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2-Methoxyestradiol: New perspectives in colon carcinoma treatment

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ABSTRACT

Colon carcinoma represents a major problem in oncology, since this type of cancer responds poorly to conventional chemotherapy. Many groups are actively involved in the search of new experimental strategies to bypass this problem. We investigated the effects of 2-methoxyestradiol (2-ME), which derives from the NADPH-dependent cytochrome P450 metabolism of 17β-estradiol. This compound has raised much interest in the past few decades for its inhibitory effects on the growth of cancer cells of different origin; however, little is known about its use on colon carcinoma-derived cell lines. In the present study, we investigated the effects of 2-ME on cell proliferation and cell cycle of two human colon carcinoma cell lines, namely HCT116 and SW613-B3. Our results showed a net anti-proliferative effect of 2-ME on both cell lines, which is accompanied by cell cycle arrest; moreover, we demonstrated that 2-ME is able to induce apoptosis as well as autophagy. This body of evidence points out that 2-ME could be considered as a promising tool against colon carcinoma.

1. Introduction

Resistance toward chemotherapy remains one of the principal obstacles to the effective treatment of cancer. Among the different therapeutic strategies adopted to beat cancer, a positive role of estrogens has been reported. Biological activity of estrogens is mainly mediated by their binding to estrogen receptors (ER) -α and -β, that belong to the steroid/thyroid hormone receptor superfamily of nuclear receptors. They are activated upon binding of the ligand, being able to interact with cis-regulatory elements of target genes either by the direct association to estrogen-response elements (EREs) or through the interaction with other DNA-bound transcription factors, such as activator protein 1 (AP-1) and stimulating protein-1 (SP-1) (Matthews and Gustafsson, 2003).

The present research focuses on the use of 2-methoxyestradiol (2-ME) to block colon cancer cell proliferation. Once thought to be an inactive metabolite, 2-ME derives from the NADPH-dependent cytochrome P450 metabolism of 17β-estradiol and has been described to inhibit cancer proliferation. A pioneering study reported the ability of 2-ME to inhibit cell proliferation of a bovine brain-derived capillary endothelial cell line (Fotsis et al., 1994). Thereafter, a similar effect on various tumor cell lines was described (Pribluda et al., 2000; Qadan et al., 2001; Bu et al., 2002). 2-ME was found to inhibit tubulin polymerization by interacting at the colchicin-binding site and to disrupt microtubule dynamics (Kamath et al., 2006; Bhati et al., 2007). It is generally considered that low (nanomolar range) concentrations of 2-ME could be effective on cell proliferation, depending on cell types, while the anti-mitotic property requires micromolar concentrations.

Many studies have reported the ability of 2-ME to induce apoptosis in various human cancer cell lines, triggering both the extrinsic and intrinsic pathways (Mooberry, 2003; Sutherland et al., 2007). 2-ME was also found to exert a strong anti-angiogenic effect (reviewed in Pribluda et al., 2000; Mooberry, 2003; Dubey and Jackson, 2009), possibly correlated to its ability to inhibit Hypoxia Induced Factor-1 (HIF-1) expression by altering microtubule assembly dynamics (Mabjeesh et al., 2003; Mooberry, 2003). A recent proteomic survey has identified new mechanisms of action of 2-ME in pancreatic cells, involving glucocorticoid receptor and NF-κB (Basu and Haldar, 2009). Taken together, the reported effects allow the identification of 2-ME as a promising anti-cancer drug.

Few studies have been conducted on the effect of 2-ME on colon carcinoma (Carothers et al., 2002; Zou et al., 2006), a type of cancer that responds poorly to therapies, and represents a major problem in clinical oncology. The present investigation aims at depicting the effects of 2-ME on cell proliferation, cell cycle, apoptosis and autophagy of two human colon carcinoma-derived cell lines: the highly tumorigenic HCT116 and the non-tumorigenic/drug-resistant, SW613-B3. Our results demonstrated that 2-ME exerts a strong anti-proliferative activity, promotes cell cycle arrest and induces apoptosis as well as autophagy.

2. Materials and methods

2.1. Cell culture and treatments

Human colon carcinoma HCT116 and SW613-B3 cells, HeLa (from uterine cervix carcinoma), and FO46 normal fibroblasts were grown at 37 °C under a 5%

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CO_2 atmosphere, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 0.1 mg/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine and 2% sodium pyruvate (all reagents were from Celbio, Milano, Italy). Twenty-four hours after seeding, cells were treated for 24 h either with etoposide (Sigma Aldrich, Milano, Italy, stock solution: 50 mM in DMSO) or 2-methoxyestradiol (2-ME, Sigma, stock solution: 10 mM in DMSO), followed by 24 h-recovery in drug-free medium. Drug concentrations are specified for each assay. In most experiments, the final concentration of DMSO in culture medium was less than 0.2% (v/v) and it did not have any effect on the activities tested in this study. Under some conditions, a fraction of 2-ME-treated cells tended to detach; this population was analyzed separately or in combination with attached cells, as specified for each assay.

