Distinct Tumor Stage–Specific Inhibitory Effects of 2-Methoxyestradiol in a Breast Cancer Mouse Model Associated with Id-1 Expression

Jung-Im Huh, Alfonso Calvo, Rhonda Charles, and Jeffrey E. Green

Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, NIH, Bethesda, Maryland

Abstract

2-Methoxyestradiol (2ME2), a metabolite of 17-β-estradiol, inhibits angiogenesis and has additional antitumor activities. We have analyzed the tumor stage–specific effects of 2ME2 in the C3(1)/Tag transgenic mouse model for breast cancer, which spontaneously develops estrogen receptor–negative mammary tumors following a predictable progression of lesion formation. When given either as a therapeutic agent in established tumors (late intervention study) or in mice with pre-invasive mammary lesions (early intervention study), tumor growth was reduced by 60% compared with untreated controls and was associated with an induction of apoptosis. In a prevention study, a significant reduction in mammary intraepithelial neoplasia (MIN) lesions was observed in animals beginning treatment at 6 weeks of age, before the appearance of histopathologic abnormalities. However, although 2ME2 reduced the number of MIN lesions in the prevention study, a paradoxical increase in tumor multiplicity and growth rate was observed. This was associated with unusual cystic tumor formation, in which significant central necrosis was observed, surrounded by an outer region of proliferative tumor cell growth. The characteristics of the cystic tumor formation in mice treated with 2ME2 at early ages are consistent with an impaired angiogenic response as observed in mice deficient for inhibitor of differentiation (Id-1). We further show that Id-1 expression is negatively regulated by 2ME2, which may be an additional mechanism for the antiangiogenic effect of 2ME2. Although 2ME2 significantly reduced tumor growth at late stages, these results also suggest that altered tumor morphology and accelerated tumor growth may occur if 2ME2 is administered in a prevention setting for prolonged periods. (Cancer Res 2006; 66(7): 3495-503)

Introduction

It is estimated that one in nine women in the United States will develop breast cancer, resulting in >40,000 deaths each year (1). Both environmental and genetic factors seem to contribute to the risk of developing breast cancer (2). The prognosis is better for patients with hormone-responsive disease, where antiestrogens, such as tamoxifen, are used as adjunct therapy for estrogen receptor (ER)–positive tumors (3). However, treatment of ER-negative breast cancer remains quite problematic without widely effective therapies.

2-Methoxyestradiol (2ME2), a naturally occurring metabolite of 17-β-estradiol, is produced following hydroxylation and methylation reactions catalyzed primarily in the liver (4). 2ME2 has been shown to be an effective antitumor agent for reducing tumor volume and neovascularization in several xenograft models and in a transgenic model for prostate cancer (5, 6), but tumor stage specificity has not been investigated, and the mechanisms through which 2ME2 inhibits tumorigenesis are not fully understood. Although a metabolite of estradiol, 2ME2 has low affinities for ERα and ERβ (7, 8), and its antitumor activity is independent of the presence of ERs (8, 9). 2ME2 is a potent inhibitor of angiogenesis and has been shown to reduce endothelial cell proliferation and migration (10). 2ME2 induces changes in the levels and activities of many proteins involved in the regulation of the cell cycle, including stress kinases, cell division kinases, cyclin B, and regulators of cell cycle arrest and apoptosis (6). It also increases the expression of p53 and death receptor 5 and activates downstream caspases in human tumor and endothelial cells (11), leading to increased apoptosis. A recent study showed that 2ME2 depolymerizes microtubules and down-regulates hypoxia-inducible factor-α protein levels and transcriptional activity in an oxygen-independent and proteasome-independent manner (12). Additional studies have shown that 2ME2 inhibits the activity of superoxide dismutase, leading to free radical–mediated damage to mitochondrial membranes and the release of cytochrome c, inducing apoptosis (13). Importantly, 2ME2 has been well tolerated both clinically and in preclinical studies, and neither dose-limiting toxicity nor a maximum tolerated dose has been established (14).

In this study, we have analyzed the tumor stage–specific effects of 2ME2 in the C3(1)/Tag transgenic mouse model of mammary cancer. This model recapitulates important histopathologic and molecular alterations in mammary cancer development over a highly predictable time course (15). Low-grade mammary intraepithelial neoplasia (MIN) lesions develop at about 8 weeks of age and progress to high-grade MIN (similar to human ductal carcinoma in situ) by 12 to 14 weeks of age. ERα-negative invasive carcinomas are observed at about 15 to 16 weeks of age (15, 16).

