Effect of Arsenic Trioxide in TRAIL (Tumor Necrosis Factor-related Apoptosis Inducing Ligand)-Mediated Apoptosis in Multiple Myeloma Cell Lines

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Purpose: The potential therapeutic application of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), in the treatment of multiple myeloma (MM), was recently proposed. However, there have been some problems with the use of TRAIL, due to the appearance of TRAIL-resistant cells in MM. The effect of arsenic trioxide (As2O3) on the rate of apoptosis induced by TRAIL was evaluated in MM cells.

Materials and Methods: Using TRAIL-sensitive (RPMI-8226) and TRAIL-resistant (ARH-77 and IM-9) MM cell lines, the cell viability, induction of apoptosis, and change in the caspases were examined after treatment with TRAIL alone, or in combination with various concentrations of As2O3.

Results: Incubating the cell lines with As2O3 augmented the TRAIL-induced apoptosis in the MM cell lines, according to the As2O3 concentration. Apoptosis was mediated through caspase activation. When TRAIL was used alone, caspase8 was activated in the RPMI-8226 cell lines, but not in the ARH-77 and IM-9 cell lines. When As2O3 was added to TRAIL, caspase-9 was activated in the ARH-77 and IM-9 cells.

Conclusion: The use of As2O3, in combination with TRAIL, would help enhance the level of TRAIL-induced apoptosis, and overcome the TRAIL-resistance, in MM cells. (Cancer Research and Treatment 2003;35:472-477)

Key Words: Arsenic trioxide, Multiple myeloma, Apoptosis, Caspase
toxicities, showing neither severe bone marrow depression, nor other severe clinical side effects. The mechanism of action has been shown to be associated with the induction of apoptosis and differentiation (13,14). Recent reports suggest that the apoptotic effect of As$_2$O$_3$ is not specific to APL cells, but can be observed in various cell lines of either plasma cells or lymphoid neoplasms (15,16).

In this study, an experiment was performed to determine whether As$_2$O$_3$ could enhance TRAIL-induced apoptosis.

**MATERIALS AND METHODS**

1) MM cell lines

The RPMI-8226 human MM cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). The ARH-77 and IM-9 human MM cell lines were obtained from the American Type Tissue Culture Collection (Manassas, VA). All the MM cell lines were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL, Grand Island, NY) and 1% penicillin-streptomycin (Sigma, St Louis, MO), at 37°C, in a 5% CO$_2$ incubator.

2) Measurement of cell viability

The cell viability was assessed using an MTT based cell proliferation kit (Roche, Roche Diagnostics GmbH, Mannheim, Germany). After suspending $5\times10^5$ cells in each well of a 96-well plate, 100 ng/ml of TRAIL (ALEXIS Co. San Diego, CA), or in combination with various concentrations of As$_2$O$_3$ (0.5, 1 & 2µM) (Sigma), they were cultured at 37°C, for 24 hours. After 4 hours of the reaction, 10µl of MTT labeling reagent was added into each well, along with 100µl of a solubilization solution. The mixture was cultured for 18~24 hours. The absorbance was read using an ELISA reader, at a wavelength of 562 nm.

3) Measurement of apoptosis

A TUNEL assay, to detect the fragmented DNA in situ, was performed on the cell cytopsins using the apoptag™ in situ apoptosis detection kit, fluorescein (INTERGEN, Manhattan, NY). 50µl of a fixed cell suspension, in 4% neutral buffered formalin, was dried on a microscope slide. The slide was washed with PBS. An equilibration buffer was then applied, and the slide incubated with working strength TdT enzyme, at

![Graphs](image_url)

**Fig. 1.** MM cells cultured with 100 ng/mL TRAIL, various concentrations of As$_2$O$_3$, or a combination of both. The viability was measured by an MTT assay, and the values are given as a percentage of the untreated control cells. Data points show the average for triplicate results.
37°C, for 1 hour, washed with stop/wash buffer, and incubated with working strength anti-digoxigenin-fluorescein, at room temperature, for 30 minutes. The slide was subsequently washed with PBS, counterstained with propidium iodide, and observed using fluorescent microscopy.

