Role of Mitogen-Activated Protein Kinases in Activation of Human Neutrophils by Antineutrophil Cytoplasmic Antibodies

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Abstract. Antineutrophil cytoplasmic antibodies (ANCA) may be important in the pathophysiology of necrotizing vasculitis. ANCA activate human neutrophils primed with tumor necrosis factor-α (TNF-α) in vitro. TNF-α priming results in translocation of ANCA antigens to the cell surface, where they are recognized by the antibodies. The signaling mechanisms involved in TNF-α priming and subsequent ANCA-induced activation were investigated. TNF-α–primed neutrophils were stimulated with monoclonal antibodies (MAb) to human myeloperoxidase (MPO) and proteinase 3 (PR3), and with preparations of human ANCA (three patients with PR3-ANCA and two patients with MPO-ANCA). Respiratory burst was measured with superoxide dismutase-inhibitable ferricytochrome C reduction and using dihydro-rhodamine-1,2,3. Phosphorylation of p38 mitogen-activated protein kinase (p38-MAPK) and the extracellular signal-regulated kinase (ERK) were assessed by immunoblotting. ANCA-antigen translocation was studied by flow cytometry. The tyrosine phosphorylation inhibitor genistein, but not calphostin or staurosporin, resulted in a reduction and using dihydro-rhodamine-1,2,3. Phosphorylation of p38 mitogen-activated protein kinase (p38-MAPK) and the extracellular signal-regulated kinase (ERK) were assessed by immunoblotting. ANCA-antigen translocation was studied by flow cytometry. The tyrosine phosphorylation inhibitor genistein, but not calphostin or staurosporin, resulted in a significant dose-dependent superoxide generation inhibition (11.6 ± 1.7 nmol O₂⁻ to 2.1 ± 0.5 for PR3-ANCA, and 16.0 ± 2.8 to 3.3 ± 1.3 for MPO-ANCA). The p38-MAPK inhibitor (SB202190) and the ERK inhibitor (PD98059) diminished PR3-ANCA–mediated superoxide production dose dependently (11.6 ± 1.7 nmol O₂⁻ to 1.9 ± 0.6 with 50 μM SB202190 and 4.0 ± 0.6 with 50 μM PD098059, respectively). For MPO-ANCA, the results were similar (16.0 ± 2.8 nmol to 0.9 ± 1.0 nmol with SB202190 and 6.4 ± 2.4 nmol with PD98059, respectively). Western blot showed phosphorylation of both p38-MAPK and ERK during TNF-α priming. The p38-MAPK inhibitor and the ERK inhibitor showed the strongest effect on respiratory burst when added before TNF-α priming, further supporting an important role for both signaling pathways in the priming process. Flow cytometry showed that p38-MAPK inhibition decreased the translocation of PR3 (by 93 ± 2%) and of MPO (by 64 ± 2%). In contrast, no such effect was seen when ERK was inhibited. Thus, p38-MAPK and ERK are important for the TNF-α–mediated priming of neutrophils enabling subsequent ANCA-induced respiratory burst. However, both pathways show differential effects, whereby p38-MAPK controls the translocation of ANCA antigens to the cell surface.
kinases (MAPK). MAPK are activated via phosphorylation of threonine and tyrosine residues by upstream dual-specificity kinases and provide potent inflammatory signaling pathways (29,30). Furthermore, the activation of MAPK activity in neutrophils can be inhibited by genistein (31–33). Therefore, we tested the hypothesis that the p38-MAPK and the extracellular signal-regulated kinase (ERK) are intimately involved in activation of human neutrophils by ANCA.

