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Induction of Apoptosis or Necrosis in Human Endometrial Carcinoma Cells by 2-Methoxyestradiol

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Abstract. *Background: We investigated the effects of 2-methoxyestradiol (2-ME), an endogenous estrogenic metabolite, on human endometrial cancer HEC-1-A and RL-95-2 cell lines. Materials and Methods: After exposure of HEC-1-A and RL-95-2 cells to 2-ME, the morphological changes were evaluated by acridine orange staining and transmission electron microscopy. Cell cycle progress, apoptosis and necrosis were assessed by flow cytometry, DNA fragmentation and Western blot. Results: 2-ME inhibited cell growth by blocking the S- and G2/M-phase in both cell lines, by inducing apoptosis in HEC-1-A cells and by causing necrosis in RL-95-2 cells. Apoptosis, on HEC-1-A cells, was accompanied by an increased expression of iNOS and STAT1. This apoptotic effect was prevented by the iNOS inhibitor 1400W and eliminated by the caspase inhibitor Z-VAD-FMK. Necrosis, on RL-95-2 cells, was due to a severe disruption of the mitochondrial membrane potential. 2-ME had no significant effect on normal human endometrial cells. Conclusion: The data suggest that 2-ME has an antitumor effect on human endometrial carcinoma cells (HEC-1-A and RL-95-2) and may contribute as a new therapeutic agent for endometrial cancer patients.*

Endometrial cancer is the most common gynecological malignancy and the fourth most common cancer in women (1). The prognosis for patients with more advanced or recurrent endometrial disease is poor, with a median survival of less than 1 year (2). The treatment is usually limited to systemic therapy – endocrine therapy and chemotherapy. The chemotherapy is associated with frequent severe toxicity. As endometrial cancer is most common in elderly women, the tolerability of treatment is important and a better tolerated and more effective treatment is needed.

2-Methoxyestradiol (2-ME) is a metabolite of endogenous estradiol-17β and the oral contraceptive agent 17-ethylestradiol, with low binding affinity to estrogen receptors α and β and androgen receptor. It has been shown that 2-ME is an effective inhibitor of tumor growth, including induction of apoptosis (3-5), inhibition of the microtubules (6-9) and antiangiogenesis (10-12). 2-ME appears to be tumor-specific, since no or very little toxicity was observed in normal tissues and in *in vivo* experiments (11,13,14). The action of 2-ME on human endometrial cancer is unknown.

The purpose of the present study was to examine the effects of 2-ME on the growth of the human endometrial carcinoma HEC-1-A and RL-95-2 cell lines.

Materials and Methods

Materials. 2-ME, estradiol-17β and acridine orange were purchased from Sigma Chemical (St. Louis, MO, USA), the AnnexinV-EGFP kit from Clontech Laboratories (Palo Alto, USA) and the Depsipher™ kit from R&D Systems (Minneapolis, MN, USA). Monoclonal antibodies iNOS and STAT1 were obtained from BD Transduction Laboratories (Lexington, KY, USA) and sheep anti-mouse horseradish peroxidase linked second antibody from Life Science (USA). iNOS inhibotor 1400W was purchased from Alexis Biochemicals (San Diego, CA, USA) and Caspase inhibitor Z-VAD-FMK from Calbiochem (San Diego, CA, USA). Human endometrium cancer cells (HEC-1-A and RL-95-2) were purchased from ATCC (Manassas, VA, USA).

Cell culture. HEC-1-A (derived from moderately-well-differentiated carcinoma) was cultured in McCoy's 5A medium supplemented with 10% FBS and RL-95-2 (derived from moderately-differentiated adeno-squamous carcinoma) was cultured in RPMI-1640 supplemented with 10% human serum, in a humidified environment of 5% CO_2 and 95% air at 37˚C.

Normal human endometrial cells were obtained from the endometrium of 4 patients, aged 37-49 years, who underwent abdominal hysterectomy because of uterine myoma. The study was

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approved by the Ethics Committee of Uppsala University, Sweden, and all patients gave their informed consent. The cells were isolated using an enzymatic solution (2.5 mg/ml collagenase, 50 μg/ml deoxyribonuclease and 200 μg/ml hyaluronidase) and cultured in RPMI-1640 with 10% FBS. The cells contained both stromal and glandular epithelial cells, as determined on the basis of morphology.

Growth assay. Cells were seeded into 10-cm culture dishes at a density 1 x 10^6/dish and incubated for 48 hours. After treatment with 2-ME, the cells were enzymatically harvested at 12, 24, 36, 48, 60 and 72 hours, respectively, and directly counted in a hemocytometer. The total cells were calculated.