2.2. Morphological analysis

For microscope observation, cells grown in 3.5-cm diameter Petri dishes (5×10^4/ml) were treated with 10 μM 2-ME for 24 h, followed by 24 h-recovery in drug-free medium. At the end of the treatment, cells were observed using an Olympus IX71 microscope equipped with a 10x objective and images were acquired with a digital camera Cool $SNAP_{ES}$ (PhotoMetrics, CA, USA), using the MetaMorph acquisition software; Adobe Photoshop 9.0.2 was used as elaborating software.

2.3. Viability assays

The effect of 2-ME on cell proliferation was evaluated by two different procedures (Giansanti et al., 2009): 1) the MTT metabolic viability assay, which measures mitochondrial activity, and 2) the measurement of the amount of DNA released from cells after alkaline lysis that is proportional to the cell number. (1) MTT assay: Cells were seeded in 96-multiwell plates at a density of 10^3/100 μl/well. Twenty-four hours later, cells were treated for 24 h with 1 μM or 10 μM 2-ME, followed by 24 h-recovery in drug-free medium. In addition to untreated controls, parallel samples were incubated under the same conditions with 0.1% DMSO to evaluate the possible effect of 2-ME solvent. At the end of the incubation, 20 μl of CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Reagent (Promega Italia, Milano, Italy) were added to each well. The plates were then maintained for 4 h at 37 °C and analyzed with a microplate reader (Gio.De Vita, Roma, Italy) at 492 nm. Experiments were performed in quadruplicate and repeated three times. This procedure allows the evaluation of viability of the whole cell population. Data of treated cells are expressed in comparison to control samples ± S.D. (2) DNA release assay: Cells were seeded in 6-cm diameter Petri dishes at a density of 5×10^4 cells/ml and, 24 h later, treated for 24 h with 10 μM 2-ME, and further incubated for 24 h in drug-free medium. Untreated controls, and samples incubated under the same conditions with 0.1% DMSO were also considered. Detached cells were discarded, while attached cells were washed in PBS, trypsinized, centrifuged for 5 min at 1500 rpm and lysed with 1 ml of 0.1 M NaOH; samples were accurately mixed and heated for 30 min at 50 °C, then allowed to reach room temperature and kept at 4 °C until the spectrophotometric analysis. The amount of released DNA, which is proportional to the number of cells, was measured as the absorbance at 260 nm. Optical density of treated samples was compared to the values of control cells. Three independent experiments were carried out. Statistical analysis was performed and data are presented as mean ± S.D.

2.4. Clonogenic assay

To evaluate colony-forming ability, 2.5×10^2 cells/ml were seeded in 6-cm diameter Petri dishes and, 24 h later, treated for 24 h with 2-ME. Given that this assay implies the administration of 2-ME to single cells, a low concentration, i.e. 5 μM was used. After drug removal, attached cells were washed with PBS and further grown in complete medium for 10 days, to allow colony formation by surviving cells. Untreated controls and samples incubated with the vehicle 0.05% DMSO were set up in parallel. Colonies were then washed with PBS, fixed for 10 min with 100% methanol, and stained with Coomassie Blue (0.1% Page Blue G-90, 50% methanol and 7.4% acetic acid). Colonies with more than 50 cells were counted. The percentage of colonies formed after treatment relative to untreated cells was determined. Experiments were performed in duplicate and repeated three times.

2.5. Flow cytometry

Cells were seeded in 10-cm diameter Petri dishes (10^6/dish), grown in complete medium for 24 h and treated with 10 μM 2-ME for 24 h. After the treatment, detached cells were recovered and added to the attached cells once the latter were trypsinized. The whole cell sample was centrifuged for 5 min at 1500 rpm at 4 °C and resuspended with cold 0.9% NaCl (10^6 cells/ml); cold 100% ethanol was added to cell suspension to a final concentration of 70%. Samples were then incubated for 30 min at room temperature with 30 μg/ml propidium iodide (Sigma) and 2 mg/ml RNase A (Sigma), kept overnight at 4 °C and finally analyzed using a Coulter Epics XL II flow cytometer (Beckman Coulter, Milano, Italy). For each sample, 10^4 cells were measured and data were analyzed. The fluorescence intensity was converted in histograms, and the percentage of cells in each phase of the cell cycle was calculated with XL II software. The applied procedure allows the evaluation of cell cycle distribution of the whole cell population. Experiments were repeated three times.

