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Induction of Retinoic Acid Receptor-α by Granulocyte Macrophage Colony-stimulating Factor in Human Myeloid Leukemia Cell Lines[1]

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ABSTRACT

We reported previously that treatment with all-*trans* retinoic acid (ATRA) and granulocyte macrophage colony-stimulating factor (GM-CSF) induces differentiation of human myeloblastic leukemia ML-1 cells to granulocytes, whereas treatment with ATRA alone induces practically no differentiation of these cells. To investigate the mechanism of the synergistic effect of these factors, we examined the effect of GM-CSF on retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in ML-1 cells. We reveal that GM-CSF induces the expression of RARα mRNA and protein and stimulates the binding of nuclear proteins to direct repeat 5, a consensus sequence with high affinity for RAR-RXR heterodimers. Furthermore, expression of CD38 mRNA mediated through RARα is induced synergistically by treatment with ATRA + GM-CSF. These results suggest that GM-CSF stimulates transcriptional activity mediated via RARα in ML-1 cells. The induction of RARα by GM-CSF may therefore be a mechanism for stimulation by GM-CSF. The induction of RARα by GM-CSF was also detected in other myeloid leukemia cell lines (THP-1 and KG-1) that showed a synergistic effect similar to that seen in ML-1 cells in response to ATRA + GM-CSF. We also found that GM-CSF induced the expression of RARα in blood cells obtained from patients with acute myeloid leukemia. This activity of GM-CSF may serve as a useful adjunct to differentiation therapy for retinoic acid-nonresponsive leukemias.

INTRODUCTION

The retinoids, particularly RA,[3] exert a wide range of biological effects related to cell proliferation and differentiation. Cellular responses to RA are mediated by two families of transcription factors that include the RARs and the RXRs. These proteins belong to a superfamily of intracellular receptors that, on ligand activation, function as dimeric transcription factors to control the expression of target genes by binding to specific DNA sequences termed RAREs (1–4). Previous studies have confirmed that the differentiative effects of RA are mediated through the retinoid receptors. A subclone of the RA-responsive murine P19 embryonal carcinoma cell line carrying a mutation in the RARα gene was found to be RA resistant (5). Similarly, in HL-60 cells, RA resistance was shown to be associated with a dominant negative, structurally altered RARα in a resistant subclone (6). Therefore, RARs and RXRs play an important role in the regulation of RA signal transduction.

RA is a known inducer of cell differentiation into granulocytes. In particular, it is known that ATRA promotes granulocytic maturation of the human cell line HL-60 (7) and of hematopoietic cells from patients with APL (8, 9). ATRA is effective in differentiation therapy for APL (10, 11). However, retinoids generally do not promote granulocytic maturation in AML subtypes, with the exception of APL. Study of the mechanism underlying the cellular responses to ATRA in AML cells may lead to the application of differentiation therapy using ATRA. ML-1 cells, derived from a patient with acute myeloblastic leukemia, are at an earlier stage of differentiation than HL-60 cells and can be easily differentiated into the macrophage but not the granulocytic pathway by various inducers such as tumor necrosis factor (12), IFN-γ (13), and 12-*O*-tetradecanoylphorbol-13-acetate (14, 15). We recently succeeded in inducing differentiation toward granulocytes in ML-1 cells by treatment with ATRA + GM-CSF (16). On the basis of this result and the results of other previous reports (7–9), we speculate that GM-CSF may promote the differentiative effects of RA. We predicted that activation of RARs and RXRs is the mechanism underlying this promoting effect; therefore, we investigated the effect of GM-CSF on RARs and RXRs in ML-1 cells. We found that GM-CSF induces the expression of RARα and stimulates the binding of nuclear proteins to RAREs in these cells. Using other AML cell lines, we found a correlation between induction of RARα and the synergistic effect of ATRA and GM-CSF on granulocytic differentiation.

MATERIALS AND METHODS

Cell Culture and Reagents. ML-1 (a human myeloblastic leukemia cell line), KG-1 (a human myeloblastic leukemia cell line), THP-1 (a human monoblastic leukemia cell line), HL-60 (a human promyelocytic cell line), and K562 (a human erythroleukemia cell line) were maintained as suspension cultures in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Filtron Ltd., Victoria, Australia). Incubation was carried out at 37°C in a humidified 5% CO_2 incubator. Fresh peripheral blood cells from AML patients were kindly provided by Dr. Tsuyoshi Nakamaki (Showa University School of Medicine, Tokyo, Japan). ATRA (Sigma Chemical Co., St. Louis, MO) was added to the culture medium at a final concentration of 10^{-7} M, and recombinant human GM-CSF (Sandoz, Wien, Austria) was added to the culture medium at the desired concentrations.

