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Arsenic Trioxide Inhibits Neuroblastoma Growth *in Vivo* and Promotes Apoptotic Cell Death *in Vitro*

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Recent clinical studies have shown that inorganic arsenic trioxide (As_2O_3) at low concentrations induces complete remission with minimal toxicity in patients with refractory acute promyelocytic leukemia (APL). Preclinical studies suggest that As_2O_3 induces apoptosis and possibly differentiation in APL cells. Like APL cells, neuroblastoma (NB) cells are thought to be arrested at an early stage of differentiation, and cells of highly malignant tumors fail to undergo spontaneous maturation. Both APL and NB cells can respond with differentiation to retinoic acid (RA) treatment *in vitro* and probably also *in vivo.* For that reason we investigated the effect of As_2O_3 alone and in combination with RA on NB cell lines. *In vitro,* the number of viable NB cells was reduced at As_2O_3 concentrations around 1 μM after 72 h exposure. The IC50 in six different cell lines treated for 3 days was in the 1.5 to 5 μM concentration interval, the most sensitive being SK-N-BE(2) cells derived from a chemotherapy resistant tumor. The combined treatment with RA (1 and 3 μM) showed no consistent additional effect with regard to induced cell death. The effect of As_2O_3 on NB cell number involved As_2O_3-induced apoptotic pathways (decreased expression of Bcl-2 and stimulation of caspase-3 activity) with no clear evidence of induced differentiation. The *in vivo* effect of As_2O_3 on NB growth was also investigated in nude mice bearing tumors of xenografted NB cells. Although tumor growth was reduced by As_2O_3 treatment, complete remission was not achieved at the concentrations tested. We suggest that As_2O_3, in combination with existing treatment modalities, might be a treatment approach for high risk NB patients. © 2000 Academic Press

Key Words: **animal tumor model; apoptosis; arsenic trioxide; differentiation; neuroblastoma.**

Abbreviations used: APL, acute promyelocytic leukemia; FCS, fetal calf serum; GAP-43, growth-associated protein-43; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; MTT, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; NB, neuroblastoma; NPY, neuropeptide tyrosine; PBS, phosphate-buffered saline; RA, retinoic acid; RAR, retinoic acid receptor; TPA, 12-O-tetradecanoyl phorbol-13-acetate; TH, tyrosine hydroxylase.

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Advanced NB still remains one of the most challenging problems in pediatric oncology despite aggressive treatment, and the need for new or additional treatment strategies is obvious. More than 60% of the children over 1 year of age with neuroblastoma (NB) present with disseminated disease at diagnosis (1). Intensive therapeutic interventions including multiple agent chemotherapy, surgery, radiotherapy, and autologuous bone marrow transplantation have resulted in minor improvement on the overall survival for these patients, especially for patients older than two years (2, 3). *In vitro,* retinoic acid (RA) induces maturation of cultured NB cells (4–6), but until recently positive *in vivo* effects of RA have not been reported. However, high-dose RA treatment after autologous bone marrow transplantation showed encouraging results in a recent combined Children Cancer Group/Pediatric Oncology Group double randomized study (7).

Acute promyelocytic leukemia (APL) is characterized by the translocation t (15, 17) which generates a PML/RARα fusion protein involving the retinoic acid receptor (RARα), a nuclear transcription factor normally contributing to myeloid differentiation (8, 9). For almost ten years, patients with APL have been treated with RA, which induces differentiation by revoking the transcriptional repression of the fusion protein, thus reflecting a successful example of translational medicine (10, 11). Some years ago it was reported that low doses of arsenic trioxide (As_2O_3) are effective in the treatment of patients with relapsed APL (12) and this was recently confirmed in an independent clinical

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study (13). Two children, 9 and 13, were included in this study and the treatment was given without severe side effects (13). *In vitro*, the 50% survival rate of APL cells after As_2O_3 treatment for 3 days lies in a concentration interval between 1 to 2 μM (14). At these concentrations, As_2O_3 induces apoptosis in APL cells by down-regulation of the Bcl-2 protein (14) and activation of caspases 1 and 3 (13). In early reports it was shown that As_2O_3 downregulates PML/RARα (15, 16), and based on morphology and differentiation markers it was suggested that low concentrations of As_2O_3 induce differentiation of these APL cells (15). However, other studies have concluded that As_2O_3 does not induce differentiation of APL cells (16), and that the effect of As_2O_3 is independent on the down-regulation of PML or the RAR fusion protein (17), and at present the effect of As_2O_3 on APL cell differentiation appears to be an open issue.

