Involvement of STAT3 in the Granulocyte Colony-stimulating Factor-induced Differentiation of Myeloid Cells*

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Granulocyte colony-stimulating factor (G-CSF) stimulates proliferation and differentiation of the progenitor cells of neutrophilic granulocytes. The binding of G-CSF to its receptor specifically activates JAK1 and JAK2 kinases, as well as STAT3, a signal transducer and activator of transcription (STAT). To examine the role of STAT3 in G-CSF receptor-mediated signal transduction, two different forms of the dominant negative STAT3 were introduced into a mouse myeloid cell line that exogenously expresses the mouse G-CSF receptor. In response to G-CSF, the parental myeloid cells grew for about 4 days, and then they stopped dividing and differentiated into cells with lobulated nuclei. During this period, the expression of the myeloperoxidase (MPO) gene was induced, while c-myc gene expression was down-regulated. In contrast, in the cells expressing the dominant negative STAT3, G-CSF could induce neither growth arrest nor morphological change. However, the induction of the MPO gene by G-CSF was not affected by the dominant negative STAT3. These results indicate that STAT3 activation is responsible for part of the G-CSF-induced differentiation of neutrophils but that another pathway, involving the expression of the MPO gene, that does not utilize the activated STAT3, is also required to fully differentiate the cells.

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein of 20 kDa and stimulates the proliferation, survival, and maturation of cells committed to the neutrophilic granulocyte lineage (1–4). Mice lacking the G-CSF gene exhibit chronic neutropenia (5), suggesting an essential role of G-CSF in granulopoiesis. A variety of G-CSF activities are mediated by a specific receptor for G-CSF, which is expressed in mature neutrophils as well as their progenitors (3, 4). The G-CSF receptor (G-CSFR) is a type I membrane protein that belongs to the homotypic growth factor receptor superfamily (6) and transduces the signals for proliferation and differentiation of myeloid precursor cells (7, 8). The membrane-proximal half of the G-CSFR cytoplasmic region is sufficient for transducing the proliferation signal, whereas its membrane-distal half of the cytoplasmic region is essential for the differentiation signal (8, 9). The G-CSF-induced differentiation signals include the induction of neutrophil-specific genes such as myeloperoxidase (MPO) (8) as well as of the morphological changes appropriate for neutrophils (9, 10).

Studies on the signal transduction mediated by cytokine receptors have recently revealed that various cytokines specifically activate the Janus family kinases (JAKs) and signal transducers and activators of transcription (STAT) family proteins (11, 12). JAK are non-receptor-type tyrosine protein kinases that become activated upon ligand-induced receptor dimerization. The activated JAKs phosphorylate STATs on a conserved tyrosine residue (13), which induces STAT dimerization. The dimerized STATs then translocate into the nucleus and regulate gene expression (14, 15).

Several groups have constructed dominant negative forms of STAT proteins, and by expressing them in various cells, it was shown that STAT5 is, at least in part, involved in the interleukin (IL)-3-induced proliferation signal (16), while STAT3 is involved in the IL-6-induced differentiation of mouse M1 cells (17, 18).

G-CSF also activates JAKs, specifically JAK1 and JAK2, and STAT3 (19–21). The membrane-proximal half of the G-CSFR cytoplasmic region (up to 76 amino acids) is sufficient to activate JAK1 and JAK2 (19, 20). However, four additional amino acids including Tyr-703 are required to activate STAT3 (22), suggesting that the extra region works as a docking site for STAT3, as found for gp130, the signal transducer of the IL-6 receptor system (23, 24). The cytoplasmic region of G-CSFR carries four tyrosine residues, all of which are in the distal region of the receptor (7). Previously, we mutated each tyrosine residue to phenylalanine and reported that the tyrosine residues at 703 and 728 and/or their surrounding regions are essential to transduce differentiation signals (10). Since the region around Tyr-703 is essential to activate STAT3 as described above, we proposed that STAT3 mediates the differentiation signals from G-CSFR. In this report, we introduced dominant negative STAT3 into a mouse myeloid cell line that responds to G-CSF by proliferation and differentiation, and showed that STAT3 plays an essential role in G-CSF-induced growth arrest and neutrophilic morphological change but that it is not required for the G-CSF-induced MPO gene expression.