NBT-reducing Ability. NBT-reducing activity was assayed microscopically by the modified method of Bachner and Nathan (17). Briefly, 3×10^5 cells were suspended in 96-well microplates with reagents and then incubated at 37°C. After 2 days of incubation, 0.1% NBT dye and 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate were added to each well and incubated at 37°C for 30 min in a 5% CO_2 incubator. After incubation, the reaction was terminated by adding 50 μl of 2 N HCl to each well of a 96-well microplate, which was then cooled on ice for 30 min. The medium was then discarded, the formazan deposits were dissolved by adding 0.1 ml of DMSO, and the dissolved formazan measured at 595 nm by a spectrophotometer for the 96-well microplates (Microplate Reader model 550; Bio-Rad, Tokyo, Japan).

Morphological Changes. The appearance of morphological changes was assessed until day 9 of incubation. Cells were prepared on glass slides by centrifugation in a Cytospin (Shandon Southern Products, Ltd., Cheshire, United Kingdom). The cells were stained with May-Grüenwald's solution for 3 min and then stained with diluted Giemsa solution for 30 min. Cells with the morphological characteristics of granulocytes were counted under the microscope.

RT-PCR. Total cellular RNA was isolated using Isogen (Wako, Osaka, Japan). Reverse transcription of total RNA into cDNA was performed using

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[3] The abbreviations used are: RA, retinoic acid; GM-CSF, granulocyte macrophage colony-stimulating factor; ATRA, all-*trans* retinoic acid; APL, acute promyelocytic leukemia; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; AML, acute myeloid leukemia; NBT, nitroblue tetrazolium; RT-PCR, reverse transcription-PCR; DR, direct repeat; poly(A)$^+$ RNA, polyadenylated RNA; G-CSF, granulocyte colony-stimulating factor.

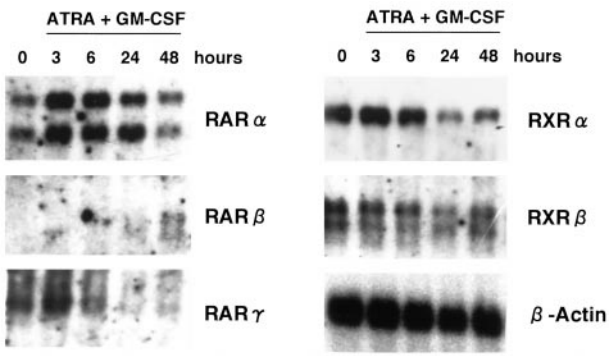

Fig. 1. Expression levels of RARs and RXRs during the granulocytic differentiation of ML-1 cells. Poly(A)$^+$ RNA samples were prepared from cells treated with 10^{-7} M ATRA and 0.1 ng/ml GM-CSF for the times indicated. Northern blot analysis using 4 μg of poly(A)$^+$ RNA was performed as described in "Materials and Methods." Radioactivities of the bands on autoradiograms were measured using a BAS1500 image analyzer (Fuji Film, Kanagawa, Japan).

Bulk First-Strand cDNA Reaction Mix (Amersham Pharmacia Biotech, San Francisco, CA). Briefly, 1 μg of total RNA was reverse transcribed using pd(N)6 primer in a final volume of 15 μl. The total reaction product was amplified by PCR for each cycle at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. Amplification was carried out for 32 cycles for RARα and 28 cycles for glyceraldehyde-3-phosphate dehydrogenase. PCR primers were as follows: (*a*) RARα, 5′-CAACAGCTCAGAACAACGTG-3′ (sense primer) and 5′-GATCTCCATCTTCAGCGTGA-3′ (antisense primer); and (*b*) glyceraldehyde-3-phosphate dehydrogenase, 5′-ATCATCAGCAATGCCTCCTG-3′ (sense primer) and 5′-CTGCTTCACCACCTTCTTGA-3′ (antisense primer).

Northern Blot Analysis. Total cellular RNA was isolated using Isogen. Poly(A)$^+$ RNA was purified using Oligotex dT30 (Takara, Kyoto, Japan). The poly(A)$^+$ RNA (4 μg) was electrophoresed on 1.2% agarose-formaldehyde gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech). Hybridizations were carried out according to the manufacturer's instructions. DNA fragments used as probes were amplified by RT-PCR using the following primers: (*a*) RARα, 5′-CAACAGCTCAGAACAACGTG-3′ (sense primer) and 5′-GATCTCCATCTTCAGCGTGA-3′ (antisense primer); (*b*) RARβ, 5′-TACTGTCGACTCCAGAAGTG-3′ (sense primer) and 5′-GTGCTTCCAG-CAATGGTTCT-3′ (antisense primer); (*c*) RARγ, 5′-CATCACCAAGGT-CAGCAAAG-3′ (sense primer) and 5′-TCTCCAGCATCTCTCGGATT-3′ (antisense primer); (*d*) RXRα, 5′-TCAATGGCGTCCTCAAGGTC-3′ (sense primer) and 5′-CGCCTCCAGCATCTCCATAA-3′ (antisense primer); (*e*) RXRβ, 5′-GAAGATGTGAGCCACCAGT-3′ (sense primer) and 5′-GCT-CAGGGTACTTCTGTTTG-3′ (antisense primer); (*f*) RXRγ, 5′-ATGAAC-TACCCATCCACCAG-3′ (sense primer) and 5′-CGGATACTTCTGCTTG-GTGT-3′ (antisense primer); and (*g*) CD38, 5′-AGTTGGGAACTCA-GACCGTA-3′ (sense primer) and 5′-TCCTGGC ATAAGTCTCTGGA-3′ (antisense primer).

