Arsenic Trioxide as an Inducer of Apoptosis and Loss of PML/RARα Protein in Acute Promyelocytic Leukemia Cells

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Background: **Retinoids, which are derivatives of vitamin A, induce differentiation of acute promyelocytic leukemia (APL) cells *in vitro* and in patients. However, APL cells develop resistance to retinoic acid treatment. Arsenic trioxide (As_2O_3) can induce clinical remission in patients with APL, including those who have relapsed after retinoic acid treatment, by inducing apoptosis (programmed cell death) of the leukemia cells. In this study, we investigated the molecular mechanisms by which As_2O_3 induces apoptosis in retinoic acid-sensitive NB4 APL cells, in retinoic acid-resistant derivatives of these cells, and in fresh leukemia cells from patients.** ***Methods:*** **Apoptosis was assessed by means of DNA fragmentation analyses, TUNEL assays (i.e., deoxyuridine triphosphate labeling of DNA nicks with terminal deoxynucleotidyl transferase), and flow cytometry. Expression of the PML/RARα fusion protein in leukemia cells was assessed by means of western blotting, ligand binding, and immunohistochemistry. Northern blotting and ribonuclease protection assays were used to evaluate changes in gene expression in response to retinoic acid and As_2O_3 treatment.** ***Results and Conclusions:*** **As_2O_3 induces apoptosis without differentiation in retinoic acid-sensitive and retinoic acid-resistant APL cells at concentrations that are achievable in patients. As_2O_3 induces loss of the PML/RARα fusion protein in NB4 cells, in retinoic-acid resistant cells derived from them, in fresh APL cells from patients, and in non-APL cells transfected to express this protein. As_2O_3 and retinoic acid induce different patterns of gene regulation, and they inhibit the phenotypes induced by each other. Understanding the molecular basis of these differences in the effects of As_2O_3 and retinoic acid may guide the clinical use of arsenic compounds and provide insights into the management of leukemias that do not respond to retinoic acid. [J Natl Cancer Inst 1998;90:124–33]**

Retinoids are a group of natural and synthetic derivatives of vitamin A proven to have dramatic activity in the treatment of acute promyelocytic leukemia (APL) *(1,2).* Retinoids have been shown to induce cytodifferentiation and maturation of APL cells *in vitro* and in patients *(3).* APL is characterized by a reciprocal chromosomal translocation, t (15;17), which fuses the PML gene with the retinoic acid receptor α (RARα) gene, leading to the production of a chimeric oncoprotein, PML/RARα *(4–8).* PML/RARα plays a dual role in the phenotype of APL. It blocks myelocytic differentiation in a dominant-negative manner but permits maturation in the presence of pharmacologic levels of retinoic acid *(9,10).* Immunohistochemical studies of the PML and PML/RARα proteins show that they colocalize in an APL-specific microparticulate structure, whereas, in normal cells, PML is localized within specific subnuclear structures called nuclear bodies or PML oncogenic domains *(11–13).* Treatment with retinoic acid reconstitutes the normal PML nuclear body pattern in APL cells *(12).* Two groups *(14,15)* recently reported that retinoic acid induces a specific degradation of PML/RARα protein, possibly through a proteasome pathway. These data led to the hypothesis that retinoids induce APL cells to mature by selectively targeting the oncogenic PML/RARα protein.

Despite an excellent initial response, APL cells develop resistance to retinoic acid, and relapse occurs in patients with APL who are treated with retinoic acid alone *(4,16,17).* Retinoic acid-resistant human APL cells may provide *in vitro* systems to study the molecular and cellular mechanisms underlying resistance to retinoic acid and to test other potential therapeutic agents that may overcome retinoid resistance. We and others have developed several retinoic acid-resistant subclones of the human APL cell line, NB4, that vary in their expression of PML/RARα. These subclones were selected after exposure either to mutagens and retinoic acid or to retinoic acid alone *(18–20).*

Recent reports *(21,22)* showed that arsenic trioxide (As_2O_3) is a very effective treatment for patients with APL, even for those who relapsed after all-*trans*-retinoic acid (t-RA)-induced clinical remission. *In vitro* experiments demonstrated that As_2O_3 induces apoptosis in NB4 cells, but it is less effective in other myeloid leukemia cell lines *(21).* The substantial effect of As_2O_3 on APL cells and in relapsed patients with APL *(21,22),* along with reports *(9,23,24)* that artificial expression of PML/RARα

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See ''Notes'' following ''References.''

prevents apoptosis in U937 myelocytic leukemia cells and TF-1 erythroleukemia cells, led us to examine the effects of As_2O_3 on cell growth and PML/RARα expression in a variety of retinoic acid-resistant NB4 subclones. We now report that As_2O_3 induces loss of the fusion protein PML/RARα but not of its messenger RNA (mRNA) in both retinoic acid-sensitive and retinoic acid-resistant cell lines, leading to apoptosis without morphologic differentiation. Thus, in cells that express PML/RARα protein, PML/RARα may be a common target for both retinoic acid and As_2O_3, and loss of PML/RARα regulation by retinoic acid does not prevent its regulation by As_2O_3. We examined the effects of As_2O_3 in U937 cells stably transfected with PML/RARα as well as in fresh leukemia cells derived from patients with APL. To develop hypotheses explaining why the As_2O_3-induced loss of PML/RARα leads to apoptosis while retinoic acid treatment leads to differentiation, we investigated the effects of both compounds in combination, and we examined alterations in gene expression induced by retinoic acid or As_2O_3.

Methods

Cell culture and compounds. The parental NB4 promyelocytic leukemia cell line, five retinoic acid-resistant subclones of this line, and the U937 myeloid leukemia cell line were grown in RPMI-medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Upstate Biotechnology Inc., Lake Placid, NY). Peripheral blood was obtained after verbal consent from newly diagnosed patients with APL whose disease was classified as M3 or M3-variant by use of morphologic criteria (French–American–British classification) and who presented with an initial percentage of circulating blast cells that was more than 80%. Confirmation of the presence of the PML/RARα gene was performed as previously described *(25)*. Leukemia cells were isolated and enriched on Ficoll–Hypaque density gradients (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), washed twice with calcium and magnesium-free phosphate-buffered saline (PBS), and cultured as described above. All cell cultures were incubated at 37 °C in humidified air supplemented with 5% CO_2. A 10-m*M* stock solution of t-RA (Sigma Chemical Co., St Louis, MO) was prepared in dimethyl sulfoxide. As_2O_3 (Sigma Chemical Co.) was dissolved in 1 *M* NaOH, and diluted further to 10 m*M* in PBS.

Viable cell counts and assays of differentiation. Cell lines were grown in the medium described above in the presence of varying concentrations of As_2O_3 for 5 or 7 days. Cells were cultured at 25 000/mL, and duplicate samples were counted by use of a hemocytometer. The trypan blue dye (Sigma Chemical Co.) exclusion method was used to count viable cells. Cell differentiation was evaluated on the basis of cellular morphology, changes in cell surface antigen expression, and the nitroblue tetrazolium (NBT) assay (Sigma Chemical Co.). Giemsa-stained cytospin slides were analyzed for cell morphology by means of light microscopy. Analysis of cell surface antigens was performed by means of flow cytometry (Faxflo cytometer; Becton Dickinson Labware, Bedford, MA); aliquots of 1×10^6 cells were stained with antibodies CD11b, CD11c, CD13, and CD15 *(26)*. The percentage of NBT-positive cells was determined (at least 200 cells per experimental condition) as previously described *(27)*.

