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2-Methoxyestradiol-Induced Cell Death in Osteosarcoma Cells is Preceded by Cell Cycle Arrest

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Abstract 2-Methoxyestradiol (2-ME), a naturally occurring mammalian metabolite of 17β-Estradiol (E_2), induces cell death in osteosarcoma cells. To further understand the molecular mechanisms of action, we have investigated cell cycle progression in 2-ME-treated human osteosarcoma (MG63, SaOS-2 and LM8) cells. At 5 μM, 2-ME induced growth arrest by inducing a block in cell cycle; 2-ME-treatment resulted in 2-fold increases in G1 phase cells and a decrease in S phase cells in MG63 and SaOS-2 osteosarcoma cell lines, compared to the appropriate vehicle controls. 2-ME-treatment induced a threefold increase in the G2 phase in LM8 osteosarcoma cells. The results demonstrated steroid specificity, as the tumorigenic metabolite, 16α-hydroxyestradiol (16-OHE), did not have any effect on cell cycle progression in osteosarcoma cells. The cell cycle arrest coincided with an increase in expression of the cell cycle markers p21, p27 and p53 proteins in 2-ME-treated osteosarcoma cells. Also, MG63 cells, transiently transfected with cDNA for a 'loss of function mutant' RNA-dependent protein kinase (PKR) protein, were resistant to 2-ME-induced cell cycle arrest. These results suggest that 2-ME works in concert with factors regulating cell cycle progression, and cell cycle arrest precedes cell death in 2-ME-treated osteosarcoma cells. J. Cell. Biochem. 104: 1937–1945, 2008.

Key words: estrogen metabolite; MG63 cells; cell cycle arrest; PKR

17β-Estradiol (E2) is metabolized by alternative hydroxylation pathways resulting in the formation of 2-,4- and 16α-hydroxylated derivatives [Zhu and Conney, 1998a]. 16α-Hydroxyl derivatives are estrogen agonists in cultured osteoblasts and in ovariectomized rats [Robinson et al., 1997; Lotinun et al., 2001]. In contrast, 2-hydroxylated metabolites are neither estrogen agonists nor antagonists on bone cells in cell culture or in whole animals [Robinson et al., 1997; Lotinun et al., 2001]. 2-Hydroxyestradiol (2-OHE) undergoes further metabolism prior to clearance. 2-OHE is *O*-methylated at peripheral sites to form 2-methoxyestradiol (2-ME), which is present in human blood and urine [Gelbke and Knuppen, 1976; Fotsis et al., 1994]. The 4- and 16-α-hydroxylation pathways are tumor promoting, whereas the 2-hydroxylation pathway has been shown to be tumor inhibitory [Zhu and Conney, 1998a]. 2-ME is highly cytotoxic to a wide range of tumor cells, but is harmless to most normal cells including normal osteoblasts [Schumacher et al., 1999; Maran et al., 2002]. However, 2-ME inhibits endothelial cell proliferation and angiogenesis [Fotsis et al., 1994; Yue et al., 1997; Zhu and Conney, 1998a], as well as vascular invasion of growth plate cartilage [Sibonga et al., 2002].

2-ME is a potential therapeutic agent for treatment of bone cancer [Maran et al., 2002; Shogren, 2007]. The molecular mechanism of action of 2-ME on osteosarcoma cells is not well understood, but appears to involve RNA-dependent protein kinase (PKR) signaling [Shogren, 2007]. To further understand the actions of 2-ME and determine whether 2-ME exerts similar effects on various osteosarcoma cell lines, we have investigated the effects of 2-ME on cell cycle progression in low metastatic

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(MG63 and SaOS-2) and high metastatic (LM8) osteosarcoma cell lines.

MATERIALS AND METHODS

Cell Culture

Human osteosarcoma cells (MG63, SaOS-2 and LM8) were grown in DMEM/F12 medium containing 10% charcoal-stripped fetal bovine serum (FBS), penicillin and streptomycin and maintained at 37°C under 5% CO_2.

