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Dependency of 2-methoxyestradiol-induced mitochondrial apoptosis on mitotic spindle network impairment and prometaphase arrest in human Jurkat T cells

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ABSTRACT

The present study sought to determine the correlation between 2-methoxyestradiol (2-MeO-E_2)-induced cell cycle arrest and 2-MeO-E_2-induced apoptosis. Exposure of Jurkat T cell clone (JT/Neo) to 2-MeO-E_2 (0.5–1.0 μM) caused G_2/M arrest, Bak activation, $\Delta\psi m$ loss, caspase-9 and -3 activation, PARP cleavage, intracellular ROS accumulation, and apoptotic DNA fragmentation, whereas none of these events except for G_2/M arrest were induced in Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2). Under these conditions, Cdk1 phosphorylation at Thr-161 and dephosphorylation at Tyr-15, up-regulation of cyclin B1 expression, histone H1 phosphorylation, Cdc25C phosphorylation at Thr-48, Bcl-2 phosphorylation at Thr-56 and Ser-70, Mcl-1 phosphorylation at Ser-159/Thr-163, and Bim phosphorylation were detected irrespective of Bcl-2 overexpression. Concomitant treatment of JT/Neo cells with 2-MeO-E_2 and the G_1/S blocking agent aphidicolin resulted in G_1/S arrest and abrogation of all apoptotic events, including Cdk1 activation, phosphorylation of Bcl-2, Mcl-1 and Bim, and ROS accumulation. The 2-MeO-E_2-induced phosphorylation of Bcl-2 family proteins and mitochondrial apoptotic events were suppressed by a Cdk1 inhibitor, but not by an Aurora A kinase (AURKA), Aurora B kinase (AURKB), JNK, or p38 MAPK inhibitor. Immunofluorescence microscopic analysis revealed that 2-MeO-E_2-induced mitotic arrest was caused by mitotic spindle network impairment and prometaphase arrest. Whereas 10–20 μM 2-MeO-E_2 reduced the proportion of intracellular polymeric tubulin to monomeric tubulin, 0.5–5.0 μM 2-MeO-E_2 increased it. These results demonstrate that the apoptogenic effect of 2-MeO-E_2 (0.5–1.0 μM) was attributable to mitotic spindle defect-mediated prometaphase arrest, Cdk1 activation, phosphorylation of Bcl-2, Mcl-1, and Bim, and activation of Bak and mitochondria-dependent caspase cascade.

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1. Introduction

2-Methoxyestradiol (2-MeO-E_2), an endogenous metabolite of 17β-E_2, has been examined as a promising anticancer drug candidate [1]. Recently, 2-MeO-E_2 has received great attention due to its anticancer activity along with its few undesirable side effects. The majority of tumor cell lines appear to be sensitive to the in vitro anti-proliferative properties of 2-MeO-E_2 at concentrations ranging from 0.08 μM to 5.0 μM [2,3]. Numerous studies have reported that the anticancer effects of 2-MeO-E_2 at pharmacological concentrations are exerted by inducing apoptosis, arresting the cell cycle at the G_1/S boundary and/or the G_2/M boundary, and potentially inhibiting angiogenesis [4–9].

The 2-MeO-E_2-induced apoptosis of tumor cells appears to be mediated by several different mechanisms, including up-regulation of the death receptor (DR5), p53, and p21, down-regulation of Bcl-2, phosphorylation of Bcl-2 and Bcl-xL, generation of reactive oxygen species (ROS), activation of c-Jun N-terminal kinase (JNK), and mitochondrial cytochrome *c* release in an estrogen receptor (ER)-independent manner [3,10–13]. With respect to 2-MeO-E_2-induced cell cycle arrest, its interference in cellular microtubule formation, which occurs via reducing the tubulin polymerization

