a time course identical to that shown for JNK1<sub>km</sub> (Fig. 4B). However, MKK7 induced much stronger phosphorylation than did MKK4. Immunodetection of immunoprecipitated MKK4 and MKK7 confirmed that within each experiment, equivalent amounts of...

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**Fig. 2. Role of adherence in JNK activation.** A, autoradiograph depicting JNK activity under two conditions that promote adherence. Phosphorylation of the substrate c-Jun-(1–79) was determined by JNK<sub>in vitro</sub> kinase assay on JNK1 immunoprecipitates from neutrophils preincubated in one of two ways: (i) under stationary conditions in a test tube for 55 min (cell-cell adherence); or (ii) in a tube for 30 min and then plated in a well for 25 min (cell substratum). Following preincubation cells were stimulated with TNF-α (10 ng/ml for 15 min), lysed, and immunoprecipitated with anti-JNK. B, buffer and T, TNF-α. B, graph represents mean c-Jun-(1–79) phosphorylation ± S.E. from PhosphorImager analyses of phosphorylated c-Jun-(1–79) band under the conditions described above. By using the Student t test, we determined a lack of statistically significant differences in JNK activation. C, graph depicts TNF-α-induced up-regulation of CD11b. Neutrophils, exposed to TNF-α (10 ng/ml) for 15, 30, and 60 min or buffer and stained with anti-CD11b, were analyzed by fluorescence analysis on the FACScan (Becton Dickinson) for surface expression of CD11b. CD11b up-regulation was expressed as a relative fluorescence index (RFI), buffer, ■, TNF-α.

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**Fig. 3. β<sub>2</sub> integrin involvement in JNK activation.** A, autoradiograph showing JNK activation following preincubation with anti-CD11b. Neutrophils were pretreated with either buffer (−), isotype control antibody, mouse IgG 20 μg/ml (IgG), or the β<sub>2</sub> integrin blocking antibody, anti-CD11b10 μg/ml, 20 μg/ml (CD11b) for 55 min at 37 °C, stimulated with buffer (B) or TNF-α (T, 1 ng/ml for 15 min). JNK activity was determined on JNK immunoprecipitates by *in vitro* kinase assay, in which c-Jun-(1–79) was used as substrate. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and the phosphorylated c-Jun-(1–79) visualized by autoradiography. B, graph depicting JNK activity under the same conditions demonstrated in A. JNK activation was determined from c-Jun-(1–79) phosphorylation by the protocol described for samples from (Fig. 2A) and was quantified by PhosphorImager analysis. The graph depicts mean JNK activity ± S.E. of three experiments. Statistical analysis employing a one-way ANOVA determined that in the presence of 20 μg/ml anti-CD11b, JNK activation was significantly reduced compared with control, * (p < 0.01). C, graph depicts TNF-α-induced increase in actin assembly in the absence and presence of anti-CD11b. Neutrophils were pretreated in the absence (□) and presence (■) of anti-CD11b (10 μg/ml), stimulated with TNF-α (10 ng/ml) for 0, 2, 5, and 15 min, and then stained with NBD-phallacidin. The fluorescence was analyzed by flow cytometry on an Epics (Coulter) for determination of actin assembly as described previously (45).
in vitro kinase assay. Autoradiograph of JNK_Km phosphorylated directly following MEKK1 activation in neutrophils. Confirming that JNK1 is itself a substrate for recombinant MKK4. Phosphorylation of wild type recombinant JNK1 was also detected.

The phosphorylation of c-Jun-(1–79) and JNK1wt induced by these immunoprecipitates is shown (Fig. 5C). We observed greatly decreased phosphorylation of both the c-Jun-(1–79) and JNK1wt species in TNFα-stimulated neutrophils that had been pretreated with anti-CD11b, suggesting that the integrin-dependent activation of JNK occurs at, or upstream of, MEKK1.

