The Role of STAT3 in Granulocyte Colony-stimulating Factor-induced Enhancement of Neutrophilic Differentiation of Me2SO-treated HL-60 Cells

GM-CSF INHIBITS THE NUCLEAR TRANSLLOCATION OF TYROSINE-PHOSPHORYLATED STAT3

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The role of granulocyte colony-stimulating factor (G-CSF) on neutrophilic differentiation of Me2SO-treated HL-60 cells was studied. G-CSF augmented the functional maturation of Me2SO-treated HL-60 cells in terms of both O2-generating ability and expression of the formyl-methionyl-leucyl-phenylalanine receptor. G-CSF induced enhancement of cell growth in Me2SO-treated HL-60 cells. These results indicate that G-CSF is a potent enhancer for the differentiation and proliferation of Me2SO-treated HL-60 cells. G-CSF caused the activation of p70 S6 kinase but not mitogen-activated protein (MAP) kinase. On the other hand, G-CSF rapidly induced tyrosine phosphorylation of signal transducers and activators of transcription-3 (STAT3), but did not induce serine727 phosphorylation. From the analysis of confocal laser scanning fluorescence microscopy and differential centrifugation, it was clearly demonstrated that G-CSF induced nuclear translocation of tyrosine-phosphorylated STAT3. The G-CSF-dependent enhancement of neutrophilic differentiation in Me2SO-HL-60 cells was reversely inhibited by granulocyte-macrophage colony-stimulating factor (GM-CSF). Notably, in the presence of GM-CSF, G-CSF induced the tyrosine phosphorylation of STAT3 but failed to induce the nuclear translocation of tyrosine-phosphorylated STAT3. GM-CSF induced activation of not only p70 S6 kinase, but also of MAP kinase. Furthermore, GM-CSF caused the rapid serine727 phosphorylation of STAT3, both in the presence and absence of G-CSF. PD98059, an MEK1 inhibitor, inhibited the G-CSF-dependent serine727 phosphorylation of STAT3 and blocked the inhibitory effect of GM-CSF on G-CSF-dependent nuclear translocation of STAT3. These results suggest that G-CSF-dependent nuclear translocation of STAT3 coordinates with the promotion of neutrophilic differentiation in Me2SO-treated HL-60 cells.

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that is critical for supporting normal neutrophil production and maturation (1, 2). G-CSF stimulates the proliferation, survival, and maturation of cells committed to neutrophilic lineage (3, 4). The diverse biological effects of G-CSF are mediated through a single class of cell-surface receptor protein that forms homodimeric complexes on ligand binding (5). The human G-CSF receptor is a member of the hematopoietin receptor superfamily (6, 7). On the other hand, granulocyte-macrophage colony-stimulating factor (GM-CSF) is also thought to play an important role in the proliferation and maturation of neutrophil and macrophage lineage cells (6, 7).

Promyelocytic leukemia HL-60 cells are known to differentiate into macrophages or neutrophils in response to several stimuli. Dimethyl sulfoxide (Me2SO), retinoic acid (RA), and Bt2cAMP cause the neutrophilic differentiation of HL-60 cells, whereas interferon-γ and phorbol ester cause the differentiation of macrophages (8–10). Because of these advantages, many studies have been performed on HL-60 differentiation as a model of proliferation and maturation of myelogenic lineage cells. Several reports have suggested that although G-CSF cannot itself induce neutrophilic differentiation of HL-60 cells, it can potentiate the neutrophilic differentiation of RA and Me2SO-treated HL-60 cells in terms of the O2-generating ability (11–13). The molecular mechanism of G-CSF role on the neutrophilic differentiation of HL-60 cells has not been fully elucidated. Conversely, GM-CSF has been shown to directly induce the monocytic differentiation of HL-60 cells (14), and also to induce the re-proliferation in Me2SO-treated HL-60 cells in which growth has been suppressed (15). The effect of GM-CSF on the neutrophilic-differentiation of HL-60 cells remains unclear.