Materials and Methods

Materials

Plasmagel was obtained from Cellular Products Inc. (Buffalo, NY), and Ficol-Hypaque was obtained from Sigma (Deisenhofen, Germany). Hanks balanced salt solution (HBSS), trypan blue, and phosphate-buffered saline (PBS) were obtained from Seromed (Berlin, Germany). Recombinant TNF-α was obtained from Genzyme (Rüsselsheim, Germany). Genistein, SB202190, PD98059, staurosporin, and calphostin C were purchased from Calbiochem (Bad Soden, Germany). Formyl-methionyl-leucyl-phenylalanine (FMLP), daidzein, bovine erythrocyte superoxide dismutase (SOD; 2500 to 7000 U/mg protein), ferricytochrome C, cytochalasin B, and phospholipid myristate acetate (PMA) were from Sigma. Dihydro-1,2,3 (DHR) was from Molecular Probes (Eugene, OR). The polyclonal rabbit antibodies to tyrosine phosphorylated p38-MAPK and ERK were acquired from New England Biolabs, Inc. (Beverly, MA), horseradish peroxidase-labeled donkey anti-rabbit IgG was from Amersham (Braunschweig, Germany), and FITC-conjugated F(ab)2-fragment of goat anti-mouse IgG was from Dako (Hamburg, Germany). The 96-well microtiter plates were from TPP-Company (Munich, Germany). Endotoxin-free reagents and plastic disposables were used in all experiments.

Isolation of Human Neutrophils

Polymorphonuclear leukocytes (PMN) of healthy human volunteers were isolated from heparinized whole blood by red blood cell sedimentation with plasma gel, followed by Ficol-Hypaque density gradient centrifugation. Erythrocytes were lysed by incubation with hypotonic saline for 15 s. PMN were spun down (200 g, 7 min) and reconstituted in HBSS with calcium and magnesium (HBSS++). PMN (10 μl) in suspension were incubated with 40 μl of trypan blue for 5 min at room temperature. Cells were counted in duplicate using a hemocytometer and considered viable if able to exclude trypan blue. The cell viability was detected in every cell preparation and found to be greater than 99%. The percentage of PMN in the suspension was determined by a Wright-Giemsa staining and by light microscopy.

Preparation of Immunoglobulins

Human IgG was prepared from Wegener’s granulomatosis (three PR3-ANCA) and microscopic polyangiitis (two MPO-ANCA) patients and from three healthy controls. Samples were obtained from freshly drawn blood and kept at −20°C. Plasma was filtered through a 0.2-μm syringe filter (Gelman Sciences, Ann Arbor, MI) and applied to a protein G affinity column (Pharmacia, Uppsala, Sweden). Bound immunoglobulins were eluted with 0.1 M glycine-HCl buffer, pH 2.75 (elution buffer). After the antibodies emerged from the column, the pH was immediately adjusted to pH 7.0 using 1 M Tris-HCl, pH 9.0. A mouse monoclonal anti-PR3 antibody (IgG1κ) previously used in our laboratory (13) was concentrated from tissue culture supernatant by using dia-flo ultrafilter YM 100 (Amicon, Beverly, MA) and purified by protein G column chromatography. A mouse monoclonal to MPO (MPO-7, IgG1k) and an isotype-matched control (IgG1κ) were purchased from Dako.

Superoxide Generation Assay

Superoxide was measured using the assay of SOD-inhibitable reduction of ferricytochrome C as described by Pick and Mizel (34). Briefly, neutrophils were pretreated with 5 μg/ml cytochalasin B for 15 min at 4°C. Cells (1 × 10⁶) were primed with 2 ng/ml TNF-α for 15 min at 37°C before stimuli or buffer control was added. The final concentrations were 5 μg/ml for the MAb to MPO, 15 μg/ml for the MAb to PR3, 75 μg/ml for purified IgG preparations, 1 μM FMLP, and 50 ng/ml PMA. All experiments were set up in duplicates. When indicated, cells were preincubated with inhibitory compounds or buffer control for 30 min on ice before cells were primed with 2 ng/ml TNF-α. The mixture was incubated in 96-well plates at 37°C for up to 120 min, and the absorption of samples with and without 300 U/ml SOD was scanned repetitively at 550 nm using a Microplate Auto-reader. No activating effect was seen when human and mouse control antibodies were used or when cells were primed with 2 ng/ml TNF-α. The baseline activity of TNF-α-treated neutrophils was determined in every experiment and was factored for each condition. In each independent experiment using human ANCA-IgG preparations, three different PR3-ANCA and two different MPO-ANCA preparations were tested.

