Commitment of Neutrophilic Differentiation and Proliferation of HL-60 Cells Coincides with Expression of Transferrin Receptor

EFFECT OF GRANULOCYTE COLONY STIMULATING FACTOR ON DIFFERENTIATION AND PROLIFERATION

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To examine the regulatory mechanisms of proliferation and maturation in neutrophilic lineage cells, we have tried to sort dimethyl sulfoxide (Me₂SO)-treated HL-60 cells into transferrin receptor (Trf-R) positive (Trf-R⁺) and negative (Trf-R⁻) cells. Differentiated Trf-R⁺ cells expressed more formyl-Met-Leu-Phe receptor (fMLP-receptor) and ability of O₂⁻ generation, as markers of differentiation, than Trf-R⁻ cells, and Trf-R⁻ cell differentiation was markedly accelerated by the incubation with granulocyte colony stimulating factor (G-CSF). On the other hand, Trf-R⁺ cells had a tendency to proliferate rather than differentiate, and proliferation was enhanced by G-CSF. These results indicate that Trf-R expression coincides with the commitment to proliferate or differentiate of HL-60 cells, and G-CSF accelerates these commitments. G-CSF-induced tyrosine phosphorylation of STAT 3 in Trf-R⁺ cells much more than in Trf-R⁻ cells. Protein 70 S6 kinase expression was higher in Trf-R⁺ cells than in Trf-R⁻ cells. Furthermore, p70 S6 kinase was hyperphosphorylated by G-CSF in Trf-R⁺ cells, but not in Trf-R⁻ cells. Rapamycin, an inhibitor of p70 S6 kinase activity, inhibited G-CSF-dependent proliferation of Trf-R⁺ cells and increased fMLP-R expression on these cells. These results suggest that commitment to proliferation and differentiation in Me₂SO-treated HL-60 cells is preprogrammed and correlated with Trf-R expression, and G-CSF potentiates the commitment. Therefore, we hypothesized that heterotypic Trf-R expression in neutrophilic lineage cells may promote proliferation and negatively regulate neutrophilic differentiation.

Neutrophils which kill bacteria or invading microorganisms constitute a major population of white blood cells. The development of mature neutrophils in the bone marrow occurs via the differentiation of multipotent stem cells into progenitor cells that are committed to neutrophilic lineages (1, 2). As cells develop the characteristics of mature neutrophils, their proliferative ability decreases. However, the mechanism regulating proliferation and maturation of neutrophilic lineage cells remains unclear.

Promyelocytic leukemia HL-60 cells differentiate into macrophages or neutrophils in response to various stimuli. Dimethyl sulfoxide (Me₂SO),¹ retinoic acid (RA), and Bț-CAMP cause neutrophilic differentiation of HL-60 cells, whereas interferon and phorbol ester cause differentiation to macrophages (3–5). These characteristics have made HL-60 cells extremely useful for studying proliferation and maturation of neutrophilic lineage cells. Developmental processes of neutrophilic lineage cells are thought to be controlled by several cytokines, which act synergistically in a homotypic or heterotypic manner. One of these cytokines, granulocyte colony-stimulating factor (G-CSF), specifically works on cells restricted to the neutrophilic granulocyte lineage (6). Several reports have suggested that while G-CSF itself cannot induce neutrophilic differentiation of HL-60 cells, G-CSF potentiates the neutrophilic differentiation of RA- and Me₂SO-treated HL-60 cells (7–9).

A variety of G-CSF activities are mediated by a specific receptor for G-CSF (G-CSF-R). G-CSF-R is a member of the hematopoietic growth factor receptor family (10), and had no intrinsic kinase domain (11–13). It is reported that G-CSF stimulation results in rapid activation of Janus tyrosine kinases (JAKs) (14–18) and signal transducer and activator of transcription (STAT) 1 (19) and STAT 3 (14, 19, 20). Using dominant-negative STAT 3, STAT 3 activation was shown to play an important role in myeloid cell line differentiation (21, 22).

The transferrin receptor (Trf-R) plays an essential role in cell proliferation, as demonstrated by the fact that blocking receptor function causes cells to arrest near the G₁-S phase boundary (23–25). Trf-R is a homodimeric glycoprotein highly expressed in many proliferating normal and malignant cells as well as in hemoglobin-synthesizing cells. In many types of cells, the expression of the Trf-R increases when the cells are stimulated to proliferate but decreases upon cessation of the cell growth (26, 27). It is reported that the expression of Trf-R on neutrophilic lineage cells gradually decreases during maturation. Therefore, we hypothesized that heterotypic Trf-R expression in neutrophilic lineage cells correlate with the commitment to proliferate or differentiate.

The importance of p70 S6 kinase in cell cycle progression of numerous cells has been reported (28). However, it has not been reported whether or not G-CSF signaling cascade involves p70 S6 kinase. Furthermore, it remains unclear how this sig-
naling pathway coincides with proliferation and differentiation of neutrophilic lineage cells.

