Laminin Promotes Differentiation of NB4 Promyelocytic Leukemia Cells With All-trans Retinoic Acid

By Pamela S. Becker, Zhifang Li, Tamara Potselueva, Joseph A. Madri, Peter E. Newburger, and Nancy Berliner

The promyelocytic leukemia cell line, NB4, was derived from a patient with acute promyelocytic leukemia and carries the t(15;17) translocation resulting in the joining of the PML oncogene to the gene encoding the retinoic acid receptor α (RARα). PML is part of a macromolecular nuclear organellae believed to function as a promoter-specific transcriptional repressor. The PML-RARα fusion protein acts as a dominant negative inhibitor of PML and disrupts the normal nuclear structures that contain PML.

Treatment with all-trans retinoic acid (ATRA) results in commitment and maturation along the myeloid lineage in nearly all patients who carry molecular evidence of this translocation. Moreover, RA restores normal PML distribution and function to native cells from patients with acute promyelocytic leukemia or to the cell line, NB4.

Early in vitro studies suggested that retinoids could have a therapeutic benefit in leukemia. For example, RA was shown to inhibit growth of several myeloid leukemia cell lines, including KG-1 acute myeloblastic leukemia and HL-60 acute promyelocytic leukemia cell lines as well as the in vitro clonal growth of leukemia cells from patients with acute myeloid leukemia (AML). Further investigation showed that RA induced morphologic changes consistent with terminal maturation of both HL-60 cells and cells derived from some patients with AML.

ATRA binds to nuclear RAR. These receptors form heterodimers with the retinoid X receptors (RXR), resulting in the RAR/RXR dimer capable of transactivating responsive genes. Synthetic ligands have been developed that can bind selectively to the RAR/RXR heterodimer or the RXR/RXR homodimer, enabling the receptors critical for a particular function to be identified. Using these ligands with myeloid cell lines, it was determined that the differentiating effects of the retinoids were mediated through the RAR/RXR heterodimers.

Two NB4 cell lines have been isolated from a patient in relapse that do not mature in response to ATRA. These have been designated NB4-RA R1 and R2. The R1 resistant subline upregulates CD11c/CD18 in response to ATRA and does exhibit enhanced growth in low-dose ATRA (15 to 150 nmol/L), although maturation is not achieved. The R2-resistant subline exhibits none of these responses to ATRA.

Elevation of adenosine-3′,5′-cyclic monophosphate (cAMP) did not express secondary granule proteins such as lactoferrin or neutrophil collagenase. In addition, growth on laminin abolished cell proliferation in the presence of ionomycin. Growth on laminin and/or with ATRA induced new expression of α5 integrin, a laminin receptor, as assessed by reverse transcription-polymerase chain reaction. Different conditions of growth (laminin or differentiation agent) resulted in specific patterns of expression of the α5a and α5b isoforms. Treatment with ATRA also resulted in the acquisition of high-level surface expression of α5 integrin, as assessed by flow cytometry. Thus, treatment of NB4 promyelocytic leukemia cells with ATRA induced expression of α5 integrin (a laminin receptor α-chain) and enabled more advanced maturation when the cells were grown on the extracellular matrix component, laminin, compared with tissue culture plastic.

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cell line was grown and maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine 2 mM/L (LG), penicillin 100 μg/mL, and streptomycin 100 U/mL (PS). HL-60 cells were obtained from the American Type Tissue Collection (Rockville, MD) and maintained in RPMI-1640 medium supplemented with 20% heat-inactivated FCS, LG, and PS. Umbilical cord blood mononuclear cells were obtained by centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The umbilical cord blood mononuclear cells were incubated for 20 hours in the same tissue culture medium as the NB4 cell line with or without interleukin-3 (IL-3) (R & D Systems, Minneapolis, MN). Bacteriologic Petri dishes (35 mm) were coated with purified human extracellular matrix proteins (originally, Telios, then GIBCO), collagen types I, III, or IV, fibronectin, or laminin (rat or murine). All coating was performed by the method of Basson et al.18 Laminin purified from EHS sarcoma cells as described18 was also used to confirm the data obtained from the commercial preparations. Uncoated tissue culture plastic was used for comparison. Cells were placed on extracellular matrix components for 12 to 16 hours before the addition of ATRA (Sigma, St Louis, MO) at a final concentration of 5 μmol/L. Cells were then observed for 72 to 96 hours. For histology, cytospin smears were prepared and stained with Wright-Giemsa stain. 12-O-Tetradecanoylphorbol-13-acetate (TPA) (Sigma) was used at a concentration of 10 ng/mL. Cells remaining adherent to the plate after three washes comprise those designated “adherent.”

