Article

Down-Regulation of CD44 Contributes to the Differentiation of HL-60 Cells Induced by ATRA or HMBA

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CD44 is highly expressed in human acute myeloid leukemia (AML) cells. Some experiments had shown that it was possible to reverse differentiation blockage in AML cells by CD44 ligation with specific antibodies, indicating that CD44 was closely related to the differentiation of leukemia cells. The differentiation of acute promyelocytic leukemia cell line HL-60 cells could be induced by all trans-retinoic acid (ATRA) and hexamethylene bisacetamide (HMBA), but so far the mechanism was not demonstrated clearly. In the present study, we investigated whether ATRA or HMBA induced the growth arrest of HL-60 cells by down-regulating the expression of CD44. The results indicated that the proliferation of HL-60 cells was obviously inhibited and the differentiation was induced by both ATRA and HMBA. The decreased expression of CD44 and cyclin E mRNA, and the increased expression of p27 and p21 at mRNA levels were observed. Furthermore, there was a negative correlation between the expression of CD44 and p27. It was concluded that ATRA and HMBA played a role in the differentiation induction of HL-60 cells, which was mediated by the down-regulation of CD44, accompanied by down-regulation of cyclin E, and up-regulation of p27 and p21 mRNA. *Cellular & Molecular Immunology*. 2007;4(1):59-63.

Key Words: CD44, HL-60 cell, differentiation, cell cycle modulator

Introduction

Adhesion molecule CD44 is a cell surface transmembrane glycoprotein encoded by single gene. As a receptor for hyaluronic acid (HA), CD44 is involved in lymphocyte activation, recirculation and homing, adhesion of extracellular matrix, angiogenesis, cell proliferation, cell differentiation and migration (1). All these biological properties are essential to the physiological activities of normal cells, but they are also associated with the pathologic activities of cancer cells. Elevated CD44 expression was correlated with poor prognosis in many solid tumors, such as lung cancer (2), breast cancer (3), colorectal cancer (4), gastrointestinal neuroendocrine tumor (5), and so on. In recent years, scholars pay more attention to the association of CD44 with hematological malignancies. They presumed that CD44 played an important role in normal myelopoiesis because anti-CD44 antibodies profoundly alter *in vitro* myelopoiesis in long-term bone marrow cultures (6). In the context of human acute myeloid leukemia (AML), experiments have shown that ligation of CD44 with some specific monoclonal antibodies can reverse the differentiation blockage of AML cells (7), indicating new possibilities for the development of CD44-targeted differentiation therapy in leukemia. The CD44 modulation maybe play important role in differentiation of leukemia cells.

The differentiation of acute promyelocytic leukemia cell line HL-60 could be induced by all trans-retinoic acid (ATRA) or hexamethylene bisacetamide (HMBA) respectively, but so far the mechanism was not demonstrated clearly (8, 9). In the present study, the HL-60 cells were treated with ATRA or HMBA for the sake of detecting whether ATRA or HMBA induced the growth arrest of HL-60 cells by down-regulating the expression of CD44, which was related to the modulation of cell cycle modulators. The results showed that the proliferation of HL-60 cells was obviously inhibited and the differentiation was induced in HL-60 cells by both ATRA and HMBA. The down-regulated expression of CD44 and cyclin E, and up-regulated expression of p27 and p21 at mRNA levels were observed by RT-PCR. Furthermore, there was a negative correlation between the expression of CD44 and p27 mRNA.

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Table 1. Primers and annealing temperature

Gene	Sense	Antisense	Size	Annealing temperature
CD44	5'-GACAGACACCTCAGTTTTTCTGGA-3'	5'-TTCCTTCGTGTGTGGGTAATGAGA-3'	845 bp	62°C
p27	5'-ATGTCAAACGTGCGAGTGTC-3'	5'-TCTGTAGTAGAACTCGGGCAA-3'	270 bp	55°C
p21	5'-CCCTCCTGGCTCTTGATACCC-3'	5'-TGCCCTTCTTCTTGTGTGTCCC-3'	341 bp	58°C
cyclin E	5'-ATACAGACCCACAGAGACAG-3'	5'-TGCCATCCACAGAAATACTT-3'	301 bp	55°C
β-actin	5'-GTGGGGCGCCCCAGGCACCA-3'	5'-CTCCTTAATGTCACGCACGATTTC-3'	540 bp	55°C

Materials and Methods

Cell line and reagents

HL-60 cells were maintained in our laboratory. ATRA, HMBA, RPMI 1640, MTT, DMSO, NBT and TPA were obtained from Sigma Co. PCR primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd. PE-conjugated CD11b antibody was purchased from Pharmingen Co.