The addition of G-CSF causes the activation of several signaling pathways in myelogenic cells, including the JAK and STAT pathway, as well as the Ras-mitogen-activated protein (MAP) kinase cascade (16), and in particular the rapid activation of STAT3 and STAT1 (17). Activation of MAP kinase has been reported in the cells that proliferate in response to G-CSF (18, 19). Nicholson et al. (19) have suggested that phosphorylation of MAP kinase was correlated with both proliferative response and JAK2 activation. On the other hand, the importance of p70 S6 kinase in cell cycle progression of numerous cells has been reported (20). However, it has not yet been studied whether or not G-CSF signaling cascade involves p70 S6 kinase. Furthermore, it also remains unclear how these signaling pathways coincide with the proliferation and differentiation of neutrophil lineage cells.

In the present study, we analyzed the G-CSF-dependent enhancement of differentiation of Me2SO-treated HL-60 cells, and observed the cross-talk of G-CSF and GM-CSF on this
determination. Furthermore, to clarify the molecular mechanism of the role of G-CSF on neutrophil differentiation, G-CSF-dependent activations of STAT3, MAP kinase, and p70 S6 kinase were studied in Me2SO-treated HL-60 cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human recombinant G-CSF (Chugai Pharmaceutical Co., Tokyo Japan) and human recombinant GM-CSF (Dainippon Pharmaceutical Co., Osaka Japan) were obtained. Anti-STAT3 (monoclonal antibody (mAb), Transduction Lab., S21320), and anti-tyrosine-phosphorylated STAT3 (polyclonal antibody (pAb), New England Biolab Inc., 9138), anti-activity 727-phosphorylated STAT3 (pAb, New England Biolab Inc., 9134S), and anti-phosphorylated MAP kinase (New England Biolab Inc., 9100) antibodies were purchased from the aforementioned companies. Anti-human C3bi receptor mAb was purchased from Nichirei Co. (Tokyo, Japan) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and anti-rabbit IgG were obtained from Jackson Immuno Research Lab., Inc. (West Grove, PA). FITC-N-formyl Met-Leu-Ph-e-Lys (fMLP) was purchased from Peninsula Lab. Inc. (Belmont, CA). Horse heart ferricytochrome c, MLF, zymosan A, and bovine erythrocyte superoxide dismutase were obtained from Sigma. Zymosan particles were opsonized by gently mixing with freshly prepared guinea pig serum (50 mg of zymosan to 5 ml of serum) for 30 min at 37 °C. Red blood cells were washed several times with HEPES-buffered saline (HBS, 122 mm NaCl, 4.9 mm KCl, 1 mm MgCl2, 16.7 mm HEPES, adjusted to pH 7.4 with NaOH). The opsonized zymosan particles were suspended in HBS (50 mg/ml) and used as a stimulant for O2 generation at a final concentration of 2.5 mg of zymosan equivalent/ml.

**Cell Culture—**HL-60 cells were kindly supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in a RPMI 1640 culture medium containing 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin at 37 °C in moisturized air containing 5% CO2. To standardize the quality of cells throughout this series, frozen cells of the same lot were thawed every three months and used for experiments. Differentiation was initiated by dispersing the cells in a fresh culture medium containing 1.25% (v/v) Me2SO to make a final cell density of about 2.5 × 106 cells/ml. G-CSF and/or GM-CSF were added to the cell suspension 2 days after the addition of Me2SO. After culturing for 5 days, the cells were collected by centrifugation at 600 × g for 5 min and were washed several times with HBS. The resultant cells were resuspended in HBS and kept in an ice-cold bath until used for analyses. The viability of the cells was evaluated by the trypan blue dye-staining exclusion method, and more than 95% viability was confirmed in HL-60 cells treated with or without differentiating agents.

**O2-generating Activity—**O2 generation by differentiated cells was measured by ferricytochrome c reduction assay (21). The cells (1.0 × 106) were preincubated in a substrate solution (50 μM ferricytochrome c, 5 mM d-glucose, and 0.5 mM CaCl2 in HBS) for 10 min at 37 °C, and the assay was initiated by the addition of a stimulant. The rate of O2 generation was measured by continuously recording the increase in absorbance at 550–540 nm using a Hitachi 557 double-beam spectrophotometer. In the assay with insoluble stimulants, such as opsonized zymosan particles, the assay mixture was constantly stirred by a windmill mixer as described previously (22).

