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Granulocyte Colony-Stimulating Factor–Induced Terminal Maturation of Human Myeloid Cells Is Specifically Associated with Up-Regulation of Receptor-Mediated Function and CD10 Expression

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Abstract

The acute promyelocytic leukemia cell line NB4 was differentiated by all-*trans* retinoic acid (ATRA), which enhanced the superoxide-producing capacity stimulated by the chemotactic peptide and phorbol ester in this cell line. Granulocyte colony-stimulating factor (G-CSF) by itself had no effect on NB4 cells but exerted additional enhancing effects on the respiratory burst activity in the presence of ATRA. This finding was not due to the induction of G-CSF receptor by ATRA, because NB4 cells expressed abundant G-CSF receptor with or without ATRA. Unlike ATRA, G-CSF enhanced superoxide release stimulated by the chemotactic peptide but not by phorbol ester. In addition, G-CSF but not ATRA attenuated cell death and enhanced survival during differentiation. Cell surface expression of the chemotactic peptide receptors CD33 and CD10 but not of CD11b and CD11c was up-regulated by ATRA plus G-CSF far more profoundly than by ATRA alone. Fundamentally identical but slightly different phenomena for the cell surface expression of CD33 and CD10 were observed in the normal human bone marrow mononuclear cells; G-CSF induced CD10 even in the absence of ATRA and down-regulated CD33 in normal cells. The present results indicate that G-CSF–induced terminal maturation of human myeloid cells is associated with up-regulation of receptor-mediated function and CD10 expression. *Int J Hematol.* 2003;77:142-151.

Key words: Granulocyte colony-stimulating factor; Differentiation; All-*trans* retinoic acid; CD10; Chemotactic peptide

1. Introduction

Granulocytic differentiation of human hematopoietic cells is a multistep process that is essential for the production of normal white blood cells in vivo [1]. Physiological regulation of normal granulopoiesis appears to be conducted via physiological hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) [1,2]. This type of differentiation of the normal hematopoietic cells is usually associated with proliferation [2].

In addition, there exists a type of granulocytic differentiation, namely differentiation induction of the leukemic granulocyte by differentiation inducers such as all-*trans* retinoic acid (ATRA) and dimethyl sulfoxide [3,4]. In contrast to the differentiation of normal hematopoietic cells by hematopoietic growth factors, this type of differentiation is associated with growth suppression and/or apoptosis of the cells [3,4].

Although important, exact evaluation of the difference and the interaction between these 2 distinct types of granulocytic differentiation has not been fully performed because of the lack of a human in vitro culture system to study both kinds of differentiation. There seems to be no report describing a good differentiative response of normal human bone marrow myeloid cells to differentiation inducers, and almost all human myeloid leukemia cell lines do not undergo sufficient differentiation in response to G-CSF. Difficulty in the study of human granulocytic differentiation also may be due to parameters that exactly represent terminal granulocytic differentiation.

Differentiation of granulocytic cells is characterized by morphological change, up-regulation of granulocytic enzymes, acquisition of effector functions, and cell surface expression of functional molecules [5-8]. It is well known

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that the respiratory burst activity is one of the most important functional parameters of myeloid differentiation [5-7]. There are also several cell surface antigens specific for myeloid lineage, such as CD11b and CD33, and these cell surface molecules develop during differentiation [6,9,10]. However, these functions and cell surface molecules seem to reflect a relatively early stage of myeloid differentiation rather than terminal granulocytic maturation [6,9,10]. On the other hand, CD10, well known as common acute lymphoblastic leukemia antigen [11], has been reported to be expressed in mature neutrophils [12-14]. This finding suggests the possibility that CD10 could be a good marker of terminal myeloid differentiation.

In the present study, we used human promyelocytic cell line NB4 to study the effects of ATRA and G-CSF on differentiation of this cell line. ATRA, although a differentiation inducer, is a physiological agent related to vitamin A and a naturally occurring substance acting via specific nuclear transcriptional receptors [15]. Both ATRA and G-CSF are of great importance in a clinical setting, because both agents are used in patients with hematological diseases, including acute promyelocytic leukemia [16]. Our results indicated that G-CSF, compared with ATRA, affects human hematopoietic cells at a more terminal level or stage of granulocytic differentiation. The receptor-mediated functions and cell surface expression of unique molecules were specifically induced by G-CSF.

2. Materials and Methods

2.1. Reagents

Highly purified recombinant human G-CSF produced by *Escherichia coli* was provided by Kirin Brewery, Tokyo, Japan. ATRA, N-formyl-methionyl-leucyl-phenylalanine (FMLP), and phorbol myristate acetate (PMA) were purchased from Sigma Chemical, St. Louis, MO, USA. RPMI 1640 medium was from Gibco Laboratories, Grand Island, NY, USA, and fetal calf serum was from JRH Bioscience, Lenexa, KS, USA. The monoclonal antibodies R-phycoerythrin (PE)-conjugated anti-CD10, fluorescein isothiocyanate (FITC)-conjugated anti-CD33, PE-conjugated anti-CD11b, and PE-conjugated anti-CD11c antibodies were from Beckman Coulter, Tokyo, Japan. Monoclonal anti–G-CSF receptor (G-CSFR) antibody was from Cosmobio, Tokyo, Japan. FITC-conjugated $F(ab')_2$ fragment goat anti-mouse immunoglobulin G (IgG) was from Beckman Coulter, Tokyo, Japan, and FMLP-FITC was from Phoenix Pharmaceuticals, Mountain View, CA, USA. Other reagents for cell culture, fluorescence-assisted cell sorter (FACS) analysis, and determination of superoxide release were purchased from Sigma.

