Mechanism of Apoptotic Cell Death by 2,4,3',5'-Tetramethoxystilbene in Human Promyelocytic Leukemic HL-60 Cells

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Abstract – We have previously shown that 2,4,3',5'-tetramethoxystilbene (TMS), a synthetic *trans*-stilbene analogue acting as a potent inhibitor of human cytochrome P450 1B1, induces apoptotic cell death in human cancer cells. In the present studies, we report the mechanisms of apoptotic cell death by TMS in human promyelocytic leukemic HL-60 cells. We found that treatment of HL-60 cells with TMS suppressed the cell growth in a concentration-dependent manner with IC₅₀ value of about 0.8 μ M. Immunoblot experiments revealed that DMHS-induced apoptosis was associated with cleavage of poly (ADP-ribose) polymerase. The release of cytochrome *c* from mitochondria into the cytosol was significantly increased in response to TMS. TMS caused activation of caspase-3 in a concentration-dependent manner and TMS-mediated caspase-3 activation was partially prevented by the caspase inhibitor, zVAD-fmk. Interestingly, we found that the cytotoxic effect of anticancer drugs such as paclitaxel, docetaxel, or etoposide was enhanced in the presence of TMS. Simultaneous treatment with TCDD also significantly increased cytotoxic effects of TMS alone or TMS and anti-cancer agents. Taken together, our present results indicated that TMS leads to apoptotic cell death in HL-60 cells through activation of caspase-3 activity and release of cytochrome *c* into cytosol. The ability of TMS to increase cytotoxic effect of anticancer drugs may contribute to its usefulness for cancer chemotherapy.

Keywords □ Tetramethoxystilbene, HL-60 cells, caspase-3, cytochrome *c*

INTRODUCTION

Apoptosis is described by its morphological characteristics, including plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies (Wyllie, 1997). It plays an important role as a protective mechanism in the organism by removing damaged cells or over-proliferating cells by an improper mitotic stimulus. However, inappropriate regulation of apoptosis may cause many serious disorders such as neural degeneration, AIDS, autoimmune disease, and cancers. Many anticancer drugs or cancer chemopreventive agents may act through the induction of apoptosis to prevent tumor promotion and progression. Apoptosis is mediated by activation of caspases, a family of cysteine proteases (Earnshaw *et al.*, 1999). Caspases are synthesized as relatively inactive precursor forms, and an apoptotic signal converts the precursors to active enzymes. Once acti-

vated, caspases cleave a variety of intracellular polypeptide, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases (Nagata, 1997).

Previously, our work (Lee *et al.*, 2002) and data from other laboratories (Huang *et al.*, 1999; Clement *et al.*, 1998; Wolter *et al.*, 2002; Estrov *et al.*, 2003) provide evidences that many *trans*-stilbene compounds can induce apoptotic cell death in various human tumor cells. 3,4,3',5'-Tetrahydroxystilbene (piceatannol) was also considered as a specific inhibitor of protein tyrosine kinase p72^{Syk} (Geahlen and McLaughlin, 1989). Recently, we found that 2,4,3',5'-tetramethoxystilbene (TMS), a methoxy derivative of oxyresveratrol acting as a potentially selective inhibitor of CYP1B1 (Chun *et al.*, 2001; Kim *et al.*, 2002; Chun and Kim, 2003) is able to block cell proliferation in human cancer cells such as MCF-7 and HL-60 cells in a concentration- and time-dependent manner (Chun *et al.*, 2005). In these studies, the biochemical mechanism of cytotoxic signaling in response to TMS was studied and we suggest that TMS induces apoptotic cell death through a caspase-3-dependent signal pathway in HL-60 cells.

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MATERIALS AND METHODS

Materials

TMS (Fig. 1) was obtained as described previously (Chun *et al.*, 2001). Anti-poly(ADP-ribose) polymerase (PARP) antibody was from Roche Molecular Biochemicals (Mannheim, Germany). Anti-cytochrome *c* antibody was purchased from GE Healthcare (Piscataway, NJ). ZVAD-fmk was obtained from Enzyme Systems Products (Livermore, CA).

