

Brief report

All-*trans*-retinoic acid induces CD52 expression in acute promyelocytic leukemia

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It is well known that all-*trans*-retinoic acid (ATRA) can induce myeloid cell differentiation in acute promyelocytic leukemia (APL) cells. In this study, we found that ATRA treatment of the APL cell line NB4 induced the expression of CD52, both at transcriptional and translational levels. CD52 is a 21- to 28-kDa nonmodulating cell surface glycosylphosphatidylinositol-linked glycoprotein expressed on lympho-

cytes and monocytes, but not in human myeloid cells. The ATRA-dependent induction of CD52 expression was not observed in non-promyelocytic leukemia cell lines such as K562, U937, and HL-60, suggesting that induction of CD52 by ATRA may be specific to leukemic cells that express promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) or are at the promyelocytic stage of myeloid

development. Antibodies against CD52 are used therapeutically against lymphocytes in certain leukemias and in patients undergoing transplantation. An ATRA-induced high level of CD52 expression might potentially serve as a novel therapeutic target in treatment of APL. (Blood. 2003;101:1977-1980)

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Introduction

Acute promyelocytic leukemia (APL) accounts for more than 10% of all acute myeloid leukemia cases. It is characterized by the unique chromosomal translocation t(15;17) that results in the formation of the chimeric promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) oncogene.¹ Expression of this oncogene leads to blocking the differentiation of bone marrow cells and arresting cells at the promyelocytic stage of myeloid development. The APL blasts are known to be exquisitely sensitive to all-*trans*-retinoic acid (ATRA), a physiologically active derivative of retinoic acid, to undergo granulocytic differentiation.²

The APL cell line, NB4, was derived from the leukemia cells of an APL patient with the characteristic chromosomal translocation t(15;17) and expresses the PML-RAR α long isoform.³ Numerous studies have used this cell line to study ATRA-induced cell differentiation and signaling pathways.^{4,5} To determine gene expression in ATRA-induced APL differentiation, in a separate study (L.Y. et al, manuscript in preparation) we analyzed NB4 global gene expression using cDNA microarray technology. One of the genes expressed at a significantly higher level in ATRA-treated NB4 cells was the CD52 gene, which increased its expression 1.77-, 3.06-, 3.88-, 4.51-, and 6.49-fold at 12, 24, 48, 72, and 96 hours of ATRA exposure, respectively.

CD52 is a 21- to 28-kDa nonmodulating cell surface glycosylphosphatidylinositol (GPI)-linked glycoprotein.⁶ It is abundantly expressed on most normal and malignant T and B lymphocytes,⁷ monocytes,⁸ macrophages,⁹ and eosinophils.¹⁰ The other known site of expression of CD52 is the male genital tract where it has been found in seminal vesicles, seminal plasma, epididymal cells, and mature sperm.¹¹ The protein core of lymphocyte and sperm CD52 is identical and the product of a single copy gene located in chromosome 1.¹² However, the glycan chain and

GPI-anchor structure are different.¹³ The function of CD52 is unknown. A humanized anti-CD52 monoclonal antibody (alemtuzumab) has been used as a therapeutic agent in a number of lymphoproliferative diseases¹⁴⁻¹⁶ and in transplantation.¹⁷ In this study, we report that CD52 antigen was inducible by ATRA in the APL cell line NB4 in a time-dependent manner, and that ATRA-dependent induction of CD52 expression occurs in NB4 cells, but not in K562, U937, and HL-60 leukemia cells, suggesting that induction of CD52 by ATRA may be specific for leukemia cells that express PML-RAR α or at the promyelocytic stage of myeloid development.

Study design

Cell lines

NB4 cells were a generous gift from Dr James R. Downing (St Jude Children's Research Hospital, Memphis, TN). The other leukemic cell lines (K562, U937, and HL-60) were purchased from American Type Culture Collection (Manassas, VA) and cultured under the same condition as NB4 cells. They were cultured in RPMI 1640 medium plus 10% fetal calf serum. Cells (1×10^6) were treated with or without 1 μ M ATRA (Sigma, St Louis, MO) for 12, 24, 48, 72, and 96 hours at 37°C in a 5% CO₂ incubator in darkness. All experiments were repeated 3 times.