2.2. Culture of the Cells

NB4 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). For induction of differentiation, NB4 cells were seeded in a 6-well plate at 2×10^4 cells/mL and grown in the presence or absence of 1 μM ATRA and/or 50 ng/mL G-CSF for 1 to 10 days. Frozen human bone marrow mononuclear cells from healthy volunteers were obtained from Biowhittaker, Walkersville, MD, USA, and were melted in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). After 6 days of precultivation in the medium, the bone marrow mononuclear cells were seeded at 2×10^4 cells/mL and grown in the presence or absence of 10 μM ATRA and/or 50 ng/mL G-CSF for 6 days.

2.3. Determination of Superoxide Release

Superoxide was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome C, and the continuous assay was performed in a Hitachi 556 spectrophotometer (double-wavelength spectrophotometer; Hitachi, Tokyo, Japan), equipped with a thermostatted cuvette holder (37°C), as described elsewhere [17]. The reduction of cytochrome C was measured at 550 nm with a reference wavelength at 540 nm, and the time course of cytochrome C reduction (absorbance change at 550-540 nm) was followed on the recorder. The amount of superoxide release was calculated from cytochrome C reduced for 5 minutes after the addition of FMLP and from the linear portion of the cytochrome C reduction for PMA.

2.4. Flow Cytometry Analysis

Cell cycle analysis was performed as described elsewhere [18]. Cells were washed in phosphate-buffered saline solution and resuspended in 70% ice-cold ethanol for fixing. After treatment with RNase (100 μg/mL) for 30 minutes at 37°C, DNA was stained with 50 μg/mL propidium iodide (PI) for 30 minutes on ice. Determination of cell cycle distribution was performed by FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan), and the number of cells in the area corresponding to the sub-G1 region was calculated with the CellQuest (Nippon Becton Dickinson Co) and ModFit LT (Verity Software House, Topsham, ME, USA) systems. For flow cytometry analyses for expression of CD10, CD33, CD11b, CD11c, and G-CSFR or binding assay of FITC-labeled FMLP, cells were incubated on ice for 30 minutes in staining buffer (phosphate-buffered saline solution containing 5% fetal calf serum and 0.05% sodium azide) with anti-CD10, anti-CD33, anti-CD11b, anti-CD11c, and anti–G-CSFR monoclonal antibodies or with FITC-labeled FMLP in the presence of 1 μg/mL PI. In cases of anti–G-CSFR antibody staining, secondary antibody (FITC-conjugated $F(ab')_2$ fragment goat anti-mouse IgG) was used. After gating out of PI-positive dead cells, 1-color and 2-color analyses were performed with the FACSCalibur system. Bone marrow cells were analyzed within a granulocyte gate. In some experiments, expression was quantified by measurement of the geometrical mean fluorescence intensity for each sample.

2.5. Morphological Evaluation of the Cells

Light microscopic examination of morphology was performed to determine the differentiation of NB4 cells. Cells suspended in Hanks balanced salt solution were adhered to slides by centrifugation with a Cytospin 2 system (Shandon,

Pittsburgh, PA, USA). Cytospin slides then were subjected to Wright-Giemsa staining for light microscopic examination of the cells.

2.6. Statistical Analysis

The Student *t* test was used to determine statistical significance.

3. Results

3.1. Effects of ATRA and G-CSF on Functional Capacity and Survival of NB4 Cells

In the initial experiment, we evaluated the effects of the optimal concentration of ATRA (1 μM), optimal concentration of G-CSF (50 ng/mL), and ATRA plus G-CSF on respiratory burst activity (as determined by superoxide [O_2^-]-producing capacity) of NB4 cells during the 10 days of cultivation. As shown in Figure 1A, ATRA by itself had potent ability to induce superoxide-producing capacity in NB4 cells during 10 days of cultivation. A significant effect was detected after 2 days of culture with ATRA, and the maximal effect was obtained after 4 days. This functional capacity declined thereafter. G-CSF alone did not induce capacity for producing superoxide in NB4 cells (Figure 2A, right). It was interesting that combined stimulation of NB4 cells with ATRA plus G-CSF did show additional effects in comparison with the effects of ATRA alone (Figure 1A). This effect was maintained for up to 10 days, resulting in a marked difference in this function between ATRA alone and ATRA plus G-CSF on day 10 ($P < .05$ on days 4 and 6, $P < .001$ on day 8, and $P < .05$ on day 10).

Figure 1B shows the growth curve of NB4 cells during differentiation induction. Without ATRA and G-CSF, NB4 sells proliferated rapidly in an exponential manner, and addition of G-CSF (50 ng/mL) alone did not affect the proliferation (control culture on day 4, $6.4 \pm 0.9 \times 10^6$ cells/well; G-CSF culture on day 4, $5.4 \pm 1.2 \times 10^6$ cells/well). Data are expressed as mean ±SD from 4 determinations, and starting cell number is 2×10^5 cells/well. ATRA (1 μM) by itself suppressed the exponential growth of NB4 cells (Figure 1B), and G-CSF had no additional effect on this growth curve when viable cell count was performed by trypan blue dye exclusion test (Figure 1B). However, the results were different when cell viability was evaluated by PI staining (Figure 1C). A significant difference in the proportion of viable cells was detected on days 6, 8, and 10 ($P < .05$ on day 6, $P < .01$ on days 8 and 10). Thus G-CSF not only potentiated the functional capacity of NB4 cells but also enhanced the survival of the cells. In addition, the findings from Figures 1A and 1C together suggest that maintenance of function by G-CSF after 4 to 10 days of culture (Figure 1A) is closely associated with G-CSF–induced enhancement of survival in the same period.

