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Expression of α_1-Acid Glycoprotein and Inflammatory Cytokines during Differentiation of HL-60 Cells

Il-Ha Lee, In-Sook Kim* and Soo-Young Lee
Chemistry Section, Department of Natural Sciences, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

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In order to understand the role of AGP on the differentiation of promyelocytic leukemia cells, the AGP expression and its relation to cytokines were investigated during granulocytic or monocytic differentiation of HL-60 cells. When HL-60 cells were treated with all-*trans*-retinoic acid (ATRA) for 5 days, the cells were fully differentiated into granulocytes, and the AGP mRNA and protein levels were continuously increased up to 5 days in a dose- and time- dependent manner. However, in the case of the monocytic differentiation of HL-60 cells by tetradeanoyl phorbol acetate (TPA), the AGP gene expression was not induced. In addition, IL-1α, IL-1β, IL-6 and TNF-α mRNAs were also enhanced during granulocytic differentiation. These cytokine transcripts showed a peak level 3 days after the ATRA treatment. It decreased gradually thereafter. However, direct addition of recombinant cytokines (IL-1β, IL-6 and TNF-α) and dexamethasone to the HL-60 cell cultures showed no AGP induction. These findings suggest that the AGP and proinflammatory cytokines are expressed in ATRA-treated promyelocytic cells. However, these cytokines do not act as autocrine inducers on AGP expression. This fact implies that the AGP expression during granulocytic differentiation of HL-60 cells is induced through a signal pathway different from hepatocyte signaling in inflammation.

Keywords: All-*trans* retinoic acid, α_1-Acid Glycoprotein, Cytokines, Differentiation, HL-60 Cells

Introduction

α_1-acid glycoprotein (AGP, orosomucoid) is a typical acute phase protein in which the serum level is increased in inflammation, infection, pregnancy and malignancy (Baumann and Gauldie, 1994). One of the structural features of AGP is that it has a high carbohydrate content (over 40%) as well as a high acidity due to the presence of a large amount of sialic acid (11%) (Bories *et al.*, 1990; Pos *et al.*, 1990). In general, AGP binds to many drugs and modulates their metabolism (Kremer *et al.*, 1988). It is known that a major source for AGP biosynthesis is hepatocytes. AGP is induced by several cytokines, such as IL-6, IL-1, TNF-α and glucocorticoids (Baumann *et al.*, 1993). However, recent reports demonstrated that the AGP gene expression could also be induced in extrahepatic cells including monocytes and epithelial cells. (Boutten *et al.*, 1992).

All-*trans*-retinoic acid (ATRA) has been introduced as a therapeutic agent for acute promyelocytic leukemia (APL). It induces the terminal differentiation of APL cells both *in vitro* and *in vivo*. HL-60 cells have been used as an *in vitro* model system for differentiation of promyelocytic leukemic cells, because the cells can be induced with granulocytic differentiation by ATRA and dimethylsulfoxide (DMSO), or monocytic differentiation by 1α, 25-dihydroxyvitamin D_3 (Vit D_3) and tetradecanoyl phorbol acetate (TPA) (Collins *et al.*, 1979; Rovera *et al.*, 1979; Breitman *et al.*, 1980; Miyaura *et al.*, 1981).

Grande *et al.* (1995) reported that ATRA-treated HL-60 cells expressed inflammatory cytokines, which were known to be inducers of various acute phase proteins in hepatocytes. In addition, we found that ATRA induces haptoglobin (one of the acute phase proteins) in various myelocytic leukemia cells (unpublished data). Therefore, it is interesting to study whether or not acute phase proteins can be induced during myeloid cell differentiation, and their expressions are regulated by cytokines. Because AGP is a well-known acute phase protein, we selected it from among other acute phase proteins to investigate in detail.

In this report the expressions of AGP and proinflammatory cytokines during differentiation of the HL-60 cell line were investigated in order to understand the role of AGP on the differentiation of promyeloleukemic cells as well as the relationship between cytokines and AGP induction in these cells.