Cell culture

Human promyelocytic leukemic HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were counted using a hemocytometer and viability was measured by trypan blue exclusion method. For treatment, 5×10^5 cells were plated in 1 ml of culture medium and incubated for 1-3 days, as indicated. After incubation, the cells were harvested by scrapping in ice-cold 0.1 M potassium phosphate buffer (pH 7.4). Cells were centrifuged at 1,000 $\times$ g for 5 min at 4°C and the pellets were resuspended in the same buffer. The cells were sonicated for 30 s at 4°C and stored at -70°C.

MTT Assay

Cells were plated onto 96-well plates and incubated at 37°C in a 5% CO₂ atmosphere. After incubation for the designated time, 10 μ g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added per well. After incubating at 37°C for 4 h, cells were centrifuged at 1,000 $\times$ g for 5 min, the medium was removed by aspiration, and then MTT formazan crystal formed was dissolved by adding 0.15 ml of DMSO and shaking for 15 min. The absorbance at 540 nm was measured using a microplate reader. The percentages of cells surviving from each group relative to control, defined as 100% survival, were calculated.

Preparation of Mitochondrial and Cytosolic Extracts

Cells were harvested by centrifugation at 2,000 $\times$ g for 5 min, washed twice with ice-cold phosphate-buffered saline, and resuspended in buffer A (20 mM Hepes buffer (pH 7.5) containing 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 μ g/ml chymostatin). Cells were disrupted by homogenization. Homogenates were centrifuged at 1,000 $\times$ g

for 10 min at 4°C. After centrifugation, supernatants were further centrifuged at 10,000 $\times$ g for 20 min at 4°C. The resulting supernatants were stored at -20°C. The 1,000 $\times$ g pellets were resuspended in buffer A and centrifuged at 10,000 $\times$ g for 20 min at 4°C. Mitochondrial pellets were resuspended in 50 μ l of ice-cold 10 mM Tris-acetate buffer (pH 8.0) containing 0.5% NP-40 and 5 mM CaCl₂ and stored at -20°C.

Western Blot Analysis

Protein samples were fractionated by SDS-PAGE and the separated proteins were transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau S to confirm equal loading and transfer of proteins. Membranes were blocked with 5% (w/v) non-fat dry milk in 20 mM Tris-HCl (pH 7.4) containing 8 mg/ml NaCl and 0.05% (w/v) Tween 20 (TBS) at room temperature overnight and incubated with primary antibodies at room temperature for 2 h. The membranes were washed three times with TBS and blotted with secondary antibodies against PARP or cytochrome *c* conjugated with horse-radish peroxidase at room temperature for 1 h, followed by three washes in TBS. Immunoreactive proteins were visualized by the enhanced chemiluminescence (ECL) procedure according to the manufacturer's protocol (GE Healthcare).

Caspase-3 Assay

Cell lysates were incubated with 100 μ M of the colorimetric substrate, Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) to measure caspase-3 activity. Reaction mixtures contained 100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 0.5 mM EDTA, 100 μ M DEVD-pNA substrate, and 50 μ g of protein samples. Plates were incubated at 37°C for 1 h. Release of free pNA, which absorbs at 405 nm, was monitored continuously.

RESULTS AND DISCUSSION

Previous our studies have shown that TMS is a potentially selective inhibitor of P450 1B1 (Chun *et al.*, 2001; Kim *et al.*, 2002; Chun and Kim, 2003). TMS showed strong inhibition of

Fig. 1. Structure of 2,4,3',5'-tetramethoxystilbene (TMS).