Measurement of Respiratory Burst by Oxidation of DHR to Rhodamine

The generation of reactive oxygen radicals was additionally assessed using DHR. This method is based on the fact that reactive oxygen radicals cause an oxidation of the nonfluorescence DHR to the green fluorescence rhodamine. In brief, neutrophils (1 × 10⁷/ml HBSS) were placed in polypropylene tubes and kept for 5 min at 37°C in a water bath. Cells were loaded with DHR (1 μM) for 10 min at 37°C. After 15 min of priming with 2 ng/ml TNF-α, cells were divided and 5 × 10⁵ cells were incubated with the stimuli in a total assay volume of 100 μl. When indicated, cells were preincubated with inhibitory compounds or buffer control for 30 min on ice before the priming. After 45 min, the reactions were stopped by adding 900 μl ice-cold PBS/1% bovine serum albumin. Samples were kept on ice and analyzed using a FACSscan (Becton Dickinson, Heidelberg, Germany). Neutrophils were identified in the scatter diagram, and data were collected from 10,000 cells per sample. The shift of green fluorescence in the FL-1 mode was determined. For each condition, the mean fluorescence intensity (MFI, representing the amount of generated reactive oxygen radicals) is reported.

Western Blot Analysis for Phosphorylated p38-MAPK and ERK

PMN were stimulated with TNF-α 2 ng/ml or buffer for 15 min followed by stimulation with the MAb to MPO, isotype control, and buffer control, respectively. Samples were harvested at the indicated time points, and cell lysates were prepared by resuspending 2 × 10⁶ cells in 20 μl of ice-cold lysis solution (20 mM Tris-HCl [pH 8.0] containing 138 mM NaCl, 1% Triton X-100, 2 mM ethylenediaminetetraacetate, 10% glycerol, 0.2 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM quercetin, 5 mM Iodoacetamide). Samples were stored for 20 min on ice and centrifuged at 12,000 × g for 5 min at 4°C. Supernatant was recovered and protein concentration was estimated by bicinchoninic acid protein assay (Pierce, Munich, Germany). Samples were incubated for
5 min at 95°C in loading buffer (250 mM Tris-HCl [pH 6.8] with 4% sodium dodecyl sulfate, 20% glycerol, 0.01% bromphenol blue, 6% β-mercaptoethanol) and 25 μg of protein per lane were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and blotted onto polyvinylidene difluoride membrane by semidy technique. Membrane was blocked in 5% skim milk/0.05% Tween/PBS overnight at 4°C. Phosphotyrosine was detected using specific polyclonal rabbit antibodies to phospho-p38 and phospho-ERK1/2, respectively (1:1,000 dilution) and a horseradish peroxidase-labeled donkey anti-rabbit IgG (1:1,000). Blot was developed by incubation in a chemiluminescence substrate (ECL, Amersham) and exposed to a x-ray film. Densitometry of phospho-p38 and phospho-ERK1/2 was performed with scanned x-ray films and the NIH image program.

Assessment of ANCA-Antigen Expression by Flow Cytometry

Flow cytometry was used to evaluate the effect of genistein, the p38-MAPK inhibitor (SB202190), and the ERK-inhibitor (PD98059) on PR3 and MPO expression on neutrophils. Immunostaining was performed as described previously (13). Cells were preincubated with 50 μM of the inhibitors or buffer control for 30 min on ice, followed by treatment with 2 ng/ml TNF-α or buffer control for 15 min at 37°C. Cells were pelleted at 200 × g for 5 min at 4°C and resuspended in 1 ml ice-cold PBS/0.5% paraformaldehyde. After the cells were washed in HBSS without Ca²⁺/Mg²⁺, they were incubated with dilutions of MAb to PR3, MPO, or an isotype control followed by a secondary FITC-conjugated F(ab)₂-fragment of goat anti-mouse IgG. Flow cytometry was performed on the same day using a FACSscan, and 10,000 events per sample were collected.