Involvement of Tyrosine Kinases in the β2 Integrin-mediated JNK Activation—Since studies in adherent neutrophils exposed to TNFα have shown that phosphorylation and activation of tyrosine kinases are associated with integrin activation, we sought to determine whether tyrosine kinases were involved in the TNFα–induced JNK activation in adherent neutrophils. Activation of JNK1 from TNFα–stimulated neutrophils was assessed following pretreatment with genistein, an inhibitor of tyrosine kinases, and wortmannin, a PI3-kinase inhibitor of tyrosine kinases, and wortmannin, a PI3-kinase inhibitor.
In initial studies, the tyrosine kinase activities of Syk and Lyn were determined in vitro by autophosphorylation. Anti-Syk immunoprecipitates from TNFα-stimulated adherent neutrophils exhibited tyrosine kinase activity with greatly enhanced autophosphorylation of a 40-kDa species, consistent with the almost universal proteolytic cleavage product of Syk (59) (Fig. 7A). Incubation with piceatannol, a Syk-specific inhibitor (60), inhibited this autophosphorylation in a dose-dependent fashion. In contrast to the augmented Syk activation, we observed minimal activation of the Src family kinase, Lyn, in TNFα-stimulated adherent neutrophils (Fig. 7B). Additionally, the autophosphorylation activity of immunoprecipitated Lyn was diminished only slightly at the highest concentration of piceatannol.

Since integrin signaling may proceed through Syk activation (19), we next questioned whether Syk activation in TNFα-stimulated neutrophils was also integrin-dependent. The Syk autophosphorylation activity, determined on Syk immunoprecipitates, was diminished by preincubation of neutrophils with anti-CD11b antibody prior to TNFα exposure (Fig. 7C). This observation provided a link between β2 integrins and Syk activation in our system, raising the possibility that Syk may be a component of the integrin-signaling pathway leading to JNK1 activation.

To test this hypothesis, we studied the effects of a Syk-specific inhibitor, piceatannol, on the TNFα-induced activation of MEKK1 and JNK1 in adherent neutrophils. Piceatannol pretreatment of TNFα-stimulated neutrophils resulted in reduced MEKK1 activation (Fig. 8A) as determined by the diminished phosphorylation of both the c-Jun-(1–79) and JNK1<sub>act</sub> species. Similarly, piceatannol pretreatment induced a concentration-dependent inhibition of JNK1 activation in TNFα-stimulated neutrophils (Fig. 8B). Pretreatment of neutrophils with 10 μM piceatannol, a concentration that significantly inhibited Syk autophosphorylation but had no effect on Lyn autophosphorylation, decreased JNK1 activation by 66%.

Recent studies have linked activation of another tyrosine kinase, Pyk2, a FAK family member, to TNFα-induced JNK signaling in PC12 cells (31), whereas β2 integrin-mediated Pyk2 phosphorylation in adherent neutrophils, exposed to TNFα (23) has been also shown. Furthermore, in the latter system, Pyk2 has been shown to lie downstream of Syk and Lyn (30). Hence, we questioned whether Pyk2 was phosphorylated under our experimental conditions and, if so, was this linked to the JNK activation we had observed earlier. Enhanced tyrosine
pretreated with 0.1% Me2SO (control) or piceatannol (3, 10, 30, and 100 μM) for 5 min. Anti-CD11b stimulation attenuated this increase that was significant at 2 h post-TNFα.

**FIG. 8.** Syk involvement in JNK pathway activation. A, MEKK activation following piceatannol pretreatment. Neutrophils, pretreated in the absence (−) and presence (+) of piceatannol (30 μM), were stimulated with TNFα (10 ng/ml for 5 min), and anti-MEK1 immunoprecipitates were exposed to coupled in vitro kinase assay (as described under “Experimental Procedures”). Samples were subjected to SDS-PAGE and transferred to nitrocellulose. Phosphorylated substrates were visualized by autoradiography (B, buffer and T, TNFα). B, JNK activity following pretreatment with piceatannol. Neutrophils, pretreated with 0.1% Me2SO (control) or piceatannol (3, 10, 30, and 100 μM) for 55 min, were stimulated in the absence (Δ) or presence of TNFα (A), 10 ng/ml, for 15 min. JNK1 immunoprecipitates were subjected to in vitro kinase assay using c-Jun-(1–79) as substrate, and samples separated by SDS-PAGE were transferred to nitrocellulose. Phosphorylated c-Jun-(1–79) was quantified by PhosphorImager analysis. Graph represents mean c-Jun phosphorylation ± S.E. of three consecutive experiments.

Phosphorylation of Pyk2 was detected in Pyk2 immunoprecipitates from TNFα-stimulated neutrophils compared with the unstimulated controls (Fig. 9A). Tyrophostin A9, a tyrosine kinase inhibitor, has been shown to inhibit specifically Pyk2 phosphorylation in TNFα-stimulated neutrophils (25). Adherent neutrophils, pretreated with tyrophostin A9 and stimulated with TNFα, exhibited reduction in JNK activation compared with nonpretreated controls (Fig. 9B).