Flow cytometry analysis. Cell cycle analysis was performed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, USA) as described by Vindelöv *et al.* (15). The sub-G1 fraction cells (usually as a marker of apoptosis and/or necrosis) and the viable cells of G_0/G_1-phase, G_2/M-phase and S-phase were determined as percentage of the total population by the computer program Cell-Quest (Becton Dickinson).

Cell membrane phosphatidylserine (PS) was detected, according to the kit instruction, by Annexin V-EGFP (Clontech Laboratories). Mitochondrial membrane potential (MMP) disruption was measured by using the mitochondrial activity marker Depsipher™ (5, 5', 6, 6', -tetrachloro-1, 1', 3, 3' -tetraethylbenzimidazolyl) (R&D Systems).

Morphological assessment of apoptotic and G_2/M-arrested cells. To observe cells undergoing apoptosis, the cell monolayer was fixed with fresh 3.7% formalin-PBS for 10 minutes. After washing with PBS, the cold 70% methanol was added and incubated at -20˚C for 5 minutes. Following another washing with PBS and addition of fresh 0.1% Triton-100-PBS for 5 minutes, the cells were stained with acridine orange (5 mg/ml). The characteristic morphological features of apoptosis and G_2/M-arrested cells were observed by fluorescence microscopy.

Transmission electron microscopy (TEM). Cancer cells were fixed with 2.5% glutaraldehyde in 0.1 M sodiumcacodylate buffer overnight at 4˚C. Then the cells were washed and post-fixed with 2% OSO_4 for 1 hour at 4˚C. The cells were dehydrated with graded alcohols and propylene oxide, as described previously (16). The cells were removed from the culture wells with propylene oxide and embedded in pure plastic. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and observed by TEM.

DNA fragmentation analysis. After treatment with 2-ME, cells (5-10x10^6) were collected and lysed in lysis buffer (contains 50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, pH 8.0) and keep on ice for 20 minutes. The lysates were incubated with RNAse A (20 mg/ml) for 1 hour and proteinase K (0.1 mg/ml) for 1.5 hours. After incubation, DNA was gently extracted with phenol/chloroform/isoamyl alcohol and electrophoresed on a 1.2% agarose gel containing ethidium bromide. DNA fragments were visualized under ultraviolet light.

Western blot analysis. After treatment, the cells were lysed by incubation with 100 ml lysis buffer (containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.02% sodium azide, 100 mg/ml phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin, pH 8.0) for 1 hour on ice. Aliquots of lysate were centrifuged for 30 minutes at 13,000 g at 4˚C and the supernatant was assayed for protein concentration (Lowry). Cell lysate proteins (20 μg) were boiled for 5 minutes in sample buffer and resolved by 6% SDS-PAGE. The proteins were electroblotted onto nitrocellulose filters, which were then blocked with 5% fetal bovine serum for 1 hour and incubated with the primary antibodies of iNOS and STAT1 at 4˚C overnight. The blots were washed and incubated with HRP-labelled second antibody for 1 hour, then developed by using an enhanced chemiluminescence detection system (ECL, Amersham Corp, UK).

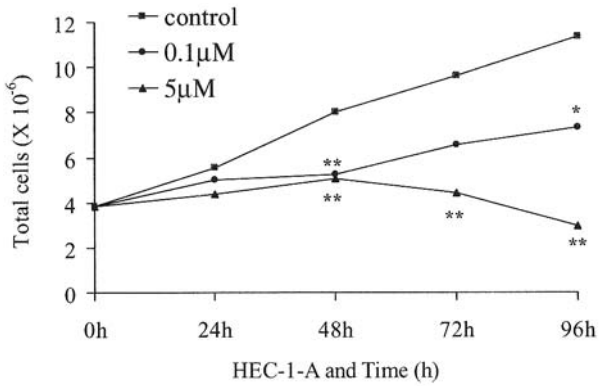

Figure 1. *Effect of 2-ME on cell proliferation. HEC-1-A cells were treated with 0.1 and 5 mM 2-ME for 96 h. The total cells were calculated (n=3). Vehicle cells were added and equal volume of DMSO as 2-ME-treated cells. * $p<0.05$, ** $p<0.01$.*

Results

Effects of 2-ME on cell growth. Growth inhibition was observed on HEC-1-A cells exposed to 0.1-5 μM 2-ME after 48-hour treatment (Figure 1, n=3). However, on RL-95-2 cells, a similar inhibitory effect was observed at a concentration of 10 μM after 60-hour treatment (data not shown).