Preparation of Nuclear Protein Extracts. Nuclear extracts were isolated according to the following method. The cells were lysed in lysis buffer [10 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 10 mM NaCl, and 0.5% NP40] and the nuclei were collected, resuspended in nuclear resuspension buffer [250 mM Tris-HCl (pH7.8), 60 mM KCl, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride], and lysed by three cycles of freeze/thaw. The nuclear extracts were spun, and supernatants were frozen immediately and stored at −80°C. Protein concentration was determined by using the Bio-Rad Protein Assay.

Western Blot Analysis. Western blot analysis was performed using the method described previously (18). The denatured nuclear extracts were analyzed by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blots were blocked at 4°C using 5% nonfat milk overnight in PBST (10 mM PBS and 0.05% Tween 20) and incubated for 2 h at room temperature with the monoclonal anti-RARα antibody from Affinity Bioreagents and the polyclonal anti-RXRα antibody (D-20) from Santa Cruz Biotechnology. The blots were then washed three times and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Gel Mobility Shift Assay. Nuclear protein (2 μg) was incubated with binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.5% glycerol, and 3 μg of salmon sperm DNA] in a total volume of 20 μl at 20°C for 15 min. One ng of ^{32}P-labeled double-stranded probe DR5 (5′-AGCT-TCAGGTCACCAGGAGGTCAGAGAGCT-3′) was added, and the reaction mixture was incubated for another 20 min. For the competition experiment, a 50-fold excess of unlabeled RAR gel shift oligonucleotides (sc-2559 or sc-2560; Santa Cruz Biotechnology) was added to incubations with the labeled fragments. For antibody experiments, samples were preincubated with the anti-RARα or anti-RXRα antibody (Santa Cruz Biotechnology) for 15 min before the addition of probe. DNA-protein complexes were electrophoresed on native 6% polyacrylamide gels in 0.25× Tris-borate EDTA buffer. Gels were vacuumed, dried, and exposed to Fuji X-ray film at −70°C.

RESULTS

Alterated Expression of RAR and RXR Genes in ML-1 Cells Treated with ATRA and GM-CSF. To investigate effect of GM-CSF on RARs and RXRs, we examined the expression of RARs and RXRs during granulocytic differentiation of ML-1 cells in response to treatment with ATRA + GM-CSF using Northern blot analysis. As shown in Fig. 1, the expression of RARα markedly increased (3-fold) 3 h after treatment with ATRA + GM-CSF. RARγ and RXRα also increased (1.4- and 2-fold, respectively) at 3 h, returning to control levels after 24 h. RARβ was not detected under control conditions but was induced slightly after treatment for 2 days. RXRβ was expressed

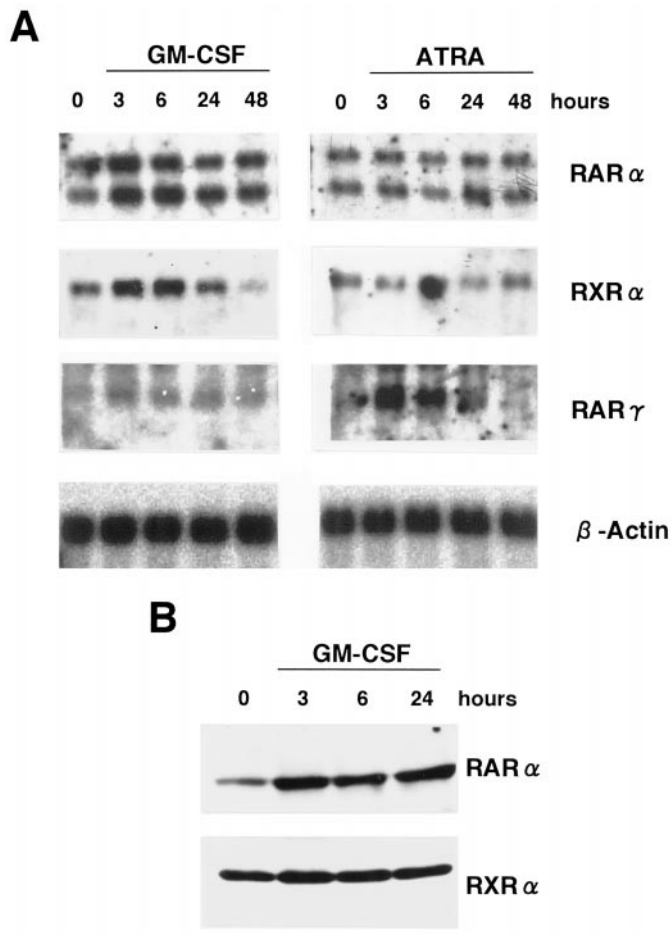

Fig. 2. *A*, expression level of RARα, RARγ, and RXRα in ML-1 cells treated with 10^{-7} M ATRA or 0.1 ng/ml GM-CSF for the times indicated. Northern blots were performed as described in Fig. 1. *B*, Western blotting analysis of RARα and RXRα in ML-1 cells treated with GM-CSF. Cultures were treated with 0.1 ng/ml GM-CSF for the times indicated. Protein lysates were prepared, and Western blots were performed as described in "Materials and Methods."

