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Induction of Apoptosis and G2/M Arrest by 2-Methoxyestradiol in Human Cervical Cancer HeLaS3 Cells

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Abstract. *Background: It has been demonstrated that 2-Methoxyestradiol (2-ME), one of the estrogen metabolites, induces apoptosis in many different tumor cell lines. In the present study, the effects of 2-ME on human cervical cancer HeLaS3 cells and on normal cervical epithelial cells were evaluated. Materials and Methods: Acridine orange staining, DNA fragmentation arrays and flow cytometry were used to measure the apoptosis and cell cycle progression. In addition, the effect of 2-ME on expression of iNOS was measured by Western blot. Results: 2-ME inhibited the growth of HeLaS3 cells. This growth inhibition was accompanied by apoptosis and G_2/M cell cycle arrest. 2-ME increased the expression of iNOS in parallel with apoptosis. Moreover, apoptosis was prevented by the iNOS inhibitor 1400W. 2-ME treatment resulted in a slight increase of the G_2/M population, but no apoptosis, in normal cervical epithelial cells. There was no synergetic effect between E_2 and 2-ME. Conclusion: 2-ME induced apoptosis via the iNOS pathway and caused G_2/M cell cycle arrest in human cervical cancer HeLaS3 cells, but showed only slight effects on normal cervical epithelial cells. These data suggest that 2-ME might be an adjuvant agent in the treatment of cervical cancer.*

2-Methoxyestradiol (2-ME), an endogenous metabolite of 17β-estradiol, has been shown to possess potent antitumor activity and to induce apoptosis in a wide range of tumor cell lines (1-5). The cytotoxic effects of 2-ME in cancer cells are attributed, in part, to effects on DNA synthesis, mitosis, faulty spindle formation and chromosome distribution (6-8).

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Studies *in vivo* have demonstrated an inhibitory effect of 2-ME on angiogenesis and metastasis of solid tumors (2, 9-11). Unlike the toxicity usually associated with conventional chemotherapeutic agents, 2-ME did not result in hair loss, gastrointestinal disturbance, or leukocyte reduction in bone marrow and thymus (9,10,12).

Carcinoma of the cervix is the second most common neoplasm in women (13) and comparable cure rates have been documented by radical hysterectomy and pelvic radiation. In addition, cervical cancer is responsive to cisplatin/cisplatin-based combination chemotherapy which has prompted evaluation of the role of chemotherapy in the overall management of high risk early stage cervical cancer, of locally advanced higher stage disease, and of recurrent or metastatic disease. However, most agents are associated with frequent severe toxicity and there is a growing demand for new agents.

2-ME's antitumor activity and its minimal toxicity stimulated our interest in its use in managing human cervical carcinoma. We chose HeLaS3 cells, adenocarcinoma cell lines, as a research model, since recently the incidence of invasive 'pure' adenocarcinomas and mixed adenosquamous cancers have risen in proportion to squamous cancers, a phenomenon especially marked in younger women (14).

Materials and Methods

Reagents. 2-ME, E_2 and acridine orange (AO) were purchased from Sigma Chemicals (St. Louis, MO, USA). Cell proliferation ELISA, BrdU (colorimetric) kit was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Monoclonal antibody iNOS was obtained from BD Transduction Laboratories (Lexington, KY, USA) and sheep anti-mouse horseradish peroxidase-linked second antibody was from LIFE Science (USA). The iNOS inhibitor 1400W was purchased from Alexis Biochemicals (San Diego, CA, USA) and HeLaS3 cells from ATCC (Manassas, VA, USA).

Cell culture. HeLaS3 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS in a humidified environment of 5% CO_2 and 95% air at 37˚C.