4) TRAIL receptors expression and caspases activation

For western blot analysis, the cells were lysed in ice cold PBS, containing 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2.0g/mL aprotinin and a protease inhibitor cocktail (Roche Diagnostics GmbH). The lysed cells were then centrifuged at 12,000 rpm, to remove the cellular debris, and the protein concentration of the extracts determined by colorimetric biotin chromogenic acid analysis (micro BCA protein assay reagent kit, Pierce, Rockford, IL). 20µg of the proteins were separated by 10% SDS-PAGE, and the proteins electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% nonfat dried milk in TTBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% (v/v) Tween 20), for 1 hour, at room temperature, and incubated in the primary antibodies, diluted to 1 : 1,000 with 5% non-fat dried milk in TTBS, for 4 hours, at room temperature. DR4 and DR5 were detected using the rabbit polyclonal antibodies for human DR4 (Serotec Inc, Raleigh, NC) and DR5 (ALEXIS Co.San Diego, CA). Caspases-3, -8, and -9 were detected using the rabbit polyclonal anticaspase-3, -8, and -9 antibodies (BD Pharmingen, Mountain View, CA). The membrane was then washed 3 times with TTBS for 15 minutes and subsequently incubated in HRP-conjugated goat anti-rabbit immunoglobulins (DAKO, Denmark), diluted at 1 : 3,000 with 5% non-fat dried milk in TTBS, for 1 hour, at room temperature. The membrane was then washed three times, as described above, and developed using an ECL detection system (Amersham Corp., Arlington Heights, IL).

RESULTS

1) As2O3 enhanced TRAIL-induced apoptosis in MM cell lines

In the first set of experiments, the effect of TRAIL was examined by measuring the viability of the RPMI-8226, ARH-77, and IM-9 cells. When 100 ng/ml TRAIL was added, the cell viabilities were 71.6±11.7, 90.8±7.3 and 94.7±4.4% in the RPMI-8226 cells, ARH-77, and IM-9 cells, respectively. In order to assess the effect of As2O3, various concentrations of As2O3 (0.5, 1.0 or 2.0μM) were added to the TRAIL. After the addition of As2O3, the cell viabilities were 58.8±4.2, 56.6±6.2 and 42.4±5.8% in the RPMI-8226 cells, 78.2±3.8, 68.0±4.8, and 45.9±8.5% in the ARH-77, and 88.1±7.5, 76.4±4.1, and 51.7±9.2% in the IM-9 cells, respectively. Therefore, the cell viability of each cell line decreased, according to the increases in the As2O3 concentration (Fig. 1). Cell death was mainly due to apoptosis, as shown by the TUNEL method (Fig. 2).

2) Change in TRAIL receptor expression following TRAIL and As2O3 treatment

Western blot analysis was performed to determine the expression of the TRAIL receptor in the MM cell lines. The death signaling receptors, DR4 and DR5, were expressed in the MM cell lines. Although a combination of As2O3 and TRAIL
Fig. 3. Changes in the protein expressions of DR4 and DR5. MM cells were exposed to 100 ng/ml TRAIL, 1.0µM As2O3, or combination of both, for 24 hours. The cell lysates were prepared from each cell type, and an equal amount of protein loaded on a 10% SDS-PAGE gel. Western blot analysis was performed with anti-DR4 and DR5 antibodies. Actin was used as an internal control (C: Control, T: TRAIL 100 ng/ml, A: As2O3 1.0µM, T+A: TRAIL+As2O3).

3) Caspase activation

In order to confirm that TRAIL-induced apoptosis was the result of the activation of the caspases, the caspase activation was assessed by western blot in the MM cell lines treated with 100 ng/ml TRAIL, 1µM As2O3, or a combination of both, for 24 hours.

With TRAIL only, the initiator, caspases 8, was activated during TRAIL-induced apoptosis in the RPMI-8226 cells, but was not activated in either the ARH-77 or IM-9 cells. The caspases 3 and 9 were activated in all three cell lines. However, in the ARH-77 and IM-9 cells, the detection levels were low. The precursor form of caspase 3 was cleaved to 17-kDa fragments after treatment with TRAIL.