In this paper, we sorted MeSO-treated HL-60 cells into Trf-R positive (Trf R1) and negative (Trf-R2) cells, and demonstrated that Trf-R1 cells tend to proliferate and Trf-R2 cells tend to undergo neutrophilic differentiation. Furthermore, based on the analysis of G-CSF effects on Trf-R1 and Trf-R2 cells, we suggest that STAT 3 positively regulates the differentiation of Me2SO-treated HL-60 cells into neutrophils, while p70 S6 kinase promotes proliferation and negatively regulates neutrophilic differentiation.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant human G-CSF was a kind gift from Chugai Pharmaceutical Co. (Tokyo, Japan). The magnetic cell sorting kit, MACS was from Miltenyi Biotec (Gladbach, Germany). MeSO was from Pierce (Rockford, IL). All-trans-retinoic acid (RA) was from Sigma. The biotin-conjugated mouse anti-human G-CSF-R monoclonal antibody and the mouse anti-human Trf-R (CD 71) monoclonal antibody were purchased from Pharmingen (San Diego, CA). The mouse anti-Met (hepatocyte growth factor (HGF) receptor) extracellular domain monoclonal antibody was from Upstate Biotechnology Inc. (Lake Placid, NY). Fluorescein isothiocyanate (FITC)-conjugated streptavidin was from Life Technologies, Inc. (Grand Island, NY). The FITC-conjugated formyl-Met-Leu-Phe (fMLP) was from Funakoshi (Tokyo Japan). The mouse anti-STAT 3 monoclonal antibody was from Transduction Laboratories (Lexington, KY). The rabbit anti-tyrosine phosphorylated STAT 3 (Tyr705) polyclonal antibody and the rabbit anti-serine phosphorylated p70 S6 kinase (Ser411) polyclonal antibody were from New England Biolabs, Inc. (Beverly, MA). The horseradish peroxidase-conjugated sheep anti-mouse Ig antibody was from Amersham Life Science Corp. (Little Chalfont, United Kingdom). The horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Bio-Rad (Germany). Rapamycin was purchased from Calbiochem-Novabiochem Intl. (San Diego, CA).

Cell Culture and Differentiation to Neutrophilic Granulocyte Lineage—HL-60 cells were kindly supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 30 mg/liter kanamycin sulfate at 37 °C under moisturized air containing 5% CO2. In order to ensure the same quality of cells for a series of experiments, frozen cells of the same lot were thawed every 3 months and used for experiments. MeSO or RA were used to differentiate HL-60 cells into neutrophilic granulocytes. The centrifuged cells were resuspended in 10% fetal bovine serum medium containing 1.25% MeSO or 1 mM RA at a density of 2.5 × 106 cells/ml. Two days after the addition of differentiating agent, magnetic cell sorting was carried out.

Magnetic Cell Sorting—About 3 × 107 cells per 2 days were collected by centrifugation. The supernatant was filtered to avoid unintentional activation of MeSO-treated HL-60 cells.
FIG. 3. The effect of G-CSF on fMLP-R expression and $O_2^-$ generating activity in differentiated Trf-R$^+$ and Trf-R$^-$ cells. After magnetic cell sorting by Trf-R expression, both cell populations were cultured for 5 days with or without G-CSF (60 ng/ml). fMLP-R expression (A) and $O_2^-$ generating activity stimulated by fMLP$^+$ (B) or opsonized zymosan (C) were examined. For the analysis of fMLP-R expression, Trf-R$^+$ and Trf-R$^-$ cells were incubated with FITC-conjugated fMLP. Washed cells were analyzed by flow cytometry. For the analysis of $O_2^-$ production, ferricytochrome c reduction assay was performed using 200 nM fMLP or 1.25 mg/ml opsonized zymosan as stimulants. Open columns denote Trf-R$^+$ cells and striped columns Trf-R$^-$ cells. Columns and bars represent the mean ± S.D. of triplicate wells (*, $p < 0.05$; **, $p < 0.01$).

by cell debris. The filtrated supernatant was kept on ice during magnetic cell sorting as conditioned medium and soon used for subsequent culture. The pelleted cells were washed with sort solution (PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA)), and then incubated with 100 μl of sorting solution containing mouse anti-human Trf-R antibody diluted 5-fold at 4 °C for 15 min. After washing, cells were incubated with 100 μl of sorting solution containing 75 μl of goat anti-mouse IgG microbeads (Miltenyi Biotec) at 4 °C for 15 min. After resuspension in 0.5 ml of sort solution, cells were applied to the separation column. The cells not adsorbed by the separation column were collected as Trf-R$^+$ cells, and the adsorbed cells were collected as Trf-R$^-$ cells. The expressions of Trf-R and G-CSF-R on cells from Trf-R$^+$ and Trf-R$^-$ fractions were analyzed by flow cytometry. After resuspension in conditioned medium at a density of approximately 2.5–5.0 × 10$^5$ cells/ml, cells were subsequently cultured for 5 days with or without 60 ng/ml G-CSF. The expression of fMLP-R was also analyzed by flow cytometry. In the study of the effect of rapamycin on differentiation of HL-60 cells, 20 ng/ml rapamycin was preincubated with cells for 30 min before the addition of G-CSF.