Cell cycle progress regulated by 2-ME. The effect of 2-ME on cell cycle progression was evaluated by using flow cytometry analysis. 2-ME caused sub-G1 cell accumulation and induced cell cycle arrest at S-phase and G_2/M-phase in a dose- and time-dependent manner on both HEC-1-A and RL-95-2 cell lines. The G_2/M-phase arrest occurred earlier than sub-G1- and S-phase cell accumulation, indicating that the block of G_2/M-phase was the first phenomenon after 2-ME treatment (Figure 2, n=3).

Effect of 2-ME on HEC-1-A cells. The morphological features of HEC-1-A cells were observed under light microscopy (Figure 3A). 2-ME (5 μM) reduced the amount of cells and increased the nonadherent cells (Figure 3A). Acridine orange staining showed that cells arrested at G_2/M-phase were indicated by bright yellow color with abnormal spindles and condensed nucleus (Figure 3B).

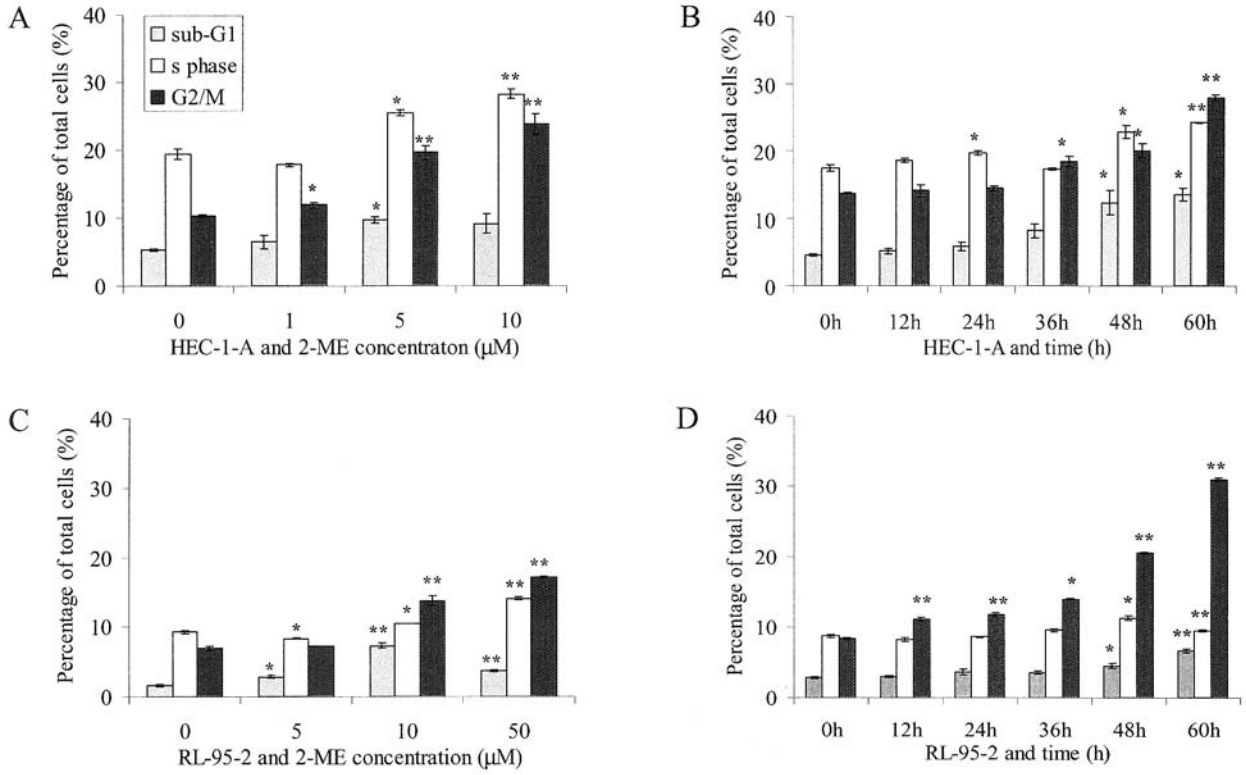

Figure 2. *Cell cycle progress analyzed by flow cytometry. (A) HEC-1-A cells were treated with different concentrations of 2-ME for 48h. (B) HEC-1-A cells were treated with 5 mM 2-ME for different times. (C) RL-95-2 cells were treated with different concentrations of 2-ME for 60 h. (D) RL-95-2 cells were treated with 10 μM 2-ME for different times. n=3. *p<0.05 **p<0.01.*

The cell membrane PS was measured by flow cytometry. Annexin V-positive cells (apoptotic cells) were increased from 4.0% to 6.7% and PI-positive cells (dead cells) were reduced from 38.0% to 23.0% after 5 μM 2-ME treatment for 48 hours (Figure 3C, n=2).

