Abstract. 2-Methoxyestradiol (2-ME) has been found to possess antitumor activity in vivo and in vitro. It has been suggested that 2-ME induces apoptosis resulting in G2/M arrest of tumor cells. In this study, the effect of 2-ME was evaluated in rat osteosarcoma and malignant fibrous histiocytoma (MFH) cell lines. 2-ME was used at final concentrations of 100 nM to 2 μM. The effect of 2-ME on cell growth was measured by the MTS assay. Induction of apoptosis and activation of caspase-3 were investigated along with apoptosis-related gene expression. The data showed that 2-ME significantly inhibited cell growth, inducing apoptosis. The activity of caspase-3 was increased at 20 h and 40 h in both cell lines. 2-ME induced p16 expression, which was possibly involved in the apoptotic process. These results suggested that the 2-ME-induced apoptosis of rat osteosarcoma and rat MFH cells was accompanied by caspase-3 activation through p16 induction.

2-Methoxyestradiol (2-ME) is a natural estrogenic metabolite formed by the hydroxylation of estradiol followed by its O-methylation in the liver (1). It is a highly potent anticancer agent that effectively induces apoptosis in tumor cells, both in vivo and in vitro (2). It is orally active in inhibiting tumor growth and metastatic spread in several tumor models, at doses that show no clinical signs of toxicity (3). Although 2-ME is formed from 17ß-estradiol (4), its anticancer action is not dependent on estrogen receptor binding (5). Previous studies have shown that one mechanism for the cytotoxic effect of 2-ME is the interference with the stability of microtubules and subsequent arrest of cells in the G2/M phase of the cell cycle (6, 7). The pro-apoptotic action of 2-ME has been linked to the stimulation of the production of cellular reactive oxygen species, resulting in release of cytochrome c from the mitochondria and activation of caspases (8, 9). In some studies, several molecular mechanisms have been attributed to 2-ME-mediated cell death and apoptosis.

Many drugs induce cancer cell death through apoptotic pathways. Apoptosis is initiated by two main pathways – the mitochondria-mediated and the death receptor-mediated. The mitochondria-mediated pathway is initiated by various changes in mitochondrial function including increased mitochondrial membrane permeability, reduced mitochondrial membrane potential, and release of cytochrome c and of the apoptosis-inducing factor. Interaction of the released cytochrome c with dATP and APAF-1 results in activation of the initiator caspase, caspase-9, which in turn triggers activation of the executor caspases of cell death (10, 11). The death receptor-mediated pathway is initiated following the binding of a specific ligand to a TNF receptor superfamily member (Fas, TNF RI, TNF RII, TRAIL, DR4, DR5). This binding is followed by the recruitment of the adaptor molecule FAS-associated death domain (FADD). Subsequently, FADD uses its death effector domain to recruit proximal caspase-8 or -10. This results in the formation of a death effector signaling complex with the subsequent release of the activated forms of these caspases. Activation of caspase-8 or -10, in turn, triggers activation of executor caspases, which mediate cell death (12, 13).

An effect of 2-ME has been shown in many cancer cells, but only few studies have shown an effect in sarcomas. We investigated the effect of 2-ME in COSINR (rat osteosarcoma) and MFH1NR [rat malignant fibrous histiocytoma (MFH)] cell lines, both of which were established in our laboratory, along with the induction of apoptosis.
Materials and Methods

Cells and cell culture. The cell lines used in this study were 4-hydroxy(amino)quinoline 1-oxide (4-HAQO)-induced rat osteosarcoma COS1NR (14) and 4-HAQO-induced rat MFH MFH1NR (15), both of which were established in our laboratory. All cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were incubated at 37°C in humidified 5% CO2.

Drug treatment. 2-ME (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol to give a 20 mM stock solution and stored at –20°C. 2-ME was diluted in culture medium and used at final concentrations of 100 nM to 2 μM for 24-48 h and 72 h.

Assessment of cell viability. The effect of 2-ME on cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) colorimetric dye reduction assay, which closely correlated with viable cell numbers under our experimental conditions. Both cell lines were inoculated into 96-well microtiter plates (2,000 cells/well) allowed to attach to the plates for 4 h at 37°C, and then exposed to different concentrations of 2-ME (final concentration 100 nM to 2 μM). After 24, 48 and 72 h incubation, cell viability was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI, USA). In brief, 20 μl of MTS solution were added into each well, the plates were incubated for 1 h under a 5% CO2 atmosphere at 37°C, and cell proliferation was then measured at an emission wavelength of 492 nm.

Detection of apoptosis by ELISA. Apoptosis induced by 2-ME in COS1NR and MFH1NR cells was determined with an Apostrand® ELISA Apoptosis Detection kit (BIOMOL International, Plymouth Meeting, PA, USA), according to the manufacturer’s instructions.

Caspase-3 activity assay. Cells were grown in 96-well dishes and treated with 1 μM of 2-ME, then caspase-3 activity was determined with a colorimetric ELISA assay kit (Sigma, MO, USA). In brief, after treatment with 2-ME, the cells were washed in phosphate-buffered saline (PBS) and assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, diluted assay buffer) was added to each well, then 10 μl of caspase-3 substrate were added with shaking to mix. The plate was covered and incubated at 37°C overnight and the absorbance at 405 nm was then measured.