2.6. Western blotting

HCT116 and SW613-B3 cells were treated for 24 h with 100 μM 2-ME. Attached (A) and detached (D) cell populations were harvested separately. Protein expression was evaluated by Western blotting according to Donà et al. (2008). After running and transfer of proteins onto nitrocellulose, membranes were incubated overnight at 4 °C, or 3 h at room temperature, with MAbs against the following proteins: PARP-1 (C2-10 Alexis Biochemicals, Vinci-Biochem, Firenze, Italy, diluted 1:1000); cyclin A (Cy-A1 Sigma, diluted 1:500); cyclin B1 (GNS1 Santa Cruz Biotechnology, Tebu-Bio, Magenta, Italy, diluted 1:1000); p53 (DO-1 Santa Cruz Biotechnology, diluted 1:2000); caspase-3 (31A1067 Alexis, diluted 1:250); γ-tubulin (GTU-88 Sigma, diluted 1:10,000). In some experiments, polyclonal antibodies against the following proteins were used: cyclin D1 (Upstate Biotechnology, Prodotti Gianni, Italy, diluted 1:500); p21 (C-21, Santa Cruz Biotechnology, diluted 1:1000); cleaved caspase-8 (Asp391 Cell Signaling Technology, Celbio, diluted 1:1000); cleaved caspase-9 (Asp315 Cell Signaling Technology, diluted 1:1000). Then, HRP-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch, Suffolk, UK, diluted 1:10000) was applied for 45 min at room temperature. All antibodies were diluted in TBS (140 mM NaCl, 100 mM Tris–HCl, pH 7.5), containing 5% skim milk and 0.1% Tween-20. Visualization of immunoreactive bands was achieved using a chemiluminescent substrate (Immun-Star™ WesternC™ Chemiluminescent Kit, Bio Rad Laboratories, Segrate, Italy). Three independent experiments were performed. Densitometric analysis of immunoreactive bands was achieved with BioRad Quantity One software.

2.7. Internucleosomal DNA degradation

For DNA ladder analysis, control and treated samples containing 2.5×10^6 cells were processed as described in Giansanti et al. (2009). HCT116 and SW613-B3 cells were treated for 24 h with 100 μM 2-ME. Attached (A) and detached (D) cell populations were harvested separately. HeLa cells treated with 100 μM etoposide for 24 h (E) or growth factor-deprived (long-term, LT) were used as positive DNA ladder control (Torriglia et al., 1999). Pictures were taken with a photographic digital camera Kodak DC290 (Rochester, NY, USA).

2.8. Immunofluorescence experiments

Cells were seeded on coverslips (5×10^4 cells/ml) and treated 24 h later either with 10 μM 2-ME or RS2780 (kindly provided by Prof. R. Silvestri, University of Roma La Sapienza, Italy) for 24 h. After treatment, attached cells were fixed with cold paraformaldehyde (2% in PBS) for 20 min and then post-fixed overnight with 70% ethanol at −20 °C. The day after, cells were permeabilized on ice with 0.1% Triton X-100 in PBS, incubated for 30 min with PTN (PBS containing 10% newborn calf serum and 0.1% Tween-20) and then for 1 h at room temperature with the polyclonal antibody to AIF (apoptosis-inducing factor) (Cell Signaling Technology, diluted 1:50), the MAb to mtHSP70 (JG1 Alexis, diluted 1:50) or with the anti-phosphorylated-H3 (P-H3) polyclonal antibody (Ser10 Upstate, diluted 1:20) in PTN. After washes with PBS, cells were incubated for 1 h at room temperature with rhodamine- or fluorescein-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) and then stained for 10 min with 0.2 μg/ml Hoechst 33258 in PBS in the dark. Finally, coverslips were mounted on slides with anti-fade solution (90% glycerol, 20 mM Tris–HCl pH 7.5, 0.1% DABCO diluted in PBS). Samples were analyzed using an Olympus IX71 microscope equipped with a 63x objective; images were acquired as described above. Three independent experiments were performed.

2.9. Autophagy markers

For the evaluation of autophagy, cells were treated with 100 μM 2-ME for 24 h, stained with 1 μg/ml Acridine Orange (AO, Sigma) for 15 min at 37 °C, then observed with an Olympus BX51 microscope. Images were taken with an Olympus C4040 camera. The autophagic markers Beclin-1, Atg 5-12, LC3 were monitored by Western blot (as described in section 2.6) by using the "Autophagy antibody kit" (Cell Signaling) under manufacturer's conditions. *In situ* conversion of LC3 form I to form II was visualized by immunofluorescence with the following protocol: control and treated cells were fixed with cold paraformaldehyde (4% in PBS) for 15 min, washed with PBS and permeabilized with cold acetone for 5 min. After washings with PBS, samples were incubated with bovine serum albumin (4% in PBS) for 10 min and with the polyclonal antibody to LC3 (see the above kit, diluted 1:100) for 1 h at 37 °C. Samples were then processed as in section 2.8 and observed with the above-described microscope.

3. Results

3.1. 3.1 2-ME treatment affects colon carcinoma cell viability

After setting the experimental conditions using HeLa cells (Supplementary Fig. 1), we analyzed the effects of 2-ME on human colon carcinoma cell lines HCT116 and SW613-B3. As shown in