Research Article

2-Methoxyestradiol Inhibits Barrett's Esophageal Adenocarcinoma Growth and Differentiation through Differential Regulation of the β-Catenin–E-Cadherin Axis

Suman Kambhampati1,2, Snigdha Banerjee1,2, Kakali Dhar1,2, Smita Mehta1,2, Inamul Haque1,2, Gopal Dhar1,2, Monami Majumder1,2, Gibanananda Ray1, Peter J. Vanveldhuizen1,2, and Sushanta K. Banerjee1,2

Abstract

The purpose of this study was to evaluate whether 2-methoxyestradiol (2-ME2), a promising anticancer agent, modulates Barrett’s esophageal adenocarcinoma (BEAC) cell growth and behavior through a cellular pathway involving β-catenin in partnership with E-cadherin, which seems to play a critical role in the induction of antitumor responses in cancer cells. We found that 2-ME2 markedly reduced the BEAC cell proliferation through regulating apoptotic machinery such as Bcl-2 and Bax. It may nullify the aggressive behavior of the cells by reducing the migratory behavior. Expressions of β-catenin and E-cadherin and binding of these two proteins is activated in a 2-ME2-dependent fashion in Bic-1 cells. Moreover, overexpressions of these two proteins may be due to the stabilization of these proteins by 2-ME2. We found that 2-ME2-induced anti-migratory effects are mediated through the β-catenin–E-cadherin signaling pathways. In view of these results, we determined whether 2-ME2 reduces BEAC tumor growth. Administration of 2-ME2 significantly decreased the growth of BEAC cells xenografted on the flank of nude mice. The evidence presented points out that the effect of 2-ME2 on β-catenin–E-cadherin–β-catenin axis being orchestrated signal transduction plausibly plays a multifaceted functional role to inhibit the proliferation and cell migration of 2-ME2-treated malignant cells and it could be a potential candidate in novel treatment strategies for Barrett’s esophageal adenocarcinoma. Mol Cancer Ther; 9(3); 523–34. ©2010 AACR.

Introduction

Barrett's esophagus–associated esophageal adenocarcinoma (BEAC) involves the mucosa of distal esophagus and gastroesophageal junction damaged by inflammatory stimuli (1). Within the last three decades, BEAC has become one of the fastest growing tumors in the Western world (2, 3). It is understood that the evolution of BEAC is a progressive multistep process, characterized by intestinal metaplasia, dysplasia, high-grade dysplasia, and invasive cancer (4). Unless detected at an early, surgically resectable stage, the treatment options for inoperable esophageal cancer are limited and the results are dismal due to significant treatment-related toxicities and poor long-term survival (5, 6). There is a desperate need for new and improved therapies in BEAC.

2-Methoxyestradiol (2-ME2) is an endogenous byproduct of 17β-estradiol (E2) with antitumor effects against different tumor subtypes (7). Previous work from our laboratory and others have shown that the antimalignancy properties of 2-ME2 are not only independent of estrogen receptors (8, 9) but involve the activation of diverse signal transduction circuits (10–12), inhibition of tubulin polymerization (13), and G2-M phase cell cycle arrest through the disruption of mitotic spindle apparatus (7, 13–16). 2-ME2 is currently undergoing phase I and II testing under the commercial name Panzem (17). Early evidence indicates that 2-ME2 is an orally bioavailable drug that causes selective inhibition of tumor cell growth and proliferation without any of the usual chemotherapy-induced toxicity. Consequently, the clinical interest in 2-ME2 is growing. Yet the mechanisms of action of 2-ME2 and the molecular circuits central to the tumor-specific growth-inhibitory properties of 2-ME2 are poorly understood.

Cancers arise through several cellular events. Notable among many cancers causing pathways is the deregulation of β-catenin activity that has been shown to augment tumor proliferation and survival and propagate cancer...
metastases (18–21). Expectably, anticancer therapies that correct the aberrant β-catenin functions are emerging as novel chemotherapeutics against a subset of tumors. To that end, in an effort to better understand the antitumor mechanisms of 2-ME2 and to establish a scientific rationale that could introduce 2-ME2 as an innovative chemotherapeutic agent against BEAC, we divided our research into three parts. First, we investigated the cellular status of β-catenin in BEAC-derived Bic-1 and OE33 cells, as there is insufficient reporting on β-catenin signaling in BE-stimulated adenocarcinogenesis. β-Catenin is a ubiquitous protein that possesses dual properties of being a positive and negative regulator of cell survival and fate (18–21). Second, the cellular functions of β-catenin are dependent on its intracellular location. These reports led us to subsequently examine if β-catenin and/or its membrane-bound partner E-cadherin participate in 2-ME2–activated signaling module in BEAC cells, and finally, we investigated whether the manipulation of β-catenin and E-cadherin genes by experimental techniques would have any effect on 2-ME2–directed antitumor responses. Based on these results, we designed in vitro experiments of 2-ME2 in BEAC xenografts.