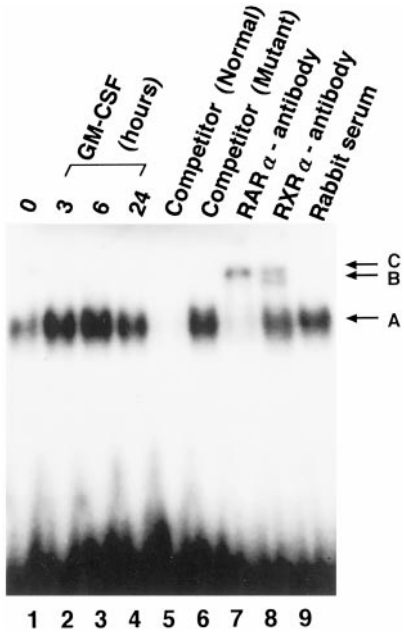

Fig. 3. Gel mobility shift assay using a labeled DR5 probe. The labeled probe was incubated with nuclear extracts from untreated ML-1 cells (*Lane 1*) or from ML-1 cells treated with GM-CSF for 3 (*Lanes 2, 5, 6, 7, 8,* and 9), 6 (*Lane 3*), and 24 (*Lane 4*) h. Competition experiments were performed by incubation with cold RAR gel shift oligonucleotide (*Lane 5*) or mutant oligonucleotide (*Lane 6*) in a 50-fold excess. For the supershift experiment, nuclear extracts were treated with RARα antibody (*Lane 7*), RXRα antibody (*Lane 8*), and rabbit serum (*Lane 9*). *Bottom arrow* (*A*) indicates the position of the DNA-protein complexes. *Upper arrows* (*B* and *C*) indicate the shifted complex formed in the presence of each antibody.

basally and decreased slightly during differentiation. RXRγ was not detected in differentiation-induced cells or in untreated cells (data not shown).

GM-CSF Induces RARα mRNA and Protein Expression in ML-1 Cells. To investigate whether these changes in expression are induced by GM-CSF, we examined the expression levels of RARα, RARγ, and RXRα in ML-1 cells treated with GM-CSF or ATRA. The expression of RARα and RXRα genes was increased by GM-CSF but not by ATRA. In contrast, RARγ expression was increased by ATRA but was minimally altered by treatment with GM-CSF (Fig. 2*A*). To determine whether the increased gene expression by GM-CSF results in corresponding increases in the respective proteins, we performed Western blot analysis with specific antibodies, and we detected induction of RARα but not RXRα protein 3 h after treatment with GM-CSF (Fig. 2*B*).

GM-CSF Stimulates Binding of Nuclear Proteins to DR5 in ML-1 Cells. The receptors for ATRA serve as *trans*-acting factors that regulate transcription by binding to responsive elements on the target. Because GM-CSF increases the levels of RARα in ML-1 cells, it is likely that the amount of receptor bound to RARE is also modulated after GM-CSF treatment. To investigate the effect of GM-CSF on the binding of nuclear receptors to RAREs, nuclear proteins were extracted from untreated and GM-CSF-treated ML-1 cells for gel mobility shift assay. The DR5 sequence that encodes two copies of AGGTCA in a DR separated by 5 nucleotides was used as a probe. DR5 is preferentially bound by RAR/RXR heterodimers. As shown in Fig. 3, the total amount of nuclear protein bound to DR5 was increased after 3 h of GM-CSF treatment. GM-CSF-stimulated binding was blocked by the addition of excess cold RAR gel shift oligonucleotide but not by the addition of a mutant oligonucleotide. Furthermore, the DNA and protein complexes were shifted to a position indicating slower migration when the extracts were treated with specific antibodies against RARα and RXRα. This shift was not detected when normal rabbit serum was used. Thus, these observations suggest that GM-CSF increases the amount of RAR-RXR binding to DR5 in ML-1 cells.

Synergistic Induction of the CD38 Gene in ML-1 Cells by Treatment with ATRA + GM-CSF. ATRA is a potent and highly specific inducer of CD38 antigen in myeloid leukemia cells, and the induction of CD38 is mediated through RARα. CD38 induction may be a useful marker for studying retinoid action in myeloid cells. To investigate the effect of GM-CSF on transcriptional activity via RARα, we examined the expression of the CD38 gene in ML-1 cells treated with GM-CSF and/or ATRA. CD38 gene expression was induced synergistically by treatment with GM-CSF + ATRA in these cells. Induction of gene expression began within 3 h of combined treatment and occurred faster than that induced by treatment with ATRA alone. Moreover, the level of CD38 mRNA in ML-1 cells treated with both regents was 2–4-fold higher than that seen in response to ATRA alone (Fig. 4). These observations indicate the possibility that GM-CSF stimulates transcriptional activity via RARα in ML-1 cells.