With the combination of TRAIL and As2O3, the expression...
level of the cleaved caspase-3 was higher after the treatment with TRAIL and As₂O₃, compared with TRAIL or As₂O₃ alone, which was probably the result of the enhancing effect of As₂O₃ (Fig. 4A). Also, the expression of procaspase-9 was more decreased after treatment with TRAIL and As₂O₃, than with TRAIL alone, but the caspase-8 activity was unaffected (Fig. 4B, 4C). These results suggest that rapid activation of the caspase cascade occurs after the TRAIL treatment in some MM cells, and caspase-8 is likely to function as an apical caspase in the TRAIL-induced signaling MM cells. In contrast, caspase-8 cleavage, and the ensuing downstream events, were absent in the others. In these cells, when As₂O₃ was added to TRAIL, caspase-9 cleavage, and the ensuing downstream events, occurred.

**DISCUSSION**

This study demonstrated that As₂O₃ could enhance the TRAIL-induced apoptosis in MM cells. The focus on TRAIL, as a potential therapeutic agent, became obvious as a surprising differential sensitivity to TRAIL-stimulated apoptosis was observed between normal and cancerous cells. Approximately 80% of human cancer cell lines, representing colon, lung, breast, skin, kidney and brain tumors, are sensitive, at least to some extent, to TRAIL. Conversely, most normal cells are TRAIL resistant (17). Furthermore, the advantage of TRAIL, as a pro-apoptotic receptor ligand, is that it does not appear to have the extreme liver toxicity that has precluded the in vivo testing of related death-inducing ligands, such as CD95/FasL and TNF-α/TNFR1, which both cause massive hemorrhagic necrosis of various tissues, including the liver (18).

Apoptosis can occur in response to a broad range of stimuli, but ultimately this suicide response results in the activation of the intracellular proteinases, known as caspases, the cleavage of the caspase substrates, and finally cell demise. The two best-characterized pathways for achieving caspase activation are termed ‘intrinsic’ and ‘extrinsic’. In the extrinsic pathway, the apoptotic machinery is triggered by an interaction of the death ligands of the TNF family, such as FasL/CD95 ligand and TRAIL/Apo2L, with their corresponding death receptors, such as FasL/CD95, DR4 and DR5. These receptors recruit the adapter proteins to their cytosolic domains that bind the specific initiator caspases (19). The intrinsic pathway centers on the mitochondria. These organelles release cytochrome-c into the cytosol, where it participates in caspase activation. The Bcl-2 family proteins regulate mitochondrial apoptosis. Although the intrinsic and extrinsic pathways for caspase activation can participate independently, an interaction between these pathways occurs in several steps (20).

The mechanisms of the apoptotic effects of As₂O₃ have revealed that it disrupts the mitochondrial transmembrane potential (21,22), which result in cytochrome-c being released from the mitochondria. Cytochrome-c activates the caspases by binding to the apoptosis protease activating factor-1, and inducing it to associate with procaspase-9, thereby triggering caspase-9 activation and initiating the proteolytic cascade that culminates in apoptosis.

In this study, it has been demonstrated that As₂O₃ could enhance TRAIL-induced apoptosis in MM cells. This effect is probably induced by different pathways of caspase activation. RPMI-8226 cells, known as TRAIL-sensitive, proceed rapidly through the cleavage of the downstream caspases by caspase-8. In contrast ARH-77 and IM-9 cells, known as TRAIL-resistant, mainly proceed through the cleavage of the downstream caspases, by caspase-9, when As₂O₃ is added to TRAIL. Caspase-3, effector caspase, was also increased when As₂O₃ was added to TRAIL. These studies are consistent with TRAIL helping to achieve apoptosis, through caspase-8, in the TRAIL-sensitive MM cell lines, where the caspase-8 activity was delayed, or not present at all, in the TRAIL-resistant MM cell lines (23).

Anti-cancer agents could be used as a method for enhancing TRAIL-induced apoptosis. Some of these agents have been reported to up-regulate the expressions of the DR4 and DR5 mRNAs, by both p53-dependent or independent mechanisms (24). This up-regulation was proposed to account for the synergistic activity, of TRAIL and the chemotherapeutic drugs, described in several tumor cell lines (25), suggesting that the anti-cancer agents could be used to overcome TRAIL resistance. In this study, although As₂O₃ did not change the expressions of the death DR4 and DR5, it enhanced apoptosis through an intrinsic pathway.

**CONCLUSION**

As₂O₃ was found to play a significant role in the TRAIL-induced apoptosis in MM cells. A combination of As₂O₃ and TRAIL could enhance the level of TRAIL-induced apoptosis, and overcome the TRAIL-resistance in MM cells.

**REFERENCES**