**Expression of C3bi and fMLP Receptors on the Cell Surface—**The expression of stimulant-receptors on the cell surface was examined by flow cytometry with a Cyto-ACE-15 Auto Cell Screener (JASCO Co., Tokyo, Japan). For the detection of C3bi receptor, cells (106 cells/100 μl of phosphate-buffered saline, PBS) were incubated with anti-human Mac-1 monoclonal antibody for 1 h at 4 °C. The cells were then washed several times in PBS, and incubated with FITC-conjugated goat anti-mouse IgG in 100 μl of PBS for 1 h at 4 °C. The cells were again washed several times with PBS and were finally suspended in an aliquot of PBS and subjected to flow cytometry.

The FITC-labeled chemotactic peptide (FITC-fMLP) was utilized to estimate the expression of the fMLP receptor on the cell surface (23, 24). Approximately 106 cells were incubated with 0.1 μM FITC-fMLP in HBS containing 2% (w/v) bovine serum albumin (BSA) for 30 min at 4 °C. The experiment was performed in the absence of the specific binding of the peptide to the receptor. The cells were preincubated with 10 μM fMLP for 15 min at 4 °C, and then 0.1 μM FITC-fMLP was added. After the incubation with the FITC-labeled peptide, the cells were collected by centrifugation at 600 × g for 5 min and washed twice with HBS containing 0.2% (w/v) BSA. The washed cells were suspended in 1 ml of HBS containing 0.2% (w/v) BSA and subjected to flow cytometry.

**Immunoblot Analysis—**Fifty nanograms/ml G-CSF and/or GM-CSF were added to Me2SO-treated HL-60 cells, and then three cells were incubated for 5 to 60 min at 37 °C. After the appropriate incubation time, the cells were mixed with chilled PBS containing a mixture of phosphatase and protease inhibitors and were transferred into an ice bath. The mixed cells were centrifuged and dissolved with radiimmune precipitation assay buffer to 107 cells/0.1 ml (25). The extract of the cells was prepared by short time sonication and subjected to Western blot analysis of Map kinase, p70S6 kinase, and STAT3. 10 to 30 μg of the extracted protein was separated onto an SDS-PAGE gel and transferred to a nitrocellulose membrane (Highbond ECL™ film; Amersham Pharmacia Biotech). Membranes were incubated with the primary antibody: anti-STAT3-ERK (1:2000, pAb prepared in rabbit), anti-p70 S6 kinase (1:2000, pAb prepared in rabbit), anti-tyrosine-phosphorylated STAT3 (1:1000) and anti-serine 727-phosphorylated STAT3 and then visualized with secondary antibodies coupled to peroxidase and an ECL reagent (Amersham Pharmacia Biotech).

In order to analyze nuclear translocation of tyrosine-phosphorylated STAT3, after the incubation with G-CSF and/or GM-CSF, HL-60 cells were collected in a hypotonic buffer containing phosphatase and protease inhibitors by differential centrifugation as previously reported (26). After vortexing for 10 s, nuclei were sedimented by centrifugation at 10,000 rpm for 5 min. Nuclear fractions were dissolved in SDS-sample buffer containing Benzon nuclease. The nuclear extracts were subjected to Western blotting analysis with anti-tyrosine-phosphorylated STAT3. The analysis of the alteration of STAT3 localization in Me2SO-treated HL-60 cells by G-CSF and/or GM-CSF was examined by confocal laser-activated microscopy (ACAS ULTIMA, Meridian Instruments and Zeiss LSM410, Carl Zeiss Inc.). The cells were treated with 50 ng/ml G-CSF and/or GM-CSF for 30 or 60 min. After the incubation, the cells were fixed with an equal volume of 3.8% paraformaldehyde in PBS and then washed with PBS. After treatment with ethanol for 5 min, the fixed cells were incubated with anti-STAT3 (1:100, mAb) or anti-tyrosine-phosphorylated STAT3 antibodies for 1 h and visualized with secondary antibody coupled to FITC. Cells were examined using an ACAS ULTIMA or Zeiss LSM410 microscope, with an extinction wavelength of 488 nm, emission of 530/50 nm.

