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Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3

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Arsenic trioxide (As_2O_3) has shown considerable efficacy in treating hematological malignancies with induction of programmed cell death (PCD) type I, apoptosis. However, the mechanisms underlying the antitumor effect of As_2O_3 on solid tumors are poorly defined. Previously, we reported that As_2O_3 induced autophagic cell death (PCD type II) but not apoptosis in human malignant glioma cell lines. The purpose of this study was to elucidate the molecular pathway leading to autophagic cell death. In this study, we demonstrated that the cell death was accompanied by involvement of autophagy-specific marker, microtubule-associated protein light chain 3 (LC3), and damage of mitochondrial membrane integrity, but not by caspase activation. Analysis by cDNA microarray, RT–PCR, and Western blot showed that cell death members of Bcl-2 family, Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) and its homologue BNIP3-like (BNIP3L), were upregulated in As_2O_3-induced autophagic cell death. Exogenous expression of BNIP3, but not BNIP3L, induced autophagic cell death in malignant glioma cells without As_2O_3 treatment. When upregulation of BNIP3 induced by As_2O_3 was suppressed by a dominant-negative effect of the transmembrane-deleted BNIP3 (BNIP3ΔTM), autophagic cell death was inhibited. In contrast, BNIP3 transfection augmented As_2O_3-induced autophagic cell death. These results suggest that BNIP3 plays a central role in As_2O_3-induced autophagic cell death in malignant glioma cells. This study adds a new concept to characterize the pathways by which As_2O_3 acts to induce autophagic cell death in malignant glioma cells.

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Introduction

Malignant gliomas are the most common and lethal tumors in the central nervous system (Burton and Prados, 2000; Brandes *et al*., 2001). Despite significant advances in molecular analysis and several promising therapies for malignant gliomas, multimodality therapy including surgery, radiation, and chemotherapy is still the mainstay of the treatment. Even the most intensive combinations are not curative and yield only a modest impact on survival for most patients. The average life expectancy of the most malignant type, glioblastoma multiforme, is usually less than 1 year. Therefore, we need to explore new therapeutic agents or alternative therapeutic approaches.

Arsenic trioxide (As_2O_3) has a long history of use as a pharmaceutical agent (Miller, 2002; Miller *et al*., 2002). However, As_2O_3 has had a mainly unfavorable reputation due to its association with a poison or an environmental toxicity. Recently, As_2O_3 has shown considerable efficacy in treating patients with acute promyelocytic leukemia (APL), inducing apoptosis or differentiation (Shen *et al*., 1997; Soignet *et al*., 1998; Niu *et al*., 1999). Initially, studies focused on the role of the aberrant promyelocytic leukemia-retinoic acid receptor (PML-RAR) α fusion protein in mediating response to As_2O_3 (Chen *et al*., 1996). However, subsequent investigations have demonstrated that As_2O_3 broadly affects signal transduction pathways and causes many alterations in cellular functions leading to apoptosis (Miller, 2002; Miller *et al*., 2002). Recent studies demonstrate that not only hematological cancers other than APL but solid tumors derived from several tissues are susceptible to As_2O_3 (Akao *et al*., 1999; Zhu *et al*., 1999; Kitamura *et al*., 2000; Grad *et al*., 2001; Ishitsuka *et al*., 2002; Maeda *et al*., 2002). However, the mechanisms underlying the antitumor effect of As_2O_3 on solid tumors have yet to be clarified. More recently, we have demonstrated that As_2O_3 induces autophagic cell death (programmed cell death (PCD) type II) but not apoptosis (PCD type I) in malignant glioma cells (Kanzawa *et al*., 2003). Autophagy is a process in which subcellular membranes undergo dynamic morphological changes for degradation and turnover of cytoplasmic organelles (Klionsky and Ohsumi, 1999; Kim and

Klionsky, 2000; Klionsky and Emr, 2000). Although autophagy plays a key role in development, cellular response to stress or starvation, or human disease, the precise mechanisms regulating autophagy induced by cancer therapy still remain unclear.

In this study, we demonstrate that mitochondria-associated cell death proteins of Bcl-2 family, Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) (Boyd *et al*., 1994; Yasuda *et al*., 1998) and its homologue BNIP3-like (BNIP3L) (Imazu *et al*., 1999), are upregulated in As_2O_3-induced autophagy in malignant glioma cells. Interestingly, BNIP3, but not BNIP3L, plays a pivotal role in autophagic cell death. These results bring to light novel pathways of autophagic cell death induced by As_2O_3.

Results

Sensitivity of malignant glioma cells to As_2O_3

To determine the antitumor effect of As_2O_3, malignant glioma U373-MG, U87-MG, and T98G cells were treated with As_2O_3 at clinically obtainable concentrations (1.0–4 μM) for 96 h. As shown in Figure 1a, As_2O_3 inhibited the viability of tumor cells in a dose-dependent manner. The viable cell number of tumor cells treated with As_2O_3 (2 or 4 μM) for 96 h decreased below the initial cell number (5×10^3) (Figure 1b). These results indicate that treatment with As_2O_3 (2 or 4 μM) for 96 h not only inhibited cell viability but also induced cell death in malignant glioma cells.

Induction of autophagy in malignant glioma cells

To determine if treatment with As_2O_3 (4 μM) induces autophagy, the ultrastructure of treated U373-MG cells was analysed by electron microscopy. As shown in Figure 2Aa and b, nuclear structure appeared normal in untreated or treated tumor cells. Autophagic vacuoles such as autophagosomes or autolysosomes were often observed in As_2O_3-treated U373-MG cells (Figure 2Ac). Additionally, the development of acidic vesicular organelles (AVOs), which is characteristic of autophagy, in As_2O_3-treated U373-MG cells was determined by a flow cytometric analysis. As shown in Figure 2Ba and b, As_2O_3 treatment increased red fluorescence (*y*-axis) in U373-MG cells from 5.8 to 25.4%, indicating the induction of AVOs. 3-Methyladenine (3-MA, 2 mM), an inhibitor of autophagy (Kanzawa *et al*., 2004), suppressed the development of AVOs in untreated or As_2O_3-treated U373-MG cells (2.6 or 14.5%) (Figure 2Bc and d). Similar results were observed in U87-MG and T98G cells treated with As_2O_3 (4 μM) for 96 h (data not shown). Microtubule-associated protein 1 light chain 3 (LC3) is a mammalian homologue of Apg8p/Aut7p essential for amino-acid starvation-induced autophagy in yeast (Kabeya *et al*., 2000). To assess if LC3 is implicated in As_2O_3-induced autophagy, U373-MG cells expressing LC3 fused green fluorescent protein (GFP-LC3) were treated with As_2O_3. Under the fluorescence microscope, GFP-LC3-transfected U373-MG cells showed diffuse distribution of green fluorescence in the absence of As_2O_3 (Figure 2Ca). In contrast, treatment with As_2O_3 (4 μM) for 96 h demonstrated increased punctate pattern of GFP-LC3 (Figure 2Cb). This indicates that LC3 is recruited to autophagosome membrane in As_2O_3-induced autophagy. 3-MA suppressed the autophagic expression patterns of LC3 (Figure 2Cc and d). To quantify the induction of autophagy, we counted 200 GFP-positive cells untreated or treated with As_2O_3 (4 μM) for 96 h and scored the percentage of tumor cells exhibiting autophagic status. As shown in Figure 2D, 53% of U373-MG cells treated with As_2O_3 showed autophagy, while autophagic cells

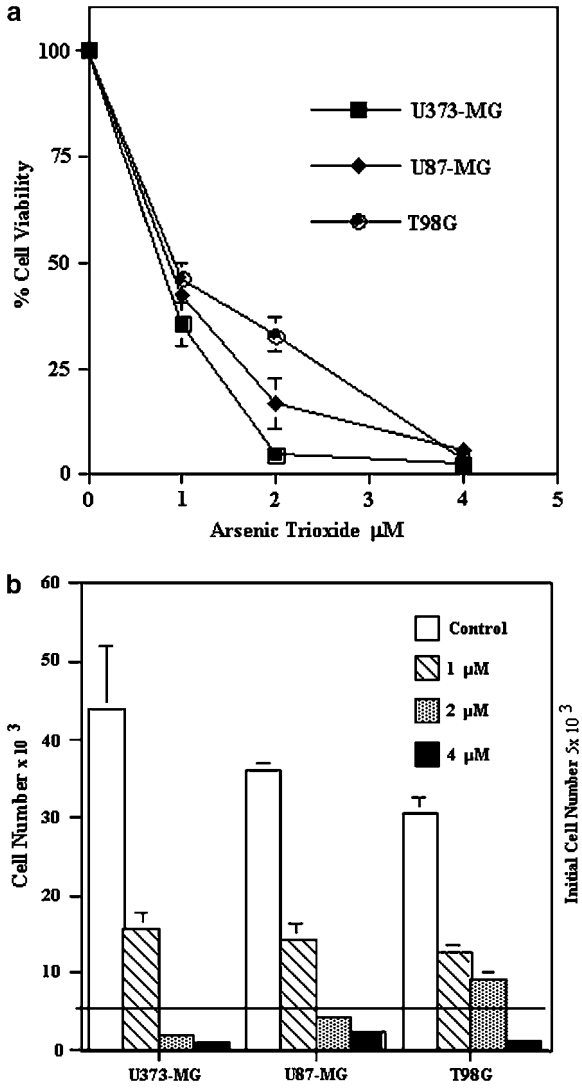

Figure 1 Sensitivity of malignant glioma cells to As_2O_3. U373-MG, U87-MG, and T98G cells were seeded at 5×10^3 cells per well (0.1 ml) in 96-well flat-bottomed plates and incubated overnight at 37°C. After exposure to As_2O_3 (1, 2, or 4 μM) for 96 h, the cells were trypsinized and the number of viable cells was counted. (**a**) Cell viability of three tumor cell lines. The viability of the untreated cells was regarded as 100%. (**b**) Actual cell number of three tumor cell lines. Results shown are the mean$\pm$s.d. of three independent experiments

