NB4 Cells Show Bilineage Potential and an Aberrant Pattern of Neutrophil Secondary Granule Protein Gene Expression

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**NB4 is an acute promyelocytic leukemia cell line that has been shown to be inducible to terminal neutrophil maturation with all-trans retinoic acid (ATRA). HL60 cells are differentially inducible with 12-O-tetradecanoylphorbol-13-acetate (TPA) or dimethyl sulfoxide (DMSO) to monocytes or granulocytes, respectively. HL60 cells induced with DMSO undergo defective neutrophil maturation, manifested by a coordinate failure of secondary granule protein gene expression. We observed a similar defect in granulocytic maturation in ATRA-induced NB4 cells. In addition, because normal promyelocytes are known to have bilineage potential, we have investigated differentiation along monocytic lines induced with TPA. We observed a striking phenotypic change along monocytoid/macrophage lines with TPA induction.**

**During normal granulocytic differentiation, the myeloblast becomes committed to maturation along either the neutrophil or the monocyte/macrophage pathway.**

**NB4 is a relatively newly described promyelocytic cell line established from a patient with acute promyelocytic leukemia (APL). Unlike HL60 cells, this cell line carries the specific cytogenetic abnormality, t(15;17), pathognomonic of M3 acute myelogenous leukemia. This nonrandom translocation creates a hybrid gene between the C-terminus of the RA receptor-α (RARα) and the amino-terminus of the promyelocytic leukemia (PML) locus, resulting in a PML-RARα fusion gene; the mRNA products of this fusion have been detected in patients with APL.**

**It has been hypothesized that the PML-RARα gene product represents a dominant-negative oncogene product that is capable of disrupting the expression of genes participating in the normal developmental program for granulocyte maturation.**

**RA, a potent morphogen, has been shown to play a role in granulocytic maturation. The administration of all-trans RA (ATRA) has been effectively used to achieve complete remission in patients with APL. Furthermore, Warrel et al have suggested that ATRA-induced remission is the result of differentiation of the leukemic cells rather than a consequence of cytoreduction.**

**Both NB4 and HL60 cells have been induced to undergo granulocytic maturation after RA induction. ATRA-induced NB4 cells have been proven to show a number of markers of the granulocytic lineage, such as the expression of CD15, the increased expression of CD11b and CD11c, and an enhanced respiratory burst as measured by the nitroblue tetrazolium reduction assay. However, the expression of neutrophil secondary granule along monocytoid/macrophage lines with TPA induction. Flow cytometry showed a TPA-induced increase in HLA-DR expression, and Northern blot analysis showed induction of expression of CD18, c-fos, and human neutrophil gelatinase (HNG). HNG is unique among the neutrophil secondary granule protein genes in that it is expressed in both the neutrophil and monocyte lineages. This again parallels our findings in TPA-induced HL60 cells, which retain the ability to express HNG. These findings confirm bilineage potential in NB4 cells. They also support the hypothesis of coordinate secondary granule protein gene expression and a defect in this control as part of the leukemic phenotype.**

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proteins, a hallmark of normal granulocyte maturation, has not been investigated in this cell line.

The objectives of this study were twofold. First, we wished to determine whether ATRA-induced maturation of NB4 cells results in the expression of secondary granules and their content proteins. We then undertook to determine whether the NB4 promyelocytes retain their bilineage potential and are capable of maturation along the monocytic/macrophage lineage after TPA induction.

MATERIALS AND METHODS

Cell culture and differentiation conditions. NB4 cells were the gift of M. Lanotte (INSERM, Paris, France). The cells lines were grown and maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 100 μg/mL penicillin and 100 U/mL of streptomycin. The cells were incubated at 37°C in a humidified air atmosphere supplemented with 5% CO2.

Cells were grown to a concentration of 1 x 10⁶/mL under the conditions described above and induced with 5 μmol/L ATRA (Sigma, St Louis, MO), 3,000 U/mL granulocyte colony-stimulating factor G-CSF (Neupogen; Amgen, Thousand Oaks, CA) or 10 ng/mL of TPA (Sigma, St Louis, MO). Cells were induced from 1 to 9 days and harvested for further analysis as previously described. Maturation was monitored by Wright staining. Adherent TPA-treated NB4 cells were fixed with methanol on the petri dish before Wright staining.

