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G-CSF Induces Apoptosis of a Human Acute Promyelocytic Leukemia Cell Line, UF-1: Possible Involvement of Stat3 Activation and Altered Bax Expression

Miyako Yoshinari,[1] Masue Imaizumi,[1,2] Atsushi Sato,[1] Masayoshi Minegishi,[4] Kunihiro Fujii,[1] Hoshiro Suzuki,[1] Toshihiko Miura,[3] Tadao Funato,[3] Toshiaki Saito,[1] Shuji Chikaoka,[1] Takeshi Rikiishi,[1] Masahiro Kizaki[5] and Kazuie Iinuma[1]

[1]*Department of Pediatrics,* [2]*Department of Pediatric Hematology and Oncology,* [3]*Department of Clinical and Laboratory Medicine, Tohoku University School of Medicine, Sendai 980-8574,* [4]*Department of Pediatric Oncology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, and* [5]*Division of Hematology and Clinical Laboratories, Keio University School of Medicine, Tokyo 160-8582*

Yoshinari, M., Imaizumi, M., Sato, A., Minegishi, M., Fujii, K., Suzuki, H., Miura, T., Funato, T., Saito, T., Chikaoka, S., Rikiishi, T., Kizaki, M. and Iinuma, K. *G-CSF Induces Apoptosis of a Human Acute Promyelocytic Leukemia Cell Line, UF-1: Possible Involvement of Stat3 Activation and Altered Bax Expression.* Tohoku J. Exp. Med., 1999, **189** (1), 71-82 —— Granulocyte colony-stimulating factor (G-CSF) is a cytokine that regulates the proliferation, differentiation and survival of cells in the granulocytic lineage. In this study, however, we found that G-CSF or interleukin-6 (IL-6) induced UF-1, a human acute promyelocytic leukemia cell line, into apoptosis that was confirmed by morphological features and DNA fragmentation. This rare response is demonstrated for the first time with human acute promyelocytic leukemia cell line. The apoptosis induced by G-CSF or IL-6 was not preceded by terminal differentiation characterized by morphological maturation, capability to reduce nitroblue tetrazolium, or surface CD11b expression. Interestingly, Western blot analysis revealed that the stimulation of UF-1 with either G-CSF or IL-6 resulted in excessive activation of both signal transducer and activator of transcription 3α (Stat3α) and Stat3β. Furthermore, an additional 18 kDa Bax-related protein was expressed by the stimulation of G-CSF or IL-6, while Bcl-2 and Bcl-X proteins

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Address for reprints: Masue Imaizumi, M.D., Department of Pediatric Hematology and Oncology, Tohoku University School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan.

e-mail: mimaizumi@ped.med.tohoku.ac.jp

remained unchanged. These findings suggest that UF-1 may be a valuable tool in investigating the aberrant regulation of apoptosis, especially the Stat3 involvement in the mechanism of apoptosis induction. ——— human APL cell line; apoptosis; G-CSF; IL-6; Stat3

Apoptosis is a physiological cell suicide process that is essential in maintenance of tissue homeostasis and elimination of genetically altered or infected cells (Miyajima et al. 1999). It is often regulated through the stimulation of cytokines, the depletion of which may turn off anti-apoptotic signaling or actively induce cell death. Thus, alterations in the mechanisms of cytokine signaling for cell cycle progression and anti-apoptotic function are implicated in hematological disorders (Miyajima et al. 1999).

In regard to the pathogenesis of acute myeloblastic leukemia (AML), granulocyte colony-stimulating factor (G-CSF) signaling pathway is of particular interest, because G-CSF is known to regulate the proliferation, differentiation and survival of myeloid progenitor cells (Liu et al. 1996). Noteworthy, G-CSF and interleukin-6 (IL-6) share a common signal transduction pathway, as the G-CSF receptor (G-CSFR) shares a strong similarity to the gp130 subunit of the IL-6 receptor. Moreover, G-CSF or IL-6 activates Janus tyrosine kinase 1 (Jak1) and Jak2, resulting in tyrosine phosphorylation of the signal transducer and activator of transcription 3 (Stat3) and Stat1 proteins. Subsequently, Stat proteins forming stable homodimers and heterodimers translocate to the nucleus, where they activate target genes (Tian et al. 1994; Guschin et al. 1995; Shimoda et al. 1997). However, little is known about how apoptosis is regulated by activation of tyrosine kinase pathways and what mediators of signal transduction are involved in the G-CSF signaling pathway.