The effect of low doses of As_2O_3 on tumor cell survival is not restricted to APL cells, As_2O_3 inhibits cell survival in a variety of cancer cells including cells of solid tumors (18–20). Like in APL, the NB tumor cells are blocked at an immature differentiation stage and in cultured NB cells this differentiation block can in some cell lines be released by drugs or combinations of growth factors (4–6, 21, 22). Furthermore, NB cells are derived from the sympathetic nervous system (23–25), and like sympathetic neuroblasts, NB express Bcl-2 (26–29). It is likely that down-regulation of Bcl-2 in NB cells will promote induction of an apoptotic process, and in NB tumors there appears to be a positive correlation at the cellular level between apoptotic cell death and low Bcl-2 expression (26, 27). The similarities in the biology of APL and NB cells, including Bcl-2 expression, a potential indirect target for As_2O_3, prompted us to investigate the effect of As_2O_3 on NB cell survival and differentiation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Drugs and chemicals. As_2O_3 (Sigma Chemicals, Inc, Milwaukee, WI) was dissolved in 1 M NaOH and kept as a stock of 30 mM As_2O_3 for up to 14 days. This stock was further diluted in Eagle's minimal essential medium for cell culture studies and in phosphate buffered saline (PBS) for animal injections. RA (Sigma) was prepared in a 2 mM stock in absolute ethanol and added to the culture medium to a final concentration of 1 or 3 μM. The phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Sigma), was dissolved in absolute ethanol and kept as a 1.6 mM stock.

Cell lines and culture conditions. Human NB cell lines SK-N-BE(2), IMR-32, SMS-MSN, and SH-SY5Y were cultured in minimal essential medium supplemented with 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (50 μg/ml) in a humidified incubator containing 95% air + 5% CO_2 atmosphere. The NB cell lines LA-N-1, and LA-N-2 were grown in RPMI-1640 medium and 10% FCS with the same supplements as above. For cell morphology and differentiation assays 10^6 cells/10 cm culture dish were exposed to different concentrations of As_2O_3 alone or in combination with RA. Morphological changes were followed by phase contrast microscopy. SK-N-BE(2) cells used for the animal studies were routinely harvested by trypsinization of confluent cultures, washed in cold PBS and kept on ice until subcutaneous injection in nude mice.

MTT reduction assay. The assay is based on the uptake of MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Promega, Madison, WI) by viable cells and its conversion to an insoluble dye via the action of succinate dehydrogenase, a reaction used to quantify number of viable cells. For the MTT assays 10,000 cells were seeded in 96-well microculture plates and three wells containing cells with no drugs were kept for measuring control cell viability. Six concentrations of As_2O_3 ranging from 0.3 to 40 μM with and without 1 μM RA were tested in triplicates. The cells with additives were grown in a cell incubator for 72 h before addition of MTT solution. The cells were incubated at 37°C for 4 h and the reaction was developed according to the supplier's protocol (Promega). The absorbance at 570 nm was recorded using an ELISA microplate reader.

RNA and protein hybridization analyses. NB cell lines exposed for three days to 1 or 3 μM As_2O_3 with and without 1 μM RA, were harvested and total RNA was extracted according to standard procedures. Ten micrograms of total RNA was electrophoretically separated on agarose gels containing formaldehyde and blotted onto filters as described (22). The filters were hybridized with ^{32}P-dCTP-labeled cDNA probes of *neuropeptide Y (NPY), growth-associated protein-43 (GAP-43)* and *glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).* Hybridizing mRNA was visualized by autoradiography and the bands were scanned using Fuji Film Science Imaging Systems LAS-100. The results were quantified using Science Lab 97 Image Gauge Ver. 3.0 relating *NPY* and *GAP-43* mRNA to the corresponding *GAPDH* mRNA levels.

