Arsenic trioxide induces apoptosis in neuroblastoma cell lines through the activation of caspase 3 in vitro

Yukihiro Akao[a,*], Yoshihito Nakagawa[b], Kiyotaka Akiyama[c]

[a] *Gifu International Institute of Biotechnology, Mitake-cho, Kani-gun, Gifu 505-0116, Japan*
[b] *The Second Department of Internal Medicine, Osaka Medical College, Daigaku-cho, Takatsuki, Osaka 569-8686, Japan*
[c] *Pharmaceutical Frontiers Research Laboratories, Japan Tobacco, Inc., 6-2 Umegaoka, Yokohama 227-0052, Japan*

Received 8 April 1999; received in revised form 11 June 1999

Abstract Arsenic trioxide (As_2O_3) induces clinical remission in acute promyelocytic leukemia, even in all-*trans* retinoic acid-refractory cases, with minimal toxicity at low (1–2 μM) concentration. We exposed various neuroblastoma cell lines to As_2O_3 at a concentration of 2 μM: as a result, seven of 10 neuroblastoma cell lines underwent apoptosis characterized by morphological changes and nucleosomal DNA fragmentation. As_2O_3-induced apoptosis in neuroblastoma cells was shown to occur through the activation of caspase 3, as judged from Western blot analysis and apoptosis inhibition assay. It seemed that the sensitivity of neuroblastoma cells to As_2O_3 was inversely proportional to their intracellular level of reduced glutathione. Taken together these results indicate that As_2O_3 would be a candidate as a therapeutic agent for treatment of neuroblastoma, which is a solid tumor, not only by systemic therapy but also by local therapy.

Key words: Arsenic trioxide; Neuroblastoma; Apoptosis; Caspase 3; Glutathione

1. Introduction

Recently, arsenic trioxide (As_2O_3) at a low dose (1–2 μM) was shown to have a therapeutic effect against acute promyelocytic leukemia (APL) cells, even in patients resistant against all-*trans* retinoic acid (ATRA) or conventional chemotherapy [1,2], by induction of apoptosis of APL cells [2]. As for the mechanism of As_2O_3-induced apoptosis on APL cells, arsenic induces apoptosis directly by the down-regulation of Bcl-2 protein and modulation of promyelocytic leukemia (PML)-retinoic acid receptor α (RARα) fusion protein specific for APL cells with the t(15;17) translocation [3] and/or modulation of PML protein [4]. Furthermore, the activation of caspases was also involved in As_2O_3-induced apoptosis in APL cells [2].

Wang et al. reported that As_2O_3 and melarsoprol, the organic arsenical, brought about apoptosis in myeloid leukemia cell lines such as HL60, U937, and KG-1 through the down-regulation of Bcl-2 protein [5]. We also reported that As_2O_3 induced apoptosis through the down-regulation of Bcl-2 protein and activation of caspases in B-cell leukemia cell lines [6]. Thus, arsenic is cytotoxic not only to APL cells but also to other types of leukemia cells by induction of apoptosis through the down-regulation of Bcl-2 protein and/or activation of caspases.

Recently, it has been reported that reactive oxygen species (ROS) such as hydrogen peroxide might play a role as a mediator to induce apoptosis through the activation of caspase 3 [7,8]. Furthermore, intracellular reduced glutathione (GSH) content might be an indicator of sensitivity of As_2O_3 in leukemia cells [9], because arsenic toxicity results from forming reversible bonds with thiol groups of regulatory proteins including GSH [10].

On the other hand, chronic toxication of arsenic compounds has been evidenced in lung and skin cancers, noncancerous skin lesions, peripheral nerve effects, and cardiovascular changes [11]. Especially the nervous system effects progress to become widespread.

Based on these findings, we exposed 10 various neuroblastoma cell lines to 2 μM As_2O_3 and assessed its apoptotic effect.

2. Materials and methods

2.1. Reagents

As_2O_3 (Sigma) prepared in phosphate-buffered saline (PBS) at a concentration of 1 mM was used at a concentration of 2 μM in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). *N*-Acetylcysteine (NAC) (Sigma) was dissolved in PBS and used at a concentration of 10 mmol/l.

2.2. Cell culture and morphological study

Human cell lines were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS (CSL Ltd.) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO_2 at 37°C.

The neuroblastoma cell lines used in this study were the following: SH-SY5Y, LA-N-1, LA-N-2, LA-N-5, NB9, NB16, NB19, IMR-32, TNB-1, and CHP134 [12]. Exponentially growing cells were treated with As_2O_3 and/or other agents.