UF-1 is an acute promyelocytic leukemia (APL) cell line derived from a human APL patient who was clinically resistant to retinoic acid (RA), and is of particular interest as the first permanent cell line with spontaneous RA-resistant APL cells (Kizaki et al. 1996). Moreover, UF-1 cells exhibit a decreased sensitivity to the action of RA to induce terminal differentiation in vitro as compared with that of HL-60 and NB4 cell lines (Wang et al. 1989; Lanotte et al. 1991; Kizaki et al. 1996).

In the present study, we elucidated cytokine effects on regulation of proliferation, differentiation and apoptosis of UF-1. Interestingly, our results demonstrated that UF-1 cells, but not HL-60 or NB4 cells, were directly induced into apoptosis by the stimulation of either G-CSF or IL-6, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-3 did not influence their survival. Furthermore, we revealed an excessive activation of Stat3 in UF-1 cells stimulated by either G-CSF or IL-6, suggesting a possible involvement of the Stat3 activation in the aberrant regulation of apoptosis.

Materials and Methods

Cell lines and cytokines

Cells were cultured in RPMI-1640 medium supplemented with 10% of fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. For the stimulation with cytokines, cells were cultured in the presence of different concentrations of G-CSF, GM-CSF, IL-6, or IL-3 for various periods as indicated. The G-CSF and GM-CSF were kindly supplied by Chugai Pharmaceutical (Tokyo), IL-3 and IL-6 by Kirin Brewery (Tokyo). All were reconbinant human cytokines. Viable cell numbers were determined by trypan blue exclusion and counted in a hemocytometer.

Nitroblue tetrazolium reduction assay

Functional maturation was evaluated by nitroblue tetrazolium (NBT) reduction assay as described (Imaizumi et al. 1994). Briefly, 1.0×10^6 cells were suspended in 1 ml of RPMI-1640 medium containing 10% FBS, 0.5 mg/ml of NBT (Sigma Chemical, St. Louis, MO, USA) and 162 nM 12-O-tetradecanoylphorbol-13-acetate (Sigma Chemical), and incubated at 37°C for 25 minutes. Subsequently, cytospin slides were prepared with Wright-Giemsa staining and cells with black-blue formazan granules were detected as positive for NBT reduction.

Cell morphology

Cytospin preparations of both untreated and treated cells were stained with Wright-Giemsa stain for morphological characterization. Five hundred cells were scored as differentiated for cells showing metamyelocytic or more mature forms, and as apoptotic for cells exhibiting highly condensed and/or fragmented nuclei.

Immunophenotypic analysis

After cultivation with G-CSF, GM-CSF, IL-6, or IL-3 for 4 days and 7 days, expressions of CD11b on UF-1 cells were studied by flow cytometric analysis using anti-CD11b monoclonal antibody (Becton Dickinson, San Jose, CA, USA) as described (Imaizumi et al. 1987).

DNA fragmentation

Total genomic DNA was extracted according to standard phenol/chloroform protocol (Bessho et al. 1994). Six μg of undigested DNA was separated on 1.5% agarose gel, stained with ethidium bromide, and photographed.