Assays of apoptosis. For DNA fragmentation analysis, cells were pelleted and resuspended in lysis buffer (10 m*M* Tris–HCl [pH 7.8], 10 m*M* EDTA, 100 m*M* NaCl, 0.5% sodium dodecyl sulfate (SDS), and 200 ng/μL proteinase K) followed by incubation at 37 °C overnight. After phenol:chloroform (1:1) extraction, DNA was precipitated with 2 vol of 100% ethanol and washed twice with 75% ethanol. The DNA pellet was resuspended in TE buffer (10 m*M* Tris–HCl [pH 8.0] and 1 m*M* EDTA), and 8 μg of DNA was electrophoresed in an ethidium bromide-stained 1.5% agarose gel for the visualization of laddering (caused by internucleosomal cleavages). The TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate [dUTP] nick-end labeling) assay was performed by use of the *In Situ* Cell Death detection kit (Boehringer Mannheim, Laval, Québec, Canada). DNA strand breaks generated by cleavage of genomic DNA during apoptosis can be identified in this assay by the labeling of free 3′-OH termini with fluorescein isothiocyanate (FITC)-conjugated dUTP. Stained cells were analyzed by means of fluorescence microscopy. For samples from patients with APL, the percentage of apoptotic cells among APL blast cells was evaluated by means of double staining with FITC-labeled Annexin-V and propidium iodide (PI) (R&D Systems, Inc., Milan, Italy). Briefly, 5×10^4 cells were washed twice with cold PBS and resuspended in 250 μL binding buffer (HEPES-buffered saline solution [Life Technologies, Inc.] supplemented with 0.25 m*M* $CaCl_2$); 10 μL FITC-conjugated Annexin-V and 10 μL PI reagent were then added to the cells, and the mixture was gently agitated on a vortex mixer and incubated for 15 minutes at room temperature in the dark. Within 1 hour, the cells were analyzed at 488 nm in a FACSCAN cytometer (Becton Dickinson Labware).

SDS–polyacrylamide gel electrophoresis and western blot analysis. Nuclear extracts were prepared from 1 to 5×10^8 control cells and treated cells as described previously *(26,27)*. Equal amounts of protein were electrophoresed in 10% polyacrylamide gels containing 0.1% SDS and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with an antibody directed against the RARα F domain [provided by P. Chambon *(28)*] (1:1000 antibody dilution), and antibody binding was detected with the ECL chemiluminescence system (Amersham Life Science Inc., Arlington Heights, IL).

Assay for ligand binding activity. NB4, NB4.606, and U937/PR9 cells were treated with or without 1 μ*M* As_2O_3 for 16 hours. Nuclear extracts were prepared from 1 to 5×10^8 cells and incubated for 18 hours at 4 °C with 10 n*M* [^{3}H]t-RA (50.7 Ci/m*M;* Du Pont NEN, Boston, MA), as previously described *(25,27)*. The extracts were subsequently fractionated at 4 °C by means of high-performance liquid chromatography (HPLC) using a superose 6 HR 10/30 size-exclusion column (Pharmacia LKB Biotechnology AB). The flow rate was 0.4 mL/minute, fractions of 0.4 mL were collected, and radioactivity was measured with the use of a liquid scintillation counter. The HPLC system was calibrated with a series of molecular weight (MW) markers, including the following: blue dextran, MW 2 000 000; thryoglobulin, MW 669 000; β-amylase, MW 200 000; bovine serum albumin, MW 66 000; and ovalbumin, MW 45 000.

Immunohistochemistry. Cells were cytocentrifuged and fixed in 3.7% paraformaldehyde in PBS at room temperature for 10 minutes, followed by an incubation in methanol for 2 minutes. PML immunostaining was performed by use of the monoclonal antibody PGM3 *(29)* for 1 hour at room temperature. The cells were subsequently stained with a rhodamine-conjugated, anti-mouse secondary antibody (1:600 dilution in PBS) for 30 minutes. After washing with PBS, antibody complexes were detected by use of a fluorescence microscope (Aristoplan-Leitz, Wetzlar, Germany).

Ribonuclease (RNase) protection assay and northern blot analysis. Total cytoplasmic RNA was isolated, and RNase protection analysis was performed as previously described *(30–32)*. Hybridization of complementary RNA probes was carried out at 45 °C overnight, followed by the addition of 300 μL RNase digestion buffer containing 40 μg/mL RNase A and 700 U/mL RNase T1. RNase digestion was performed at 25 °C for 1 hour, and Rnase-resistant fragments were resolved by means of electrophoresis in 6% polyacrylamide sequencing gels that contained 7.6 *M* urea. A glyceraldehyde-3-phosphate dehydrogenase probe (Ambion Inc, Austin, TX) was included in all analyses as a control for RNA loading. As approximate size markers, ^{32}P-labeled *Msp* I-digested fragments of the plasmid pBR322 were run in all gels. Northern blot analysis was performed as previously described *(20)*.

Transglutaminase assay. Control or treated cells (approximately $1–5 \times 10^6$) were collected by means of centrifugation and washed twice in calcium and magnesium-free PBS containing 1 m*M* EDTA and 1 m*M* phenylmethylsulfonyl fluoride. Cell pellets were resuspended in 200 μL of the same solution and sonicated at 4 °C. After taking an aliquot for total protein determination, dithiothreitol was added to the remaining sonicate to a final concentration of 10 m*M*. The sonicate was then centrifuged at 4 °C for 5 minutes at 10 000*g*, yielding soluble (supernatant) and particulate (pellet) fractions. Transglutaminase activity assays were performed as previously described *(33)*.

Results

Inhibition of Cell Growth Induced by As_2O_3 in Retinoic Acid-Resistant APL Subclones

We examined the effects of As_2O_3 on a variety of retinoic acid-resistant NB4 subclones. MR6, R4, and NB4.006 are three

resistant subclones developed by maintaining NB4 cells in retinoic acid-containing media *(19,20).* The subclones NB4.306 and NB4.606 were developed by means of mutagenesis with low-dose radiation, followed by culturing in retinoic acid-containing media *(19).* PML/RARα expression can be seen in western blots of the proteins from all resistant subclones except for NB4.306, although several subclones demonstrate altered ligand binding to PML/RARα *(20).* The cells were treated with different doses of As_2O_3, and viable cells were counted via the trypan blue dye exclusion method. All five retinoic acid-resistant cell lines showed growth inhibition induced by As_2O_3 in a dose-dependent manner, comparable to or greater than the inhibition induced in NB4 cells (Fig. 1).