Expression of RARα in Other Leukemia Cells Treated with GM-CSF. To investigate the relation between the induction of RARα by GM-CSF and the synergistic effect of granulocytic differentiation by ATRA + GM-CSF, we examined the alteration of NBT-reducing activity by ATRA and/or GM-CSF and the induction of RARα by GM-CSF in other leukemia cell lines. As shown in Fig. 5*A*, the KG-1 and THP-1 cell lines showed a synergistic effect in response to

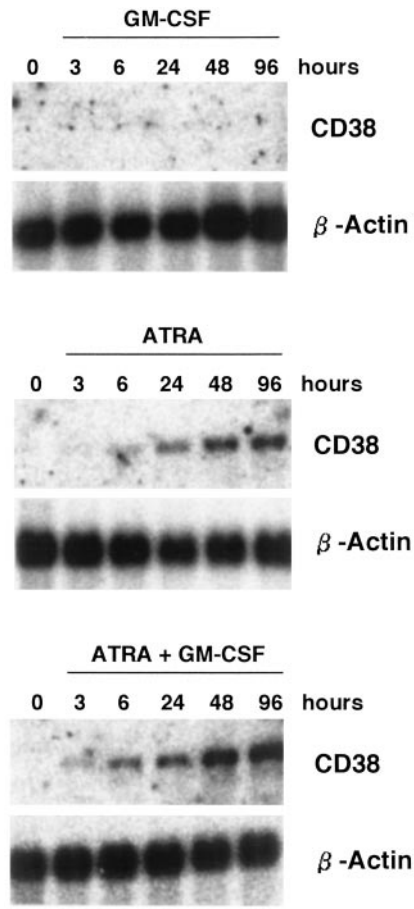

Fig. 4. Expression level of CD38 in ML-1 cells treated with 10^{-7} M ATRA and/or 0.1 ng/ml GM-CSF for the times indicated. Northern blot analyses using 4 μg of poly(A)$^+$ RNA were performed as described in Fig. 1. The exposure time is 6 h. All filters were exposed to the imaging plate at the same time.

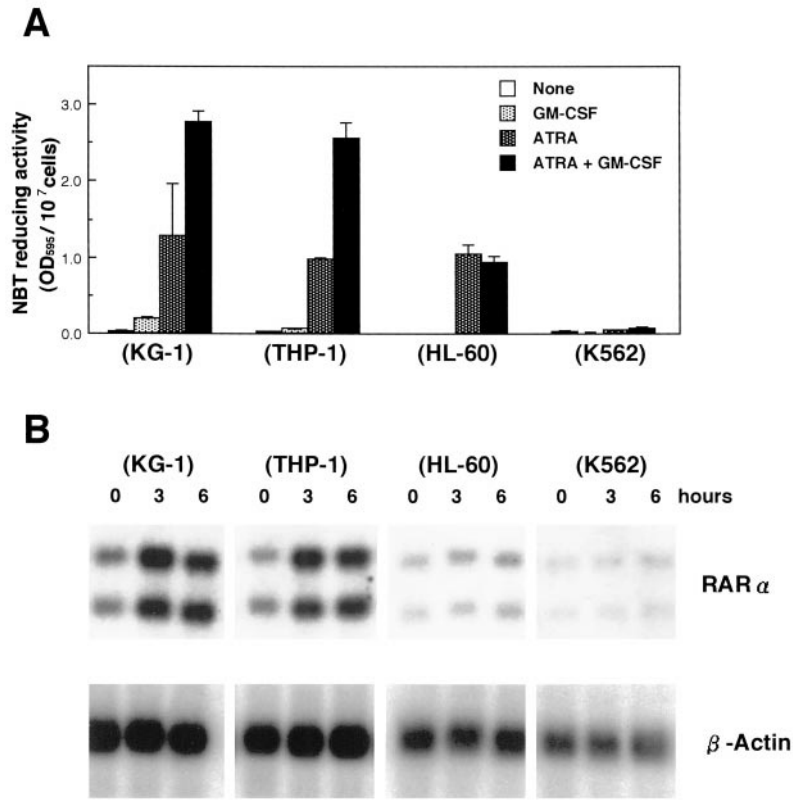

Fig. 5. NBT-reducing activity and expression level of RARα in various cell lines treated with 10^{-7} M ATRA and/or 0.1 ng/ml GM-CSF. *A*, effect of 10^{-7} M ATRA and/or 0.1 ng/ml GM-CSF on the induction of NBT-reducing activity of various cell lines. NBT-reducing activity was determined as described in "Materials and Methods." Values are the means ± SE of triplicate determinations. *B*, expression level of RARα in various cell lines treated with 0.1 ng/ml GM-CSF for the times indicated. Northern blots were performed as described in Fig. 1.