Western blot analysis

Antibodies used were as follows: Anti-Stat3 antibody (Transduction Labs,

Lexington, KT, USA) directed against both Stat3α and Stat3β; anti-phosphorylated Stat3 antibody (Phospho-Stat3 [Tyr705] Antibody) (New England Biolabs, Beverly, MA, USA) directed against phosphorylated tyrosine 705 of Stat3; anti-Bcl-2 and anti-Bax monoclonal antibodies (Medical and Biological Labs, Nagoya); and anti-Bcl-$x_{S/L}$ antibody, S-18 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Whole cell lysates were mixed with 3× sodium dodecyl sulfate (SDS)-sample buffer (150 mM Tris-HCl, pH 6.8; 6% SDS; 30% glycerol; 15% 2-mercaptoethanol) at 1 : 2 ratio and were heated for 5 minutes at 100°C. Protein loaded on each lane was 100 μg for Stat3 and phophorylated Stat3 detection, or 60 μg for Bcl-2, Bcl-X and Bax detection, respectively. They were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Waltham, MA, USA). Prestained molecular weight markers (Kaleidoscope Prestained Standards; Bio-Rad, Hercules, CA, USA) were included in each gel. Membranes were blocked for 60 minutes in Tris-buffered saline (TBS: 100 mM Tris-HCl, pH 7.5 and 154 mM NaCl) with 0.05% Tween-20 (TBS-T) and 5% skim milk (Wako Pure Chemical Industries, Osaka). After blocking, membranes were incubated with Phospho-Stat3 antibody for overnight at 4°C, or with other primary antibodies for 1 hour at room temperature, respectively. After washing the membranes three times with TBS-T, they were incubated with horseradish peroxidase conjugated secondary antibody in TBS-T for 60 minutes. Subsequently, membranes were washed three times with TBS-T and developed using the enhanced chemiluminescence (ECL) detection system (Amersham Life Sciences, Arlington Heights, IL, USA). Where indicated, membranes were stripped, blocked, and reprobed following the same protocols as described above.

Immunoprecipitation

Total cell lysates (1×10^7 cells) were immunoprecipitated with anti-Stat3 antibody, C-20 (Santa Cruz Biotechnology), which was directed against Stat3α, and subjected to 10% SDS-PAGE. The membrane was probed with anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology, Lake Placid, NY, USA), and visualized with ECL detection system. After stripping, the membrane was immunoblotted with anti-Stat3 antibody, C-20, to confirm the equal volume loading.

Results

Growth inhibition of UF-1 cells by G-CSF or IL-6 stimulation

We studied the effects of different cytokines, such as G-CSF, GM-CSF, IL-3 and IL-6, on the growth pattern of UF-1 and NB4 cells. Cell growth and viability of NB4 cells did not change between conditions with or without each cytokine, indicating that these cytokines alone did not affect growth of NB4 cells (Figs. 1b and 1d). By contrast, growth inhibition and cell death of UF-1 cells