EXPERIMENTAL PROCEDURES

Factors, Cell Lines, and Transformants—The production of mouse recombinant IL-3 has been described (25). Human recombinant G-CSF was provided from Chugai Pharmaceutical Co. The biological activities of G-CSF and IL-3 were determined by measuring their ability to stimulate 3H-thymidine incorporation into mouse IL-3-dependent FDC-P1 cells expressing G-CSFR (7). One unit of activity represents the concentration of CSF required for half-maximal stimulation with 5 × 10⁵ cells/100 μl. The purified human G-CSF had about 1.0 × 10⁵ units/mg protein.

The mouse myeloid cell line GM-162M, which is an LGM-1 transformant expressing the mouse G-CSFR (10), was grown in RPMI 1640...
medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 45 units/ml of mouse IL-3. The expression plasmids, pCAGGS-HA-STAT3F and pCAGGS-HA-STAT3D, for the dominant negative mouse STAT3 were described previously (17). The dominant negative STAT3 are tagged with the influenza hemagglutinin (HA)-epitope at the N terminus of mouse IL-62M cells. The parental GM-I62M cells were transduced with the expression plasmids using a Gene Pulser (Bio-Rad). Cells were cultured in 15 ml of growth medium in 96-well microtiter plates (0.1 ml/well) for 14 days, and the puromycin-resistant clones were selected in a medium containing a 0.75 µg/ml of puromycin. The expression of the dominant negative STAT3 proteins in individual clones was analyzed by immunoprecipitation with rabbit anti-STAT3 antibody (Santa Cruz Biotechnology), followed by Western blotting with the anti-HA monoclonal antibody (Boehringer Mannheim). Twelve independent clones expressing the dominant negative STAT3 were obtained. Three clones were randomly picked and used in this study.

Factor Stimulation, Immunoprecipitation, and Western Blotting—Cells were washed with factor-free medium and grown in medium lacking 10% serum for 1 day. After stimulation for 10 min at 37 °C, 150 units/ml G-CSF or 45 units/ml IL-3, the cells (1 × 106 cells) were lysed with 1 ml of lysis buffer (50 mM HEPES buffer, pH 7.8, 150 mM NaCl, 1.5 mM MgCl2, 0.1% Triton X-100, 1.2 mM EDTA, 20 mM β-glycerophosphate, 100 mM NaF, 0.1 mM Na3VO4, 0.1 mM phenylmethylsulfonyl fluoride hydrochloride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin (26). For immunoprecipitation with the rabbit anti-STAT3 antibody, 0.1 µg of rabbit anti-STAT3 antibody was added to 1 ml of lysate and incubated for 12 h at 4 °C. The immune complex was adsorbed to protein A-Sepharose (30 µl, Pharmacia Biotech Inc.) by incubating at 4 °C for 2 h. The beads were washed with washing buffer (50 mM HEPES buffer, pH 7.8, 150 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100), dissolved in Laemmli sample buffer (62.5 mM Tris-HCl, 1% sodium dodecyl sulfate, 10% glycerol, 100 mM NaF, 0.1 mM Na3VO4, 0.1 mM phenylmethylsulfonyl fluoride hydrochloride), and heated at 95 °C for 5 min. After centrifugation at 3000 rpm for 1 min, the supernatant was removed from the tube by aspiration and subjected to electrophoresis on a 4–20% gradient polyacrylamide gel (Daichi chemical). Proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore Corp.) at 35 V overnight. After blocking with Block Ace (Dainippon Seiyaku), the filters were incubated with 3 ml of phosphate-buffered saline supplemented with 0.1% Tween 20, 10% Block Ace, and 1 ml of mouse- or human-originated STAT3, a DNA fragment containing the STAT3 binding site (17). In brief, the cells were suspended in lysis buffer (50 mM HEPES buffer, pH 7.8, 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 0.1 mM Na3VO4, 0.1% Tween 20, 10% glycerol, 1% dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride hydrochloride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin), and lysed by sonication. After centrifugation at 15,000 × g for 15 min, the supernatant was recovered as whole-cell extracts. A set of complementary oligonucleotides, hSIE (5'-GTGCCATTTCCGTAATCTTGTC-3'), that carry the GAS binding site (27) was custom-synthesized in Vector Request. After annealing, the oligonucleotides were end-labeled with α-32P]dCTP (3000 Ci/mmol, Amersham Corp.) using the Klenow fragment of E. coli DNA polymerase. The reaction mixture for the gel shift assay contained 20 mM HEPES buffer, pH 7.8, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 2 µg poly(dI-dC), 40 µg of the whole-cell extracts, and 62.5 fmol of probe DNA (43,000 cpm) in a total volume of 17 µl. After incubation at room temperature for 20 min, the products were resolved by electrophoresis on 4.5% polyacrylamide gels in 0.25 × TBE buffer as described (17, 22).