The normal cervical cells were isolated from 3 female patients aged 36-44 years old who underwent abdominal hysterectomy because of uterine myoma. The study was approved by the Ethics Committee of Uppsala University, Sweden and all patients gave their informed consent. The cervical tissues were taken from different parts of the cervix to obtain columnar, stratified squamous and transitional epithelial cells. The tissues were cut into small pieces and digested in an enzymatic solution (0.1% trypsin and 0.02% EDTA) for 60 minutes. The isolated cells were cultured in Epilife medium (Cascade Biologics) with human keratinocyte growth supplement, 60 μM calcium, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The characteristics of epithelial cells were determined on the basis of morphology.

Growth assay. The effect of 2-ME on cell proliferation was measured by cell proliferation ELISA, BrdU (colorimetric) Kit according to the manufacturer.

Flow cytometry assay. Cell cycle progress was measured by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, USA) as described previously (15). The sub-G1 fraction cells, as a marker of number of apoptotic cells, and cell cycle compartments of viable cells including G_0/G_1-phase, G_2/M-phase and S-phase of cells were determined as percentage of the total population by the computer program Cell-Quest (Becton Dickinson).

Morphological assessment. The morphological features of apoptotic cells (chromatin condensation and fragmentation) and metaphase blocked cells (abnormal spindles, uneven chromosomes) were monitored by fluorescence microscopy. At least 500 cells from randomly selected fields were counted in each dish and 6 dishes were used in each group.

The cell monolayer in 35-mm dishes was fixed with fresh 3.7% formalin-PBS for 10 minutes. After washing with PBS, cold 70% methanol was added and incubated at -20˚C for 5 minutes, again washed with PBS and then subjected to fresh 0.1% Triton-100-PBS for 5 minutes. Finally, the cells were stained with 5 μg/ml acridine orange.

DNA fragmentation analysis. To detect apoptosis DNA gel electrophoresis was used. Cells (5-10 x 10^6) were collected and lysed in lysis buffer (containing 50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, pH 8.0) and kept on ice for 20 minutes. The lysates were incubated with RNAse A (20 mg/ml) for 1 h and proteinase K (0.1 mg/ml) for 1.5 h. After incubation, DNA was gently extracted with phenol/chloroform/isoamyl alcohol and electrophoresed on a 1.2% agarose gel, containing ethidium bromide. DNA ladders were visualized under ultraviolet light.

Western blot analysis. The change in expression of iNOS caused by 2-ME was examined using Western blot as previously reported (16). The band was quantified by Scion Image (Scion Corporation, USA) and expressed as area x density.

Statistical analysis. Experimental data are presented as the mean±SEM of measurements from at least three culture wells. Student's *t*-test was used for unpaired comparisons between two means of treatment and vehicle groups.

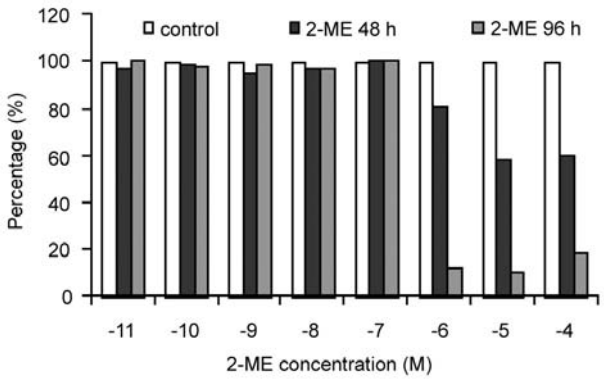

Figure 1. *Effect of 2-ME on HeLaS3 cells. Cells were treated with 2-ME (10^{-11} M to 10^{-4} M) for 48 and 96 h. The absorbance of control cells was regarded as 100%. The absorbance of treated cells reduced largely from 10^{-6} M to 10^{-4} M (n=4).*

Results

Growth inhibition by 2-ME. Growth inhibition was observed in HeLaS3 cells exposed to 2-ME at a concentration of 10^{-6}-10^{-4} M after 48-h treatment ($p<0.05$) and a stronger effect was found after 96-h treatment ($p<0.01$, Figure 1).