We show that 2ME2 can affect tumorigenesis at both early and late stages, via different mechanisms. When 2ME2 is administered in late stages to animals with lesions in the C3(1)/Tag mouse model, it inhibits mammary tumor growth and multiplicity by significantly increasing apoptosis in mammary tumor epithelial cells. In early stages, when 2ME2 is administered at 6 weeks of age before significant lesion formation, an unusually high rate of cystic tumor formation is observed, consistent with a strong inhibition of tumor angiogenesis. However, a paradoxical increase in tumor multiplicity.
and tumor size occurs in mice in the early treatment group, where rates of apoptosis are not increased. The cystic tumor formation observed with 2ME2 treatment is similar to the cystic changes reported in a mammary breast cancer model deficient for the transcription factor Id-1 (17). Id-1 has been shown to inhibit differentiation, and its expression has been correlated with increased angiogenesis and endothelial growth (18). In this study, we show for the first time that 2ME2 down-regulates Id-1 expression in mouse and human mammary tumor and endothelial cell lines.

These observations suggest that different biological responses to 2ME2 may predominate (antiangiogenic or increased apoptosis), depending upon the stage at which 2ME2 is administered during mammary tumor development. Although 2ME2 may be a useful adjunct in established ER- tumor models, caution may be warranted for its prolonged use as an agent to prevent breast cancer or breast cancer recurrence.

Materials and Methods

Cell culture. The mammary tumor epithelial cell line M6 was previously derived from C3(1)/Tag mammary gland tumors (19, 20). M6 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 50 units/mL of penicillin G sodium/50 μg/mL of streptomycin sul fate, SV/40 T-antigen transformed mouse endothelial cells, SVEC-4, were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 10 mmol/L Hepes, 2 mmol/L glutamine, 50 units/mL of penicillin G sodium, and 50 μg/mL of streptomycin sulfate. MDA MB 231 cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum. Human microvascular endothelial cells-1 (HMEC-1) were grown in MCDB 131 supplemented with 15% FBS, 10 mmol/L/L-glutamine, 50 units/mL of penicillin G sodium, and 50 μg/mL of streptomycin sulfate (21). All supplements were purchased from Invitrogen.

In vitro proliferation assay. Detection of DNA synthesis was done using the bromodeoxyuridine (BrdUrd) cell proliferation colorimetric ELISA kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. For BrdUrd assays, 2,500 cells per well were seeded in a 96-well plate, allowed to attach overnight, and were then incubated with the compound in the growth medium for 48 hours.

In vitro apoptosis assay. Apoptosis was measured by examination of the presence of cytoplasmic histone-associated DNA fragments according to the manufacturer’s instructions (Roche, Indianapolis, IN). Briefly, 10,000 cells per well were seeded in a 24-well plate and after 24 hours were exposed to 2ME2 at concentrations ranging from 0 to 10 μmol/L for 48 hours. The detection of cytoplasmic histone associated DNA fragments was quantitated as per manufacturer’s protocol.

Measurement of caspase-3 enzymatic activity. Caspase activity was measured using a caspase fluorometric protease assay kit (BioVision Research Products, Palo Alto, CA). Briefly, after treatment of cells with 5 μmol/L 2ME2 for the indicated periods of time, cells were harvested, pelleted, and frozen on dry ice. This concentration was selected based on our previous results studying the effects of 2ME2 on apoptosis (8). Cell lysis buffer was added to the cell pellets, and the protein concentration was determined using the Bradford assay. Two hundred micrograms of each cell lysate was incubated with 5 μL of 1 mmol/L stock of fluorescently labeled tetrapeptide caspase substrate at 37°C for 1 hour. The release of 7-amino-4-trifluoromethyl coumarin was measured with a fluorometer (Wallac Victor, Perkin-Elmer Life Sciences, Boston, MA) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase activity was assayed in duplicate samples.

Animals and experimental schedule. Transgenic female mice heterozygous for the C3(1)/Tag transgene in the FVB/N background were generated as previously described (15). Three sets of experiments were done: early and late intervention studies and a prevention study. The scheme of the study is depicted in Fig. 2.

