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Tyrosine Phosphorylation of p38 but Not Extracellular Signal-Regulated Kinase in Normal Human Neutrophils Stimulated by Tumor Necrosis Factor: Comparative Study with Granulocyte-Macrophage Colony-Stimulating Factor

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We investigated the cytokine-specific involvement of two members of the microtubule-associated protein kinase family, extracellular signal-regulated kinase (ERK)(1 and 2) and p38, in normal human neutrophils. Both tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) induced tyrosine phosphorylation of a 42-kDa protein in human neutrophils, though the time course of its phosphorylation and its band pattern in electrophoresis differed for each of the cytokines. In addition, GM-CSF, but not TNF, induced a mobility shift of 42-kDa ERK2 in human neutrophils. By using immunoprecipitation followed by immunoblotting, we clarified that GM-CSF, but not TNF, induced tyrosine phosphorylation of ERK2 and that TNF, but not GM-CSF, induced tyrosine phosphorylation of p38. Results of a combined stimulation study showed that tyrosine phosphorylation of ERK2 and that of p38 do not interfere or interact with each other at least in human neutrophils. These results indicate cytokine specific involvement and an independent activating system of ERK and p38 in normal human neutrophils stimulated by two cytokines which share many biological activities in these cells.

Intracellular kinases and phosphorylation of their substrate proteins plays critical roles in signaling pathways in many kind of cells. Among them, microtubule-associated protein kinases(MAPK) have been intensively studied by many investigators of cytokine signaling(1). Recent studies clarified several distinct but related molecules in MAPK family. These include extracellular signal-regulated kinase(ERK), c-Jun N-terminal kinase(JNK), and p38(2). In regard to stimulus specificity of these three molecules, ERK has been proposed to be involved in signaling pathways after growth factor receptor, whereas osmolarity change and inflammatory cytokines such as tumor necrosis factor(TNF) and interleukin 1(IL-1) are known to induce activation of JNK and p38. However, agonist specificity of these MAPK family is not so simple, particularly in TNF. For example, it has been reported that TNF does phosphorylate and activate both 44-kDa ERK1 and 42-kDa ERK2 in human fibroblast(3). In regard to hematopoietic cells, TNF is known to phosphorylate and activate 42-kDa ERK2 in human myeloblastic cell line HL-60(4). Finally, TNF has been reported to phosphorylate 42-kDa ERK2 in normal human neutrophils(5). On the other hand, TNF seems to utilize both ERK and p38 to stimulate human endothelial cells(6).

We have previously reported qualitative similarity of the effects of TNF and granulocyte-macrophage colony-stimulating factor(GM-CSF) on neutrophil functions and signaling pathways(7-9), i. e. both cytokines rapidly primed neutrophils for enhanced release of superoxide upon stimulation, by themselves triggered superoxide release for prolonged time-courses, induced no changes in transmembrane potential and cytoplasmic concentration of free Ca^{2+}, and induced sustained increase in pHi(cytoplasmic alkalinization) with lag time of 2-5 min. In addition, we observed both TNF and GM-CSF induced tyrosine phosphorylation of 42-kDa protein (p42) within 10 min in human neutrophils(9). All these findings suggest that these two cytokines

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Abbreviations used: MAPK, microtubule-associated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; IL-1, interleukin 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; p42, 42-kDa protein.

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stimulate human neutrophils via identical or very similar mechanisms or signaling pathways.

Thus, the aim of the present study is to clarify the qualitative differences in signaling pathways of TNF and GM-CSF by using common target cells of these two cytokines, particularly focused on MAPK family. Results clearly shows agonist-specific involvement of MAPK family in normal human mature neutrophils.

MATERIALS AND METHODS

Reagents. Highly purified recombinant human TNF and GM-CSF produced by *Escherichia coli* were provided by Dainippon Pharmaceutical Co. Ltd., Osaka, Schering Plough Co., Ltd., Osaka, Kirin Brewery Co. Ltd., Tokyo, and Dainippon Pharmaceutical Co. Ltd., Osaka, Japan, respectively. Contamination of lipopolysaccharide was less than 100 pg/mg protein, as determined by limulus amebocyte lysate assay. Conray from Mallinckrodt Inc., St. Louis, MO; and Ficoll from Pharmacia Fine Chemicals Inc., Piscataway, NJ. The monoclonal anti-phosphotyrosine antibody(PY20) were purchased from ICN Biomedicals, Inc., Costa Mesa, CA; polyclonal anti-ERK 1, ERK 2, p38 antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; alkaline phosphatase-conjugated anti-mouse IgG and alkaline phosphatase-conjugated anti-rabbit IgG antibodies from Promega, Madison, WI; and nitrocellulose membrane and protein A-Sepharose from Bio-Rad, Richmond, CA. Other reagents for electrophoresis, Western blotting, and immunoprecipitation were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of cells. Human neutrophils were prepared from healthy adult donors as described(7), using dextran sedimentation, centrifugation with Conray-Ficoll, and hypotonic lysis of contaminated erythrocytes. Neutrophil fractions were suspended in Hanks' balanced salt solution, and contained >95% neutrophils.