Long Term Cell Growth Assay and Morphological Examination—To determine their long term growth potential, cells were incubated in factor-free medium containing either 30 units/ml of human G-CSF or 45 units/ml of mouse IL-3. The medium was replenished every 2 days to reduce the cell density to 1 × 104 cells/ml. Viable cells were counted after staining with trypan blue. To analyze the morphological changes, cells were cytospun onto glass slides and stained with Wright-Giemsa.

Northern Blotting—Cells grown in the presence of IL-3 were washed with factor-free medium, transferred to medium containing 30 units/ml G-CSF, and cultured for 24, 48, and 72 h. Total RNA was extracted using guanidine isothiocyanate-phenol-chloroform (28) and fractionated by electrophoresis through 1% agarose gels. Northern hybridization was conducted under high stringency conditions as described previously (17). The primers for mouse CD44, GAPDH, c-myc, and human EF-1α cDNA (31) were labeled with 32P using a random primer labeling kit (Boehringer Mannheim).

**RESULTS**

Establishment of Transformants Expressing the Dominant Negative STAT3—Mouse myeloid LGM-1 cell transformants (GM-I62M) expressing G-CSFR respond to G-CSF by showing growth arrest, morphological differentiation, and the induction of myeloid-specific genes such as MPO (10). To examine the role of STAT3, which is activated by G-CSF in various myeloid cells, two different dominant negative forms of mouse STAT3 (STAT3F or STAT3D) tagged with the influenza HA (17) were introduced into GM-I62M cells. STAT3F expresses a replacement of the tyrosine residue at 705 with phenylalanine and should not be phosphorylated, whereas two glutamic acids at the residues of 434 and 435 were mutated to alanines in STAT3D, which should prevent the binding of STAT3 to the target DNA. Either form of STAT3 inhibits the IL-6-induced activation of the endogenous STAT3 (17).

Several independent transformant clones expressing STAT3F or STAT3D were obtained and named GM-S3F or GM-S3D, respectively. Analyses by immunoprecipitation with anti-STAT3 antibody, followed by Western blotting with anti-HA antibody, indicated that all the clones expressed a similar amount of STAT3F or STAT3D. The expression level of the dominant negative STAT3 was comparable or slightly higher than that of the endogenous STAT3 (Fig. 1A).

The parental GM-I62M cells are factor-dependent, requiring IL-3 for their growth. This property did not change when cells expressed the dominant negative STAT3; i.e. when IL-3 was removed from the medium, GM-S3F and GM-S3D cells died within 4 days, whereas the transformants continued to grow in the presence of IL-3 (see below), confirming that STAT3 is not involved in the IL-3-induced proliferation signal (32). To examine whether the dominant negative STAT3 could inhibit the G-CSF-induced activation of STAT3, GM-I62M, GM-S3F, and GM-S3D cells were starved for 5 h and then stimulated with either IL-3 or G-CSF. As shown in Fig. 1A, the stimulation of GM-I62M cells with G-CSF but not IL-3 induced the tyrosine phosphorylation of STAT3. A similar level of tyrosine phosphorylation of STAT3 was observed in GM-S3D cells upon G-CSF stimulation, whereas the G-CSF-induced phosphorylation of STAT3 was greatly diminished in GM-S3D cells.