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the NB4 cells by guanidine isothiocyanate extraction and centrifugation on cesium chloride gradients.19 The RNA was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo dT priming. α-Chain–specific primers20 were used as follows: α1 sense: position 1817-1835, α1 antisense: position 2590-2607; α2 sense: position 2082-2106, α2 antisense: position 3275-3299; α3 sense: position 516-535, α3 antisense: position 1666-1691, α4 antisense: position 2988-2924; α5 sense: position 1657-1681, α5 antisense: position 2900-2924; α6 sense: position 2159-2182, α6 antisense: position 2959-2983. The α1 primers were derived from rat sequence; all others were human sequence. The sizes of the expected PCR products were 790, 1,217, 1,189, 1,047, 1,267, 824 bp for α1 through α6, respectively. The primers that demonstrate the alternatively spliced isoforms, αα and αβ are the following: sense: 5’-CTAACGGAGTCTGCAACTC-3’ (position 2656-2675), antisense: 5’-ACTCTGAATCTACTCAGC-3’ (position 3480-3499). The conditions for PCR were as follows: 94°C denaturation for 1.5 minutes, 55°C annealing for 2 minutes, and 72°C extension for 3 minutes, for 30 cycles. RNA from NB4 cells resistant to ATRA (sublines R1 and R2) was kindly provided by Dr M. Lanotte. The RNA was isolated from cells exposed to ATRA for 0, 24, and 72 hours. In addition, RNA was provided from cells “primed” in ATRA for 3 weeks, then grown in the presence of 100 μmol/L 8-CPT-cAMP for 24 or 72 hours.

Flow cytometry. The NB4 cells grown under the different conditions of differentiation and extracellular matrix were labeled with murine antihuman α3 or α6 (GIBCO) at a dilution of 1:500, or rat-antihuman α6 integrin antibody (CLB, Amsterdam, The Netherlands) at a dilution of 1:100 for 30 minutes on ice. After three washes, the cells were then suspended in the secondary antibody, phycoerythrin.

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**Table 1. Expression of β, Integrin Class α-Chains by NB4 Cells (RT-PCR)**

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Abbreviations: PI, plastic; Ln, laminin; Adh, adherent; TPA, tetradecanoylphorbol acetate; X, detection of PCR product.
anti-IgG for 30 minutes on ice. Controls included cells stained with secondary antibody alone and cells stained with isotype control antibody (IgG2A) and secondary phycoerythrin anti-IgG. After three washes in phosphate-buffered saline (PBS)/1% fetal bovine serum/0.1% azide, the cells were washed in PBS and fixed in 1.5% paraformaldehyde, then analyzed by flow cytometry. For comparison, umbilical cord blood mononuclear cells were examined by two-color flow cytometry for coincident expression of CD34 and integrins. The anti-CD34 antibody was 12.8 (IgM), kindly provided by Dr I. Bernstein (Fred Hutchinson Cancer Research Center, Seattle, WA). The antibody directed against \( \alpha_6 \) integrin was identical to that described above. Secondary fluorescein isothiocyanate (FITC)-anti IgM and phycoerythrin-anti IgG were used to stain the cells for flow cytometry as described above. The histograms and statistics were obtained using PC-Lysis software (Becton Dickinson, San Jose, CA).