Our studies categorically establish the in vitro and in vivo antitumor efficacy of 2-ME2 against BEAC cells and tumors. We have established that the cytotoxic effects of 2-ME2 occur in parallel with increased expression of membranous β-catenin and enhanced β-catenin–E-cadherin association at the plasma membrane of 2-ME2–treated cells. We also describe that by selecting the β-catenin–E-cadherin membranous complex as a specific drug target, 2-ME2 efficiently inhibits cell motility of BEAC cells. Collectively, these studies advance our current understanding of the signaling defects underlying BE-induced carcinogenesis and act as a precursor to future translational studies involving 2-ME2 in BE-associated cancers.

Materials and Methods

Animals, Cell Lines, and Reagents

About 8-wk-old athymic male and female mice (nu/nu) were obtained from Charles River Laboratories and used for xenograft experiments. The BEAC cell line Bic-1 was a kind gift from Dr. David G. Beer, University of Michigan, Ann Arbor, MI. All other epithelial cancer cell lines derived from breast carcinoma (MCF-7, MDA-MB-231), prostate (PC-3), and pancreatic cancer (Mia-Paca2) were purchased from the American Type Culture Collection and cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (HyClone) and antibiotics (Sigma). Human OE33 cell line was purchased from Sigma and cultured in the same medium described above. 2-ME2 was purchased from Sigma. Mouse monoclonal antibody against β-catenin and E-cadherin were obtained from BD Biosciences. Mouse monoclonal anti–Bcl-2 antibody was obtained from Oncogene Research Products, and Polyclonal anti–Bax and secondary antibodies, such as goat anti–rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology. Protein A/Protein G Immunoprecipitation kit was purchased from KPL, Inc., and MEM-PER® eukaryotic membrane protein extraction reagent kit was obtained from Pierce. All other chemical were obtained either from Sigma or Fisher’s Scientific.

Cell Proliferation Analysis by Cell Counting

Tumor cells (10,000 cells per well in 3 mL medium) were plated onto six-well tissue culture plates containing DMEM with 10% fetal bovine serum. After reaching approximately 60% to 70% confluent growth, cells were treated with different dosages of 2-ME2 for 24 h. After completion of the experiments, cells were stained with 0.2% trypan blue solution for 5 min and the viable cells ( unstained) were counted using an automatic cell counter (Nexcelom). In each experiment set, cells were plated in quadruplicates.

Apoptosis Assay

Photometric enzyme immunoassay for quantitative in vitro determination of apoptotic cell death was determined as previously described (16).

Xenograft Model

Bic-1 cells (2.5 × 10⁶) were injected into the right hind leg of each mouse for the development of tumor. The mice were divided into two groups (four mice per group) with a control group and 2-ME2 treatment group. To remove any gender differences in 2-ME2 actions on BEAC xenografts, we included two female and two male mice per group. The mice were maintained in a specific pathogen-free facility at VA Medical Center, Kansas City, Missouri. Kansas City VA Medical Center Animal Research Committee approved all the animal experiments. To determine the inhibitory effect of 2-ME2 on tumor volume, nude mice bearing xenografts of Bic-1 cells were given 2-ME2 doses (75 mg/kg/d) by orogastric feeding or vehicle (control) after tumor growth of ~100 mm³ was noted in the hind leg of animals. The doses of 2-ME2 have been previously reported in the literature by us (15, 21) and others (22). 2-ME2 was dissolved in DMSO + peptamen (milk) in a 1:2 ratio. We used DMSO + peptamen (1:2 ratio) as a vehicle control. Tumor growth was monitored for 16 d by measuring two perpendicular diameters twice weekly. Tumor volume was calculated according to the formula $V = \frac{a \times b^2}{2}$, in which $a$ and $b$ are the largest and smallest diameters, respectively.