*To whom correspondence should be addressed.
Tel: 82-2-590-1269; Fax: 82-2-592-7068
E-mail: ikim@cmc.cuk.ac.kr

Materials and Methods

Cell culture and differentiation The HL-60 human promyelocytic leukemia cell line and the HepG2 human hepatoblastoma cell line were obtained from American Type Culture Collection (Rockville, USA). They were grown in a RPMI-1640 medium (GIBCO BRL, Rockville, USA) that was supplemented with 10% fetal bovine serum (Hyclone, Logan, USA).

To induce cell differentiation, the HL-60 cells were plated at a density of 1×10^5/ml in a medium containing 1 μM of ATRA (Sigma, St. Louis, USA), 1.3 % of DMSO (Sigma) or 50 nM of TPA (Sigma) and then cultured for 5 days. The cell differentiation was evaluated by morphological changes in Giemsa staining (Breitman *et al.*, 1980) and the reduction of c-myc gene expression (Grosso *et al.*, 1984).

Confluent monolayer of HepG2 cells were washed twice with PBS and incubated with 200 U/ml of IL-6 for 2 days in a serum-free RPMI 1640 medium. The IL-6-treated HepG2 cells were used for the positive control experiment for AGP expression.

Total RNA preparation and RT-PCR Total cellular RNA was extracted using a RNAzol B solution (Tel-test, Friendswood, USA) based on the modified single step guanidinum isothiocyanate lysis method (Chomczynski and Sacchi, 1987). The RT-PCR reactions were carried out using a mRNA Selective PCR Kit (TaKaRa, Shiga, Japan) according to the procedures outlined by the manufacturer. Briefly, 5 μg of total RNA that were extracted from each sample were reverse-transcribed for 30 minutes at 42°C in a 1 × PCR buffer containing 5 mM $MgCl_2$, 1 mM dNTP/analog mixture, RNase inhibitor, oligo dT primer and 0.1 U/μl AMV reverse transcriptase in a total volume of 50 μl. Then 10 μl of the RT reactant was amplified in a total volume of 50 μl. The amplification was performed for 30 cycles under the following reaction conditions: 5 mM $MgCl_2$, 1 mM dNTP/analog mixture, 0.4 μM primers (Table 1) (Grande *et al*, 1995), 0.1 U/μl *Taq* DNA polymerase; denaturation, 85°C for one minute; annealing, 55°C for one minute; extension, 72°C for one minute. The 10 μl of the RT reaction was then electrophoresed on a 1.5% agarose gel and stained with EtBr (Kim *et al.*, 2000).

Western blot analysis Equal amounts (30 μg) of proteins were analyzed by electrophoresis on 12% SDS-polyacrylamide gels in duplicate (Laemmli, 1970). After electrophoresis one gel was stained with Coomassie blue and the other gel was transferred to a nitrocellulose membrane using a XCell™ blot module (Novex, San Diego, USA). The membrane was sequentially incubated with an anti-human AGP antibody (Sigma) and a peroxidase-conjugated anti-goat IgG (Sigma). After washing the blot was analyzed with the ECL system (Amersham, Buckinghamshire, England) (Baek *et al.*, 1999).

Northern blot analysis Total cellular RNAs were isolated from the HL-60 cells that were treated with or without AGP-inducers and HepG2 cells that were treated with IL-6 (200 U/ml). Six μg of the RNA sample was electrophoresed on 1% agarose-formaldehyde gel and transferred to a nylon membrane. The AGP mRNA was analyzed by the DIG chemiluminescent detection method according to the procedure recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The probes, incorporated with DIG-11-dUTP, were prepared by PCR amplification of human AGP cDNA and β-actin cDNA.

Results and Discussion

Induction of differentiation of HL-60 cells It is well known that the HL-60 promyelocytic leukemia cells can be differentiated into granulocytes by ATRA and DMSO, or into monocytes/ macrophages by Vit D_3 and TPA (Collins *et al.*, 1979; Rovera *et al.*, 1979; Breitman *et al.*, 1980; Miyaura *et al.*, 1981). First, we confirmed the induction of differentiation of HL-60 cells by various inducers. When the HL-60 cells were incubated with 1 μM ATRA, or 1.3% DMSO for 5 days, the cell growth was apparently decreased by 41% to 60% of the growth of untreated control cells, respectively. Moreover, the c-myc gene, which is overexpressed in undifferentiated HL-60 cells, was down-regulated time-dependently in ATRA-treated cells (Fig. 1B). The HL-60 cells that were treated with 50 nM TPA adhered to the bottom of the culture dish. According to the Giemsa staining results (Fig. 1A), the