Statistical Analysis

Results are given as mean ± SEM. Comparisons were made by t test or ANOVA as appropriate.

Results

Signal Transduction Inhibitors and Respiratory Burst

We first studied the effect of the classical signal transduction inhibitors genistein, staurosporin, and calphostin on the superoxide generation of human neutrophils primed with TNF-α and activated by ANCA. Neutrophils were preincubated with signal transduction inhibitors before the priming with TNF-α and the subsequent stimulation with ANCA. Using the assay of SOD-inhibitable reduction of ferricytochrome C, our results demonstrate that genistein decreased superoxide production by 30 min, whereas staurosporin and calphostin did not (Figure 1A, n = 3). Increasing concentrations of genistein (10⁻⁷ to 10⁻⁴ M) had a progressively greater effect (Figure 1B). We next tested the effect of the inhibitors in experiments using human ANCA as stimulators. Figure 2 indicates that 50 μM genistein also inhibited the respiratory burst when neutrophils were activated with human PR3- and MPO-ANCA, respectively. In each of the five experiments using human PR3 ANCA, ANCA preparations from three different patients were tested, and in each experiment using human MPO-ANCA, ANCA preparations from two different patients were tested. Cells activated with PR3-ANCA released 11.6 ± 1.7 nmol O₂⁻/10⁶ cells per 60 min. Cells that were preincubated with 50 μM genistein released only 2.1 ± 0.5 nmol (P < 0.001). These values for MPO-ANCA without genistein were 16.0 ± 2.8 nmol. Preincubation with genistein resulted in a release of 3.3 ± 1.3 nmol (P < 0.001). To exclude the possibility that genistein resulted in toxic effects that compromised the respiratory burst, we determined the generation of superoxide in PMA-treated cells in the absence and in the presence of 50 μM genistein. Neutrophils were incubated with 50 μM genistein or buffer control for 30 min on ice before the stimulation with 50 ng/ml PMA. At 60 min, cells that were pretreated with buffer control generated 47.7 ± 6.0 nmol O₂⁻/10⁶ cells, and pretreatment with genistein resulted in 45.7 ± 5.0 nmol (n = 3). These data indicate that genistein used at 50 μM did not affect the functional activity to generate superoxide. In addition, we excluded that the observed effect of genistein on ANCA-stimulated respiratory burst was nonspecific by showing that daidzein, an inactive analog of genistein, did not inhibit respiratory burst. After 60 min, cells that were pretreated with...
buffer control and stimulated with the MAb to MPO (5 μg/ml) generated 27.8 ± 2.2 nmol O$_2^-$; Pretreatment with genistein decreased this amount to 11.3 ± 3.2 nmol, whereas daidzein resulted in 30.5 ± 2.5 nmol (n = 3). As demonstrated for the activation with the MAb to MPO, staurosporin and calphostin also had no effect on neutrophil activation by human ANCA (data not shown), whereas calphostin inhibited the superoxide generation in response to PMA (at 60 min from 41.6 ± 5.6 nmol O$_2^-$/10^6 cells to 4.5 ± 3.0 nmol; n = 3), and calphostin decreased superoxide release in neutrophils stimulated with FMLP (at 60 min from 18.1 ± 4.6 nmol to 5.9 ± 2.8 nmol; n = 3), demonstrating that both inhibitors were functioning. These data indicate that tyrosine kinase activation plays a role in the respiratory burst of human neutrophils primed with TNF-α and activated by ANCA.