Cell Migration Assay

Cell migration assays were done as described earlier (27). Briefly, Bic-1 cells (2 × 10⁴ cells/well) seeded on 8.0-μm pore Transwell filter insert (Becton Dickinson). To the upper chamber, 5 μmol/L concentration of 2-ME2 was added to the medium according to the experimental design. Cell migration was allowed to proceed for 24 h. After 24 h of incubation, the cells of the upper side...
of the membranes were removed by gently wiping with a cotton swab, and the cells that had migrated to the lower surface of the membranes were fixed with methanol, stained with Giemsa, and counted.

**Cell Lysis, Immunoprecipitations, and Immunoblotting**

Cells were treated for the indicated times with 5 μmol/L of 2-ME2, unless stated otherwise, and lysed in phosphorylation lysis buffer as previously described (22–24). Immunoprecipitations and immunoblotting using an enhanced chemiluminescence method were done as previously described (22–24).

**Short Hairpin RNA Insert Preparation and Transfection in Bic-1 Cells**

E-cadherin and β-catenin–specific short hairpin RNA (shRNA) and mismatched shRNA were designed, synthesized, and cloned into pSilencer 1.0-U6 expression following the instruction provided by the manufacturer (Ambion, Inc.) and as previously described (23, 24). E-cadherin and β-catenin–specific shRNA sequences are provided in Supplementary Table S1. Expression vectors, with mismatched or specific shRNA inserts, were transformed into the competent cells, DH5α. Plasmids were purified by the QIAprep Spin Miniprep kit (Qiagen). The transfection procedure was the same as that previously described (23, 24).

**Statistical Analysis**

All data are expressed as the mean ± SD. Statistically significant differences between groups were determined by using the paired Student’s two-tailed t test. A value of *P* < 0.05 was considered statistically significant.

**Results**

**2-ME2 Inhibits the Proliferation of Barrett’s Adenocarcinoma Cells and Activates Apoptosis**

The objective of this study was to evaluate if 2-ME2 is able to exhibit an antiproliferative effect on Bic-1 and OE33 cells. To test the hypothesis, Bic-1 cells were exposed to 1 and 5 μmol/L doses of 2-ME2 for 24 hours. Cell numbers were counted electronically using the Cellometer Auto T4 Cell Counter (Nexcelom) by trypan blue method. Compared with untreated controls, 2-ME2 significantly decreased Bic-1 cell numbers with 5 μmol/L dose at 24 hours (Fig. 1A). 2-ME2 also produces similar cytolytic effects at 5 μmol/L dose on OE33 cells at 24 hours (Fig. 1A).

To study whether the inhibition of cell growth by 2-ME2 is due to the apoptosis, we performed ELISA-based apoptosis assay. We found that 2-ME2 (5 μmol/L) significantly enhanced the apoptotic cell death in Bic-1 and OE33 cells (Fig. 1B). Next, we measured the expression of Annexin V and propidium iodide (PI), considered to be an early and late apoptotic marker, respectively. As shown in Fig. 1C, 2-ME2–treated Bic-1 cells show pronounced immunofluorescence staining with Annexin V and PI, indicating that both early and late cell death programs are activated within 24 hours of 2-ME2 treatment. Because the induction of proapoptotic Bax and inhibition of antiapoptotic Bcl-2 has previously been shown to activate the cellular suicide programs (16, 25), we, subsequently, sought to determine the levels of Bax and Bcl-2 protein expression in Bic-1 and OE33 cells following 2-ME2 (5 μmol/L) treatment. We found that Bax and Bcl-2 proteins were differentially expressed in the total lysates of Bic-1 and OE33 cells and the ratio of Bax to Bcl-2 was significantly increased in treated groups (Fig. 2), which is the hallmark of apoptosis (26). These findings suggest that the common denominator in 2-ME2–triggered programmed cell death is elevating of the Bax/Bcl-2 protein ratio.

