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Report

Experimental down-regulation of intermediate biomarkers of carcinogenesis in mouse mammary epithelial cells

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Summary

The polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) is a metabolism-dependent procarcinogen whose tumorigenicity is modified by dietary and endocrine manipulations *in vivo*. DMBA initiates molecular and cellular alterations in the mammary tissue, while dietary components and estrogens affect the post-initiational phase of tumorigenic transformation. The mechanism(s) responsible for modulation of tumorigenic transformation remain unclear. This study examines the effects of selected tumor suppressing agents and estradiol (E_2) metabolites on *in vitro* DMBA carcinogenesis utilizing a newly established mouse mammary epithelial cell line C57/MG. Alteration in DNA repair synthesis, metabolism of E_2 via the C2- and C16α-hydroxylation pathways, and acquisition of anchorage-independent growth were utilized as molecular, endocrine, and cellular biomarkers to quantitate the cellular transformation by DMBA and its modulation by tumor suppressing agents and E_2 metabolites. A single 24 hr exposure of 0.78 μM DMBA to C57/MG cells resulted in a 193.9% increase in DNA repair synthesis and a 73.1% decrease in C2/C16α hydroxylation of E_2. The DMBA treated C57/MG cells also exhibited increased anchorage-independence *in vitro* prior to tumorigenesis *in vivo*. A simultaneous treatment of cells with DMBA and with the highest non-cytotoxic doses of the tumor suppressing agents 5 μM N-(4-hydroxyphenyl) retinamide (HPR), 50 μM indole-3-carbinol (I3C), or 1 μM tamoxifen (TAM) resulted in a 35.6% to 63.9% decrease in DNA repair synthesis, a 23.8% to 1347.6% increase in C2/C16α hydroxylation of E_2, and a 53.8% to 72.4% decrease in anchorage-independent growth. The E_2 metabolites at the highest non-cytotoxic doses of 0.76 μM estrone (E_1), 0.69 μM 2-hydroxyestrone (2-OHE_1), and 0.66 μM 2-methoxyestrone (2-$MeOHE_1$) suppressed DMBA-induced DNA repair synthesis by 56.0% to 68.8%. These tumor suppressing agents and E_2 metabolites also effectively suppressed post-initiational, anchorage-independent growth by 24.9% to 72.4%. These results indicate that DMBA induces cellular transformation in part by causing DNA damage, altering C2/C16α hydroxylation in favor of C16α-hydroxylation, and inducing anchorage-independent growth prior to tumor development. Effective downregulation of these genotoxic, endocrine and proliferative end points by prototypic tumor suppressing agents and by E_2 metabolites generated via the C2-hydroxylation pathway suggest that these agents may influence mammary tumorigenesis by inhibiting early occurring initiational and/or post initiational events.

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Abbreviations: DMBA – 7,12-dimethylbenz(a)anthracene; HPR – N-(4-hydroxyphenyl) retinamide; I3C – indole-3-carbinol; TAM – tamoxifen; E_2 – 17β-estradiol; E_1 – estrone; 2–OHE_1 – 2-hydroxyestrone; 2-$MeOHE_1$ – 2-methoxyestrone; 16α-OHE_1 – 16α-hydroxyestrone; E_3 – estriol; DME/F12 – Dulbecco's modified Eagle's medium; F12 – Ham's medium; HU – hydroxyurea; PBS – phosphate buffered saline; NaOH – sodium hydroxide; SDS – sodium dodecyl sulfate; TCA – trichloroacetic acid; [C2-^{3}H] E_2 – estradiol labeled at C2 position; [C16α-^{3}H] E_2 – estradiol labeled at C16α position; ANOVA – analysis of variance

Introduction

The type and amount of macro- and micronutrients in the diet have been noted to modulate experimental mammary cancer in rodents. Alterations in the type of fat, amounts of vitamins A, C, or E, or micronutrients zinc and selenium suppress tumor incidence and multiplicity, and prolong the latent period of carcinogen-induced, spontaneous and transplantable tumors in rats and mice [1–4]. Most, if not all of these *in vivo* studies have documented a modulatory influence of diet on tumor progression.