Northern blot analysis for secondary granule proteins. RNA was prepared from NB4 cells as described above. Control RNA from peripheral blood of patients with chronic myelogenous leukemia was prepared as previously described. Ten micrograms of total RNA from cells grown with or without induction of differentiation, on plastic or on laminin, was loaded on each lane. RNA was also isolated from cells adherent to laminin. The following cDNA probes were used to hybridize the blots: a 2.4-kb full-length lactofenin cDNA, a 2.4-kb fragment of human neutrophil collagenase, a 330-bp fragment of human neutrophil gelatinase, and a \( \gamma \)-actin probe as a control.

Nitroblue tetrazolium assay. Superoxide generation was assed qualitatively by observing the reduction of soluble yellow nitroblue tetrazolium (NBT) (Sigma) to insoluble blue formazan in the presence of PMA (1 \( \mu \text{g/mL} \)) as described. The cells were counterstained with safranin.

Discontinuous cytochrome C reduction assay. Superoxide generation was quantitatively assessed using a discontinuous cytochrome C reduction spectrophotometric assay, as described. Cells were stimulated with phorbol myristate acetate 1 \( \mu \text{g/mL} \). The samples contained ferricytochrome C (50 nmol), cells (2.5 \( \times \) 10⁶), and PMA. The analysis was performed at 15 minutes. Reference samples also contained superoxide dismutase (0.01 mg/mL).

Ionomycin treatment. Cells, 4 \( \times \) 10⁶, were placed in 100-mm dishes, uncoated or coated with laminin. Ionomycin was added to a final concentration of 100 \( \mu \text{mol/L} \), and the cells were examined at 72 hours by Wright-Giemsa staining of cytospin smears and reduction of NBT.

RESULTS

Morphology of NB4 cells induced with ATRA on laminin. Wright-Giemsa staining of cytofilm smears of NB4 cells induced with ATRA on plastic- or laminin-coated plates showed morphologic evidence for acquisition of a more differentiated phenotype for cells induced with ATRA on laminin compared to plastic (Fig 1). For cells induced on laminin, the chromatin pattern appeared more condensed, with indentation resembling metamyelocytes or narrow in the pattern of bands (see ref 29 for histology of normal blood and bone marrow cells). In addition, multiple cells were observed with segmented nuclei, including bilobed, trilobed (see arrow in Fig 1F), or even multilobed forms (see arrow in Fig 1E). Such nuclei with multiple segments were never observed for cells grown on tissue culture plastic with ATRA in the absence of laminin (Fig 1C), although the chromatin pattern did appear to be more condensed in those cells. The \( \beta_1 \) activating antibody kindly provided by Dr N. Kovach (Haborview Medical Center, Seattle, WA) did not reproduce the morphologic change (data not shown); nor did antibodies to \( \alpha_2 \) through \( \alpha_6 \). With more prolonged incubation, some
cells acquired a spindle shape and became adherent. These changes did not occur on collagen types I, III, IV, or fibronectin, although some adherence to fibronectin was observed.

**Expression of β integrin class α-chains by NB4 cells: RT-PCR and flow cytometry.** As assessed by RT-PCR using oligonucleotide primers specific to the various α-chains, the β1 class integrins were variably expressed, depending on differentiation conditions and Ln, as shown in Table 1. By RT-PCR, α6 was expressed under all conditions of differentiation and extracellular matrix. However, the surface expression of αe modulated with exposure to ATRA and for laminin, as assessed by flow cytometry (Fig 2; Table 2). Nearly the entire cell population became positive for α6 after ATRA treatment on plastic (85%) or laminin (93%). There was moderately increased expression of α6 with laminin alone (41%), compared with growth on tissue culture plastic (22%) or the isotype control antibody (18%). For comparison, umbilical cord blood CD34+ cells also express α6, and the expression did not change after 20 hours’ exposure of the cells to IL-3 (Fig 3). The percent of cells exhibiting coincident staining for CD34 and α6 was 37%, and 35% after 20-hour exposure to IL-3, compared with 4% for control cells lacking primary antibodies. In contrast, the majority of cells did not express α2 by flow cytometry analysis, and there was a very small population that became positive after ATRA induction. Flow cytometry analysis for α2 showed a very minor population of positive cells that diminished with ATRA on plastic, and increased slightly with ATRA on laminin. Thus, there was little effect of ATRA on expression of the other integrin laminin receptor α chains, α2 and α5.