Assessment of cell differentiation

ATRA-induced NB4 cell differentiation was evaluated using cell morphology, expression of cell surface antigens related to granulocytic differentiation and maturation such as CD11b and CD18 integrins¹⁸ by flow cytometry (see "Flow cytometry analysis"), and the nitroblue tetrazolium (NBT) reduction test.¹⁹

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RT-PCR

For reverse transcription–polymerase chain reaction (RT-PCR) studies, total RNA was isolated from NB4 cells treated with or without ATRA. The cDNA was synthesized in a 20- μ L reaction mixture containing 5 μ g RNA, 5 mM deoxyribonucleoside phosphate (dNTP), 0.1 μ g random primers, and 200 U reverse transcriptase, and the mixture was incubated at 42°C for 1 hour. The cDNA was amplified for 30 cycles with denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute. The primers used for CD52 amplification were forward 5'-GCCACGAAGATCCTACCAAA-3' and reverse 5'-GCTTGGCCCCTA-CATCATTA-3' (385 bp). Amplified products were sequenced and confirmed by comparison with sequence data from GenBank.

Western blotting analysis

Cell lysates were separated on 4% to 20% gradient sodium dodecyl sulfate–polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a semidry electroblot chamber. The membrane was blocked overnight with 5% dry milk in Tris (tris(hydroxymethyl)aminomethane)–buffered saline and then incubated with anti-CD52 antibody (Serotec, Raleigh, NC) for 1 hour at room temperature. The binding of primary antibody was detected using peroxidase-coupled second antibody (Sigma). Bound peroxidase was detected by the Super Signal Substrate (Pierce, Rockford, IL).

Flow cytometry analysis

ATRA-treated cells (1×10^6) were stained with CD18–fluorescein isothiocyanate (FITC; Becton Dickinson Biosciences, San Jose, CA), CD11b–phycoerythrin (PE), and CD52–FITC antibodies (both from Serotec) following the manufacturer's instructions. Multicolor analysis of cell surface CD52, CD18, and CD11b molecules was performed by a FACScan flow cytometer and data were analyzed with FCS Express 2 software (De-Novo Software, Thornhill, ON, Canada). Controls consisted of FITC- and PE-conjugated isotype-matched immunoglobulin. Samples were analyzed in triplicate. For every sample 3×10^4 cells were acquired.

Results and discussion

ATRA-induced NB4 cell differentiation

To confirm ATRA-induced NB4 cell differentiation in our system, the cells were treated with 1 μ M ATRA for 24, 48, 72, and 96 hours, and cell differentiation was evaluated by cell morphology changes, CD11b and CD18 expression, and the NBT reduction test. There was no obvious morphologic change in the NB4 cells at the end of 24 hours of ATRA treatment. However, after 24 hours of ATRA exposure, the ratio of cytoplasm to nucleus increased, chromatin condensation occurred gradually, and sequential maturation of myeloid cells was observed with progression to band forms and some segmented granulocytes (data not shown). In addition, the number of NBT⁺ cells paralleled the changes of cell morphology (data not shown). Furthermore, the cell surface differentiation markers, CD11b and CD18, began to emerge at 24 hours and reached maximal expression (~95%) 48 to 96 hours following ATRA treatment (see "Cell specificity of CD52 expression induction by ATRA").