It is well established that the multiphasic cascade of events leading to the development of overt tumor involves early-occurring events of initiation and promotion. These are associated with preneoplastic transformation preceding the appearance of mammary carcinoma [5]. A spectrum of intermediate biomarkers has provided specific and sensitive end points to measure the extent of preneoplastic transformation at the molecular, metabolic, endocrine, and cellular levels. Quantifiable perturbation in these biomarkers has been detected in response to diverse initiators prior to the appearance of overt cancer [6–8]. Experimentally-induced downregulation of perturbed biomarkers should provide specific end points for chemopreventive intervention. Effective inhibition, suppression, and/or retardation of preneoplastic transformation by prototypic tumor suppressing components of the diet may then provide an optimized experimental system in which to evaluate the mechanism(s) of action, as well as the preventive efficacy of various naturally-occurring agents.

The mammary epithelial cell line C57/MG, established from the mammary tissue of the C57BL/6J strain of mouse, has been utilized in a recent study [9] to examine whether selected metabolites of E_2 generated via the C16α-hydroxylation pathway induce cellular transformation. In that study the prototypic mammary carcinogen DMBA was used as a positive control. In the present study spontaneously immortalized, non-tumorigenic C57/MG cells are utilized as an *in vitro* model to examine the direct effects of known tumor suppressing agents and of selected metabolites of E_2 on initiational and post initiational aspects of DMBA carcinogenesis. The specific biomarkers utilized as quantitative end points include: DNA repair synthesis (unscheduled DNA synthesis), an indirect measure for genotoxic DNA damage, as a molecular marker; altered ratio of C2/C16α-hydroxylation of estradiol as an endocrine marker; and acquisition of anchorage-independent growth as a cellular marker.

Materials and methods

C57/MG cell line

This cell line is established from mammary tissue of the 6–8 week old virgin female C57BL/6J strain of mouse [9]. This strain of mouse does not express the murine mammary tumor virus, and consequently has a less than 1% incidence of spontaneous mammary tumors [10]. The C57BL mouse, however, is highly susceptible to chemical carcinogen-induced mammary tumors [11]. Cells were routinely maintained in DME/F-12 (Sigma Chemical Co., St. Louis, MO) supplemented with heat inactivated 10% fetal bovine serum, 4 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, and 5 μg/ml insulin (Eli Lilly, Indianapolis, IN). Cells at passage 20–30 were used for the experiments. The morphological characteristics and morphogenetic potential as observed from *in vitro* and *in vivo* studies as well as the biochemical properties as determined from the relative extents

of *in vitro* E_2 metabolism of C57/MG cells are presented in Table 1.

Chemicals

DMBA, I3C, TAM, and HU were purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-thymidine (specific activity: 40–60 Ci/mmol) was obtained from DuPont NEN (Boston, MA), and HPR was obtained from McNeil Pharmaceutical Co. (Spring House, PA). The E_2 metabolites, E_1, 2-OHE_1 and 2-$MeOHE_1$ were obtained from Steraloids, Wilton, NH. Stock solutions (1000 ×) of DMBA and HPR were prepared in DMSO, and stored in the dark to minimize the photochemical destruction. Stock solutions of I3C, TAM, E_1, 2-OHE_1 and 2-$MeOHE_1$ were made in 100% ethanol. HU was dissolved in DME/F-12 medium. All the stock solutions were appropriately diluted in the culture medium to obtain the final concentrations.

Treatment with DMBA

Approximately 5×10^6 C57/MG cells were plated in a T-75 cm^2 flask. After an initial attachment period of 18–24 hrs, new medium containing 200 ng/ml (0.78 μM) DMBA was added, and the cultures were incubated for 24 hr [9]. Treated cells were washed with PBS, and were used for the experiments on estradiol metabolism without further DMBA treatment.