For Western blot analyses, cells were seeded day 0 at a density of 2×10^6/10 cm dish and one day later As_2O_3 was added. After 3 days in As_2O_3 the medium was removed and the dishes were washed twice with ice-cold PBS before harvest. The cells were lysed in RIPA-buffer (10 mM Tris–HCl, 160 mM NaCl, 1% Triton X100, 1% Na deoxycholate, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, and protease inhibitors ("Complete, EDTA-free," Boehringer-Mannheim), and protein content in the cleared supernatant was determined by the method of Bradford. For SDS–PAGE, 120 μg of each sample were loaded on a 10% polyacrylamide gel. After blotting, the filter was blocked with 5% fat-free dried milk in Tris–HCl pH 8.0, 150 mM NaCl and 0.05% Tween 20. The anti-Bcl-2 antiserum (Santa Cruz Biotechnology) was used in a 1/500 dilution and the anti-tyrosine hydroxylase (TH) antibody (Boehringer-Mannheim) was diluted 1/500. The secondary anti-rabbit and anti-mouse horseradish peroxidase-linked antibodies (Amersham) were diluted 1/5000. The peroxidase reaction was visualized with Super Signal (Pierce) by exposure of X-ray film.

Fluorometric assay for caspase-3 activity. Caspase-3 activity was measured in cell lysates from non-treated and arsenic trioxide treated NB cells. Cells were grown in 6-well plates, 0.5×10^6 cells/well, for 48 and 72 h with and without As_2O_3 in increasing concentrations. Cleavage of the fluorogenic caspase-3 substrate DEVD-AMC (Upstate Biotechnology, Lake Placid) was performed according to manufacturer's instructions, using a Fluorostar plate reader and excitation and emission wavelengths of 390 and 460 nm. The caspase-3 inhibitor DEVD-CHO (3 μM; Pharmingen, San Diego) was used to confirm assay-specificity.

Experimental animals. Athymic mice of the NMRI strain (nu/nu) (Bogstad, Denmark) were housed in controlled environment and fed *ad libidum*. Cages were autoclaved and changed weekly. All procedures were carried out in accordance with the regional ethical committee for animal research, which approved the study (M101-99). Female mice 6 weeks of age weighing 20–25 g at arrival were used. After one week of acclimatization, the mice were anesthetized with Halothan and injected on the left side of upper back with 5 to 10×10^6 SK-N-BE(2) cells suspended in PBS to a volume of 200 μl. Groups of 5 mice were housed in each cage. The tumor take was high in the presented experiments, 39 of 40 animals had tumor-take after 7–12

days. At a tumor size of approximately 0.1 cm^3, As_2O_3 diluted in PBS to 400 μl was given intravenously daily in the tail vein. Control mice received 400 μl PBS without drug. The animals were randomized in three groups receiving 0, 200, or 400 μg As_2O_3. Tumor volume was estimated every second day measured when the mouse was secured in a Plexiglass holder. After three weeks the mice were sacrificed with CO gas, and animals and dissected tumors were weighed.

Tissue preparation, immunohistochemistry, and TdT-dUTP nick end labeling TUNEL). Mouse tumors were immediately fixed in 4% buffered formaldehyde at 4°C and processed for paraffin embedding before sectioning. Four to five micrometer sections were deparaffinized in xylene, rehydrated and incubated in 0.3% H_2O_2 and methanol for 20 min to block endogenous peroxidase activity. To enhance immunohistochemical staining the sections were microwaved to 95°C in 0.01 mM sodium citrate buffer (pH 7.3) for 10–15 min. After blocking of nonspecific binding with 1% bovine serum albumin for 20 min the optimally diluted primary antibody was incubated for 1 h at room temperature. Slides were rinsed and incubated 30 min with avidin-biotin-peroxidase conjugated secondary antibody. Diaminobenzidine was used as chromogen. The primary antibodies and dilutions were as follows: anti-human monoclonal anti-TH (Boehringer Mannheim) at 1:25 dilution, anti human monoclonal anti-Ki-67 (Dako) at 1:100 dilution, anti-human monoclonal anti Bcl-2 (Dako) at 1:100 dilution, and anti-mouse polyclonal anti CD 34 (Chemicon) at 1:100 dilution. The TUNEL staining was performed according to the manufacturer's protocol (Boehringer-Mannheim).

