2-Methoxyestradiol induces cell cycle arrest and apoptosis of nasopharyngeal carcinoma cells

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ABSTRACT

AIM: To investigate 2-methoxyestradiol induced apoptosis and its mechanism of action in CNE2 cell lines. **METHODS:** CNE2 cells were cultured in RPMI-1640 medium and treated with 2-methoxyestradiol in different concentrations. MTT assay was used to detect growth inhibition. Flow cytometry and DNA ladders were used to detect apoptosis. Western blotting was used to observe the expression of p53, $p21^{WAF1}$, Bax, and Bcl-2 protein. **RESULTS:** 2-methoxyestradiol inhibited proliferation of nasopharyngeal carcinoma CNE2 cells with IC_{50} value of 2.82 μmol/L. The results of flow cytometry showed an accumulation of CNE2 cells in G2/M phase in response to 2-methoxyestradiol. Treatment of CNE2 cells with 2-methoxyestradiol resulted in DNA fragmentation. The expression levels of protein p53 and Bcl-2 decreased following 2-methoxyestradiol treatment in CNE2 cells, whereas Bax and $p21^{WAF1}$ protein expression were unaffected after treatment with 2-methoxyestradiol. **CONCLUSION:** These results suggest that 2-methoxyestradiol induced cell cycle arrest at G2/M phase and apoptosis of CNE2 cells which was associated to Bcl-2 down-regulation.

INTRODUCTION

2-methoxyestradiol, an estrogen derivative that cannot bind the estrogen receptor, has recently emerged as a very promising agent for cancer treatment. It can inhibit tumor growth at doses showing no clinical signs of toxicity[1]. 2-methoxyestradiol targets both the tumor cells and blood vessel formation at several stages in the angiogenic cascade. Moreover, the ability of 2-methoxyestradiol to inhibit metastatic spread in several models adds to its therapeutic value for cancer treatment at various stages of the disease. Huang P *et al* reported that 2-methoxyestradiol inhibited superoxide dismutases (SOD) activity and induced apoptosis in cancer cells[2]. Carothers AM *et al* reported that 2-methoxyestradiol induced p53-associated apoptosis of colorectal cancer cells[3]. Qadan LR *et al* reported that 2-methoxyestradiol was a powerful growth inhibitor of LNCaP, Du145, PC-3, and ALVA-31 prostate cancer cells, and induced a marked accumulation of cells in the G_2/M phase of the cell cycle and an increase in the sub-G1 fraction (apoptotic)[4]. Bu S *et al* investigated the mechanism of 2-methoxyestradiol-induced apoptosis in prostate cancer cells[5]. They found that 2-methoxyestradiol led to an activation of c-Jun N-terminal kinase and phosphorylation of Bcl-2, which proceded the induction of apoptosis.

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Nasopharyngeal carcinoma (NPC) occurs with a high incidence in Southern China and Southeast Asia, which is a malignancy of epithelial origin with overexpression of epidermal growth factor (EGF) receptor. Radiotherapy is the predominant treatment for NPC. No effective chemotherapy is available[6]. NPC is the leading cause of death from cancer in the South of China. The human nasopharyngeal carcinoma cell line CNE2 was established at Hunan Medical College in China from the tumor of a patient[7]. CNE2 cells harbor dysfunctional p53[8]. To exploit the therapeutic effect of 2-methoxy-estradiol on nasopharyngeal carcinoma, CNE2 was treated with 2-methoxyestradiol. (Our investigation showed that 2-methoxyestradiol could inhibit cell proliferation, induce cell cycle arrest in G2/M phase and apoptosis associated to Bcl-2 protein downregulation was observed[9]).

MATERIALS AND METHODS

Drugs and reagents 2-methoxyestradiol was from Sigma company and initially dissolved in 100 % Me_2SO and stored at -20 ºC. MTT was purchased from Janssen Chimica Co. RPMI-1640 medium and Me_2SO were purchased from Sigma Co. Anti-Bax antibody, anti-Bcl-2, anti-p53, and anti-p21 antibodies were purchased from Santa Cruz. CNE2 cells was grown in RPMI-1640 medium and cultured in an incubator at 37 ºC under 5 % CO_2 in air.