Gene expression of CD52 antigen in leukemic cells

To confirm the results obtained from microarray studies, the kinetics of *CD52* gene expression were studied using RT-PCR assays. As shown in Figure 1A, there was no expression of *CD52* gene in untreated NB4 cells, but rapid expression induction was observed in ATRA-treated NB4 cells. The level of expression of *CD52* message increased proportionally to the time of ATRA

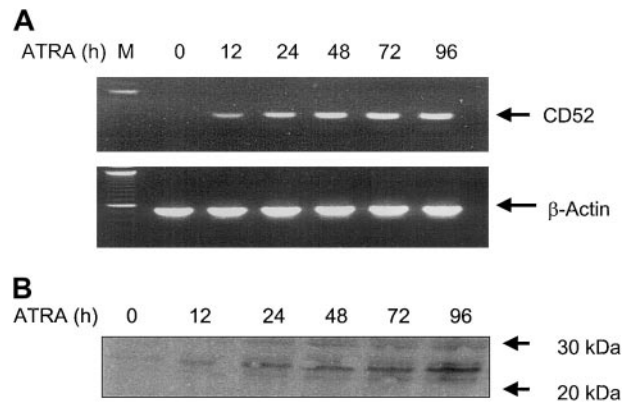


Figure 1. Induction of CD52 expression by ATRA in NB4 cells. (A) RT-PCR analysis of CD52 mRNA and (B) Western blotting analysis of CD52 protein induction in NB4 cells treated with ATRA at different time points. CD52 mRNA and protein were not detected in NB4 cells untreated with ATRA at 0 hours. Expression of β -actin confirmed similar loading in each lane.

exposure. Additionally, Western blotting analysis (Figure 1B) supported the direct relationship between CD52 expression and ATRA treatment of APL cells. The multiple protein bands of 21 to 28 kDa, which are characteristic of CD52 protein,²⁰ were detected only in ATRA-treated cells.

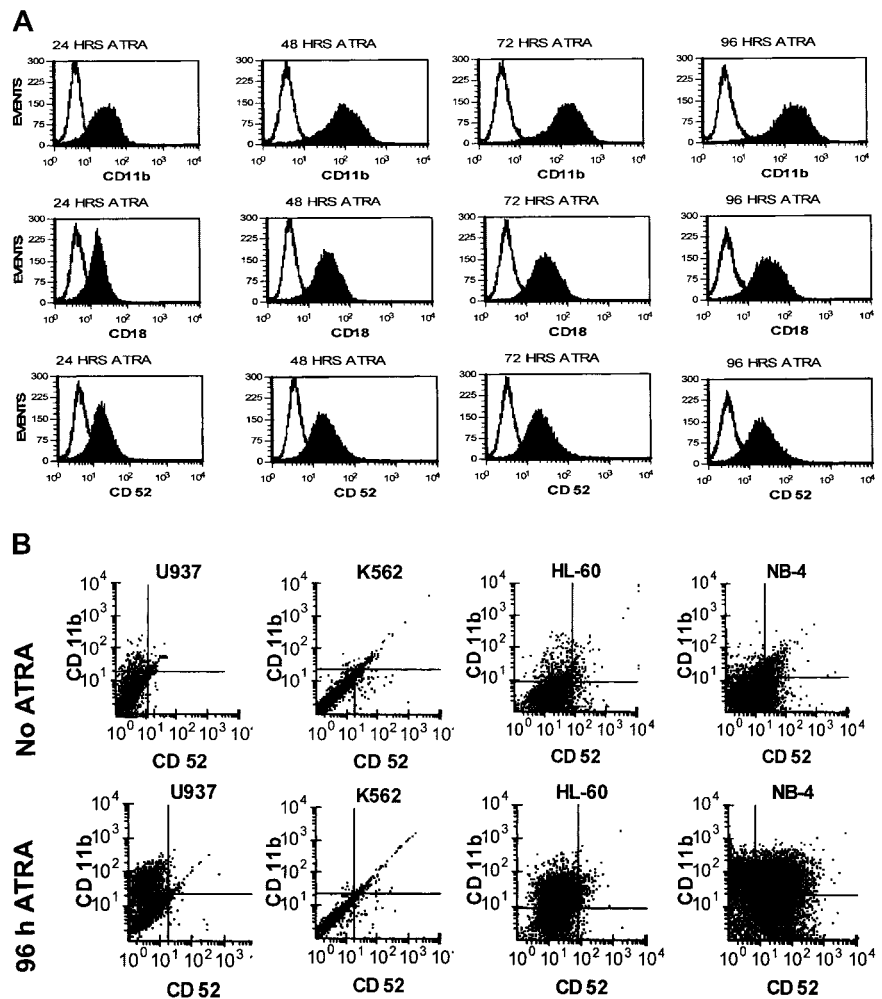
Cell surface expression of CD52 in ATRA-treated NB4 cells