An enhancing effect of G-CSF on superoxide-producing capacity was significant ($P < .01$) and evident when FMLP was used as a triggering agonist of superoxide release, whereas the effect of G-CSF was not detected when PMA was used as an agonist (Figure 2A, left). This finding suggested that the effect of G-CSF is limited to the receptor-mediated route of responses. These findings resemble short-term (10 minutes) priming of normal human neutrophils, in which superoxide release stimulated by FMLP but not by PMA was enhanced by the incubation of normal human neutrophils with G-CSF for 10 minutes at 37°C [19]. However, an additional effect of G-CSF in NB4 cells was not priming, because superoxide release stimulated by both FMLP and

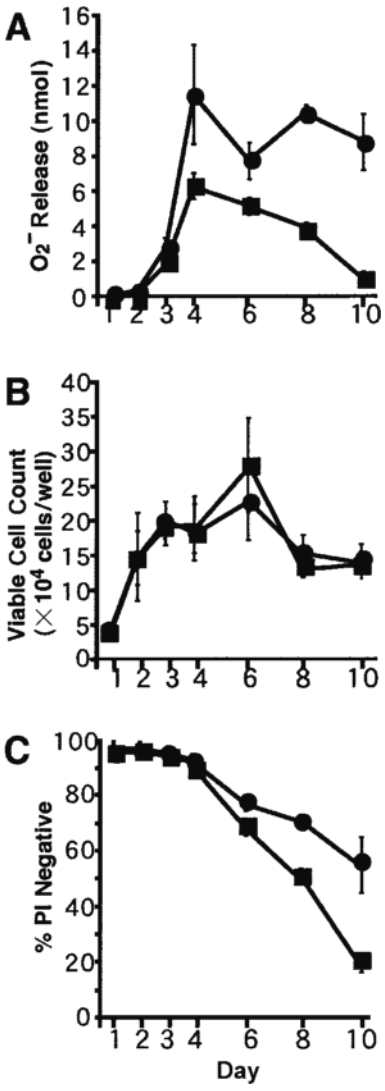

Figure 1. Time course of differentiation, proliferation, and viability of NB4 cells with all-*trans* retinoic acid (ATRA) and/or granulocyte colony-stimulating factor (G-CSF). A, NB4 cells were cultured with 1 μM ATRA alone (squares) or 1 μM ATRA plus 50 ng/mL G-CSF (circles) for up to 10 days. The functional capacity, cell number, and viability of the cells were determined at the indicated time point. Each point represents means ±SD of triplicate cultures. A representative result of 2 to 4 experiments is shown. A, Superoxide (O_2^-) release stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP) (1 μM) was determined by reduction of cytochrome C and is expressed as nmol/5 minutes per 1×10^6 cells. B, Viable cell count was performed by trypan blue dye exclusion test. C, Propidium iodide (PI) negativity was determined by flow cytometry.

PMA was not enhanced by incubation of ATRA-induced NB4 cells with G-CSF for 10 minutes at 37°C (Figure 2B).

3.2. Effects of ATRA and G-CSF on Cell Surface Expression of G-CSFR in NB4 Cells

The results described above showed an additional effect of G-CSF on NB4 cells only in the presence of ATRA, suggesting the possibility that NB4 cells do not express G-CSFR and that expression of this receptor was induced by ATRA. To address this problem, we studied the effect of ATRA and/or G-CSF on cell surface expression of G-CSFR in NB4 cells.

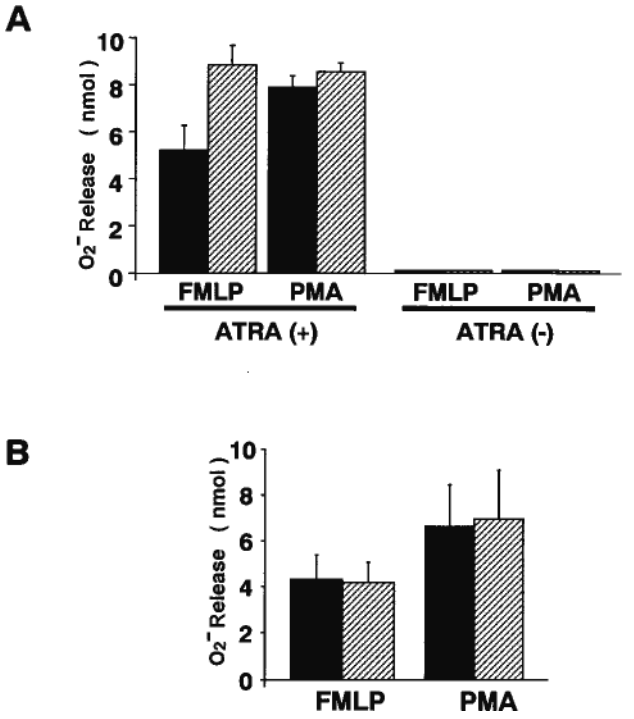

Figure 2. Effects of all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) on the respiratory burst activity of NB4 cells. A, left, NB4 cells were cultured with 1 μM ATRA alone (closed column) or 1 μM ATRA plus 50 ng/mL G-CSF (shaded column) for 4 days. Superoxide (O_2^-) release stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP) (1 μM) and phorbol myristate acetate (PMA) (100 ng/mL) was determined by reduction of cytochrome C and is expressed as nmol/5 minutes per 1×10^6 cells for FMLP and nmol/minute per 2.5×10^5 cells for PMA. Results are shown as means ±SD of 3 experiments. Right, NB4 cells were cultured with (shaded column) or without 50 ng/mL G-CSF (black column) in the absence of ATRA for 4 days. Superoxide (O_2^-) release is expressed as nmol/5 minutes per 1×10^6 cells for FMLP and nmol/minute per 1×10^6 cells for PMA. Results are shown as mean ±SD of 3 experiments. B, NB4 cells were cultured with 1 μM ATRA alone for 4 days and then incubated in the presence (shaded column) or absence (black column) of 50 ng/mL G-CSF for 10 minutes at 37°C. Superoxide (O_2^-) release stimulated by FMLP (1 μM) and PMA (100 ng/mL) was determined by reduction of cytochrome C and is expressed as nmol/5 minutes per 1×10^6 cells for FMLP and nmol/minute per 2.5×10^5 cells for PMA. Results are shown as means ±SD of 3 experiments.