Apoptosis and cell cycle regulation by 2-ME. Five μM 2-ME treatment resulted in an increase of sub-G1 (apoptotic cells), G_2/M population and a decrease in G_0/G_1 population in HeLaS3 cells ($p<0.01$, respectively). There was no significant difference between vehicle and 2-ME-treated cells in the percentage of S-phase cells (Figure 2a). During the first 5-h treatment, the percentage in increase of the ratio of G_2/M cells was greater than that of apoptotic cells, indicating that the G_2/M block occurred prior to apoptosis ($p<0.01$, Figure 2b).

Morphological assessment of apoptosis was performed with fluorescence microscopy. A large number of abnormal cells with uneven chromosome distribution were observed after exposure to 5 μM 2-ME for 20 h (Figure 3b), indicating that G_2/M arrest was due to a metaphase block. Some features of apoptotic cells, such as chromatin condensation and nuclear fragmentation, were also found following 2-ME treatment (Figure 3c). To show the total effect of 2-ME on HeLaS3 cells, the percentage of apoptotic and metaphase-blocked cells were calculated and presented as a line chart. 2-ME induced apoptosis and blocked metaphase in a dose- and time-response manner (Figure 3d and 3e).

The DNA ladder fragmentation, the hallmark of apoptosis, was examined by gel electrophoresis to confirm the apoptosis. A time- and dose-dependent change in the amount of oligonucleosomal-length DNA fragmentation was detected after application of 2-ME on HeLaS3 cells (Figure 4).