**Expression of secondary granule proteins by Northern blot analysis.** We have previously reported that ATRA-induced NB4 cells do not express neutrophil-specific granule proteins. Despite improved morphologic maturation on laminin, induced cells still failed to express lactoferrin or neutrophil collagenase, as assessed by Northern blot analysis (Fig 4). Gelatinase was expressed in cells grown on plastic, and this property was lost with ATRA induction, as previously shown.16

**Superoxide generation by NB4 cells induced to differentiate with ATRA.** All cells induced with ATRA acquired the ability to reduce NBT (Fig 5), regardless of whether the cells were grown on plastic or laminin. Uninduced cells did not reduce NBT, whether grown on plastic or laminin. To quantify the findings of the NBT assay, a cytochrome C reduction assay was performed. The cells induced with ATRA on plastic generated 0.53 ± 0.10 nmol superoxide/min/10⁶ cells, and those induced with ATRA on laminin generated 0.47 ± 0.04 nmol superoxide/min/10⁶ cells. Similar to the result of the NBT reduction assay, cytochrome C reduction was not detectable for uninduced cells on plastic or laminin.

**Laminin regulates proliferation induced by ionomycin calcium ionophore.** Ionomycin, a calcium ionophore, has been shown by others to induce NADPH oxidase activity in mature neutrophils31 and to stimulate T-lymphoblast binding to extracellular matrix via β1 integrins.32 Another, calcium ionophore, A23187, was shown by another group to increase the adherence of mature neutrophils to extracellular matrix proteins, fibronectin and laminin.33 Therefore, we examined the effect of ionomycin on the differentiation of NB4 cells on laminin. Ionomycin did not induce differentiation on plastic or laminin (data not shown), and the NBT reduction assay was negative. However, there was regulation of proliferation on laminin, in that the cell number on laminin with ionomycin only increased 1.2-fold, compared to 23-fold on tissue culture plastic with ionomycin and 20-fold without ionomycin.

**Expression of α6A versus α6B isoforms by NB4 cells and RA-resistant sublines.** Regarding the expression of α6 isoforms (Fig 6), there was equal expression of α6A and α6B for cells grown on plastic, with slightly more α6A after ATRA induction on plastic or without induction on laminin. The sizes were identical to those reported for the human teratocarcinoma cell line,32 844 and 714 bp for A and B, respectively. The cells induced with ATRA on laminin exhibited equivalent expression of the two isoforms, with a predominance in the 6A type for the adherent cells. For both adherent and nonadherent NB4 cells induced with TPA, there was only expression of the 6A type. NB4 cell lines resistant to RA (sublines R1 and R235) were also studied for expression of α6 isoforms at the mRNA level by RT-PCR using the primers for α6A and α6B. RNA was kindly provided by Dr M. Lanotte, but the cells were not yet available, so that the effect of ATRA plus laminin could not be ascertained, nor could the morphology of resistant cells grown on laminin be studied. Expression of α6 was observed for resistant cells.
LAMININ PROMOTES MATURATION OF NB4 CELLS

A

B

C

D

Fig 4. Northern blot analysis for secondary granule proteins. 32P-Labeled cDNA probes for the following were used to hybridize the blots: (A) lactoferrin, (B) neutrophil collagenase, (C) gelatinase, (D) γ-actin control. 1, Ln; 2, Ln-adh; 3, Ln-ATRA; 4, Ln-ATRA-adh; 5, plastic; 6, PI-ATRA; 7, CML. Adh, adherent cells. There is no expression of lactoferrin or neutrophil-specific collagenase under any conditions of growth.

by RT-PCR after 72 hours of exposure to ATRA (barely detectable or not seen before this time), and this expression was indistinguishable from control NB4 cells that responded to ATRA, with the amount of 6A slightly greater than 6B. Interestingly, there was similar expression by resistant R1 cells primed with ATRA, which was abolished by the addition of 8-CPT-cAMP, an agent that causes activation of the cAMP-dependent protein kinase and enables override of the RA resistance. The finding that the cell lines resistant to RA in terms of maturation induced α6 expression with ATRA, and that the agent which overcomes resistance abolished expression, suggests that the mechanisms of action and signal transduction mediated by ATRA for myeloid maturation are distinct from those involved with induction of α6 expression.