**Expression Pattern of β-Catenin and Its Regulation by 2-ME2 in BEAC-Derived Bic-1 and OE33 Cells**

Ample studies have shown the involvement of β-catenin overexpression in the development of various cancers by increased tumor proliferation and inhibition of apoptosis (18–21). Our goal was to evaluate whether β-catenin could be a target molecule of 2-ME2 to exert its catastrophic effect on Barrett’s adenocarcinoma cells. To do so, we first determined the level of β-catenin protein in Bic-1 cells jointly with a panel of other adenocarcinoma cell lines, including breast carcinoma (MCF-7; MDA-MB-231), prostate (PC-3), and pancreatic (Mia-Paca2) cancer cells, by immuno–Western blot analysis using a mouse monoclonal against β-catenin. As shown in Fig. 3A, the highest levels of β-catenin protein were observed in the lysates of MCF-7 breast cancer cells (lane 2) and Bic-1 cells (lane 4). Contrastingly, there was an insignificant β-catenin expression in the whole-cell lysates of invasive carcinoma cells, including the Mia-Paca2 cells (lane 1), PC-3 cells (lane 5), and MDA-MB-231 cells (lane 3). In studies in which β-catenin expression was evaluated in Bic-1 cell lysates, we consistently found β-catenin expressed as a multimeric protein complex, which agrees with previous work (27).

The differential accumulation of β-catenin within intracellular compartments has been linked to tumor formation (18–21, 28), as well as a positive regulator of apoptosis and cellular differentiation (29, 30). These reports led us to investigate whether 2-ME2 would alter the cellular levels of β-catenin in Bic-1 cell lysates to achieve its antitumor effects. We found that 5 μmol/L 2-ME2 treatment markedly increased the total cellular protein levels of β-catenin by 5.2-fold at 24 hours in Bic-1 cells (Fig. 3A, right). This was an unexpected result as we speculated that the inhibition of β-catenin by 2-ME2 would constitute the fundamental molecular mechanism underlying the anticancer actions of 2-ME2 on cells that express abundant β-catenin.

As a result of our unforeseen observations showing that 2-ME2 effectively induces rather than reduce the total intracellular β-catenin protein expression in Bic-1...
cell lysates (Fig. 3A), we hypothesized that 2-ME2 treatments may change the β-catenin distribution intercellularly to mediate its antiproliferative actions. By function, the membrane-bound β-catenin acts in partner with E-cadherin to facilitate cytoskeletal attachment and thereby has antitumor properties, whereas nuclear-associated β-catenin binds with Tcf-3/4 and activates the gene expression involved in tumor multiplication and spread (19–21, 28). With that background in view and to test our objective, Bic-1 cells were stimulated for 24 hours with 5 μmol/L of 2-ME2; following that, the quantitative subfractionation of nuclear, cytoplasmic, and membrane-associated β-catenin was studied using immuno–Western blotting. In nontreated Bic-1 cells, high baseline levels of cytoplasmic and nuclear β-catenin was observed but the membrane-bound β-catenin was undetectable (Fig. 3B). Upon stimulation of Bic-1 cells with 5 μmol/L of 2-ME2, β-catenin expression was significantly increased in the membrane fraction, whereas the β-catenin levels in cytosolic and nuclear fractions were unchanged (Fig. 3B). Taken together, these data indicate that 2-ME2 exclusively upregulates β-catenin expression in Bic-1 cells at the plasma membrane fractions, and that event could be responsible for the growth-inhibitory properties of 2-ME2 on malignant cells.

Multiple studies have shown that the accumulated β-catenin in the nucleus needs to bind to its cognate receptor, Lef/Tcf transcription factors, to activate target genes (18–21, 28). Therefore, the status of Lef/Tcf was determined in 2-ME2–exposed or 2-ME2–unexposed Bic cells. Our results illustrate that Tcf-3/4 protein levels remain unaltered even after 24 hours of 2-ME2 treatment (Supplementary Fig. S1).

**β-Catenin Binds to E-Cadherin in a 2-ME2–Dependent Fashion**

Anticancer strategies that modulate the subcellular distribution of β-catenin–E-cadherin to promote cell-to-cell
adhesion are increasingly being viewed as novel, especially in the setting of invasive cancers with deregulated β-catenin signaling (31–34). Within that context and to further expand on our findings that 2-ME₂ induces preferential expression of membranous β-catenin in Bic-1 cells, we explored the hypothesis whether 2-ME₂ treatments could promote β-catenin–E-cadherin binding in Bic-1 cells. To test this, we conducted the coimmunoprecipitation experiments on membrane extracts of Bic-1 cells that were incubated with either β-catenin or an E-cadherin antibody for 24 hours. We found that β-catenin–E-cadherin binding enhances significantly in 2-ME₂-exposed cells, whereas the binding of these two proteins was undetected or minimally detected in membranous lysates of unstimulated Bic-1 cells (Fig. 3C).