Metabolite Treatment and Cell Proliferation

The human osteosarcoma cells were plated at 5×10^4 cells per well into 24-well plates containing 1 ml/well medium. After allowing the cells to attach overnight, the media in the wells were replaced with fresh 1 ml medium. The metabolites were added into each well containing the test compounds or carrier and maintained for 72 h. At the end of treatment, the cell growth was measured by MTS-based cell viability assay systems as per the manufacturer's protocol (Promega, Madison, WI).

17β-Estradiol (E_2), 2-methoxyestradiol (2-ME) and 16α-hydroxyestradiol (16-OHE) were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of the metabolites at their respective concentrations were made in 95% ethanol.

Analysis of Cell Cycle Distribution

The human (MG63, SaOS-2 and LM8) osteosarcoma cells (1×10^6 cells) were plated in T-75 flasks and maintained for 24 h. The next day media were changed, and the cells were treated with vehicle (ethanol) or 10 μM E_2, 2-ME and 16-OHE. After 24 h of treatment, the cells were trypsinized, spun and washed with PBS. Each sample was then fixed on ice by adding 300 μl of cold 95% ethanol drop-wise for 5 min. After allowing the cells to fix on ice for 1 h, they were then washed with PBS three times and resuspended in RNase A solution and stained with propidium iodide (50 μg/ml). The samples were then analyzed, and the percentage of cells in each phase of the cell cycle was determined using a FACS Scan unit (Becton Dickinson, San Jose, CA). The relative proportions of cells in the G1, S, and G2/M cell cycle phases were estimated by compartment analysis of DNA fluorescence using cell cycle analysis software from the manufacturer.

Transient Transfection

MG63 cells plated in T-75 flasks were transfected at 60% confluence with 5 μg of pcDNA3-PKR-K296R or empty vector pcDNAneo using the transfection agent lipofectamine, as described in the manufacturer's protocol (Invitrogen, Carlsbad, California). Twenty-four hours post-transfection, the cells were treated with vehicle and 2-ME. Cells were processed for cell cycle studies after 24 h of treatment, as described above.

Preparation of Cytoplasmic Extract and Protein Analysis

Cytoplasmic extracts were prepared as described [Kennedy et al., 2005]. Briefly, cells harvested after 2-ME treatment were lysed by suspending in 1× cell lysis buffer (Cell Signaling, Danvers, MA). After centrifugation at 10,000*g* for 10 min, the resultant supernatant containing cytoplasmic extract (100 μg protein) was analyzed by Western blot hybridization using anti-p21, anit-p53 (Cell Signaling, Danvers, MA), anti-p27(Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin (Sigma) antibodies.

RESULTS

2-ME Induces a Cell Cycle Block in MG63 Osteosarcoma Cells

To assess the effect of various estrogenic compounds on cell cycle progression, we have performed flow cytometry analyses in Veh, 2-ME, E_2 and 16-OHE-treated osteosarcoma cells. The results show that 2-ME treatment contributed to a 2-fold increase and a 2.3-fold decrease in percentage of cells in G1 phase and S phase, respectively (Fig. 1B) compared to the vehicle control (Fig. 1A). The tumorigenic metabolite16α-OHE did not induce similar effects in G1 and S phase cells (Fig. 1D). The parent estrogen, 17β-estradiol had minimal effects on the cells in G1 phase, but decreased the cells in S phase by twofold.