Induction of Apoptosis by As_2O_3 Without Differentiation in Retinoic Acid-Resistant Cell Lines and in Fresh APL Cells From Patients

NB4 and retinoic acid-resistant subclones were treated with 1 μ*M* As_2O_3 for up to 6 days. Fixed, cytospun samples of each cell line were stained and examined by means of light microscopy, which showed no evidence of morphologic differentiation (data not shown). We then analyzed the expression of three surface antigens after 4 days of culture in 1 μ*M* As_2O_3 or 1 μ*M* t-RA. The percentage of cells from NB4 and three retinoic acid-resistant sublines (R4, NB4.306, and NB4.606) expressing surface differentiation antigens after 4 days of exposure to either compound is shown in Table 1. The increased levels of CD11b and CD11c and the reduced level of CD13 in NB4 cells following treatment with 1 μ*M* retinoic acid are consistent with the known sensitivity of these cells to retinoic acid-induced differentiation *(34).* The three resistant cell lines showed minimal expression of CD11b and CD11c with or without retinoic acid treatment. CD13 expression remained unchanged following treatment with retinoic acid in R4 and NB4.306, although NB4.606 displayed some degree of CD13 reduction. All cell lines, including NB4, showed no alteration in surface antigen expression in the presence of 1 μ*M* As_2O_3. Thus, consistent with the morphologic studies, As_2O_3 does not induce markers of cell differentiation.

We then performed assays characterizing cell apoptosis. Cells

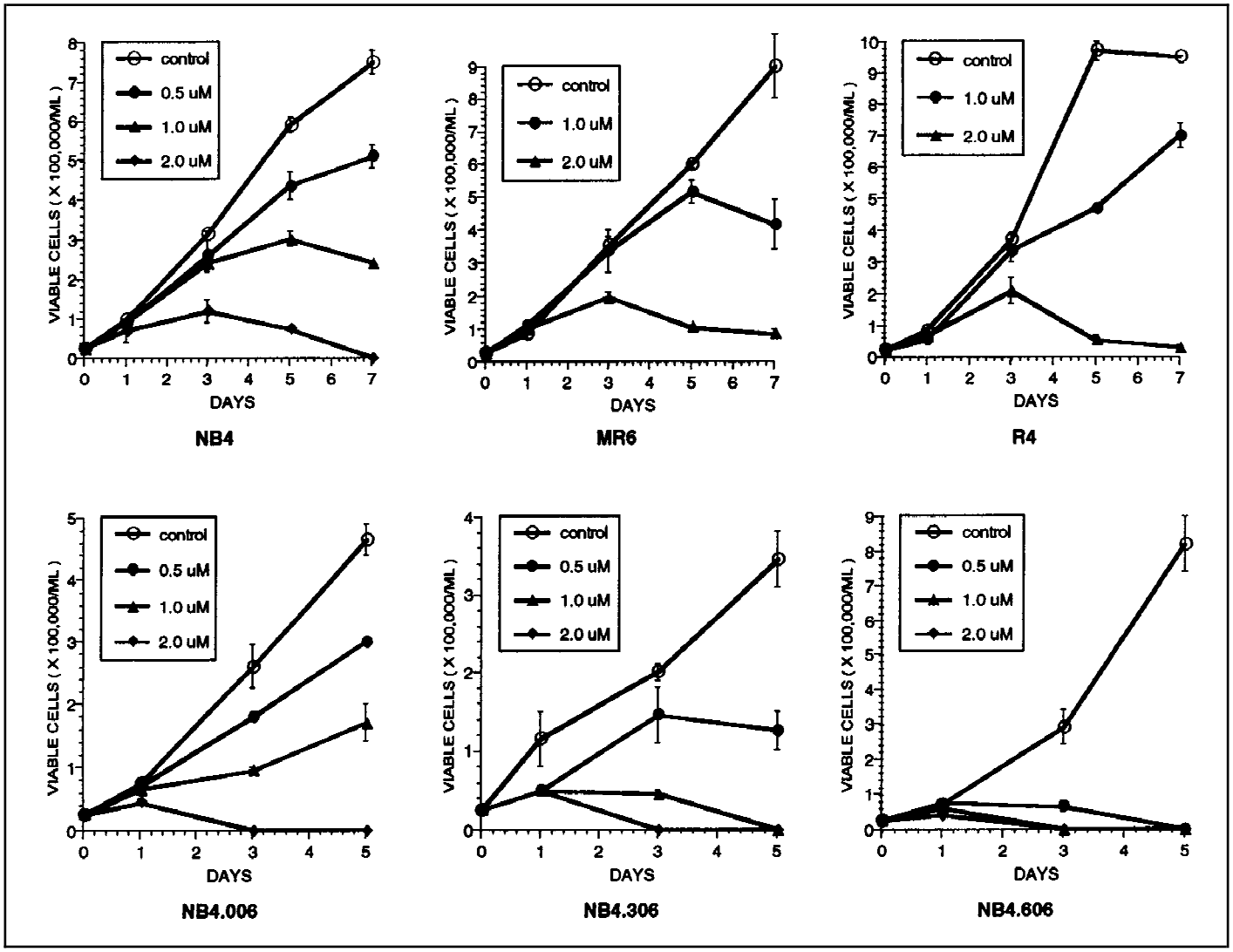

Fig. 1. Growth curves for NB4 human promyelocytic leukemia cells and a variety of retinoic acid-resistant subclones. Cells were treated with indicated concentrations of arsenic trioxide (As_2O_3), and viable cells were counted by use of the trypan blue dye exclusion method. Control cultures received no As_2O_3. Each data point represents the mean of two independent experiments, and standard deviations are indicated by vertical lines through the data points.

Table 1. Cytofluorometric analysis of surface marker expression: percentages of cells expressing specific surface markers*

Markers	Control	1 μ*M* t-RA	1 μ*M* As_2O_3	Control	1 μ*M* t-RA	1 μ*M* As_2O_3
	NB4 cells			***R4 cells***		
CD11b	1.5	43.0	1.4	0.9	1.5	8.6
CD11c	0.1	42.1	3.8	0	0	0.1
CD13	98.5	12.7	90.7	93.1	91.5	97.5
	NBA.306 cells			***NB4.606 cells***		
CD11b	3.8	2.4	1.6	1.8	2.8	0.3
CD11c	0	1.1	0.1	0	3.8	0.2
CD13	98	93.9	96.1	98.5	48.8	94.6

*Cells were incubated for 4 days in culture medium alone (control) or in culture medium supplemented with either 1 μ*M* all-*trans*-retinoic acid (t-RA) or 1 μ*M* arsenic trioxide (As_2O_3). R4, NB4.306, and NB4.606 are retinoic acid-resistant subclones of the NB4 cell line. *See* text for additional details.

were treated with 1 μ*M* t-RA or 1 μ*M* As_2O_3, and DNA was isolated and electrophoresed for fragmentation analysis. NB4 cells showed no indication of apoptosis induced by t-RA up to day 3, whereas after 1 day of treatment with 1 μ*M* As_2O_3, NB4 and the two retinoic acid-resistant lines, MR6 and R4, showed a distinctive DNA laddering pattern (data not shown). We confirmed the induction of apoptosis by As_2O_3 at the single cell level via the TUNEL assay. This assay labels *in situ* DNA strand breaks that are produced during apoptosis and these labeled breaks can be detected by means of immunohistochemistry. Cells were treated with 1 μ*M* As_2O_3 and assayed after 1 day. Approximately 2.4% of the untreated NB4 cells showed the positive fluorescent staining that is indicative of apoptosis, whereas 10% of the cells exhibited apoptotic staining after 1 day of As_2O_3 treatment. The three retinoic acid-resistant cell lines, MR6, R4, and NB4.606, showed comparable levels of cell apoptosis induced by As_2O_3 (data not shown).