ATRA + GM-CSF. In contrast, HL-60, a human promyelocytic leukemia cell line that shows granulocytic differentiation in response to treatment with RA or DMSO, showed high NBT-reducing activity in response to treatment with ATRA alone and no synergistic effect in response to treatment with ATRA + GM-CSF. K562 cells did not show induction of NBT-reducing activity after treatment with ATRA and/or GM-CSF. To investigate the differentiation levels of KG-1 and THP-1 cells, we examined the morphological change induced by GM-CSF and/or ATRA in KG-1 and THP-1 cells. As shown in Table 1, treatment with ATRA + GM-CSF synergistically induced morphological changes in KG-1 and THP-1 cells similar to those seen in ML-1 cells after ATRA + GM-CSF treatment. This result suggests that treatment with ATRA + GM-CSF synergistically induces differentiation of KG-1 and THP-1 cells to granulocytes. We also examined the expression of RARα in these cell lines after treatment with GM-CSF alone (Fig. 5*B*). Interestingly, the KG-1 and THP-1 cell lines that showed synergistic differentiation were induced to express the RARα gene by GM-CSF. In contrast, HL-60 cells that did not show a synergistic effect were induced slightly to express the gene as compared to KG-1 and THP-1 cells. K562 cells were not induced to express the gene. These results raise the possibility that the expression of the RARα gene by GM-CSF is associated with a synergistic effect of ATRA + GM-CSF. Finally, to investigate the effect of GM-CSF on primary AML cells, we examined the induction of RARα by GM-CSF in short-time cultured peripheral blood cells from two AML patients (FAB-M2 and FAB-M5a) using RT-PCR. As in ML-1 cells, GM-CSF induced the expression of RARα mRNA in these two primary AML cell cultures (Fig. 6).

Table 1 *Morphological changes in KG-1 and THP-1 cells after treatment with ATRA and/or GM-CSF for 9 days*

Morphological maturation was classified according to the nuclear shape as blast, myelocyte, banded, and segmented. Differential counts were performed under a microscope among a minimum of 200 cells. Morphological changes were determined as described in "Materials and Methods." Values are the mean ± SD of triplicate determinations.

	Myeloid cell type (% of total cells ± SD)			
Inducer	Blasts	Myelocytes	Banded neutrophils	Segmented neutrophils
KG-1				
None	85.8 ± 2.0	14.2 ± 2.0	0.0	0.0
GM-CSF (0.1 ng/ml)	88.0 ± 3.6	12.0 ± 3.6	0.0	0.0
ATRA (10^{-7} M)	24.7 ± 2.4	50.0 ± 2.9	16.0 ± 2.7	9.3 ± 0.6
GM-CSF + ATRA	7.0 ± 1.8	21.0 ± 1.8	29.7 ± 2.1	42.3 ± 2.4
THP-1				
None	98.7 ± 0.2	1.3 ± 0.2	0.0	0.0
GM-CSF (0.1 ng/ml)	90.7 ± 0.8	9.3 ± 0.8	0.0	0.0
ATRA (10^{-7} M)	12.7 ± 2.2	31.1 ± 1.3	38.5 ± 2.9	17.7 ± 0.6
GM-CSF + ATRA	4.7 ± 2.1	17.2 ± 3.0	28.3 ± 4.3	49.8 ± 3.2

DISCUSSION

ATRA effectively induces complete remission of APL but is ineffective in the treatment of other AMLs. Stimulation of cellular responses to RA in AML cells may make possible the application of differentiation therapy using ATRA for various subtypes of AML. Given our recent finding of the induction of granulocytic differentiation by combined ATRA + GM-CSF treatment (16), analysis of the role of GM-CSF in this system may contribute new information regarding the molecular mechanism underlying the cellular response to ATRA. By examining the effect of GM-CSF on RARs and RXRs in ML-1 cells, we demonstrated that GM-CSF stimulates the expression of RARα in these cells, and we showed a correlation between the induction of RARα and retinoid sensitivity in various cell lines. Data indicating a correlation between the expression level of RARs and cellular retinoid sensitivity have been reported previously. High levels of RARα in estrogen receptor-positive mammary carcinoma cells are correlated with stronger retinoid-mediated growth-inhibitory effects

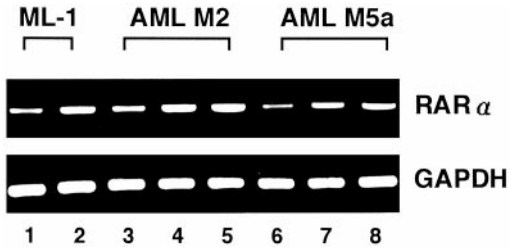

Fig. 6. Alteration of expression level of RARα by GM-CSF in peripheral blood cells from AML patients. Total RNA samples were prepared from nontreated cells (*Lanes 1, 3,* and *6*) and cells treated with 0.1 (*Lanes 2, 4,* and *7*) or 1 ng/ml (*Lanes 5* and *8*) GM-CSF for 3 h. RT-PCR was performed as described in "Materials and Methods." The PCR products were separated on a 1.5% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide.