Because tyrosine kinase activation can result in stimulation of MAPK-dependent pathways, we examined the role of p38-MAPK and ERK in ANCA-mediated superoxide release of human neutrophils. Using the MAb to MPO, we titrated a dose-response curve for the effect of pretreatment of neutrophils with the specific p38-MAPK inhibitor (SB202190) and the ERK inhibitor (PD98059). Figure 3 demonstrates that SB202190 inhibits already at lower concentrations of 1 μM, whereas the inhibitory effect of PD98059 required higher concentrations of 50 μM. Both compounds strongly inhibit at 50 μM and were selected for the experiments with human ANCA and the MAb to MPO and PR3, respectively. In each of the six experiments using human PR3-ANCA, ANCA preparations from three different patients were tested, and in each of the six experiments using human MPO-ANCA, ANCA preparations from two different patients were tested. For clarity, although tested in a continuous superoxide assay, the data are given for the representative 60-min time point of activation (Figure 4). Both components resulted in a significant inhibition of the respiratory burst. Activating neutrophils with MPO-ANCA resulted in 16.0 ± 2.8 nmol O$_2^-$/10^6 cells, whereas preincubation with SB202190 decreased this amount to 0.9 ± 1.0 nmol (P < 0.001) and preincubation with PD98059 to 6.4 ± 2.4 nmol (P < 0.01). PR3-ANCA stimulated 11.6 ± 1.7 nmol O$_2^-$, and this amount was decreased by SB202190 to 1.9 ± 0.6 nmol O$_2^-$ (P < 0.001) and by PD98059 to 4.0 ± 0.6 nmol O$_2^-$ (P < 0.01). These results indicate that p38-MAPK and ERK are important for the respiratory burst of human neutrophils primed with TNF-α and activated by ANCA.

Using the dihydrodihromine oxidation test, we studied the effect of genistein, SB202190, and PD98059 on ANCA-mediated respiratory burst in human neutrophils by a second independent method. The MFI was 11 ± 1 in untreated cells and 20 ± 1 in cells primed with 2 ng/ml TNF-α. The MFI value increased to 578 ± 139 in TNF-α-primed neutrophils activated with the MAb to MPO. This number was decreased to 18 ± 4 by 50 μM genistein, to 18 ± 5 by 50 μM SB202190, and to 267 ± 89 by 50 μM PD98059 (n = 3). A typical experiment is shown in Figure 5. These results confirm the assay of SOD-inhibitable reduction of ferricytochrome C, showing that all three inhibitors decreased the respiratory burst in response to ANCA.
MAPK in Activation of Human Neutrophils by ANCA

Phosphorylation of p38-MAPK and ERK in TNF-α–Primed Neutrophils Activated by ANCA

We performed Western blot analysis to study tyrosine phosphorylation of p38-MAPK and ERK, respectively. Using antibodies that detect only the phosphorylated forms of both kinases, we analyzed the effect of TNF-α priming as well as the effect of the subsequent ANCA stimulation. Figure 6 shows representative immunoblots of phosphorylated p38-MAPK and phosphorylated ERK (Figure 6A) and the corresponding densitometric analysis (Figure 6, B through E). Minimal phosphorylation was seen at 0 time, whereas a significantly increased phosphorylation was observed after priming with TNF-α, reaching a maximum at 5 min. The data showed no significant additional effect of the MAb to MPO on the phosphorylation of neither p38-MAPK nor ERK. With the use of antibodies that recognize the nonphosphorylated forms of both kinases, no change in expression was observed (data not shown). These data suggest an important role for both the p38-MAPK and ERK pathways, during TNF-α–mediated priming of human neutrophils.

We determined the effect of genistein and the specific inhibitors of ERK and p38-MAPK on the tyrosine phosphorylation of ERK and p38-MAPK (Figure 7). Daidzein, an inactive analog of genistein, was used as control. PMN were preincubated with 50 μM of the inhibitors, primed with 2 ng/ml TNF-α and after 15 min activated with 5 μg/ml of the MAb to MPO. On the basis of the kinetics of tyrosine phosphorylation seen in Figure 6, samples were analyzed after 5 min of priming with TNF-α and after 5 min of stimulation with the MAb to MPO, respectively. Our data indicate that genistein and PD98059 but not daidzein inhibited phosphorylation of ERK, whereas none of the inhibitors diminished the phosphorylation of p38-MAPK.