RESULTS

As_2O_3 Induces Cell Death of Cultured Human NB Cells

The effects of As_2O_3 on NB cell morphology and survival were tested on six different NB cell lines, IMR-32, LAN-1, LAN-2, SK-N-BE(2), SH-SY5Y, and SMS-MSN, which were grown in the presence of 0.3 to 40 μM As_2O_3. The morphology of the treated cells was assessed over a time period of 72 h. Exposure of the cells to As_2O_3 led to a concentration-dependent increase in floating cells, and consequently a loss of adherent cells. Cells of all tested cell lines were affected at As_2O_3 concentrations down to 1 μM in that the adhering cells began to round up and later detach. No signs of induced differentiation, like neurite outgrowth, was noted at the As_2O_3 concentrations tested. The combined effect of 1 or 3 μM RA and As_2O_3 on cell morphology was also tested. RA-treatment alone did induce neurite outgrowth in all cell lines except for LA-N-2 cells, but no additional positive effect on neurite outgrowth was seen when combined with As_2O_3 (data not shown). To quantify the effect of As_2O_3 ± RA on cell viability, MTT assays of 3 day cultures were performed. The concentration of As_2O_3 needed for 50% reduction of viable cells compared to control differed between cell lines, and was approximately 1.5 μM for SK-N-BE(2), 2.5 μM for IMR-32 and LA-N-1, and 5 μM for SH-SY5Y, SMS-MSN and LA-N-2 cells (Fig. 1 and data not shown). Treatment by As_2O_3 in combination with 1 μM RA did not affect the viability of the tested NB cells with the exception of IMR-32 cells. At high As_2O_3 concentrations these cells survived better in the presence of RA, while RA had the opposite effect at low concentrations of As_2O_3 (Fig. 1).