2-ME Affects the Growth of SaOS-2 and LM8 Osteosarcoma Cells

The MTS-based cell viability assay carried out at 72 h after 2-ME treatment shows a dose-dependent effect on cell survival in several osteosarocoma cell lines (Fig. 2). In the case of SaOS-2 cells, the cell survival was reduced

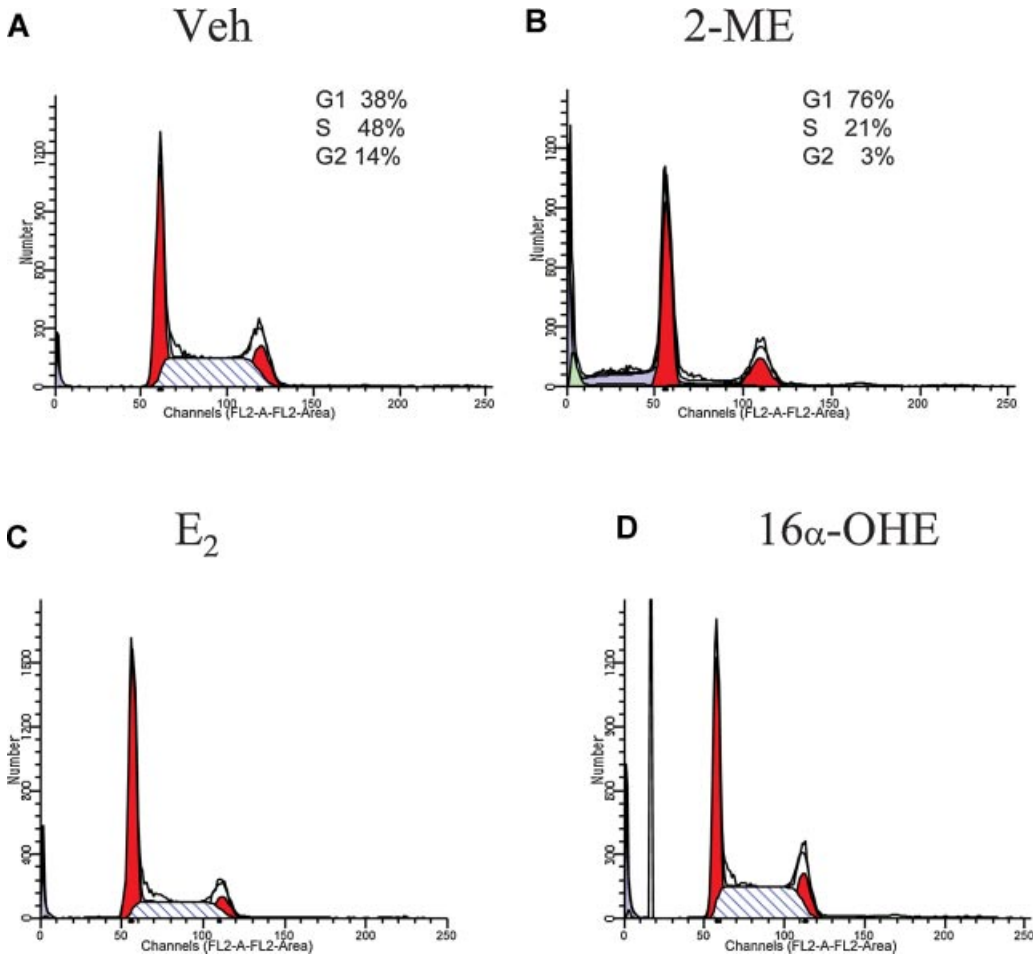

Fig. 1. Ligand specific cell cycle arrest in osteosarcoma cells. MG63 cells were treated with Veh, vehicle (**A**), 10 μM of 2-ME (**B**), E2, 17β-estradiol (**C**) and 16α-OHE, 16α-hydroxyestradiol (**D**) for 24 h and analyzed using a FACS Scan unit. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to 83%, 77%, 38%, 34%, 33%, and 33%, respectively, in the presence of 1, 2, 5, 10, 20, and 50 μM 2-ME compared to vehicle control (Fig. 2A). Similarly, the results show that LM8 cell survival was reduced to 36%, 32%, 26%, 21%, 19%, and 17%, respectively, in the presence of 1, 2, 5, 10, 20, and 50 μM 2-ME (Fig. 2B) compared to the vehicle control.