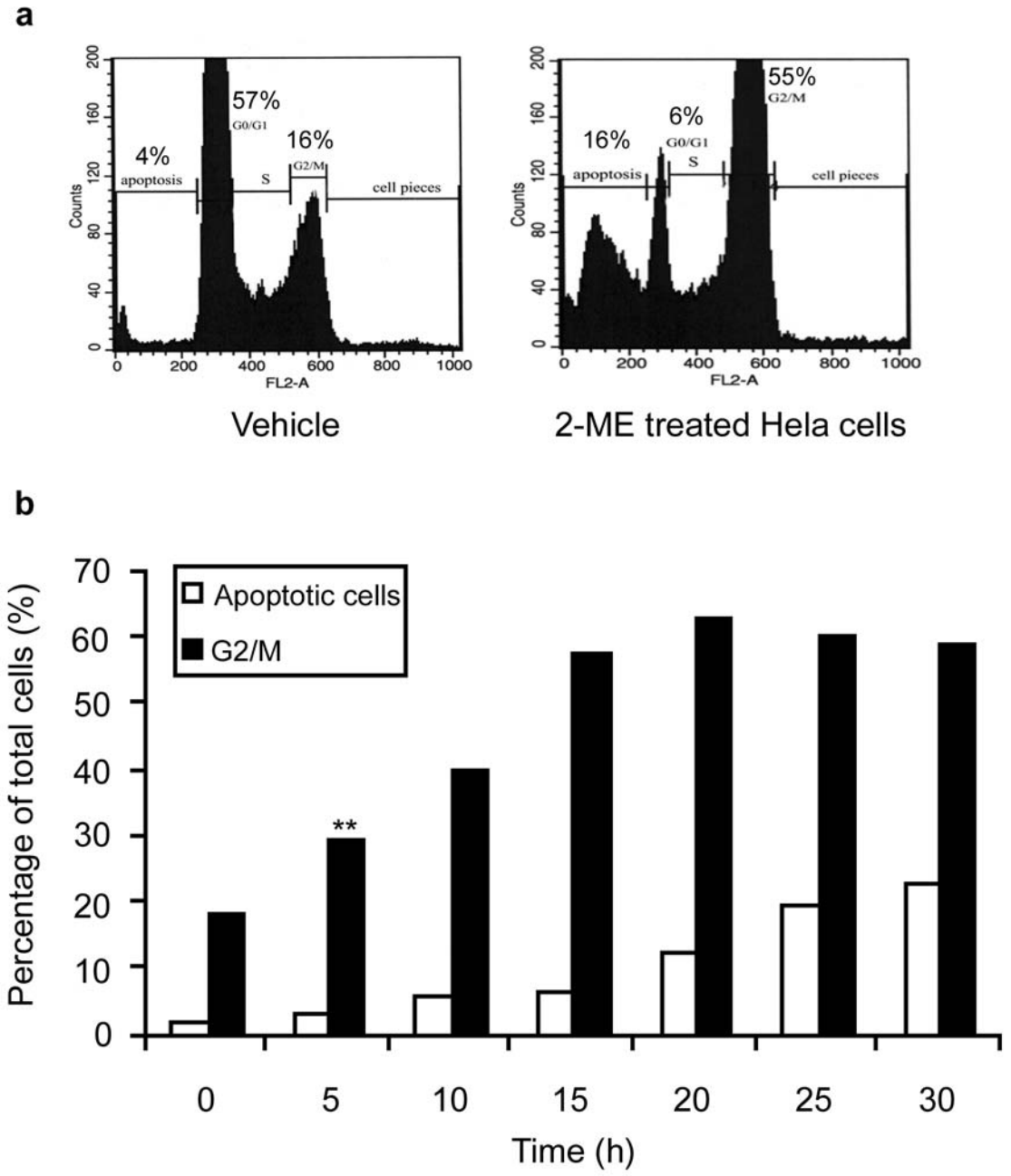

Figure 2. *Effects of 2-ME on cell cycle progress. (a) Cells were incubated with DMSO or 5 μM 2-ME, respectively for 20 h. (b) Cells were incubated with 5 μM 2-ME at different times. During the first 5-h treatment, the increase in the ratio of G_2/M cells was greater than that of apoptotic cells ($p<0.01$, $n=6$).*

Effects of 2-ME on iNOS expression. The expression of iNOS increased gradually after exposure to 2-ME for 5 h and peaked at 25 h in HeLaS3 cells (Figure 5a). A prominent band was observed at the concentration of 10 μM (Figure 5b). The average density of each band was analyzed and showed the time- and concentration-dependent effects of 2-ME. When cells were pre-treated with the iNOS inhibitor 1400W, apoptosis induced by 2-ME was prevented as detected by DNA gel electrophoresis (Figure 5c).

Effects of 2-ME on normal cervical epithelial cells. After exposure to 5 μM 2-ME for 20 h, some normal cervical columar cells displayed abnormal distribution of chromosomes (Figure 6a). Similar morphological changes