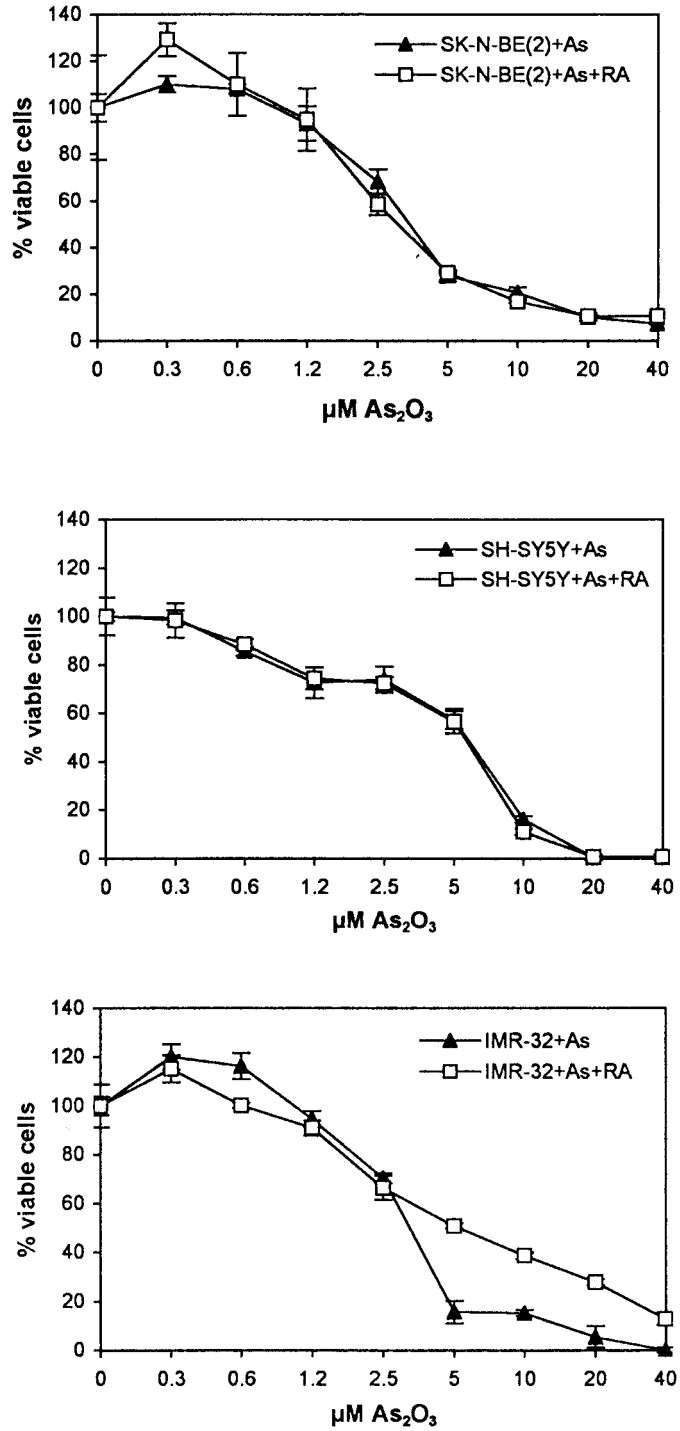

FIG. 1. Cell viability analysis of NB cells treated with arsenic trioxide alone or in combination with RA. Plated NB cells (SK-N-BE(2), SH-SY5Y and IMR-32) were treated with the indicated concentrations of As_2O_3 (As) for 3 days, without (filled triangles) or with (open squares) 1 μM RA. The amount of viable cells were then assessed using the MTT-assay. The result is presented as percentage of values obtained from treated compared to non-treated cultures ± SD.

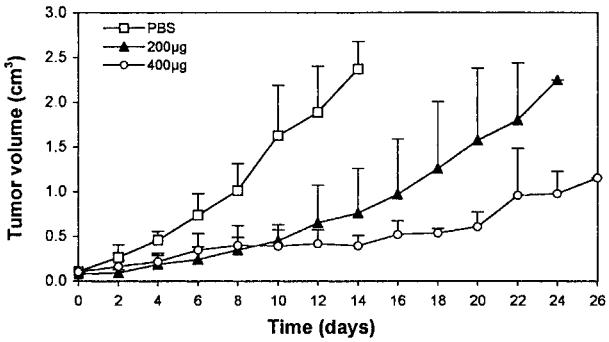

FIG. 2. *In vivo* growth of xenotransplanted human NB cells in nude mice and treatment with arsenic trioxide. SK-N-BE(2) cells were injected subcutaneously into the mice. When tumors had reached the volume of approximately 0.1 cm^3, treatment with solvent vehicle (PBS; open squares), or 200 (filled triangles), and 400 μg (open circles) As_2O_3 was initiated. In each treatment group 6 to 7 animals were used. Tumor sizes are given in cm^3 ± SD.

As_2O_3 Represses in Vivo Growth of Xenotransplanted Human NB Cells

In our hands, all cultured human NB cells so far tested form localized solid tumors when xenotransplanted into nude mice (data not shown). To test the *in vivo* effects of As_2O_3 on NB growth, mice were injected subcutaneously with SK-N-BE(2) cells, which form the most aggressively growing tumors of the NB cell lines tested. The animals were randomly assigned to three different groups when the tumors reached a volume of approximately 0.1 cm^3. Group 1 animals received PBS only, group 2 received 200 μg As_2O_3, and group 3 received 400 μg As_2O_3, as daily injections in the tail vein. Tumors in control mice receiving PBS grew rapidly, and these animals were sacrificed after 10 to 14 days of treatment due to large tumors (Fig. 2). Mice receiving As_2O_3 showed a dose-dependent inhibition of tumor growth compared to the control mice as shown in Fig. 2. Mice treated with 200 μg As_2O_3 did not show signs of severe side effects, except that they seemed slightly apathetic 1 to 2 min after injection of As_2O_3. No animal in this group lost weight during the As_2O_3 treatment, as compared to the control animals injected with PBS alone. Animals treated with 400 μg As_2O_3 suffered from side effects (anorexia, transient edema) resulting in weight loss at the end of treatment.

In an attempt to mechanistically understand the As_2O_3-induced repressed growth of the NB xenograft tumors, differences in number of proliferating and apoptotic cells, and the differentiation status of tumor cells in the dissected tumors, were assessed. Sections of tumors from non-treated and treated animals were immunohistochemically stained with antibodies directed towards Ki-67, Bcl-2, and TH, respectively, and sections were also stained with the TUNEL technique. None of these stainings resulted in statistically significant differences in the number of positive cells between treated and non-treated tumors, and based on CD 34 immunohistochemistry there were no apparent differences in the vascularization of the tumors (data not shown).