A Northern blot analysis. Total RNA was extracted from NB4 cells at various time points after induction as previously described. Control granulocyte RNA was obtained from peripheral blood neutrophils from a patient with chronic phase chronic myelogenous leukemia (CML) or from a patient with APL in remission after therapy. Blood was separated with MONO-POLY Ficoll-Hypaque resolving medium (Flow Laboratories, ICN Biomedicals Inc, Costa Mesa, CA) as per the manufacturer's instructions, and total RNA was prepared from the granulocyte layer. A total of 10 μg of each sample was electrophoresed in 1% agarose/formaldehyde gels, blotted onto nitrocellulose (Schleicher and Schuell, Keene, NH), and hybridized overnight in 50% formamide at 42°C to cDNA probes. The following cDNA probes were used in this study: a 2.4-kb full-length LF cDNA; a 2 kb cDNA fragment of myeloperoxidase (MPO; kindly provided by Dr Susan Weil, Thomas Jefferson University, Philadelphia, PA), a 2.4-kb fragment of HNC; a 330-bp cDNA fragment of HNG generated by polymerase chain reaction (PCR) as previously described; an 800-bp EcoRI fragment of CD18 (kindly provided by Dr Daniel Tenen, Beth Israel Hospital, Boston, MA); a 300-bp cDNA fragment of the monocytic-specific 72-kD gelatinase gene; a cDNA fragment of the third exon of the c-mye gene (kindly provided by Dr Philip Leder, Harvard University, Boston, MA), and the full-length cDNA clone for the c-fos gene.

Cell surface markers. NB4 cells were induced with ATRA and TPA as described above for 48 hours. Flow cytometry was performed on the cell suspensions after indirect immunofluorescence staining as previously described. Antigen-specific monoclonal antibodies (Becton Dickinson, Mountain View, CA) were incubated with fluorescein isothiocyanate-conjugated F(ab); goat antimouse IgG (Cappel, Malvern, PA) at a dilution of 1:100 at 4°C for 45 minutes. Cells were washed and resuspended in RPMI plus 1% paraformaldehyde. Cell-surface fluorescence was then determined on a Becton Dickinson FACScan.

Reverse transcription-PCR (RT-PCR). A total of 2 μg of total RNA obtained from both TPA-treated and untreated NB4 cells was subjected to the RT reaction using avian myeloblastosis virus (AMV)-RT (Boehringer Mannheim) in the presence of random hexamers (Boehringer Mannheim) by previously described methods. The resulting cDNA was then amplified in two separate PCR reactions, using pairs of oligonucleotides specific for HNG (92 kD) in one case and for the monocytic-specific 72-kD gelatinase in the other. The sequences for these oligonucleotides primers have been detailed previously. The PCR products were then electrophoresed in a 1% agarose gel, visualized after ethidium bromide staining, and photographed. As a control, RT-PCR was performed on a CML cDNA library, previously described.

RESULTS

Morphology of ATRA-induced NB4 cells. The morphology of Wright-stained NB4 cells after exposure to ATRA does not show strong evidence of maturation. Uninduced cells had the appearance of promyelocytes. Induced cells showed some of the characteristics of myelocytes, such as nuclear indentation; however, they remained large with prominent nucleoli (Fig 1A and B). However, in concordance with the observations of Lanotte et al., growth arrest was noted within 48 hours of ATRA induction, and the induced cells acquired nitroblue tetrazolium positivity (data not shown). NB4 cells exposed to 5 μmol/L ATRA for 3 days lacked visible secondary granules, as is evident in Fig 1B. Longer exposure (to 10 days) of these cells to varying concentrations (1 to 5 μmol/L) of ATRA yielded essentially the same result (data not shown).