Time-Dependent Inhibition of Superoxide by Genistein, the p38-MAPK Inhibitor, and the ERK Inhibitor

To analyze further the role of p38-MAPK and ERK during TNF-α priming and during the subsequent ANCA stimulation, we studied the effect of the p38-MAPK inhibitor, the ERK inhibitor, and genistein when added at different time points. Figure 8 shows that the effect of all three inhibitory compounds is time dependent. Adding the p38-MAPK inhibitor SB202190 and daidzein 15 min before TNF-α and within in the 15-min time period of TNF-α priming resulted in a strong decrease of superoxide release. Both substances demonstrated a similar degree of inhibition. The inhibiting effect of the ERK inhibitor PD98059 was observed only when the component was added before TNF-α. All three inhibitors did not decrease superoxide release significantly when added 15 min after the MAb to MPO. These experiments provide further support for an important role of tyrosine kinases and particularly for the p38-MAPK and ERK pathway during TNF-α priming.

Effect of the p38-MAPK Inhibitor and the ERK Inhibitor on Translocation of ANCA Antigens

We finally investigated a possible mechanism by which the p38-MAPK and the ERK pathway may control ANCA-stimulated respiratory burst in TNF-α–primed neutrophils. We explored the hypothesis that p38-MAPK and ERK control the TNF-α–mediated translocation of ANCA antigens from the intracellular granules to the cell surface. Using flow cytometry, we showed in eight parallel experiments that inhibiting p38-MAPK with SB202190 (50 μM) resulted in a decreased TNF-α–induced translocation of PR3. Untreated cells showed an MFI for PR3 membrane expression of 31.4 ± 4.4. That number was increased to 66.7 ± 8.6 by 2 ng/ml TNF-α. The TNF-α effect was almost abrogated by SB202190 (34.1 ± 6.4; P < 0.01). MPO membrane expression increased from 4.8 ± 1.1 in untreated cells to 14.3 ± 2.8 after TNF-α treatment and was diminished to 8.2 ± 2.2 by SB202190 (P < 0.05). In contrast, no such inhibitory effect was seen when the ERK pathway was inhibited (50 μM PD98059). A representative FACS analysis for PR3 membrane expression is shown in Figure 9A with the corresponding statistics in Figure 9B. In addition, we assessed the effect of 50 μM genistein on the TNF-α–induced increase in expression of PR3 and MPO (n = 5). The PR3 expression in untreated cells was 27.5 ± 6.1 MFI, and genistein decreased the TNF-α–induced increase of PR3 expression from 69.2 ± 14.0 MFI to 46.4 ± 9.5 MFI (not significant). The MPO expression was 4.2 ± 0.8 MFI for untreated cells, and genistein decreased the TNF-α–induced MPO expression from 11.3 ± 2.4 to 7.3 ± 1.9 (P < 0.05).
indicate that tyrosine phosphorylation and p38-MAPK control the TNF-α–mediated translocation of ANCA antigens to the cell surface.

**Discussion**

TNF-α–pretreated human neutrophils can be activated by ANCA in vitro. The results of this study show that tyrosine phosphorylation plays an important role in this process. Tyrosine kinase activation can result in stimulation of mitogenic signal transduction pathways, including p38-MAPK and ERK. Our data indicate that inhibiting either of these two pathways results in decreased respiratory burst. Furthermore, our data demonstrate that both signaling pathways are crucial for ANCA activation by controlling TNF-α–mediated priming. We show that both kinases act by different mechanisms. P38-MAPK controls the translocation of ANCA antigens to the cell surface. ERK does not seem to be specifically involved in this translocation.

Several groups have shown that MAb to human ANCA antigens and human ANCA itself can activate TNF-α–primed neutrophils and cause the release of reactive oxygen species, toxic-granule components, cytokines, and leukotrienes (7,10–16,35,36). These effector functions may play an important role in inducing inflammation in ANCA-associated vasculitis. Several investigations were performed to determine which part of the ANCA molecule is important for the neutrophil activation, e.g., F(ab')2 fragments versus Fc part (10–13). However, the underlying intracellular signaling events in ANCA-induced activation have not been elucidated in detail. An earlier report indicated that activation of cytokine-primed neutrophils by ANCA IgG results in tyrosine phosphorylation of multiple proteins, including p39, p41, and p46 kD bands, respectively (28). Our observation that genistein abrogates superoxide generation in ANCA-activated TNF-α–primed neutrophils is in agreement with these findings. In addition, our results indicate a functional significance of tyrosine phosphorylation in this activation process.