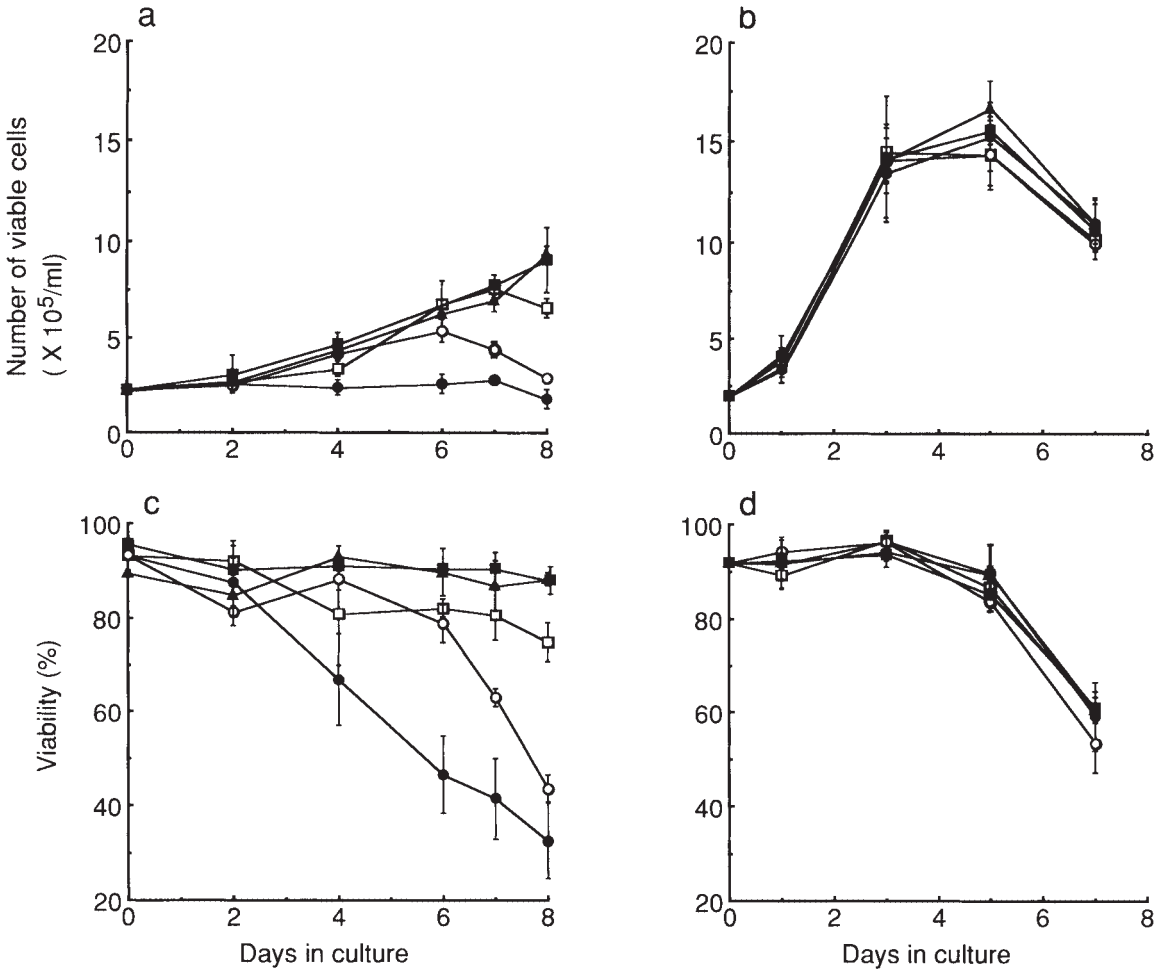

Fig. 1. Effects of various cytokines on the growth and viability of UF-1 (a, c) and NB4 cells (b, d). Cells were cultured in 10%-FBS-containing RPMI 1640 without cytokine (□) or in the presence of 10 ng/ml of G-CSF (●), IL-6 (○), IL-3 (▲), or GM-CSF (■). Number of viable cells (a, b) and viability (c, d) were counted at the indicated time.

were induced in response to either G-CSF or IL-6 at 10 ng/ml or higher concentrations, while GM-CSF or IL-3 had no effect on the growth of UF-1 cells. This phenomenon was observed in a dose-dependent manner (data not shown), and more pronounced with G-CSF than that with IL-6 (Figs. 1a and 1c). Thus, G-CSF or IL-6 inhibited the cell growth of UF-1 cells and induced their cell death.

Evidence of apoptosis in UF-1 cells stimulated with G-CSF or IL-6

Apoptosis of UF-1 cells was assessed by cell morphology and DNA fragmentation. Morphologically, chromatin condensation, fragmentation of nuclei and formation of apoptotic bodies were observed, indicating the presence of apoptotic cells (Fig. 2). Cells with apoptotic features were 10 to 15% of whole cell population after 4 days with G-CSF or IL-6 treatment (Fig. 3c). DNA fragmentation was prominent in UF-1 cells with G-CSF or IL-6 stimulation (Fig. 4). Thus, the cell death of UF-1 was confirmed to be due to apoptosis.