Within 12 hours of TPA induction, the majority of NB4 cells were found to adhere to the plastic of the petri dish. Increased adhesiveness after TPA induction is a well-documented characteristic of monocytic cells. After 48 hours of TPA exposure, Wright staining of adherent NB4 cells showed a monocytic/macrophage-like morphology (Fig 1C).

Aberrant expression of secondary granule protein genes in ATRA-induced NB4 cells. NB4 cells were exposed to ATRA (5 μmol/L) for 1 to 4 days, and the isolated total RNA was subjected to Northern blot analysis. Figure 2a through c shows Northern blots of these RNA samples that have been consecutively probed for the presence of MPO (a primary granule protein); the secondary granule content proteins LF, HNG, HNC, and TC1; CD18, a β-integrin subunit present on the plasma membrane and on the secondary granule membrane; and c-myc, a nuclear oncogene.

Contrary to the findings of Lanotte et al., ATRA treatment of NB4 cells appeared to result in decreased expression of MPO so that, by day 3 of ATRA induction, no MPO transcript was detected (Fig 2a). This result is similar to that observed in HL60 cells, where MPO mRNA levels rapidly decrease on induction of terminal myeloid differentiation. No transcripts for three of the four secondary granule proteins, i.e., LF (Fig 2a), HNC, and TC1 (Fig 2b), were detected in total RNA isolated from uninduced and ATRA-induced
NB4 cells. The transcript for HNG, the fourth secondary granule protein, was observed in uninduced NB4 cells. However, a gradual decrease in the level of HNG mRNA was observed after 6-hour to 4-day ATRA exposure of the cells (Fig 2a). CD18 mRNA levels appear to increase in ATRA-treated NB4 cells (Fig 2c), a finding which parallels the previous report of upregulation of CD11b/CD18 mRNA observed during granulocyte or monocyte differentiation in HL60 cells.

The c-myc transcript is abundantly represented in uninduced NB4 cells, but on ATRA induction, its abundance appears to diminish (Fig 2b). HL60 cells, which have a 40-fold amplification of the c-myc gene and greatly amplified expression of the proto-oncogene, have a dramatic and rapid decrease in c-myc expression when the cells are induced to differentiate along the granulocytic pathway. By comparison, the rate at which the c-myc transcript decreases in NB4 cells on induction is slower and less dramatic than in HL60 cells. This may reflect the fact that HL60 cells undergo growth arrest and show phenotypic maturation in response to DMSO and RA much more quickly than do NB4 cells.

Of the four secondary granule proteins, none have been shown to be expressed in either uninduced or DMSO-treated HL60 cells at the RNA level. However, TPA-induced HL60 cells that are programmed to differentiate along the monocytic lineage do express HNG. The finding that uninduced and TPA-induced NB4 cells express HNG implies that these cells probably have a monocytic potential and prompted us to further investigate this possibility.

**TPA induction of NB4 cells.** NB4 cells were treated with TPA, as detailed in Materials and Methods, and incubated for a period of 4 days. Total RNA isolated daily from the treated cells was analyzed by Northern blot analysis. As shown in the time course in Fig 3a, TPA treatment of NB4 cells dramatically increases the level of HNG mRNA. The cells were also induced with ATRA, with G-CSF, and with a combination of these two inducing agents for up to 72 hours (Fig 3b). The level of the HNG transcript in G-CSF-treated cells remains virtually the same as in the uninduced cells. As shown in Figs 2a and 3b, 72-hour ATRA-treated NB4 cells express negligible levels of the HNG message. Furthermore, treatment of NB4 cells with both ATRA and either G-CSF or TPA greatly reduces the expression of HNG mRNA as compared with the transcript levels without ATRA (Fig 3b). This suppression of HNG expression may possibly be attributable to a previously reported negative regulatory effect caused by the interaction of a TPA-responsive element (TRE)-binding protein complex, activation protein-I (AP-1), and the RAR through a direct protein-protein interaction. The abundant expression of HNG in TPA-treated NB4 cells suggests that these cells are capable of being induced along monocytic lines after treatment with TPA.