when compared with estrogen receptor-negative breast carcinoma cells (19). In addition, RA-resistant HL-60 cells that carry a defective RARα can be rendered RA responsive by the introduction of RARα, RARβ, RARγ, or RXRα (20). These results suggest that GM-CSF induction of RARα in ML-1 cells increases cellular retinoid sensitivity and substantially advances differentiation toward the granulocytic pathway in combination with ATRA. We also demonstrated that GM-CSF increases the binding of nuclear proteins to RAREs and stimulates expression of CD38 mRNA via RARα in a ligand-dependent manner in ML-1 cells. These results indicate the possibility that GM-CSF stimulates RAR-RXR transcriptional activity in a ligand-dependent manner through the induction of RARα expression. This may provide a novel pathway for GM-CSF action in granulocytic differentiation. We also demonstrated the induction of RARα by GM-CSF in peripheral blood cells from AML patients. This activity of GM-CSF may therefore be useful as an adjunct for differentiation therapy using ATRA in various AML patients.

Investigation of the mechanism of induction of the RARα gene by GM-CSF is important because the signal or molecules related for induction may provide targets for the stimulation of cellular response to RA. We have little information regarding the mechanism of induction of the RARα gene by GM-CSF. Chen *et al.* (21) reported that AP-1, a transcription factor, plays an important role in signaling the expression of the RARα, RARγ, and RXRα genes stimulated by transforming growth factor β1. The induction of RARα by GM-CSF may be associated with AP-1 because GM-CSF induces c-fos and c-jun, which form AP-1. The expression of RARα is regulated by tyrosine kinase signaling pathways (22), and estrogen increases the level of RARα mRNA and protein (23). To examine whether the induction is transcriptional or posttranscriptional, we performed a preliminary examination using a run-on assay. However, we could not detect the increase in the RARα transcription to account for the large increase in RARα mRNA. We have not examined the alteration of RARα mRNA half-time by treatment with GM-CSF; however, it may be that RARα induction by GM-CSF is due to the effect on the posttranscriptional level of the mRNA.

To investigate the effect of other cytokines on RARα, we examined the expression of RARα in ML-1 cells treated with other cytokines related to granulocytic differentiation. Expression did not change after treatment with various cytokines including G-CSF, macrophage colony-stimulating factor, interleukin 3, and interleukin 6 (data not shown). However, these observations may result from differences in expression of each cytokine receptor in these cells because regulation of RARs and RXRs by some cytokines has been reported. Chen *et al.* (21) reported that transforming growth factor β1 transcriptionally stimulates the expression of RARα, RARγ, and RXRα genes. Another group of researchers (24) observed that IFN-γ increases the expression of the RARγ gene. These results suggest that the expression of RAR and RXR genes is stimulated by some cytokines. Regulation of nuclear hormone receptors by cytokines may be a way to control cell growth and differentiation.

In this report, we noted the possibility that induction of the RARα gene by GM-CSF is part of the mechanism underlying synergistic granulocytic differentiation by ATRA + GM-CSF. The synergistic effect of cytokines and hormones has been investigated by others, who have reported similar results concerning the mechanism of synergistic effect. Widschwendter *et al.* (24) proposed that an IFN-γ-mediated increase in RARγ may play a role in the synergistic inhibition of proliferation in breast cancer cell lines after treatment with retinoids + IFN-γ. Another group suggested that up-regulation of the G-CSF receptor by RA may account for the synergistic effect of G-CSF and RA in the differentiation of APL cells (25). Cell growth and differentiation are probably regulated by a complicated interaction between cytokines and hormones. In future studies, the synergistic effect of combinations of various cytokines and hormones should be studied. It is also important to examine the expression of various receptors in cancer cells. Because the sensitivity of the cell to cytokines and hormones is affected by the expression level of appropriate receptors, further information on the expression pattern of receptors in target cells and on the dosages of cytokines and hormones that are optimum for expression will probably be required for advances in clinical treatment of APL and other forms of leukemia or, eventually, all types of cancers. We expect the application of combined treatment to lead to improved clinical efficacy.