2-ME₂ Attenuates the Motility/Migration of Bic-1 Cell That Is Enhanced by Small Interfering RNA–Induced Silencing against E-Cadherin and β-Catenin

As noted earlier, the augmentation of β-catenin–E-cadherin binding and/or correction of E-cadherin deficiency in tumor cells lead to the inhibition of tumor cell motility. To assess the effects of 2-ME₂ on the migration of Bic-1 cells, a modified Boyden chamber assay was utilized. As shown in Figure 3B, 2-ME₂ treatment significantly reduced the migration of Bic-1 cells compared to the untreated control group.
migration, a hallmark of invasive phenotype. Thus, in this study, we determined if 2-ME₂ modulates the migration of tumor cells. To test this, we designed cell culture experiments to optically observe the movement of Bic-1 cells across a cell-free zone created using a “scratch” technique. As shown in Fig. 4A, under serum-free conditions, Bic-1 cells show migratory properties at 24 hours by voyaging into the cell-free zone created by the scratch. In contrast, the width of the scratch-induced cell-free zone remained relatively unperturbed in 2-ME₂-treated Bic-1 cells, signifying 2-ME₂-induced arrest on cell motility.

In the in vitro setting, increased tumor cell mobility combined with an enhanced capacity of the tumor cells to invade across the basement membrane, which is facilitated by the proteosomal degradation of Matrigel by cancer cells, underlies the development of tumor metastases. Hence, after showing the effect of 2-ME₂ on Bic-1 cell motility, we choose to perform Matrigel assays to investigate the transmigratory capacity of 2-ME₂-treated and 2-ME₂-nontreated Bic-1 cells across an intact Matrigel membrane. In parallel, we determined the role of β-catenin and its binding partner, E-cadherin, on the migration of Bic-1 cells. First, we transiently transfected Bic-1 cells with pSilencer vectors containing small interfering RNA (shRNA) constructs against E-cadherin or scrambled DNA for 24 hours. Transfection efficiency