Table 1. Oligodeoxynucleotides used in the RT-PCR reaction

Oligomer	Nucleotide sequence (5'-3')
IL-1 α direct primer	CCAGACATGTTTGAAGACCTG
IL-1 α reverse primer	GAGTGGGCCATAGCTTACATG
IL-1 β direct primer	ATGGCAGAAGTACCTAAGCTCGC
IL-1 β reverse primer	ACACAAATTGCATGGTGAAGTCAGTT
IL-6 direct primer	ATGAACTCCTTCTCCACAAGCGC
IL-6 reverse primer	CATAAGTTCTGTGCCCAGTGG
TNF-α direct primer	ATGAGCACTGAAAGCATGATCCGG
TNF-α reverse primer	GCAATGATCCCAAAGTAGACCTGCCC
β-actin direct primer	ATCTGGCACCACACCTTCTACAATGAGCTGCG
β-actin reverse primer	CGTCATACTCCTGCTTGCTGATCCACATCTGC

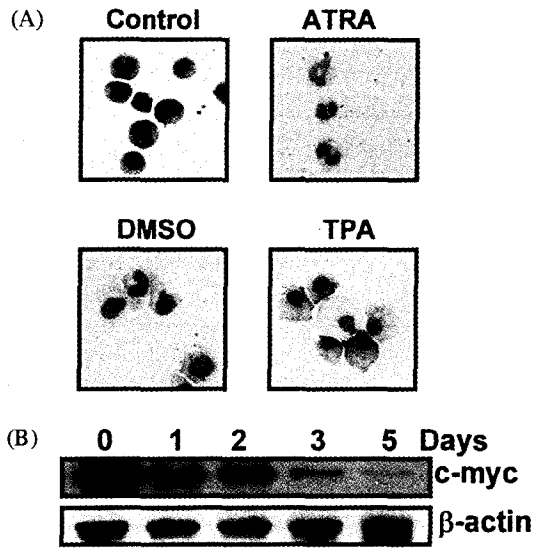

Fig. 1. Induction of differentiation of HL-60 cells. 1×10^5/ml of HL-60 cells were incubated with 1 μM ATRA, 1.3% DMSO or 50 nM TPA for induction of differentiation into granulocytes or monocytes/macrophages, respectively. (A) After 5 days, the cells were stained with a Giemsa solution and examined with a microscope in order to confirm the morphological changes of differentiated cells. (B) Down-regulation of the c-myc gene expression in ATRA-treated cells (a criterion for differentiation of HL-60 cells) was also observed using the Northern blot technique. Detailed experimental procedures were described in "Materials and Methods".

ATRA- or DMSO-treated cells showed bending and segregated nuclei. These were similar to that of granulocytes. On the other hand, TPA-treated cells showed a larger cell size than the control cells and big cytosols which contain many vacuoles. These findings indicate that the HL-60 cells were successfully differentiated into granulocytes by ATRA and DMSO, or monocytes/macrophages by TPA.

AGP synthesis during granulocytic differentiation of HL-60 cells In order to investigate the gene expression of AGP during the differentiation of HL-60 cells, Northern blot analysis was carried out with total RNAs extracted from the differentiation-induced cells. As shown in Fig. 2, ATRA at 1 μM continuously increased the AGP transcript level up to a 5 day period. The size of the AGP transcript in HL-60 cells was the same as that in HepG2 hepatocarcinoma cells which were known to express the AGP gene (positive control). The 1.3% DMSO also induced AGP gene expression. However, TPA at 50 nM failed to induce this gene (Fig. 2), although terminal differentiation into macrophages could be induced by this agent (Fig. 1A). Therefore, it seems that the AGP gene expression during myelocytic differentiation is specific to differentiation-lineage, especially to granulocytic differentiation. These findings are similar to the results of other studies demonstrating that thrombospondin-1 was induced during granulocytic differentiation by ATRA, but was