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REFERENCES

1. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. RXR β: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell, *67:* 1251–1266, 1991.
2. Bugge, T. H., Pohl, J., Lonnoy, O., and Stunnenberg, H. G. RXR α, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J., *11:* 1409–1418, 1992.
3. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Garnier, J. M., Mader, S., and Chambon, P. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell, *68:* 377–395, 1992.
4. Durand, B., Saunders, M., Leroy, P., Leid, M., and Chambon, P. All-*trans* and 9-*cis* retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. Cell, *71:* 73–85, 1992.
5. Pratt, M. A. C., Kralova, J., and Mcburney, M. W. A dominant negative mutation of the α retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. Mol. Cell. Biol., *10:* 6445–6453, 1990.
6. Collins, S. J., Robertson, K. A., and Mueller, L. Retinoic acid-induced granulocytic differentiation of HL-60 myeloid leukemia cells is mediated directly through the retinoic acid receptor (RAR-α). Mol. Cell. Biol., *10:* 2154–2163, 1990.
7. Breitman, T. R., Selonich, S. E., and Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA, *77:* 2936–2940, 1980.
8. Chomienne, C., Ballerini, P., Balitrand, N., Daniel, M. T., Fenaux, P., Castaigne, S., and Degos, L. All-*trans* retinoic acid in acute promyelocytic leukemias. II. *In vitro* studies: structure-function relationship. Blood, *76:* 1710–1717, 1990.
9. Nilsson, B. Probable *in vitro* induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukemia. Br. J. Haematol., *57:* 365–371, 1984.
10. Huang, M., Ye, H-C., Chen, S-R., Chai, J-r., Lu, J-X., Zhoa, L., Gu, L-j., and Wang, Z-y. Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Blood, *72:* 567–572, 1988.
11. Castaigne, S., Chomienne, C., Daniel, M-T., Ballerini, P., Berger, R., Fenaux, P., and Degos, L. All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. Blood, *76:* 1704–1790, 1990.
12. Takeda, K., Iwamoto, S., Sugimoto, H., Takuma, T., Kawatani, N., Noda, M., Masaki, A., Morise, H., Arimura, H., and Konno, K. Identity of differentiation inducing factor and tumour necrosis factor. Nature (Lond.), *323:* 338–340, 1986.
13. Takei, M., Takeda, K., and Konno, K. The role of interferon-γ in induction of differentiation of human myeloid leukemia cell lines ML-1 and HL-60. Biochem. Biophys. Res. Commun., *124:* 100–105, 1984.

14. Takeda, K., Minowada, J., and Bloch, A. Kinetics of appearance of differentiation-associated characteristics in ML-1, a line of human myeloblastic leukemia cells, after treatment with 12-*O*-tetradecanoylphorbol-13-acetate, dimethyl sulfoxide, or 1-β-D-arabinofuranosylcytosine. Cancer Res., *42:* 5152–5158, 1982.
15. Saito, Y., Hatta, H., Mano, Y., Taira, N., Shimizu, T., and Takeda, K. Vitamin D_3 analogue KH1060 combined with TPA synergistically induces mature macrophages in human myeloblastic leukemia ML-1 cells. Anticancer Res., *19:* 1069–1076, 1999.
16. Oka, Y., and Takeda, K. Retinoic acid combined with GM-CSF induces morphological changes with segmented nuclei in human myeloblastic leukemia ML-1 cells. Anticancer Res., *17:* 1951–1956, 1997.
17. Bachner, R. L., and Nathan, D. G. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N. Engl. J. Med., *278:* 971–976, 1986.
18. Shimizu, T., Oka, Y., Awai, N., and Takeda, K. Hypophosphorylation of pRB and repression of cyclin D3 and cdc25A during the granulocytic differentiation of human myeloblastic ML-1 cells. Leukemia Res., *23:* 901–907, 1999.
19. Leede, B. J., Folkers, G. E., Brink, C. E., Saag, P. T., and Burg, B. Retinoic acid receptor α1 isoform is induced by estradiol and confers retinoic acid sensitivity in human breast cancer cells. Mol. Cell. Endocrinol., *109:* 77–86, 1995.
20. Robertson, K. A., Emami, B., Mueller, L., and Collins, S. J. Multiple members of the retinoic acid receptor family are capable of mediating the granulocytic differentiation of HL-60 cells. Mol. Cell. Biol., *12:* 3743–3749, 1992.
21. Chen, Y., Takeshita, A., Ozaki, K., Kitano, S., and Hanazawa, S. Transcriptional regulation by transforming growth factor β of the expression of retinoic acid and retinoid X receptor genes in osteoblastic cells is mediated through AP-1. J. Biol. Chem., *271:* 31602–31606, 1996.
22. Flicker, S. H., Schneider, S. M., Offterdinger, M., Dittrich, E., Fazeny, B., Valenta, R., Huber, H., Dittrich, C., and Grunt, T. W. Tyrosine kinase signaling pathways control the expression of retinoic acid receptor-α in SK-BR-3 breast cancer cells. Cancer Lett., *115:* 63–72, 1997.
23. Sun, G., Porter, W., and Safe, S. Estrogen-induced retinoic acid receptor α1 gene expression: role of estrogen receptor-sp1 complex. Mol. Endocrinol., *12:* 882–890, 1998.
24. Widschwendter, M., Daxenbichler, G., Dapunt, O., and Marth, C. Effect of retinoic acid and γ-interferon on expression of retinoic acid receptor and cellular retinoic acid-binding protein in breast cancer cells. Cancer Res., *55:* 2135–2139, 1995.
25. Tkatch, L. S., Rubin, K. A., Ziegler, S. F., and Tweardy, D. J. Modulation of human G-CSF receptor mRNA and protein in normal and leukemic myeloid cells by G-CSF and retinoic acid. J. Leukocyte Biol., *57:* 964–971, 1995.

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