To confirm the presence of the CD52 molecule on the cell surface, we examined its membrane expression using flow cytometric analysis. Figure 2A shows CD52 protein expression on ATRA-treated leukemic cells. Untreated NB4 cells did not express CD52. After treatment with ATRA, CD52 protein was expressed in a time-dependent manner (Figure 2A). It appeared at 24 hours and reached maximal expression (~90%) 48 to 96 hours following ATRA treatment. As ATRA induced cell differentiation, CD11b and CD18, markers of granulocytic differentiation,² were also up-regulated, paralleling the CD52 expression (Figure 2A). The analysis of bivariate plots showed that although at 96 hours of ATRA exposure approximately 80% of the cell population coexpressed CD11b and CD52, approximately 5% of the cells expressed only CD52, whereas approximately 10% cells expressed only CD11b (Figure 2B). These results indicate that up-regulation of CD52 and CD11b might be mediated by different pathways.

Cell specificity of CD52 expression induction by ATRA

To determine the cell specificity of ATRA-induced CD52 expression, we tested other leukemic cell lines using the same conditions as with the NB4 cells (Figure 2B). ATRA did not induce either CD52 or CD11b expression in the erythroblastic leukemia line K562. In the monocytic leukemia cell line U937, ATRA only slightly increased the expression of CD11b, but not CD52. Interestingly, CD52 was also not up-regulated in ATRA-treated HL-60 cells, a human myeloblastic leukemia lacking t(15;17) (Figure 2B). Our results thus suggest that the induction of expression of the *CD52* gene by ATRA may be specific for cells with the *PML-RAR α* gene or arrested at the promyelocytic stage of myeloid development. Of interest, previous studies have demonstrated that both HL-60 and NB4 cells could differentiate into neutrophils in the presence of ATRA.² Unlike NB4 cells, however, ATRA induced CD11b but no CD52 expression in HL-60

Figure 2. Time-dependent induction of CD52, CD11b, and CD18 expression in ATRA-treated NB4 cells and cell specificity of CD52 expression. (A) Flow cytometric analysis of CD11b, CD18, and CD52 surface expression in NB4 cells during treatment with ATRA at 24, 48, 72, and 96 hours. The kinetics of CD11b, CD18, and CD52 expression induced by ATRA was similar. Open and filled histograms represent untreated and treated APL cells by ATRA, respectively. (B) Expression of CD11b and CD52 on U937, K562, HL-60, and NB4 cells treated with or without ATRA for 96 hours. Induction of CD11b and CD52 expression in each type of cell was analyzed on 3×10^4 cells/sample. Up-regulation of CD52 by ATRA occurred only in NB4 cells.



(Figure 2B), providing evidence that mature granulocytes derived from these 2 cell lines following exposure to ATRA may be different.

Because CD52 is an excellent target for complement-mediated cell lysis and antibody-dependent cellular cytotoxicity, the anti-CD52 monoclonal antibody alemtuzumab has been used clinically to treat lymphoproliferative disorders and to deplete lymphocytes from organ transplants.¹⁴⁻¹⁷ Alemtuzumab has no effect on myeloid leukemia, most likely because myeloid cells express little, if any, CD52.²¹ Also, previous studies showed that normal neutrophils do

not express CD52 antigen¹⁰ and that alemtuzumab does not affect the biologic function of normal neutrophils.²² Our preliminary observations suggest that ATRA treatment of a patient's acute promyelocytic leukemic cells in vitro induced CD52 expression on the leukemic cells. However, this interesting phenomenon needs further investigation. Provided that our data on CD52 expression by NB4 cells after ATRA-induction can be confirmed in leukemic promyelocytes from actual patients, this antigen may become a new target for antibody therapy in patients with APL.

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