Neutrophils that are challenged with various stimuli including ultraviolet irradiation, growth factors, and cytokines respond with an activation of the MAPK cascade. MAPK are activated via phosphorylation of threonine and tyrosine residues by upstream dual-specificity kinases and phosphorylate substrates on serine or threonine adjacent to proline residues (29,30). Three MAPK pathways have been described; however, only p38-MAPK and ERK play a role in human neutrophils. No stimulus resulting in activation of Jun N-terminal MAb to MPO (anti-MPO) after preincubation with buffer, 50 μM genistein (Gen), 50 μM SB202190 (SB), and 50 μM PD98059 (PD), respectively. The increase in fluorescence intensity represents the oxidation of the nonfluorescent dihydrorhodamine to the green fluorescent rhodamine reflecting the amount of generated reactive oxygen radicals. A typical of three independent experiments is shown. Preincubation with all three inhibitory compounds decreased the respiratory burst in TNF-α–primed neutrophils that were challenged with the MAb to MPO.

**Figure 5.** Using the dihydrorhodamine oxidation test, the effect of genistein (A), the p38-MAPK inhibitor SB202190 (B), and the ERK inhibitor PD98059 (C) on the respiratory burst of TNF-α–primed neutrophils activated with 5 μg/ml MAb to MPO was assessed in parallel. Each graph shows the results from unstimulated cells (cells), TNF-α–primed cells (TNF), and TNF-α–primed cells treated with...
kinases/stress-activated protein kinase in neutrophils has been reported. With the use of in vitro kinase activity assays, it was shown that bacterial phagocytosis, granulocyte-macrophage colony-forming unit, and TNF-α stimulate ERK and p38-MAPK activation in human neutrophils. This effect was inhibited by the specific inhibitors PD98059 and SB202190 and by the tyrosine phosphorylation inhibitor genistein (31–33). In contrast, cross-linking of the FcγII-receptor resulted in a genistein-independent activation of MAPK (37). Carefully established dose-response experiments for the specific inhibitors PD98059 and SB202190 show a good dose-response curve from 0.1 μM up to 200 μM with a maximal effect observed at approximately 50 to 100 μM (38–41). Here, we demonstrate that genistein and, more important, the specific ERK and p38-MAPK inhibitors PD98059 and SB202190 decreased ANCA-induced respiratory burst in TNF-α-primed neutrophils. These findings indicate an important role of both MAPK pathways for the activation of neutrophils by ANCA. Conceivably, the p39, p41, and p46 kD bands observed by Radford et al. (28) in fact reflect p38-MAPK and ERK (p42/44).