MTT assay CNE2 cells were placed in 96-well plate at a density of 2000 cells per well. The stock of 2-methoxyestradiol was diluted, and added to the wells for the desired final assay concentration. After 3-d exposure to 2-methoxyestradiol, 10 μL of MTT (5 mg/L) was added to each well and incubated for another 4 h, and liquid in the wells was evaporated. Me_2SO 100 μL was added to each well. The absorbance was detected in the microplate reader 550 model with 565 nm wavelength. Growth inhibition was expressed as a percentage of absorbance detected in control wells that were treated with 0.1 % Me_2SO alone. Me_2SO controls were not different from cells in regular growth medium. IC_{50} value was determined using a Bliss Software.

Cell cycle analysis Cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in cold PBS, and fixed with 70 % ethanol. After overnight refrigeration at -20 ºC and subsequent rehydration in PBS for 30 min at 4 ºC, the cell nuclei were stained for 30 min in dark with 50 mg/L propidium iodide containing 125 kU/L protease-free RNase, both diluted in PBS. Cells were filtered through 95-μm pore size nylon mesh and a total of 15 000 stained nuclei were analyzed in a FACS. DNA histograms were modeled off-line using ModFitLT software.

Internucleosomal DNA damage The integrity of DNA was assessed by agarose gel electrophoresis. Cells (1×10^6) were centrifuged at 3000×*g* for 3 min. Cells were washed once with PBS, and cell pellets were solubilized in 100 μL of lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L tetraacetic acid, 0.4 % SDS, 0.5 g/L proteinase K). Pellets were incubated for 8 h at 50 ºC, then 10 μL of 0.5 g/L RNaseA was added. The samples were incubated for 1 h at 50 ºC and heated to 70 ºC for 5 min, then 100 μL of phenol:chloroform: isopropanol (25:24:1) was added. After centrifugation, supernatants were transferred to new tubes, and twice-fold volume ethanol (ice cold) was added. After centrifugation, the pellets were suspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L tetraacetic acid) and loaded on 1.8 % agarose gel for electrophoresis. The gel was stained with ethidium bromide, and photographed with UV illumination.

Western blot analysis Lysates were prepared from 1×10^6 cells by dissolving cell pellets in 100 μL of lysis buffer (20 mmol/L Na_2PO_4 (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 % aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 g/L leupeptin, 100 mmol/L NaF, and 2 mmol/L Na_3VO_4). Lysates were centrifuged at 12000 r/min in for 10 min. The supernatant was collected. The protein content was determined using the Bio-Rad protein assay (Bio-Rad labortories, Hercules CA). SDS-PAGE sample buffer (10 mmol/L Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 0.2 mol/L DTT) was added to lysates. Lysates were heated to 100 ºC for 5 min, and 40 μg of protein was loaded in each well of a 10 % SDS-PAGE gel. Resolved proteins were electrophoretically transferred to nitrocellulose and incubated sequentially with primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (or anti-rabbit-IgG, Amersham Life Sciences). After washing, the bound antibody complex was detected using an ECL chemiluminescence reagent and XAR film (Kodak) as described by the manufactures (Amersham).

RESULTS

Growth inhibition of CNE2 cells by 2-methoxyestradiol Treatment of CNE2 cells for 3 d with

0.625, 1.25, 2.5, 5.0, and 10 μmol/L of 2-methoxyestradiol resulted in inhibition of cell proliferation in a dose-dependent manner. The inhibitory rates of 2-methoxyestradiol on cell growth of CNE2 cells were 16.48 %, 34.07 %, 68.13 %, 72.53 %, and 74.73 %, respectively. IC_{50} value was 2.82 μmol/L. Inhibition of cell proliferation could be the results of the induction of apoptosis, cell growth arrest and/or inhibition of growth. Thereby, we investigated whether 2-methoxyestradiol could induce cell cycle arrest and apoptosis in CNE2 cells (Fig 1).