**RESULTS**

**Effects of G-CSF and GM-CSF on the O2-generating Ability of HL-60 Cells Differentiating to Neutrophil-like Cells—**The neutrophilic differentiation of HL-60 cells was initiated by incubation with 1.25% (v/v) Me2SO. Two days after the addition of Me2SO, 50 ng/ml G-CSF and/or 50 ng/ml GM-CSF were added to Me2SO-treated cells, and then the cells were incubated for 5 days. Fig. 1 shows the O2-generating activities of the differentiated HL-60 cells. The O2 generation from the G-CSF-treated HL-60 cells in response to fMLP or OZ was found to be much higher than that of the cells treated with Me2SO alone, as reported previously (13). On the other hand, the incubation with GM-CSF reduced the fMLP or OZ-induced O2-generating ability of HL-60 cells. It was noted that GM-CSF markedly reduced the G-CSF-dependent enhancement of neutrophilic differentiation of HL-60 cells in terms of O2-generating ability. As previously reported (13), nondifferentiated HL-60 cells did not produce O2 in the presence of fMLP or OZ (data not shown).

**G-CSF but Not GM-CSF Enhances the Expression of the fMLP Receptor in HL-60 Cells during Neutrophilic Differentiation—**Expressions of the fMLP and OZ (C3bi) receptors on the surface of HL-60 cells during the neutrophilic differentiation were analyzed by flow cytometry. As previously reported (13), the fluorescent signal representing the C3bi receptor remarkably increased in HL-60 cells from the treatment of Me2SO (data not shown). The expression of the C3bi receptor in the Me2SO-treated HL-60 cells, however, was not altered by incubation with either G-CSF or GM-CSF. On the other hand, the expression of the fMLP receptor in these cells markedly changed after incubation with either G-CSF and/or GM-CSF (Fig. 2). The expression of fMLP-receptor (fMLP-R) in Me2SO-differentiated HL-60 cells was characterized by both a high and a low level population, whereas the nondifferentiated cells ex-
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Differentiation of HL-60 cells was initiated by dispersing the cells in a fresh culture medium containing 1.25% (v/v) Me2SO to establish a final cell density of about 2.5 × 10⁶ cells/ml. Two days after the addition of Me2SO, G-CSF and/or GM-CSF were added to the cell suspension and then the cells were incubated for 5 days. The O₂⁻ generation from the cells was measured by means of a ferricytochrome c reduction assay. The cells (1 × 10⁶) were preincubated in a substrate solution (50 μM ferricytochrome c, 5 mM d-glucose, and 0.5 mM CaCl₂ in HBS) for 10 min at 37 °C, and the assay was initiated by the addition of either 500 nM fMLP (a) or 1.25 mg/ml opsonized zymosan (b). Data represent the mean ± S.D. from four experiments. *, p < 0.01 versus control.

**FIG. 1.** Superoxide generation by differentiated HL-60 cells. Differentiation of HL-60 cells was initiated by dispersing the cells in a fresh culture medium containing 1.25% (v/v) Me2SO to establish a final cell density of about 2.5 × 10⁶ cells/ml. Two days after the addition of Me2SO, G-CSF and/or GM-CSF were added to the cell suspension and then the cells were incubated for 5 days. The O₂⁻ generation from the cells was measured by means of a ferricytochrome c reduction assay. The cells (1 × 10⁶) were preincubated in a substrate solution (50 μM ferricytochrome c, 5 mM d-glucose, and 0.5 mM CaCl₂ in HBS) for 10 min at 37 °C, and the assay was initiated by the addition of either 500 nM fMLP (a) or 1.25 mg/ml opsonized zymosan (b). Data represent the mean ± S.D. from four experiments. *, p < 0.01 versus control.

**FIG. 2.** Expression of fMLP receptor and C3bi on the differentiated HL-60 cells. Differentiation of HL-60 cells was induced in the same manner as in Fig. 1. Approximately10⁵ cells were incubated with 0.1 μM fMLP-FITC in HBS containing 2% (v/v) bovine serum albumin for 30 min at 4 °C. For the evaluation of the specific binding of the peptide to the receptor, the cells were preincubated with 10 μM fMLP for 15 min at 4 °C prior to adding 0.1 μM fMLP-FITC. After the incubation with the FITC-labeled peptide, the cells were collected by centrifugation at 600 × g for 5 min and then washed twice with HBS containing 0.2% (w/v) bovine serum albumin. The washed cells were suspended in 1 ml of HBS containing 0.2% (w/v) bovine serum albumin and subjected to flow cytometry. For the detection of a C3bi receptor, the cells (10⁶ cells/100 μl of PBS) were incubated with 5 μl of diluted mouse ascitic fluid containing anti-human-C3bi receptor monoclonal antibody for 1 h at 4 °C. Washing with PBS several times, the cells were incubated with FITC-conjugated goat anti-mouse IgG in 100 μl of PBS for 1 h at 4 °C.