Phenotypic Effects of As_2O_3 on Cultured NB Cells

To further investigate possible mechanisms behind the *in vivo* effects of As_2O_3, cultured NB cells were exposed for 3 days to 1 and 3 μM As_2O_3 without or in combination with 1 μM RA and examined for potential effects on the differentiation status of these cells. Expression of two well-characterized markers of sympathetic ganglionic differentiation, *NPY* and *GAP-43* (21, 24), was analyzed by Northern blot hybridization. In SK-N-BE(2) and IMR-32 cells a discrete up-regulation of *NPY* mRNA was observed with 1 μM As_2O_3, while the *GAP-43* mRNA levels were unchanged or reduced (Fig. 3). In the presence of 1 μM RA *GAP-43* expression increased in both cell lines, which is in accordance with the morphological differentiation seen, while only the SK-N-BE(2) cells had increased *NPY* expression. The combination of RA and As_2O_3 consistently did not affect the expression levels of these marker genes when compared to the expression induced by RA alone (Fig. 3). The expression levels of another differentiation marker gene for the sympathetic lineage, *TH*, was not affected by low As_2O_3 concentrations, while treatment with 2 and 4 μM As_2O_3 reduced the TH levels in the SH-SY5Y and IMR-32 cells (Fig. 4). Based on morphology and marker gene expression data, we conclude that exposure of human NB cells to doses of As_2O_3 affecting the survival of these cells, did not induce differentiation in the cell lines investigated.

Neuroblasts of the developing human sympathetic nervous system express *Bcl-2* and the expression increases with developmental age (26). In these neuroblasts, *Bcl-2* expression is positively correlated to cell survival, which was demonstrated *in vitro*, and in a transgenic mouse model where overexpression of *Bcl-2* under the influence of the neuron-specific enolase promoter resulted in overgrowth and enlargement of sympathetic ganglia (30, 31). Based on the similarities in gene expression in sympathetic neuroblasts and in NB cells, we analyzed the Bcl-2 protein levels in NB cell lines treated with As_2O_3 at concentrations affecting the survival of these cells. As shown in Fig. 4, in two of three tested cell lines the Bcl-2 protein concentration began to decrease at 1 to 2 μM As_2O_3, with SK-N-BE(2) cells being more sensitive than SH-SY5Y cells. Phorbolester-differentiated SH-SY5Y cells served as a positive control with increased Bcl-2 expression in the sympathetically differentiated, TPA-treated, cells (26). The third cell line, IMR-32, did not show an appreciable decrease in Bcl-2 protein when treated with up to 4 μM As_2O_3 (Fig. 4), despite that As_2O_3 at this concen-

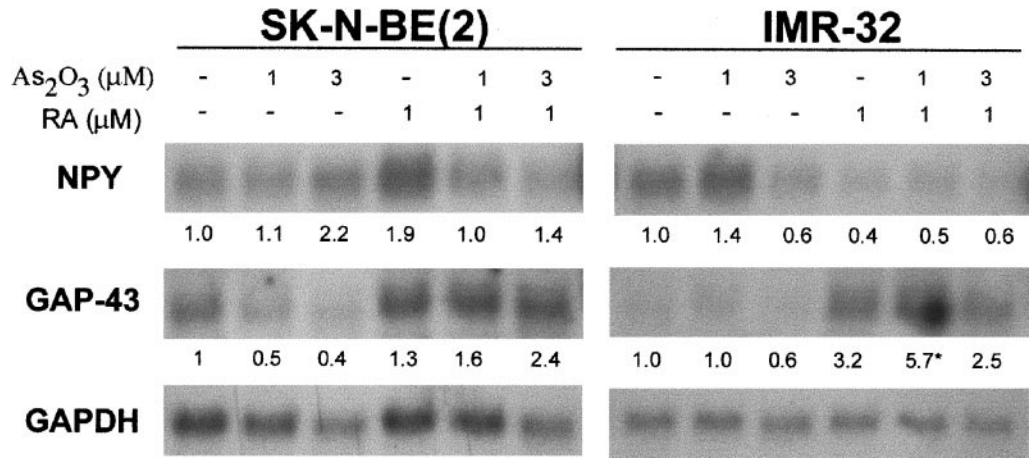

FIG. 3. Expression of sympathetic neuronal differentiation markers in arsenic trioxide and RA-treated NB cells. SK-N-BE(2) and IMR-32 NB cells were treated for 3 days with 1 or 3 μM As_2O_3 with or without 1 μM RA as indicated in the figure. Total RNA was prepared and the expression of *NPY* and *GAP-43* was analyzed by Northern blot hybridizations. *GAPDH* served as RNA loading control. *NPY* and *GAP-43* expression was quantified by scanning the autoradiographs, and the numbers in the figure give the expression relative to non-treated cells, after compensation for the *GAPDH* signal.

tration had a considerable capacity to kill IMR-32 cells (see Fig. 2).