Figure 6. Phosphorylation of p38-MAPK and ERK in response to TNF-α priming and to the subsequent ANCA stimulation was studied by immunoblotting. Cell aliquots were obtained after 0, 2, 5, and 15 min of TNF-α priming (T) with 2 ng/ml and after 2, 5, and 15 min of subsequent treatment with buffer control (C), isotype control (I), and 5 μg/ml MAb to MPO (M), respectively. At each time point, phosphorylated p38-MAPK and ERK was determined by immunoblotting, and a representative example is shown (A). The corresponding densitometric analysis is shown in B through E (n = 5). (B) The effect of TNF-α priming on ERK phosphorylation with the effect of the subsequent antibody treatment in C. (D) The effect of TNF-α priming on p38-MAPK phosphorylation with the effect of the subsequent antibody treatment in E. These data show that p38-MAPK and ERK activation is predominantly a feature of TNF-α priming. (Co) –x–, untreated controls; (T) –○–, TNF-α priming; (M) –□–, MAb to MPO; (I) –△–, isotype control buffer; (C) –○–, control.
We found a rapid tyrosine phosphorylation of ERK and p38-MAPK in response to small priming concentrations of TNF-α peaking at 5 min and an additional, although not significant, increase during the subsequent ANCA stimulation. The strongest effect of the p38-MAPK and ERK inhibitors, as well as of the tyrosine kinase inhibitor genistein, on superoxide generation occurred when the inhibitory compounds were added before TNF-α priming. These data indicate the significance for tyrosine kinases and, in particular, both MAPK pathways in TNF-α-mediated neutrophil priming rather than in the consecutive ANCA stimulation. Other investigators also reported that TNF-α phosphorylates p38-MAPK and ERK observing the highest phosphorylation or kinase activities after 10 min of priming. ERK activation by TNF-α was also demonstrated by Rafiee et al. (32). However, there is some controversy regarding this issue. Other investigators reported that TNF-α activates only p38-MAPK (43,44). We showed that PD98059, the specific inhibitor of the ERK pathway, blocked tyrosine phosphorylation of ERK, whereas the specific inhibitor of the p38-MAPK pathway, SB202190, did not prevent tyrosine phosphorylation of p38-MAPK. This finding is in agreement with reports from the literature; others demonstrated that PD98059 inhibits the upstream MAPK/ERK kinase preventing the phosphorylation and therefore the activation of ERK, whereas SB202190 inhibits the p38-MAPK pathway by competing with ATP for the ATP-binding site on p38-MAPK without affecting phosphorylation of p38-MAPK itself (41,43,45). It is interesting that our data also indicate that genistein prevents phosphorylation of ERK but not of p38-MAPK.

We demonstrated that p38-MAPK and ERK inhibit TNF-α-mediated neutrophil priming via different mechanisms. P38-MAPK inhibition but not ERK inhibition abrogated TNF-α-mediated translocation of ANCA antigens from cytoplasmic granules to the cell surface. Several investigators showed that treatment of resting neutrophils with TNF-α results in increased expression of PR3 and MPO. This process enables an interaction of ANCA with the expressed target antigens, resulting in a full-blown neutrophil activation. Witko-Sarsat et al. (46) showed that within the normal population, the percentage of neutrophils that express PR3 is variable and that a high PR3 expression is significantly more frequent in patients with ANCA-associated vasculitis. Additional activation resulted in a further increase in the amount of PR3 expressed. Conceivably, high membrane PR3-expressing neutrophils could favor the occurrence or the progression of vasculitic inflammation. Thus, understanding the mechanisms that control PR3 and MPO expression is important. To our knowledge, our report is the first indicating that the TNF-α–induced translocation of ANCA antigens from cytoplasmic granules to the cell surface is controlled by p38-MAPK.

Our data demonstrate a similar effect of genistein and the p38-MAPK inhibitor SB202190 on the respiratory burst to ANCA. The strongest inhibition occurred when given before and during TNF-α priming, whereas the ERK inhibitor PD98059 affected only superoxide generation when given before TNF-α. We showed that genistein and SB202190 reduced respiratory burst activity in the entire population of cells, whereas PD98059 decreased the activity in only a portion of...
the neutrophils. In addition, SB202190 and genistein but not PD98059 decreased TNF-α–induced ANCA antigen translocation. Together, these data support our hypothesis that tyrosine phosphorylation and activation of p38-MAPK are important early steps in the translocation of ANCA antigens and activation of neutrophils by ANCA, whereas ERK seems to act by a different mechanism that will be explored in future studies.

In summary, our data indicate that tyrosine phosphorylation and, in particular, MAPK play an important role in the activation of human neutrophils by ANCA. Inhibiting either of these pathways results in decreased respiratory burst, mainly by abrogating TNF-α–mediated priming. Both kinases induce different mechanisms, whereby p38-MAPK but not ERK controls the translocation of ANCA antigens to the cell surface. Pharmacologic blockade of p38-MAPK and ERK may limit inflammatory damage caused by ANCA-activated PMN.

References