To examine the effects of As_2O_3 on fresh APL cells, leukemia blast cells were isolated from four patients newly diagnosed with APL, and these blast cells were incubated in cell culture medium alone (control) or in medium supplemented with 1 μ*M* As_2O_3, 1 μ*M* t-RA, or both agents combined. After 7 days of culture, cytospun samples of the cells were stained with May–Grünwald–Giemsa and examined by means of light microscopy. Cells treated with t-RA showed morphologic features of granulocytic maturation, whereas no evidence of morphologic differentiation was observed in control cells or in cells exposed to As_2O_3. Cells treated with both agents exhibited morphologic signs of granulocytic differentiation but at a level less pronounced than that observed for cells exposed to t-RA alone. In parallel, we evaluated the expression of several membrane antigens in fresh leukemia cells cultured under the same conditions as described above. As shown in Table 2, we observed the following: 1) cells cultured in the absence of any agent did not undergo changes in surface marker expression; 2) cells cultured in the presence of t-RA acquired the expression of several membrane antigens (CD11b, CD11c, CD18, and CD15) typically expressed in mature phagocytes; 3) As_2O_3-treated cells did not develop expression of the majority of membrane-differentiation antigens (CD11b, CD11c, and CD18), with the exception of CD15; and 4) consistent with our morphologic findings, cells cultured in the presence of both agents showed a level of expression of membrane-differentiation antigens lower than that observed in cells exposed to t-RA alone. Thus, *in vitro* treatment of fresh APL cells with As_2O_3 does not induce phenotypic differentiation and diminishes the level of differentiation induced by retinoic acid.

We also evaluated the effects of t-RA and As_2O_3 in combination on apoptosis in APL blast cells. APL cells isolated from patients and cultured *in vitro* in RPMI-1640 medium containing 10% FBS exhibited a moderate and progressive increase in the rate of spontaneous apoptosis from day 0 to day 7 of culture; this

Table 2. Cytofluorometric analysis of cell surface antigen marker expression: percentages of positive cells among leukemia blast cells derived from four patients with acute promyelocytic leukemia*

Markers	C0	C7	t-RA	As_2O_3	t-RA + As_2O_3	C0	C7	t-RA	As_2O_3	t-RA + As_2O_3
	Patient 1					***Patient 2***				
CD11b	30	19	86	13	42	68	12	89	17	NE
CD11c	2	1	74	8	13	4	3	69	11	NE
CD15	22	34	99	97	97	10	37	95	92	NE
CD18	10	4	59	3	NE	4	8	57	23	NE
	Patient 3					***Patient 4***				
CD11b	10	2	51	3	5	10	4	79	3	31
CD11c	0	0	51	2	32	0	0	54	1	17
CD15	2	29	100	98	99	3	6	99	69	99
CD18	54	48	94	58	50	37	33	95	33	79

*All patients had the t(15;17) translocation. The blast cells were cultured for 7 days in medium alone (control) or in the presence of 1 μ*M* all-*trans*-retinoic acid (t-RA), 1 μ*M* arsenic trioxide (As_2O_3), or a combination of both agents. C0 = control at day 0 of culture; C7 = control at day 7 of culture; NE = not evaluated. *See* text for additional details.

Table 3. Effect of all-*trans*-retinoic acid (t-RA) and/or arsenic trioxide (As_2O_3) on apoptosis: percentages of positive cells among leukemia blast cells derived from two patients with acute promyelocytic leukemia*

	Patient 2				Patient 4			
Days	C	t-RA	As_2O_3	t-RA + As_2O_3	C	t-RA	As_2O_3	t-RA + As_2O_3
0	3	3	3	3	2	2	2	2
1	3	2.5	17	8	3	3	10	4
2	7	7	20	14	7	4	15	6
5	10	10	30	16	NE	NE	NE	NE
7	14	12	33	19	20	33	65	36

*Both patients had the t(15;17) translocation. The blast cells were cultured as reported in Table 2, and the percentages of apoptotic cells were determined by use of the Annexin-V labeling method (*see* text for additional details). C = control culture (not treated); NE = not evaluated.

phenomenon was not modified by the addition of t-RA, but it was greatly enhanced by treatment with As_2O_3 (Table 3). Interestingly, cells cultured in the presence of both t-RA and As_2O_3 exhibited a percentage of apoptotic cells lower than that observed for cells cultured in As_2O_3 alone.

As_2O_3-Induced Down-regulation (loss) of PML/RARα Protein in NB4 Cells, in Retinoic Acid-Resistant NB4 Subclones, and in APL Cells From Patients

We have reported *(14)* that retinoic acid induces a loss of the PML/RARα protein in retinoic acid-sensitive APL cells. Since PML/RARα inhibits apoptosis in several systems *(9,23,24)*, we tested whether As_2O_3 would regulate the expression of this protein (Fig. 2, A). NB4 and two resistant cell lines, R4 and MR6, were incubated with 1 μ*M* t-RA or 1 μ*M* As_2O_3 for 24 hours. Treatment with t-RA induced a down-regulation of PML/RARα in NB4 cells but not in R4 or MR6 cells (Fig. 2, A; lanes 2, 5, and 8). However, expression of PML/RARα was completely lost after treatment with As_2O_3 in NB4 cells and in cells of the two resistant cell lines (Fig. 2, A; lanes 3, 6, and 9), whereas RARα protein expression remained unchanged. The unaltered expression of RARα confirms, as in the case of retinoic acid-treated NB4 cells, the selective regulation of the fusion protein. PML/RARα expression following As_2O_3 treatment was also analyzed in the retinoic acid-resistant subclones NB4.006 and NB4.606; similar down-regulation was observed in both cell lines (data not shown).

Cells from a patient with APL were treated *in vitro* with 1 μ*M* t-RA or 1 μ*M* As_2O_3 for 4 days, and the percentage of differentiated cells was determined by use of the NBT reduction assay (Fig. 2, B). More than 60% of the t-RA-treated cells were NBT positive, whereas As_2O_3-treated cells were not differentiated. Nuclear extracts were prepared and subjected to western blot analysis (Fig. 2, C). Consistent with the *in vitro* results, the PML/RARα protein level was down-regulated following both retinoic acid treatment and As_2O_3 treatment.