**FIG. 3.** G-CSF and/or GM-CSF induce cell proliferation in Me2SO-treated HL-60 cells. The differentiation of HL-60 cells was induced in the same manner as in Fig. 1. After 2 days of incubation, 50 ng/ml G-CSF and/or GM-CSF were added to HL-60 cell suspension, and the cells were subsequently cultured for various periods. The number of cells was determined at each indicated time. Open circle, Me2SO alone; closed circle, with G-CSF; open triangle, with GM-CSF; closed triangle, with G-CSF and GM-CSF. Data represent the mean ± S.D. from three experiments. *, p < 0.01 versus control; **, p < 0.01 versus G-CSF.

**FIG. 4.** Time After Addition of DMSO (Days)

level fMLP-R-expressing cells that were treated with Me2SO alone. Furthermore, in the presence of GM-CSF, G-CSF failed to enhance high level fMLP-R-expressing cells. These results, therefore, suggest that G-CSF augments the neutrophilic differentiation of Me2SO HL-60 cells, whereas GM-CSF inhibits neutrophilic differentiation of HL-60 cells.

**Effect of G-CSF and GM-CSF on Signal Transduction**—The previously mentioned results indicate that G-CSF acts as an accelerator on the maturation of HL-60 cells to neutrophils, whereas this action is inhibited by GM-CSF. Accordingly, we examined the cross-talk of these cytokines in terms of the signal transduction pathways.

Fig. 4 shows the effects of G-CSF and/or GM-CSF on the activation of MAP kinase and p70 S6 kinase. Within 5 min, GM-CSF clearly induced a mobility shift of MAP kinase, indicating the phosphorylation of MAP kinase. The GM-CSF-dependent MAP kinase activation gradually ceased within 30 min (Fig. 4c). The phosphorylation of MAP kinase by GM-CSF was confirmed by Western blot using anti-phosphorylated MAP kinase antibody (Fig. 4b). An SDS-PAGE mobility shift assay indicated that GM-CSF also induced a delayed phosphorylation of p70 S6 kinase relative to that of MAP kinase (Fig. 4c).

G-CSF, on the other hand, did not induce the activation of MAP kinase but caused a similar delayed activation of p70 S6 ki-
The mobility shift of ERK by phosphorylation was analyzed by dispersing the cells in a fresh culture medium containing 1.25% Me2SO in RPMI 1640 containing 10% fetal bovine serum. G-CSF and/or GM-CSF were added to the cell suspension 2 days after the addition of Me2SO. After incubation for the indicated time, the cells were mixed with ice-chilled PBS and transferred into an ice bath. Then, the cells were sedimented and dissolved with radioimmune precipitation assay buffer. Ten to 30 μg of the extracted protein was separated on an SDS-PAGE gel and transferred onto a nitrocellulose membrane. All lanes in each panel have the same total protein. The arrowhead indicates the mobility shift of ERK by phosphorylation.

The addition of both G-CSF and GM-CSF caused the rapid activation of MAP kinase and the delayed activation of p70 S6 kinase.

Next, we examined whether STAT3 is activated upon the addition of G-CSF and/or GM-CSF. As shown in Fig. 5b, G-CSF induced a tyrosine phosphorylation of STAT3 in Me2SO-treated HL-60 cells, whereas GM-CSF alone did not. G-CSF-dependent tyrosine phosphorylation of STAT3 continued for 30 min. These results are consistent with those of the G-CSF- and GM-CSF-coupled JAK-STAT pathways (16, 26). On the other hand, Sengupta et al. (27) reported that tyrosine phosphorylation of STAT3, which was induced by IL-6, was partially inhibited by GM-CSF. In the present experiment, G-CSF-induced tyrosine phosphorylation of STAT3 was not affected by GM-CSF in the Me2SO-treated HL-60 cells, as shown in Fig. 5. Several investigators have reported that maximal activation of transcription by STAT3 requires both tyrosine and serine phosphorylation (28–31). Serine-phosphorylated STAT3 shows a mobility shift on SDS-PAGE (32). GM-CSF induced the rapid SDS-PAGE mobility shift of STAT3. However, G-CSF caused a delayed SDS-PAGE mobility shift of STAT3. As shown in Fig. 5c, the experiment using anti-serine 727-phosphorylated STAT3 antibody showed that GM-CSF caused rapid serine 727 phosphorylation in STAT3, which corresponded to the SDS-PAGE mobility-dependent assay; however, G-CSF did not induce the serine 727 phosphorylation of STAT3.