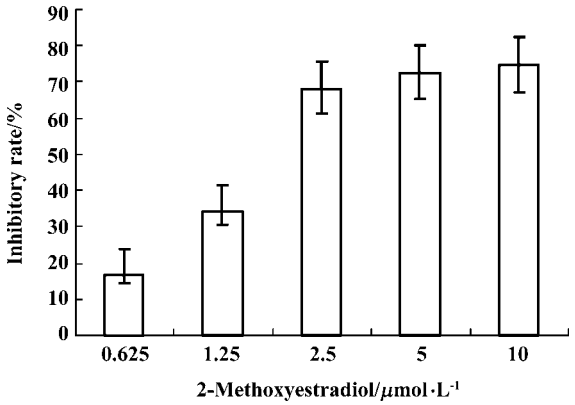

Fig 1. Effect of 2-methoxyestradiol on proliferation of CNE2 cells. CNE2 cells were treated with various concentrations of 2-methoxyestradiol as indicated for 3 d. Growth inhibition was determined with MTT assay and shown as inhibitory rate.

Effect of 2-methoxyestradiol on cell cycle distribution in CNE2 cells We tested the cell cycle distribution under 0, 2.5, 5.0, 10.0 μmol/L of 2-methoxyestradiol for different times. The results showed that the percentages of cells in G_1 and S phase decreased, the percentages of cells in G_2/M phase and the sub-G_1 fraction (apoptotic) increased (Tab 1-3).

Tab 1. Cell cycle distribution of CNE2 cells following 2-methoxyestradiol treatment for 48 h.

2-Methoxyestradiol/ μmol·L^{-1}	Percentage/% G_1	S	G_2M
0	57.0	33.6	9.3
2.5	17.1	40.2	42.8
5.0	9.9	37.5	52.6
10.0	2.5	23.1	74.4

Tab 2. Cell cycle distribution of CNE2 cells following 2-methoxyestradiol treatment for 72 h.

2-Methoxyestradiol/ μmol·L^{-1}	Percentage/% G_1	S	G_2M
0	60.4	34.2	5.4
2.5	8.1	18.4	73.5
5.0	1.6	15.1	83.4
10.0	0	0	100

Tab 3. SubG1 fraction of CNE2 cells following 2-Methoxyestradiol treatment.

SubG1 fraction/%	2-Methoxyestradiol/μmol·L^{-1} 0	2.5	5.0	10.0
6	3.1	1.4	2.9	5.6
12	3.9	7.9	3.9	2.3
24	3.7	24.2	21.2	24.9
48	8.5	41.2	35.5	44.7
72	12.0	51.8	61.0	70.2

Internucleosomal DNA damage in 2-methoxyestradiol-treated CNE2 cells The results of flow cytometry above showed that 2-methoxyestradiol increased subG1 fraction. This indicated that 2-methoxyestradiol could be able to induce apoptosis in CNE2 cells. In order to confirm the apoptosis induction by 2-methoxyestradiol in CNE2 cells, DNA fragmentation was detected using agarose gel electro-phoresis. Genomic DNA was prepared from 1×10^6 -2×10^6 CNE2 cells that had been incubated in the absence or presence of different concentrations of 2-methoxyestradiol for 72 h. The integrity of the DNA was assessed by agarose gel electrophoresis. Internucleosomal DNA damage in CNE2 cells was readily detected after treatment with 2-methoxyestradiol for 72 h (Fig 2).

Effect of 2-methoxyestradiol on the expression levels of protein p53, $p21^{WAF1}$, Bcl-2, and Bax The results shown above indicated induction of cell cycle arrest and apoptosis of CNE2 cells by 2-methoxyestradiol. It is unclear how 2-methoxyestradiol induced cell cycle arrest and apoptosis in CNE2 cells. The cells treated with 10 μmol/L of 2-methoxyestradiol for indicated times were lysed and resolved in 10 % SDS-PAGE and Western blot analysis was performed using anti-