Figure 3. 2-ME₂ induces total and membranous β-catenin expression in Bic-1 cells. A, total β-catenin expression was detected in different cancer cell line extracts by Western blotting using β-catenin-specific monoclonal antibodies (left) and 2-ME₂-induced alteration of total β-catenin protein levels in Bic-1 cell extracts (right). Bic-1 were incubated with 2-ME₂ (5 μmol/L) for the indicated times. Equal amounts of total cell lysates (100 μg/lane) were analyzed by immunoblotting with an antibody against β-catenin. The blots were stripped and reprobed with β-actin antibody as a control for loading. *, P < 0.05 versus controls; ns, nonsignificant. B, distribution of β-catenin protein in different cellular compartments of Bic-1 cells before and after exposure to 5 μmol/L of 2-ME₂ for 24 h. Cells were fractionated into membranous (mem), cytosolic (cyto), and nuclear (nucl) fractions. Equal aliquots of all three cellular fractions were resolved by immunoblotting for β-catenin. Subsequently, the blot was stripped and reprobed with β-actin antibody as a control for loading of cytosolic and nuclear fractions, and calnexin antibody as a control for loading of membrane fraction. C, increased interactions of β-catenin and E-cadherin were observed in Bic-1 cells following 2-ME₂ stimulation. Cells were treated with 5 μmol/L 2-ME₂ for 24 h or left nontreated. Whole-cell lysates were harvested and used for immunoprecipitation (IP)/Western blot (WB) analysis with antibodies as indicated. Immunoprecipitated β-catenin and E-cadherin have been normalized to IgG and their fold inductions are shown in the figure.
Figure 4. Contribution of E-cadherin and β-catenin to 2-ME2–mediated effects on Bic-1 cell motility. A, representative images of the scratch test show nontreated Bic-1 cells grown in serum-free conditions (control) at 0 or 24 h after scratch, and 2-ME2–treated Bic-1 cells at similar time points. In the control test, the distance between the borderlines becomes infiltrated with motile Bic-1 cells 24 h after scratch, whereas it is still preserved in 2-ME2–treated samples (middle). Immunofluorescence images (right) of green fluorescent protein–tagged Bic-1 cells in 2-ME2–treated and nontreated cultures. B, reduced expression of E-cadherin (left) and β-catenin (right) by gene-directed shRNAs. E-cadherin level was determined by Western blotting in mismatched shRNA-transfected and E-cadherin shRNA-transfected Bic-1 cells. β-Catenin protein expression in Bic-1 cells was analyzed by immunofluorescence technique using a monoclonal antibody against β-catenin (red). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). a, mismatched shRNA-transfected cells; b, β-catenin-shRNA–transfected cells; and c, β-catenin-shRNA–transfected cells and 2-ME2–treated (5 μmol/L) cells. C, contribution of E-cadherin (left) and β-catenin (right) to the 2-ME2–mediated effects on Bic-1 cell migration. E-cadherin or β-catenin–transfected Bic-1 cells were placed in the upper chamber of the Boyden chamber in the presence or absence of 2-ME2, and cell migration toward serum was allowed to proceed for 24 h. Treatment series are control (1), control + 2-ME2 (2), scrambled control (3), scrambled control + 2-ME2 (4), E-cadherin/β-catenin-shRNA (5), and E-cadherin/β-catenin-shRNA + 2-ME2 (6). Columns, mean number of migrated cells to the undersurface of the Matrigel membrane from three independent experiments; bars, SD. *, P < 0.05 versus controls; **, P < 0.01 versus shRNA; ***, P < 0.001 versus control/scrambled control; #, P < 0.01 versus control; ##, P < 0.001 versus 5.
was determined by immunoblotting. We found that all three synthetic shRNA oligonucleotides targeted against different sequences within the coding region of the human E-cadherin gene (designated shRNA E-cad#1-3) yielded marked reduction in E-cadherin protein expression compared with scramble shRNA or native Bic-1 cells (Fig. 4B). Based on the degree of E-cadherin inhibition measured by Western blotting, we selected the most potent shRNA-E-cad#1 clones for subsequent biological studies. shRNA-transfected Bic-1 cells (15,000 per well) were seeded on the upper chamber of a modified Boyden chamber containing regular DMEM in the presence or absence of 2-ME2 (5 μmol/L) for 24 hours. Subsequently, cell migrations were studied according to our previous method (35). Upon shRNA-mediated inhibition of E-cadherin expression, in the absence of 2-ME2, the migratory mode of shRNA-transfected Bic-1 cells at 24 hours was augmented compared with mismatch vector–transfected and naïve Bic-1 cells, respectively (Fig. 4B). These shRNA experiments indicate that the downregulation of E-cadherin boosts the trans-basement membrane excursion of Bic-1 cells. In contrast, the migration of 2-ME2–exposed (5 μmol/L) cells was appreciably decreased at 24 hours compared with 2-ME2 unexposed cells (Fig. 4B). Next, we designed to examine the effect of β-catenin silencing by shRNA on cell migration. We found that silencing of the β-catenin gene in Bic-1 cells promoted their cell migration compared with the

Figure 5. 2-ME2 restores E-cadherin protein in shRNA-transfected Bic-1 cells. A, transfected Bic-1 cells were treated with 5 μmol/L of 2-ME2 for 24 h or left nontreated. Equal amounts of cell lysates (100 μg/lane) were analyzed by Western blotting with an antibody against E-cadherin. Quantification of each band was done by a densitometry analysis software. *, P < 0.005 versus mismatched controls. **, P < 0.01 versus shRNA alone transfected cells. B, E-cadherin and β-catenin shRNA-transfected Bic-1 cells were exposed to 5 μmol/L of 2-ME2 for 24 h, and β-catenin (left) and E-cadherin (right) mRNA expressions were determined by Northern blotting using nonradioactive DIG-labeled probe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as loading control.
Repression of E-Cadherin and β-Catenin by shRNA Is Restored by 2-ME₂ Treatment

From results from previous studies, we reasoned whether 2-ME₂ could restore the protein levels by overcoming the shRNA-directed repression of E-cadherin and β-catenin genes in Bic-1 cells. As shown in Fig. 4B, β-catenin protein levels enhanced significantly in 2-ME₂–exposed β-catenin-shRNA–transfected Bic-1 cells at 24 hours. Similar results were obtained when E-cad–shRNA–transfected cells were treated with 2-ME₂ for 24 hours (Fig. 5A). Altogether, these results support the claim that the repressions of E-cadherin and β-catenin protein by shRNAs are restored by 2-ME₂.