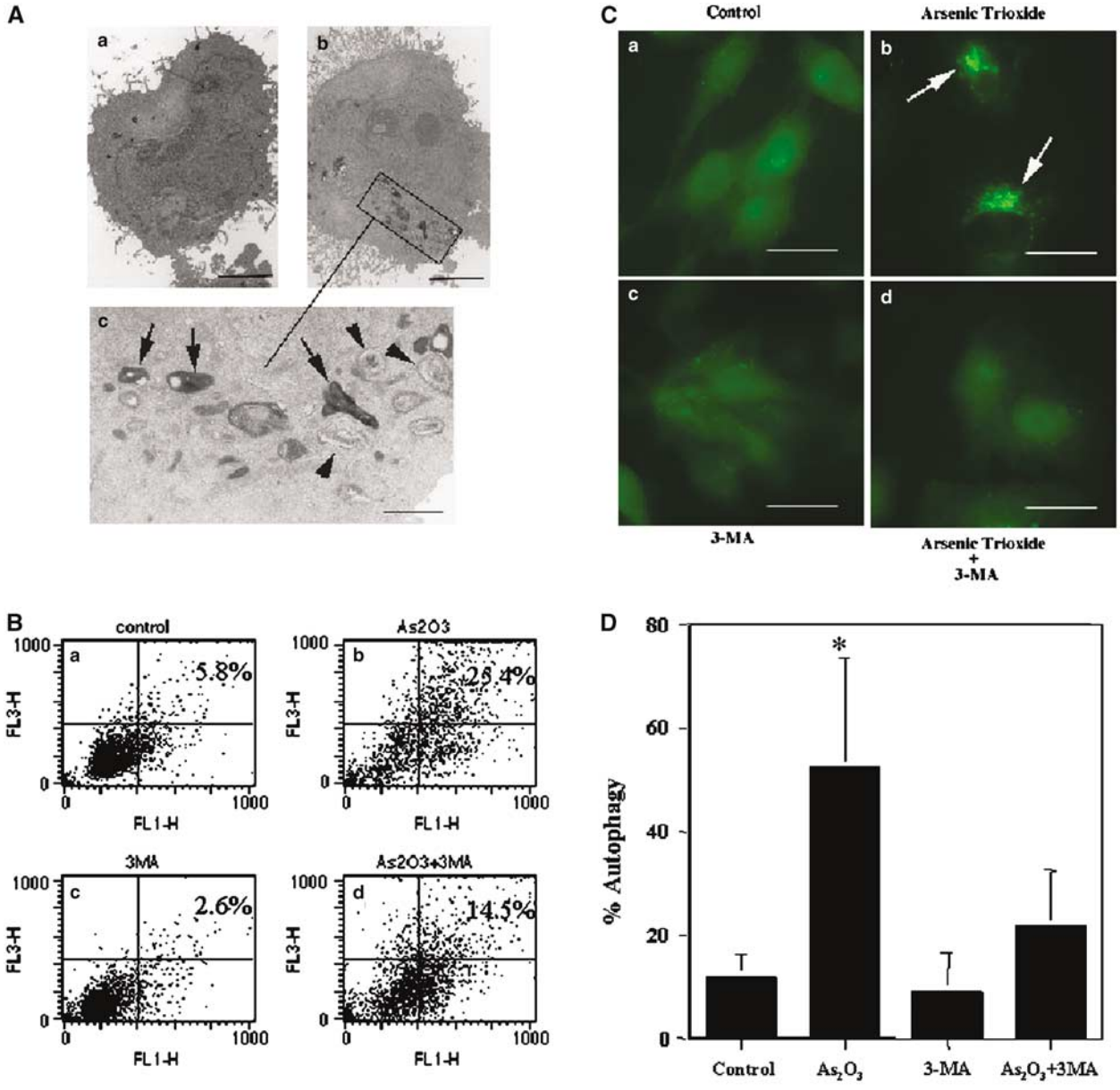

Figure 2 Induction of autophagy in malignant glioma cells by As_2O_3. (**A**) Ultrastructural features of As_2O_3-treated U373-MG cells for 96 h. (a) Control; (b) 4 μM As_2O_3 treatment; (c) high magnification of typical autophagic vacuoles. The arrows indicate autolysosomes and the arrowheads autophagic vacuoles. Bar: (a, b) 3.4 μm; (c) 1 μm. (**B**) Development of AVOs in As_2O_3-treated U373-MG cells. Detection of green and red fluorescence in acridine orange-stained cells using FACS analysis. Tumor cells were exposed to 4 μM As_2O_3 for 72 h and/or 3-MA (2 mM) was added one day after addition of As_2O_3. FL1-H indicates green color intensity (cytoplasm and nucleus), while FL3-H shows red color intensity (AVO). (a) Control; (b) 4 μM As_2O_3 treatment; (c) 3-MA (2 mM) treatment; (d) 4 μM As_2O_3 and 3-MA (2 mM) treatment. (**C**) Punctate signal of GFP-LC3 in As_2O_3-treated U373-MG cells. GFP-LC3-transfected U373-MG cells were exposed to 4 μM As_2O_3 for 72 h and/or 3-MA (2 mM) was added 1 day after addition of As_2O_3. (a) Control; (b) 4 μM As_2O_3 treatment; (c) 3-MA (2 mM) treatment; (d) 4 μM As_2O_3 and 3-MA (2 mM) treatment. Cells were examined by fluorescence microscopy. The arrows indicate typical autophagic cells with LC3 aggregation. Bar: (a–d) 14 μm. (**D**) Quantification of autophagic cells in As_2O_3-treated U373-MG cells. The percentage of autophagy was quantified by counting the number of cells showing the punctate pattern of LC3-GFP in 200 GFP-positive cells. Results shown are the mean $\pm$ s.d. of three independent experiments. *$P<0.05$

were detected in only 10% of untreated U373-MG cells. The incidence of autophagy in untreated or As_2O_3-treated tumor cells was decreased to 7 or 23% by 3-MA, respectively. As_2O_3-induced autophagy was significantly suppressed by 3-MA ($P<0.05$). These results together indicate that As_2O_3 induced autophagy in malignant glioma cells.

Involvement of caspase activation and mitochondrial damage in As_2O_3-induced cytotoxicity

To determine whether apoptosis is not occurring in malignant glioma cells treated with As_2O_3, we assessed the involvement of caspase activation by using caspase inhibition assay. As shown in Figure 3A, 50 μM

z-VAD-fmk (pan-caspase inhibitor) did not significantly influence the cell viability of U373-MG cells treated with As_2O_3 (4 μM) for 48 h. On the other hand, z-VAD-fmk inhibited caspase-dependent apoptosis induced by cisplatin (Kondo *et al*., 1995). As_2O_3 has been reported to induce apoptosis in leukemia cells via mitochondrial disruption (Kitamura *et al*., 2000). Therefore, to determine if mitochondrial membrane integrity is damaged in autophagic cell death induced by As_2O_3, mitochondrial membrane potential was measured using the tetramethylrhodamine, ethyl ester, per chlorate (TMRE) staining. As shown in Figure 3B, As_2O_3 treatment induced the membrane potential loss from 5.4 to 22.3% in U373-MG cells. The permeability transition (PT) pore inhibitor cyclosporin A (Sullivan *et al*., 1999) partially blocked mitochondrial membrane disruption in As_2O_3-treated U373-MG cells from 22.3 to 14.1%. Similar results were observed in U87-MG and T98G cells treated with As_2O_3 (data not shown). These results suggest that As_2O_3-induced autophagic cell death was associated with mitochondrial dysfunction, although caspase activation was not involved.