**FIG. 9.** TNFα-induced Pyk2 activation and association with the JNK pathway. A, Western blot analysis of tyrosine-phosphorylated Pyk2. Neutrophils, stimulated with either buffer (B) or TNFα (T), 10 ng/ml, for for 5 and 15 min were immunoprecipitated (IP) with anti-Pyk2. Samples, separated by SDS-PAGE, were transferr to nitrocellulose and membranes probed with anti-phosphotyrosine. B, autoradiography showing dose response of tyrophostin A9 inhibition on JNK activation. Neutrophils, pretreated with piceatannol (10 μM), tyrophostin A9 (1 μM, 5 μM), or solvent control (0.1% Me2SO) were either stimulated with either buffer (B) or TNFα (T), 10 ng/ml, for 15 min. A JNK in vitro kinase assay was performed on JNK1 immunoprecipitates, and the phosphorylation of a c-Jun-(1–79) fragment substrate was visualized by PhosphorImager analysis.

Caspase-3 cleavage has been widely described in cells undergoing apoptosis, including neutrophils. We observed a time-dependent cleavage of pro-caspase-3 (32 kDa) to a caspase-3 cleavage product (17 kDa) in lysates from adherent neutrophils that had been exposed to TNFα (Fig. 10B). When neutrophils were pretreated with tyrophostin A9 (the Pyk2-associated tyrosine kinase inhibitor shown in Fig. 9B to inhibit JNK activation) and then stimulated with TNFα, the cleavage of caspase-3 was greatly reduced (Fig. 10C).

**DISCUSSION**

Regulation of cellular responses to specific stimuli occurs in part through selective activation of MAPK-signaling cascades. In human neutrophils, chemotactic-induced activation of c-Raf and MEKK1 leads to the activation of p42/p44 ERK MAPK pathways (40, 41, 61, 62), whereas chemotaxtants and lipopolysaccharide activate p38 MAPK as well (63, 64). In contrast, studies to date in human neutrophils have not as yet provided evidence for activation of JNK and its associated signaling partners. This study, performed in primary human neutrophils, describes key elements leading to activation of the JNK pathway following stimulation with TNFα under adherent conditions. Our data support the conclusion that integration of two signals, one from integrin-mediated adherence and the other through the TNF receptor(s), regulates a pathway in which activation of tyrosine kinases, Syk and Pyk2, is linked to activation of a MAPK module consisting of MEKK1, MKK4/7, JNK1, and associated with cleavage of caspases-3 and externalization of phosphatidylserine (Fig. 11). We propose that induction of apoptosis, a critical mechanism for maintaining a self-limiting inflammatory response, may be associated with activation of these elements. This pathway not only constitutes the first report of activation and utilization of a JNK pathway in human neutrophils but joins a very limited group of studies on apoptosis induced through stimulation of the JNK pathway.

JNK activation in response to TNFα exposure has been reported for a variety of cell types (65). Ten different human JNK isoforms, found as either 46- or 55-kDa proteins, have been described, arising from the alternative splicing of the three genes jnk1, jnk2, and jnk3. We detected by Western blot anal-
TNFα induces annexin V binding. Unstimulated neutrophils (□) or neutrophils exposed to TNFα (10 ng/ml, for 1, 1.5, and 2 h) in the absence (■) and presence (▲) of anti-CD11b F(ab')₂ fragments (2 μg/ml) were incubated with a mixture of annexin V fluorescent and propidium iodide, and their fluorescence was analyzed by flow cytometry. (10,000 cells were analyzed, and the percent of cells binding annexin V was determined from the propidium iodide-negative staining population.)

Statistical analysis was carried out by two-way ANOVA. * designates comparison of (i) TNFα/buffer with p < 0.0136 at 1.5 h and p < 0.0001 for 2 h and (ii) TNFα-CD11b/buffer with p < 0.002. ** represents (p < 0.01) for samples stimulated with TNFα for 2 h in the presence and absence of anti-CD11b F(ab')₂ fragments. B. TNFα induces caspase-3 cleavage in neutrophils. Anti-caspase-3 immunoblot shows the appearance of the 17-kDa cleavage product with time. Triton-soluble lysates were prepared from neutrophils stimulated with either buffer (B) or TNFα (T) 10 ng/ml for 0, 60, 90, and 120 min. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-caspase-3. The 32-kDa proenzyme is cleaved into a heterodimer of a 17-kDa caspase-3. The 32-kDa proenzyme is cleaved into a heterodimer of a 17-kDa caspase-3.