As shown in previous studies, 2ME2 causes very low signs of toxicity when administered in vivo. Doses of 75 mg/kg body weight did not cause any clinical toxicity in mice (22, 23). Based on previous studies using several mouse models (14) that showed that doses of 2ME2 ranging from 50 to 150 mg/kg/d are effective but not toxic against various cancer types, such as melanoma (5), neuroblastoma (24), and lung metastasis (23), we used the highest dose, 150 mg/kg body weight for our study in the C3(1)/Tag transgenic breast cancer model.

For the intervention experiments, mice were randomly selected for a control group (n = 12) and an experimental group (n = 12). Animals in the experimental group received 150 mg/kg/d 2ME2 daily in 0.2 mL 0.5% methylcellulose by oral gavage. For the late intervention group, treatment was initiated when mice developed tumors 0.5 cm in diameter and was continued for 6 weeks or until the tumors reached a maximum diameter of 2 cm.

For the early intervention experiments, designed to show inhibition from MIN to invasive tumor formation (typically occurring at 16-20 weeks of age), another cohort of mice was randomly separated into a control (n = 10) and an experimental group (n = 10). The same dosing regimen was used as above, but treatment was initiated at 12 weeks of age and continued for 6 weeks.

The effect of 2ME2 on preneoplastic mammary glands was analyzed in a prevention experiment. Six-week-old mice were divided into two groups with treatment continuing until the mice were euthanized or the tumors reached 2 cm in diameter: (a) a control group that received vehicle (0.2 mL of 1% hydroxypropylcellulose in sterile water, n = 22) and (b) a group that received 150 mg/kg/d 2ME2 (formulated with 1% hydroxypropylcellulose in sterile water, 0.2 mL, n = 24). From this group, 10 mice (5 controls and 5 mice treated with 2ME2) were sacrificed at 12 and 15 weeks for mammary gland analysis. The formulation of 2ME2 used in the prevention experiment (done after the intervention studies) was altered based upon data, indicating improved pharmacokinetics and activity with this formulation in other tumor models (25).

Body weights were checked weekly for all mice. Mammary tumor size was measured by caliper, and tumor volume was calculated using the formula: the largest diameter × (the smallest diameter)2 × 0.4 (26). All mice were treated in accordance with the guidelines of the Animal Care and Use of Laboratory Animals (NIH publication no. 86-23, 1985) under an approved animal protocol.

Antibodies. The following primary antibodies were used: SV40 Tag (for Western blot: 1:80, Ab-2; Oncogene, Boston, MA; for immunohistochemistry: 1:25, PAB011; PharMingen, San Diego, CA), mouse monoclonal anti-Ki-67 (DAKO, Carpinteria, CA; 1:10,000), and mouse monoclonal anti-proliferating cell nuclear antigen (anti-PCNA; DAKO; 1:1,000). Mouse monoclonal antibody 1:120, specific to SV40 Tag and Id-1 polyclonal antibody (1:1,000) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Histology, immunohistochemistry, and terminal deoxynucleotidyl transferase–mediated nick end labeling analysis. Mammary tissues and tumors were excised, fixed in 4% paraformaldehyde, embedded in paraffin, cut at a thickness of 4 μm, and stained with H&E.

For immunohistochemical analysis, sections were heated in a microwave oven in 0.1 mol/L sodium citrate for antigen retrieval, hybridized with primary antibody, and processed using the avidin-biotin complex method (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). Using a ×10 objective, the number of MIN lesions counted in five fields of the #4 mammary gland from each of five mice in each group at 15 weeks of age were determined in the intermediate prevention study and reported as the average number of MIN lesions per mouse. Apoptotic cells were identified using the TUNEL in situ end-labeling technique (ApopTag, Oncor, Gaithersburg, MD). Cells staining positively for Ki-67, PCNA, and terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay were quantified using a Zeiss Axiosplan microscope and ImagePro software (Media Cybernetics, L.P., Silver Spring, MD). At least 1,000 cell nuclei per section of mammary gland were counted in randomly selected fields in non-necrotic areas. Final data were expressed as a mean percentage of positively stained epithelial cells with respect to the total number of epithelial cells examined. Mammary glands from five animals in the 2ME2-treated and control groups from the intervention experiment were used for quantification.