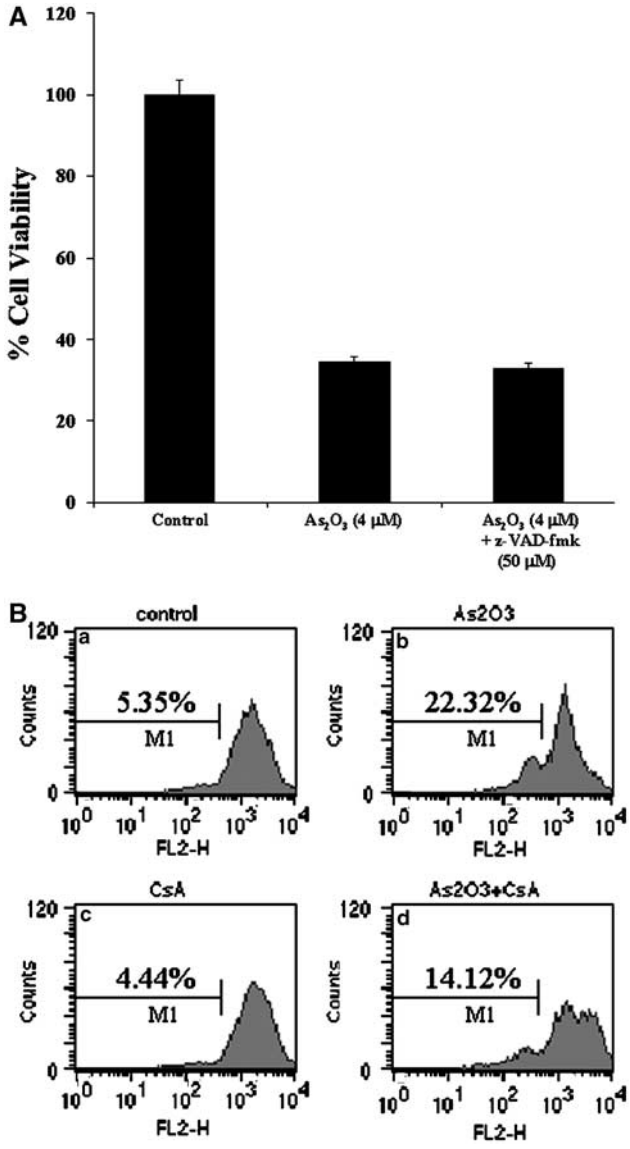

Figure 3 Involvement of caspase activation and mitochondrial damage in As_2O_3-induced cytotoxicity. (**A**) Effect of pan-caspase inhibitor, z-VAD-fmk, on the cell viability of U373-MG cells treated with As_2O_3. z-VAD-fmk (50 μM) was added to cells 1 h before the treatment with As_2O_3 (4 μM). After 2 days, the cell viability was determined. The viability of untreated U373-MG cells in the presence of diluted DMSO was regarded as 100%. Results shown are the mean $\pm$ s.d. of three independent experiments. (**B**) Disruption of mitochondrial membrane potential in U373-MG cells by As_2O_3. Mitochondrial membrane potential was measured by TMRE fluorescence using FACS. After treatment with As_2O_3 for 24 h, the cells were collected and stained with TMRE. (a) Control; (b) 4 μM As_2O_3 treatment; (c) cyclosporin A (5 μM) treatment; (d) 4 μM As_2O_3 and cyclosporin A (5 μM) treatment

Microarray analysis for As_2O_3-induced autophagic cell death

To screen which genes are associated with As_2O_3-induced autophagic cell death, we performed the cDNA microarray assay by using U373-MG cells treated with As_2O_3 (4 μM) for 0, 5, 10, 24, or 48 h. As shown in Figure 4, 173 genes among 14 285 genes were selected as differentially expressed based on variance structure. The left panel shows 173 genes with their expression profiles ordered by a hierarchical clustering algorithm. Each column represents a sample and each row represents a gene. The right panel shows a zoom-in sub-branch of the hierarchical tree along with gene names. Detailed annotations for genes showing altered expression are provided in Supplementary Information Table 1.

Upregulated genes in U373-MG cells treated with As_2O_3

Out of 173 genes, 54 genes were constantly upregulated with functional clustering. As for cell death-associated genes, only two genes, BNIP3L and protein phosphatase 1 regulatory subunit 15A (PPP1R15A), were increased during autophagic cell death. BNIP3L is a mitochondria-associated cell death protein showing significant homology with BNIP3 (Imazu *et al*., 1999). BNIP3L and BNIP3 possess the BH (Bcl-2 homology) 3 domain, which is a short stretch of sequences shared by all Bcl-2 family proteins (both the antiapoptotic and proapoptotic members) (Boyd *et al*., 1994; Yasuda *et al*., 1998; Imazu *et al*., 1999). It has recently been shown that BNIP3L and BNIP3 have proapoptotic activity in some types of cells (Imazu *et al*., 1999; Vande Velde *et al*., 2000; Guo *et al*., 2001) and hypoxia induces upregulation of these proteins (Sowter *et al*., 2001). PPP1R15A is one of the protein phosphatases 1 (PP1) that dephosphorylate proapoptotic BAD and allow BAD to interact with antiapoptotic Bcl-X_L and initiate cell death (Ayllon *et al*., 2000). However, the role of PPP1R15A in cell death was not fully understood. Vande Velde *et al*. (2000) propose that BNIP3 is a gene that mediates a nonapoptotic or necrosis-like cell death with autophagic features. Therefore, we assessed the detailed role of BNIP3 and BNIP3L in autophagic cell death as described below. Regarding signal transduction-associated genes, adrenomedullin and prostate

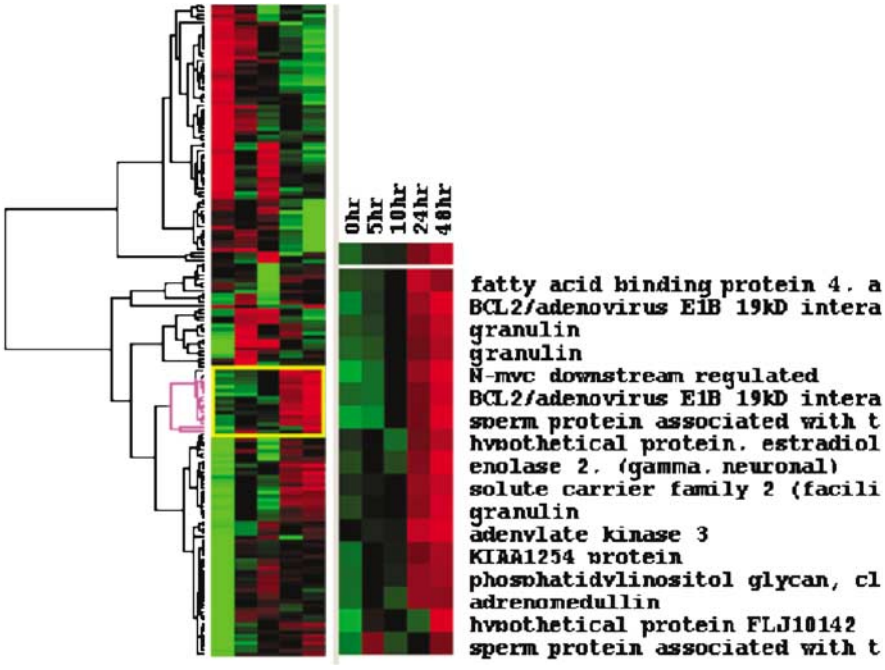

Figure 4 Hierarchical clustering of differentially expressed genes. RNA was isolated from U373-MG cells treated with As_2O_3 (4 μM) for 0, 5, 10, 24, or 48 h. The left panel shows 173 genes with their expression profiles ordered by a hierarchical clustering algorithm. Each column represents a sample and each row represents a gene. The right panel shows a zoom-in sub-branch of the hierarchical tree along with gene names. Coloring scheme: above median shown in red, below median in green, missing value in gray with brightness showing the scale. Black represents median expression level

differentiation factor, which are induced by hypoxic stress (Kitamuro *et al*., 2000; Albertoni *et al*., 2002), were upregulated. Therefore, these changes might be due to stress induced by As_2O_3 treatment. An increase in granulin, a human glioma-associated growth factor gene (Liau *et al*., 2000), might indicate the possibility that tumor cells might try to escape from cell death by stimulating their growth. As responses to external stimulus, several genes including N-myc downstream regulated gene 1, which was classified in databases as a tumor suppressor (Hunter *et al*., 2003), and heavy metal-response protein were upregulated. Upregulation of microtubule-associated protein light chain 3B among cell growth and/or maintenance-related genes indicated activation of autophagic pathway (Kabeya *et al*., 2000). An increase in heme oxygenase (decycling) 1 as antioxidation and stress response and metallothionein 1E as heavy metal ion transporter was consistent with recent investigation using arsenic for kidney cells (Zheng *et al*., 2003).