For morphological examination of apoptosis, cells were stained with Hoechst 33342 (5 μg/ml) at 37°C for 30 min and observed by fluorescence microscopy.

2.3. Assessment of nucleosomal DNA fragmentation

Cellular DNA was extracted from whole cells. RNase was added to the DNA solution at a final concentration of 20 μg/ml, and the mixture was incubated at 37°C for 30 min. Electrophoresis was performed on a 2.0% agarose gel. After electrophoresis, DNA was visualized by ethidium bromide staining.

2.4. Western blot analysis

The activation of caspases such as caspase 1, 3, 8 and 9, and the change of amount of Bcl-2 family members such as Bcl-2 and Bax were studied by Western blot analysis. Each cell protein lysate was prepared in lysis buffer (2×PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride), and then homogenized with an ultrasonic homogenizer. Thirty micrograms of protein of each homogenized sample was separated by SDS-PAGE using the appropriate percent polyacrylamide gel and electrophoresed onto a PVDF membrane (Dupont). After blockage of nonspecific binding sites for 1 h by 5% non-fat milk in TPBS (PBS and

*Corresponding author. Fax: (81) (574) 67-6627.
E-mail: giib21@kani.or.jp

PII: S0014-5793(99)00841-8

Table 1
Intracellular reduced glutathione levels of neuroblastoma cells

Cell line	LA-N-1	LA-N-2	LA-N-5	NB9	NB16	NB19	IMR-32	CHP134	TNB-1	SH-SY5Y
Glutathione content[a]	50.1 ± 2.25[b]	41.5 ± 2.36	37.2 ± 2.16	112.6 ± 4.25	36.2 ± 1.92	36.2 ± 2.25	46.3 ± 3.15	39.6 ± 2.12	33.6 ± 2.02	22.5 ± 1.42

[a]The glutathione content is expressed as nanomoles per miligram protein.
[b]The values was a average of the values in three independent experiments. Mean ± S.D. is given ($n = 6$).

0.1% Tween 20), the membrane was incubated overnight at 4°C with anti-human caspase 1 (Santa Cruz Biotechnology), anti-human caspase 3 (Transduction Laboratories), anti-human caspase 8 (Medical and Biological Laboratories), anti-human caspase 9 (Santa Cruz Biotechnology), anti-human Bcl-2 (100) (Santa Cruz Biotechnology) or anti-human Bax antibody (Medical and Biological Laboratories). The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega) at room temperature, and then washed three times with TPBS. The immunoblot was visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs).

2.5. Inhibition of apoptosis by caspase 3 inhibitor

For the study of inhibition of apoptosis, the tetrapeptide caspase 3 inhibitor acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic aldehyde (Ac-DEVD-CHO) (Peptide Institute) was added 12 h before As_2O_3 treatment. The optimal concentration of the inhibitor was determined from dose-response curves for the extent of cell death. Inhibition of apoptosis by caspase 3-like protease inhibitor was evaluated by the blockage of the process of nucleosomal DNA fragmentation.

2.6. Measurement of intracellular reduced glutathione

Intracellular GSH content was measured using the Glutathione Assay Kit (Calbiochem). Briefly, $2–3 \times 10^6$ cells were homogenized in 5% metaphosphoric acid using a Teflon pestle (Racine). The homogenate was centrifuged and its supernatant was used for GSH measurement according to the manufacturer's instruction. The pellet was dissolved in 1 N NaOH and used for the measurement for protein content [13]. The GSH content was expressed as nmol per mg protein. The values are the mean of three independent experiments.

3. Results

We used As_2O_3 at a concentration of 2 μM, which is clinically effective in APL patients [2]. Of the 10 neuroblastoma cell lines, seven (LA-N-2, LA-N-5, NB16, NB19, CHP143, TNB-1, and SH-SY5Y) underwent apoptosis as identified by nucleosomal DNA fragmentation, which is the biochemical hallmark of apoptosis, at a concentration of 2 μM As_2O_3 (Fig. 1 and Fig. 2A). Morphological findings of apoptosis such as cell shrinkage, chromatin condensation and nuclear segmentation were observed in all seven of the affected neuroblastoma cell lines (data not shown). As a representative example, nucleosomal DNA fragmentation and morphological characteristics of apoptosis of SH-SY5Y cells after treatment with 2 μM As_2O_3 are demonstrated in Fig. 2A and Fig. 3, respectively. The amount of nucleosomal DNA fragments gradually increased with the time of exposure to As_2O_3. Western blot analysis confirmed that the apoptotic process elicited by the exposure to As_2O_3 was executed through the activation of caspase 3, which cleaves the inhibitor of caspase-activated deoxynuclease, after which the caspase-activated deoxynuclease degrades the chromosomal DNA to nucleosomal units (Fig. 4) [14]. The time course of caspase 3 activation corresponded to that of nucleosomal DNA fragmentation. The formation of nucleosomal DNA fragments was significantly blocked by adding caspase 3 inhibitor to the medium, even at