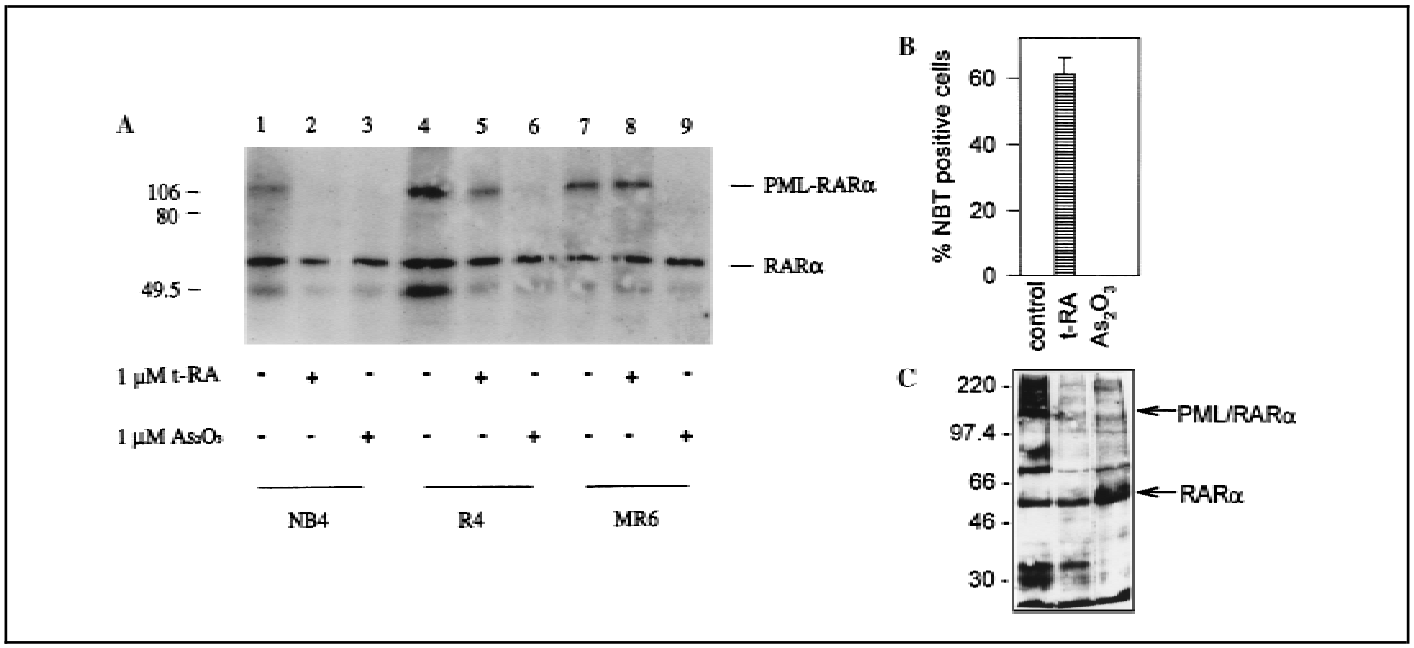

Fig. 2. Arsenic trioxide (As_2O_3) and the down-regulation (loss) of PML/RARα protein. **A**) NB4 cells and two retinoic acid-resistant cell lines (R4 and MR6) were not treated or were treated with either 1 μ*M* all-*trans*-retinoic acid (t-RA) or 1 μ*M* As_2O_3 for 24 hours. A specific anti-RARα (retinoic acid receptor α) antibody was used in this western blot analysis; the positions of PML/RARα and RARα in the blot are indicated. **B** and **C**). Leukemia cells were isolated from one newly diagnosed patient with acute promyelocytic leukemia and treated with either 1 μ*M* t-RA or 1 μ*M* As_2O_3 for 4 days. B) Percentages of differentiated cells were determined by means of the NBT (nitroblue tetrazolium)-reduction assay; the values shown represent averages of the percentages of positive cells evaluated in at least 10 microscopic fields; the error bar above the t-RA data indicates the standard deviation. C) A western blot analysis was performed with the same anti-RARα antibody as in A; PML/RARα protein was down-regulated by both t-RA and As_2O_3 treatment.

As_2O_3 Regulation of Retinoid Binding Capacity in Nuclear Extracts of NB4 Cells, Retinoic Acid-Resistant NB4.606 Cells, and U937/PR9 Cells

To provide additional confirmation of the selective loss of PML/RARα protein induced by treatment with As_2O_3, we analyzed the binding of [^{3}H]t-RA to nuclear and cytosolic extracts from NB4 cells and from the retinoic acid-resistant subclone NB4.606, treated with or without 1 μ*M* As_2O_3 (Fig. 3, A–D). The size-exclusion HPLC profiles of both NB4 and NB4.606 nuclear extracts show three specific t-RA-binding components with apparent MWs of 50, 110, and 669 kd, representing endogenous RARα, PML/RARα monomer, and a high molecular weight complex involving PML/RARα, respectively (Fig. 3, A and B) *(8,27,35).* After 16 hours of treatment with 1 μ*M* As_2O_3, the binding components with MWs of 110 and 669 kd, but not of 50 kd, disappeared from both cell extracts (Fig. 3, C and D). Thus, ligand binding to PML/RARα monomer or multimeric complexes is lost after treatment with As_2O_3, consistent with the results of western blot analysis. HPLC profiles of cytosolic extracts from these cells show a peak at 16 kd, representing the cytosolic retinoic acid-binding proteins, that, like the 50-kd peak attributed to unrearranged RAR, is not altered by As_2O_3 treatment (data not shown).