When STAT is activated by tyrosine phosphorylation, it translocates into the nucleus and binds to the DNA element (11, 14). To confirm the inhibition of the G-CSF-induced STAT3 activation in the cells expressing the dominant negative STAT3, a DNA fragment containing the STAT3 binding site (GAS) was prepared, and the cell extracts were analyzed using a gel shift assay. As shown in Fig. 1B, the cell extracts from GM-I62M cells stimulated by G-CSF produced a shifted band(s) that could be supershifted by the anti-STAT3 antibody (data not shown). However, no shifted band was observed in the extracts from IL-3-activated cells. These results agree with previous observations that G-CSF specifically activates STAT3 (33, 34), while IL-3 does not activate STAT3 (32). On the other hand, when the cell extracts from G-CSF-stimulated GM-S3F or GM-S3D cells were analyzed, the intensity of the shifted band was greatly diminished. These results indicate that the G-CSF-induced binding of the endogenous STAT3 to the target DNA element was inhibited by the dominant negative STAT3.

**Effect of the Dominant Negative Form of STAT3 on G-CSF-
induced Cell Growth and Differentiation—To examine the effect of the dominant negative STAT3 on G-CSF-induced growth arrest and terminal differentiation, GM-I62M, GM-S3F, and GM-S3D cells were cultured in medium containing either IL-3 or G-CSF, and their growth properties were monitored. As shown in Fig. 2A, parental GM-I62M cells and their transformants of the dominant negative STAT3 grew in the presence of IL-3 with a similar doubling time (14 h), but all cells died within 4 days in the absence of IL-3. When GM-I62M cells were grown in the G-CSF-containing medium, they started to proliferate with a doubling time of 19 h, which is slightly lower than that observed with these cells in IL-3. Four days after the shift to the G-CSF-containing medium, the cells stopped growing, and at day 10, the cell number in the culture became more than 100 times higher than that observed with GM-I62M cells (Fig. 2B).

We then examined the morphological changes of these cells. As shown in Fig. 3, when GM-I62M, GM-S3F, and GM-S3D cells were cultured with IL-3, they exhibited immature myeloblastic features with a large nucleus. However, when GM-I62M cells were cultured in the medium containing G-CSF, the nuclei became lobulated after 10–12 days. At day 12, about 65% of the GM-I62M cells had lobulated nuclei, which is typical for neutrophilic granulocytes. In contrast, both GM-S3F and GM-S3D cells maintained myeloblastic morphology in the medium containing G-CSF, and the percentage of cells carrying the lobulated nuclei was similar to that observed in the medium containing IL-3 (Fig. 3B). These results indicate that STAT3 activation is crucial for the G-CSF-induced growth arrest and morphological change to neutrophilic granulocytes.

Effect of the Dominant Negative STAT3 on Gene Expression—During the G-CSF-induced differentiation of neutrophils, the expression of many genes is modulated (8, 35). For example, the expression of MPO and neutrophilic elastase is up-regulated, while the expression of the c-myc oncogene is down-regulated (8, 36). To examine whether the expression of these genes is regulated by STAT3, GM-I62M, GM-S3F, and GM-S3D cells were cultured in the presence of G-CSF, and the expression levels of MPO and c-myc were followed by Northern hybridization. As shown in Fig. 4, G-CSF gradually induced the MPO gene expression in GM-I62M cells, while the expression of the c-myc gene was down-regulated. However, in both GM-S3F and GM-S3D cells, G-CSF could stimulate the MPO gene expression but did not down-regulate the c-myc expression. These results indicate that G-CSF-induced repression of the c-myc gene expression is mediated by activated STAT3, but the MPO expression is not.
gene induction by G-CSF does not require the activation of STAT3.

**DISCUSSION**

Cytokines stimulate the tyrosine phosphorylation of JAKs and activate the STAT proteins. For example, IL-3 activates JAK2 and STAT5 (16, 37), while IL-6 and G-CSF activate JAK1 and JAK2, and STAT3 (21, 38–40). Using dominant negative forms of STAT proteins, Mui et al. (16) have reported that STAT5 is, at least in part, involved in the IL-3-induced proliferation signal, whereas STAT3 seems to be involved in the IL-6 or LIF-induced differentiation of mouse M1 cells (17, 18, 24) and to mediate the antiapoptotic function of IL-6 by activating Bcl-2 (41). In this report, we have shown that dominant negative STAT3 inhibits the G-CSF-induced morphological changes of mouse LGM-1 cells. However, there was no effect on the G-CSF-induced growth: the cells expressing the dominant negative STAT3 continued to grow in response to G-CSF. These results indicate that STAT3 activation is not necessary for the G-CSF-induced proliferation of the myeloid cell precursor.