Downregulated genes in U373-MG cells treated with As_2O_3

Out of 173 genes, 38 genes were constantly downregulated with functional clustering. Pleiotrophin, an angiogenic and mitogenic growth factor, contributes to the malignancy of gliomas (Mentlein and Held-Feindt, 2002). Treatment with As_2O_3 reduced the expression of pleiotrophin, indicating that proliferation of U373-MG cells was suppressed. A decline in chemokine (C-X-C motif), hypothelical protein, and insulin-induced gene 1 detected in this study was consistent with a report by Zheng *et al* (2003). Recently, it has been shown that thrombospondin 1, a natural inhibitor of angiogenesis, is downregulated in malignant glioma cells by anoxia (Tenan *et al*., 2000). Therefore, inhibition of thrombospondin 1 might be due to hypoxic stress following exposure of tumor cells to As_2O_3.

mRNA and protein expression of BNIP3 or BNIP3L in As_2O_3-induced autophagy in malignant glioma cells

The cDNA microarray analysis indicated that BNIP3L might be upregulated during As_2O_3-induced autophagic cell death in malignant glioma cells. To confirm the result, semiquantitative RT–PCR was performed. As shown in Figure 5a, the expression of BNIP3L mRNA in U373-MG cells was increased in a time-dependent manner after exposure to As_2O_3. To determine whether the result obtained from U373-MG cells can be generalized to malignant glioma cells, we performed RT–PCR for BNIP3L mRNA using other glioma U87-MG and T98G cells. As shown in Figure 5a, treatment with 4 μM As_2O_3 increased the mRNA expression of BNIP3L gene in U87-MG and T98G cells. Moreover, we examined whether BNIP3L's homologue, BNIP3, is affected by treatment with As_2O_3. Figure 5a revealed that the expression level of BNIP3 mRNA was also increased in all tumor cells like BNIP3L mRNA. These results indicate that both BNIP3L and BNIP3 were transcriptionally upregulated in U373-MG, U87-MG, and T98G cells following As_2O_3 treatment. Then we performed Western blot analysis to determine whether

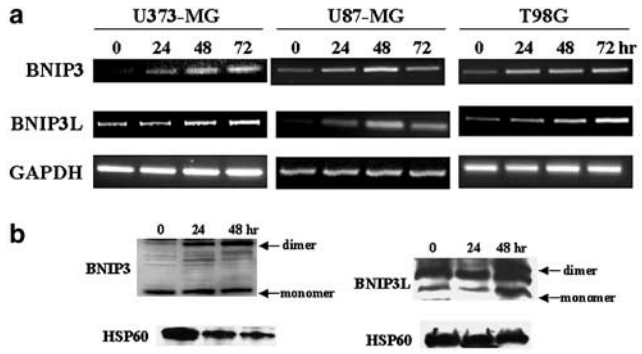

Figure 5 Expression of BNIP3 or BNIP3L in malignant glioma cells treated with As_2O_3. (**a**) Semiquantitative RT–PCR analysis of BNIP3 or BNIP3L mRNA in malignant glioma cells treated with As_2O_3. U373-MG, U87-MG, or T98G cells were treated with As_2O_3 (4 μM) for 0, 24, 48, or 72 h. RNA was reverse transcribed and amplified with specific primers for BNIP3, BNIP3L, or GAPDH. (**b**) Expression of BNIP3 or BNIP3L protein in U373-MG cells treated with As_2O_3. Mitochondrial fractions (40 μg each) from U373-MG cells treated with As_2O_3 (4 μM) for 0, 24, or 48 h were applied to Western blot using antibodies against BNIP3 or BNIP3L. Anti-HSP60 antibody was used as a loading control of mitochondrial protein

BNIP3 or BNIP3L protein expression is increased by As_2O_3 treatment. Because BNIP3 and BNIP3L are mitochondria-associated proteins (Boyd *et al*., 1994; Yasuda *et al*., 1998; Imazu *et al*., 1999), mitochondrial protein fraction was prepared for Western blot analysis. As shown in Figure 5b, treatment with As_2O_3 (4 μM) increased the expression of dimer but not monomer form of BNIP3 protein in U373-MG cells. On the other hand, As_2O_3 increased the dimer and monomer forms of BNIP3L in U373-MG cells. Most of Bcl-2 family proteins including BNIP3 and BNIP3L work with dimer formation in mitochondria (Yasuda *et al*., 1998). Therefore, an increase in the dimer expression of BNIP3 and BNIP3L proteins is supposed to correlate with an increase in their activities. Based on the results of cDNA microarray, RT–PCR, and Western blot analysis, we suggest that BNIP3 and BNIP3L might play an important role in As_2O_3-induced autophagic cell death in malignant glioma cells.

Overexpression of BNIP3 induced autophagy in malignant glioma cells

Based on the above results, we speculated that As_2O_3 activates BNIP3 and BNIP3L, and subsequently induces autophagic cell death in malignant glioma cells. To test our hypothesis, we determined if exogenous expression of BNIP3 or BNIP3L induces autophagy in tumor cells without As_2O_3 treatment by using the expression vectors of wild-type BNIP3 and BNIP3L. As shown in Figure 6a, BNIP3-transfected U373-MG cells exhibited autophagic change with aggregation pattern of GFP-LC3 48 h after BNIP3 gene transfection. Interestingly, the BNIP3 immunostaining of transfected cells showed punctate pattern. Because the Western blot analysis indicated that BNIP3 protein increased in mitochondria (Figure 5b), it is possible that exogenous expression of BNIP3 is colocalized with mitochondria. In contrast, β-galactosidase- or BNIP3L-transfected U373-MG cells did not show significant autophagic pattern of GFP-LC3 expression. To quantify these findings, we counted 200 BNIP3-, BNIP3L-, or β-galactosidase-positive tumor cells and scored the percentage of autophagic cells (Figure 6b). When the control β-galactosidase gene was transduced, 22% of β-galactosidase-positive U373-MG cells showed autophagy, indicating that the vector transfection itself induced autophagy to some extent. On the other hand, overexpression of BNIP3 or BNIP3L induced autophagy in 68 or 34% of U373-MG cells, respectively. The ability of BNIP3 to induce autophagy was significantly higher than that of β-galactosidase or BNIP3L gene ($P<0.01$). Exogenous expression of BNIP3 induced autophagy in 52% of U87-MG cells and in 60% of T98G cells, while autophagic cells were detected in 7% of U87-MG cells or in 28% of T98G cells after transfection with BNIP3L vector (Figure 6b). BNIP3 also significantly induced autophagy in U87-MG and T98G cells compared to β-galactosidase or BNIP3L ($P<0.01$). In addition, we determined whether over-expression of BNIP3 or BNIP3L induces cell death in malignant glioma cells. As shown in Figure 6c, BNIP3 expression induced cell death in 17% of U373-MG cells 2 days after the transfection. On the other hand, there was no significant difference in cell death incidence between expressions of β-galactosidase and BNIP3L. These results indicate that BNIP3, but not BNIP3L, induces autophagic cell death in malignant glioma cells without As_2O_3 treatment.

Effect of wild-type or dominant-negative BNIP3 on As_2O_3-induced autophagic cell death

To further assess the link between As_2O_3-induced autophagic cell death and BNIP3 upregulation, U373-MG cells expressing GFP-LC3 were transiently transfected with the wild-type BNIP3 vector or BNIP3ΔTM containing the transmembrane-deleted BNIP3 cDNA, which acts as a dominant-negative protein (Vande Velde *et al*., 2000; Kubasiak *et al*., 2002), prior to the treatment with As_2O_3 (4 μM). β-Galactosidase gene was cotransfected to detect wild-type BNIP3- or BNIP3ΔTM-transfected cells. Tumor cells expressing β-gal protein alone underwent autophagic cell death with punctate pattern of GFP-LC3 following a 24-h As_2O_3 treatment (Figure 7Aa and b). In contrast, BNIP3ΔTM-transfected cells exhibited no autophagic feature 24 h after exposure to As_2O_3 (Figure 7Ac and d). As shown in Figure 7B, downregulation of BNIP3 by BNIP3ΔTM inhibited As_2O_3-induced autophagy from 54 to 17%, whereas upregulation of BNIP3 by wild-type BNIP3 increased the incidence of As_2O_3-induced autophagy up to 74%. Then we determined the effect of BNIP3 or BNIP3ΔTM expression on the mitochondrial damage caused by As_2O_3. As shown in Figure 7C, U373-MG cells transfected with BNIP3, but not with BNIP3ΔTM, in the absence of As_2O_3 lost TMRE staining, indicating that BNIP3 overexpression itself