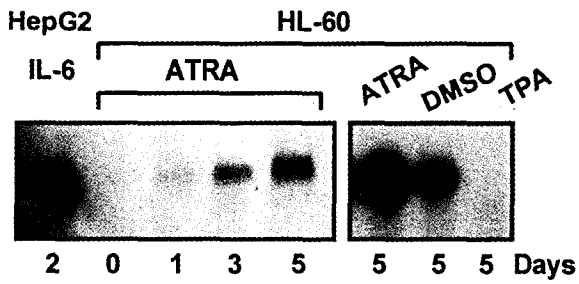

Fig. 2. Induction of the AGP gene expression during granulocytic differentiation of acute promyelocytic leukemia cells. Total cellular RNAs were isolated from the HL-60 cells treated with 1 μM ATRA, 1.3% DMSO and 50 nM TPA for the indicated times. The 6 μg of the RNA sample was analyzed by the Northern blot method. The human AGP cDNA was labeled with DIG-11-dUTP by PCR amplification and used as a probe. Note that the AGP gene expression was induced during granulocytic differentiation by ATRA and DMSO, but not induced during the monocytic differentiation by TPA.

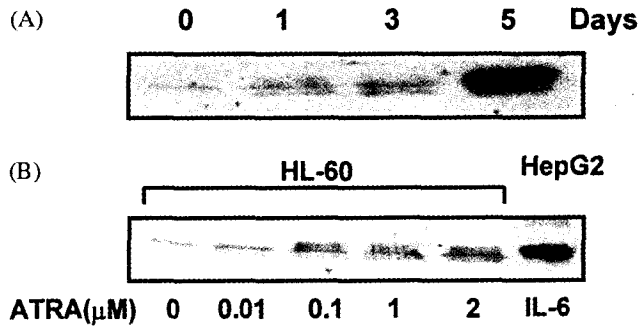

Fig. 3. Biosynthesis of AGP protein during differentiation of HL-60 cells. The lysates (30 μg of protein) from HL-60 cells treated with ATRA were analyzed by Western blot method using the anti-human AGP antibody. AGP biosynthesis was increased in a time- (A) and a dose-dependent (B) manner. HepG2 cells, which were treated with 200 U/ml of IL-6 for 2 days, were used for a positive control experiment.

not induced during monocytic differentiation by TPA (Touhami *et al.*, 1997). According to the Western blot analysis of the cell lysates, AGP protein synthesis was also increased in a time- and a dose-dependent manner during ATRA-induced differentiation (Fig. 3A-B).

Correlation between the expression of inflammatory cytokines and the AGP synthesis during differentiation of HL-60 cells The relationship of cytokines to the differentiation of myeloid leukemic cells has been studied. Grande *et al.* (1995) reported that ATRA stimulated simultaneously granulocytic differentiation and expression of IL-1α, IL-1β, IL-3, IL-6 and TNF-α. Peck and Bollag (1991) demonstrated an enhanced differentiation of HL-60 cells by the combined treatment of ATRA and cytokines.

These cytokines are known to be inducers of AGP in hepatocytes, which are the main sources for AGP biosynthesis

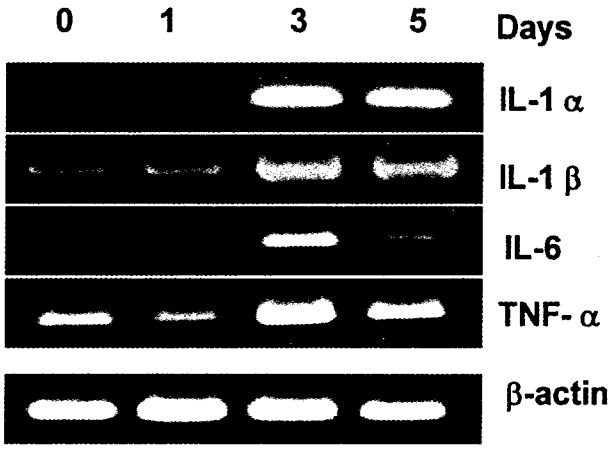

Fig. 4. Gene expression of proinflammatory cytokines in ATRA-treated HL-60 cells. The HL-60 cells were treated with 1 μM ATRA for the indicated times, then the total cellular RNAs were isolated. For the RT-PCR reaction, 5 μg of total RNA that was extracted from each sample was reverse-transcribed for 30 minutes at 42°C in a 1 × PCR buffer in a total volume of 50 μl. Then 10 μl of the RT reactant was amplified in a final volume of 50 μl for 30 cycles. All transcripts showed maximum levels 3 days after ATRA treatment and declined thereafter.