Flow Cytometric Analysis of Trf-R, HGF-R, G-CSF-R, fMLP-R, and Cell Cycle—The expression of Trf-R and HGF-R in Me$_2$SO-treated HL-60 cells was determined by flow cytometric methods. About 1 × 10$^6$ cells pretreated with Me$_2$SO for various periods were collected and incubated with 50 μl of 0.5% BSA/PBS containing mouse anti-human Met antibody or mouse anti-human Trf-R antibody at 4 °C for 15 min. After washing, cells were labeled with FITC-conjugated goat anti-mouse IgG (F(ab)2) fragment. After the incubation, cells were washed and resuspended in 0.5% BSA/PBS. Flow cytometric analysis was performed with a Cyto ACE-150 Auto Cell Screener (JASCO Co., Tokyo, Japan).

The expressions of Trf-R and G-CSF-R on sorted cells was determined as follows. For Trf-R expression assay, cells which had been already labeled with mouse anti-human Trf-R antibody, were incubated with 50 μl of sorting solution containing FITC-conjugated goat anti-mouse IgG antibody at 4 °C for 15 min. Washed cells were next resuspended in sorting solution, and then subjected to flow cytometric analysis. For G-CSF-R expression assay, cells were washed and incubated with 100 μl of sorting solution containing biotin-conjugated mouse anti-human G-CSF-R monoclonal antibody at 4 °C for 30 min. They were then washed once more and incubated with 100 μl of sorting solution containing FITC-conjugated streptavidin at 4 °C for 30 min. Washed cells were then resuspended, and subjected to flow cytometric analysis.

For the formyl-Met-Leu-Phe receptor (fMLP-R) expression assay, sorted cells were subsequently cultured with or without G-CSF and/or rapamycin for 5 days. Cells were collected and washed with 1.5 ml of 0.5% BSA/PBS. They were then incubated with 50 μl of 0.5% BSA/PBS containing 100 nM FITC/MLP at 4 °C for 30 min. Washed cells were resuspended in 0.3 ml of 0.5% BSA/PBS, and then subjected to flow cytometric analysis. For cell cycle analysis, sorted cells were subsequently cultured in conditioned media with or without G-CSF and/or rapamycin for 22 h. Cells were collected and washed with 5 ml of PBS(-), then fixed with 5 ml of 70% ethanol for 30 min. After centrifugation, they were treated with 0.5 mg/ml RNase A (Wako Pure Chemical Industries Ltd.), stained with 0.5 ml of 50 μg/ml propidium iodide (Sigma) at 4 °C for 10 min, and then subjected to flow cytometric analysis.

$O_2^-$ Generating Activity—After cell sorting, Trf-R$^+$ and Trf-R$^-$ cells were cultured in conditioned media with or without G-CSF for 5 days. The $O_2^-$ generating activity of the differentiated cells was measured in terms of ferricytochrome c reduction assay. The cells (0.4–0.7 × 10$^5$) were suspended in substrate solution (50 μM ferricytochrome c, 5 mM d-glucose, and 0.5 mM CaCl$_2$ in HEPES-buffered saline) and the assay was initiated by addition of 200 nM fMLP or 1.25 mg/ml opsonized zymosan. The absorbance increase at 550–540 nm was continuously recorded by a Hitachi 557 double-beam spectrophotometer. For the end point assay, incubation for 6 min was terminated by addition of an equal volume of chilled PBS(−) and the assay mixture was centrifuged at 1,500 × g for 10 min at 4 °C. The absorbance increase of the supernatant at 550–540 nm was measured.