As shown in Figure 3, NB4 cells abundantly expressed G-CSFR even before differentiation induction by ATRA, and ATRA did not exert a detectable effect on this basal expression of G-CSFR. G-CSF neither up-regulated nor down-regulated G-CSFR expression during differentiation induction by ATRA. Thus the effect of G-CSF alone and the additive or synergistic effects of ATRA and G-CSF could not be explained by G-CSFR level in NB4 cells.

3.3. Effects of ATRA and G-CSF on Cell Cycle in NB4 Cells

ATRA alone but not G-CSF alone suppressed the proliferation of NB4 cells (data not shown), and the combination of the two resulted in the effect of ATRA alone (Figure 1B). We then studied the effects of ATRA and G-CSF on the cell cycle distribution of NB4 cells. As shown in Figure 4, ATRA (1 μM) reduced the S and G2/M phases, the result being mild G1 arrest, and induced accumulation of the sub-G1 region, particularly after 4 days of cultivation. These findings con-

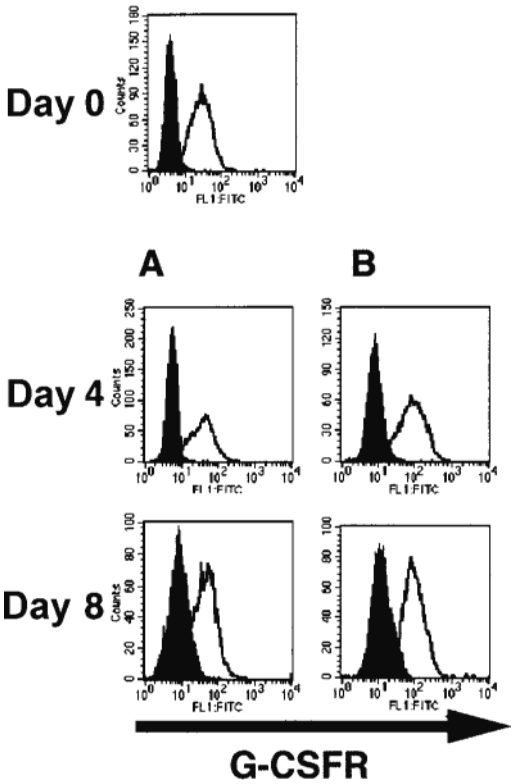

Figure 3. Effects of all-*trans* retinoic acid and granulocyte colony-stimulating factor (G-CSF) on the expression of G-CSFR in NB4 cells. Cell surface expression of G-CSF receptor (G-CSFR) on NB4 cells was measured by flow cytometry. NB4 cells were incubated with 1 μM all-*trans* retinoic acid (ATRA) (A) or 1 μM ATRA plus 50 ng/mL G-CSF (B) for 4 or 8 days. Data on untreated NB4 cells are presented at the top of the figure. Closed and open areas represent cells stained with secondary antibody alone and cells stained with anti–G-CSF antibody plus secondary antibody, respectively. A representative result of 2 or 3 experiments is shown. FITC indicates fluorescein isothiocyanate.