The apoptotic effect of 2-ME was confirmed by DNA fragmentation. A time- and dose-dependent change in amounts of oligonucleosomal-length DNA fragmentations was detected after application of 2-ME to HEC-1-A cells. The greatest effect was reached at a concentration of 5 mM (Figure 3D, n=3). These results indicate that the increased cell accumulation at sub-G1-phase was mainly due to the apoptosis of HEC-1-A cells.

17β-estradiol (E_2), at concentration of 10 nM, had no significant effect on HEC-1-A cells. There was no difference in cell cycle progress between cells treated with 2-ME and treated with 2-ME in combination with E_2 (data not shown).

Effect of 2-ME on RL-95-2 cells. Ten mM 2-ME treatment resulted in an increase in the sub-G1, S and G2/M cell populations in RL-95-2 cells (data not shown). G2/M cell cycle arrested cells were found by acridine orange staining as indicated by the yellow fluorescence (Figure 4A). In addition, cell membrane PS measurement showed that the apoptotic cells were changed from 4% to 6.6% ($p>0.05$) and the dead cells were enhanced markedly from 4.4% to 9.8% ($p<0.05$) after 2-ME treatment (Figure 4B, n=3). However, no apoptosis was detected by DNA electrophoresis. These data suggest that the increased sub-G1 population by 2-ME was due to the induction of necrosis on RL-95-2 cells.

Ultrastructural changes by 2-ME. Under TEM, the ultrastructure of untreated HEC-1-A cells showed a large nucleus and little glycogen in the cytoplasm, indicating great metabolic activity (Figure 5.1). After treatment with 5 mM 2-ME for 24 hours, the metaphase cells increased substantially. The condensed chromosomes were arrayed in a disorderly fashion in the middle of the cells, indicating the dysfunction of the mitotic spindle. The mitochondria had a higher electric density suggesting that they had condensed and the endoplasmic reticulum (ER) had expanded markedly (Figure 5.2). Apoptotic cells contained numerous DNA fragments (Figure 5.3) and nuclear condensation (Figure 5.4). All cells had a complete cytoplasm (Figure 5.4).