STAT3 is thought to be activated at the plasma membrane by tyrosine phosphorylation and translocated into the nucleus to induce the transcription of target genes. We, therefore, analyzed the cytokine-dependent nuclear translocation of STAT3 in Me2SO-treated HL-60 cells by using confocal microscopy. As shown in Fig. 6a (G-CSF), G-CSF caused the translocation of STAT3 into nuclei within 60 min. It was, however, demonstrated that in the presence of GM-CSF, G-CSF failed to cause the nuclear translocation of STAT3, even when the STAT3 was tyrosine-phosphorylated (Fig. 5b). Furthermore, GM-CSF alone did not alter the cellular location of STAT3. We therefore hypothesized that the activation of the GM-CSF-specific signal cascade disturbs the nuclear translocation of STAT3, and therefore examined the effect of MEK1 inhibitor, PD98059, on STAT3 translocation. As shown in Fig. 6 (PD-98059+G/GM-CSF), in the presence of PD98059, the addition of both G-CSF and GM-CSF caused the nuclear translocation of STAT3.

G-CSF-dependent nuclear translocation of STAT3 was analyzed using anti-tyrosine 705-phosphorylated STAT3 pAb. As shown in Fig. 6b, whereas control cells and GM-CSF-treated cells did not show any response to anti-tyrosine-phosphorylated STAT3 antibody, G-CSF resulted in translocation of tyrosine-phosphorylated STAT3. In the presence of GM-CSF, G-CSF prompted the tyrosine phosphorylation of STAT3 but failed to induce the translocation of tyrosine-phosphorylated STAT3 into a nuclear portion. In the presence of PD98059, upon the addition of both cytokines, tyrosine-phosphorylated STAT3 was translocated into a nuclear portion.

On the other hand, a rapid mobility shift of STAT3 induced by GM-CSF was clearly inhibited by PD98059 (Fig. 7a). In the presence of either G-CSF alone or both cytokines, tyrosine-phosphorylated STAT3 exhibited an SDS-PAGE mobility shift as compared with that in the presence of PD98059. In the presence of PD98059, the addition of G-CSF or both cytokines caused a delayed SDS-PAGE mobility shift in tyrosine-phosphorylated STAT3, suggesting that the G-CSF-dependent delayed serine (or threonine) phosphorylation may be catalyzed by kinases other than MAP kinase, or its downstream kinase. Furthermore, the SDS-PAGE mobility shift pattern of G-CSF-dependent tyrosine-phosphorylated STAT3 differed from that of STAT3 with G-CSF, suggesting that a small amount of STAT3 was phosphorylated to tyrosine residue upon the addition of G-CSF. Using an anti-serine 727-phosphorylated STAT3 antibody, GM-CSF induced a rapid serine 727 phosphorylation of STAT3, which was markedly inhibited by the addition of PD98059, whereas G-CSF alone did not induce the serine 727 phosphorylation, suggesting that the delayed serine (or threonine) phosphorylation of STAT3 induced by G-CSF may be a result other than from the serine 727 residue. In the presence of PD98059, the addition of GM-CSF did not induce the activation of MAPK (data not shown).

To confirm the nuclear translocation of STAT3, we prepared a nuclear fraction from HL-60 cells and determined the content of tyrosine-phosphorylated STAT3. As shown in Fig. 7d, tyrosine-phosphorylated STAT3 was detected in the nuclear fraction in G-CSF-treated cells but not that in both cytokine-
treated cells. Furthermore, in the presence of PD98059, tyrosine-phosphorylated STAT3 was detected in nuclear fractions in G-CSF-treated cells and both cytokine-treated cells. These results indicate that the GM-CSF inhibits G-CSF-dependent nuclear translocation and PD98059 blocks the inhibition.
and a nuclear fraction was prepared as described under “Experimental whole cell extracts. For the analysis of nuclear translocation of tyrosine-performed as in Fig. 5. Panels with or without PD98059 for 30 min, G-CSF and/or GM-CSF were added to the cell suspension, and then the extraction of cells was performed as in Fig. 5. Panels a, b, and c indicate the Western blots of whole cell extracts. For the analysis of nuclear translocation of tyrosine-phosphorylated STAT3, cells were disrupted with a hypotonic solution, and a nuclear fraction was prepared as described under “Experimental Procedures” (d).