Morphology—After cell sorting, Trf-R$^+$ and Trf-R$^-$ cells were resuspended with conditioned media with or without G-CSF. Five days after the addition of G-CSF, cells were collected and spun by HEG-SP2 (Omrorn, Kyoto, Japan) to make a cytosin smear, stained by HEG-ST.
Preparation of Cell Lysates and Immunoblotting—After cell sorting, Trf-R\(^2\) and Trf-R\(^1\) cells resuspended in conditioned medium were stimulated with G-CSF (60 ng/ml) for 30 min before being treated with G-CSF. The reaction was terminated by adding an ice-cold mixture of protease and phosphatase inhibitors (2 mM EDTA, 0.2 mM ammonium molybdate, 20 mM sodium fluoride, 0.2 mM iodoacetamide, 0.2 mM benzamine, and 0.5 mM Na\(_3\)VO\(_4\) in PBS) and allowed to stand for 10 min on ice. After centrifugation, cells were treated with lysis buffer (10 mM K\(_2\)HPO\(_4\), 1 mM EDTA, 50 mM 
EGTA, 10 mM MgCl\(_2\), 50 mM \(\beta\)-glycerophosphate, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 1% Triton X-100, and 1% deoxycholate). The cells were disrupted at 40 watts for 10 s twice with a Branson Sonifier. After the centrifugation, the supernatant was mixed with an equal volume of 2x sample buffer containing 20 mM Tris (pH 6.8), 5% lithium lauryl sulfate, 4 mM EDTA, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromphenol blue, and boiled at 100 °C for 1 min. Samples were stored at -20 °C before Western blotting analysis. Cell-free lysates (1–2 \times 10^5 cell extract/line) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose (Hybond-ECL, Amersham Pharmacia Biotech, United Kingdom). Filters were blocked by incubation with 1.0% BSA and 0.1% Tween 20 in Tris-buffered saline (TBS-T; 20 mM Tris, pH 7.6, 150 mM NaCl) overnight at 4 °C. After washing with TBS-T, cells were incubated with various antibodies. The antibodies used for Western blotting were mouse anti-STAT 3 monoclonal antibody, rabbit anti-tyrosine-phosphorylated STAT 3 (Tyr705) polyclonal antibody, rabbit anti-p70 S6 kinase polyclonal antibody, rabbit anti-serine phosphorylated p70 S6 kinase (Ser\(^{411}\)) polyclonal antibody, and rabbit anti-MAPK polyclonal antibody (29). After washing in TBS-T three times, filters were probed with horseradish peroxidase-conjugated sheep anti-mouse Ig antibody or horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, then subjected to an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech). For reprobing with different antibodies, blots were stripped in 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol at 50 °C for 30 min and reblocked with 1% BSA in TBS-T. The bands that appeared on x-ray films were scanned and the density of each band was determined on a Macintosh model computer using the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet within the linear range for quantitation.

DNA Fragmentation—To detect DNA fragmentation, sorted cells (5 \times 10^5) were subsequently cultured with or without G-CSF (60 µg/ml) and/or rapamycin (20 ng/ml) at 37 °C for 5 h. As a positive control, HL-60 cells (5 \times 10^5) treated with Me2SO for 2 days were incubated with 1 µg/ml actinomycin D or 1 µM A23187 in RPMI 1640 at 37 °C for 5 h. The cellular DNA was extracted, electrophoresed in 2% agarose gel, stained with SYBR Green ITM using the Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries, Ltd.) and then subjected to Fluor Imager 595 (Molecular Dynamics Japan, Tokyo) analysis. Statistical analysis was performed using the unpaired t test.

**FIG. 4.** The effect of G-CSF on cell proliferation and cell cycle in Trf-R\(^{-}\) and Trf-R\(^{+}\) cells. After magnetic cell sorting by Trf-R expression, both cell populations were cultured in conditioned media with or without G-CSF (60 ng/ml). Proliferation (A) and cell cycle (B) were examined. For the proliferation assay, cell numbers of each sample were counted using a Sysmex F300 automatic microcell counter at 1, 3, and 5 days after magnetic cell sorting. Open symbols denote control cells, and closed symbols denote G-CSF-treated cells. The results represent the mean ± S.D. of triplicate wells. For the cell cycle analysis, sorted cells were subsequently cultured in conditioned media with or without G-CSF for 22 h. Cells were stained with propidium iodide and subjected to flow cytometric analysis. The x axis represents the fluorescence intensity and the y axis the relative cell number.
RESULTS

Time Course of expression of Trf-R and HGF-R—Human myeloid leukemia cell line, HL-60, cells are able to differentiate into neutrophilic cells in response to Me2SO or RA. The alterations of cell surface receptor expression of Trf (CD 71) and HGF (c-Met) in differentiating cells were determined. After incubation with Me2SO for 1 day, HL-60 cells expressed a single peak of Trf-R, whose intensity was less than that on untreated control HL-60 cells (Fig. 1A). During the incubation with Me2SO for 2–6 days, the Trf-R expression on HL-60 cells was gradually reduced and divided into two peaks. The lower peak of Trf-R was virtually identical to the results obtained by incubation without anti-Trf-R antibody, suggesting that this cell population consisted of Trf-R<sup>−</sup> cells. We categorized the cell population that expressed the higher peak of Trf-R as Trf-R<sup>+</sup> cells. HGF plays a crucial role as a hematopoietic regulator in the proliferation and differentiation of multi-hematopoietic progenitors (30). In contrast to the Trf-R expression, Me2SO treatment had no effect on HGF-R expression on HL-60 cells (Fig. 1B). The same phenomenon was also observed in HL-60 cells treated with RA (data not shown).