To test whether the decrease in Bcl-2 protein seen in As_2O_3-treated SK-N-BE(2) and SH-SY5Y cells was linked to an induced apoptotic process, caspase-3 activity was measured in the arsenic trioxide-treated NB cells. In both SK-N-BE(2) and SH-SY5Y cells an increased caspase-3 activity was recorded after 48 and 72 h. Also in IMR-32 cells a slight increase in caspase-3 activity was detected (Fig. 5, 48 h data not shown). Thus, the reduced Bcl-2 protein levels and the activation of caspase-3 in As_2O_3-treated NB cells, together with lack of data supporting that these cells differentiate, suggest that the reduced number of viable cells seen *in vitro* (Fig. 1) at least in part is explained by apoptotic cell death, and not by differentiation-induced growth arrest.

DISCUSSION

In traditional Chinese medicine arsenic has been used as treatment of different cancers for centuries. Purified As_2O_3 suitable for intravenous administration, to overcome the side effects associated with oral administration, has been used in China since 1970 and has been proven to be an effective second line drug for patients with APL. Daily infusions of 10 mg As_2O_3 in adult patients with relapse of APL, give peak arsenic plasma levels of 4–6 μM in these patients (32). *In vitro*, 1 to 2 μM As_2O_3 is needed to obtain a 50% APL cell death after 3 days of treatment (15, 16). In the present study, sensitivity to As_2O_3 was found in all six tested NB cell lines, with 50% cell death obtained between 1.5 to 5 μM As_2O_3 depending on cell line tested. The drug had an inhibitory effect on tumor growth *in vivo* at As_2O_3 doses giving no apparent, early, side effects. Thus, similar to APL cells, NB cells appear to be sensitive to As_2O_3 both *in vitro* and *in vivo* at clinically achievable doses.

The studied NB cell lines are established from highly malignant tumors, and all but one, (SH-SY5Y) have an amplified *N-myc* gene. The cell lines established from untreated primary tumors or infiltrated bone marrow (IMR-32, LA-N-1, LA-N-2) were not more sensitive to As_2O_3 than those derived from patients with residual disease or relapse after treatment with chemotherapy (SK-N-BE(2), SH-SY5Y, a neuroblastic clone of the SK-N-SH cell line) (33–36). We chose to use the SK-N-BE(2) cell line in the animal model studies, a cell line forming very aggressively growing tumors in nude mice without signs of metastatic spread. This cell line was established from a 2-year-old boy with residual disease after induction therapy and these cells are more resistant to chemotherapy than those of a corresponding cell line established at diagnosis (33). Furthermore, these cells have a high p53 protein levels (data to be published) and in keeping with this, a mutated *p53* (37). As demonstrated here, the SK-N-BE(2) cells were sensitive to As_2O_3 suggesting minimal cross-resistance between As_2O_3 and chemotherapy resistance (36).

NB cell lines frequently respond to RA by differentiation and inhibited growth. Combination of RA with As_2O_3, did not consistently affect As_2O_3-induced cell death, and RA-induced phenotypic changes, like increased *GAP-43* expression, were unaltered by As_2O_3. We further conclude that As_2O_3 alone does not alter the differentiation status of the cultured NB cells tested. Thus, the reduced number of viable cells in cultures treated with As_2O_3 alone or in combination with RA is

not the result of a differentiation-induced growth inhibition. Instead, data suggest a toxic effect of arsenic trioxide involving mechanisms normally protecting NB cells from apoptotic cell death. At a molecular level this is seen as an As_2O_3-induced caspase-3 activation in all three cell lines tested, although weak in the treated IMR-32 cells. These results are in agreement with a recent study showing that 2 μM As_2O_3 induce apoptotic cell death in several NB cell lines, including SH-SY5Y and LA-N-2 cells, as determined by a DNA fragmentation assay (19). Interestingly, in that study As_2O_3-treated IMR-32 did not result in detectable DNA-fragmentation, which also is in agreement with the caspase-3 and Bcl-2 data presented here. While Bcl-2 expression in As_2O_3-treated IMR-32 was unchanged, the drug induced a concentration dependent reduction of Bcl-2 protein in the two other tested cell lines without an apparent general toxic effect on protein synthesis. Although SK-N-BE(2) cells were more sensitive to As_2O_3 than SH-SY5Y cells (Fig. 1), and the As_2O_3 concentration needed to significantly reduce the Bcl-2 levels were lower for the SK-N-BE(2) than the SH-SY5Y cells (Fig. 4), IMR-32 cells exemplifies that down-regulation of Bcl-2 is not a prerequisite for As_2O_3-evoked NB cell death. Whether this discrepancy reflects two different mechanisms by which As_2O_3 kills NB cells is an open question.