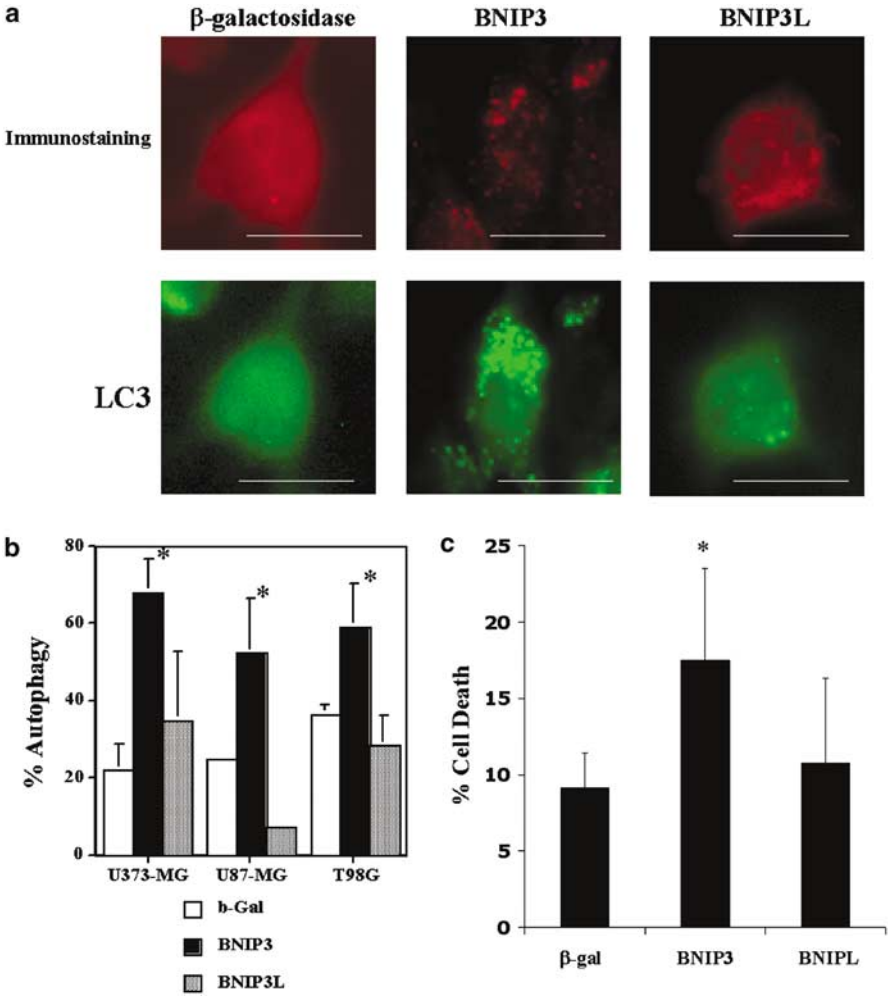

Figure 6 Overexpression of BNIP3 induces autophagy in malignant glioma cells. (**a**) U373-MG cells were cotransfected with the GFP-LC3 and β-gal, BNIP3, or BNIP3L expression vector. LC3 was detected with green fluorescence. β-gal, BNIP3, and BNIP3L proteins were stained immunohistochemically and detected with red fluorescence. Bar, 10 μm. (**b**) Quantification of autophagic cells transfected with the BNIP3 or BNIP3L expression vector. The number of cells showing a punctate pattern of GFP-LC3 in 200 β-galactosidase-, BNIP3L-, or BNIP3L-positive cells was counted. Results shown are the mean ± s.d. of three independent experiments. $*P<0.01$. (**c**) Cell death in malignant glioma cells by BNIP3 or BNIP3L expression. U373-MG cells were transiently transfected with the BNIP3 or BNIP3L expression vector in the presence of β-galactosidase expression vector and 2 days later, cell death was assessed. Results shown are the mean ± s.d. of three independent experiments. $*P<0.05$

induces loss of mitochondrial membrane potential. This observation was consistent with a previous report (Vande Velde *et al*., 2000). On the other hand, BNIP3-transfected U373-MG cells treated with As_2O_3 (4 μM) for 24 h lost TMRE staining more than As_2O_3-treated cells without BNIP3 transfection (Figure 7C), indicating that overexpression of BNIP3 augmented As_2O_3-caused mitochondrial damage. In contrast, BNIP3ΔTM-transfected U373-MG cells maintained TMRE staining even after exposure to As_2O_3 (4 μM) for 24 h, suggesting that As_2O_3-induced mitochondrial damage was rescued to some extent by BNIP3ΔTM expression. These results using wild-type and dominant-negative BNIP3 expression vectors indicate that As_2O_3-induced autophagic cell death was augmented by BNIP3 upregulation and was inhibited by downregulation of BNIP3. On the basis of the results demonstrating that As_2O_3-induced autophagic cell death and mitochondrial damage were closely related to BNIP3 expression, we conclude that BNIP3 plays a central role in As_2O_3-induced autophagic cell death rather than BNIP3L.

Discussion

As_2O_3 activates numerous intracellular signal transduction pathways, resulting in induction of apoptosis, promotion of differentiation, or inhibition of angiogenesis (Chen *et al*., 1996; Shen *et al*., 1997; Soignet *et al*., 1998; Niu *et al*., 1999; Miller *et al*., 2002; Miller, 2002). These multiple actions of As_2O_3 highlight the need to