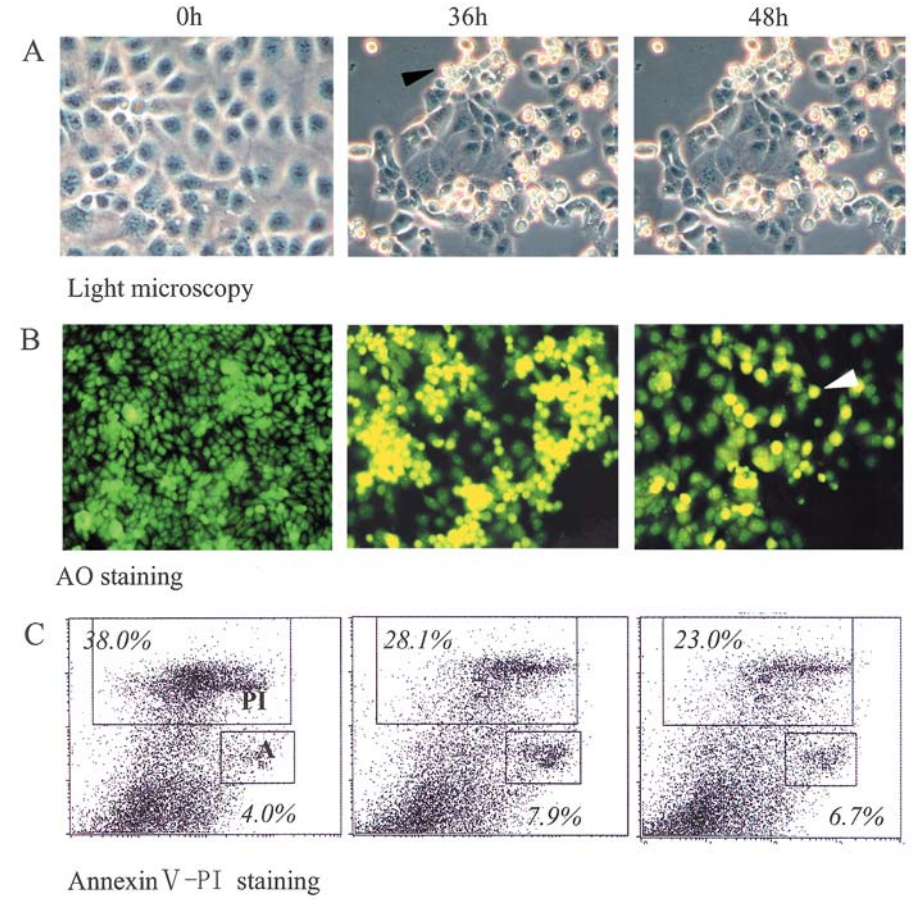

Figure 3. *Effects of 2-ME on HEC-1-A cells. (A) Cells under light microscopy. Arrow shows the non-adherent cells. (B) Cells stained with AO and observed by fluorescent microscopy (x200). Arrow shows the unusual metaphase cells. (C) AnnexinV-PI measurement by flow cytometry. A: Annexin V, PI: propidium iodide. (D) 2-ME-induced apoptosis was confirmed by electrophoretic analysis of internucleosomal DNA fragmentation.*

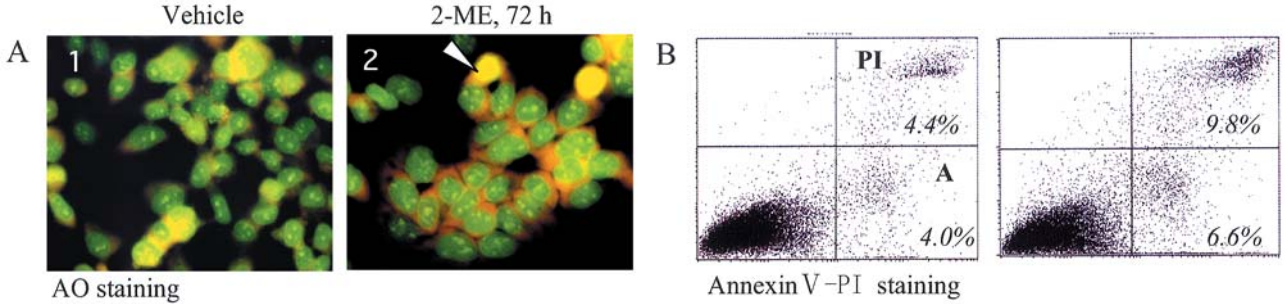

Figure 4. *Effects of 2-ME on RL-95-2 cells. (A) Cells with AO staining under fluorescent microscopy (x400). Arrow indicates the G2/M cell cycle-arrested cells (unusual metaphase cells). (B) AnnexinV-PI staining and flow cytometry show the alternations on apoptotic and necrotic cells. A: Annexin V, PI: propidium iodide.*