The MAP kinases kinases reported to be directly upstream of JNK are MEKK1, MEKK4, and MEKK7 (49, 50, 70, 71). Additionally, or alternatively, other MEKK family members such as MEKK5 (51), and NIK (73). We have focused on MEKK1, which phosphorylates and activates MEKK4/MKK7 in vitro (49). Overexpression of MEKK1 in a variety of cell types results in activation of both MEKK4 and JNK(s) (49, 74) and also leads to apoptosis in fibroblasts (75). In this study, we demonstrate, with the use of recombinant constituents of the JNK pathway, MEKK1 activation in adherent neutrophils following exposure to TNFα. In light of our previous findings that formyl-methionyl-leucyl-phenylalanine induces MEKK1-associated p42/44 ERK MAPk activation (conditions in which MEKK4 is not activated (40)), we speculate that the neutrophil may respond to specific stimuli by using the same upstream kinase but allowing different downstream effectors to be activated. The yeast accomplishes such specificity through the use of scaffolding proteins (76). Additionally, or alternatively, other MEKK family members may also activate MEKK4 and MKK7 in response to TNFα.

Signaling events upstream of MEKK remain to be elucidated, although the pleiotropic actions of TNFα (7) are known to be mediated by two TNF receptors, RI and RII, that belong to a superfamily of cell surface receptors including Fas and...
CD40 (77). Although most of the biological effects of TNFα are ascribed to TNFRI (78, 79), neutrophils express both types. Binding of TNFα induces receptor aggregation and recruitment of TNF-associated proteins (80–82), including TRAF2 and RIP, thought to be points of divergence that lead to many downstream events, including activation of the JNK pathway and of the transcription factor NF-κB (83).

In spite of the many activities linked to activation of TNFRI and -II, we demonstrate that an additional component, neutrophil adherence, is necessary for JNK1 activation in TNFα-stimulated neutrophils. Although neutrophils utilize a variety of adhesion molecules, β2 integrins are fundamentally important in both homotypic and cell-substratum adhesive events, and neutrophil interactions with the extracellular matrix have been shown to require integrins (16). In our studies, both the JNK and MEKK1 activation in TNFα-stimulated adherent neutrophils could be blocked by pretreatment of cells with an antibody to CD11b, the α subunit of the β2 integrin, Mac-1 (CD11b/CD18, aMβ2). Although JNK activation following integrin activation alone has been reported (84), we found that in neutrophils adherence in the absence of TNFα stimulation was insufficient to initiate activation of JNK1. By using an isotype control antibody, we demonstrated that the anti-CD11b blocking was indeed specific. Additionally, we present evidence that anti-CD11b does not block the TNFR itself by demonstrating that TNFα-induced actin assembly in neutrophils is an integrin-independent function (Fig. 2C). Initial actin assembly in the neutrophil in response to variety of agonists occurs in suspension and is independent of β2 integrin blockade. Hence, this provides a system in which integrin-independent signaling events downstream of the TNFR and its expression can be assessed.

Neutrophil interactions with the extracellular matrix have been shown to induce phosphorylation of specific cytoplasmic tyrosine kinases, including the Src family member p58Fgr (19–22). Fuortes and colleagues (20) have demonstrated that TNFα activation of adherent neutrophils resulted in tyrosine phosphorylation of several protein species that were not seen in suspended cells. Our studies show that the tyrosine kinase inhibitor, genistein, attenuated TNFα-induced actin assembly in neutrophils and is an integrin-independent function (Fig. 2C). Initial actin assembly in the neutrophil in response to variety of agonists occurs in suspension and is independent of β2 integrin blockade. Hence, this provides a system in which integrin-independent signaling events downstream of the TNFR and its expression can be assessed.