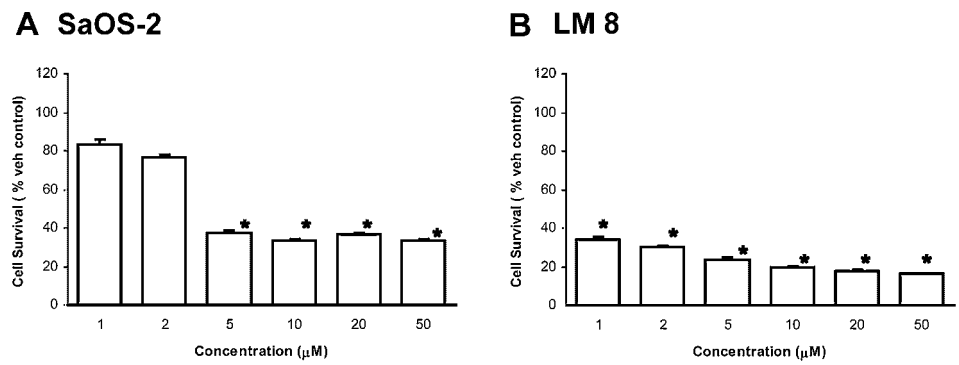

Fig. 2. 2-ME induces cell death in SaoS-2 (**A**) and LM8 (**B**) osteosarcoma cell lines. Osteosarcoma cells were treated with Veh or 5 μM of 2-ME for 72 h. The cells were harvested and the proliferation was determined by MTS assay. Values are the mean ± SE (N = 3 replicate cultures). The absence of error bar denotes a line thickness greater than the error. $^*P \leq 0.05$ versus Veh.

To determine whether the 2-ME-mediated cell death is preceded by cell cycle block in SaOS-2 and LM8 osteosarocoma cells, we have analyzed the effect of 2-ME on cell cycle progression by flow cytometry analysis in SaOS-2 and LM8 osteosarcoma cells. 2-ME treatment increased the cells in G1 phase by 2-fold in SaOS-2 compared to the vehicle control (Fig. 3A,B). In the case of LM8 cells, 2-ME treatment did not increase G1 phase, but resulted in a 2.3-fold increase in G2 phase cells compared to the vehicle (Fig. 3C,D).

2-ME Alters the Expression of Proteins Involved in Cell Cycle Regulation

In order to determine whether 2-ME-induced cell cycle block is accompanied by changes in proteins involved in cell cycle regulation, we have studied the expression of p21, p27 and p53 proteins in various osteosarcoma cell lines. Figure 4A shows that 2-ME treatment leads to an increase in p21 protein levels in MG63, SaOS-2 and LM8 cells compared to the vehicle control. 2-ME treatment also induces the expression of p27 protein in MG63 and SaoS2 osteosarcoma cells, but does not affect the expression in LM8 cells (Fig. 4B). p53 level increases in 2-ME-treated MG63 cells whereas it does not change with 2-ME treatment in SaOS2 and LM8 cells (Fig. 4C). Our results show that the control actin protein does not change with the 2-ME treatment in these cells (Fig. 4D).

A Dominant Negative Mutant, PKR, Inhibits 2-ME-Dependent Cell Cycle Arrest

Our earlier studies showed that RNA-dependent protein kinase, PKR, is involved in 2-ME-mediated cell death [Shogren, 2007]. PKR protein is regulated in a cell cycle-dependent manner. To understand the role of PKR protein in 2-ME-mediated growth arrest in osteosarcoma cells, we have investigated MG63 cells that transiently express the dominant negative PKR mutant protein, which has been shown to block endogenous PKR protein functions. 2-ME is ineffective in inducing growth arrest and G1/S block in the presence of dominant negative mutant PKR protein expression (Fig. 5B) compared to the vehicle