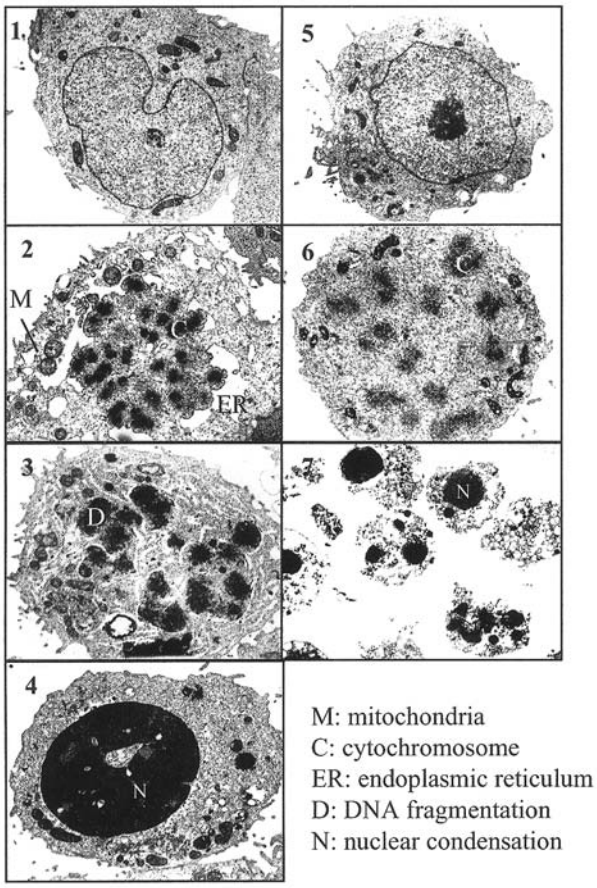

Figure 5. *Ultrastructure of HEC-1-A and RL-95-2 cells before and after treatment with 2-ME. (1-4) HEC-1-A cells. (1) Without treatment (x5000). (2) Unusual metaphase cell with abnormal chromosomes array after 24-h treatment (x5000). (3) Apoptotic cells contained numerous DNA fragments, (x5000). (4) The cells with broken nucleus, but a complete cytoplasm after 48 h treatment (x6000). (5-7) RL-95-2 cells. (5) Without treatment (x6000). (6) Unusual metaphase cell with abnormal chromosomes array after 24-h treatment (x5000). (7) Necrotic cells with broken cytoplasm membrane, condensed or broken nucleus after 60-h treatment (x2000).*

Figure 5.5 shows RL-95-2 cells without 2-ME treatment. Abnormal metaphase cells were found after treatment with 10 mM 2-ME for 24 hours (Figure 5.6). After 48-hour treatment, necrotic cells were observed as broken plasma membrane, broken nucleus and expanded cytoplasm organelles (Figure 5.7). No anaphase or telophase cells were observed in either HEC-1-A or RL-95-2 cells, indicating that the cells were blocked in metaphase.

Disruption of mitochondrial membrane potential (MMP) by 2-ME. Depsipher^{TW} was used to mark the activity of mitochondria. After 48-hour treatment by 5 μM 2-ME, on HEC-1-A cells, the MMP destruction was increased from 17.0% to 26.6% (Figure 6A, n=4) and on RL-95-2 cells from 12.2% to 34.8% (Figure 6B, n=4).

Effects of 2-ME on iNOS and STAT1 expression of HEC-1-A cells. To further investigate the mechanism of apoptosis on HEC-1-A cells, Western blot was used. The expression of iNOS increased quickly after exposure to 2-ME for 12 hours and peaked at 48 hours, then started to reduce at 72 hours (Figure 7A). A prominent band was observed at the concentration of 1 mM (Figure 7B). The increased amount of STAT1 after 2-ME treatment was time- and concentration-dependent and the peak occurred at 24 hours (Figure 7A and 7B). The amount of oligonucleosomal-length DNA was prevented by the iNOS inhibitor 1400W (Figure 7C) and eliminated by the caspase inhibitor Z-VAD-FMK (Figure 7D).

Effects of 2-ME on normal endometrial cells. 2-ME (5 μM, 48 hours) had no effects on the morphological characteristics and cell cycle progress of normal human endometrial cells (including stromal and grandular epithelial cells) (data not shown).

Discussion

In the present study, we investigated the effect of 2-ME on human endometrial cancer cells (HEC-1-A and RL-95-2 cells) and normal endometrial cells. The data showed that 2-ME inhibited the growth of both cancer cells through different mechanisms. On HEC-1-A cells, the growth inhibition was caused by blocking cell cycle progress in the S- and G_2/M-phase, and by induction of apoptosis. On RL-95-2 cells, inhibition was caused by blocking cell cycle progress in the S- and G_2/M-phase, and by induction of necrosis.

Flow cytometry analysis showed that both HEC-1-A and RL-95-2 cells were arrested in the G_2/M-phase of the cell cycle after 2-ME exposure. Moreover, acridine orange staining and TEM suggested that G_2/M blocking occurred in metaphase. Previous studies suggested that the metaphase arrest by 2-ME is caused by its toxicity to microtubules or to the regulators of cell cycle progression. 2-ME may destabilize (11,17) or stabilize the microtubules (12, 18). p34cdc2 (p34 cell division cycle 2) kinase complex is a regulator of cell cycle, which modulates the progression of cells from G2- into M-phase. It has been demonstrated that 2-ME inactivated the cyclinB/ p34cdc2 complex by decreasing p34cdc2 and cyclin B1 protein levels (19), by increasing the accumulation of phosphorylated p34cdc2 (20) and by causing phosphorylation of cdc25 C (21), which is an activator of p34cdc2.

S-phase makes up the interphase, during which DNA and other cellular macromolecules are synthesised. In the present study, 2-ME caused S-phase arrest in both types of endometrial cancer cells, but the mechanism is still unclear.