Preparation of cell lysate. Human neutrophils(1×10^7/ml) suspended in Hanks' balanced salt solution were prewarmed for 10 min and were then stimulated with various agonists for indicated periods at 37°C. The reactions were terminated by rapid centrifugation, and the pellets were frozen in liquid nitrogen after aspiration of the supernatant. For Western blotting, the cell-pellets were resuspended in ice-cold solution containing 50 mM Hepes(pH. 7.4), 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 10 μg/ml leupeptin. The cell suspension was mixed with 1:1 with 2× sample buffer(4% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol, and a trace amount of bromphenol blue dye in 125 mM Tris-HCl, pH 6.8), heated at 100 °C for 5 min and then frozen at −80 °C until use. For immunoprecipitation, the cell-pellets were resuspended in ice-cold solution containing 50 mM Hepes(pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenymethylsulfonyl fluoride, 100 μg/ml aprotinin, and 10 μg/ml leupeptin, and lysed for 20 min at 4 °C. Insoluble materials were removed by centrifugation for 15 min at 10000g at 4°C.

Western blotting. Samples were subjected to 7.5-11% sodium dodecyl sulfate gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm^2 for 2 hrs at 4°C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffer(150 mM Tris-HCl, pH 7.6) containing 3% bovine serum albumin for 1 hr at 25°C. The blots were then washed in PBS containing 0.05% Tween 20. For Western blotting of phosphotyrosine-containing proteins, the membranes were incubated with monoclonal anti-phosphotyrosine antibody, PY20(1 μg/ml in Tris-buffer containing 1% BSA) for 4 hrs at 25°C. The primary antibody was removed and the blots were washed three times in PBS containing 0.05% Tween 20. To detect antibody reactions, the blots were incubated for 2 hrs with alkaline phosphatase-conjugated anti-mouse IgG antibody diluted 1:2000 in Tris-buffer containing 1% bovine serum albumin, washed three times with PBS containing 0.05% Tween 20, and then placed in a buffer containing 100 mM Tris-HCl(pH 9.7), 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 200 μg/ml nitroblue tetrazolium, and 200 μg/ml 5-bromo-4-chloro-3-indolylphosphate for 10-30 min at 25°C. Enzymatic color development was stopped by rinsing the membranes in deionized water. For Western blotting of MAPK, the membranes were sequentially incubated with a polyclonal anti-MAPK antibodies and alkaline phosphatase-conjugated anti-rabbit IgG antibody. After incubation, enzymatic color development was performed as described above.

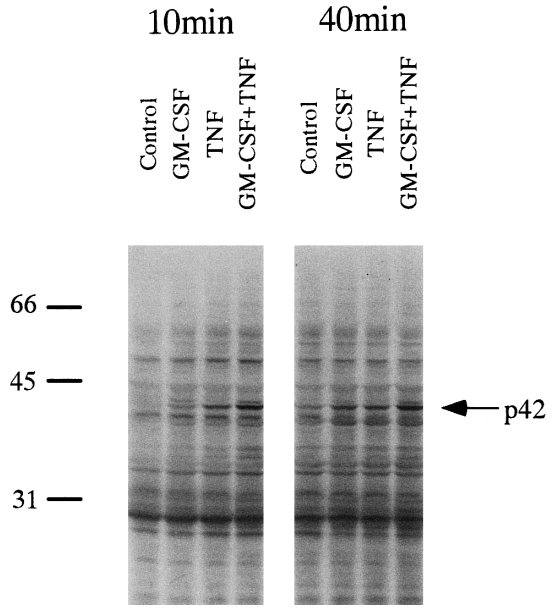

FIG. 1. Tyrosine phosphorylation of proteins in human neutrophils stimulated by GM-CSF and TNF. Cells were stimulated with the optimal concentrations of GM-CSF(5 ng/ml), TNF(50 ng/ml), or GM-CSF plus TNF for 10 min or 40 min at 37°C. Tyrosine phosphorylation of proteins was analyzed by immunoblotting using monoclonal anti-phosphotyrosine antibody.