Cell culture and induction

HL-60 cells were cultured in RPMI 1640 standard medium containing 2 mmol/L L-glutamine and supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in 5% CO_2. Exponentially growing cells (1×10^7) were incubated with 0.1 μmol/L ATRA, 2 mmol/L HMBA, 0.1% alcohol or RPMI 1640 for 1 to 3 days, respectively.

Proliferation assay

Cell proliferation was determined by MTT (methyl thiazolyl tetrazolium) assay. HL-60 cells (5×10^5/well) were incubated in 96-well microplates, with 0.1 μmol/L ATRA, 2 mmol/L HMBA, 0.1% alcohol or RPMI 1640 for 20 to 68 h, then, 10 μl MTT (5 mg/m1) was added to each well for an additional 4 h. The reaction was stopped by adding 150 μl DMSO and the absorbance (A) at 570 nm were determined on a multi-well plate reader (Bio-Rad). Each group was in triplicate samples. Cell inhibition rate = 100% × (experimental group A values - control group A values) / control group A values.

Differentiation assay

Differentiation of HL-60 cells was assessed by the NBT (nitroblue tetrazolium) reduction test and cellular surface differential antigen CD11b assay by flow cytometry. Brief, 100 μl HL-60 cells (1×10^6/m1) were induced by 0.1 μmol/L ATRA or 2 mmol/L HMBA for 1 to 3 days in 96-well microplates, respectively. Then added 100 μl NBT (1 mg/ml) and 200 ng TPA (1 mg/ml) to the wells, incubated at 37ºC in 5% CO_2 for 1 h, centrifuged for 5 min, and Wright's staining. When NBT was phagosomed by the cells, intracellular dye converts it into an insoluble blue crystalline form (formazan crystals). Two hundred cells were observed and the positive cells with the formazan crystals were counted under immersion objective. RPMI 1640 was used as blank control and alcohol as dissolvent control.

For detection of cell differentiation antigen CD11b, 1×10^6 cells were washed twice with PBS, incubated with PE-conjugated CD11b antibody or isotype control (PE-conjugated IgG1), at 4°C for 30 min. Samples were analyzed by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) and the CellQuest software. The expression rate of positive cells was counted in 10^5 cells.

Cell cycle analysis

Briefly, cells were induced with 0.1 μmol/L ATRA, 2 mmol/L HMBA, 0.1% alcohol or RPMI 1640 for indicated time, washed twice in PBS and injected into cold 70% ethanol, then kept overnight at 4°C. Subsequently, cells were rinsed with PBS and treated with 0.03 mg/ml trypsin (Sigma Co.) for 10 min at room temperature. Then, RNase A (Sigma Co., 0.08 mg/ml) and trypsin inhibitor (Sigma Co., 0.5 mg/ml) were added, and cells were incubated for additional 30 min at room temperature. Finally, prepared nuclei were stained with propidium iodide (PI, Sigma Co.) at a final concentration of 20 μg/ml. Stained nuclei were analyzed by flow cytometry and MacCycle software.

RT-PCR

At the indicated time points, 1×10^7 cells of each experimental group were taken, and total cellular RNA was isolated using Trizol reagent (Sigma Co.) according to the manufacturer's instructions. The concentration of prepared RNA was assayed by UV absorption at 260 nm. cDNA synthesis was performed using the M-MLV (TaKaRa Co.) reverse transcriptase. PCR was performed as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, specific annealing temperature for 1 min, and 72°C for 1 min, and with an additional extension at 72°C for 10 min in a final volume of 100 μl. The primer sequences and reannealing temperature of CD44, p27, p21, cyclin E and β-actin genes are showed in Table 1. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light and photographed with GELDos1000 image analysator. A gel thinlayer scan for semi-quantitative assay was done. The relative content of each expected product and β-actin was determined by the ratio of corresponding optical density.