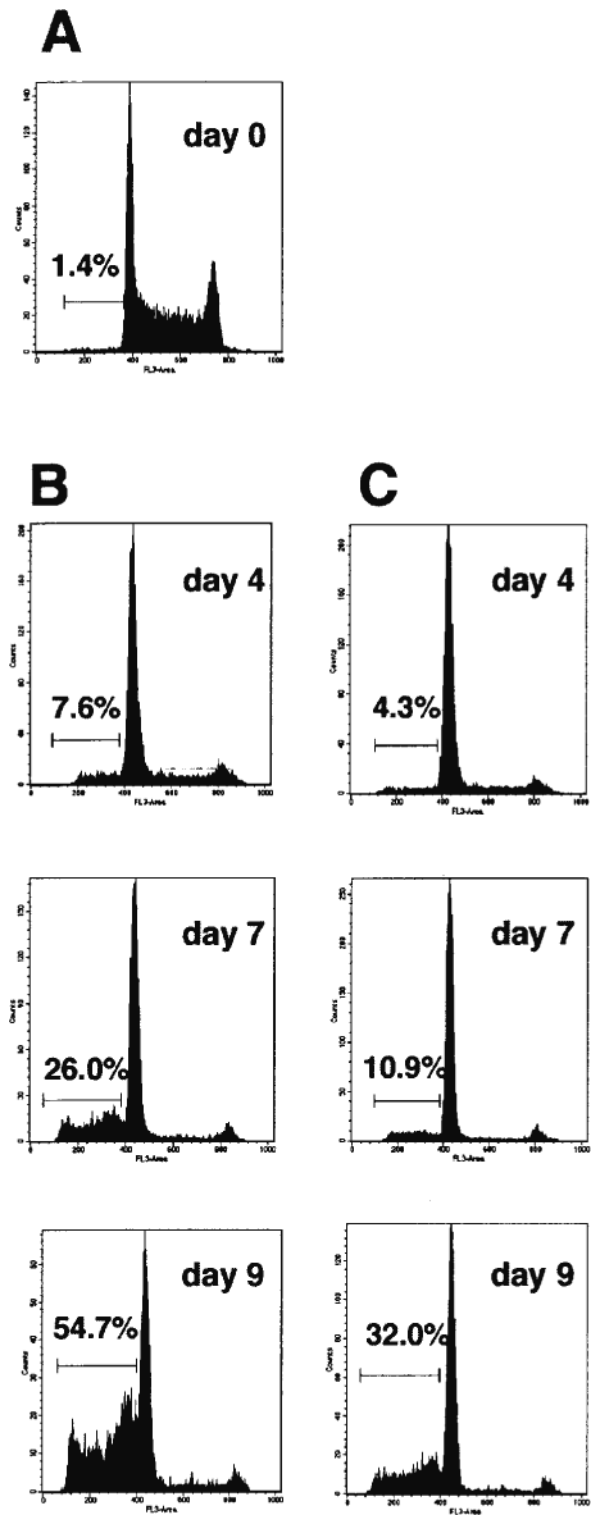

Figure 4. Cell cycle analysis of NB4 cells during differentiation induction. Cell cycle analyses were performed with propidium iodide (PI) staining by flow cytometry. Percentage of sub-G1 region is shown in each graph. A representative result of 3 experiments is shown. A, NB4 cells before induction. B, NB4 cells after induction with 1 μM all-*trans* retinoic acid (ATRA) alone for 4, 7, and 9 days. C, NB4 cells after induction with 1 μM ATRA plus 50 ng/mL G-CSF for 4, 7, and 9 days.

firmed the ATRA-induced apoptotic cell death of NB4 cells (Figure 1C). G-CSF (50 ng/mL) alone did not induce detectable alteration of cell cycle distribution in NB4 cells (data not shown). Combined stimulation of NB4 cells with ATRA and G-CSF resulted in a cell cycle state fundamentally identical to that of ATRA alone, except that reduction in the sub-G1 region was observed in coculture with G-CSF. This finding is compatible with the antiapoptotic effect of G-CSF shown in Figure 1C.

3.4. Effects of ATRA and G-CSF on Cell Surface Expression of FMLP Receptors in NB4 Cells

The specific effect of G-CSF on FMLP-induced superoxide release (Figure 2) suggests possible up-regulation of FMLP receptor by G-CSF. We studied the cell surface expression of FMLP receptors in NB4 cells during differentiation induction using binding study of FITC-labeled peptide for flow cytometric analysis. The effect of ATRA (1 μM) alone on cell surface expression of FMLP receptors in NB4 cells was very rapid, and the maximal expression was evident even after 1 day of incubation (Figure 5), when FMLP-induced superoxide release was still undetectable (Figure 1A). This finding indicated the postreceptor signaling pathway or superoxide-producing system was not equipped with the cells after 1 day of differentiation induction. It was interesting that G-CSF (50 ng/mL) apparently exerted an additional enhancing effect on ATRA-induced up-regulation of FMLP receptor expression after 4 days of culture (Figure 5). This effect of G-CSF was maintained for up to 8 days, and even on day 8, ATRA alone did not produce the expression level equivalent to ATRA plus G-CSF on day 4. Thus G-CSF–induced up-regulation of FMLP receptor might explain, at least in part, G-CSF–induced enhancement of FMLP-induced superoxide release in ATRA-induced NB4 cells.

3.5. Effects of ATRA and G-CSF on Cell Surface Expression of CD33, CD10, CD11b, and CD11c in NB4 Cells

The results in Figure 5 indicate the significant role of up-regulation of cell surface molecules during differentiation, particularly in G-CSF–induced additional maturation effect. We analyzed the cell surface expression of 4 differentiation-related leukocyte-specific markers during differentiation induction of NB4 cells. NB4 cells substantially expressed CD33 and CD10 without differentiation induction (Figure 6). After 4 and 6 days of differentiation induction by ATRA (1 μM), cell surface expression of CD33 and CD10 was not or only slightly up-regulated (Figure 6). G-CSF (50 ng/mL) alone had no effect on the cell surface expression of these 2 molecules (Figure 6A). It was important that cell surface expression of both CD33 and CD10 was additionally and markedly up-regulated by G-CSF in the presence of ATRA (1 μM) (Figure 6). Particularly, the effect on CD10, which is well known as common acute lymphoblastic leukemia antigen [11], was highly specific to G-CSF. In contrast, G-CSF (50 ng/mL) did not exert detectable effects on the cell surface expression of CD11b and CD11c antigens

even in the presence of ATRA (1 μM) under the condition in which ATRA alone induced marked increments of cell surface expression of both molecules (data not shown).

3.6. Effects of ATRA and G-CSF on Morphology of NB4 Cells

We determined another important marker of differentiation, that is, morphology. After 4 days of differentiation induction by ATRA (1 μM), NB4 cells showed a detectable level of morphological change, such as slight convolution of

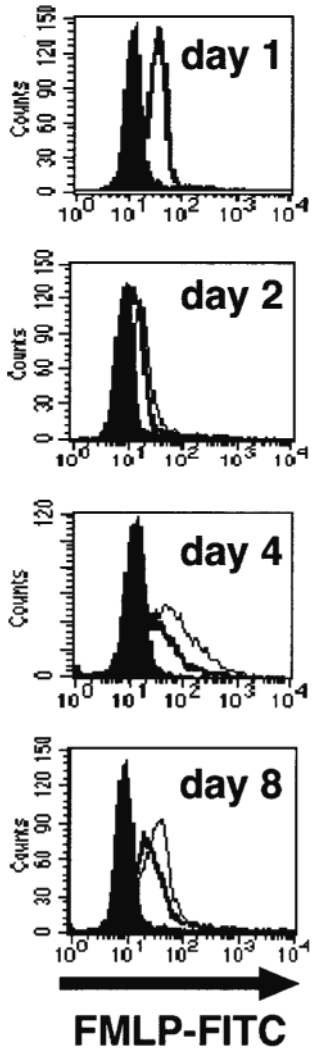

Figure 5. Effects of all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) on the expression of FMLP receptor in NB4 cells. NB4 cells were cultured with 1 μM ATRA alone or 1 μM ATRA plus 50 ng/mL G-CSF for 1, 2, 4, and 8 days. Cell surface expression of N-formyl-methionyl-leucyl-phenylalanine (FMLP) receptor was determined by binding of fluorescein isothiocyanate (FITC)-labeled FMLP by flow cytometry. Data for NB4 cells before induction (closed area) and for the cells induced with 1 μM ATRA alone (thick line) or 1 μM ATRA plus 50 ng/mL G-CSF (thin line) are presented. A representative result of 2 experiments is shown.