RNA extraction and semiquantitative reverse transcription-PCR. Total RNA was isolated using Trizol reagent (Invitrogen) from mammary...
Inhibition of Mammary Cancer by 2ME2

We first evaluated the effects of 2ME2 on proliferation and apoptosis in M6 and SVEC-4 cells in vitro. M6 is a tumorigenic cell line isolated from C3(1)/Tag mammary tumors and previously characterized (19, 20). SVEC-4 cells are SV40 T-antigen transformed mouse endothelial cells that have been previously described (27). 2ME2 caused a dose-dependent inhibition of cell proliferation for both M6 and SVEC-4 cells (Fig. 1A). The IC50 value was ~4 μmol/L. Levels of apoptosis were assessed by detection of cytoplasmic histone-associated DNA fragments and caspase-3 activation. 2ME2 induced apoptosis in M6 and SVEC-4 cells as evidenced by the induction of DNA fragmentation at ≥1 μmol/L concentrations (Fig. 1B). To investigate the kinetics of apoptosis in response to treatment with 2ME2 in these cell lines, we evaluated caspase-3 activation (Fig. 1C). Caspase-3 activation was detectable in both M6 and SVEC-4 cells treated with 5 μM 2ME2 beginning after about 16 hours of treatment and markedly increased by 24 hours of treatment (Fig. 1C). These data show that 2ME2 inhibits cell proliferation and increases apoptosis of M6 and SVEC-4 cells in vitro.

Stage-Specific Effects of 2ME2 on Mammary Tumor Growth, Tumor Multiplicity, and Survival in C3(1)/Tag Transgenic Mice

To evaluate the tumor stage–specific effects of 2ME2 in C3(1)/Tag mice, we did three independent in vivo experiments: (a) a late intervention study, (b) an early intervention study, and (c) a prevention study. The experimental scheme is depicted in Fig. 2.

**Figure 1.** Effect of 2ME2 on proliferation and apoptosis in vitro in M6 (C) and SVEC-4 cells (E). A and B, antiproliferative (A) and apoptotic (B) activities of increasing concentrations of 2ME2 for 48 hours. C, kinetics of 2ME2-induced caspase-3 activation in M6 and SVEC-4 cells. Increased caspase-3 activation was observed after 16 hours of treatment with 5 μmol/L 2ME2. Points, average of three independent experiments; bars, SD. Proliferation, apoptosis, and caspase assays were done as described in Materials and Methods.
Late intervention study. The late intervention experiment was designed to determine whether 2ME2 could retard tumor growth or result in tumor regression of established mammary tumors in C3(1)/Tag mice. For this purpose, we treated mice bearing 0.5 cm tumors with 150 mg/kg/d 2ME2 for 6 weeks (or until tumor size reached 2 cm, requiring euthanasia of the animal). Three weeks after the initiation of treatment, a significant decrease (*, P < 0.05) in tumor burden was observed in the 2ME2-treated mice compared with the control mice (Fig. 3A). After 6 weeks of treatment, tumor burden was reduced by ~60% (**, P < 0.01) in animals treated with 2ME2 compared with the control mice.

Tumor multiplicity was significantly decreased in the mice treated with 2ME2 compared with the controls (P < 0.001 after 3 weeks). The mean number of tumors per mouse (MNT) in control mice was 2.1 after 2 weeks of treatment, whereas in the 2ME2-treated mice, the MNT was 1.2, a 45% reduction. After 6 weeks of treatment, the MNT for the control group was 5.8 compared with 2.5 for the mice treated with 2ME2, a 57% reduction in the 2ME2-treated group (Fig. 3C). Animals receiving 2ME2 showed a modest but statistically significant 2-week increase in survival compared with the control mice (P = 0.0072; Fig. 3E). To determine the mechanism of tumor inhibition and increased survival, we evaluated proliferation and apoptosis. In the late intervention group, the apoptotic index was significantly increased by ~2-fold in tumors from the 2ME2-treated group compared with the control group (Fig. 3F). The proliferation index, as determined by Ki67 and PCNA staining, was not statistically different between the control and treated mice (data not shown) in any of the intervention experimental groups.