**DISCUSSION**

It is known that HL-60 cells possess the ability to differentiate into neutrophilic cells by treatment with Me2SO (8) or RA (9). Several reports have suggested that G-CSF cannot itself induce neutrophile differentiation in nondifferentiated HL-60 cells, but it can potentiate the differentiation into neutrophils initiated by Me2SO or RA (11–13). However, the role of G-CSF in the development of functional maturation during the differentiation of HL-60 cells into neutrophilic cells has not been extensively elucidated. We confirmed that G-CSF enhanced the neutrophile differentiation of Me2SO-treated HL-60 cells (Figs. 1 and 2). However, whereas GM-CSF has been shown to be a potential inducer of monocytic differentiation of human myeloid leukemia cells, including HL-60 cells (33), the effect of GM-CSF on neutrophile differentiation of HL-60 cells remains unclear. In this paper, we showed that GM-CSF inhibited the neutrophile differentiation of Me2SO-treated HL-60 cells in terms of O2- generating ability and fMLP-R expression. Furthermore, the G-CSF-dependent enhancement of neutrophile differentiation in Me2SO-treated HL-60 cells was markedly inhibited by GM-CSF, suggesting that GM-CSF acts as an antagonist of G-CSF in regard to the neutrophile differentiation of HL-60 cells.

As a result of this data, we postulated that a G-CSF-specific signal transduction pathway may be implicated in the enhancement of neutrophile differentiation of HL-60 cells. It has been reported that G-CSF activates STAT3 through the activation of JAK2, whereas GM-CSF activates STAT5 through the activation of JAK2 (17, 19, 26, 34–37). In a study using dominant negative STAT3, Minami et al. (37) speculated that STAT3 activation is a critical step in gpl30-mediated terminal differentiation and growth arrest in a myeloid cell line. In this paper, we showed that G-CSF activated STAT3 by phosphorylation of tyrosine residue, whereas GM-CSF alone did not. Sengupta et al. (27) reported that GM-CSF partially inhibited interleukin-6 (IL-6)-induced tyrosine phosphorylation of STAT3. In this study, the addition of GM-CSF did not inhibit the tyrosine phosphorylation of STAT3 in Me2SO-treated HL-60 cells. This discrepancy may be caused by a difference of cell type-specific activation of STAT3.

In the presence of GM-CSF, however, G-CSF failed to induce the nuclear translocation of STAT3, even though G-CSF induced the tyrosine phosphorylation of STAT3. The inhibitory effect of GM-CSF on the nuclear translocation of tyrosine-phosphorylated STAT3 was restored by the addition of PD98059 (Fig. 6 and 7). Through the activation of MAP kinase, GM-CSF induces the serine phosphorylation of STAT3, which interrupts the nuclear translocation of STAT3 in HL-60 cells. It remains unclear why the serine-phosphorylated STAT3 does not translocate into the nucleus in HL-60 cells, as it can in other cells (34, 38). Further research is required to clarify the molecular mechanism in the inhibition of nuclear-translocation of STAT3.

GM-CSF inhibited the G-CSF-dependent enhancement of neutrophile differentiation in Me2SO-treated HL-60 cells (Figs. 1 and 2). We postulated that G-CSF-dependent tyrosine phosphorylation and nuclear-translocation of STAT3 may be associated with the promotion of neutrophile differentiation in Me2SO-treated HL-60 cells and that the inhibition of G-CSF-dependent neutrophile differentiation by GM-CSF may be because of the interruption of nuclear translocation of STAT3, as discussed above. In regard to the effect of PD98059 on the differentiation of Me2SO-treated HL-60 cells, our preliminary results indicated that the decrease in fMLP receptor expression by GM-CSF was restored by the addition of 100 μM PD98059. However, cell growth and O2 generating activity were markedly inhibited by PD98059, both in the presence and absence of G-CSF and/or GM-CSF (data not shown). Thus, a different approach is necessary to clarify the role of MAP kinase on the inhibitory effect of GM-CSF.