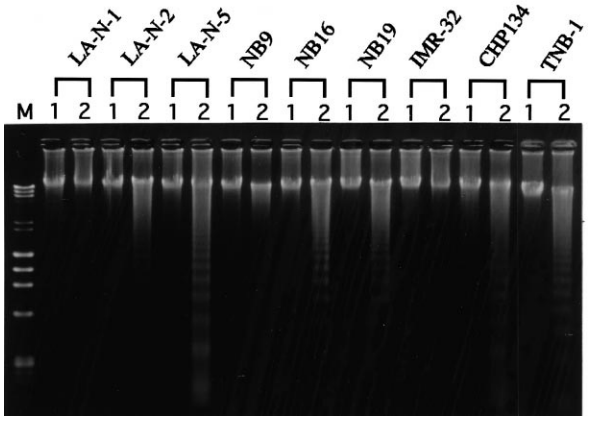

Fig. 1. Nucleosomal DNA fragmentation 72 h after treatment of nine neuroblastoma cell lines with 2 μM As_2O_3. Every two lanes are arranged pairwise. Lane 1 is DNA from untreated cells; lane 2, DNA from cells treated with As_2O_3 for 72 h. Lane M is a DNA size marker. The names of cell lines are also indicated.

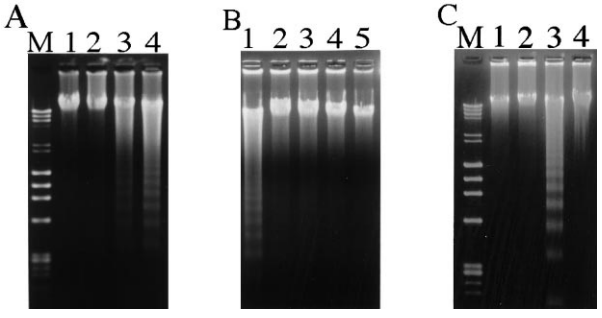

Fig. 2. Arsenic-induced apoptosis (A), its blockage by caspase 3-like proteases inhibitor (B), and treatment with *N*-acetylcysteine (C) in SH-SY5Y cells. 3 μg of DNA was applied in each lane. Nucleosomal DNA fragmentation was visualized by agarose gel electrophoresis. A: Lane M, DNA size marker; lane 1, DNA from untreated cells; lane 2, DNA from cells treated with 2 μM As_2O_3 for 24 h; lane 3, DNA from cells treated with 2 μM As_2O_3 for 48 h; lane 4, DNA from cells treated with 2 μM As_2O_3 for 72 h. B: The inhibitor, Ac-DEVD-CHO for caspase 3-like proteases, was added 12 h before As_2O_3 exposure. The rescue of cell death was evaluated at 72 h after As_2O_3 exposure by the block of formation of nucleosomal DNA fragments at each concentration of the inhibitor. Lane 1, DNA from cells treated with 2 μM As_2O_3 for 72 h (the same sample as A, lane 4); lane 2, DNA from cells treated with 50 μM inhibitor; lane 3, DNA from cells treated with 100 μM inhibitor; lane 4, DNA from cells treated with 200 μM inhibitor; lane 5, DNA from cells treated with 400 μM inhibitor. C: 10 mmol/l NAC was added 6 h before As_2O_3 exposure. The rescue of cell death was evaluated at 72 h after 2 μM As_2O_3 exposure or 2 μM As_2O_3 exposure, together with 10 mmol/l NAC by the block of formation of nucleosomal DNA fragments. Lane M, DNA size marker; lane 1, DNA from untreated cells; lane 2, DNA from cells treated with 10 mmol/l NAC for 72 h; lane 3, DNA from cells treated with 2 μM As_2O_3 for 72 h; lane 4, DNA from cells treated with 2 μM As_2O_3 and 10 mmol/l NAC for 72 h.