DISCUSSION

This study was undertaken to determine whether extracellular matrix components could overcome the defect in differentiation observed in NB4 cells as shown by lack of expression of secondary granule proteins. Other investigators had described that growth of murine erythroleukemia cells on fibronectin promoted maturation to the reticulocyte stage when the cells were induced with dimethyl sulfoxide (DMSO). We found that growth of NB4 cells on the extracellular matrix component, laminin, induced a profound morphologic change to a more mature phenotype, including fully segmented forms, compared with growth on uncoated tissue-culture plastic.

We had previously reported that NB4 cells treated with ATRA failed to express secondary granule proteins. We now report that despite improved morphologic maturation on laminin, we find that ATRA-induced cells still do not express lactoferrin or neutrophil collagenase mRNA by Northern blot analysis. In addition, NB4 cells treated with ATRA on either plastic or Ln acquired the ability to reduce nitroblue tetrazolium, indicating that the necessary superoxide generating enzymes had been acquired. There was no significant difference in the quantitative assay of superoxide generation between plastic and laminin.

The observed effects on morphologic appearance are likely to be the result of ligation of integrin proteins. Integrin expression modulates with hematopoietic differentiation in normal progenitors, as well as keratinocyte and hepatocyte differentiation. Here we find that there is extensive modulation of integrin expression of the β1 class in NB4 cells, depending on conditions of growth on laminin and or induction of differentiation with ATRA or TPA. Interestingly, the laminin receptor, α6, appears to be expressed at the mRNA level in these cells under all growth conditions, as assessed by RT-PCR, and it is laminin that appears to have an effect on the morphologic phenotype.

We also observe α6 integrin expression by HL60 cells by RT-PCR with or without induction with DMSO or TPA (data not shown), by umbilical cord blood CD34+ cells, and (Fig 3) by murine hematopoietic stem cell populations obtained by lineage depletion and fluorescence activated cell sorting for Sca+ or Hoechst 33342/Rhodamine 123+ cells. Moreover, flow cytometric analysis of β1 integrin expression revealed that ATRA induction was associated with a uniform upregulation of α6 surface expression in the entire cell population. In addition, there appeared to be differences in the expression of α6 isoforms under different conditions of extracellular matrix and inducers of differentiation, with a preponderance of the 6A isoform for cells induced with ATRA or TPA. In comparison, embryonal stem cells only express the 6B isoform; coexpression with the 6A isoform is seen only after differentiation is induced with RA. Although both α2 and α3 can serve as laminin receptors as well, their expression was not modulated by treatment with ATRA.

ATRA induces expression of α6 integrin isoforms, with 6A in greater quantity than 6B, even in cells that are resistant to its maturational effects, the R1 and R2 sublines. Similarly,
Fig 5. NBT reduction by NB4 cells, with or without induction of differentiation, on plastic or laminin. (A) Plastic, (B) Ln, (C) plastic-ATRA, (D) Ln-ATRA. In the absence of ATRA, the cells are negative for superoxide production. NB4 cells exposed to ATRA acquire the ability to reduce NBT.

the R1 subline increases expression of the integrin CD11c/CD18 in response to ATRA, even though it does not mature. However, when the cAMP agonist 8-CPT-cAMP is used in R1 cells previously primed with ATRA, the α6 expression is abolished. This latter finding suggests that the mechanism of action of ATRA leading to α6 expression is quite distinct from that leading to maturation, and is actually antagonized by cAMP elevation. Therefore, it is likely that laminin is exerting its effect on maturation by a distinct mode of action related to signal transduction mediated through the integrin pathway.

Thus, the NB4 acute promyelocytic leukemia cell line exhibits acquisition of surface expression of α6 integrin with ATRA, and growth on the ligand for α6β1 integrin, laminin, permits more extensive maturation in the presence of ATRA, with full segmentation of the nucleus achieved.

ACKNOWLEDGMENT

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