Early intervention study. To evaluate whether 2ME2 inhibits progression from pre-invasive MIN (ductal carcinoma in situ like) lesions to palpable, invasive tumors in C3(1)/Tag mice, animals were treated beginning at 12 weeks of age for a total of 6 weeks. We have previously shown that at 12 weeks of age, MIN lesions are found in the mammary glands of C3(1)/Tag mice, but few invasive tumors and no palpable tumors are seen. Although onset of palpable tumors was not significantly delayed in mice treated with 2ME2 compared with controls in the early intervention study (data not shown), tumor burden (cumulative tumor volume) was significantly decreased in animals that received 2ME2 compared with the control mice (Fig. 3B). The degree of tumor reduction was similar to the late intervention experiment (50-60%). The MNT was also statistically lower in mice treated with 2ME2 compared with controls (Fig. 3D). The effect in the late intervention study was more pronounced than in the early intervention experiment (compare Fig. 3B and D). However, in the early intervention study, survival was not significantly increased in the 2ME2-treated animals compared with the control mice (data not shown). Unlike the late intervention study, the apoptotic index was not changed in MIN lesions of 15-week-old mice in the early intervention group.

Prevention study. Animals received 2ME2 beginning at 6 weeks of age until mice were euthanized either from tumor burden (diameter of 2 cm) or because animals seemed moribund. Surprisingly, tumor burden seemed to be increased in animals that received 2ME2 compared with the control mice in this experiment (Fig. 4A; *, P < 0.05), but this seemed due to the cystic nature of the tumors that developed, as discussed below. At 20 weeks of age, tumor size in the 2ME2-treated group was 2.3-fold larger than the control group.

The MNT seemed to be higher in mice treated with 2ME2 compared with controls (Fig. 4B; *, P < 0.05). At 20 weeks of age, the MNT in the control group was 0.5, whereas it was 2.2 in the 2ME2-treated group, an almost 4.4-fold induction of MNT compared with the control group.

Apoptosis was not increased in the preinvasive lesions, and the proliferation index was not decreased in the prevention study, similar to the early intervention study. The overall survival in the 2ME2-treated group compared with the control group was reduced due to the accelerated cystic tumor expansion requiring earlier euthanasia (data not shown).

These results suggest that increased survival in the intervention study is likely due to increased apoptosis that was not observed in the 2ME2-treated mice in the prevention group.
2ME2 Altered the Phenotype of Tumors during Mammary Cancer Progression in the Prevention Study

To further understand the nature of the cystic tumor formation induced by 2ME2 in the prevention study, several histologic analyses were done using five mice from each group (Fig. 4C). Tissues were analyzed histologically, and the number of MIN lesions was quantitated.

At all ages, dilated ducts filled with proteinaceous material were noted in all the treated mice, but only in one of the mice from the control group (Fig. 4C, left). At early stages, small lesions with evidence of early central necrosis were observed within the lumens of ducts in the 2ME2-treated mice (Fig. 4C, middle). By 20 to 21 weeks of age, when palpable mammary tumors were developing, marked necrosis in the central regions of many of the tumors were observed in the 2ME2-treated group (Fig. 4C, bottom right), but a similar degree of central necrosis was not observed in the control group (Fig. 4C, top right). Eventually, most 2ME2-treated mice developed multiple cystic tumors filled with secreted fluid with significantly dilated mammal ductal architecture (Fig. 4C, bottom right), whereas tumors in the control mice remained primarily solid (Fig. 4C, top right). Overall, cystic tumors were observed in 71% of the mice treated with 2ME2 but rarely seen in the mice in the control group, where only an occasional tumor with small cysts was observed.

The average number of MIN lesions per mouse was 15.25 ± 3.3 in the control group compared with 6.6 ± 2.9 in the 2ME2-treated group (P < 0.01). This result indicates that overall, 2ME2 inhibited MIN lesion development, although large cystic tumors still developed. It is likely that the cystic nature of the tumors led to larger tumor sizes compared with the controls, and detection of these smaller tumors was more apparent as they expanded from the cystic fluid that would not have been palpable in the untreated mice.

To exclude the possibility that these effects mediated by 2ME2 were a consequence of changes in transgene expression, we did immunohistochemical staining for T-antigen. The distribution of expression and the intensity of staining of T-antigen in the 2ME2-treated animals were similar to those found in the control mice (data not shown).