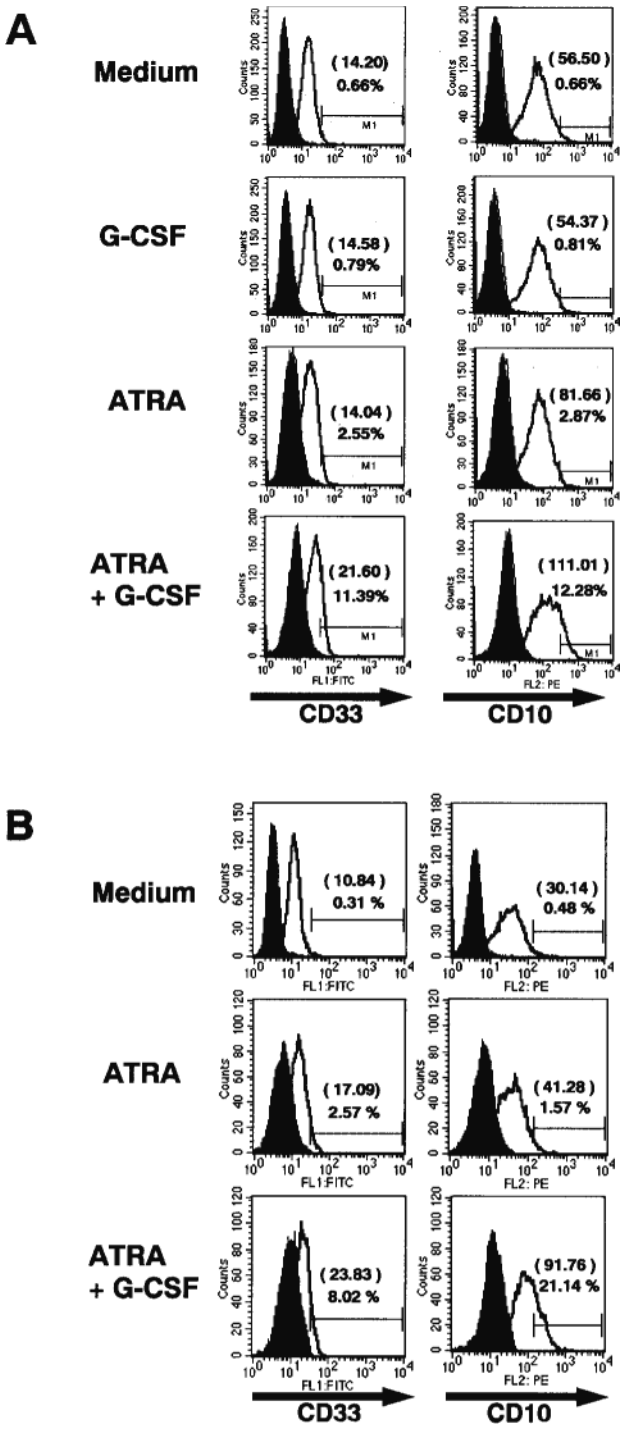

Figure 6. Effects of all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) on the expression of CD10 and CD33 in NB4 cells. Cell surface expression of CD10 and CD33 on NB4 cells was measured by flow cytometry. NB4 cells were incubated with medium alone, 1 μM ATRA or 1 μM ATRA plus 50 ng/mL G-CSF for 4 days (A) or 6 days (B). Data for 50 ng/mL G-CSF alone on day 4 also are presented (A). Closed and open areas represent nonstained and antibody-stained cells, respectively. Both percentage of positive cells and mean fluorescence intensity (in parentheses) are shown in each panel. A representative result of 2 or 3 experiments is shown.

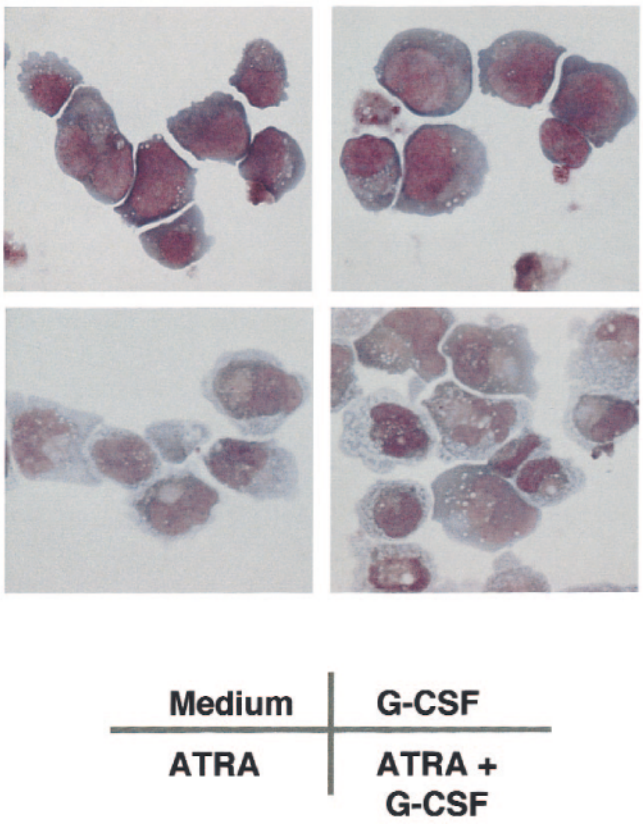

Figure 7. Effects of all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) on morphology of NB4 cells. NB4 cells were incubated with medium alone, 1 μM ATRA, 50 ng/mL G-CSF, or 1 μM ATRA plus 50 ng/mL G-CSF for 4 days. After fixation to slides, cells were subjected to Wright-Giemsa staining for light microscopic examination.

nuclei (Figure 7). In contrast, G-CSF had no effect on morphology of NB4 cells in the presence or absence of ATRA (Figure 7).