Effect of 2ME₂ on β-Catenin and E-Cadherin mRNA

To investigate the mechanisms responsible for the 2ME₂–induced upregulation of E-cadherin and β-catenin, we used the shRNA constructs designed for our earlier experiments to first knock down β-catenin and/or E-cadherin genes in the Bic-1 cells. The shRNA-transfected cells were then grown in either the presence or absence of 2-ME₂ (5 mmol/L) for 24 hours at 37°C. Total mRNA levels of β-catenin and E-cadherin were determined by Northern blot analysis. As depicted in Fig. 5B, 2-ME₂ is unable to rescue the shRNA-mediated inhibition of β-catenin and E-cadherin mRNA expression in these cells. Therefore, this study suggests that the 2-ME₂–mediated upregulation of the expressions of these proteins could be the result of the stabilization of these proteins. However, further studies are warranted.

2-ME₂ Inhibits the Growth of BEAC Xenografts

When Bic-1 cells are implanted into nude mice, they are remarkably tumorigenic. To illustrate that point, measurable xenografts were noted within a week of Bic-1 inoculation into the hind leg of nude mice, whereas OE33 cells produced comparable xenografts at 3 to 4 weeks after implantation (Supplementary Fig. S2). These studies suggest that both cell lines are capable of producing xenografts in immunocompromised mice, although at varying time points. Given the rapid tumor-forming potential of Bic-1 cells, we subsequently sought to determine the in vivo effects of 2-ME₂ on Bic-1–generated xenografts. To determine the inhibitory effect of 2-ME₂ on tumor volume, four athymic nude (two female and two male) mice in each group were treated with or without 2-ME₂ (75 mg/kg/d) after bearing a minimum xenograft size of ∼100 mm³ (day 0). Tumor growth was monitored for 16 days by measuring two perpendicular diameters twice weekly. Our data show that 2-ME₂ significantly inhibits the growth of Bic-1–induced tumors in nude mice (Fig. 6). However, like in humans, interanimal variability was noted in tumor responses (range of tumor reduction was 38–71%).

Discussion

Therapeutic advances in BEAC have lagged behind other cancers due to the paucity of reliable in vitro and in vivo models. Although previous studies have established Bic-1 and OE33 cells as BEAC-derived cell lines, there is limited information characterizing the ongoing molecular events in these cells. Previous studies suggest that both Bic-1 and OE33 cells have mutated p53 gene (36, 37). Despite that concordance in p53 status, there are molecular and behavioral differences between Bic-1 and OE33 cells. We found a striking difference in the level of villin expression in Bic-1 (high expression) and OE33 (low expression) cells (Supplementary Fig. S3). Villin is a Ca²⁺–dependent actin-binding protein and a useful marker for intestinal cell differentiation and for recognition of malignant tumors of colonic origin (38). Therefore, our observations make us speculate two possible scenarios to explain the differential expression of villin in BEAC cell lines. First, in the villin overexpressing Bic-1 cells, the microvilli architecture could be better preserved. Second, while taking into account the epithelial heterogeneity of BEAC, the presence of colonocyte-like cells in Bic-1 may be responsible for the preferential expression of villin in Bic-1 cells. Moreover, when Bic-1 cells are implanted into nude mice, they...
become remarkably tumorigenic within a week. OE33 cells also produce measurable xenografts, however, at 3 to 4 weeks after implantation (Supplementary Fig. S2). Collectively, these studies suggest that these two cell lines are phenotypically and behaviorally distinct, and therefore, we selected these two cell lines for the present study.

The poor understanding of BEAC pathobiology is partly responsible due to the meager therapeutic targets available against BEAC. Research efforts geared to introduce targeted and novel treatments are therefore desperately needed to improve patient outcomes in BEAC (5, 6). 2-ME2 is being increasingly recognized as a novel chemotherapy drug due to its propensity to activate a wide array of anticaner targets with a relative sparing of the normal tissues (17, 39, 40). We have carried out studies to determine the therapeutic efficacy of 2-ME2 against adenocarcinoma and to identify surrogate biomarkers that would be useful in predicting its anticancer responses. Our studies clearly establish that 2-ME2, at a concentration of 5 mmol/L, attenuated the in vitro proliferation of BEAC-derived Bic-1 and OE33 cells through the induction of apoptosis. Differential regulations of Bax and Bcl-2 may promote apoptosis (Figs. 1 and 2).