2-ME induced apoptosis on HEC-1-A cells and necrosis on RL-95-2 cells, which was confirmed by Annexin V-PI staining, acridine orange staining, DNA fragmentation and

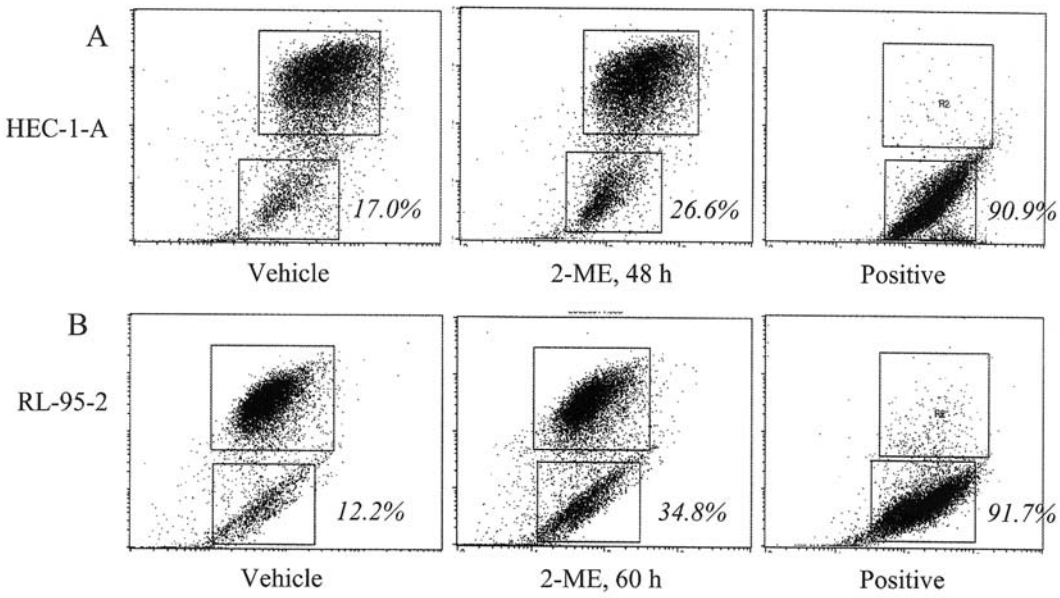

Figure 6. *Effects of 2-ME on disruption of mitochondrial membrane potential (MMP). (A) HEC-1-A cells, the MMP disrupted cells changed after treatment with 5 μm 2-ME for 48 h ($p>0.05$, $n=4$). (B) RL-95-2 cells, the MMP disrupted cells increased after treatment with 10 μM 2-ME for 60 h ($p<0.05$, $n=4$). Positive control, valinomycin 100 nM for 16 h.*