DNA repair (unscheduled DNA synthesis, UDS)

The extent of DNA damage caused by DMBA, and the effect of tumor suppressing agents and of E_2 metabolites on the genotoxicity of DMBA, was assessed by measuring unscheduled DNA synthesis in the presence of 5 mM HU + 5 uCi/ml ^{3}H-thymidine in all the treatment groups. The control group was treated with 0.78 μM DMBA alone, while the experimental groups were treated with DMBA + the test compound at the maximum non-cytotoxic concentrations. After a 24 hr treatment, the cells were washed twice with cold PBS and lysed in 1 ml of 1 N NaOH + 1% SDS. Two hundred μl aliquots of cellular lysate were taken to precipitate the macromolecules by 10% TCA. HU-insensitive ^{3}H-thymidine uptake was measured by determining the TCA-precipitable ^{3}H radioactivity [9, 12, 13]. The extent of HU-insensitive ^{3}H-thymidine uptake in the solvent treated group was used as the control to quantitate UDS in the DMBA-treated group. The modulation in UDS by the test compounds was evaluated by using UDS in DMBA treated group as the control.

Estrogen metabolism

The alteration in estrogen metabolism due to exposure of the cells to DMBA and to the test compounds was determined by a radiometric assay [9, 14]. The cultures of C57/MG cells were either treated with DMBA alone (control group) or were co-administered DMBA + the individual test compounds at the highest non-cytotoxic concentrations (experimental groups). The extent of C2-hydroxylation and C16α-hydroxylation pathways of estradiol metabolism were measured by ^{3}H exchange

Table 1. Biological characteristics of C57/MG cells

Origin	Morphological and biochemical properties
Mammary tissue from virgin, female C57/BL mouse	*In vitro* morphology: polygonal cells, form epithelial colonies at low seeding density, no growth in anchorage-independent conditions. *In vivo* morphology: form non-hyperplastic ducts in parenchyma-free mammary fat pads. Non-tumorigenic in syngeneic or athymic mice. Estradiol metabolism: high conversion of E_2 to E_1 and to 2-OHE_1. Low conversion of E_2 to 16α-OHE_1. Minimal conversion of androgens to E_2 via aromatization.

from stereospecifically labeled estradiol to form ^3H_2O. The amount of ^3H_2O formed is an indirect measure of conversion of estradiol 2-hydroxyestrone (2-OHE_1) or to 16α-hydroxyestrone (16α-OHE_1) in a stoichiometric manner [15]. Cells were incubated with [C2-^{3}H]E_2 or [C16α-^{3}H]E_2 (5×10^6 dpm, specific activity ~ 20 Ci/mmol), and the test compounds for 48 hrs. Five hundred μl of the medium were diluted to 3.5 ml with water, lyophilized, and the sublimed water was counted in a liquid scintillation counter for ^3H_2O formation. The extent of this metabolism was corrected for the non-specific ^{3}H exchange obtained from incubations without cells.

Anchorage-independent growth

To examine the effect of tumor suppressing agents and E_2 metabolites on the post-initiational stages of DMBA carcinogenesis, DMBA-initiated cells at passage five were used for the anchorage-independent growth assay [9]. Aliquots of 2 ml of 0.6% agar (Agar Nobel, Difco, Detroit, MI) containing the culture medium were plated into each well of a 6 well plate to make the basal layer. Two ml of the culture medium containing 0.33% agar, the test compounds, and 1×10^5 cells were then overlaid in each well. The cultures were incubated for 14 days at 37° C in a humidified atmosphere of 5% CO_2: 95% air, and the number of colonies ≥ 400 μm formed were counted under 10 x magnification. The relevance of anchorage-independent growth *in vitro* to tumorigenesis *in vivo* was ascertained by injecting the DMSO-treated (solvent controls) and DMBA-treated (initiated) C57/MG cells into mammary parenchyma-free fat pads of syngeneic recipients, and examining the outgrowths at the transplant sites 14–26 weeks after transplantation [16].