Figure 3. A and B, tumor stage–specific effects of 2ME2 on mammary tumor burden in the late intervention and early intervention studies. A, tumor burden was significantly (**, P < 0.01) inhibited by ~60% after 6 weeks of treatment in the late intervention study compared with the control. B, tumor burden was significantly (**, P < 0.01) decreased in mice that received 2ME2 in the early intervention study. C and D, tumor stage–specific effects of 2ME2 on mammary tumor multiplicity. C, mean number of tumors was significantly decreased (***, P < 0.001) after 6 weeks of 2ME2 treatment in the late intervention study compared to control. D, mean number of tumors is significantly lower (*, P < 0.05) in the 2ME2–treated mice compared with the control group in the early intervention study. E, a modest but significant (P = 0.0072) increase in survival was observed in 2ME2–treated mice compared to controls in the late intervention study. F, 2ME2 induced apoptosis in mammary tumors in the late intervention study. Quantification of apoptosis in tumors showed increased apoptosis in the 2ME2–treated group compared with the control group (*, P < 0.05). Arrows, termination of treatment in (B) and (D).
2ME₂ Down-regulates Id-1 Expression in Mammary Tumor and Endothelial Cells in a Dose-Dependent Manner

Id-1 expression has been correlated with aggressive breast cancer and poor prognosis (28, 29). Interestingly, the tumor phenotype from our prevention study seemed very similar to that observed for MMTV-neu mice deficient for Id-1, in which tumors exhibited central necrosis and were quite cystic (17). The lack of Id-1 in that setting resulted in impaired angiogenesis. Therefore, we investigated the expression of Id-1 in response to 2ME₂ treatment. Id-1 was down-regulated by 2ME₂ in M6 cells [which are derived from C3(1)/Tag mammary tumors] and HMEC-1 in a dose-dependent manner at the RNA levels (Fig. 5A). Similar results in protein levels were observed using M6 cells, MDA MB 231, and SVEC-4 (Fig. 5B). Tumors from vehicle-treated C3(1)/Tag mice showed inconsistent levels of Id-1 expression, perhaps due to tumor heterogeneity (data not shown), and thus did not allow for the adequate evaluation of this in vivo.

The transcriptional regulation of Id-1 has been well characterized in breast cancer cell lines by other groups (30, 31). Based upon the results above, we studied the transcriptional regulation of Id-1 by 2ME₂ using three different cell lines, in which luciferase reporter constructs containing various lengths of the Id-1 5' regulatory region were assayed with or without 2ME₂. All these cell lines showed a consistent reduction (maximum 50%) in transcriptional activity of Id-1 in a dose-dependent fashion (Fig. 6, A and B).

Discussion

This is the first report evaluating the effects of 2ME₂ on several stages of tumor development in a transgenic breast cancer mouse model. An important feature of the C3(1)/Tag model used in this study is that mammary tumor progression can be monitored and tumor stage-specific responses can be evaluated. Several studies have shown tumor stage-specific effects of antiangiogenic agents using different mouse models (32, 33). We took advantage of the C3(1)/Tag model in designing experiments to address how 2ME₂ might affect different stages of mammary tumorigenesis in a model that develops ER-negative mammary tumors. Different phenotypic responses were observed depending upon the stage of tumor development when 2ME₂ was administered, and these results may be due to different mechanisms of action.

Transgenic mice that were treated with 2ME₂ following the development of MIN (lesions similar to human ductal in situ carcinoma) in the early intervention study, or once palpable tumors of \( \sim 0.5 \) cm were observed in the late intervention study, showed a significant reduction in tumor growth and tumor multiplicity associated with increased apoptosis in vivo. Our in vitro studies using M6 mammary tumor cells from C3(1)/Tag transgenic mice and mouse endothelial SVEC-4 cells showed that 2ME₂ could result in a significant reduction in proliferation and increased apoptosis associated with increased DNA fragmentation and elevated caspase-3 activity. However, when similar variables were studied in tumors arising spontaneously in vivo and treated with 2ME₂, no reduction in cell proliferation was observed, but a 2-fold increase in the rate of apoptosis of tumor
cells was observed. This suggests that at the late stage of tumorigenesis studied in vivo, 2ME₂ exerted an inhibitory effect, at least in part through increased apoptosis. The tumor inhibitory effects we observed with 2ME₂ in the early intervention study were more modest and showed a reduction in tumor growth but not complete eradication of tumor development. No significant difference in tumor cell proliferation or apoptosis was observed in the early lesions from either the early intervention or prevention studies (data not shown). These results suggest that 2ME₂ is able to significantly decrease mammary tumor size and number in established yet relatively small invasive tumors.