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rate, has been implicated [14]. Additionally, the G_2/M-promoting doses of 2-MeO-E_2 and paclitaxel (a microtubule-polymerizing drug) have been shown to exert similar effects on the cell cycle and apoptosis in human prostate cancer cells [15]. These previous studies suggest that the mechanism underlying 2-MeO-E_2-induced cell cycle arrest may be similar to those of microtubule-targeting drugs, which commonly induce the disruption of mitotic spindles and loss of microtubule function, leading to arrest at the M phase due to activation of the mitotic spindle assembly checkpoint [16,17]. However, several studies have reported that following treatment with 2-MeO-E_2, tumor cells undergo cell cycle arrest at the G_1/S phase or late G_2 phase rather than the M phase, along with apoptosis [4–6,9]. In addition, 2-MeO-E_2 appears to inhibit tubulin polymerization by interacting with its colchicine-binding site, and the *Ki* value of 2-MeO-E_2 for inhibition of colchicine binding appears to be 22 μM, which is a much higher concentration than that which is required to induce apoptosis [14]. Furthermore, it has been reported that Bcl-2 overexpression in Jurkat T cells via retroviral transduction can prevent 2-MeO-E_2 (0.5–1.0 μM)-induced apoptosis via $p27^{Kip1}$-mediated G_1/S arrest and NF-κB activation, suggesting that Jurkat T cells might arrest at the G_1/S phase prior to undergoing apoptosis in the presence of 2-MeO-E_2 (0.5–1.0 μM) [9]. Although these previous studies raised the possibility that 2-MeO-E_2 at low doses (0.5–1.0 μM) could induce apoptosis independently of microtubule damage and subsequent mitotic arrest, the correlation between cell cycle arrest and apoptosis in tumor cells following 2-MeO-E_2 treatment requires further investigation in order to clarify the anticancer activity of 2-MeO-E_2.

Recently, to obtain direct evidence for a causal link between 2-MeO-E_2-induced cell cycle arrest and 2-MeO-E_2-induced apoptosis, we also decided to take advantage of Bcl-2 overexpression, which can inhibit 2-MeO-E_2-induced apoptosis. Bcl-2 overexpression has previously been utilized to determine the correlation between p53-mediated G_1 arrest and p53-mediated apoptosis in murine M1 myeloid leukemia cells, in which p53-mediated G_1 arrest was not detectable unless the simultaneous induction of p53-mediated apoptosis was delayed by Bcl-2 overexpression [18]. In this study, to examine whether 2-MeO-E_2 arrests cell cycle progression at the G_1/S phase and/or the G_2M phase and the mechanism by which 2-MeO-E_2-induced cell cycle arrest activates the apoptotic death pathway, we investigated the apoptogenic mechanism of 2-MeO-E_2 (0.1–1.0 μM) using Jurkat T cell clone stably transfected with an empty vector (JT/Neo) or a *Bcl-2* expression vector (JT/Bcl-2). To further examine the dependency of 2-MeO-E_2-induced apoptotic events on G_1/S arrest and/or G_2/M arrest, we investigated the effect of aphidicolin (APC), which is known to arrest cell cycle progression at the G_1/S border [19,20], on 2-MeO-E_2-induced apoptosis.