Magnetic Cell Sorting by Trf-R Expression—Two days after the treatment with Me2SO, cells expressing Trf-R were isolated by magnetic cell sorting. Me2SO-treated HL-60 cells were incubated with a combination of mouse anti-human Trf-R monoclonal antibody and goat anti-mouse IgG microbeads. To confirm the separation of Trf-R expressing and nonexpressing cells, both populations of sorted cells were stained with FITC-conjugated goat anti-mouse IgG antibody. Flow cytometric analysis showed that Trf-R<sup>+</sup> cells had markedly stronger fluorescence intensity than Trf-R<sup>−</sup> cells (Fig. 2A), indicating that the cell types could be successfully separated by magnetic cell sorting.

Comparison of the Ability of Trf-R<sup>+</sup> and Trf-R<sup>−</sup> Cells to Undergo Differentiation and Proliferation in the Presence or Absence of G-CSF—Since G-CSF is thought to be a cytokine that specifically works on the neutrophilic lineage cells, the reactivity of each cell population to G-CSF was examined.

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Fig. 5. Effect of G-CSF on the morphology of Trf-R<sup>+</sup> and Trf-R<sup>−</sup> cells. After cell sorting, Trf-R<sup>+</sup> and Trf-R<sup>−</sup> cells were resuspended in conditioned media with or without 60 ng/ml G-CSF. Five days after the addition of G-CSF, cells were collected and spun to make a cytospin smear, then stained with Wright stain (objectives; ×60). Stained cells were counted under a microscope (B). Values are the mean ± S.D. (n = 4) of the percentage of metamyelocytes and more immature cells (promyelocytes and myelocytes). #, control versus G-CSF on each cell of metamyelocytes, not significant. **, Trf-R<sup>+</sup> versus Trf-R<sup>−</sup> cells on control or G-CSF of metamyelocytes, significant (p < 0.0001).

### The number of metamyelocytes on Trf-R<sup>+</sup> and Trf-R<sup>−</sup> cells (percent).

|         | Metamyelocytes | Promyelocytes and myelocytes |
|---------|----------------|-----------------------------|
| **Trf-R<sup>−</sup> Cells** |                |                             |
| Control  | 30.3 ± 2.63    | 69.8 ± 2.63                 |
| G-CSF    | 29.3 ± 4.50<sup>#</sup> | 70.8 ± 4.50                 |
| **Trf-R<sup>+</sup> Cells** |                |                             |
| Control  | 5.3 ± 1.50**   | 94.8 ± 1.50                 |
| G-CSF    | 6.0 ± 1.83<sup,#,**</sup> | 94.0 ± 1.83                 |
First, G-CSF-R expression on the cell surface was determined. In contrast to the expression of Trf-R (Fig. 2A), there was no difference in the expression of G-CSF-R between Trf-R\(^+\) and Trf-R\(^-\) cells (Fig. 2B). In order to compare differentiation and proliferation of Trf-R\(^+\) and Trf-R\(^-\) cells, both cell populations were resuspended in conditioned medium as described under "Experimental Procedures." After the cells had been subsequently cultured for 5 days with or without G-CSF (60 ng/ml), the expression of fMLP-R, a marker of neutrophilic differentiation, was examined. As shown in Fig. 3A, the differentiated Trf-R\(^+\) cells expressed higher levels of fMLP-R than the Trf-R\(^-\) cells, and G-CSF potentiated the fMLP-R expression on Trf-R\(^-\) cells. Incubation with G-CSF induced fMLP-R expression to a lesser extent in the Trf-R\(^-\) cells compared with the Trf-R\(^+\) cells.

O\(_2^\cdot\) generating activity stimulated by fMLP or opsonized zymosan is shown in Fig. 3, B and C, respectively. After cell sorting, Trf-R\(^+\) and Trf-R\(^-\) cells were cultured in conditioned media with or without G-CSF for 5 days. The extent of fMLP-stimulated O\(_2^\cdot\) generation by differentiated Trf-R\(^-\) cells (Fig. 3B) was 3.3-fold that by Trf-R\(^+\) cells, which was significantly higher (**, p < 0.01). G-CSF enhanced the O\(_2^\cdot\) generating activity of Trf-R\(^+\) and Trf-R\(^-\) cells, with the amount of O\(_2^\cdot\) produced by Trf-R\(^-\) cells differentiated with G-CSF being 1.9-fold that by Trf-R\(^+\) cells cultured with G-CSF, which was significantly higher (**, p < 0.01). These results are supported by the trends in fMLP-R expression (Fig. 3A). O\(_2^\cdot\) generating activity stimulated by opsonized zymosan showed the same tendency as that stimulated by fMLP (Fig. 3C).