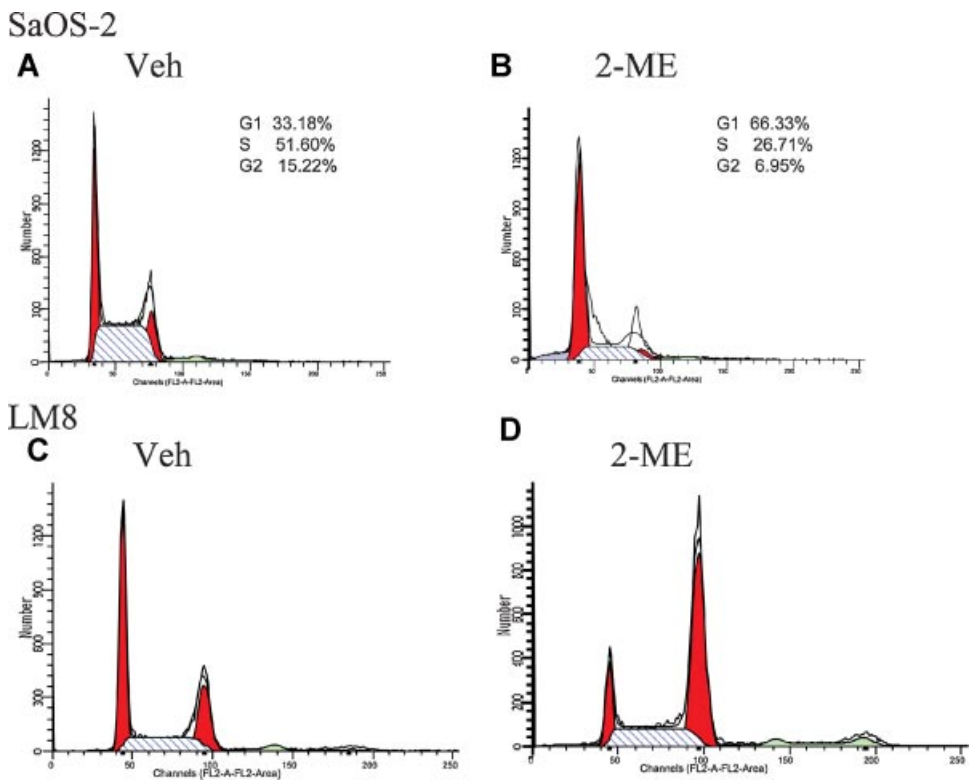

Fig. 3. Flow cytometry in osteosarcoma cells. SaOS-2 and LM8 cells were treated with Veh (**A**,**C**) or 10 μM 2-ME (**B**,**D**) for 24 h and analyzed using a FACS Scan unit. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

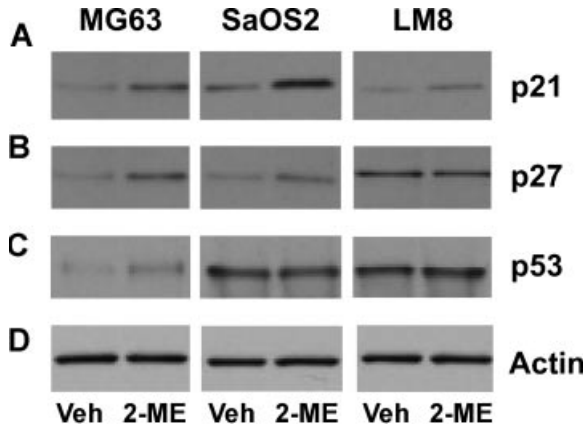

Fig. 4. 2-ME treatment increases p21 protein levels. Cytoplasmic extracts prepared from osteosarcoma cells treated with Veh and 10 μM 2-ME for 24 h were analyzed by Western blot hybridization using anti-p21 (**A**), anti-p53 (**C**) (Cell Signaling Technology) anti-p27 (**B**) (Santa Cruz Biotechnology) and anti-actin (**D**) (Sigma) antibodies.

control (Fig. 5A). However, in cells transfected with control vector, 2-ME induced the G1/S block (Fig. 5D).