Statistical analysis

All experiments were performed in triplicate unless otherwise noted. Significance of differences between mean

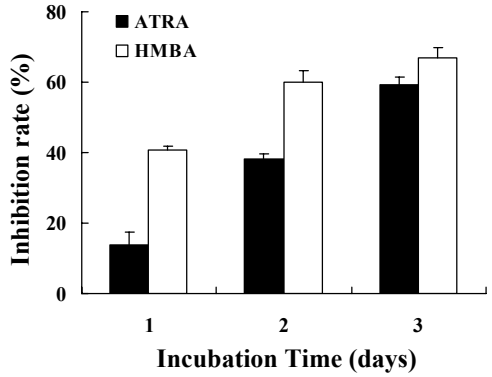

Figure 1. **The proliferation of HL-60 cells was inhibited by ATRA or HMBA.** HL-60 cells were incubated by ATRA or HMBA for 1 to 3 days, the inhibition rates of cell proliferation were detected by MTT.

values was assessed by *t*-test and ANOVA, percentage was assessed by Chi-square test and correlation analysis by Pearson correlation coefficient. $p < 0.05$ was considered to be significant. All calculations were performed by SPSS for Windows, Version 12.0.

Results

Both ATRA and HMBA inhibited the proliferation of HL-60 cells

MTT assay results showed that after incubated with 0.1 μmol/L ATRA for 1 to 3 days, the inhibition rates of HL-60 cells were 13.71 ± 1.18%, 38.15 ± 3.12% and 59.35 ± 2.96%, respectively, compared with dissolvent control ($p < 0.01$). The inhibition rates of HL-60 cells induced by HMBA were 40.58 ± 3.64%, 60.12 ± 1.55% and 66.94 ± 2.24%, compared with blank control ($p < 0.01$) (Figure 1). The inhibition rate was increased gradually with time and the effect of HMBA was stronger than that of ATRA ($p < 0.01$). The results indicated that both ATRA and HMBA could inhibit the proliferation of HL-60 cells in a time-dependent manner.

Both ATRA and HMBA induced the differentiation of HL-60 cells

As shown in Figure 2A, the percentage of NBT positive cells in HL-60 cells treated with ATRA for 1 to 3 days were 7.50 ± 1.32%, 25.17 ± 3.51% and 43.67 ± 1.15% respectively, in comparison with the dissolvent control (0.41 ± 0.25%, 0.75 ± 0.24% and 1.10 ± 0.5%) ($p < 0.01$), and with HMBA were 4.3 ± 0.57%, 17 ± 1.5% and 22.83 ± 1.04% respectively, in comparison with the blank control (0.41 ± 0.25%, 0.75 ± 0.24% and 1.00 ± 0.5%) ($p < 0.01$). The increased NBT reduction showed that the two drugs could promote the differentiation of HL-60 cells.

After treatment with ATRA or HMBA for 1 to 3 days, the expression rate of CD11b on HL-60 cells was increased obviously, indicating that both ATRA and HMBA could induce HL-60 differentiation to mature granulocytes. The