On the other hand, the proliferative activity of Trf-R\(^+\) cells was much higher than that of Trf-R\(^-\) cells (Fig. 4A). One day after magnetic cell sorting, no difference between Trf-R\(^+\) and Trf-R\(^-\) cells in proliferation was observed. Since the inoculated cell density of both cell types was 3.9 \times 10^5 cells/ml after magnetic cell sorting, Trf-R\(^+\) and Trf-R\(^-\) cells increased 2.2- and 2.0-fold after 3 days, and 2.7- and 2.3-fold after 5 days from the initial level, respectively. G-CSF enhanced the proliferation of Trf-R\(^+\) and Trf-R\(^-\) cells 1.2- and 1.1-fold after 3 days, and 1.4- and 1.2-fold after 5 days compared with that in the absence of G-CSF, respectively.

Fig. 4B shows a typical profile of cell cycle analysis using propidium iodide by flow cytometry. The area of S phase in Trf-R\(^+\) cells altered by G-CSF was largest and the cell types ranked as follows; Trf-R\(^+\) > G-CSF > Trf-R\(^-\) > Trf-R\(^+\) + G-CSF > Trf-R\(^-\). These results support the results of Fig. 4B.

Together, these data suggest that Trf-R\(^+\) cells are differentiation type cells, and Trf-R\(^-\) cells are proliferative type cells. We also sorted Trf-R\(^+\) and Trf-R\(^-\) cells from RA (1 \(\mu\)g)-pretreated HL-60 cells, and observed the same tendency of both cell types (data not shown). These results also suggest that G-CSF enhances differentiation in Trf-R\(^-\) cells and proliferation in Trf-R\(^+\) cells.

Morphology—Fig. 5 shows the morphology of differentiated Trf-R\(^+\) and Trf-R\(^-\) cells with or without G-CSF. Promyelocyte, myelocytes, and metamyelocytes were observed in both cell types. The ratio of metamyelocytes was significantly higher in Trf-R\(^+\) cells than in Trf-R\(^-\) cells (Fig. 5B, p < 0.0001), Trf-R\(^-\) cells are more mature than Trf-R\(^+\) cells. Stab cells or seg-
mmented cells, however, could not be observed. Moreover, G-CSF did not alter the morphological features of these cells.

Signal Transduction Induced by G-CSF on Both Cell Types—To clarify what molecular events were induced by G-CSF in Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells, G-CSF-dependent intracellular signaling events were analyzed by immunological methods. G-CSF is known to activate STAT 3 (20). Therefore, we examined the activation of STAT 3 in Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells using anti-tyrosine-phosphorylated STAT 3 antibody (Fig. 6A) and quantitated the density of each band (Fig. 6F). Although there was no difference in the protein level of STAT 3 between Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells (Fig. 6B), tyrosine 705 of STAT 3 in Trf-R<sup>-</sup> cells was markedly phosphorylated on the addition of G-CSF for 5 and 30 min (Fig. 6F, *p < 0.05). G-CSF also induced tyrosine phosphorylation of STAT 3 in Trf-R<sup>+</sup> cells, but at a lower level than in Trf-R<sup>-</sup> cells.

Protein 70 S6 kinase plays an important role in the progression of cells from the G<sub>1</sub> to S phase of the cell cycle (31). It has not been reported whether or not G-CSF signaling cascade involves p70 S6 kinase. So, we examined the activation of p70 S6 kinase in Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells (Fig. 6, C, D, and E). Fig. 6 shows a Western blot analysis of p70 S6 kinase. The protein level of p70 S6 kinase in Trf-R<sup>+</sup> cells was higher than that in Trf-R<sup>-</sup> cells. Upon addition of G-CSF, an electrophoretic mobility shift of p70 S6 kinase was clearly observed in Trf-R<sup>+</sup> 30 min, suggesting that G-CSF induced serine/threonine phosphorylation of p70 S6 kinase. This mobility shift was not observed in Trf-R<sup>-</sup> cells. A Western blot using anti-serine phosphorylated p70 S6 kinase (Ser<sup>411</sup>) antibody supported that in Fig. 6C. Ser<sup>411</sup> of p70 S6 kinase is located in the autoinhibitory domain and phosphorylation of this site directly affects kinase activity. Serine 411 of p70 S6 kinase in Trf-R<sup>-</sup> cells was markedly phosphorylated on the addition of G-CSF for 5 and 30 min (Fig. 6, D and G). G-CSF also induced serine 411 phosphorylation of p70 S6 kinase in Trf-R<sup>-</sup> cells, but at a lower level than in Trf-R<sup>+</sup> cells. Using anti-extracellular signal-regulated kinase (ERK) antibody, we also confirmed that there was no difference in MAPK content between Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells, and that G-CSF did not stimulate MAPK in either Trf-R<sup>+</sup> or Trf-R<sup>-</sup> cells (Fig. 6E). Considering that the expression of G-CSF-R on Trf-R<sup>-</sup> and Trf-R<sup>-</sup> cells was the same (Fig. 2B), these results indicate that there are differences in G-CSF-induced activation of STAT 3 and p70 S6 kinase downstream of the G-CSF-R in Trf-R<sup>-</sup> and Trf-R<sup>-</sup> cells.