RNA extraction and RT-PCR analysis. Total RNA was extracted using the RNeasy Total RNA system (Qiagen GmbH, Hilden, Germany) and first-strand cDNAs were synthesized from 600 ng samples with the Superscript II RNase H reverse transcriptase system (Invitrogen Life Technologies, Carlsbad, CA, USA). All PCR reactions were performed using 1 μl of a 10 μl reaction mixture as the template. The primer sequences used for amplification of the rat Fas and Fas ligand, p16INK4a, and p19ARF genes are listed in Table I. The rat glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control to adjust the amounts of template. Aliquots of 10 μl of the amplification products were separated by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

Results

Cell proliferation. Cell proliferation following treatment with 100 nM, 200 nM, 500 nM, 1 μM, or 2 μM of 2-ME is shown in Figure 1. The data show that 2-ME statistically significantly inhibited cellular growth in a dose-dependent manner (Figure 1).

Detection of apoptosis. The proportion of apoptotic cells determined by colorimetric assay increased in both cell lines in a dose-dependent manner with 2-ME treatment, while growth rates decreased (Figure 2). Thus, the induction of apoptosis was associated with cell growth suppression in both tumor cell lines.

Caspase-3 activity and Fas and Fas-ligand mRNA expression. The activity of caspase-3 measured by ELISA increased at 20 h and 40 h in both cell lines treated with 1 μM of 2-ME (Figure 3). Expression of Fas and Fas-ligand mRNA increased with 2-ME treatment at 16 h or 36 h in COS1NR. This finding correlated closely with caspase-3 activity (Figure 4), while expression of Fas and Fas-ligand mRNA was not affected by 2-ME treatment in MFH1NR.

Expression of p16 mRNA in both cell lines after treatment. Expression of p16 mRNA appears higher at 36 h in COS1NR and at both 16 h and 36 h in MFH1NR. p19 mRNA expression was unaffected in these cell lines (Figure 5).

Discussion

2-ME, an endogenous natural product of estradiol metabolism, has been shown to have activity against tumor cell lines in preclinical models. It inhibits the growth of
various cancer cells, including lung, breast, pancreatic, hepatocellular carcinoma, neuroblastoma, medulloblastoma, melanoma and gastric cancer (1, 16-18). However, only a few studies have demonstrated an effect of 2-ME on sarcomas. Golebiewska et al. reported that the effect of 2-ME varies among cell types. Although 2-ME seems to inhibit the growth of most tumor types in vitro and in vivo, the mechanisms of cell growth inhibition are multifactorial and depend on the tumor type (5).

The inhibitory effects of 2-ME have been attributed to several mechanisms, including disruption of microtubule function (19) inhibition of superoxide dismutase (20), inhibition of angiogenesis (21), and induction of apoptosis (22, 23). 2-ME has been demonstrated to cause G2/M arrest, to bind to tubulin and microtubules inhibiting both polymerization and depolymerization, and to cause abnormal spindle formation in cultured cells (4).

In this study, we found that 2-ME inhibited rat osteosarcoma and rat MFH cell growth through the induction of apoptosis. Apoptosis is initiated by two main pathways, the death receptor-mediated 'extrinsic pathway' and the mitochondria-mediated 'intrinsic pathway' (24) and both pathways are intimately connected, converging into a common pathway causing the activation of effector enzymes termed caspases (25). The death receptor-dependent pathway is mediated by a death-inducing signaling complex, which is formed by recruitment of a Fas-associated death domain and procaspase-8 to the death receptor, thereby activating caspase-8 (26). Caspase-8 directly activates caspase-3, leading to apoptosis (27). The mitochondrial 'intrinsic pathway' is mediated by Bcl-2

Figure 1: The effect of 2-ME on COS1NR and MFH1NR cell lines. Cell growth of both cell lines was inhibited by 2-ME in dose- and time-dependent manner. All values represent means from 5 replicated results and error bars show standard deviation.

Figure 2: The proportion of apoptotic cells as determined with colorimetric assay increased in both cell lines in a dose dependent fashion, while growth rates decreased with 2-ME treatment. All values represent means from 5 replicated results.

Figure 3: Caspase-3 activity was increased in a time-dependent manner after both 20 and 40 h in 2-ME-treated COS1NR and MFH1NR cell lines. All values represent means from 5 replicated results.
family proteins, and Bid-Bak heterodimers that regulate the release of cytochrome c from mitochondria and activation of caspase-3 and caspase-9 (27, 28). The extrinsic and the intrinsic pathways

In the present study, induction of apoptosis occurred with increased Fas-L expression along with caspase-3 activation when rat osteosarcoma cells were treated with 2-ME. Increased expression of p16 mRNA was also observed in both cells after 36 h of treatment with 2-ME and was possibly involved in the induction of apoptosis. Thus, the effect of 2-ME on these sarcomas might be associated with the death receptor-dependent pathway. Garcia et al. reported that oral administration of 2-ME (75 mg/kg body weight) for 4 weeks inhibited tumor growth by about 60% with no evidence of toxicity in a breast cancer model, reduced the number of metastases in a lung tumor model by 59%, and reduced tumor size in mice with angiosarcoma by 68% (29). Furthermore, Zhang et al. evaluated the efficacy of 2-ME administration orally to hormone-refractory prostate cancer patients in whom other treatments, including hormone therapy, had failed. They concluded that 2-ME was safe and well-tolerated (30). Many studies have indicated that the growth inhibitory effect of 2-ME is specific to tumor cells and does not affect normal cells. Further studies will help to elucidate the mechanism of the effect of 2-ME on tumor growth inhibition and its use in treatment might become feasible in clinical settings.

In conclusion, our results suggest that 2-ME inhibits cell growth of rat osteosarcoma and MFH in a dose- and time-dependent manner, inducing apoptosis, and is a potential treatment for these sarcomas.

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References


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