Statistical analysis

The statistical significance of the data was determined by comparing the relative extent of DNA repair, estradiol metabolism and anchorage-independent growth obtained in C57/MG cells treated with DMBA alone (control) and those treated with the tumor suppressing agents HPR, I3C and TAM. For the experiments measuring DNA repair (n = 5) and estradiol metabolism (n = 12), a two way ANOVA model was applied to the data. Because of the limited number of replicate determinations, it was not informative to assess the interexperimental variation. The experimental factor was therefore treated as one of the two fixed effects in the two way ANOVA model. In addition, a logarithmic transformation was necessary in order to promote normality of data distribution and to stabilize the error variance. An additive two way ANOVA was found to be sufficient to summarize the variation in the log data of either of the two end points. For the experiments measuring anchorage-independent growth (n = 9), two sample t test and Mann-Whitney Rank test were performed within the additive two way model. The control group was then compared with the treatment groups, to determine the statistical significance of differences.

Results

Characterization of C57/MG cells

The spontaneously immortalized C57/MG cell line at passage 20 was evaluated for the persistence of non-transformed phenotype by several morphological, morphogenetic and biochemical criteria (Table 1). These cells were unable to form tridimensional colonies under anchorage-independent conditions of growth. Upon transplantation into syngeneic recipients the cells formed non-hyperplastic ducts in parenchyma free mammary fat pads but did not form tumors. The lack of anchorage-independent growth *in vitro* and of tumorigenicity *in vivo* indicates that this immortalized cell line has not undergone spontaneous transformation.

Modulation of genotoxicity of DMBA

The ability to alter the genotoxic effect of DMBA by the tumor suppressing agents HPR, I3C, and TAM, and by estradiol metabolites E_1, 2-OHE_1, and

Table 2. Effects of tumor suppressing agents on genotoxicity of DMBA

Treatment		HU-insensitive ^{3}H-thymidine uptake[a] (cpm × 10^3/μg DNA)	DNA repair synthesis (% control)[b]
Initiator	Modulator		
None	None	8.3 ± 1.3[c]	–
DMBA	None	24.4 ± 3.6[d]	+ 193.9
DMBA	HPR	10.1 ± 1.2[e]	– 58.6
DMBA	I3C	15.7 ± 5.8[f]	– 35.6
DMBA	TAM	8.8 ± 1.5[g]	– 63.9

[a] Determined after a 24 hr incubation with 5 mM HU + 5 μCi/ml ^{3}H-thymidine.

[b] $\frac{\text{treated-control}}{\text{control}} \times 100$

[c–g] Mean ± SD, n = 5, [c–d,d–e,d–g] $P < 0.0001$, [d–f] n.s.

2-MeOHE$_1$, was evaluated by measuring DNA repair synthesis in C57/MG cultures treated with DMBA + the highest non-cytotoxic doses of individual test compounds. The cultures treated with 0.78 μM DMBA constituted the controls. From the data presented in Table 2 it is clear that exposure to DMBA resulted in a 193.9% increase in DNA repair synthesis relative to that seen in cells treated with the solvent DMSO. The extent of DMBA-induced DNA repair synthesis was suppressed by 58.6% in the presence of 5 μM HPR and by 63.9% in the presence of 1 μM TAM. Treatment with 50 μM I3C, however, was ineffective in the suppression of DNA repair synthesis. All three compounds at the highest non-cytotoxic levels by themselves did not induce DNA repair, and therefore were unlikely to be genotoxic (data not shown).

The effect of estradiol metabolites on DMBA-induced DNA repair synthesis is presented in Table 3. The three metabolites E$_1$, 2-OHE$_1$, and 2-MeOHE$_1$ were found to suppress DNA repair synthesis by 56.0%, 68.8%, and 64.9%, respectively. Similar to the tumor suppressing agents, the three metabolites of estradiol by themselves did not induce DNA repair (data not shown).

Alteration in estradiol metabolism

The relative extent of estradiol metabolism via the C2-hydroxylation and the C16α-hydroxylation pathway in response to treatment with DMBA and with the tumor suppressing agents is shown in Table 4. Exposure of C57/MG cells to DMBA resulted in an increaase in C16α-hydroxylation with a concomitant decrease in C2-hydroxylation of estradiol leading to an altered C2/C16α hydroxylation ratio in favor of C16α-hydroxylation. The C2/C16α hy-