Recent studies, however, have shown that another tyrosine kinase, the FAK family Pyk2, localizes to podosomes and focal adhesion-type structures in TNFα-stimulated adherent neutrophils (23). TNFα-induced Pyk2 phosphorylation in neutrophils is decreased in the presence of β2 integrin blocking antibodies (30) and Pyk2 integrates signals from integrins and TNFR (23). In B-cells, aggregation of β3 integrins (86) and in T-cells, β3 integrins (87) results in the phosphorylation of Pyk2. Our studies in adherent neutrophils show both phosphorylation of immunoprecipitated Pyk2 following TNFα stimulation and inhibition of JNK activation by the tyrphostin A9, shown to inhibit specifically Pyk2 (23). These observations provide preliminary evidence that Pyk2 may be associated with JNK activation in the neutrophil and are in agreement with studies in which Pyk2 overexpression in 293 cells leads to activation of co-expressed JNK, whereas dominant-negative Pyk2 inhibits JNK in PC12 (31). Pyk2 has been linked to JNK via p130Cas and p130Csk (87, 88). Whereas in THP1 cells the PI3-kinase pathway is associated with Pyk2 (89), it is not necessary for Pyk2 activation in neutrophils (30). This observation agrees with our findings that wortmannin does not affect TNFα-induced JNK activation in neutrophils. Our studies on Syk and Pyk2, together with our CD11b blocking studies in TNFα-induced JNK activation in the neutrophil, further strengthen our hypothesis that the JNK pathway may require two interacting signals, one of which involves β2 integrins whose actions are mediated in part by activation of tyrosine kinases. However, the TNFR- and integrin-mediated signals may not necessarily be independent, since they may reflect the action by TNFα to modify integrin function (19) as demonstrated by both the capability of TNFα to induce up-regulation of CD18/CD11b (Fig. 3C) and stimulate β2 integrin-mediated adherence in neutrophils (90). Since both antibodies to β2 integrins and piceatannol inhibited MEKK1 activation (Fig. 5 and Fig. 8), these data indicate that protein tyrosine kinases and β2 integrins effects impinge on the JNK pathway upstream of MEKK1.

Neutrophil apoptosis may involve different MAP kinase pathways. In the neutrophil, as in many other cells, TNFα enhances apoptosis. We have shown herein that in adherent neutrophils TNFα induces apoptosis with a rapid time course comparable to that induced by UV exposure (91). Whereas recent studies invoke a role for JNK as a co-participant of apoptosis in response to ionizing radiation, UV, TNFα (38), and trophic factor deprivation (92), the role of JNK in apoptosis may be stimulus- and cell-specific. Certain studies suggest the possibility that JNK lies upstream of caspase-3 (93). Our studies on caspase-3 cleavage and annexin V binding demonstrated the induction of apoptosis in TNFα-stimulated adherent neutrophils.

Walzog and colleagues (37) have previously suggested that integrin involvement is important in TNFα-induced apoptosis and, furthermore, that tyrosine kinase inhibitors modulate the induction of apoptosis. Similarly, it has been demonstrated that wound macrophages induce neutrophil apoptosis through a mechanism requiring both TNFα and integrin-ligand interactions (94). Of considerable interest, Coxon and colleagues (95) have reported that CD11b −/− mice exhibit an increase in elicited neutrophils, ascribed, in part, to a failure of apoptosis. Similarly, we found that preincubation of neutrophils with F(ab)2′ fragments of anti-CD11b partially inhibited annexin V binding. Furthermore, a recent report that overexpression of Pyk2 leads to apoptosis in rat and mouse fibroblasts (27) supports our studies in which caspase-3 cleavage is both attenuated and delayed following preincubation of neutrophils with tyrphostin A9. These effects on caspase-3 cleavage were induced by the same concentrations of tyrphostin A9 that inhibited JNK activation.

While TNFα alone is a potent inducer of apoptosis in transformed cells, an additional insult is required to induce apoptosis in normal cells (e.g. cycloheximide). We propose that in human neutrophils, integrin involvement is required to induce apoptosis in the presence of TNFα. Hence, unlike many cells, in which integrin-mediated detachment from substratum leads to apoptosis (anokiasis), we have found, in contrast, that the neutrophil requires integrin-mediated attachment for apoptosis to occur. As we have previously reported, UV-induced apoptosis of neutrophils involves the MAP kinase family member p38 (91). Thus, neutrophil apoptosis may involve different MAP kinase pathways.
family members for apoptotic responses under diverse conditions.

The acute inflammatory response is characterized by both large numbers of adherent neutrophils and the presence of TNFα. It is precisely these conditions, as we have demonstrated here, that allow the neutrophil to utilize the JNK pathway to act to down-regulate the intensity of the inflammatory response. Although further studies, including investigations using genetically manipulated mice, are required to determine the relationship between the JNK pathway and apoptosis, this complex activation sequence provides a mechanism for enabling the neutrophil to both acquire specific functional capabilities and to ensure they are only used transiently.

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Tumor Necrosis Factor-α Activation of the c-Jun N-terminal Kinase Pathway in Human Neutrophils: INTEGRIN INVOLVEMENT IN A PATHWAY LEADING FROM CYTOPLASMIC TYROSINE KINASES TO APOPTOSIS
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