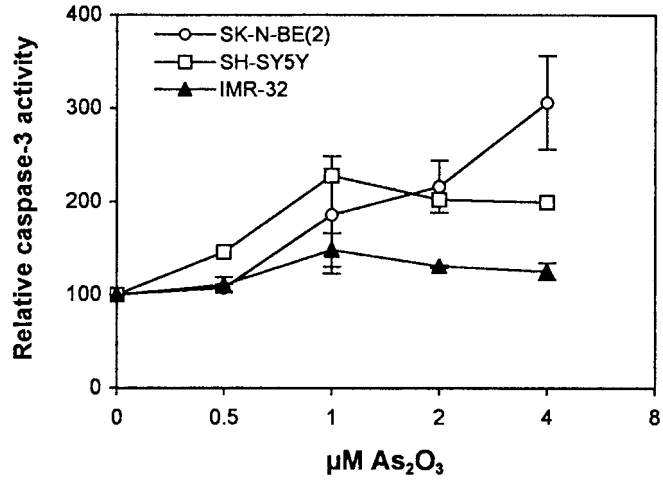

FIG. 5. Caspase-3 activity in As_2O_3-treated NB cells. Cells were treated for 72 h and lysates were assayed for caspase-3 activity using the fluorogenic substrate DEVD-AMC. The activity is expressed as % ±SD of the activity in non-treated cells (100%). SK-N-BE(2) (open circles); SH-SY5Y (open squares); IMR-32 (filled triangles). As_2O_3-induced caspase-3 activity was inhibited in all tested cell lines by 3 μM of the selective caspase-3 inhibitor DEVD-CHO.

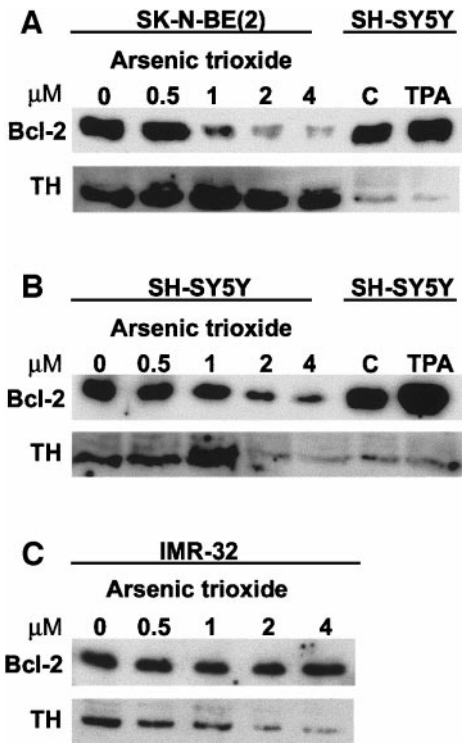

FIG. 4. Western blot analysis of Bcl-2 and TH in arsenic trioxide-treated NB cells. SK-N-BE(2), SH-SY5Y and IMR-32 NB cells were treated for 3 days with 0.5 to 4 μM As_2O_3. Floating cells were washed off and the remaining adherent cells were lysed and 120 μg total protein of each sample were subjected to SDS–PAGE and Western blotting. The protein blots were incubated with an anti-Bcl-2 antiserum and an anti-TH antibody, which detected a 26 kDa (Bcl-2) and a 60 kDa (TH) immunoreactive protein, respectively. Non-treated and 16 nM TPA-treated SH-SY5Y cells served as positive controls for BCL-2 expression.

The *in vivo* regress of NB tumors treated with As_2O_3 confirms the *in vitro* results, which suggest apoptotic cell death as a major mechanism. Compared to effective treatment doses in humans, relatively high doses of As_2O_3 were needed to achieve NB growth inhibition in mice. This is in agreement with a previous animal study on APL, where mice required higher doses than humans to obtain similar plasma levels (38). The positive toxic effect of As_2O_3 on tumor cells seemed not to be hampered by acquired drug resistance and *p53* mutation, and might suggest that As_2O_3 could be a clinically useful drug for treatment of relapsed NB.

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