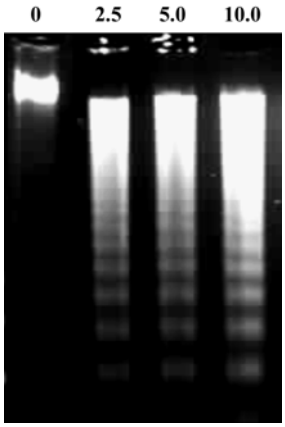

Fig 2. DNA fragmentation of CNE2 cells induced by 2-methoxyestradiol. CNE2 cells were incubated in the absence or presence of 0, 2.5, 5.0, and 10 μmol/L of 2-methoxyestradiol for 72 h. Cells were harvested. DNA was isolated and DNA fragmentation was visualized as oligonucleosome-sized fragmentation in ethidium bromide after DNA agarose gel electrophoresis. Internucleosomal DNA damage in CNE2 cells was readily detected 6 h after treatment with 2-methoxyestradiol. This figure is a representative of three experiments.

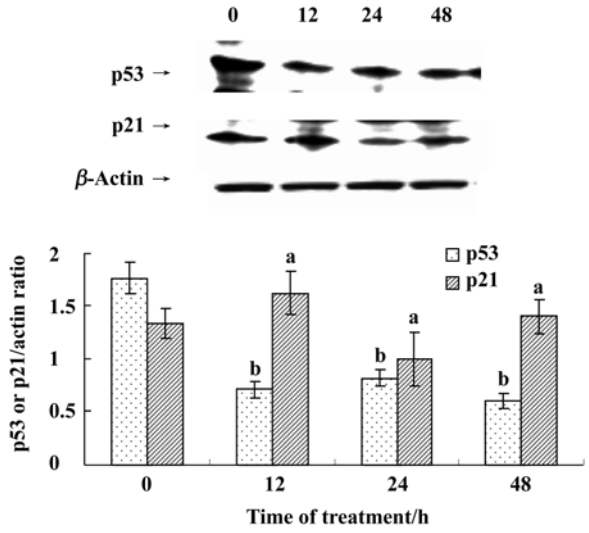

Fig 3. Effect of 2-methoxyestradiol on p53 and p21 protein levels in CNE2 cells. CNE2cells were treated with 10 μmol/L of 2-methoxyestradiol for 0 h, 12 h, 24 h, 48 h. Cells were sequentially harvested and lyzed. Western blot analysis was performed with anti-p53 and anti-p21 antibody. Lower panel shows the ratios of protein quantitation of p53 or p21 to actin. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 compared with control.

p53, anti-p21, anti-Bcl-2, and Bax antibody. Fig 3 showed that p53 reduced and $p21^{WAF1}$ had no change following 2-methoxyestradiol treatment. This indicated

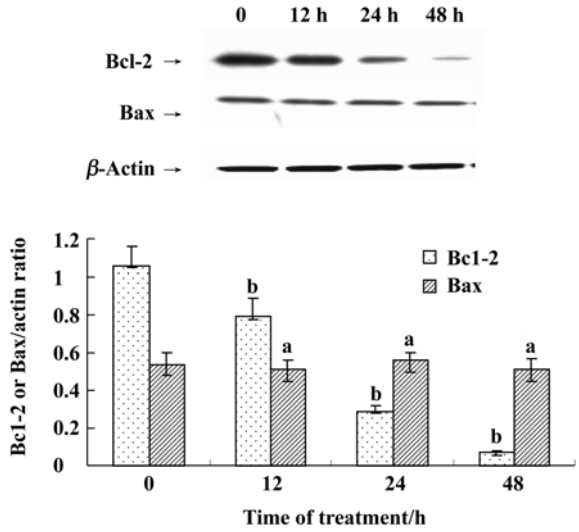

Fig 4. Effect of 2-methoxyestradiol on Bcl-2 and Bax protein levels in CNE2 cells. CNE2 cells were treated with 10 μmol/L of 2-methoxyestradiol for 0 h, 12 h, 24 h and 48 h. Cells were sequentially harvested and lyzed. Western blot analysis was performed with anti-Bcl-2 or anti-Bax antibody. The result is a representative of three experiments. Lower panel shows the ratios of protein quantitation of Bcl-2 or bax to actin. (*n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 compared with control)

that $p21^{WAF1}$ had no relationship with 2-methoxyestradiol-mediated apoptosis, but reduced dysfunctional p53 in CNE2 cells could contribute to 2-methoxyestradiol-mediated apoptosis. As shown in Fig 4, the levels of Bcl-2 protein decreased in a time-dependent manner, but Bax had no change following 2-methoxyestradiol treatment. This indicated that Bcl-2 was related to 2-methoxyestradiol-mediated apoptosis in CNE2 cells.