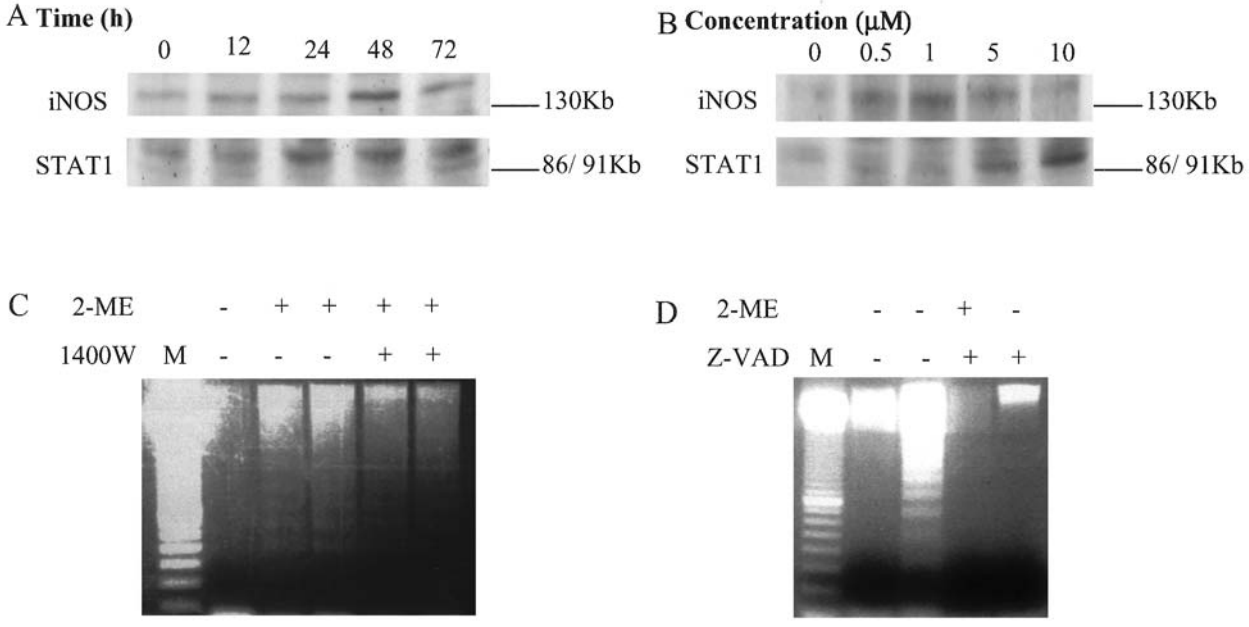

Figure 7. *Effects of 2-ME on iNOS and STAT1 expression of HEC-1-A cells. (A) The increased expression of iNOS, (B) of STAT1 was observed after 2-ME-treatment ($n=2$). (C) Apoptosis was prevented by pre-treating the cells with iNOS inhibitor 1400W (100 μM, 500 μM, 4 h). (D) Apoptosis was blocked by pre-treating the cells with caspases inhibitor Z-VAD-FMK (100 μM, 1 h).*

TEM. The mitochondria appear to be a core component of the cell death machinery (22). Mitochondrial permeability transition (PT) involves a dynamic multiprotein complex formed in the contact site between the inner and outer mitochondrial membranes. The PT complex can function as a sensor for stress and damage. Massive induction of PT causes necrosis and the plasma membrane integrity is disrupted before apoptogenic proteases come into action. In

contrast, a more subtle, regulated induction of PT allows for activation of proteases, thus giving rise to the apoptotic phenotype. The disruption of the MMP can provoke PT pore. Our data on MMP showed that mitochondria were involved in the effect of 2-ME on both the HEC-1-A and RL-95-2 cells. In addition, the disruption of MMP in RL-95-2 cells was stronger than in HEC-1-A, suggesting that the difference in cell death is determined by disruption-degree on the mitochondria.

The signal changes during apoptosis are of particular interest. The mechanisms of nitric oxide (NO)-induced apoptosis have been suggested to involve enhancement of Fas-L/Fas interactions (23), regulation of p38 mitogen-activated protein kinase (24, 25) and damage to mitochondria (26-28). A convenient way to investigate NO is to measure NOS (nitric oxide synthase). Western blot analysis was employed to study the expression of iNOS on HEC-1-A cells. An increased expression of iNOS was seen after 2-ME treatment in a dose- and time-dependent manner. These changes were parallel with apoptosis indicating a correlation between iNOS and apoptosis. In addition, the iNOS inhibitor 1400W prevented the apoptosis which further confirms that a NO-dependent pathway was involved in 2-ME-induced apoptosis in HEC-1-A cells.

The STAT proteins were identified as transcription factors, which were critical in mediating virtually all cytokine-driven signaling. These proteins are latent in the cytoplasm and become activated through tyrosine phosphorylation, which typically occurs through cytokine receptor associated kinases (JAKs) or protein tyrosine kinase (PTK). STAT1 plays an important role in growth arrest, in promoting apoptosis (29-31), and is required for the constitutive expression of some caspases (32, 33). Inhibitors of PTK (AG-126), or JAK2 (AG-490) inhibited TNF-alpha, NO production, caspase 1 activation and apoptosis (34), indicating that the JAK2/STAT1 pathway is associated with NO production. In our study, 2-ME increased the expression of STAT1 and caused apoptosis which was blocked by the caspases inhibitor Z-VAD-FMK, suggesting that both STAT1 and caspases were involved in the action of 2-ME. Our findings indicate that apoptosis by 2-ME on HEC-1-A may be mediated by the JAK/STAT1 pathway.

However, the receptors by which 2-ME activated the JAK/STAT1 pathway are not clearly identified. It is reported that 2-ME induces apoptosis by activating a putative GPCR (G protein-couples receptor) (35). In a study of thrombin in tumor cells, one of a novel GPCR family, PAR-1 (protease-activated receptor) was found to have a link with the STAT signal pathway and induction of apoptosis (36). The above findings indicated that the JAK-STAT pathway may be activated through GPCR in apoptosis by 2-ME. In addition, 2-ME induces interferon gene expression (37), which is known to activate the JAK-STAT pathway (38). Therefore, we hypothesize that 2-ME may activate the JAK-STAT1 pathway through GPCR, or changing cytokines level, then increases NO production, activates caspases and induces apoptosis.

Taken together, these results suggest that 2-ME has antitumor effects on HEC-1-A and RL-95-2 cells. The apoptosis on HEC-1-A cells involved the activation of iNOS, caspases and STAT1. 2-ME had no significant effects on normal endometrium cells. 2-ME may contribute as a new therapeutic agent for endometrial cancer patients, but its adverse effect needs to be investigated further.

Acknowledgements

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