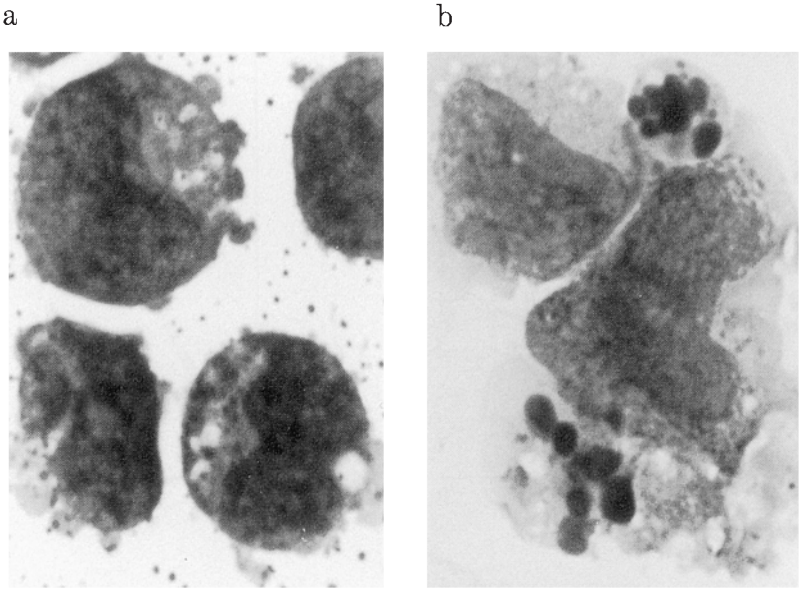

Fig. 2. Morphology of UF-1 cells before treatment (a) and after 5-days treatment with 10 ng/ml of G-CSF (b). Cells with high condensed and fragmented nuclei and vacuolated cytoplasm were considered to be apoptotic (Original magnification $\times 1000$).

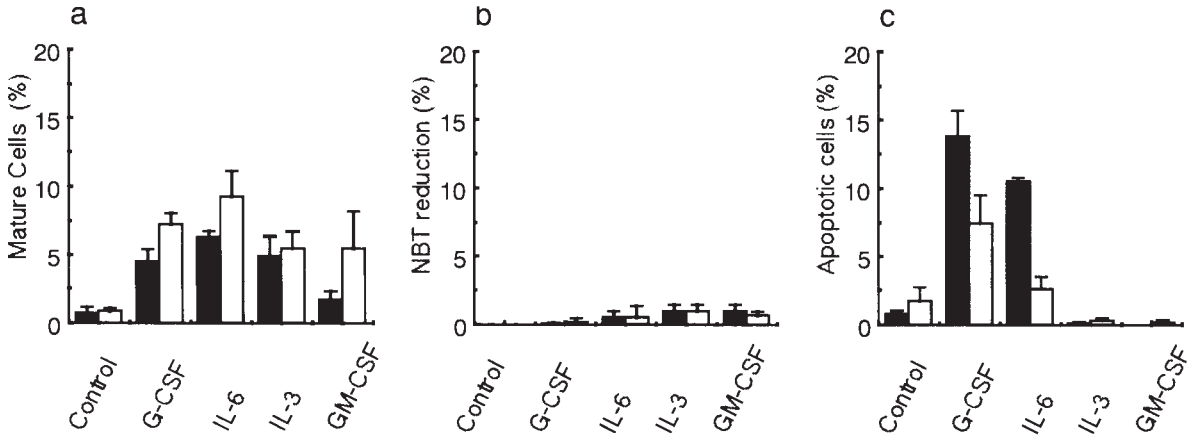

Fig. 3. Proportions of cells with mature morphology (a), NBT reduction (b), and apoptotic features assayed by Wright-Giemsa staining (c). UF-1 cells were cultured without cytokine or in the presence of indicated cytokine for 4 days (solid box) or seven days (open box). Results represent mean$\pm$S.D. of three or more experiments.

Direct induction of apoptosis without differentiation by G-CSF or IL-6 stimulation

As to the onset of apoptosis, it is important to clarify whether the cell death was resulting from or independent of terminal differentiation. To assess this point, we examined their morphology, surface expression of the maturation antigens CD11b, and capability to reduce NBT. The proportion of cells with morphological maturation beyond metamyelocytes was less than 10% of whole cell population, and there observed no difference between each cytokines (Fig. 3a). UF-1 cells treated with G-CSF or IL-6 did not show any increase in CD11b expression (data not shown), and cells with positive for NBT reduction were hardly seen in any conditions (Fig. 3b). Thus, terminal differentiation was not