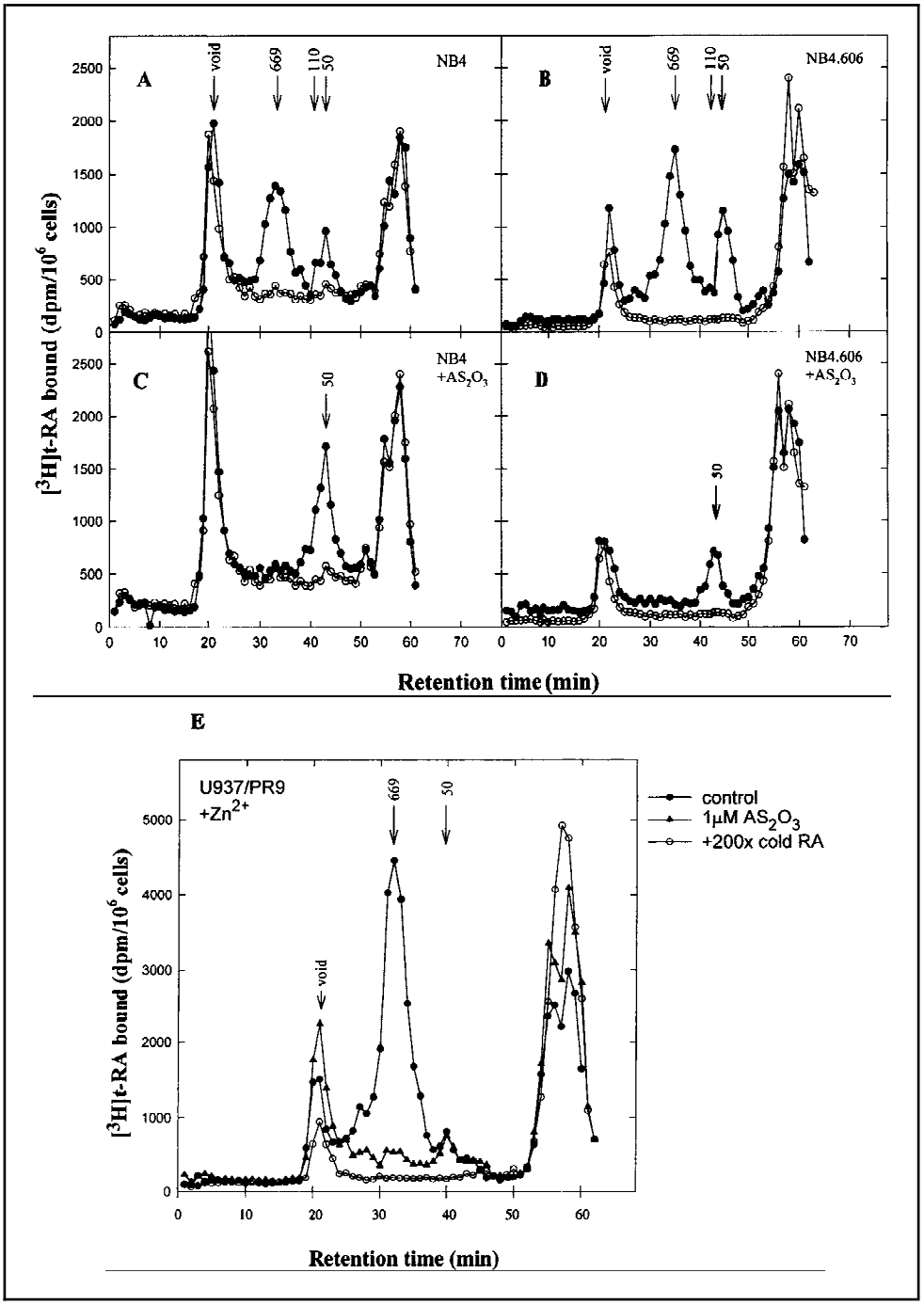

Fig. 3. Analysis of specific all-*trans*-retinoic acid (t-RA) nuclear binding activity in NB4, NB4.606, and U937/PR9 cells. NB4 cells and retinoic acid-resistant NB4.606 cells were cultured in medium alone (**A** and **B,** respectively) or treated with 1 μ*M* As_2O_3 for 16 hours (**C** and **D,** respectively). Nuclear extracts from the cells were incubated with 10 n*M* [^{3}H]t-RA in the presence (○) or in the absence (●) of a 200-fold excess of unlabeled t-RA and subsequently analyzed by means of a high performance liquid chromatography size-exclusion assay. **E)** U937/PR9 cells were induced to express PML/RARα protein in a 2-hour incubation with 100 μ*M* Zn^{2+} alone; the cells were then treated (▲) or not (●) with 1 μ*M* As_2O_3 for 16 hours, and nuclear extracts were analyzed as described above. Results for the incubation with a 200-fold excess of unlabeled t-RA (cold RA) are shown only for the extract from cells not treated with As_2O_3 (○). Arrows indicate the void peak elution times and apparent molecular weights (in thousands) of t-RA-binding species. dpm = disintegrations per minute.

To determine whether the effects of As_2O_3 on PML/RARα protein were unique to NB4 cells and their subclones, we studied the promonocytic leukemia cell line U937, which does not express the APL-specific chimeric protein PML/RARα. U937/PR9 is a stable subclone transfected with a PML/RARα complementary DNA under control of a Zn^{2+}-inducible promoter *(9).* Nuclear extracts from U937/PR9 cells induced with 100 μ*M* Zn^{2+} for 2 hours and incubated in control medium for 16 hours showed a 669-kd specific t-RA-binding complex similar to the one seen in extracts from NB4 cells (●; Fig. 3, E). However, when extracts of cells treated with 100 μ*M* Zn^{2+} for 2 hours followed by 1 μ*M* As_2O_3 for 16 hours were examined, nuclear multimeric complex binding to ligand was nearly abolished (▲; Fig. 3, E). These data demonstrate that the down-regulation of PML/RARα protein induced by As_2O_3 is specific for the fusion protein, whether it occurs in APL cells or in other hematopoietic cells artificially expressing PML/RARα protein.

Reorganization of PML Protein: As_2O_3 Versus Retinoic Acid

Since As_2O_3 causes a loss of expression of PML/RARα in retinoic acid-sensitive and retinoic acid-resistant APL cell lines, we examined whether it affects the subcellular localization of PML and PML/RARα in these cells. Immunohistochemical studies on cells before and after 6 or 18 hours of treatment with 1 μ*M* As_2O_3 were performed using a specific anti-PML antibody (Fig. 4, red stain); nuclei were localized with DAPI stain (Fig. 4, blue stain). Before As_2O_3 treatment, PML and PML/RARα co-localized in the nucleus in a typical, APL-specific, microparticulate pattern both in NB4 cells and in cells of the retinoic acid-resistant cell lines R4 and NB4.306 (Fig. 4, A–C). Six hours after As_2O_3 treatment, major decreases were observed in the amounts of nuclear PML and PML/RARα in both NB4 cells and cells of the two resistant cell lines, including NB4.306, a cell line that does not express a detectable level of PML/RARα protein, as determined by western blotting (Fig. 4, D–F). This reorganization of the PML and PML/RARα staining pattern became more dramatic with longer exposure to As_2O_3 (Fig. 4, G–I). The decreased staining observed in this immunohistochemical study is consistent with the loss of PML/RARα protein, which was demonstrated previously in the western blot analysis (Fig. 2, A), where the fusion protein was not detectable after 24 hours of As_2O_3 treatment. However, the reorganization of PML and PML/RARα induced by As_2O_3 is different from the reorganization induced by retinoic acid. Although retinoic acid fails to reconstitute the normal nuclear body pattern of PML in differentiation-resistant clones *(12)*, As_2O_3 has equal effects on NB4 and the retinoic acid-resistant NB4 subclones. Furthermore, continued treatment with As_2O_3 leads to a substantially decreased staining of nuclear bodies by the anti-PML antibody, something not seen with retinoic acid treatment. This finding suggests that expression of unrearranged PML is also lost after treatment with As_2O_3 but not with retinoic acid.

As_2O_3 and Regulation of Levels of mRNAs Encoding PML, PML/RARα, and Retinoid Receptors

We have shown that As_2O_3 down-regulates PML and PML/RARα at the protein level (Fig. 2, A; Fig. 4). To determine whether this regulation is also observed at the mRNA level, we performed a northern blot analysis using specific PML and RARα probes. The mRNA levels for PML, PML/RARα, and RARα remained unchanged following As_2O_3 treatment for 3 days (data not shown). By use of a ribonuclease protection assay, we also examined the effects of As_2O_3 on the expression of mRNAs encoding the six retinoid receptor isoforms. In NB4 cells and in the five retinoic acid-resistant subclones tested, RARα, RXRα, and RXRβ mRNAs are constitutively expressed, whereas expression of RARβ, RARγ, and RXRγ mRNAs is not seen. Although retinoic acid induces RARβ expression and down-regulates RXRα expression in retinoid-sensitive NB4 cells *(36)*, As_2O_3 has no detectable effect on RARβ and RXRα mRNA levels after 3 days. The expression level of CD38 mRNA, up-regulated (increased) during retinoic acid-induced differentiation in HL-60 cells and in retinoic acid-treated NB4 cells *(36–38)*, also remained unchanged after As_2O_3 treatment (data not shown).