Two models have been proposed for the cytokine-induced differentiation of hemopoietic cells. In the stochastic model (42–45), cytokines provide proliferative and survival signals to the differentiating hematopoietic cells, but they do not provide specific lineage commitment signals. In the instructive model (46), cytokines transmit specific signals to multipotent hemopoietic cells, thereby directing lineage commitment. We showed

**FIG. 3. Effect of dominant negative STAT3 on G-CSF-induced morphological changes.** A, the G-CSF-induced morphological changes. GM-I62M, GM-S3F, and GM-S3D cells were maintained in the presence of either IL-3 (45 units/ml) or G-CSF (30 units/ml) for 12 days. The cell morphology was visualized after Wright-Giemsa staining. Magnification was × 200. B, inhibition of the G-CSF-induced morphological changes by dominant negative STAT3. GM-I62M, GM-S3F, and GM-S3D cells were cultured for 12 days in the medium containing either 45 units/ml IL-3 or 30 units/ml G-CSF, and the cells with lobulated nuclei were scored. The cell morphology of at least 400 cells was examined. The data are presented as the percentage of the cells with differentiated morphology. The values represent the average of four independent experiments ± S.D. (bars).
here that the differentiation-competent LGM-1 cell transformants expressing the dominant negative STAT3 grow without differentiation in response to G-CSF. These results indicate that the G-CSF-induced proliferation signal alone is not sufficient to induce the terminal differentiation and that G-CSF actively transmits the differentiation signal (9, 10), supporting the instructive model for the G-CSF-induced terminal differentiation into neutrophils, at least in LGM-1 cells.

We showed that the differentiation signals evoked by G-CSF include growth arrest and the down-regulation of c-myc, both of which are mediated by activated STAT3. G-CSF induces the c-myc expression, and it is observed in cells expressing the truncated G-CSF that cannot activate STAT3 (22), suggesting that the induction and repression of the c-myc gene is regulated by different mechanisms. Thus, the intact G-CSF should transduce both the proliferation signal (activation of the c-myc gene), and the signal causing growth arrest (the repression of the c-myc expression). LGM-1 cells eventually cease growing and terminally differentiate in response to G-CSF (10), indicating that the signal for growth arrest predominates over the signal to stimulate proliferation. On the other hand, G-CSF stimulates the proliferation of FDC-P1 and BAF cells without inducing growth arrest (7, 8). Since G-CSF activates STAT3 in these cell lines, these results suggest that the downstream signaling machinery that induces growth arrest is in LGM-1 cells, which are committed to the neutrophilic granulocyte lineage, but not in the early myeloid precursor FDC-P1 or proB BAF cells. This assumption probably holds for the IL-6 and LIF systems, because IL-6 and LIF induce the differentiation of mouse myeloid M1 cells but stimulate proliferation of myeloma or embryonic stem cells (39, 40). To understand the molecular mechanisms behind G-CSF-induced growth arrest, it will be necessary to identify the protein that can be specifically activated by STAT3 in LGM-1 cells but not in FDC-P1 or BAF cells.

Unlike the growth arrest that was inhibited by the dominant negative STAT3, the G-CSF-induced MPO gene expression was not affected by the dominant negative STAT3, suggesting that the differentiation signals from the G-CSFR can be separated into two pathways. This scheme agrees with the recent report that the 32D myeloid cells overexpressing Bcl-2 undergo the morphological change into neutrophilic granulocytes without G-CSF but do not express the MPO gene (47). Previously, we mutated one of the tyrosine residues of the G-CSFSR (Tyr-703) to phenylalanine. The mutated receptor could transduce neither the signal for the G-CSF-induced morphological change nor the induction of the MPO gene, suggesting that a protein(s) that can associate with this region is responsible for these differentiation signals (10). Recently, we have identified several proteins that can bind to the phosphorylated peptide carrying Tyr-703.3 One of these was STAT3, but several other proteins specifically bound to this peptide. It is possible that some of them are responsible for activating the MPO gene. In any case, our results suggest that the G-CSF-induced differentiation into neutrophilic granulocytes is not mediated by a single pathway, rather it is mediated by the concerted action of multiple signaling pathways.

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Role of STAT3 in G-CSF-induced Myeloid Cell Differentiation

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