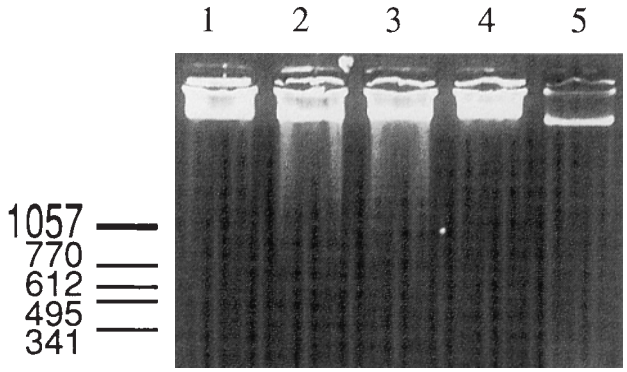

Fig. 4. DNA fragmentation patterns of unstimulated cells (lane 1) and stimulated cells for 7 days with 10 ng/ml of G-CSF (lane 2), IL-6 (lane 3), IL-3 (lane 4), and GM-CSF (lane 5) of UF-1 cell line. Six μg of DNA was loaded per lane and electrophoresed on a 1.5% agarose gel containing ethidium bromide. Molecular sizes in base pairs are indicated on the left.

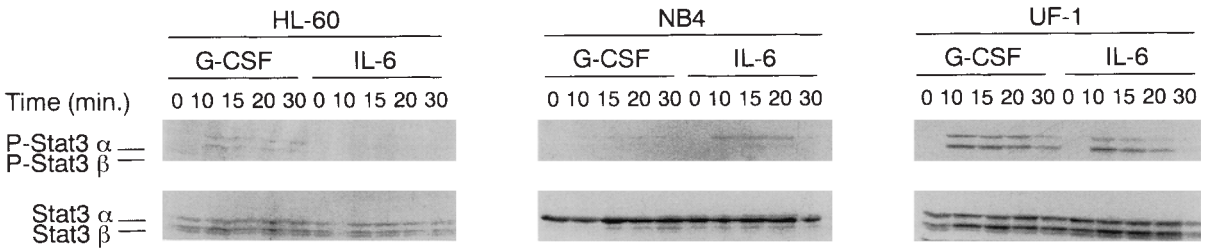

Fig. 5. Expression of Stat3α and Stat3β in HL-60, NB4, and UF-1 cell lines. Cell lysates were prepared at the indicated time in minutes with G-CSF (100 ng/ml) or IL-6 (100 ng/ml) stimulations and subjected to Western blot analysis, using antibodies directed against phosphorylated-Stat3 (P-Stat3) (upper panel) or Stat3 (lower panel). Equal amounts of 100 μg of proteins were loaded on the membrane. These were the representative results or experiments repeated more than three times.

observed in UF-1 cells with cytokine stimulation. Furthermore, apoptotic cells were observed as early as 4 days after the addition of G-CSF or IL-6, when cells did not show differentiation (Figs. 3a and 3c). These results showed that G-CSF or IL-6 stimulation directly induced apoptosis of UF-1 cells without their prior commitment to granulocytic differentiation.

Excessive activation of Stat3 in G-CSF or IL-6 stimulated UF-1 cells

We investigated expressions and tyrosine phosphorylations of Stat3 in HL-60, NB4, and UF-1 cells by Western blotting. Stat3α was constitutively expressed with or without cytokines in three cell lines, while Stat3β was expressed in HL-60 and UF-1, but not in NB4 (Fig. 5). Phosphorylations of tyrosine 705 were seen in the three cell lines only with G-CSF or IL-6 stimulation, and UF-1 showed prominent phosphorylations for both Stat3α and Stat3β, as compared to HL-60 or NB4. Especially, Stat3β represented an excessive activation within 10 minutes after stimulation of G-CSF or IL-6. As to verify the amount of Stat3α phosphorylation, cells were immunoprecipitated with anti-Stat3α antibody, C-20, and

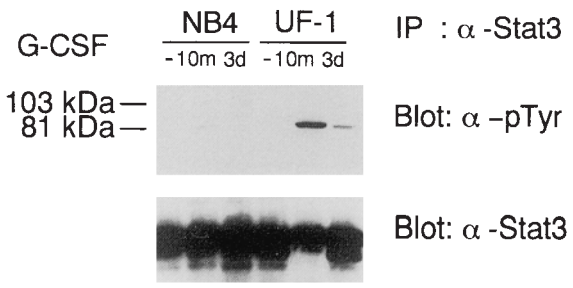

Fig. 6. Phosphorylation of Stat3 in the presence of G-CSF shown in NB4 and UF-1 cell lines. Whole cell lysates from unstimulated cells (—), and stimulated cells with 100 ng/ml of G-CSF for 10 minutes (10m) or with 10 ng/ml of G-CSF for three days (3d) were immunoprecipitated with anti-Stat3 antibody, C-20 (α-Stat3), and immunoblotted with anti-phosphotyrosine antibody, 4G10 (α-pTyr) (upper panel). The blot was reprobed with α-Stat3 to confirm equal loading (lower panel). Positions of molecular weight standards are indicated at the left.