Immunoprecipitation. The lysates from human neutrophils were precleared with protein A-Sepharose for 1 hr at 4°C. The precleared lysates were then incubated with polyclonal anti-MAPK antibodies or a monoclonal anti-phosphotyrosine antibody(PY20), and the immune complexes were collected using protein A-Sepharose. All of the immunoprecipitates were intensively washed with ice-cold solution containing 50 mM Hepes(pH 7.4), 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 10 μg/ml leupeptin.

RESULTS

In the initial experiment, we evaluated the characteristics of tyrosine phosphorylation of 42-kDa protein (p42) in human neutrophils stimulated by GM-CSF, TNF, and combination of both. As shown in Fig. 1(left panel), tyrosine phosphorylation of p42 was consis-

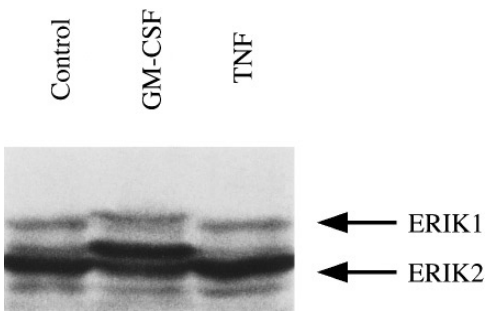

FIG. 2. Mobility shift of ERK1 and ERK2 in human neutrophils stimulated by GM-CSF and TNF. Cells were stimulated with the optimal concentrations of GM-CSF(5 ng/ml) or TNF(50 ng/ml) for 10 min at 37°C. Western blotting was performed by using specific antibody against ERK1 and ERK2.

tently more potent in TNF-stimulated cells than in GM-CSF-stimulated cells, when the cells were stimulated with the maximal concentrations of these two cytokines for 10 min at 37°C, an optimal condition for functional activation of neutrophil by these cytokines(7-9). Putative electrophoretic mobility shift of p42 was observed in GM-CSF-stimulated cells, which is consistent to the hypothesis that p42 is one member of MAPK, ERK2, which is hyperphosphorylated and mobility-shifted upon appropriate stimulation(1). On the other hand, such phenomenon was not observed in TNF-stimulated cells, and p42 in TNF-stimulated neutrophils always consisted of single band. Combined stimulation of neutrophils with GM-CSF plus TNF resulted in the almost additive level of tyrosine phosphorylation of p42. GM-CSF-induced tyrosine phosphorylation of p42 continued to increase for up to 40 min, whereas TNF-induced one retained or decreased during 40 min incubation(9), resulting in almost equal level of tyrosine phosphorylation of p42 at 40 min after the stimulation of both cytokines(Fig. 1, right panel). These findings indicated distinct characteristics of tyrosine phosphorylation of p42 according to the stimuli. Particularly, no detectable mobility shift of p42 in TNF-stimulated cells in spite of its potent tyrosine phosphorylation is of interest and of importance.

To address this question, we determined whether ERK2 in human neutrophils could be mobility-shifted by GM-CSF or TNF. As shown in Fig. 2, mobility shift of 42-kDa ERK2 was clearly demonstrated in GM-CSF-stimulated cells. On the other hand, TNF did have no effect on band pattern of ERK2(Fig. 2). Amount 44-kDa ERK1 was much less in human neutrophils than that of 42-kDa ERK2, and mobility shift of ERK1 was also observed in GM-CSF-stimulated cells, but not in TNF-stimulated cells(Fig. 2).

These findings suggest two possibilities. One is that TNF potently induce tyrosine phosphorylation of ERK2 without its mobility-shift, suggesting selective tyrosine phosphorylation of ERK2 without its serine or threonine phosphorylation by TNF. Another possibility is that TNF does not utilize ERK2 as its signaling molecule and p42 tyrosine-phosphorylated by TNF is not identical to ERK2. To answer this question, we performed immunoprecipitation of ERK2 (and ERK1) followed by immunoblotting of phosphotyrosine containing protein. As shown in Fig. 3(left panel), we confirmed mobility shift of ERK 2 in human neutrophils stimulated by GM-CSF. However, again we could not detect mobility shift of ERK2 induced by TNF(Fig. 3, left panel). Corresponding to these findings, ERK2 was tyrosine-phosphorylated by GM-CSF but not by TNF(Fig. 2, right panel). This finding clearly indicates that TNF does not utilize ERK2 and p42 in TNF-stimulated neutrophils(Fig. 1) is not ERK2, and in addition suggests that TNF phosphorylates certain 42-kDa protein other than ERK2. Combined stimulation of human neutrophils with GM-CSF plus TNF resulted in the effects of GM-CSF alone(Fig. 3). We could neither detect ERK1 itself in the immunoprecipitate nor detect its tyrosine phosphorylation(Fig. 3) probably because of less amount of ERK1 in neutrophils(Fig. 2) and/or less ability of antibody to precipitate ERK1.