DISCUSSION

Estrogen is metabolized into compounds that activate pathways which, depending upon the metabolite, can either block or induce cell growth. 2-ME, which is a product of 2-hydroxylation pathway, exerts anti-tumor effects in vivo and in vitro [Mukhopadhyay and Roth, 1997; Seegers et al., 1997; Zhu and Conney, 1998b; Schumacher et al., 1999; Pribluda et al., 2000; Maran et al., 2002]. We have previously shown that 2-ME induces cell death in osteosarcoma cells, but does not affect normal osteoblasts [Maran et al., 2002]. We have demonstrated that the mechanism of 2-ME-mediated cell killing in osteosarcoma cells involves apoptosis [Maran et al., 2002; Shogren, 2007]. In this study, we show that 2-ME blocks the progression of osteosarcoma cells at G1/S or G2/M

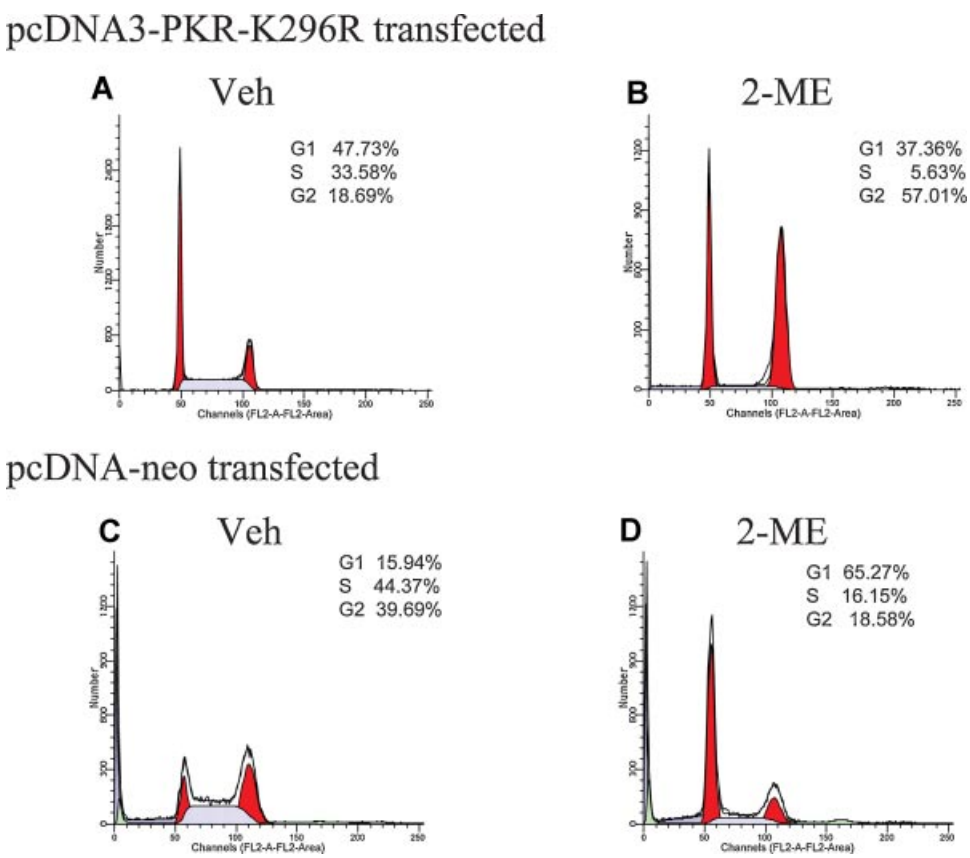

Fig. 5. Dominant negative PKR blocks 2-ME-mediated cell cycle arrest. MG63 cells were transfected with the plasmid pCDNA3-PKR-K296R (**A**,**B**) or empty vector pcDNA neo (**C**,**D**) and treated with Veh or 10 μM 2-ME for 24 h. The cells were harvested and analyzed by flow cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

boundary during cell cycle and demonstrate that growth arrest precedes cell death.