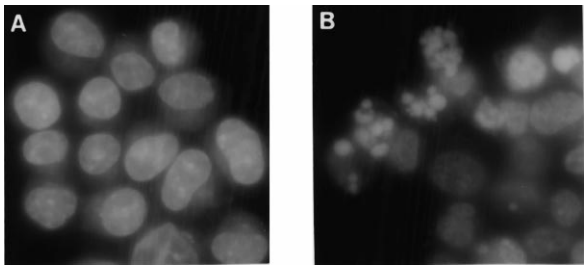

Fig. 3. Morphological aspects of Hoechst 33342-stained cells. Untreated SH-SY5Y cells (A) and SH-SY5Y cells treated with 2 μM As_2O_3 for 48 h (B).

a concentration of 50 μM, before treatment with As_2O_3 (Fig. 2B).

Activation of caspase 3 was found in all seven of the cell lines that showed nucleosomal DNA fragmentation, as judged from the results of Western blot analysis (data not shown). Activation of caspase 9 and caspase 8, which work upstream of caspase 3, was not found in As_2O_3-induced apoptosis of SH-SY5Y cells by Western blot analysis. In addition, neither down-regulation of Bcl-2 protein nor up-regulation of Bax protein was found. Thus, As_2O_3 is able to induce apoptosis in the majority of various neuroblastoma cell lines tested in this study through the activation of caspase 3.

The content of intracellular GSH was significantly high in cells of LA-N-1 and NB9, which did not undergo apoptosis, but very low in cells of SH-SY5Y, in which apoptosis was easily induced by As_2O_3 (Table 1). With regard to these three cell lines, the intracellular level of GSH was inversely correlated with the susceptibility to apoptosis elicited by exposure to As_2O_3. To examine the relationship between the content of intracellular GSH and As_2O_3-induced apoptosis, we treated SH-SY5Y cells with 2 μM As_2O_3 alone or together with 10 mmol/l NAC (a thiol-containing antioxidant capable of directly inactivating ROS as well as inducing GSH production) for 72 h. In Fig. 2C, NAC was able to protect SH-SY5Y cells from As_2O_3-induced DNA ladder formation completely.

4. Discussion

In the present study, we showed that As_2O_3 at a low dose (2 μM) induced apoptosis in seven of 10 neuroblastoma cell lines. As_2O_3 induces clinical remission in ATRA-resistant APL patients [1,2], which indicates that the mechanisms of apoptosis induced by As_2O_3 and ATRA are different. Then, the plasma concentration of As_2O_3 was sustained at 1–2 μM, which is thought to be a therapeutic and cancer-selective range in treating APL cells with minimal toxicity [1]. Earlier, we had accepted that As_2O_3 induces apoptosis in NB4 cells which have t(15;17) translocation by degrading PML-RARα fusion protein, which is directly associated with the pathogenesis of APL [3] and further seems to target especially to PML protein [4]. However, NAC and lipoic acid, which increase the content of GSH, blocked As_2O_3-induced apoptosis in NB4 cells regardless of inducing degradation of the PML-RARα chimeric protein [9]. Moreover, our results in this study indicate that As_2O_3 is also effective against solid tumors of neuroblastoma cells which have no PML-RARα chimeric protein.

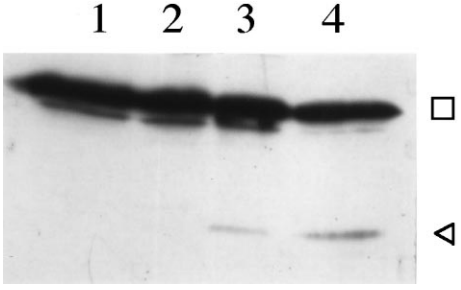

Fig. 4. Western blot analysis of caspase 3 in SH-SY5Y cells treated with 2 μM As_2O_3. 30 μg of protein of the homogenate was electrophoresed on a 12% SDS-polyacrylamide gel. Lane 1, untreated cells; lane 2, cells treated with 2 μM As_2O_3 for 24 h; lane 3, cells treated with 2 μM As_2O_3 for 48 h; lane 4, cells treated with 2 μM As_2O_3 for 72 h. Note that anti-human caspase 3 antibody recognized both the 32 kDa proenzymes (open square) and their 17 kDa active forms (open triangle).

The GSH redox system is known to modulate the growth-inhibitory effects of arsenicals [10]. Recently, Dai et al. reported that the sensitivity to As_2O_3-induced apoptosis in the several cell lines including NB4 cells was inversely correlated with the intracellular GSH content [9], which determines the sensitivity of cancer cells toward As_2O_3. In cells of low GSH content, because arsenic binds to sulfhydryl group containing compound such as GSH, the capacity to eliminate ROS is decreased, with the result that intracellular levels of ROS increase and they cause the activation of caspase 3 [7,8]. In our cases, cells with a high level of intracellular GSH such as LA-N-1 cells and NB9 cells did not undergo apoptosis, while cells with a low level of intracellular GSH such as SH-SY5Y cells, LA-N-5 cells, and TNB-1 cells were susceptible to undergo apoptosis. Moreover, NAC completely prevents the DNA ladder formation caused by exposure to As_2O_3 in SH-SY5Y cells. Thus, the intracellular GSH content would be a good indicator for application of As_2O_3 to various kinds of cancer cells.