It was reported that the maximal activation of the transcription of STAT3 requires both serine and tyrosine phosphorylation; however, serine kinase(s) that is responsible for the phosphorylation of STAT3 has not yet been clarified (28–31). The proposed MAP kinase phosphorylation site, PXSP, is conserved among members of the STAT family, suggesting a role for MAP kinase in serine phosphorylation (28, 29). Other groups have suggested that IL-6-sensitive serine kinase is responsible for the phosphorylation of STAT3 (30, 31). Whereas the addition of GM-CSF rapidly induced an SDS-PAGE mobility shift and serine 727 phosphorylation of STAT3 in either the presence or absence of G-CSF, G-CSF alone did not show the above reaction. On the other hand, G-CSF induced the tyrosine phosphorylation of STAT3 but not serine 727 (Figs. 5 and 7). The addition of PD98059 inhibited serine 727 phosphorylation induced by GM-CSF. In the presence of PD98059, G-CSF caused a delayed SDS-PAGE mobility shift of tyrosine-phosphorylated STAT3. These results suggest that G-CSF induces serine (or threonine) phosphorylation, other than serine 727, in STAT3. Furthermore, both GM-CSF-dependent MAP kinase activation and serine 727 phosphorylation of STAT3 were observed by as early as 5 min. The GM-CSF-dependent mobility shift of STAT3 was markedly reduced by the addition of PD98059, a MEK1 inhibitor (Figs. 4 and 7). Therefore, it was suggested that GM-CSF-dependent serine phosphorylation of STAT3 is induced by the activation of MAP kinase in HL-60 cells.

GM-CSF and G-CSF are thought to play an important role in the growth of myelogenic cells. As previously reported, we observed that G-CSF and GM-CSF augmented the cell number of Me2SO-treated HL-60 cells (15, 39). GM-CSF-dependent cell growth of Me2SO-treated HL-60 cells was much higher than that of G-CSF. The enhancement of GM-CSF and G-CSF on the growth of Me2SO-treated HL-60 cells was not cumulative. Therefore, it appears that the signaling pathway of GM-CSF-dependent cell growth in Me2SO-treated HL-60 cells may also include the pathway of G-CSF.

A number of hematopoietic growth factors, including GM-
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CSF, IL-3, and steel factor have been shown to activate the Ras MAP kinase pathway (40, 41). Conversely, the phosphatidylinositol 3-kinase-p70 S6 kinase pathway in several cells has also been shown to be associated with the growth signals of many cytokines (20). In the present study, we observed that GM-CSF induced a rapid electromobility shift in immunoreactive MAP kinase (ERK1 and ERK2) and a time-delayed electromobility shift in immunoreactive p70 S6 kinase. On the other hand, G-CSF was shown to induce a time-delayed activation of p70 S6 kinase but not MAP kinase. When both GM-CSF and G-CSF were added to MeSO-treated HL-60 cells, the activation pattern of both MAP kinase and p70 S6 kinase were similar to that of GM-CSF alone. In conclusion, we postulate that the activation of both kinases provides maximum activation of cell growth in MeSO-treated HL-60 cells. Furthermore, it also been shown to be associated with the growth signals of many cytokines (20). In the present study, we observed that GM-CSF induced a rapid electromobility shift in immunoreactive MAP kinase (ERK1 and ERK2) and a time-delayed electromobility shift in immunoreactive p70 S6 kinase. On the other hand, G-CSF was shown to induce a time-delayed activation of p70 S6 kinase but not MAP kinase. When both GM-CSF and G-CSF were added to MeSO-treated HL-60 cells, the activation pattern of both MAP kinase and p70 S6 kinase were similar to that of GM-CSF alone. In conclusion, we postulate that the activation of both kinases provides maximum activation of cell growth in MeSO-treated HL-60 cells. Furthermore, it appears that the activation of p70 S6 kinase in MeSO-treated HL-60 cells may be a mutual pathway for the G-CSF and GM-CSF-dependent enhancement of cell proliferation.

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