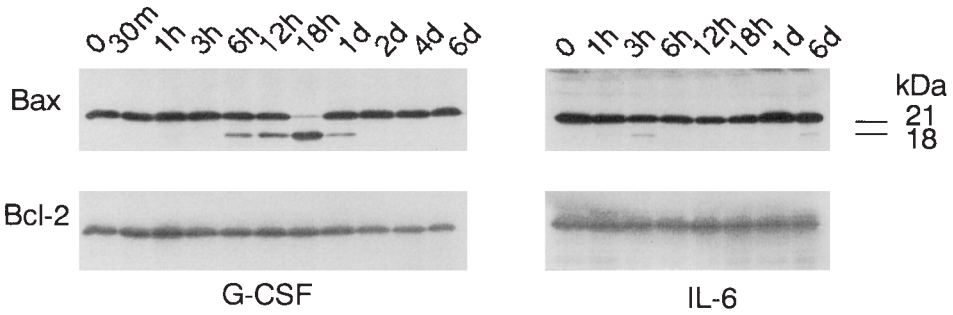

Fig. 7. Expressions of Bax and Bcl-2 proteins in UF-1 cells prepared at the indicated time with 10 ng/ml of G-CSF (left panel) or IL-6 (right panel) stimulations and subjected to Western blot analysis, using antibodies directed against anti-Bax (upper panel) or anti-Bcl-2 (lower panel). Equal amounts of 60 μg of proteins were loaded on the membrane.

blotted with anti-phosphotyrosine antibody, 4G10. Then, it was demonstrated more clearly that the amount of phosphorylated Stat3α compared to loaded protein was extremely high in UF-1 cells (Fig. 6). Because tyrosine phosphorylation of Stat3β was indicated to be much higher than that of Stat3α by Western blotting, both Stat3α and Stat3β of UF-1 cells were excessively activated by the stimulation of either G-CSF or IL-6.

Expressions of Bcl-2, Bcl-X, or Bax proteins during apoptosis induction

To determine which protein finally acted as an inducer for apoptosis, we examined Bcl-2, Bcl-X and Bax expressions by Western blotting analysis (Fig. 7 and data not shown). The extent of these proteins did not change between 30 minutes to 6 days after G-CSF or IL-6 stimulation. Of interest, anti-Bax antibody not only revealed a 21 kDa Bax protein, but also detected the transient appearance of an additional 18 kDa protein in UF-1 cells treated with either G-CSF or IL-6.

Discussion

G-CSF can prolong granulocytes survival by interfering with the physiologic process of apoptosis (Colotta et al. 1992). G-CSF was also shown to accelerate the RA-induced maturation process in vitro of fresh APL cells obtained from patients (Imaizumi et al. 1994). To our surprise, however, this study demonstrated for the first time that G-CSF mediated an opposed effect on the survival of UF-1, a human APL cell line. As reported previously, induction of cell death by G-CSF was not confined only to UF-1 cells. Bessho et al. (1994) reported similar findings with a radiation-induced murine myeloid leukemia cell line that was induced into apoptosis by G-CSF both in vitro and in vivo, thus resulting in the survival of leukemic mice. Similarly, Dong et al. (1996) demonstrated G-CSF-induced apoptosis of murine myeloid 32D cells transfected with the human G-CSFR.

The distinct regions of the cytoplasmic domain of the G-CSFR have shown to play a determinative role in G-CSF induced proliferation or differentiation (Dong et al. 1993). However, we could not detect mutations in these regions of G-CSFR in UF-1 cells (data not shown). Thus, we speculate that aberrant signal transduction leading to apoptosis may lie within the common signaling cascade of G-CSF and IL-6, such as Jak-Stat pathway, because both G-CSF and IL-6 induced UF-1 cells into apoptosis in a similar manner as demonstrated in this study.

