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 µM. 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 activated, 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 p72sk (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.
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 µg/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 x 10⁵ cells were plated in 1 ml of culture medium and incubated for 1-3 days, as indicated. After incubation, the cells were harvested by scraping in ice-cold 0.1 M potassium phosphate buffer (pH 7.4). Cells were centrifuged at 1,000 x 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 µg 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 x 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 x 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 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 µg/ml chymostatin). Cells were disrupted by homogenization. Homogenates were centrifuged at 1,000 x g for 10 min at 4°C. After centrifugation, supernatants were further centrifuged at 10,000 x g for 20 min at 4°C. The resulting supernatants were stored at -20°C. The 1,000 x g pellets were resuspended in buffer A and centrifuged at 10,000 x g for 20 min at 4°C. Mitochondrial pellets were resuspended in 50 µl 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 horseradish 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 µM 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 µM DEVD-pNA substrate, and 50 µg 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