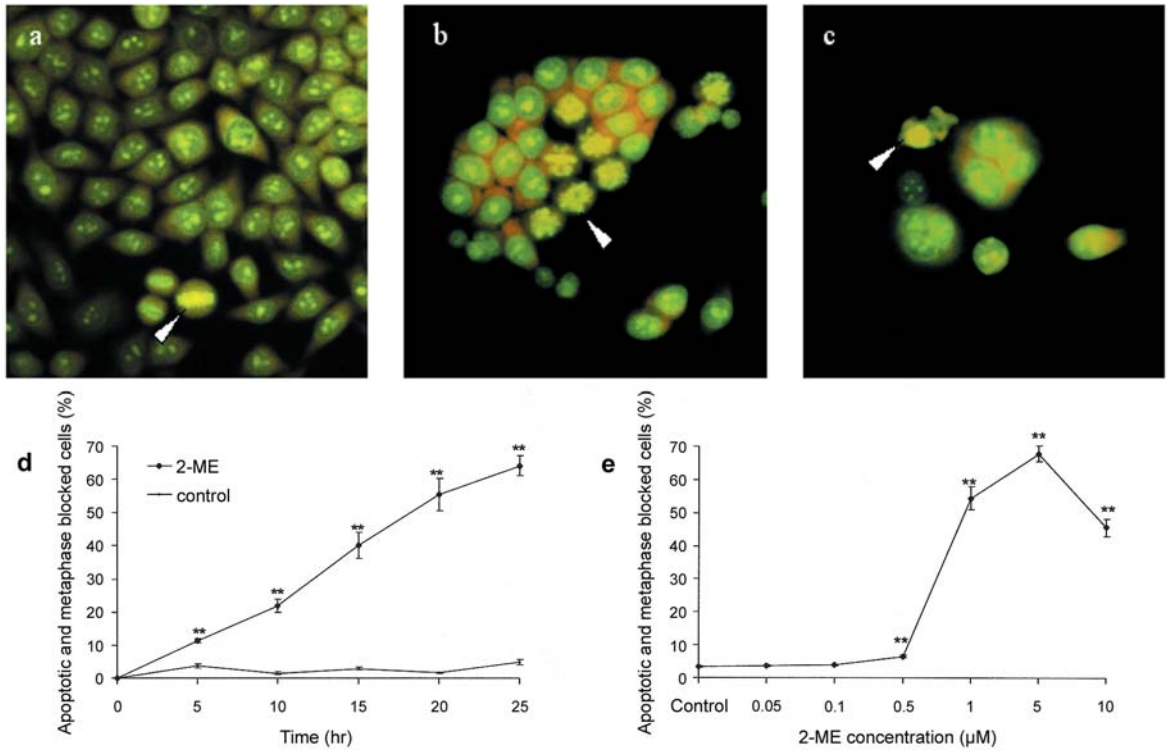

Figure 3. *Morphological changes by 2-ME. (a) Vehicle cells were incubated with 2.5 μl DMSO for 20 h. Arrow indicated the normal metaphase cells in which chromosomes arranged in line. (b) Cells were incubated with 5 μM 2-ME for 20 h. Triangle indicated unusual metaphase cells with uneven chromosome. (c) Apoptotic cell with condensed chromatin. (d) Cells were treated with 1 μM 2-ME for different times. (e) Cells were treated with indicated concentrations of 2-ME for 20 h. Apoptotic cells and G_2/M-arrested cells were quantified according to the morphological characteristics (n=6). Student's t-test * p<0.05, ** p<0.01 versus basal.*

were observed in squamous cells and transitional cells (data not shown). Cell cycle analysis supported the morphological findings, showing that 2-ME induced G_2/M-phase cell cycle arrest on columar (from 25% to 40%), squamous (from 25% to 42%) and epithelial cells as compared to vehicle (Figure 6b). However, no significant cell cycle changes were found in transitional epithelial cells. No obvious apoptosis was detected either by microscopy or cell cycle analysis and DNA gel electrophoresis (Figure 6c) on normal cervical cells.

17β-estradiol (E_2) at 10 nM had no effect on HeLaS3 cells as compared to vehicle. Furthermore no differences were found by flow cytometry (data not shown) or DNA fragmentation assay (Figure 6c) between 2-ME-treated and combined E_2 and 2-ME-treated cells.

Discussion

In the present study we found that 2-ME inhibited the growth of HeLaS3 cells in a dose- and time-dependent manner and that this inhibitory effect was due to induction of apoptosis and G_2/M cell cycle arrest. In addition, 2-ME had a slight effect on normal human cervical epithelial cells.

Morphological analysis illustrated that 2-ME induced an abnormal spindle formation at metaphase and that this proportion of cells increased with time and concentration, suggesting that G_2/M arrest was mainly due to inhibition of mitosis at metaphase. The antimitotic activity of 2-ME has been demonstrated on different cancer cells, by a decrease in the formation of cAMP in mid-mitosis (6); by inhibiting calmodulin-regulated tubulin dynamics (17); by causing mitotic phase-specific centrosome disintegrity (18); by blocking tubulin polymerization *via* interaction with colchicine binding site or by forming an ineffective tubulin polymer (7, 19).

Our results from flow cytometry showed that G_2/M cell cycle arrest is followed by subsequent apoptosis. However, no precedent report has addressed whether there is a linking mechanism between G_2/M cell cycle arrest and apoptosis.