It has been recently reported that TNF induce tyrosine phosphorylation of another member of MAPK, p38, and the molecular weight of this protein was almost

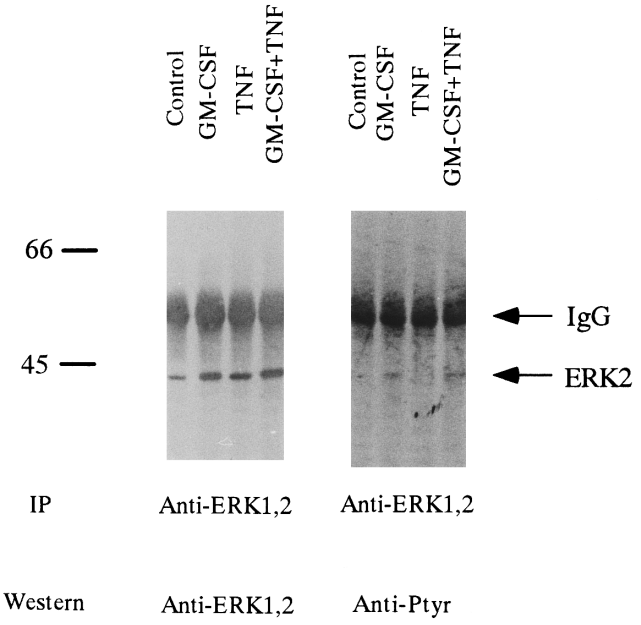

FIG. 3. Tyrosine phosphorylation of ERK2 in human neutrophils stimulated by GM-CSF and TNF. Cells were stimulated with the optimal concentrations of GM-CSF(5 ng/ml), TNF(50 ng/ml), or GM-CSF plus TNF for 10 min at 37°C, and were then solubilized and immunoprecipitated with anti-ERK antibody. Immunoprecipitated samples were blotted with anti-ERK antibody or anti-phosphotyrosine antibody.

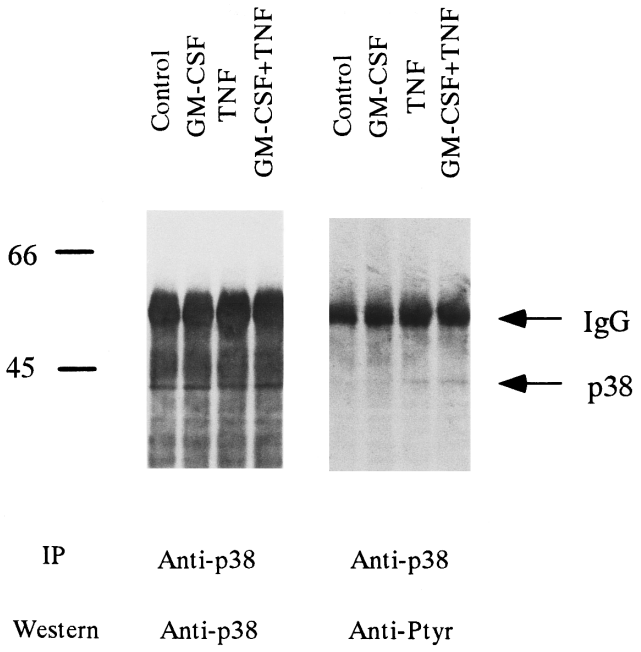

FIG. 4. Tyrosine phosphorylation of p38 in human neutrophils stimulated by GM-CSF and TNF. Cells were stimulated with the optimal concentrations of GM-CSF(5 ng/ml), TNF(50 ng/ml), or GM-CSF plus TNF for 10 min at 37°C, and were then solubilized and immunoprecipitated with anti-p38 antibody. Immunoprecipitated samples were blotted with anti-p38 antibody or anti-phosphotyrosine antibody.