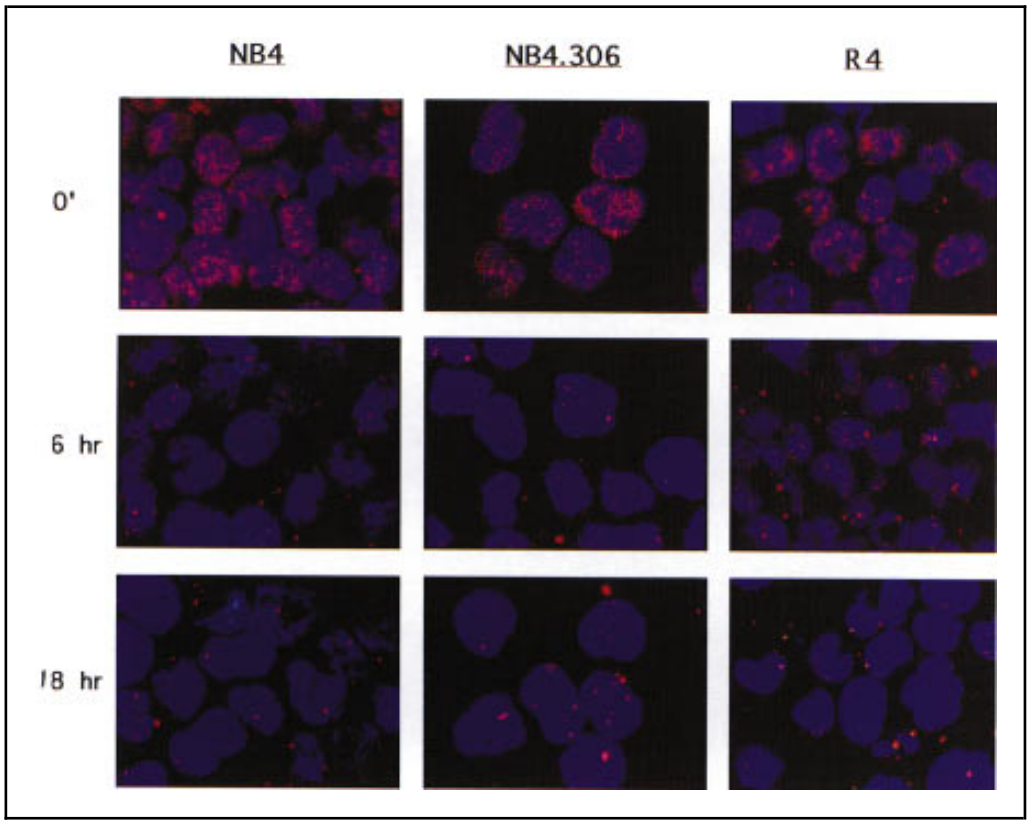

Fig. 4. Immunohistochemical analysis using a specific anti-PML antibody in NB4 cells (**A, D,** and **G**) and in cells of two retinoic acid-resistant cell lines, NB4.306 (**B, E,** and **H**) and R4 (**C, F,** and **I**). Cells were either not treated (0′)or treated with 1 μ*M* arsenic trioxide for 6 or 18 hours. Nuclear staining by DAPI (blue) and PML–PML/RARα staining by the anti-PML antibody (red) were superimposed. *See* text for additional details.

As_2O_3 Interference With Retinoic Acid-Inducible Type II Transglutaminase (Type II TGase) Activity in NB4 Cells

Studies using fresh leukemia cells from patients with APL suggest that As_2O_3 interferes with retinoic acid-induced differentiation (Table 2). We investigated, therefore, whether As_2O_3 exerts an effect on proteins associated with differentiation induced by retinoic acid. Type II TGase activity has been shown to be increased during retinoid-induced differentiation in NB4 cells, mediated through a PML/RARα signaling pathway *(39)*. Fig. 5, B, shows type II TGase activity in NB4 cells pretreated with either 1 μ*M* As_2O_3 or control medium for 16 hours, followed by the addition of different concentrations of t-RA for 3 days. The level of type II TGase

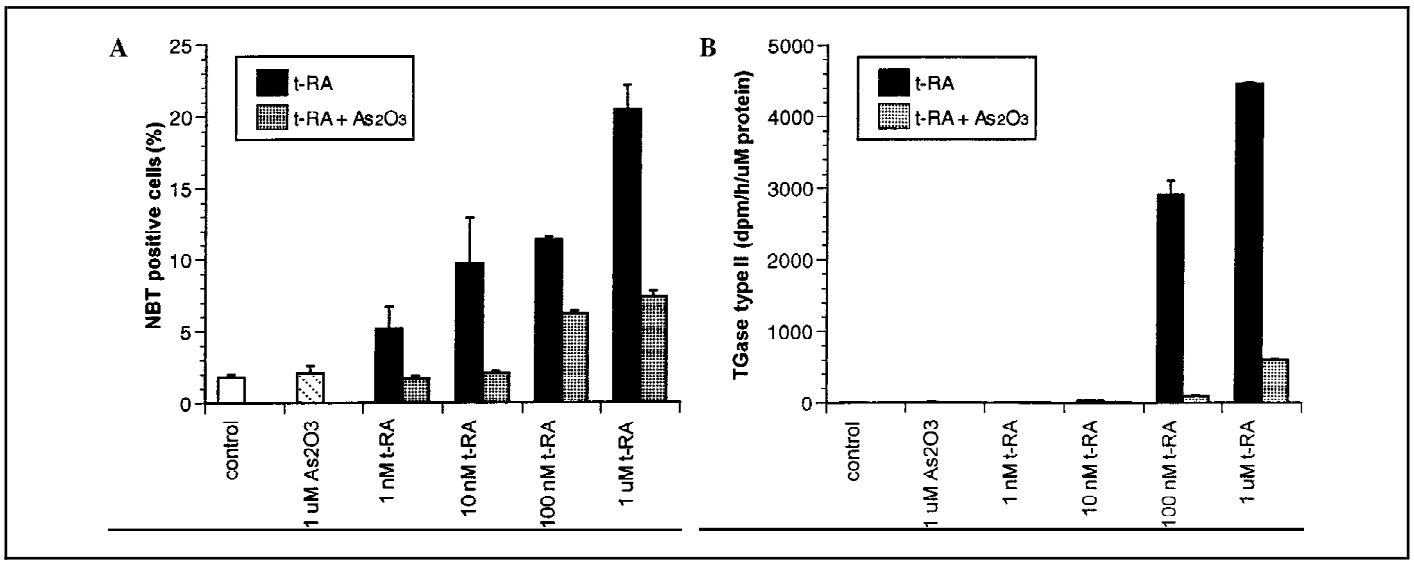

Fig. 5. Effect of arsenic trioxide (As_2O_3), all-*trans*-retinoic acid (t-RA), or both agents in combination on NB4 cell differentiation and type II transglutaminase (TGase type II) activity. NB4 cells were either not treated (control) or treated with 1 μ*M* As_2O_3 for 16 hours. In most cases, the cells were subsequently exposed to specific concentrations of t-RA for 3 days. **A**) The percentages of differentiated cells were determined by means of the NBT (nitroblue tetrazolium)-reduction assay; the results signify the average percentages of differentiated cells evaluated in at least 10 microscopic fields per experimental condition. **B**) TGase type II activity was determined as described in the ''Materials and Methods'' section. Each data point represents the average of values obtained from duplicate cultures in one experiment. In A and B, the error bars indicate standard deviations. dpm = disintegrations per minute; h = hour.

activity was not affected by As_2O_3 treatment alone, but it was increased by t-RA treatment in a dose-dependent manner. The activity of this enzyme was elevated by about 3000-fold with 100 n*M* t-RA and by about 4500-fold with 1 μ*M* t-RA. However, the induction of type II TGase activity was significantly reduced by pretreatment with As_2O_3, whether analyzed on the basis of viable cells or units of protein (data not shown). The status of cell differentiation was also evaluated either morphologically or with the NBT reduction assay (Fig. 5, A). Cell differentiation was induced by t-RA in a dose-dependent manner; co-treatment with both t-RA and As_2O_3 reduced the percentage of differentiated cells, in agreement with the decrease in type II TGase activity.