The apoptotic mechanism of 2-ME has been intensively investigated. It has been reported that 2-ME induces apoptosis by activation of SAPK/JNK (20,21,16), interfering with NFÎ B transcriptional activity (22), inducing interferon gene expression (23), inhibiting superoxide dismutase (24) and up-regulating death receptor 5 (DR5) (25). However, the apoptotic mechanism of 2-ME in HeLaS3 cells is unknown.

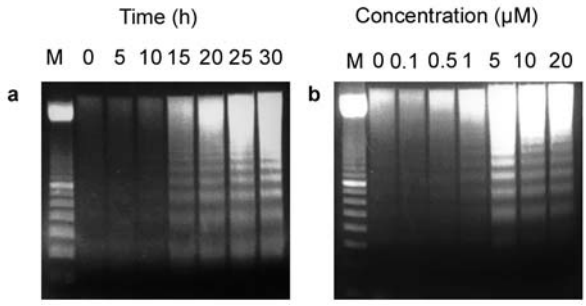

Figure 4. *Electrophoretic analysis of internucleosomal DNA fragmentation in 2-ME-treated HeLaS3 cells. (a) Time-dependency. HeLaS3 cells were treated with 5 μM 2-ME and DNA fragmentation was observed at 15-30 h. (b) Concentration-dependency. HeLaS3 cells were incubated with different concentrations of 2-ME for 25 h. DNA fragmentation was observed at 1-20 μM. Lane M indicates DNA size markers.*

Nitric oxide (NO) has emerged as a regulatory molecule involved in the control of a variety of biological processes. There are conflicting opinions about the activity of NO in apoptosis. Some toxicity studies support a potent protective effect of NO against apoptosis by increasing cGMP production, nitrosylation of caspases and inhibition of MPT (mitochondrial permeability transition) (26, 27). However, other reports indicate that NO results in apoptosis through the sequential loss of mitochondrial membrane potential, followed by caspase activation and degradation of inhibitor of caspase-activated DNAse (28, 29). Mitochondria appear as central executioners of programmed cell death (30). It is interesting to know whether NO's function is involved in the apoptosis of HeLaS3 cells treated by 2-ME. The easy way to investigate NO is to measure NOS (nitric oxide synthase). In this study we focused on the expression of iNOS (130kDa). Our data showed a dose- and time-dependent increase in the expression of iNOS after treatment with 2-ME. These changes were in accordance with apoptosis occurring, indicating that iNOS joined the activity of 2-ME. Furthermore, it was shown that the iNOS inhibitor 1400W prevented the formation of DNA fragmentation. These results suggest that iNOS is involved in the apoptotic signalling pathway in HeLaS3 cells by 2-ME.

2-ME was toxic to 55 different tumor cell lines (31), but there are few studies addressing the effects on normal cells. It has been shown that 2-ME inhibits the proliferation of human glomerular mesangial cells (32), aortic smooth muscle cells (33), adipocyte (34) and prostate epithelial cells (35). It induces apoptosis in thyroid follicular (36) and bovine vascular endothelial cells (20,37). No effect was seen in skin fibroblasts (38), breast cells (16), osteoblasts (23) and bronchial epithelial cells (1). In our study, three methods were used to measure the cytotoxic activity of 2-