Table 3. Effects of estradiol metabolites on genotoxicity of DMBA

Treatment		HU-insensitive ^{3}H-thymidine uptake[a] (cpm × 10^3/μg DNA)	DNA repair synthesis (% control)[b]
Initiator	Modulator		
None	None	8.0 ± 0.9[c]	–
DMBA	None	28.2 ± 2.7[d]	+ 252.5
DMBA	E$_1$	12.4 ± 2.9[e]	– 56.0
DMBA	2-OHE$_1$	8.8 ± 1.3[f]	– 68.8
DMBA	2-MeOHE$_1$	9.9 ± 3.8[g]	– 64.9

[a] Determined after a 24 hr incubation with 5 mM HU + 5 μci/ml ^{3}H-thymidine.

[b] $\frac{\text{treated-control}}{\text{control}} \times 100$

[c–g] Mean ± SD, n = 5, [c–d,d–e,d–g] $P < 0.0001$, [d–e] $P < 0.001$.

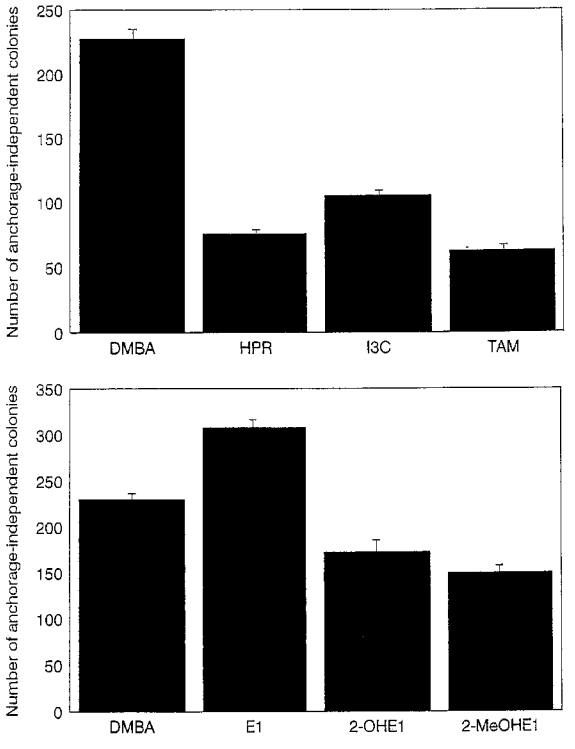

Fig. 1. Anchorage-independent growth assay to examine the effects of tumor suppressing agents and of estradiol metabolites on DMBA treated C57/MG cells. Number of colonies formed from 1.0×10^5 DMBA treated cells maintained in the presence of tumor suppressing agents 5 μM HPR, 50 μM I3C, and 1 μM TAM (1a) and estradiol metabolites 0.73 μM E_1, 0.69 μM 2-OHE_1, and 0.66 μM 2-MeOHE_1 (1b). DMBA versus HPR, I3C, and TAM $P < 0.001$. DMBA versus E_1 showed an increase $P < 0.005$; DMBA versus 2-OHE_1 and DMBA versus 2-MeOHE_1 however, showed a decrease $P < 0.001$.

droxylation ratio in the presence of HPR was substantially increased in favor of C2 hydroxylation. Treatment with TAM, however, failed to alter the ratio.

Alteration in anchorage-independent growth

The effects of tumor suppressing agents and of estradiol metabolites on modulation of DMBA-induced anchorage-independent growth are presented in Figs 1a and 1b. Treatment of DMBA-initiated cells with HPR, I3C, and TAM resulted in a 66.7%, 53.8%, and 72.4% decrease in the number of anchorage-independent colonies, respectively (Fig. 1a). Untreated C57/MG cells or those treated with HPR, I3C, and TAM did not form tridimensional colonies under anchorage-independent conditions of growth (data not shown). From amongst the estradiol metabolites tested for their ability to modulate DMBA-induced anchorage independent growth, treatment with E_1 resulted in a 33.9% increase in the number of tridimensional colonies. In contrast, treatment with 2-OHE_1 and with 2-MeOHE_1 exhibited a 24.9% and a 34.9% decrease in the number of colonies, respectively (Fig. 1b).