The majority of tumors exposed to 2ME₂ before the development of MIN lesions were remarkably cystic with a relatively thin rim of viable tumor cells in the periphery. The tumors seemed to originate as nodular lesions within the lumens of mammary ducts. As these lesions enlarge, central necrosis develops, ultimately resulting in large, fluid-filled cystic tumors. This type of cystic morphology was not observed in the tumors that arose in the control group of transgenic mice. Although cystic mammary tumors have occasionally been observed in C3(1)/Tag mice, in our long experience with this model system, we have not observed this striking morphologic characteristic of tumors in an experimental cohort of mice.

To further define the effects of 2ME₂ on lesion formation, we quantitated the average number of MIN lesions per mouse after 7 weeks of treatment in the prevention cohorts (at 13 weeks of age). The significant reduction in the number of MIN lesions in the 2ME₂-treated group suggests that 2ME₂ inhibits this type of early tumor progression in addition to altering histologic characteristics of the invasive tumors that do develop. Previous studies have also shown that 2ME₂ has potent antiangiogenic activities (5, 12, 34). In the case of the present prevention study, it seems that once the tumor has grown to a critical size, vascularization becomes limiting, and the central tumor region undergoes necrosis. However, the peripheral region of the tumor receives sufficient oxygen and nutrients and is able to continue to proliferate and survive as had similarly been observed in MMTV-neu2Id-1⁻/⁻ mice.

Our observations in the prevention study are remarkably similar to those reported by de Candia et al., where it was shown that deficiency of Id-1 and Id-3, which play an important role in tumor angiogenesis, did not prevent or delay tumor formation but did alter the morphologic phenotype of mammary tumors (17). In that study, MMTV-HER-2/neu tumors that developed in Id-deficient mice were larger, exhibited central necrosis, and became cystic with a viable rim of tumor cells surrounding a nonviable core of cellular debris (17). We checked Id-1 expression in tumors from three different studies to see if 2ME₂ could down-regulate Id-1 expression. Although expression of Id-1 in late-stage tumors was quite heterogeneous in the in vivo studies using 2ME₂, we showed that 2ME₂ does significantly inhibit Id-1 transcription in mouse and human breast cancer cell lines using Id-1 promoter-reporter constructs.

Elevated Id-1 expression is strongly associated with breast cancer and poor prognosis (28, 29). A recent report showed that Id-1 may contribute to both primary breast tumorigenicity and metastatic potential (35). Whether 2ME₂ can reduce Id-1 expression in a subset of human breast tumors will require further study.

It is not clear why cystic tumor formation was not observed in mice treated with 2ME₂ at later stages of tumor development in the intervention studies. Because 2ME₂ was administered at a point when these tumors already reached a stage with some degree of vascularization and were treated for a shorter period of time, large cystic tumors may not have been able to develop. 2ME₂ was able to reduce tumor growth and multiplicity when administered at later stages, primarily through an induction of apoptosis in the tumor cells as discussed above. It is possible that 2ME₂ may, therefore, be useful as a therapeutic agent in combination with other drugs.

![Figure 5.](image-url)
In vitro data from other studies indicate that unlike estradiol, 2ME₂ does not promote proliferation of ER-positive breast cancer cells (8, 9), and that the affinity of 2ME₂ to ER is extremely low (8). Expression of ER and progesterone receptor in mammary tumors did not change by 2ME₂ treatment (data not shown). Unlike a recent study in which increased uterine and liver weights were observed (36), we did not observe increased uterine weight in treated animals compared with controls (data not shown). These results are consistent with other studies that have shown that 2ME₂ does not have an estrogenic effect (8).

In summary, we show that 2ME₂ may inhibit tumorigenesis through different mechanisms, depending upon the stage of cancer development, which has important implications for its use in clinical settings. Although tumor growth at late stages seemed to be reduced by 2ME₂ through an increase in apoptosis, administration of 2ME₂ at very early stages may have a more profound effect on the establishment of angiogenesis, resulting in cystic tumor formation.

This is relevant because 2ME₂ is being evaluated in clinical trials. It is possible that 2ME₂ used in combination with agents that affect other pathways important in cancer growth may lead to additional strategies to prevent tumor progression or the treatment of established tumors.

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