Addition of G-CSF was unable to correct the defect in LF expression in ATRA-induced NB4 cells. As expected, no
Fig 2. Northern blot analysis of total RNA isolated from ATRA-induced NB4 cells is shown. Total RNA from control neutrophils (C), uninduced NB4 cells (0) and ATRA-induced NB4 cells (6 hours and 1, 2, 3, and 4 days) was isolated, electrophoresed on a formaldehyde agarose gel, and blotted onto a nitrocellulose filter. The filter was then probed consecutively with the following 32P-labeled cDNA fragments: MPO, LF, and HNG (2a). A second Northern blot using an aliquot of the same RNAs was probed with HNC, TC1, and c-myc (myc) (2b). A third blot using another aliquot of the same RNAs was probed with the human β2-integrin subunit, CD18 (2c). γ-Actin probe was used as a control for RNA loading. The 28S and 18S rRNA markers are indicated on the right.
LF transcripts were detected in NB4 cells induced toward monocytic differentiation. As previously observed, MPO expression was again reduced by ATRA induction; the transcript was also completely suppressed by treatment with TPA (Fig 3b).

c-fos expression in TPA-treated NB4 cells. Analysis of the HNG promoter has led to the identification of a pair of AP-1 recognition sites in the 5' flanking DNA. Therefore, we investigated whether the TPA response appeared to be mediated via the AP-1 complex by looking for an associated increase in c-fos expression. As shown in Fig 4, within 30 minutes of exposure of NB4 cells to TPA, the c-fos transcript became apparent. As expected, no c-fos message was observed in the uninduced or the ATRA-induced NB4 cells. A decrease in the level of c-fos transcript was observed in the TPA- plus ATRA-treated cells, suggesting again that ATRA may interfere directly with the TPA response.

TPA induction results in maturation along the monocytic lineage. It has been well documented that genes containing TPA-responsive elements (TREs or AP-1 binding site) are capable of being positively or negatively modulated in response to TPA induction. To confirm that TPA-treated NB4 cells were actually maturing along monocytic lines rather than displaying a nonspecific response to TPA, we examined the expression of the 72-kD type IV interstitial gelatinase in these cells. The 72-kD gelatinase is expressed in monocytes and a variety of other tissues but is not expressed in neutrophils, and no TPA-responsive element has been described in the promoter of this gene. In fact, the expression of the 72-kD gelatinase has been reported to be suppressed by TPA in HT-1080 cells. Therefore, a Northern blot of NB4 cells treated with G-CSF, ATRA, and TPA using the same conditions as described in Fig 3b. No transcript for the 72-kD gelatinase gene was observed in the control lane (C), because this represents RNA derived from normal neutrophils, which do not express the monocye-specific gelatinase gene. Both uninduced as well as G-CSF-induced NB4 cells express the 72-kD gelatinase mRNA. On the other hand, ATRA-induced cells do not. As noted above, this is in accord with the previously reported observations of the TPA-induced suppression of 72-kD gelatinase expression observed in HT-1080 cells. An increase in the level of the 72-kD collagenase transcript in TPA- plus ATRA-induced cells was also observed.

Because the signal for the interstitial (72-kD) gelatinase
mRNA was so faint, the identity of this mRNA was further confirmed by the RT-PCR technique (Fig 6). A granulocytic library derived from CML peripheral blood cells was used as a control for this study. This library, from which the secondary granule protein gene cDNAs were cloned, has been shown previously to lack monocytic sequences. In accord with this, expression of the 72-kDa gelatinase transcript was absent in the CML control (Fig 6, lane 1). Uninduced and TPA-induced NB4 cells (Fig 6, lanes 3 and 5) appear to express the 72-kD gelatinase transcript, whereas the ATRA-induced cells do not (Fig 6, lane 4). The 92-kD HNG transcript, on the other hand, is expressed in the CML control, in uninduced and TPA-induced NB4 cells, and at very low levels in ATRA-induced cells.