The validity of anchorage-independent growth as a cellular end point for tumorigenic transformation was established by determining the tumorigenicity of DMBA treated cells. Injection of DMBA treated cells resulted in a 40% incidence of tumors at the transplant site within 16 weeks. In contrast, injection of DMSO treated cells formed non-hyperplastic epithelial ducts at the transplant site. These recipients did not exhibit any tumors for the entire 24 week duration of the experiment.

Table 4. Alteration in metabolism of estradiol by tumor suppressing agents

Treatment		Estradiol metabolism (%)[a,b]		C2/C16α ratio
Initiator	Modulator	C2-hydroxylation	C16α-hydroxylation	
None	None	0.28 ± 0.08^{c}	0.18 ± 0.08^{d}	1.56
DMBA	None	0.17 ± 0.03^{e}	0.40 ± 0.06^{f}	0.42
DMBA	HPR	1.40 ± 0.04^{g}	0.23 ± 0.02^{h}	6.08
DMBA	I3C	0.68 ± 0.02^{i}	0.19 ± 0.05^{j}	3.58
DMBA	TAM	0.17 ± 0.03^{k}	0.33 ± 0.01^{l}	0.52

[a] Determined after a 48 hr incubation with [C2-^{3}H] E_2 or [C16α-^{3}H] E_2.
[b] Mean ± SD, n = 12. Normalized per 1.0×10^4 cells.
$^{c-e,e-g,g-i}$ $P < 0.0001$; $^{d-f,f-h,f-j}$ $P < 0.0001$; $^{e-k,f-l}$ n.s.

Discussion

Chemical carcinogen-induced rodent mammary tumorigenesis has been an extensively used model to examine the role of diet on cancer progression. The incidence, multiplicity; and latent period of spontaneous or carcinogen-induced mammary tumors is altered by the amount and type of dietary fat [1, 2, 17, 18]. Modifying influence of systemic and humoral factors *in vivo*, however, precludes the examination of effects of individual components of diet directly on the transformation-sensitive mammary epithelium. Thus, mechanism(s) critical for positive and/or negative growth regulation of transformation-sensitive target cells by dietary components remain to be established.

In the present study an epithelial cell culture model of non-tumorigenic mammary epithelial cells derived from the C57 strain of mice is utilized to examine the ability of known tumor suppressing agents and selected metabolites of estradiol to modulate the transforming effect of DMBA. Of the selected test compounds, HPR is a synthetic analogue of Vitamin A, I3C is a naturally occurring component of cruciferous plants, and TAM is a synthetic antiestrogen. These agents are used in the study because of their documented tumor inhibitory effects *in vivo* [1, 4, 19–24]. Of the E_2 metabolites tested, E_1 (generated by C17-oxidation of E_2) is the common precursor for C16α- and C2 hydroxylation pathways. The metabolites generated via the C16α-hydroxylation pathway, 16α-OHE_1 and E_3, possess E_2 agonistic properties [7, 15]. In addition, in our recent study on C57/MG cells, the C16α-hydroxylated metabolites are shown to induce genotoxic damage as well as aberrant growth in anchorage-independent conditions [9]. These *in vitro* results suggest that 16α-OHE_1, and to a lesser extent E_3, may function as initiators for mammary cell transformation. The E_2 metabolites generated via the C2-hydroxylation pathway, on the other hand, possess E_2 antagonistic properties *in vivo* [7, 15]. It is therefore important to examine whether these agents can down-regulate the effect of DMBA on transformation of C57/MG cells.

Our previous *in vitro* studies on mammary epithelial tissue of mouse [9, 25–28] have demonstrated that transfection with oncogenes or treatment with DMBA results in upregulation of C16α/C2 hydroxylation ratio, aberrant hyperproliferation in anchorage-dependent as well as anchorage-independent conditions of growth *in vitro*, and tumorigenicity *in vivo*. Thus, altered metabolism of E_2 and hyperproliferation that precede tumorigenesis may be considered as *in vitro* biomarkers for preneoplastic transformation of mammary epithelial cells. Downregulation of these biomarkers therefore constitutes a measure of preventive efficacy against mammary cell transformation.