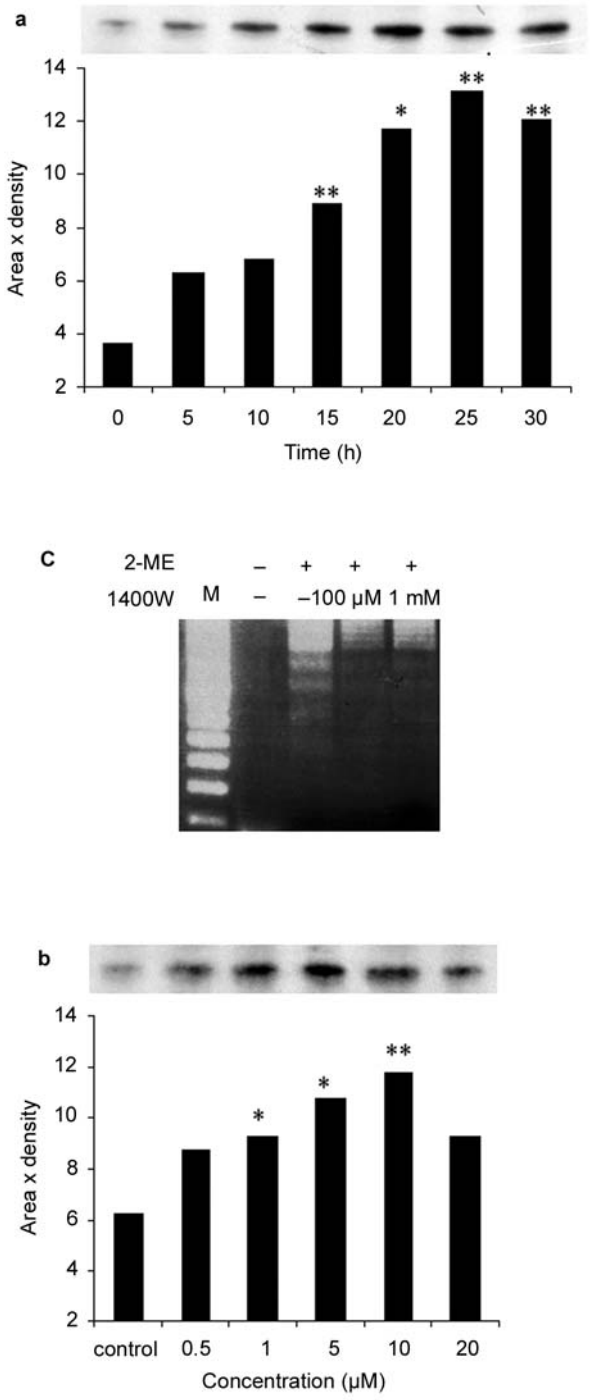

Figure 5. *iNOS expression changed by 2-ME. (a) The expression of iNOS increased gradually after treatment with 5 μM 2-ME and peaked at 25 h. (b) The expression of iNOS changed with concentrations and peaked at 10 mM. Scion Image was used to measure the density of each band and expressed as area x density. The mean value of each point was given from 3 independent experiments. * $p<0.05$, ** $p<0.01$ (c) iNOS inhibitor 1400W prevented the apoptosis induced by 2-ME.*