Analysis of cell surface markers. Flow cytometric analysis after indirect immunofluorescence staining on ATRA-treated, TPA-treated, and uninduced NB4 cells was performed (Table 1). In accordance with a previous report, 99% of the uninduced and induced cells expressed the pan-myeloid CD13 surface antigen. The granulocyte-associated CD15 antigen was expressed in 92% of uninduced and 96% of ATRA-induced NB4 cells. Expression of the two β-integrin subunits, CD11b and CD11c, was found to increase to 73% and 94%, respectively, in both ATRA-induced and TPA-induced NB4 cells. This observation is consistent with previous reports of increased CD11b and CD11c mRNA levels after myeloid differentiation. A relatively large percentage of uninduced NB4 cells (75%) express the CD45 antigen. Both ATRA and TPA induction lead to a modest (approximately 20%) increase in the number of cells expressing this cell surface marker. The HLA-DR surface antigen, a marker of early monocytic development, was expressed on only 3% of uninduced NB4 cells. On TPA-induction, 35% of NB4 cells in the population expressed this antigen on the cell surface. However, ATRA induction appeared to abolish the small population of cells expressing the HLA-DR antigen. No expression of the CD14 late monocytic cell surface marker on uninduced and induced NB4 cells was observed, an observation that has been previously reported. These findings suggest that TPA induction of NB4 cells results in the expression of only early monocytic markers.

DISCUSSION

Morphologic and cytochemical analysis of the promyelocytic cell line NB4 indicate that these cells show characteristics of early myeloid progenitor cells that have retained their bilineage potential. ATRA treatment of NB4 cells resulted in morphologic changes that were more subtle than those previously reported. Our ATRA-induced cells were primarily myelocytes; no bands or mature neutrophils were observed even after 7 to 9 days of induction. NB4 cells express a number of panmyeloid (CD45 and CD13) and granulocytic
We have provided further evidence that ATRA-mediated granulocytic differentiation associated with NB4 cells, which harbor the t(15;17) translocation, is defective. This finding is similar to the report that DMSO-induced granulocyte maturation is abnormal in HL60 cells. Both NB4 and HL60 promyelocytic cell lines appear to display a defect in the pattern of granulocytic maturation, as measured by the absence of secondary granules or the expression of their content proteins. The simultaneous absence of transcripts of all four secondary granule protein genes in ATRA-induced NB4 cells supports the previously hypothesized claim that the expression of these genes in neutrophils is coordinately regulated at the transcriptional level.

In apparent conflict with the hypothesis of shared transcriptional regulation of secondary granule protein gene expression in NB4 cells is the detectable expression of one of the four secondary granule protein genes, HNG, in both uninduced and TPA-induced cells. HNG is unusual among the secondary granule proteins, because it is the only one that is normally expressed in both monocytes and neutrophils. We have previously reported that HNG mRNA is present in HL60 cells induced along the monocytic pathway with TPA, but is not present in uninduced HL60 cells or in HL60 cells induced toward neutrophils. This observation supports the hypothesis that different regulatory mechanisms are involved in the expression of this gene in the granulocytic and monocytic lineages, and that the latter regulatory pathway may be intact in leukemic cells. Therefore, expression of HNG in TPA-induced NB4 cells is not surprising. However, the expression of HNG in uninduced NB4 cells remains unexplained. We hypothesize that it reflects a small subpopulation of NB4 cells that have a more mature monocytic phenotype, as supported by the expression of the monocytic 72-kD gelatinase and HLA-DR by a small number of uninduced cells. Alternatively, it could be an aberration associated with in vitro passage, or an anomaly resulting from the multiple chromosomal abnormalities (reported previously by Notte et al.12 and confirmed by this report's author; data not shown) in NB4 cells.