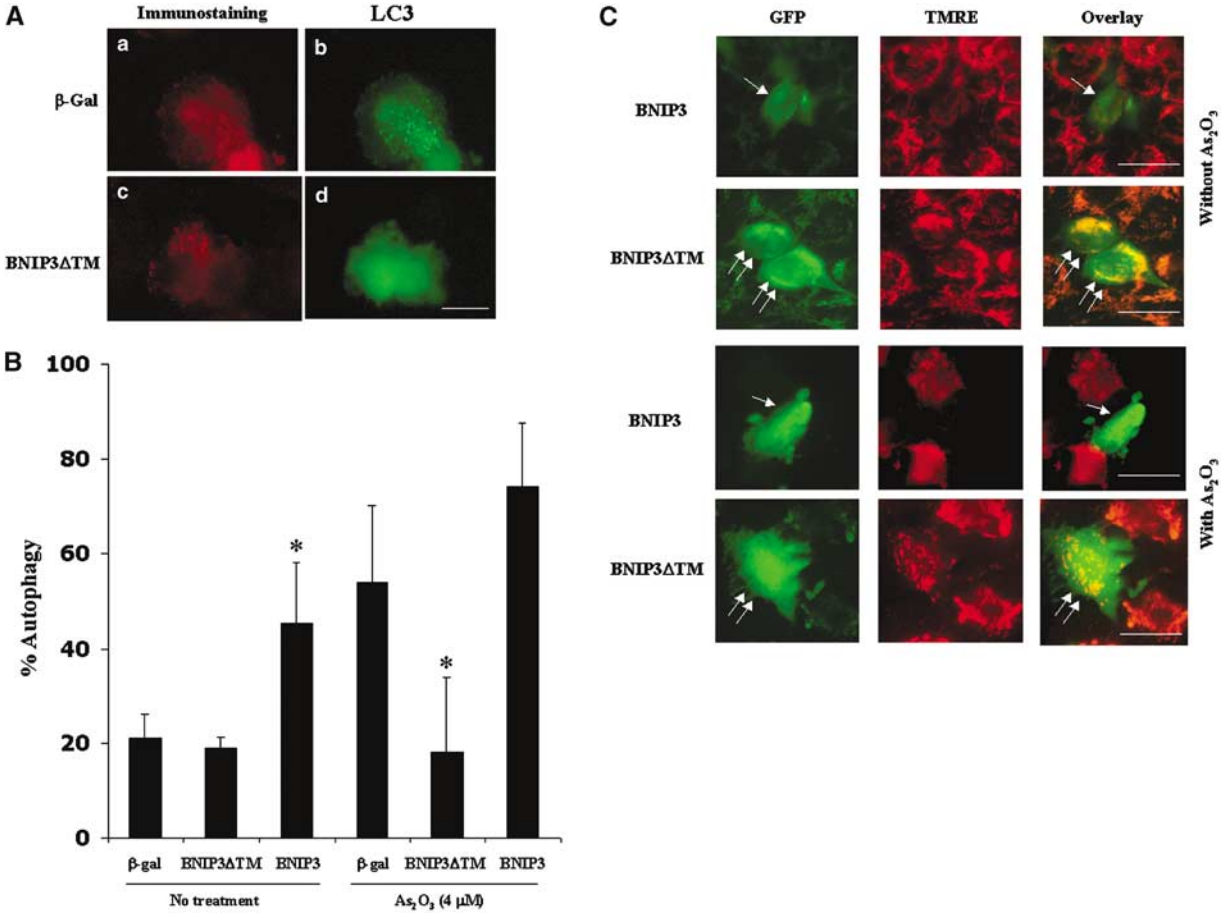

Figure 7 Effect of wild-type BNIP3 or dominant-negative BNIP3ΔTM on As_2O_3-induced autophagic cell death. (**A**) BNIP3ΔTM inhibits As_2O_3-induced autophagy. U373-MG cells were transiently transfected with the BNIP3ΔTM and/or β-galactosidase expression vector in the presence of GFP-LC3. The following day, tumor cells were treated with As_2O_3 (4 μM) for 24 h, stained with anti-β-galactosidase antibody, and the incidence of autophagy was observed. (a, b) Transfected with β-galactosidase and GFP-LC3 vectors; (c, d) transfected with BNIP3ΔTM, β-galactosidase, and GFP-LC3 vectors. Bar, 8 μm. (**B**) Effect of BNIP3 or BNIP3ΔTM on As_2O_3-induced autophagy. The incidence of autophagy was determined by counting autophagic cells among 200 β-galactosidase-positive cells treated as above. Results shown are the mean ± s.d. of three independent experiments. $^*P<0.01$. (**C**) Effect of BNIP3 or BNIP3ΔTM on As_2O_3-induced mitochondrial damage. U373-MG cells were transiently transfected with the BNIP3 or BNIP3ΔTM expression vector in the presence of GFP expression vector. After 24 h, transfected cells were treated with or without As_2O_3 (4 μM) for 24 h. Then, treated tumor cells were stained with TMRE and observed under a fluorescence microscope. Arrows and double arrows indicate BNIP3- and BNIP3ΔTM-transfected cells, respectively. Bar, 10 μm

define the molecular mechanisms mediating the effect of As_2O_3 on cancer cells. With regard to hematological malignancies, there is accumulating evidence that As_2O_3 induces apoptosis at clinically used concentrations (Chen *et al*., 1996; Shen *et al*., 1997; Soignet *et al*., 1998; Niu *et al*., 1999). However, the mechanisms by which As_2O_3 exhibits antitumor effect on solid tumors are poorly defined. Recently, we have reported that As_2O_3 induces autophagic cell death but not apoptosis in malignant glioma cells (Kanzawa *et al*., 2003). The observation prompted us to determine the signal factors regulating As_2O_3-induced autophagic cell death in malignant glioma cells. In the present study, we provide for the first time direct evidence of the involvement of BNIP3 as an inducible factor that triggers autophagic cell death in all malignant glioma cells we tested following As_2O_3 therapy.

BNIP3 was initially identified as an adenovirus E1B 19-kDa-interacting protein (Boyd *et al*., 1994; Yasuda *et al*., 1998). BNIP3 is a cell death-inducing factor and a member of the BH3-only subfamily of Bcl-2 family proteins. Proteins in this group do not possess the BH1 and BH2 domains as the other Bcl-2 family members, but binds through a common BH3 domain (Chen *et al*., 1997). A major function of this class of proteins is to determine the on/off state of the mitochondrial PT pore (MPTP) (Puthalakath and Strasser, 2002). BNIP3 expression is normally undetectable in most organs, including brain, but can be induced by hypoxia (Vande Velde *et al*., 2000). Overexpression of BNIP3 induces caspase-independent nonapoptotic or necrosis-like cell death (Vande Velde *et al*., 2000; Guo *et al*., 2001). BNIP3-induced cell death is accompanied by rapid and profound mitochondrial dysfunction characterized by

opening of the MPTP (Vande Velde *et al*., 2000), which may be consistent with our results. However, increasing evidence indicates that BH3-only proteins generate non-MPTP channels to the mitochondrial membrane (Martinou and Green, 2001) and that BH3-only proteins act mainly via multi-BH-domain proteins of the Bcl-2 family (Puthalakath and Strasser, 2002). Therefore, we further need to investigate the role of MPTP in As_2O_3-induced autophagic cell death in malignant glioma cells.

In the present study, an increase in the dimer form of BNIP3's homologue, BNIP3L, was observed in malignant glioma cells treated with As_2O_3. BNIP3L shares 56% identity with the amino-acid sequences of BNIP3 and directly targets the mitochondria to induce loss of membrane potential and release of cytochrome *c* (Imazu *et al*., 1999). Moreover, overexpression of BNIP3L induced apoptosis in some cells (Imazu *et al*., 1999). When apoptosis was induced in various cancer cells including malignant glioma cells by EGR2 expression, BNIP3L and another Bcl-2 family proapoptotic protein Bak were transactivated (Unoki and Nakamura, 2003). In the present study, overexpression of BNIP3L did not induce autophagy or cell death significantly. Therefore, BNIP3L might have other functions than serving as an autophagic cell death inducer. BNIP3 upregulation by As_2O_3 failed to be detected by the cDNA microarray experiment. One explanation for this might be that only the genes having mean log expression level (LEL) > 5.59 and standard deviation > 0.5 were detected. The lower the threshold of the LEL set, the more the genes including BNIP3 that might be detected. Indeed, microarray analysis using U373-MG cells failed to demonstrate upregulation of BNIP3 and subsequent experiments indicated the importance of BNIP3 in As_2O_3-induced autophagic cell death. Therefore, the microarray data might be de-emphasized. However, the results of the microarray analysis demonstrated that hypoxic stress-associated molecules such as adrenomedullin, prostate differentiation factor, and heme oxygenase (decycling) 1 were upregulated in U373-MG cells treated with As_2O_3. Recent investigations show that BNIP3 and BNIP3L are upregulated in hypoxia-mediated cell death (Guo *et al*., 2001; Sowter *et al*., 2001; Kubasiak *et al*., 2002). These results together suggest the possibility that As_2O_3 may induce hypoxic status in tumor cells and subsequently BNIP3 is upregulated, leading to autophagic cell death. This possibility was supported by the stimulatory effect of wild-type BNIP3 transfection and the inhibitory effect of dominant-negative BNIP3ΔTM on As_2O_3-induced autophagic cell death and mitochondrial damage. Further study is, however, necessary to determine the direct association between mitochondrial damage and the autophagic cell death pathway from initiation to execution.

Based on its morphological appearances, PCD can be subdivided into at least two groups (Schwartz *et al*., 1993; Lockshin and Zakeri, 2001). Apoptosis or PCD type I is mainly mediated by a cascade of caspases (Kondo *et al*., 1995; Reed, 1999). It has typical morphological characteristics such as cell shrinkage and chromatin condensation. As for malignant glioma cells, chemotherapeutic agents such as cisplatin or taxol are well known to induce apoptosis (Kondo *et al*., 1995; Terzis *et al*., 1997). On the other hand, autophagic cell death or PCD type II is generally independent of caspases (Vande Velde *et al*., 2000; Lockshin and Zakeri, 2002; Kanzawa *et al*., 2003). Autophagy is characterized by the accumulation of autophagic vacuoles within the cytoplasm. It has been reported that Beclin 1 or PTEN is involved in induction of autophagy (Liang *et al*., 1999; Arico *et al*., 2001). Recently, we have demonstrated that γ-irradiation, As_2O_3, or temozolomide induces autophagy but not apoptosis in malignant glioma cells (Kanzawa *et al*., 2003, 2004; Yao *et al*., 2003). However, the precise mechanisms regulating autophagic process in cancer therapy are not yet defined. Although the biochemical mechanisms of apoptosis and autophagic cell death are profoundly different (Ferri and Kroemer, 2001), some links between the two types of cell death via mitochondria are indicated (Cohen *et al*., 2002). Elmore *et al*. (2001) suggested that induction of mitochondrial membrane permeabilization (MMP) at a low level, below the threshold required for induction of apoptosis, results in sequestering damaged mitochondria in autophagic vacuoles. When MMP is sufficiently high to sustain the active execution of cell death, apoptosis is induced. Therefore, it is likely that induction of apoptotic or autophagic cell death may depend on the level of MMP.

In summary, treatment with As_2O_3 upregulated mitochondrial cell death proteins BNIP3 and BNIP3L, and damaged mitochondrial membrane integrity. Exogenous expression of BNIP3, but not BNIP3L, induced autophagic cell death in malignant glioma cells without As_2O_3 treatment. Moreover, As_2O_3-induced autophagic cell death was enhanced by BNIP3 transfection and was inhibited by BNIP3ΔTM expression. These findings suggest that As_2O_3 induced stress status and BNIP3, one of stress-associated proteins, plays a pivotal role in execution of autophagic cell death. The present study not only adds a new concept to the actions of As_2O_3 but also sheds light on the pathway of autophagic cell death induced by As_2O_3.

Materials and methods

Reagent

Arsenic trioxide (As_2O_3) solution (0.1%) was kindly supplied by Dr Samuel Waxman (Mount Sinai Medical Center, NY, USA).