3.7. Effects of ATRA and G-CSF on Cell Surface Expression of CD10 and CD33 in Normal Human Bone Marrow Mononuclear Cells

To further extend the results in Figure 6 (particularly the unexpected findings for CD10) and explore the possible roles of CD10 and CD33 during normal human myeloid differentiation, we determined the effects of ATRA and G-CSF and their combination on surface expression of CD10 and CD33 during in vitro cultivation of normal human bone marrow mononuclear cells. As shown in Figure 8, ATRA (10 μM) induced a marginal to slight increase in expression of CD33 in the absence (83% versus 85%) or presence (68% versus 78%) of G-CSF. G-CSF (50 ng/mL) did not up-regulate but rather down-regulated the expression of CD33 in the absence (83% versus 68%) or presence (85% versus 78%) of ATRA. It was interesting that G-CSF markedly up-regulated the expression of CD10 in the absence of ATRA (45% versus 84%), whereas ATRA alone did not affect CD10 expression. Thus selective up-regulation of CD10 antigen by G-CSF was a common event in both normal and leukemic human myeloid cells. Unlike NB4 cells, the cells without ATRA were affected by G-CSF alone.

3.8. Effects of ATRA and G-CSF on Morphology of Normal Human Bone Marrow Mononuclear Cells

In contrast to that of NB4 cells, cultivation of normal human bone marrow mononuclear cells with ATRA alone

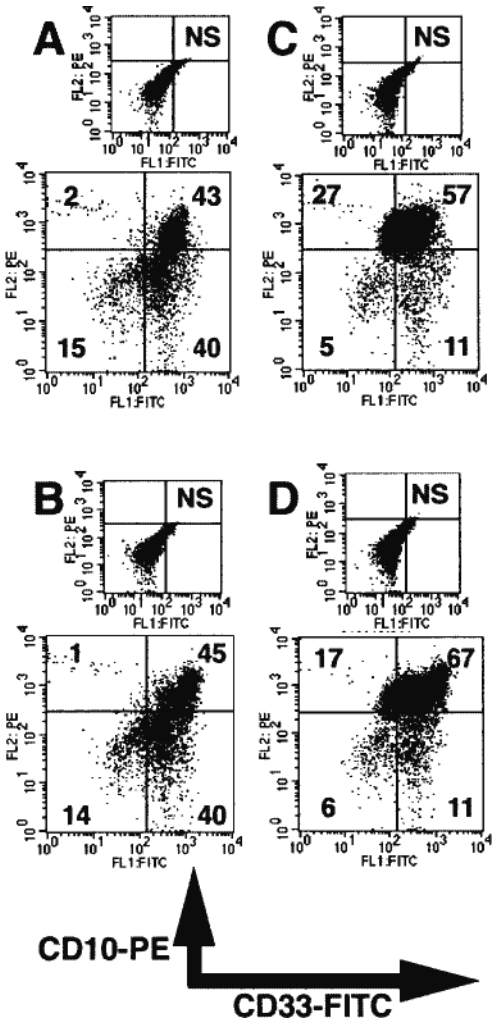

Figure 8. Effects of all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) on the expression of CD10 and CD33 in bone marrow mononuclear cells. Normal human bone marrow mononuclear cells were cultured with medium alone (A), 10 μM ATRA (B), 50 ng/mL G-CSF (C), or 10 μM ATRA plus 50 ng/mL G-CSF (D) for 6 days. Expression of CD10 and CD33 on the cells was measured by flow cytometry; each subfigure shows the results obtained when non-stained cells (NS) were presented with antibody-stained cells. Each value in the divided area of a subfigure represents the percentage of double-negative and CD10-positive and/or CD33-positive cells. A representative result of 2 experiments is shown. FITC indicates fluorescein isothiocyanate.

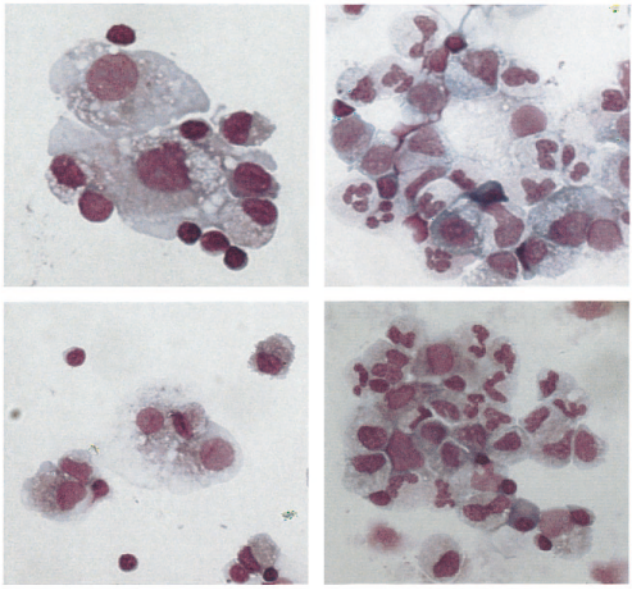

Figure 9. Effects of all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) on morphology of bone marrow mononuclear cells. Normal human bone marrow mononuclear cells were cultured with medium alone, 10 μM ATRA, 50 ng/mL G-CSF, or 10 μM ATRA plus 50 ng/mL G-CSF for 6 days. After fixation to slides, cells were subjected to Wright-Giemsa staining for light microscopic examination.

did not induce detectable changes in morphology (Figure 9). G-CSF alone induced prominent morphological alteration such as segmented nuclei in normal human bone marrow mononuclear cells, and ATRA plus G-CSF gave the same results with G-CSF alone. Thus G-CSF alone not only up-regulated CD10 but also affected morphology in normal human myeloid cells.