The anti-tumor and anti-growth mechanisms of 2-ME involve multiple, cell-specific pathways including disruption of microtubles, induction of oncogenes, cytokines and growth arrest. 2-ME-dependent tumor cell death and other anti-tumor effects (e.g., inhibition of angiogenesis) are associated with growth arrest due to the inability of the cells to progress through the cell cycle. 2-ME treatment results in G1 arrests in breast cancer cells [Lottering et al., 1992] and chondrosarcoma cells [Fong et al., 2007]. In contrast, G2/M arrest has been demonstrated in 2-ME-treated endothelial, breast cancer, prostate cancer and hepatoma cells [Reiser et al., 1998; Lin et al., 2000; Kumar et al., 2001; Qadan et al., 2001]. The results show that in the presence of 2-ME, cell cycle progression is blocked at G1/S boundary in MG63 and SaOS-2 osteosarcoma cells, but at G2/M boundary in LM8 osteosarocoma cells. Thus, 2-ME induces G2/M block in highly metastatic tumor cells, but not in low metastatic cells. This finding suggests that distinct tumor inducing pathways are involved in vivo in tumor progression by low metastatic and high metastatic cells. Thus, multiple mechanisms could be involved in 2-ME-induced growth arrests in osteosarcoma cells, which could eventually converge to a tumor inhibitory pathway. Further investigation is necessary to delineate the molecular signals associated with 2-ME-induced growth arrest at different stages of the cell cycle in osteosarcoma cells with high metastatic potential.

Cell cycle progression in eukaryotic cells involves numerous regulatory proteins that control progression of cells through each phase of the cycle. One of these factors is a cyclin-dependent kinase (CDK) inhibitor, p21 (also called WAF-1 or CIP1), which was initially thought to function as a negative regulator of cell cycle progression [el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994]. The cell cycle inhibitory effects of p21 may be attributed to its ability to bind CDKs as proliferating cell nuclear antigen (PCNA), resulting in the inhibition of cell cycle progression. p21 plays an essential role in growth arrest after DNA damage [Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994]. The present studies show that the p21 is induced with 2-ME treatment. Previous reports show that overexpression of p21 leads to G1 and G2 arrests [Ogryzko et al., 1997; Niculescu et al., 1998]. p21 is induced by both p53-dependent and -independent mechanisms [Gartel and Tyner, 1999]. Although it was originally known as a p53-targeted gene, p21 has been implicated as a downstream effector of various tumor suppressors, growth factors and cytokines including breast cancer gene BRCA-1, Wnt-1, interferon and transforming growth factor-β [Jiang et al., 1994; Datto et al., 1995; Englert et al., 1997; Somasundaram et al., 1997; Katayama et al., 2007]. p21 participates in both pro-apoptotic and anti-apoptotic functions in response to various anti-tumor agents depending upon the cellular context [Gartel and Tyner, 2002; Liu et al., 2003].

2-ME treatment is accompanied by increases in p21 protein levels in colorectal cancer, hepatocelluar carcinoma, melanoma, prostate cancer cells, non-small cell lung cancer [Huober et al., 2000; Kumar et al., 2001; Carothers et al., 2002; Ghosh et al., 2003; Schumacher et al., 2006], and, in this study, multiple osteosarcoma cell lines. The present studies show that p21 is induced in 2-ME-treated osteosarcoma cells during G1 and G2 growth arrest and suggest p21 contributes to the cell killing. However, not all of the effects of 2-ME can be attributed to increased p21 levels. In nasopharyngeal carcinoma cells, the anti-tumor effects of 2-ME are not accompanied by changes in p21 expression [Zhou et al., 2004]. In addition, 2-ME-mediated cell cycle arrest in smooth muscle cells was accompanied by a decrease in p21 levels [Barchiesi et al., 2006].