Tumor cell lines

Human malignant glioma U373-MG, U87-MG, and T98G cells were purchased from ATCC (American Tissue Culture Collection, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell viability assay

The cytotoxic effect of As_2O_3 on malignant glioma cell lines was determined by using a Trypan blue dye exclusion assay as described previously (Kanzawa *et al*., 2004). Tumor cells were seeded at 5×10^3 cells per well (0.1 ml) in 96-well flat-bottomed plates and incubated overnight at 37°C. After exposure to As_2O_3 (1, 2, or 4 μM) for 96 h, the cells were trypsinized and the number of viable cells was counted. The viability of untreated cells was regarded as 100%.

Electron microscopy

To demonstrate morphologically the induction of autophagy in As_2O_3-treated tumor cells, the cells treated with or without As_2O_3 (4 μM) for 96 h were harvested by trypsinization, washed, and fixed with glutaraldehyde (3%) for 30 min. Then, we performed the ultrastructural analysis as described previously (Kanzawa *et al*., 2003, 2004).

Detection of acidic vesicular organelles with acridine orange

To quantify the development of AVOs in As_2O_3-treated cells, we stained tumor cells with acridine orange and measured the intensity of the red fluorescence as described previously (Kanzawa *et al*., 2004). To inhibit autophagy, 2.0 mM 3-MA was added next day after addition of As_2O_3. Green (510–530 nm) and red (> 650 nm) fluorescence emission from 10^4 cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur from Becton Dickinson using CellQuest software.

Involvement of LC3

Recently, GFP-LC3-expressing cells were used to demonstrate induction of autophagy (Kabeya *et al*., 2000; Mizushima *et al*., 2001; Kanzawa *et al*., 2004). Therefore, using the GFP-LC3 expression vector kindly supplied by Dr T Yoshimori and Dr N Mizushima (National Institute for Basic Biology, Okazaki, Japan), tumor cells were transiently transfected using Gene-Porter (Gene Therapy System Inc., San Diego, CA, USA). To detect the localization of LC3, GFP-LC3-transfected cells were observed under a fluorescence microscope.

Caspase inhibition assay

To determine whether caspase is involved in As_2O_3-induced cell death, caspase inhibition assay was performed using pan-caspase inhibitor, z-VAD-fmk (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) solubilized in DMSO as described previously (Kondo *et al*., 1995). At 1 h before treatment with As_2O_3 (4 μM), 50 μM z-VAD-fmk was added. After 2 days, cell viability was determined by Trypan blue staining.

Mitochondrial membrane potential

Mitochondrial membrane integrity was measured with TMRE (Molecular Probe Inc., Eugene, OR, USA) as described previously (Kanzawa *et al*., 2004). After treatment with As_2O_3 (4 μM) for 24 h, cells were stained with 150 nM TMRE in PBS for 30 min and observed under a fluorescence microscope. To measure the mitochondrial membrane potential, treated tumor cells were collected by trypsinization, stained with TMRE, and then analysed using flow cytometry. For inhibition experiment, the PT pore inhibitor cyclosporin A (Sigma) (Sullivan *et al*., 1999) was added 2 h before exposure to As_2O_3.

Gene expression profile by microarray analysis

Total RNA was isolated at 0, 5, 10, 24, and 48 h after exposure of U373-MG cells to As_2O_3 (4 μM) by using the Quiagen RNeasy kit (Valencia, CA, USA). At least 20 μg of total RNA with Abs 260 nm/Abs 280 nm ratio between 1.6 and 2.0 for each sample was obtained. cDNA microarray analysis was performed at the Microarray & Affymetrix Facility at the University of Texas MD Anderson Cancer Center, using established methods as described previously (Clifford *et al*., 2003). Briefly, RNA from each time point was converted into double-stranded cDNA using a cDNA synthesis kit (SuperScript Choice, GIBCO). After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription (IVT) reaction (T7 MegaScript System, Ambion). Aliquots of each sample (10 μg of cRNA in 200 μl of the master mix) were hybridized to the Human Genome U133A (HG-U133A) Gene Chip Array (Affymetrix, Santa Clara, CA, USA) at 45°C for 16 h in a rotisserie oven set at 60 rpm. The HG-U133A has 22 215 probe sets, 14 285 genes, 12 397 full lengths, and 12 735 known genes. Additionally, the HG-U133A includes a set of human maintenance genes (hybridization controls, poly-A controls, normalization controls, and housekeeping controls) to facilitate the normalization and scaling of array experiments. Thereafter, the arrays were washed with $6 \times$ SSPE and $0.5 \times$ SSPE, stained with streptavidin–phycoerythrin (Molecular Probes), washed again, and read by using a confocal microscope scanner with the 560-nm long-pass filter (Molecular Dynamics). Data analysis was performed using Positional Dependent Nearest Neighbor Model (PDNN) (Zhang *et al*., 2003), which gives estimated absolute expression levels corrected for probe binding affinity. The data set was further filtered to remove absent and invariably expressed genes across all the samples. The filtering criteria were applied to LEL so that, to survive the filtering test, the mean LEL across samples for a gene should exceed 5.59 and the standard deviation of LEL across samples for the gene should exceed 0.5.

Semiquantitative RT–PCR

The expression of BNIP3 or BNIP3L mRNA in tumor cells treated with or without As_2O_3 (4 μM) for up to 3 days was analysed by semiquantitative RT–PCR amplification as described previously (Unoki and Nakamura, 2003). Total RNA was isolated from untreated or treated cells using the RNeasy kit. An equal amount of RNA was reverse-transcribed to cDNA and amplified by PCR with ThermoScript™ RT–PCR System and Platinum *Taq* DNA polymerase (Invitrogen). PCR was performed with gene-specific primers for 21–30 cycles at 94°C for denaturing, 55°C for annealing, and 72°C for extension. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blot analysis

To detect BNIP3 or BNIP3L protein, mitochondrial protein extracts were prepared as previously described (Goldstein *et al*., 2000). Tumor cells treated with As_2O_3 (4 μM) for 0 to 48 h were washed in PBS and incubated for 30 min on ice in 300 μl lysis buffer (68 mM sucrose, 200 mM mannitol, 50 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, and $1 \times$ complete protease inhibitor (Boehringer Mannheim, Germany)). Cells were then lysed with 80 strokes of a dounce homogenizer (using a B-type pestle) and centrifuged at 4°C (800 *g*). The supernatant was centrifuged at 14 000 *g* for 10 min. The supernatant (cytosol) and pellets (mitochondria) were stored at −70°C for Western blot analysis. Proteins (40 μg) were separated in Tris-HCl SDS

gel and transferred to polyvinylidene difluoride membranes. The membranes were treated with antibodies against BNIP3 (BD Bioscience Pharmingen, San Diego, CA, USA), BNIP3L (Oncogene Research Product, San Diego, CA, USA), and mitochondrial protein, heat shock protein (HSP)-60 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Transient transfection assay

To investigate if exogenous expression of BNIP3 or BNIP3L induces autophagy in malignant glioma cells without As_2O_3 treatment, malignant glioma cells were transiently transfected with the BNIP3, BNIP3L, or β-galactosidase expression vector as described previously (Vande Velde *et al*., 2000; Sowter *et al*., 2001). To detect cells undergoing autophagy, the GFP-LC3 expression vector was cotransfected. After 2 days, cells were fixed in 1% paraformaldehyde, blocked with 10% normal rat serum, stained with anti-BNIP3 or -BNIP3L antibody or anti-β-galactosidase antibody (Promega), and visualized by Texas red conjugated secondary rat anti-goat IgG antibody. The percentage of BNIP3-, BNIP3L- or β-galactosidase-positive cells exhibiting autophagic pattern of GFP-LC3 expression was determined. To determine whether BNIP3 or BNIP3L induces cell death in malignant glioma cells, tumor cells were transfected with BNIP3 or BNIP3L in the presence of β-galactosidase expression. After 2 days, we counted 200 β-galactosidase-positive cells, and flat or round green cells were designated as live or dead as described previously (Imazu *et al*., 1999).

To detect the effect of wild-type BNIP3 or dominant-negative BNIP3ΔTM on As_2O_3-induced autophagic cell death, tumor cells were transiently transfected with the BNIP3 or BNIP3ΔTM vector in the presence of β-galactosidase and GFP-LC3 vectors. The following day, tumor cells were treated with As_2O_3 (4 μM) for 24 h and the incidence of autophagy was determined. BNIP3, BNIP3ΔTM, and BNIP3L vectors were kindly supplied by Dr AH Greenberg and Dr D Dubik (University of Manitoba, Manitoba, Canada).

Statistical analysis

Experiments were performed at least three times independently. Statistical differences were assessed using an unpaired Student's *t*-test comparing each data point to untreated cells. A probability (P) <0.05 was considered to be significant.