identical to that of 42-kDa ERK2. To explore possible involvement of p38 in human neutrophils, we investigated tyrosine phosphorylation of p38 in suspended human neutrophils stimulated by GM-CSF and TNF by using immunoprecipitation of p38 followed by Western blotting of phosphotyrosine-containing proteins. As shown in Fig. 4, only TNF, but not GM-CSF, induced tyrosine phosphorylation of p38 in human neutrophils. Electrophoretic mobility shift of p38 in TNF-stimulated neutrophils was not detected(Fig. 4), a finding consonant to the result showing no electrophoretic mobility shift of TNF-stimulated p42(Fig. 1). These findings together suggest that p42 in TNF-stimulated suspended human neutrophils was identical to p38, though we can not rule out the possibility that 42-kDa phosphoproteins other than ERK2 and p38 are involved in signaling pathway after TNF-receptors.

Combined stimulation of human neutrophils with TNF plus GM-CSF induced further level of tyrosine phosphorylation of p42 as compared with TNF alone(Fig. 1). However, TNF induced no significant effect on GM-CSF-induced tyrosine phosphorylation of ERK2(Fig. 3), and GM-CSF induced no significant effect on TNF-induced tyrosine phosphorylation of p38(Fig. 4). Thus, apparent additive effect of TNF and GM-CSF on p42(Fig. 1) is highly likely to represent the sum of tyrosine phosphorylation of two different proteins with almost identical molecular weight in Western blotting.

DISCUSSION

In the present study, we demonstrated GM-CSF selectively induced tyrosine phosphorylation of ERK MAPK, whereas TNF selectively induced that of p38 MAPK in normal human suspended neutrophils. In addition, combined study clarified activating systems for these two different kinds of MAPK do not interfere nor interact with each other, suggesting independent regulation of upstream pathways of ERK and p38 at least in normal human neutrophils stimulated by GM-CSF and TNF.

It has been recently proposed that ERK family of MAPK and p38 or JNK family of MAPK mediate different signals from different agonists or stimuli and are linked to different cell biological phenomena, i.e.:ERK mainly transduces growth-promoting or differentiation-inducing signals from growth factor receptor, whereas p38 and JNK transduce apoptotic signals from cellular shock and proinflammatory cytokines such as TNF and IL-1(2). These notions, however, are not applicable to all situations. For example, TNF has been reported to activate and phosphorylate ERK in human fibroblasts(3), human myeloblastic cell line(4), and normal human neutrophils(5), and TNF activates both ERK and p38 in human endothelial cells(6). It is also known that TNF induces proliferation rather than apoptosis in human fibroblast(10). In addition, more recent report describes Ras-dependent activation of JNK in murine cell lines stimulated by granulocyte colony-stimulating factor and interleukin 3, though this JNK-related signaling pathway does not seem to be linked to cell proliferation(11). On the other hand, upstream molecule of JNK and p38 seems to contribute to cell survival rather than apoptosis in certain situations(12). All these findings together indicates complexed patterns and complicated significance of MAPK-related intracellular signaling pathways, though some of the previous findings obtained from murine or human cell lines may not be applicable to normal human system. In this paper, we are presenting data by using normal human hematopoietic cells stimulated by physiological cytokines.

Signaling pathway after TNF-receptor are heterogeneous even among normal human mature phagocytes according to the cell types. In fact, TNF did not induce tyrosine phosphorylation of any protein in human monocytes in spite of its potent stimulatory effects on monocyte functions(13). This is not because of low amount of p38 protein in monocytes, since both neutrophils and monocytes possess equivalent amount of p38, as determined by Western blotting(unpublished obser-

vation). Alternatively, these different findings between neutrophils and monocytes may represent the difference in upstream signaling pathways of p38 and/or JNK, though the critical point which explains the clear difference between neutrophils and monocytes remains to be determined.

Results for the current investigation provided evidence for qualitative differences of intracellular signaling pathways between TNF and GM-CSF in human neutrophils. On the other hand, there are no qualitative differences between these two cytokines regarding their effects on neutrophil functions, and the effects of TNF are consistently more potent than those of GM-CSF(7-9). Different roles of ERK and p38 in functional activation of phagocytes and causal relationship between MAPK-related pathways and functional activation of phagocytes are at present unclear and await further investigation. On the other hand, apoptosis-inducing effect of TNF on neutrophil(14) may be related to its effect on p38 in the same cells(present results).

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