Discussion

Arsenic compounds have been shown to act as co-mutagens or co-carcinogens *(40–42)*. However, As_2O_3 has been recently reported *(21,22)* to be effective in treating patients with APL, including those who have relapsed after t-RA treatment, by inducing apoptosis of the leukemia cells. To investigate further the mechanisms of As_2O_3 action, we tested its effect *in vitro* on both retinoic acid-sensitive and retinoic acid-resistant NB4 subclones. We found that As_2O_3, from concentrations of 0.5–2 μ*M*, is able to induce apoptosis without differentiation in NB4 cells and in its retinoic acid-resistant subclones at comparable levels. These *in vitro* concentrations are within the range found in the plasma of patients with APL who were treated with doses of As_2O_3 that were well tolerated and that led to complete clinical remission *(21,22)*. We previously reported *(14)* that selective degradation of the PML/RARα protein occurs as NB4 APL cells respond to retinoic acid. Immunohistochemical and western blot analyses have suggested that a loss of PML/RARα protein also occurs with As_2O_3 treatment in NB4 cells *(21,43)*. We find that As_2O_3 induces the loss of this fusion protein both in NB4 cells and in multiple retinoic acid-resistant subclones, as shown by western blot analysis (Fig. 2, A), PML and PML/RARα immunohistochemistry (Fig. 4), and a ligand binding assay (Fig. 3). This loss of PML/RARα protein is also observed in fresh leukemia cells from patients with APL (Fig. 2, C). Furthermore, the ability of As_2O_3 to down-regulate PML/RARα protein selectively is not limited to APL cells. In the U937/PR9 subclone stably expressing PML/RARα protein, As_2O_3 treatment leads to a loss of the exogenous fusion protein without changing the level of the endogenous RARα protein (Fig. 3, E).

These data suggest that the loss of PML/RARα protein expression may be important for As_2O_3-induced apoptosis. Expression of PML/RARα prevents the apoptosis induced by growth factor withdrawal in U937 and TF-1 cells *(9,23,24)* and leads to the transformation of chicken bone marrow cells *in vitro* and the induction of acute leukemia in chicken embryos *(44)*. Furthermore, PML has been shown to function as a growth suppressor, and co-transfection of PML/RARα inhibits the transformation-suppressor function of PML *(45,46)*. We have confirmed the report by Chen et al. *(21)* that both the U937 and HL-60 cells, which do not express PML/RARα, are less responsive to doses of As_2O_3 capable of inducing apoptosis in retinoic acid-sensitive and retinoic acid-resistant APL cells (data not shown). To determine whether PML/RARα expression directly affects the responsiveness of leukemia cells to As_2O_3, we compared the response of the U937/PR9 subclone to As_2O_3 with that of a mock-transfected control. The U937/PR subclone did not show an increased growth inhibition or apoptotic response to As_2O_3 (data not shown), despite the loss of PML/RARα protein expression. This result suggests that the loss of PML/RARα leads to apoptosis only in cells that are dependent on its expression for their leukemic growth.

NB4.306, one of the retinoic acid-resistant subclones exam-

ined in this study, shows no expression of PML/RARα protein on western blot and binding analysis. However, NB4.306 cells still exhibit a cellular PML distribution typical of APL *(19)*, suggesting that low levels of PML/RARα may be present, undetectable by western blot analysis but still able to delocalize PML. The response of NB4.306 cells to As_2O_3 suggests that either molecular mechanisms other than the degradation of PML/RARα protein are involved or that As_2O_3 causes the degradation of the remaining PML/RARα protein. The immunohistochemistry pattern of PML–PML/RARα staining in NB4.306 cells observed before and after treatment with retinoic acid or As_2O_3 is consistent with the latter possibility.

Although retinoids induce terminal cytodifferentiation and As_2O_3 induces apoptosis of APL cells, both induce the degradation of PML/RARα. Thus, PML/RARα may be a common target of both retinoic acid and As_2O_3, but with two potential pathways of cellular response. As_2O_3 induces the loss of PML/RARα in retinoic acid-resistant cells, suggesting a different mechanism of action on PML/RARα protein. Indeed, our studies on fresh APL cells *in vitro* show that the addition of As_2O_3 interferes with the capacity of t-RA to induce cellular differentiation, while the addition of t-RA reduces cell apoptosis induced by As_2O_3. This finding suggests that the downstream pathways of response to these agents may compete with each other. To begin to understand how these two agents induce overlapping but distinct pathways, we investigated whether other genes involved in the retinoic acid-signaling pathway also play a role in response to As_2O_3 treatment. We found that As_2O_3 has no effect on the expression of genes associated with granulocytic differentiation or on the expression of the six retinoid receptor isoforms. RARβ expression, however, is induced by retinoic acid treatment. Since RARβ has been linked to retinoid-induced differentiation in a variety of cellular systems and *in vivo (47–50)*, its selective induction may play a role in defining the retinoid-induced phenotype in APL cells. Furthermore, the induction of type II TGase by t-RA was impaired by pretreatment with As_2O_3, suggesting that As_2O_3 may suppress gene regulation involved in differentiation. On the other hand, the fusion protein PML/RARα disrupts the normal functions of both unrearranged PML and RARα, and our immunohistochemistry data are consistent with a very recent report *(51)* that the unrearranged PML may be selectively lost after As_2O_3 treatment. Thus, As_2O_3 may also differ from retinoic acid in its effects on still poorly defined PML pathways.

APL is a disease with abnormal cell differentiation and proliferation, caused by the fusion of the PML and RARα genes. As_2O_3 induces apoptosis without differentiation in retinoic acid-sensitive and retinoic acid-resistant APL cells. As_2O_3, like retinoic acid, induces a loss of PML/RARα protein and may be most effective in cells whose malignant growth is mediated by PML/RARα expression. However, both As_2O_3 and retinoic acid have additional distinct molecular actions and may interfere with each other's phenotypic response. This circumstance suggests that these two agents, although both effective in treating APL, may not show enhanced activity if given in combination. Further studies of the mechanisms of action of these two agents may better define their respective roles in the treatment of APL and, possibly, of other cancers.

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Notes

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