Increased expression of the AP-1-responsive HNG gene after TPA induction of NB4 cells coupled with the induction of c-fos expression is suggestive of maturation along

**Table 1. Indirect Immunofluorescence Staining on Cell Suspension**

<table>
<thead>
<tr>
<th></th>
<th>Uninduced NB4 Cells (%)</th>
<th>TPA-Induced NB4 Cells (%)</th>
<th>ATRA-Induced NB4 Cells (%)</th>
</tr>
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<tbody>
<tr>
<td>CD45</td>
<td>75</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>CD13</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>CD15</td>
<td>92</td>
<td>92</td>
<td>96</td>
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<tr>
<td>HLA-DR</td>
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<td>35</td>
<td>0.2</td>
</tr>
<tr>
<td>CD11b</td>
<td>19</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>CD11c</td>
<td>3</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td>CD14</td>
<td>—</td>
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</table>

*Percentage of cells positive for each of seven surface antigens is shown for uninduced NB4 cells, NB4 cells induced with ATRA for 48 hours, and NB4 cells induced with TPA for 48 hours.*

(CD15) cell surface antigens, the concentration of which remains virtually unchanged after induction with either ATRA or TPA. The presence of these and other myeloid cell surface markers (CD33) has been confirmed in NB4 cells.11 TPA induction of NB4 cells, on the other hand, results in increased adhesiveness as well as in a distinct monocyte/macrophage-like phenotype. Furthermore, TPA induction of NB4 cells resulted in a significant increase in the level of HLA-DR, a surface marker expressed on the monocyte-macrophage lineage.30 Relatively high levels of another monocytic marker, CD9, have also been reported in this cell line.12
monocyte/macrophage lines. The promoters of a number of genes such as interstitial collagenase and stromelysin have been shown to contain AP-1 recognition sites. AP-1 has been shown to be the prime modulator of induction of these genes by phorbol esters such as TPA. The AP-1 transcription complex is composed of the products of the c-jun and c-fos proto-oncogenes, the c-jun and c-fos proteins, and c-jun homodimers and c-jun/c-fos heterodimers are capable of recognizing the AP-1-responsive element, which in turn is known to confer pleiotropic effects on genes containing this element by affecting functions as diverse as cell growth, development, and differentiation.

The activity of AP-1 is known to be regulated not only by factors such as phorbol esters but also by cytokines and growth factors that modulate the levels of protein kinase C by mechanisms involving the transcriptional, translational, and/or posttranslational modulation of the c-jun and c-fos proto-oncogene products. The c-fos component of the AP-1 complex represents an early response gene product after induction to agents such as TPA. For example, in the human monocytic cell line U937, the c-fos transcript appears within 10 minutes of TPA induction. Evidence for the absolute requirement for c-fos expression in TPA-induced interstitial collagenase (an AP-1-responsive gene; expression has been previously reported. In fact, the investigators showed that elevated c-fos expression repressed c-fos promoter activity, which in turn abolished collagenase mRNA expression. Additionally, c-fos expression is known to be rapidly upregulated in U937 and HL60 cells induced to differentiate along the monocytic lineage after TPA induction. The c-fos transcript was detected within 30 minutes of TPA induction of NB4 cells, which is consistent with this pathway of TPA responsiveness.

ATRA treatment of TPA-induced NB4 cells resulted in relatively lowered levels of the c-fos transcript. This finding may reflect a direct steroid-induced suppression of the response to TPA. In previous studies, the AP-1 complex has been shown to block RA-induced gene expression in genes such as the human osteocalcin gene through a direct protein-protein interaction with the RAR.

We conclude that TPA-induced NB4 cells mature along the monocytic lineage, with the expression of HNG and with an increase in the surface expression of HLA-DR. The associated upregulation of c-fos suggests the involvement of the AP-1 complex in the induction of HNG gene expression. A nonspecific TPA response for this observation seems unlikely, because the monococyte-specific 72-kD interstitial gelatinase gene, which lacks an AP-1-responsive element in its promoter, was also shown to be expressed in TPA-induced cells.

Taken together, our findings suggest bilineage potential in NB4 cells. Along with HL60 cells, this cell line provides an excellent system to examine the differential expression of the HNG gene in the monocyte and neutrophil lineages. The absence of all secondary granule proteins and their respective mRNAs in ATRA-induced NB4 cells provides further support for the hypothesis of coordinate regulation of neutrophil secondary granule protein gene expression and a defect in this control as part of the leukemic phenotype.

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REFERENCES


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