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References

Akao Y, Nakagawa Y and Akiyama K. (1999). *FEBS Lett.*, **455,** 59–62.
Albertoni M, Shaw PH, Nozaki M, Godard S, Tenan M, Hamou MF, Fairlie DW, Breit SN, Paralkar VM, de Tribolet N, Van Meir EG and Hegi ME. (2002). *Oncogene*, **21,** 4212–4219.
Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P and Ogier-Denis E. (2001). *J. Biol. Chem.*, **276,** 35243–35246.
Ayllon V, Martinez AC, Garcia A, Cayla X and Rebollo A. (2000). *EMBO J.*, **19,** 2237–2246.
Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, D'Sa-Eipper C and Chinnadurai G. (1994). *Cell*, **79,** 341–351.
Brandes AA, Basso U and Pasetto LM. (2001). *Expert Rev. Anticancer Ther.*, **1,** 357–370.
Burton EC and Prados MD. (2000). *Curr. Treat. Options Oncol.*, **1,** 459–468.
Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, Saxena S, Gietz RD and Greenberg AH. (1997). *J. Exp. Med.*, **186,** 1975–1983.
Chen GQ, Zhu J, Shi XG, Ni JH, Zhong HJ, Si GY, Jin XL, Tang W, Li XS, Xong SM, Shen ZX, Sun GL, Ma J, Zhang P, Zhang TD, Gazin C, Naoe T, Chen SJ, Wang ZY and Chen Z. (1996). *Blood*, **88,** 1052–1061.
Clifford JL, Yang X, Walch E, Wang M and Lippman SM. (2003). *Mol. Cancer Ther.*, **2,** 453–459.
Cohen I, Castedo M and Kroemer G. (2002). *Trends Cell Biol.*, **12,** 293–295.
Elmore SP, Qian T, Grissom SF and Lemasters JJ. (2001). *FASEB J.*, **15,** 2286–2287.
Ferri KF and Kroemer G. (2001). *Nat. Cell Biol.*, **3,** E255–E263.
Goldstein JC, Waterhouse NJ, Juin P, Evan GI and Green DR. (2000). *Nat. Cell Biol.*, **2,** 156–162.
Grad JM, Bahlis NJ, Reis I, Oshiro MM, Dalton WS and Boise LH. (2001). *Blood*, **98,** 805–813.
Guo K, Searfoss G, Krolikowski D, Pagnoni M, Franks C, Clark K, Yu KT, Jaye M and Ivashchenko Y. (2001). *Cell Death Differ.*, **8,** 367–376.
Hunter M, Bernard R, Freitas E, Boyer A, Morar B, Martins IJ, Tournev I, Jordanova A, Guergelcheva V, Ishpekova B, Kremensky I, Nicholson G, Schlotter B, Lochmuller H, Voit T, Colomer J, Thomas PK, Levy N and Kalaydjieva L. (2003). *Hum. Mutat.*, **22,** 129–235.
Imazu T, Shimizu S, Tagami S, Matsushima M, Nakamura Y, Miki T, Okuyama A and Tsujimoto Y. (1999). *Oncogene*, **18,** 4523–4529.
Ishitsuka K, Ikeda R, Utsunomiya A, Uozumi K, Hanada S, Suzuki S, Takeuchi S, Takatsuka Y, Takeshita T, Ohno N and Arima T. (2002). *Leuk. Lymphoma*, **43,** 1107–1114.
Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T. (2000). *EMBO J.*, **19,** 5720–5728.
Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y and Kondo S. (2004). *Cell Death Differ.*, **11,** 448–457.
Kanzawa T, Kondo Y, Ito H, Kondo S and Germano IM. (2003). *Cancer Res.*, **63,** 2103–2108.
Kim J and Klionsky DJ. (2000). *Annu. Rev. Biochem.*, **69,** 303–342.
Kitamura K, Minami Y, Yamamoto K, Akao Y, Kiyoi H, Saito H and Naoe T. (2000). *Leukemia*, **14,** 1743–1750.
Kitamuro T, Takahashi K, Nakayama M, Murakami O, Hida W, Shirato K and Shibahara S. (2000). *J. Neurochem.*, **75,** 1826–1833.
Klionsky DJ and Emr SD. (2000). *Science*, **290,** 1717–1721.
Klionsky DJ and Ohsumi Y. (1999). *Annu. Rev. Cell Dev. Biol.*, **15,** 1–32.

Kondo S, Barna BP, Morimura T, Takeuchi J, Yuan J, Akbasak A and Barnett GH. (1995). *Cancer Res.*, **55,** 6166–6171.

Kubasiak LA, Hernandez OM, Bishopric NH and Webster KA. (2002). *Proc. Natl. Acad. Sci. USA*, **99,** 12825–12830.

Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H and Levine B. (1999). *Nature*, **402,** 672–676.

Liau LM, Lallone RL, Seitz RS, Buznikov A, Gregg JP, Kornblum HI, Nelson SF and Bronstein JM. (2000). *Cancer Res.*, **60,** 1353–1360.

Lockshin RA and Zakeri Z. (2001). *Nat. Rev. Mol. Cell Biol.*, **2,** 545–550.

Lockshin RA and Zakeri Z. (2002). *Curr. Opin. Cell Biol.*, **14,** 727–733.

Maeda H, Hori S, Nishitoh H, Ichijo H, Ogawa O, Kakehi Y and Kakizuka A. (2002). *Cancer Res.*, **61,** 5432–5440.

Martinou JC and Green DR. (2001). *Nat. Rev. Mol. Cell Biol.*, **2,** 63–67.

Mentlein R and Held-Feindt J. (2002). *J. Neurochem.*, **83,** 747–753.

Miller Jr WH. (2002). *Oncologist*, **7,** 14–19.

Miller Jr WH, Schipper HM, Lee JS, Singer J and Waxman S. (2002). *Cancer Res.*, **62,** 3893–3903.

Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y and Yoshimori T. (2001). *J. Cell Biol.*, **152,** 657–668.

Niu C, Yan H, Yu T, Sun HP, Liu JX, Li XS, Wu W, Zhang FQ, Chen Y, Zhou L, Li JM, Zeng XY, Yang RR, Yuan MM, Ren MY, Gu FY, Cao Q, Gu BW, Su XY, Chen GQ, Xiong SM, Zhang T, Waxman S, Wang ZY, Chen SJ, Hu J, Shen ZX and Chen SJ. (1999). *Blood*, **94,** 3315–3324.

Puthalakath H and Strasser A. (2002). *Cell Death Differ.*, **9,** 505–512.

Reed JC. (1999). *J. Clin. Oncol.*, **17,** 2941–2953.

Schwartz LM, Smith SW, Jones ME and Osborne BA. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 980–984.

Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL, Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ, Chen Z and Wang ZY. (1997). *Blood*, **89,** 3354–3360.

Soignet SL, Maslak P, Wang ZG, Jhanwar S, Calleja E, Dardashti LJ, Corso D, DeBlasio A, Gabrilove J, Scheinberg DA, Pandolfi PP and Warrell Jr RP. (1998). *N. Engl. J. Med.*, **339,** 1341–1348.

Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH and Harris AL. (2001). *Cancer Res.*, **61,** 6669–6673.

Sullivan PG, Thompson MB and Scheff SW. (1999). *Exp. Neurol.*, **160,** 226–234.

Tenan M, Fulci G, Albertoni M, Diserens AC, Hamou MF, El Atifi-Borel M, Feige JJ, Pepper MS and Van Meir EG. (2000). *J. Exp. Med.*, **191,** 1789–1798.

Terzis AJ, Thorsen F, Heese O, Visted T, Bjerkvig R, Dahl O, Arnold H and Gundersen G. (1997). *Br. J. Cancer*, **75,** 1744–1752.

Unoki M and Nakamura Y. (2003). *Oncogene*, **22,** 2172–2185.

Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R and Greenberg AH. (2000). *Mol. Cell. Biol.*, **20,** 5454–5468.

Yao KC, Komata T, Kondo Y, Kondo S and Germano IM. (2003). *J. Neurosurg.*, **98,** 378–384.

Yasuda M, Theodorakis P, Subramanian T and Chinnadurai G. (1998). *J. Biol. Chem.*, **273,** 12415–12421.

Zhang L, Miles MF and Aldape KD. (2003). *Nat. Biotechnol.*, **21,** 818–821.

Zheng XH, Watts GS, Vaught S and Gandolfi AJ. (2003). *Toxicology*, **187,** 39–48.

Zhu XH, Shen YL, Jing YK, Cai X, Jia PM, Huang Y, Tang W, Shi GY, Sun YP, Dai J, Wang ZY, Chen SJ, Zhang TD, Waxman S, Chen Z and Chen GQ. (1999). *J. Natl. Cancer Inst.*, **91,** 772–778.

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