P450 1B1-mediated ethoxyresorufin *O*-deethylation and estradiol 4-hydroxylation with an IC_{50} value of 6 nM and 90 nM, respectively. Because P450 1B1 is the most catalytically efficient estradiol 4-hydroxylase characterized to date and 4-hydroxyestradiol has been suggested to be carcinogenic (Hayes *et al.*, 1996; Shimada *et al.*, 1996, 1997, and 1999), TMS is considered as a good candidate for preventing mammary tumor formation. TMS also significantly inhibited dioxin-induced P450 1B1 gene expression in human tumor cells such as MCF-7, MCF-10A, and HL-60 (Chun *et al.*, 2005).

To examine the potential of TMS as an adjuvant agent for cancer protection, the cytotoxic effect of TMS was determined by a MTT assay. TMS exhibited a significant inhibition of HL-60 cell growth with an IC_{50} of about 0.8 mM (Fig. 2). To characterize whether the suppression of cell growth by TMS was mechanism-dependent, cleavage of PARP was determined. Within 24 h of TMS treatment, HL-60 cells exhibited significant amounts of PARP cleavage in a concentration-dependent manner (Fig. 3A). Because cytochrome *c* release from mitochondria into cytosol is a strong indicative of apoptotic cell death, the effect of TMS on the accumulation of cytochrome *c* in the cytosol was examined. Treatment with TMS caused a significant release of cytochrome *c* from mitochondria into the cytosol (Fig. 3B). These results suggested that TMS induced apoptotic cell death in HL-60 cells.

Because activation of caspases may play a central role in the execution stage of apoptosis. We questioned whether caspase-dependent signal pathways are involved in the apoptotic cell death induced by TMS in HL-60 cells. Treatment of HL-60 cells with TMS for 24 h increased caspase-3 activity in a con-

Fig. 2. Cytotoxic effect of TMS in HL-60 cells. The HL-60 cells (2.5×10^5 /ml) were treated with various concentrations of TMS for 72 h and viable cells were determined using MTT dye reduction. Each data point represents the mean $\pm$ S. D. of three independent experiments.

(A)

(B)

Fig. 3. (A) Cleavage of PARP by TMS. HL-60 cells were incubated with 1, 5, 10, or 20 μ M of TMS for 24 h. Cell lysates were prepared and were subjected to SDS-PAGE. Cleavage of PARP (85 kDa band) was determined by immunoblotting using anti-PARP antibody. (B) Translocation of cytochrome *c* in TMS-induced apoptosis. Cytosolic and mitochondrial fractions were isolated from cell lysates. Each fraction was subjected to SDS-PAGE. The amounts of cytochrome *c* in each fraction were determined by immunoblotting using anti-cytochrome *c* antibody.

centration-dependent manner (Fig. 4). Moreover, 50 μ M of zVAD-fmk, a caspase inhibitor, blocked the cell death induced by TMS (Fig. 4). These results showed that caspase-3 may play an important role in TMS-mediated apoptotic cell death in HL-60 cells.

To evaluate the effect of TMS as an adjuvant for cellular toxicities of anti-cancer agents, TMS was co-treated with paclitaxel, docetaxel, or etoposide up to 96 h (Fig. 5A). 1 μ M of

Fig. 4. Activation of caspase-3 by the TMS in HL-60 cells. HL-60 cells were treated with 1, 5, 10, or 20 μ M of TMS for 24 h in the presence and absence of caspase inhibitor zVAD-fmk (50 μ M). Caspase-3 activities were determined with cell lysates using DEVD-pNA (100 μ M) as a substrate. Each data point represents the mean $\pm$ S.D. of three independent experiments. *Significantly different from untreated control group ($p < 0.05$). #Significantly different from TMS (20 μ M)-treated group ($p < 0.05$).

Fig. 5. (A) Enhancement of cytotoxic effects of anticancer agents in HL-60 cells treated with TMS. Cells were treated with paclitaxel (50 nM), docetaxel (50 nM), or etoposide (100 nM) in the presence or absence of TMS (1 μ M) for 0, 24, 48, 72, and 96 h. MTT assays were performed to measure cell proliferation. The level in control cells at 0 h was set at 100 %. Results represent the means $\pm$ S. D. of five different experiments. *Significantly different from untreated control group ($p < 0.05$). (B) Effect of TCDD. Cells were treated with paclitaxel (50 nM), docetaxel (50 nM), or etoposide (100 nM) in the presence or absence of TMS (1 μ M) or TCDD (2 nM) for 0, 24, 48, 72, and 96 h. Results represent the means $\pm$ S. D. of five different experiments. *Significantly different from untreated control group ($p < 0.05$).