in inflammation. Therefore, we examined the potential relationship of inflammatory cytokines to the AGP synthesis in HL-60 cells. After the cells were incubated with 1 μM of ATRA, total RNAs were isolated and then the gene expression of various cytokines (IL-1α, IL-1β, IL-6 and TNF-α) were determined using the RT-PCR technique. As shown in Fig. 4, ATRA increased the transcriptions of all cytokines. It showed maximum levels at day 3 with a gradual decrease thereafter. Because of the possibility that ATRA-induced cytokines may act on the AGP expression during cell differentiation, we attempted to detect the AGP expression after recombinant cytokines (IL-1β, IL-6 and TNF-α) were directly added to the HL-60 cell cultures. However, none of these could induce AGP expression (data not shown). We concluded that the cytokines induced by ATRA do not act as autocrine inducers on AGP expression. This suggests that other factors besides these cytokines are more likely required for AGP gene regulation during HL-60 cell differentiation. Dexamethasone, which is a potent inducer of AGP in hepatocytes, could also not induce AGP expression (Fig. 5). These results reveal that a different signal pathway from that in hepatoma cells may be involved in the AGP expression during myeloid cell differentiation.

In this study we demonstrated that the AGP expression was up-regulated by ATRA during the granulocytic differentiation of HL-60 cells. The mechanism of this induction is unknown but it does not involve inflammatory cytokines, IL-1β, IL-6 and TNF-α. This is the first report to show up-regulation of the AGP gene expression during myeloid cell differentiation. Especially noted as a new finding is the role of ATRA as a inducer of AGP.

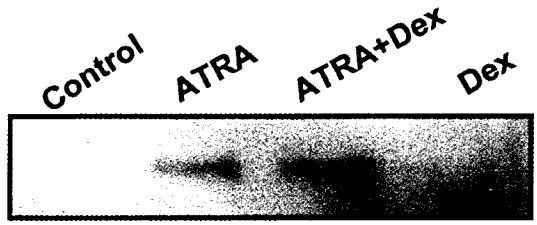

Fig. 5. Effect of dexamethasone on AGP gene expression. Northern blot analysis was performed by the same method as in Fig. 2 using total RNAs from the HL-60 cells that were incubated for 3 days with 1 μM ATRA, 1 μM dexamethasone and combination of ATRA and dexamethasone.

It seems likely that the AGP expression is not a cause, but a result of cell differentiation. This is because maximum induction appeared at day 5, after the ATRA treatment when the differentiation was nearly completed (Fig. 1A-B). We thought that AGP probably acts on a feed-back system that inhibits the hyperinflammatory effects of cytokines. Actually, several studies have pointed out the roles of AGP in the regulation of the immune system (Costello *et al.*, 1979; Bennett *et al.*, 1980; Bories *et al.*, 1990). In addition, AGP protected animals from lethal shock that is induced by TNF (Libert *et al.*, 1994). Our results also showed a decline in cytokine expressions by day 5 after the ATRA treatment, when AGP was expressed at its maximum. Therefore, AGP may play a role in the mitigation of hyperinflammatory reaction by inflammatory cytokines that are secreted during myeloid cell differentiation.

ATRA has been introduced as a therapeutic agent for acute promyelocytic leukemia, since it is capable of inducing cell differentiation *in vivo* and *in vitro*. However, ATRA also induced significant and common side effects, such as retinoic acid syndrome (RAS) which is developed in 5-25% of the patients treated with ATRA (Grignani *et al.*, 1994; Degos *et al.*, 1995). It has been understood that RAS is caused by a cytokine-induced hyperinflammatory reaction, although the exact mechanisms remain unclear. If AGP can diminish the activities of proinflammatory cytokines in myeloid cells, AGP may be applied to a mitigator of RAS. To solve the exact physiological functions of AGP that are induced during granulocytic differentiation, further study will be needed using AGP cDNA-transfected myeloid cells.

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