4. Discussion

In the present study, we showed differentiation-inducing effects of G-CSF on human leukemic myeloid cells in the presence of ATRA and clarified some differences between the effects of ATRA alone and additional effects of G-CSF. In particular, G-CSF enhanced the receptor-mediated response of NB4 cells and up-regulated cell surface molecules such as CD 10 and CD33.

The differentiation of myeloid hematopoietic cells stimulated by G-CSF has been studied mainly with murine cells and/or cell lines [20-23], and human in vitro culture models of G-CSF–induced differentiation are limited. On the other hand, the effects of retinoic acids such as ATRA on the differentiation of hematopoietic cells (and cell lines) have been intensively studied by use of human systems [3,4,15,16]. Thus the NB4 model with ATRA and G-CSF is valuable as a human model for G-CSF–induced differentiation and is useful for direct comparison of the effects of the 2 agonists and their interaction.

Two differentiation inducers, ATRA and G-CSF, exerted opposing effects with regard to survival and/or apoptosis of differentiating NB4 cells. ATRA induced apoptosis of NB4 cells, whereas G-CSF enhanced the survival of the cells, despite their common effects on differentiation induction. This finding suggested the different intracellular mechanisms for differentiation. Combined stimulation of NB4 cells with ATRA plus G-CSF substantially rescued the cells from apoptosis, particularly during the later phases of cultures. This finding suggested the apoptotic signal generated by ATRA after differentiation might be blocked at least in part by the antiapoptotic signal generated by G-CSF. In addition, such a survival signal of G-CSF contributes to the maintenance of functional capacity.

ATRA alone induced respiratory burst activity of NB4 cells during in vitro culture of the cells. This effect of ATRA on myeloid function was evident even when the superoxide-producing capacity was determined by use of phorbol ester PMA as a triggering agonist. On the other hand, additional effect of G-CSF was detected only when chemotactic peptide FMLP was used as a triggering agonist of superoxide release. Although the short-term (5-10 minutes) priming effect of G-CSF was ruled out in the present culture system, the differentiation-inducing effect of G-CSF was closely and selectively related to the receptor-mediated route of cellular events. The increment of FMLP receptor expression by itself might explain these phenomena, at least in part.

We report up-regulation (or in some instances down-regulation) of cell surface molecules such as CD10 and CD33 in leukemic and normal human myeloid cells. In these circumstances, G-CSF exerted a detectable effect only in the presence of ATRA in NB4 cells, whereas G-CSF by itself exerted substantial effects without ATRA in normal cells. In addition, G-CSF up-regulated CD33 expression in NB4 cells, whereas G-CSF down-regulated its expression in normal cells. These results seem to reflect a difference in differentiation stage of the cells used; that is, normal bone marrow mononuclear cells are fully equipped with the molecules or functions inducible by ATRA and fully express CD33, an immature myeloid antigen [9,10]. On the other hand, leukemic NB4 cells should be induced by ATRA to reach the differentiation level equivalent to normal bone marrow mononuclear cells.

In addition to the acquisition of effector functions and up-regulation of cell surface molecules, morphological changes are important phenomena during myeloid differentiation. In this study, we confirmed, by means of morphology, critical roles of G-CSF but not ATRA in terminal differentiation of normal myeloid cells. In contrast, leukemic NB4 cells showed moderate morphological change in response to ATRA but not G-CSF. These findings further support the distinct regulation of normal and leukemic cell differentiation, although the molecular mechanism remains to be determined. The absence of effect of G-CSF on morphology of NB4 cells might suggest distinct signaling pathways between morphological matura-

tion versus functional differentiation, and the mechanistic study for this problem is underway in our laboratory.

In both leukemic and normal cells, G-CSF up-regulated cell surface expression of CD10 antigen during differentiation. Although CD10 is well known as common acute lymphoblastic leukemia antigen and is reported to be expressed in immature lymphoid cells [11], other reports describe the specific and limited expression of CD10 on mature neutrophils among human myeloid cells [12-14]. Thus the present results showing up-regulation of CD10 expression during in vitro cultivation of human myeloid cells clearly indicate the terminal process of differentiation or maturation of human granulocytic cells by G-CSF.

CD10 is also called neutral endopeptidase [14] and might play some roles as an enzyme on mature myeloid cells [8]. One hypothesis is that CD10 degrades bioactive peptides such as FMLP and regulates the functional responses of neutrophils to the peptide [24,25]. If this hypothesis is applicable to the present results, expression of FMLP receptor and the acquisition of an FMLP-induced response might be negatively regulated by the simultaneous increment of CD10 expression. The exact roles of CD10 expression during myeloid differentiation await further investigation.

NB4 cells expressed an almost equivalent level of G-CSFR in the presence or absence of ATRA. In accordance with this phenomenon, a G-CSF-induced signaling pathway such as phosphorylation of Stat3 [20-23] was observed in the presence or absence of ATRA (unpublished observations). In contrast, G-CSF was able to exert differentiative effects on this cell line only in the presence of ATRA. These findings together suggest that the differentiation-inducing effect of G-CSF was controlled at the post–G-CSFR (signaling) level rather than the G-CSFR level. Such signaling molecule(s) may be induced specifically by ATRA and might be used by a G-CSFR–triggered intracellular pathway for terminal myeloid differentiation.

In the present study, we showed that G-CSF potently induced terminal differentiation and maturation as well as survival of human granulocytic cells. Such effects of G-CSF were associated with acquisition of receptor-mediated function and up-regulation of CD10 expression. This in vitro culture system for G-CSF–specific differentiation and survival might contribute to the understanding of intracellular and intercellular signaling mechanisms for human myeloid cell biology controlled by the hematopoietic growth factors.

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