In regard to the function of Stat3, its activation has been shown to correlate with Src-transformed cells (Yu et al. 1995) or to play an essential role for G-CSF or IL-6 mediated differentiation in myeloid cells (Minami et al. 1996; Shimozaki et al. 1997), while in rarer cases it correlates with apoptosis induction. In mouse pro-B cell line BAF-B03, Stat3 mediates anti-apoptotic signal through Bcl-2 expression (Fukada et al. 1996), while it induces apoptosis without terminal differentiation in Stat3-overexpressed M1 cells (Minami et al. 1996). In a pre-B-cell variant line, 1A9-M, Stat3 activation was shown to be essential for IL-6-induced apotosis, which was independent of Bcl-2 downregulation (Oritani et al. 1999). Further experiments are required to clarify these paradoxical association of the Stat3 activation with apoptosis induction.

Two isoforms of Stat3 have been identified, α (p92) and β (p83), which have distinct transcriptional and biological functions (Schaefer et al. 1997). Stat3α, but not Stat3β, contains serine 727 that has been demonstrated to be a site of phosphorylation, possibly by MAP kinase (Wen et al. 1995). Stat3β arises from alternative splicing of the Stat3 gene that lacks the C-terminal domain of Stat3α results in the loss of serine 727. Instead it contains 7 unique amino acid residues that enable it, unlike Stat3α, to interact with c-Jun (Schaefer et al. 1995). In COS cells transfected with Stat3 expression plasmids, activated Stat3β was more stable and had greater DNA-binding activity than activated Stat3α, whereas Stat3α showed greater transcriptional activity than Stat3β (Schaefer et al. 1997).

When both were co-expressed in monkey COS-1 cells, Stat3β inhibited the transactivating ability of Stat3α, acting as a negative regulator of transcription (Caldenhoven et al. 1996). UF-1 may exhibit an aberrant response to cytokine stimulation, leading to a changed balance of Stat3α and Stat3β, which thereby influences the transactivating ability.

As to elucidate a final step in an evolutionary conserved pathway for apoptosis, we examined expressions of Bcl-2 and Bcl-X, cell death antagonists, as well as the death effector Bax (Packham et al. 1998). UF-1 showed no change of the expression of Bcl-2 or Bcl-X protein, neither the upregulation of 21 kDa Bax protein during induction of apoptosis. However, it does not rule out the possibility that other Bcl-2 families as well as caspase families, Ras-MAP pathways, or PI3 kinase are involved in the mechanism of apoptosis induction. Furthermore, we found the transient expression of 18 kDa protein detected by anti-Bax antibody, which was previously shown to be involved in the induction of cell death, and therefore it may be indicated to play a role in the regulation of apoptosis (Thomas et al. 1996; Isogai et al. 1998).

Our work is the first to demonstrate the G-CSF effect inducing apoptosis of human APL cell line with a reciprocal (15 ; 17) (q21 ; q21) translocation and PML/RARα chimeric gene. This rare response may be caused in relation with chimeric PML/RARα oncoprotein or with hyperactivated Stat3 protein, or with another factor(s) which we could not identify. The PML/RARα fusion protein of APL renders hemopoietic progenitor cells resistant to apoptotic stimulations with a lack of caspase 3 activation (Wang et al. 1998). Therefore, PML/RARα oncoprotein increases cell survival leading to leukaemogenesis. In considering the role of PML/RARα in regulation of apoptosis, it would be interesting to investigate the possible correlation between G-CSF signaling cascades and PML/RARα action.

Finally, it is likely that excessive activations of Stat3 may also contribute to other disorders of granulopoiesis in which myelopoiesis is either abnormally decreased or increased. Analyses of patients with AML, myelodysplastic syndrome, and other neutropenic syndromes seem warranted. In conclusion, UF-1 may serve as a powerful tool in investigating the biological role of Stat3 and in understanding how apoptosis is regulated through G-CSF signaling in granulopoiesis.

Acknowledgments

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