The growth of Bic-1 xenografts was significantly slower in the 2-ME2 group than in animals treated with vehicle (control; Fig. 6). This effect is, however, variable. Therefore, 2-ME2 may prove to be a therapeutic agent for patients with BEAC if its poor bioavailability in humans as well in animals is addressed. The design of new formulations of 2-ME2 to improve its bioavailability is ongoing in our laboratory.

Sequential loss of membranous β-catenin and E-cadherin expression along the metaplasia-dysplasia-cancer sequence has been previously reported in clinical specimens (41). It is now customary knowledge that the proinflammatory cytokines produced by inflamed cells of BE augment the nuclear translocation of β-catenin that eventually culminates in neoplastic transformation by transcriptional activation of oncogenes. Given the importance of β-catenin nuclear translocation as a hallmark of neoplastic transformation, we determined whether 2-ME2 can be a regulator of this translocation event. We therefore examined the expression profiles of β-catenin in BEAC cells before and after the exposure of 2-ME2. We found constitutive expression of nuclear and cytosolic, instead of membranous, β-catenin in unexposed Bic-1 cells, whereas membranous fraction of β-catenin accumulated remarkably in 2-ME2-exposed Bic-1 cells (Fig. 3). Membrane-bound β-catenin plays a vital role in cell adhesion machinery, which may be particularly relevant to the prevention of cancer progression through the induction of mesenchymal to epithelial transition (42–44). Therefore, we anticipate that the 2-ME2-induced accumulation of β-catenin in the membrane may diminish the aggressive behavior of the BEAC cells.

β-Catenin–mediated cell-cell adhesion is a coordinated process that is accomplished by molecular interactions with E-cadherin, a cell adhesion molecule that acts as a tumor suppressor inhibiting the invasive front and it is frequently downregulated in aggressive cancer cells (31–34, 44, 45). Our results show that 2-ME2 treatment promotes E-cadherin expression and its association with β-catenin in the plasma membrane fraction of Bic-1 cells (Fig. 5A and B). These studies strengthen our above hypothesis and suggest that 2-ME2 may be able to restore the cell adhesion property of the BEAC cells.

The next logical step was to perform studies that would show the biological relevance of membranous β-catenin–E-cadherin induction in 2-ME2-treated cells. The picture evolving from our Transwell motility assays is that contrary to the motile and invasive phenotype of the 2-ME2-unexposed cells, the 2-ME2-treated cells exhibit a drastic inhibition of cellular migration in E-cadherin– and β-catenin–deficient Bic-1 cells. Loss of E-cadherin expression promotes cancer dissemination through local proteolysis and through enhanced motility and migration of cancer cells across the Matrigel basement membrane (46–49). However, the observation made in our shRNA knocking-down experiments is that β-catenin silencing leads to increased cell motility in a BEAC model that is original and previously unreported. These findings underscore our argument that the assembly and function of the β-catenin and E-cadherin epithelial adhesion molecules are mutually supportive in Bic-1 cells. 2-ME2, by restoring the expression level of β-catenin and E-cadherin transmembrane adhesion molecules in native and shRNA-transfected Bic-1 cells (Fig. 4), abrogates their motile and migratory behavior and acts as a potent anti-invasive drug.

Because we found that 2-ME2 enhances membrane β-catenin and E-cadherin levels in these cells even after silencing of the expressions of these two molecules, we sought to determine how does 2-ME2 nullifies the effect of the shRNA-mediated downregulation of β-catenin and E-cadherin expression? We provide evidence to suggest that the induction of membranous β-catenin protein and E-cadherin by 2-ME2 is accomplished by protein stabilization (Fig. 5). To the best of our knowledge, this study provides a firsthand report of posttranscriptional mechanisms involved in the 2-ME2-mediated protein stability of β-catenin–E-cadherin cell-cell adhesion complex.

In conclusion, after reviewing the current body of evidence, our results are the first to describe the in vitro and in vivo antitumor effects of 2-ME2 on BEAC-derived cell lines and xenografts, respectively. Because there has not been an appreciable improvement in the overall survival of patients with BEAC over the last decade (5, 6), there is an urgent need to investigate effective and less toxic chemotherapeutic options. Our data provide solid scientific merit for the broader use of 2-ME2 in chemoprevention and treating patients with BEAC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.


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