TMS alone showed significant inhibition of HL-60 cellular proliferation. We could see weak additive inhibitory effects of paclitaxel or docetaxel in HL-60 cells treated with TMS. However, TMS showed a significant inhibition of cell proliferation when cells were co-treated with 100 nM etoposide. To determine whether the induction of P450 1B1 by TCDD may influence on cell death by simultaneous treatment, the effect of TCDD on cellular proliferation of HL-60 cells co-treated with TMS and anti-cancer agents were examined. TCDD treatment significantly enhanced cytotoxicities in cells co-treated with anti-cancer agents and TMS (Fig. 5B). Interestingly, cellular toxicity of TMS alone was also strongly enhanced by TCDD.

Recently increasing interest has been paid to *trans*-stilbene compounds such as resveratrol as cancer chemopreventive agents. Various *trans*-stilbene compounds show cytotoxic effects and induce apoptotic cell death in human cancer cells. Resveratrol is a well-known inducer of apoptotic cell death (Hsieh *et al.*, 1999; Surh *et al.*, 1999; Tsan *et al.*, 2000). Previous studies showed that 3,4'-dimethoxy-5-hydroxystilbene induces apoptosis in HL-60 cells (Lee *et al.*, 2002). 3,4,3',5'-

Tetrahydroxystilbene increases apoptosis by inhibiting tyrosine kinase p72^{Syk}. (Wieder *et al.*, 2001). TMS also was considered to induce apoptosis in human cancer cells (Chun *et al.*, 2005).

In this study, to evaluate the mechanisms of apoptosis by TMS, we examined the effect of TMS on PARP cleavage, cytochrome *c* translocation, and caspase activity. The results of these experiments indicate that TMS induces apoptotic cell death in HL-60 cells and TMS-mediated apoptosis is dependent on caspase-3 activation. Caspase-3 activity was increased by TMS treatment in a concentration-dependent manner and a caspase inhibitor zVAD-fmk significantly blocked TMS-induced caspase-3 activation. The possibilities that other caspases may involve in TMS-mediated apoptosis can not be ruled out.

Previously we showed that TCDD induces P450 1B1 gene expression in HL-60 cells (Chun *et al.*, 2005). We also suggested that TMS acts as a P450 1B1 substrate and the cytotoxic effect of TMS metabolite(s) produced by P450 1B1 may be potent in human cancer cells. The present results that simultaneous treatment with TMS and TCDD significantly prevents

cell proliferation compared to TMS or TCDD alone also support our hypothesis. Because TCDD induced P450 1B1 expression in HL-60 cells, induced P450 1B1 may generate more cytotoxic TMS metabolite(s) from NADPH-dependent TMS metabolism and thus the cytotoxic effect of TMS may be enhanced by TCDD treatment. The ability of TMS to induce apoptotic cell death in human cancer cells may be beneficial. The enhancement of cytotoxic effect of anti-cancer agents to cancer cells by TMS may contribute to its usefulness for cancer chemotherapy. The precise mechanism by which TMS enhances the sensitivity of anti-cancer agents still needs to be determined. Our future studies will be focused on the simultaneous treatment with etoposide and TMS because their co-treatment showed more significant suppression of HL-60 proliferation than paclitaxel or docetaxel.

In summary, we report that TMS is a strong inducer of apoptotic cell death in HL-60 cells. Because TMS has already been known as a potentially selective inhibitor of P450 1B1, a major estrogen metabolizing enzyme, its potential to induce apoptosis takes an additional advantages as a cancer protecting agent.

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