2. Materials and methods

2.1. Reagents, antibodies, and cells

2-MeO-E_2, APC, 3,3′dihexyloxacarbocyanine iodide ($DiOC_6$), and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical (St. Louis, MO, USA). An ECL western blot kit was purchased from Amersham (Arlington Heights, IL, USA), and the Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA, USA). The anti-caspase-3 antibody was purchased from Pharmingen (San Diego, CA), and the anti-poly (ADP-ribose) polymerase (PARP), anti-Bax, anti-Bim, anti-Bcl-2, anti-Bcl-xL, anti-Mcl-1, anti-Cdk1, anti-cyclin B1, anti-Aurora A kinase (AURKA), anti-histone H1, anti-p-histone H3 (Ser-10), anti-histone H3, anti-lamin B, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-caspase-9, anti-p-Cdk1 (Tyr-15), anti-p-Cdk1 (Thr-161), anti-p-Cdc25C (Thr-48), anti-Cdc25C, anti-p-Bcl-2 (Thr-56), anti-p-Bcl-2 (Ser-70), anti-p-Mcl-1 (Ser-159/Thr-163), anti-p-AURKA (Thr-288), anti-p-c-Jun (Ser-63), and anti-α-tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), and the anti-p-histone H1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The anti-Bak (Ab-1) and anti-Bax (6A7) antibodies were obtained from Calbiochem (San Diego, CA, USA). The anti-p-Bcl-xL (Ser-62) antibody was obtained from Invitrogen (Carlsbad, CA, USA). The Cdk1 inhibitor RO3306 was purchased from Tocris Bioscience (Ellisville, MO, USA), and the AURKA inhibitor MLN8237 and Aurora B kinase (AURKB) inhibitor AZD1152-HQPA were obtained from Selleck (Huston, TX, USA). The JNK inhibitor SP600125 and the p38 MAPK inhibitor SB202190 were purchased from Biomol (Plymouth Meeting, PA, USA). The JNK inhibitor IX and the ROS sensitive probe dihydroethidium (DHE) were obtained from Santa Cruz Biotechnology. Human acute leukemia Jurkat T cell clone, stably transfected with a *Bcl-2* expression vector (JT/Bcl-2) or with an empty vector (JT/Neo) was kindly provided by Dr. Dennis Taub (Gerontology Research Center, NIA/NIH, Baltimore, MD, USA). Both JT/Neo cells and JT/Bcl-2 cells were maintained in RPMI 1640 medium containing 10% FBS, 20 mM HEPES (pH 7.0), 5×10^{-5} M β-mercaptoethanol, 100 μg/ml gentamicin, and 400 μg/ml G418 (A.G. Scientific Inc., San Diego, CA, USA). JT/Bcl-2 cells overexpressing Bcl-2 and JT/Neo cells were identified by western blot analysis. These stable clones were kept in culture for no more than 3 months before the studies, and used in numerous our previous investigations including a recent study [21]. To investigate the effect of the Cdk1 inhibitor (RO3306) [22], the AURKA inhibitor (MLN8237) [23], the AURKB inhibitor (AZD1152-HQPA) [24], the JNK inhibitors (SP600125 or JNK inhibitor IX) [25,26], and the p38 MAPK inhibitor (SB202190) [27] on 2-MeO-E_2-induced apoptotic events, JT/Neo cells were pretreated with the individual inhibitors for 1 h prior to 2-MeO-E_2 treatment for 20 h.

2.2. Flow cytometric analysis

Flow cytometric analysis to measure cell cycle state of Jurkat T cells exposed to 2-MeO-E_2 was performed on a FACS Calibur (BD Sciences, San Jose, CA, USA) as described elsewhere [28]. The extent of necrosis was detected using an Annexin V-FITC apoptosis kit as previously described [28]. The changes in the mitochondrial membrane potential ($\Delta\psi m$) following 2-MeO-E_2 treatment were measured after staining with $DiOC_6$ [29,30]. Activation of Bak and Bax in Jurkat T cells following 2-MeO-E_2 treatment was measured as previously described [31]. To measure intracellular ROS accumulation, the cells were treated with DHE at 37 °C for 30 min, and the fluorescence intensity was analyzed by flow cytometry (FACS Aria III system, BD Sciences) with an excitation wavelength of 488 nm [32].

2.3. Immunofluorescence microscopy

Immunostaining of Jurkat T cells treated with 2-MeO-E_2 was performed as previously described [33].

2.4. Preparation of cell lysates and western blot analysis

Cell lysates were prepared by suspending 5×10^6 Jurkat T cells in 300 μl of lysis buffer as described elsewhere [28]. An equivalent amount of protein lysate (20 μg) was electrophoresed on a 4–12% NuPAGE gradient gel and then electrotransferred to an Immobilon-P membrane. Protein detection was performed using an ECL western blot kit according to the manufacturer's instructions.

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