The present studies show that another kinase inhibitor, p27 protein, is increased with 2-ME treatment in osteosarcoma cells. p27 negatively regulates G1/S transition [Hershko and Shapira, 2006]. Low or absent p27 expression is frequently observed in many human cancers, including breast, prostate, gastric, lung and colorectal cancer [Catzavelos et al., 1997; Esposito et al., 1997; Loda et al., 1997; Mori et al., 1997; Tsihlias et al., 1998, 1999]. Furthermore, downregulation of p27 is strongly associated with aggressive tumor and poor prognosis. Our results provide additional support that p27 upregulation is associated with anti-tumor effects of 2-ME. Additionally, 2-ME increased p27 expression in low metastatic cells (MG63 and SaOS2), but not in high metastatic cells (LM8) suggesting the involvement of different

anti-tumor mechanisms in low and high metastatic cells.

p53 protein is a transcription factor and a tumor suppressor that has several anti-cancer mechanisms, including activation of DNA repair proteins, induction of cell cycle block and induction of apoptosis. 2-ME treatment increases expression of functional and wildtype p53 in cancer cells resulting in apoptosis. The requirement of p53 in non-2-ME-mediated and 2-ME-mediated induction of apoptosis was documented [Chen et al., 1996; Mukhopadhyay and Roth, 1997; Mukhopadhyay and Roth, 1998; Vousden, 2006; Aylon and Oren, 2007]. On the other hand, it has been shown that growth arrest and apoptosis may occur in 2-ME-treated cells independent of p53 [Schumacher et al., 1999; Pribluda et al., 2000]. The present studies show that 2-ME treatment leads to an increase in p53 protein levels in MG63 cells. In contrast, p53 expression is not affected in other osteosarcoma cell lines (SaOS2, LM8). Thus, 2-ME-dependent cell death of osteosarcoma cells depends upon cell type and further confirms that 2-ME actions involves p53-dependent and p53-independent mechanisms.

PKR is a potent negative regulator of cell growth in eukaryotic cells [Williams, 1999; Clemens, 2003; Barber, 2005]. In addition, ectopic expression of catalytically inactive mutants of PKR in NIH3T3 cells led to their transformation and subsequent formation of tumors when these cells were injected into nude mice [Koromilas et al., 1992; Meurs et al., 1993; Barber et al., 1995]. This appears to be due to the interference with the activity of endogenous wild type PKR by the overexpressed mutant protein [Koromilas et al., 1992; Meurs et al., 1993; Barber et al., 1995]. PKR regulates p53-dependent cell cycle arrest and G1/S arrest and p21-dependent transcriptional inductions were impaired in PKR−/− cells [Cuddihy et al., 1999]. Also, PKR is essential for 2-ME-induced cell death in human osteosarcoma cells [Shogren, 2007]. Current results add additional support to this conclusion and demonstrates that PKR is essential for 2-ME-mediated cell cycle regulation. Specifically, inhibition of endogenous PKR protein in the presence of dominant negative mutant PKR protein reverses 2-ME-dependent cell cycle arrest. Also, E_2 and 16-OHE do not trigger G1/S arrest. Thus, steroids that are chemically similar to 2-ME that do not induce PKR expression [Shogren, 2007], do not have any effect on cell cycle progression. These findings further link the upregulation of PKR to the cell cycle arrest at G1/S phase boundary in the presence of 2-ME treatment. p21 regulation appears to take place upstream of PKR induction as inhibition of PKR does not have any effect on p21 induction in 2-ME-treated cells (Shogren and Maran, unpublished work). Detailed investigation could delineate the various molecular steps involved in 2-ME-dependent cell cycle arrest.

In conclusion, the present studies show that the cell cycle arrest precedes cell death in 2-ME-treated osteosarcoma cells. 2-ME regulates p21, p27, p53 and PKR factors that control cell cycle progression and influence osteosarcoma cells, leading to the blockade of cell cycle progression and cell death. Further investigation of the factors that contribute to the growth arrest will lead to a better understanding of the anti-tumor mechanisms and improved therapeutic application of this promising drug, 2-ME.

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