Effect of Rapamycin on Differentiation and Proliferation of Trf-R<sup>+</sup> and Trf-R<sup>-</sup> Cells—It is well known that rapamycin, a bacterial macrolide, selectively blocks p70 S6 kinase activation. After cell sorting, Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells were cultured in conditioned media with or without G-CSF (60 ng/ml) and/or rapamycin (20 ng/ml) for 5 days. The expression of fMLP-R and proliferative activity in both cell types were examined. In the absence of rapamycin, fMLP-R expression on the cultured Trf-R<sup>-</sup> cells was higher than that on Trf-R<sup>-</sup> cells (Fig. 7, top panel, and Fig. 3A). However, there was no difference in fMLP-R expression between Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells cultured in the presence of rapamycin (Fig. 7, middle panel), suggesting that rapamycin induced an enhancement of fMLP-R expression on the differentiated Trf-R<sup>-</sup> cells. Rapamycin abrogated this G-CSF-dependent difference in fMLP-R expression on Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells (Fig. 7, bottom panel). While rapamycin did not show any significant inhibitory effect on the proliferation of either Trf-R<sup>+</sup> or Trf-R<sup>-</sup> cells, it completely inhibited G-CSF-induced proliferation of Trf-R<sup>-</sup> cells. Fig. 8B shows the cell cycle analysis using rapamycin. As shown in the left panel, while there were fewer Trf-R<sup>+</sup> than Trf-R<sup>-</sup> cells at G<sub>2</sub>/G<sub>1</sub> phase, there were more Trf-R<sup>+</sup> than Trf-R<sup>-</sup> cells at S phase and G<sub>2</sub>/M phase. G-CSF decreased the G<sub>2</sub>/G<sub>1</sub> cell populations and rapamycin increased them in both the presence and absence of G-CSF. In contrast, G-CSF increased the S phase and G<sub>2</sub>/M phase populations and rapamycin with or without G-CSF decreased them. These results support the proliferative result in Fig. 8A. Thus, G-CSF-induced proliferation coincides with an activation of p70 S6 kinase, and basal proliferation is mediated by some other pathway(s). Rapamycin had no effect on maturation of morphology even if it was added concomitantly with G-CSF (data not shown).

To confirm whether differentiation was associated with apoptotic death, DNA fragmentation was examined. After cell sorting, cells were cultured with or without G-CSF and/or rapamycin for 5 h. Apoptotic death occurred in actinomycin D- or A23187-treated cells used as a positive control. G-CSF or rapamycin, however, did not induce apoptotic death in Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells.

We also examined the effect of rapamycin on G-CSF-induced STAT 3 tyrosine phosphorylation. Trf-R<sup>+</sup> or Trf-R<sup>-</sup> cells were pretreated with rapamycin for 30 min and then were stimulated by G-CSF for 30 min. Rapamycin did not alter the extent of G-CSF-induced tyrosine phosphorylation of STAT 3 in either cell type (Fig. 9, A and E), nor that of STAT 3 protein (Fig. 9B). Under these conditions, rapamycin blocked the electrophoretic mobility shift and serine 411 phosphorylation of p70 S6 kinase.
induced by G-CSF (Fig. 9, C, D, and F). Rapamycin alone did not induce tyrosine phosphorylation of STAT 3 nor did it alter the amount of STAT 3 protein, but it alone blocked the electrophoretic mobility shift and serine 411 phosphorylation of p70 S6 kinase induced by conditioned media (data not shown).

DISCUSSION

In the present study, we examined mechanisms involved in neutrophilic differentiation, focusing specifically on transferrin receptors and G-CSF. We found that HL-60 cells, induced to undergo neutrophilic differentiation by Me2SO or RA treatment, exhibited heterogeneous expression of Trf-R and gradual decrease in Trf-R expression (Fig. 1A). The alteration of Trf-R expression was significant compared with c-Met (Fig. 1B) or G-CSF-R (Fig. 2B), which remained unchanged. Using a magnetic cell sorting system, Me2SO-treated HL-60 cells were successfully separated into Trf-R⁺ and Trf-R⁻ cells (Fig. 2A). Trf-R⁺ HL-60 cells proliferate at a higher rate than Trf-R⁻ cells (Fig. 4, A and B), but Trf-R⁻ cells are less able to undergo neutrophilic differentiation (Fig. 3, A-C). These results indicate that the expression of Trf-R in HL-60 cells correlated with the ability to undergo neutrophilic differentiation.

G-CSF is a cytokine critical for normal neutrophil production and maturation. G-CSF stimulates the proliferation, survival, and maturation of cells committed to the neutrophilic lineage, but the mechanism by which this occurs has not been elucidated. Rodel and Link (32), examining mRNA expression of cathepsin G, reported that the complete maturation of mouse 32D cells to granulocytes appears to be dependent on G-CSF. On the other hand, studies with G-CSF (33), G-CSF-R (34), and G-CSF-R⁻ interleukin-6 (35) deficient mice indicated that G-CSF-independent mechanisms of granulopoiesis must exist. Therefore, it is necessary to clarify the role of G-CSF in the regulation of neutrophilic differentiation and proliferation.