DISCUSSION

Apoptosis is the most common and distinct form of cell death involving a series of steps and acts as physiological suicide mechanism to preserve tissue homeostasis through proper cell turnover[10]. There is ample evidence that naturally occurring compounds and many chemotherapeutic agents with antitumor effects can trigger the apoptosis of cancer cells[11]. In this study, we reported that 2-methoxyestradiol induced cell cycle arrest and apoptosis in CNE2 cells demonstrated by flow cytometry and DNA fragmentation.

The present study showed that 2-methoxyestradiol induced cell cycle arrest in G_2/M phase in CNE2 cells in a dose-dependent manner. This may be associated with inhibition of mitosis[12]. It is valuable to investigate.

The p53 tumor suppressor gene is an important regulator of apoptosis. Carothers *et al* reported that treatment of CRC cells with 2-methoxyestradiol increased expression of p53 and $p21^{WAF1/CIP1}$ proteins and induced apoptosis[3]. The result of our study showed that p53 decreased but $p21^{WAF1}$ had no change following 2-methoxyestradiol treatment. In CNE2 cells p53 is dysfunctional[8]. This indicated that reduced p53 could contribute to 2-methoxyestradiol-mediated apoptosis in CNE2 cells.

Bcl-2 was originally identified at the chromosomal breakpoint of t (14;18)-beating B-cell lymphomas. Bcl-2 belongs to a growing family of proteins that regulate apoptosis or programmed cell death. The Bcl-2 family includes both death antagonists such as Bcl-2 and Bcl-x_L and death agonists such as Bax, Bak, Bid, and Bad. These related proteins share at least one of four homologous regions termed Bcl homology (BH) domains (BH1 to BH4). As a prototypic member of this family, Bcl-2 can contribute to neoplastic cell expansion by preventing normal cell turnover caused by physiological cell death mechanisms. High levels of Bcl-2 gene expression are found in a wide variety of human cancer. In addition, Bcl-2 is implicated in chemoresistance as overexpression of Bcl-2 can inhibit the cell-killing effect of many currently available anticancer drugs by blocking the apoptotic pathway. The expression levels of Bcl-2 proteins correlate with relative resistance to a wide spectrum of chemotherapeutic drugs and irradiation. Therefore, the inhibition of the protective function of Bcl-2 protein overexpressed in tumor cells is an attractive strategy for either restoring the normal apoptotic process in these cells or making these cells more susceptible for conventional chemotherapy or radiotherapy. In this regard, cell-permeable, small molecule inhibitors of Bcl-2 may represent a new class of therapeutic agents for the treatment of cancer[5,13].

It has been suggested that 2-methoxyestradiol exerts their biological effects by inhibition of mitosis and apoptosis induction[12]. Bcl-2 protein is able to repress a number of apoptotic death programs. The 21 kD protein partner, Bax, which overexpresses to counter the death repressor activity of Bcl-2, homodimerizes and forms heterodimers with Bcl-2 *in vivo*. The ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus[13-17]. The results from our study showed that 2-methoxyestradiol obviously inhibited Bcl-2 protein expression in a time-dependent manner, while Bax protein had no change in the CNE2 cells. Therefore, the ratio of Bcl-2 to Bax will decrease while apoptosis induction increased. This may be the mechanism underlying 2-methoxyestradiol-induced apoptosis in CNE2 cells.

Thus, our results from this study suggested that 2-methoxyestradiol was potential to be developed into drugs for nasopharyngeal carcinoma treatment.

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