Although it was reported by us [6] and others [3,5] that activation of caspase 3 was accompanied by the down-regulation of Bcl-2 in As_2O_3-induced apoptosis, we did not observe down-regulation of Bcl-2 in adherent SH-SY5Y cells by the analysis of Western blot. Furthermore, we did not find up-regulation of Bax and activation of caspase 9 which is associated with cytochrome *c* release from mitochondria. In samples from floating cells like leukemia cells, contamination of apoptotic cells which would not generate proteins actively for cell proliferation should be considered to assess the down-regulation of Bcl-2 by the analysis of Western blot, because it is unclear whether Bcl-2 contributes to the induction of apoptosis in early phase.

Based on these findings, it is considered that As_2O_3-induced apoptosis could be mediated by the common pathway in the process of cell death. It is still not clear whether As_2O_3 modulates the GSH redox system directly, but from our present results and previous report [6], arsenic most likely functions as a caspase 3 activator.

Thus, our results raise the possibility that As_2O_3 will be effective even against a solid tumor such as neuroblastoma and warrant clinical trials for patients with neuroblastomas not only by systemic therapy but also by local therapy.

Acknowledgements: This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] Shen, Z.X., Chen, G.Q., Ni, J.H., Li, X.S., Xiong, S.M., Qiu, Q.Y., Zhu, J., Tang, W., Sun, G.L., Yang, K.Q., Chen, Y., Zhou, L., Fang, Z.W., Wang, Y.T., Ma, J., Zhang, P., Zhang, T.D., Chen, S.J., Chen, Z. and Wang, Z.Y. (1997) Blood 89, 3354–3360.
[2] Soignet, S.L., Maslak, P., Wang, Z.G., Jhanwar, S., Calleja, E., Dardashti, L.J., Carso, D., De Blasio, A., Gabrilove, J., Scheinberg, D.A., Pandolfi, P.P. and Warrell Jr., R.P. (1998) New Engl. J. Med. 339, 1341–1348.
[3] Chen, G.Q., Zha, J., Shi, X.G., Ni, J.H., Zhong, H.J., Si, G.Y., Jin, X.L., Tang, W., Li, X.S., Xong, S.M., Shen, Z.X., Sun, G.L., Ma, J., Zhang, P., Zhang, T.D., Gazin, C., Naoe, T., Chen, S.J., Wang, Z.Y. and Chen, Z. (1996) Blood 88, 1052–1061.
[4] Zhu, J., Koken, M.H.S., Quignon, F., Chelbi-Alix, M.K., Degos, L., Wang, Z.Y., Chen, Z. and de Thè, H. (1997) Proc. Natl. Acad. Sci. USA 94, 3978–3983.
[5] Wang, Z.G., Rivi, R., Delva, L., Konig, A., Scheinberg, D.A., Gambacorti-Passerini, C., Gabrilove, J., Warrell Jr., R.P. and Pandolfi, P.P. (1998) Blood 92, 1497–1504.
[6] Akao, Y., Mizoguchi, H., Kojima, S., Naoe, T., Ohishi, N. and Yagi, K. (1998) Br. J. Haematol. 102, 1055–1060.
[7] Chen, Y.C., Lin-Shiau, S.Y. and Lin, J.K. (1998) J. Cell. Physiol. 177, 324–333.
[8] DiPietrantonio, A.M., Hsieh, T.C. and Wu, J.M. (1999) Biochem. Biophys. Res. Commun. 255, 477–482.
[9] Dai, J., Weinberg, S.R., Waxman, S. and Jing, Y. (1999) Blood 93, 268–277.
[10] Lee, T.C., Wei, M.L., Chang, W.J., Ho, I.C., Lo, J.F., Jan, K.Y. and Haung, H. (1989) In Vitro Cell Dev. Biol. 25, 442–447.
[11] Snow, E.T. (1992) Pharmacol. Ther. 53, 31–65.
[12] Nishi, Y., Akiyama, K. and Korf, B.R. (1992) Mamm. Genome 2, 11–20.
[13] Markwell, M.A.K., Hass, S.M., Tolbert, N.E. and Bieber, L.L. (1982) Methods Enzymol. 72, 296–303.
[14] Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamastu, A. and Nagata, S. (1998) Nature 391, 43–50.