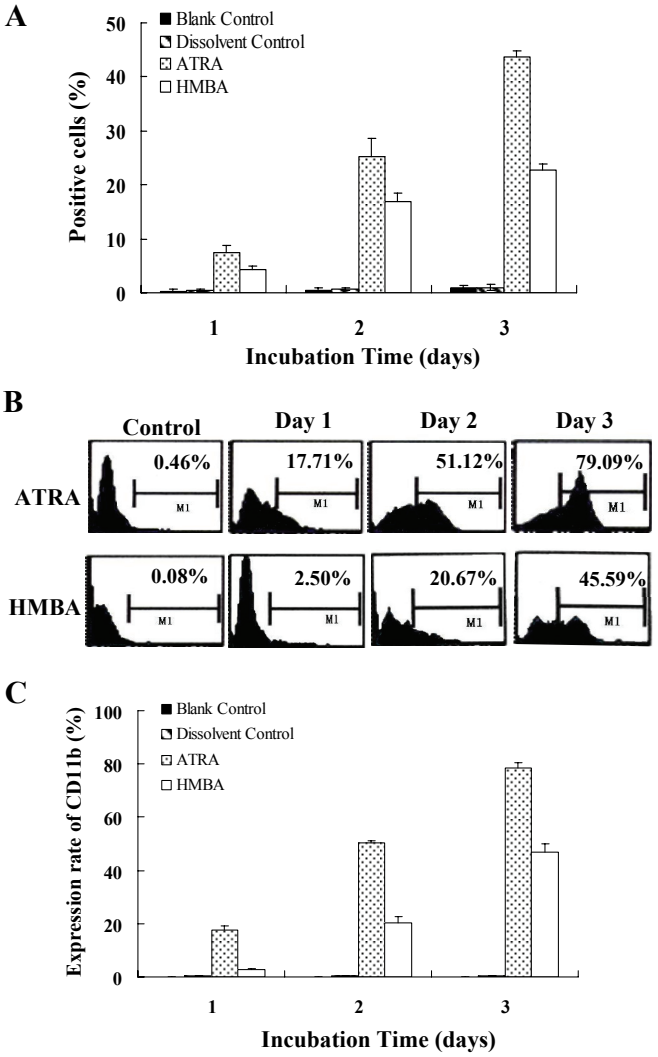

Figure 2. Effect of ATRA or HMBA on differentiation of HL-60 cells. (A) HL-60 cells were incubated with ATRA or HMBA for 1 to 3 days. The percentage of NBT positive cells was calculated. (B) CD11b expression on HL-60 cells induced by ATRA or HMBA. HL-60 cells were incubated with ATRA or HMBA for 1 to 3 days. The expression rate of CD11b on HL-60 cells was analyzed by flow cytometry. One representative example was shown. And the results were further summarized in (C).

expression rate of CD11b were 17.41 ± 1.91%, 50.39 ± 0.76% and 78.40 ± 1.94% in HL-60 cells incubated by ATRA for 1 to 3 days, in comparison with the dissolvent control of 0.46 ± 0.04%, and in HMBA-induced group were 2.69 ± 0.52%, 20.16 ± 4.46% and 46.85 ± 3.17%, in comparison with the blank control of 0.08 ± 0.02% ($p < 0.01$) (Figures 2B and 2C).

Both ATRA and HMBA caused G0/G1 arrest of HL-60 cells

Exposure to 0.1 μmol/L ATRA or 2 mmol/L HMBA for three days induced an obvious accumulation of HL-60 cells at the G0/G1 phase and a decrease at the S phase, which was consistent with the inhibition of proliferation as shown above.

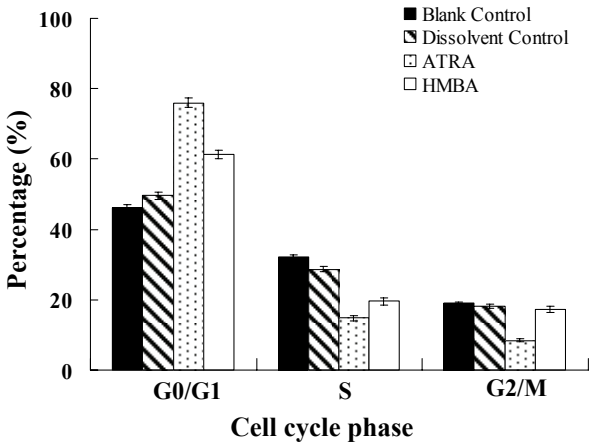

Figure 3. Cell cycle distribution in HL-60 cells after induced by ATRA or HMBA for 3 days. After incubated with ATRA or HMBA for 3 days, the percentage of HL-60 cells at G0/G1 phase was increased, and that at S phase was decreased ($p < 0.05$).

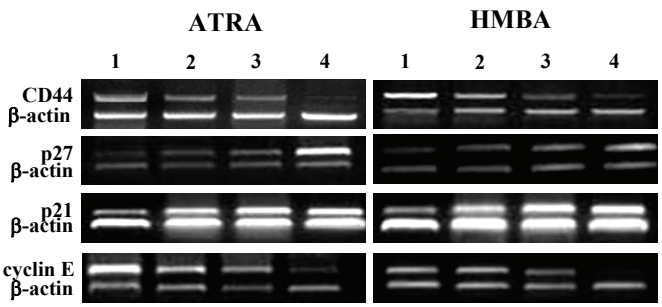

Figure 4. The mRNA changes of CD44, p27, p21 and cyclin E in HL-60 cells induced by ATRA or HMBA. Lane 1, control; Lane 2, day 1; Lane 3; day 2; Lane 4, day 3.

The ratio of G0/G1 phase cells in ATRA-induced HL-60 cells was 76.01 ± 1.40%, comparing with the dissolvent control with 49.61 ± 1.12% ($p < 0.05$). Similarly, the ratio of G0/G1 phase cells in HMBA-induced HL-60 cells was 61.4 ± 1.23%, comparing with the blank control with 46.25 ± 0.95% ($p < 0.05$, Figure 3). This indicated that the two drugs could induce HL-60 cell cycle arrest in G0/G1 phase.