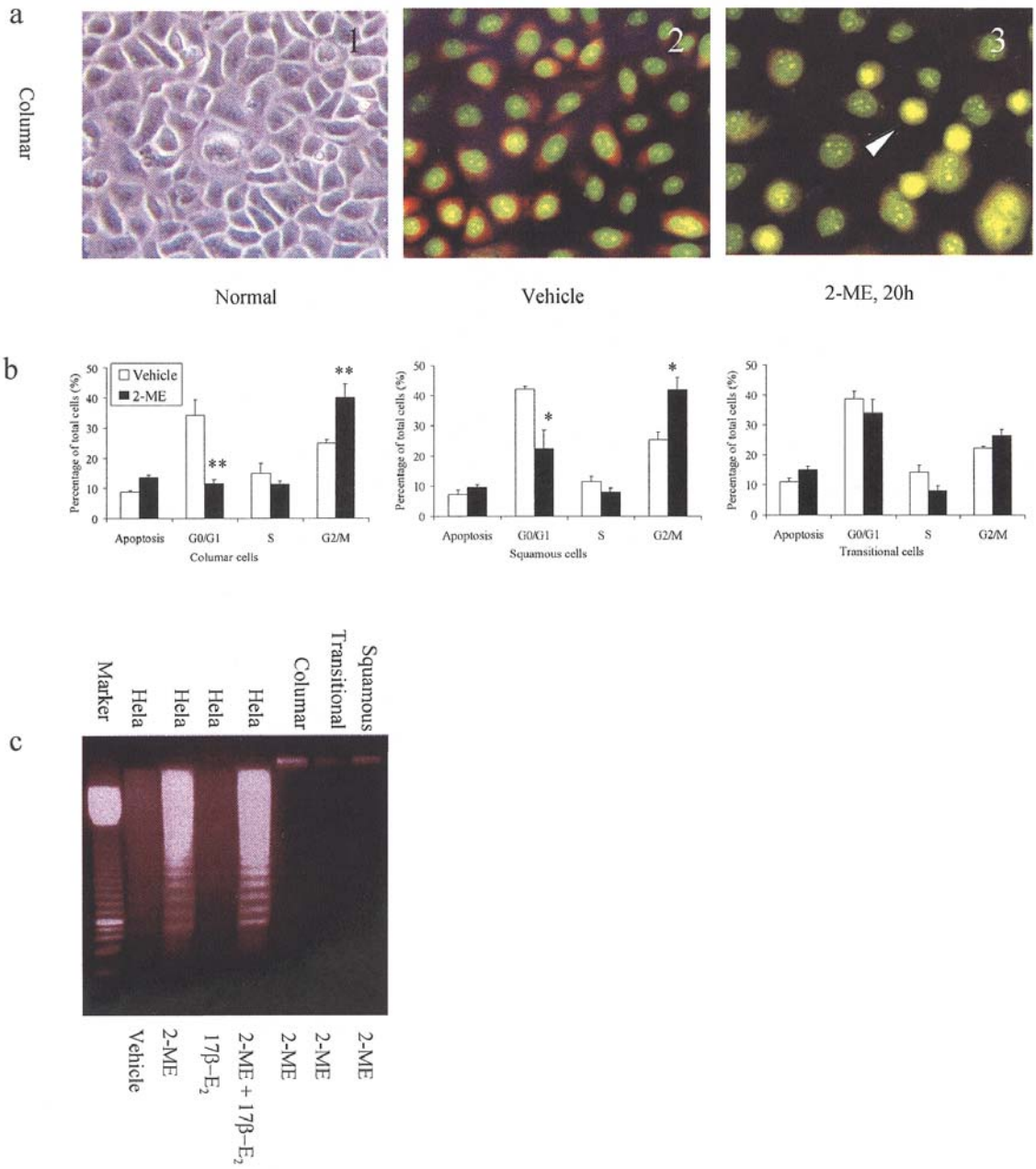

Figure 6. *Effects of 2-ME on normal cervical epithelial cells. (a1) Normal cervical columar epithelial cells under light microscopy; (a2) Normal cervical columar epithelial cells with AO staining under fluorescence microscopy; (a3) Normal cervical columar cells treated with 5 μM 2-ME for 20 h. Triangle showed the unusual mitotic cells. (b) Effects of 2-ME (5 μM, 20 h) on cell cycle progress of normal cervical epithelial cells. (c) DNA gel electrophoresis.*

ME on normal cervical epithelial cells. Morphological analysis indicated an increase of metaphase cells and flow cytometry showed an increase in G_2/M-arrested cells in columar and squamous epithelial cells. Compared with the effect of 2-ME on HeLaS3 cells, the influence on normal epithelial cervical cells was weak, indicating 2-ME's toxicity mainly on the fast dividing cells. Our results suggest that 2-ME might be used as an adjuvant agent in the treatment of cervical cancer. However, the weak effect of 2-ME on normal cervical epithelial cells should be considered for long-term clinical setting.

Taken together, our results showed that 2-ME induced apoptosis *via* the iNOS pathway and caused G_2/M arrest on HeLaS3 cells. A weak effect was also found in primary normal cervical epithelial cells. 2-ME may have a potential clinical application in the treatment of human cervical cancer.

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