Here, using fMLP-R expression and O₂⁻ generating activity as a marker of neutrophilic differentiation, we found that G-CSF markedly promoted the neutrophilic differentiation of Me2SO-treated Trf-R⁺ HL-60 cells, whereas G-CSF did not enhance the neutrophilic differentiation of Trf-R⁻ cells (Fig. 3, A-C). On the other hand, the proliferative ability of Trf-R⁺ cells was enhanced more than that of Trf-R⁻ cells by G-CSF (Figs. 4, A and B, and 8, A and B). Since there was no difference of G-CSF-R expression between Trf-R⁺ and Trf-R⁻ HL-60 cells.

![Fig. 8. Effects of rapamycin on proliferation, cell cycle, and DNA fragmentation in Trf-R⁺ and Trf-R⁻ cells.](image-url)
Neutrophilic Differentiation and Proliferation of HL-60 Cells

protein 70 S6 kinase phosphorylates the 40 S ribosomal protein S6, and is necessary for cell cycle progression to the S phase (28). Recent studies have demonstrated that p70 S6 kinase is activated by a pathway independent of p21ras (36) and acts downstream of phosphatidylinositol 3-kinase (37–40). Chou and Blenis (41) have shown that Rho family G proteins, Rac-1 and Cdc42, bind in vitro to hypophosphorylated p70 S6 kinase in a GTP-dependent fashion, and that GTPase-deficient alleles elevate p70 S6 kinase activity in vivo. Busca et al. (42) have shown that inhibition of the phosphatidylinositol 3-kinase/p70 S6 kinase induces B16 melanoma cell differentiation, and the elevation of cAMP level may contribute to the reduction of both kinases. We therefore suspected that the reduction of p70 S6 kinase may coincide with the commitment of neutrophilic differentiation in Me2SO-treated HL-60 cells. We determined that the protein level of p70 S6 kinase in Trf-R+ HL-60 cells was lower than that in Trf-R− cells (Fig. 6C), while there was no difference in MAPK expression (Fig. 6E). Furthermore, the G-CSF-dependent activation of p70 S6 kinase in Trf-R+ cells was higher than that in Trf-R− cells (Fig. 6, C, D, and G), suggesting that activation of p70 S6 kinase negatively regulates or suppresses the differentiation of HL-60 cells to neutrophilic cells.

Rapamycin is a potent and specific inhibitor of p70 S6 kinase, preventing phosphorylation and activation of p70 S6 kinase by all known external stimuli (31, 38, 43–45). In this study, rapamycin induced increased expression of fMLP-R in Trf-R− cells to levels similar to those found on Trf-R+ HL-60 cells (Fig. 7, middle). A G-CSF-dependent increase in fMLP-R expression was also observed in the presence of rapamycin in both Trf-R− and Trf-R+ cells (Fig. 7, bottom); in the absence of rapamycin, G-CSF induced only slight enhancement of fMLP-R in Trf-R+ cells (Fig. 3A). These results clearly suggest that rapamycin causes up-regulation of fMLP-R expression. On the other hand, rapamycin partially inhibited the proliferation of Trf-R− cells, and completely blocked the G-CSF-dependent enhancement of cell growth in Trf-R+ cells (Fig. 8, A and B). These results suggest that rapamycin, which blocks p70 S6 kinase activity, can block proliferation and enhance neutrophilic differentiation without inducing apoptotic death (Fig. 8C) in Trf-R+ HL-60 cells.

G-CSF treatment induced STAT 3 phosphorylation in Trf-R− cells and to a lesser extent in Trf-R+ cells. However, rapamycin had no effect on G-CSF-induced STAT 3 phosphorylation (Fig. 9, A and E). This suggests that rapamycin-induced enhancement of neutrophilic differentiation does not occur through the JAK-STAT 3 pathway(s). Although the JAK-STAT 3 pathway is activated by G-CSF (14, 19, 20) and has been implicated in cellular differentiation (21, 22), some neutrophilic differentiation events in HL-60 cells, such as fMLP-R expression, are not regulated by STAT 3 alone. Neutrophilic differentiation may also require down-regulation of the p70 S6 kinase cascade.

In conclusion, Me2SO-differentiating HL-60 cells were successfully sorted into two populations, Trf-R− and Trf-R+ cells. Analysis of G-CSF-dependent differentiation and proliferation...
in Trf-R+ and Trf-R− cells suggests that STAT 3 positively regulates the differentiation of Me_SO-treated HL-60 cells into neutrophils, while the p70 S6 kinase negatively regulates the neutrophilic differentiation and promotes cellular proliferation instead.

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