Both ATRA and HMBA down-regulated the expression of CD44 and cyclin E, and up-regulated the expression of p27 and p21 mRNA in HL-60 cells

The RT-PCR results indicated that CD44, p21 and cyclin E significantly expressed in HL-60 cells, while p27 weakly expressed. After treatment with 0.1 μmol/L ATRA or 2 mmol/L HMBA for 1 to 3 days, HL-60 cells showed a significant decreased expression of CD44 and cyclin E, and increased expression of p27 and p21 ($p < 0.05$) (Figure 4). In the cells induced by ATRA, the ratio of corresponding optical density of CD44/β-actin was decreased from 0.9037 to 0.0413, p27/β-actin increased from 0.2365 to 1.8531, p21/β-actin increased from 0.3675 to 0.7886, and cyclin E/β-actin decreased from 1.8234 to 0.1473. In the cells induced by HMBA, CD44/β-actin was decreased from 1.7673 to 0.1422, p27/β-actin increased from 0.7655 to 1.3584, p21/β-actin increased from 0.3965 to 0.8647, and cyclin E/β-actin decreased from 1.1462 to 0.1335. Furthermore, correlation analysis showed that there was an obviously negative correlation between the level of CD44 and p27 ($r = -0.686$, $p < 0.05$), as well as CD44 and p21 ($r = -0.687$, $p < 0.05$), while a positive correlation of CD44 and cyclin E ($r = 0.604$, $p < 0.05$).

Discussion

ATRA is the ramification of vitamin A, and it can inhibit the proliferation and induce the differentiation of tumor cells. As a physiological differentiation inducer, it was successfully applied in the treatment of hematological malignance and became the model of differentiation therapy (8). HMBA is an effective hybrid polar compound which can also induce differentiation of various transformed cells (9). Otherwise, their molecular mechanisms have not been demonstrated clearly.

CD44 is the most important cell surface receptor of HA. It usually promotes the development of tumors by enhancing cell migration and inducing signal transduction, through the interaction of HA (10). CD44 is expressed in all subtypes of AML. Its antibody can trigger terminal differentiation of leukemic blasts in some subtypes (11). Gadhoum reported that the specific monoclonal antibodies H90 and A3D8 directed to the CD44 cell surface antigen could trigger terminal differentiation of leukemic blasts in AML1 to AML5 subtypes, induce the differentiation of AML cell lines, inhibit their proliferation and, in some cases, induce their apoptosis (12). Charrad showed that A3D8 and/or H90 induced terminal differentiation of THP-1, HL-60, NB4 and KG1a cell lines and strongly inhibited their proliferation (13). Johnsson analyzed gene expression profiling in several cell lines (K562 leukemia, MCF-7 breast cancer and S1 colon cancer) with acquired resistance against five cytostatic drugs (14). Using cDNA microarray, it was reported that the expression of CD44 altered in the cell lines. Artus reported that CD44 ligation triggered a novel caspase-independent cell death pathway *via* calpain-dependent apoptosis-inducing factor release in erythroleukemic HEL cells (15). These results were of great theoretical value in the development of CD44-targeted differentiation therapy. On the other hand, hereby we aimed to detect whether modulation of CD44 expression partly contributes to the mechanism of ATRA or HMBA on HL-60 cells.

In the present study, the expression of CD44 down-regulated by exposure to ATRA or HMBA, which was in agreement with the cell growth arrest and differentiation induction in HL-60 cells. It indicated that down-regulation of CD44 by ATRA or HMBA maybe play an important role in differentiation of HL-60 cells. On the other hand, the abnormal regulation mechanism of cell cycle is closely

correlated with tumorigenesis. G1 cell cycle arrest and inhibition of cell proliferation is the early essential event of cell differentiation. The cyclin-dependent kinase inhibitor p27, p21 bind to cyclin-dependent kinase (CDK) or cyclin/CDK complexes and inhibit their activities, which was indicated by G1 phase arrest and negative regulation of cell cycle (16). The abnormal expression of p27 existed in all subtypes and cell lines of leukemia, and down-regulation of p27 correlated with poor prognosis (17). Gadhoum reported that CD44 ligation stabilized p27 protein, resulting in increased association with cyclin E/CDK2 complexes and inhibition of their kinase activity in NB4 cells (18). Using a p27 antisense vector, they provided direct evidence that p27 was the main mediator of cell growth arrest by CD44. CD44 ligation also led to p27 accumulation in THP-1, KG1a, and HL-60 cell lines and in primary leukemic cells, suggesting that the process was general in AML and that CD44 ligation was an efficient means to increase p27 expression in AML cells. Our study further showed that p27 and p21 mRNAs were up-regulated and had an obvious negative correlation with CD44 expression, while cyclin E was down-regulated and had a positive correlation with CD44 in HL-60 cells induced by ATRA or HMBA. In conclusion, down-regulation of CD44 by ATRA or HMBA cooperates with cell cycle modulators in induction of differentiation.

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