It is clear from the data presented in Table 1 that C57/MG cells retain their epithelial and nontumorigenic characteristics as well as estrogen responsiveness. A single 24 hr treatment with DMBA to these cells induces genotoxic damage, elevates C16α-hydroxylation with a concurrent suppression in C2-hydroxylation, and enhances growth in anchorage-independent conditions. These *in vitro* observations are essentially similar to those reported for early passage C57/MG [9], and therefore demonstrate the susceptibility of nontumorigenic C57/MG cells to DMBA-induced transformation.

Perturbation of DNA repair synthesis has been an extensively utilized approach to evaluate genotoxicity of chemicals [13, 29]. The detection of DNA repair synthesis as evidenced by increased levels of HU-insensitive thymidine incorporation in C57/MG cells therefore provides evidence for the susceptibility of nontransformed mammary epithelial cells to an established, genotoxic mammary carcinogen.

In the experiments performed to examine the modulation of DMBA-induced genotoxicity, HPR, I3C and TAM were selected as test compounds on the basis of their documented ability to inhibit mammary tumorigenesis in rodent models [1, 4, 19–24]. Suppression of genotoxicity by these agents should validate the molecular marker as an end point for efficacy of preventive intervention. The highest non-cytotoxic doses of 5 μM HPR and 1 μM TAM were effective in suppressing DMBA-induced DNA repair, while 50 μM I3C was ineffective. This differential effect of the three tumor suppressing agents raises the possibility that the three agents function via distinct mechanisms.

The estrogen metabolites E_1, 2-OHE_1, and 2-Me-OHE_1 were selected on the basis of their known estrogen antagonistic properties [7, 15]. In addition, our previous studies have shown that cellular transformation induced by chemical carcinogen or by oncogene is accompanied by a decrease in the C2-hydroxylation pathway that is essential for the formation 2-OHE_1 and to $MeOHE_1$ [9, 25, 26, 28]. It is noteworthy that all the three metabolites were able to suppress DMBA-induced DNA repair synthesis, thereby inhibiting the genotoxocity of the carcinogen.

The experiments utilizing the endocrine marker, i.e. estradiol metabolism, have clearly demonstrated that HPR and I3C were effective in antagonizing the perturbation of C16α-hydroxylation pathway that is induced by DMBA. Both these agents increased the C2-hydroxylation pathway at the expense of C16α-hydroxylation, which in turn resulted in an increased C2/C16α-hydroxylation ratio. TAM was substantially less effective in this assay. HPR is metabolized via a P450-dependent pathway prior to its receptor binding activity [30, 31], while I3C is noted to activate the synthesis of P4501A1-inducible C2-hydroxylase [22]. Thus, increased microsomal P450 may in part be responsible for the observed inhibition of the endocrine biomarker that is upregulated by DMBA. The biotransformation of estradiol is largely dependent upon oxidative reactions leading to the formation of C2-hydroxylated or C16α-hydroxylated metabolites [7, 15, 22]. Although the exact role of nuclear estrogen receptors in the metabolic conversion of estradiol remains to be elucidated, the presence of receptor appears to be obligatory for the induction of estradiol 2-hydroxylation (H.L. Bradlow, personal communication). On the other hand, the biological effects of the nonsteroidal antiestrogen TAM are in part dependent upon its binding to the nuclear estrogen receptor, rendering it inactive for estrogen-mediated proliferation signal [23, 32–34].

The acquisition of anchorage-independent growth is an *in vitro* marker for post-initiational events in the process of tumorigenic transformation [9, 26–28]. Since this growth property is specific for initiated and/or tumorigenically transformed cells, anchorage-independent growth may represent an *in vitro* cellular marker for transformation that is analogous to the clonal expansion of transformed cells preceding the appearance of tumor *in vivo*. Having demonstrated that the tumor suppressing agents as well as E_2 metabolites generated via C2 hydroxylation inhibit genotoxicity of DMBA, and that tumor suppressing agents specifically upregulate C2 hydroxylation, it was important to examine whether these agents also affect hyperproliferation in anchorage-independent conditions of growth. The results obtained from the anchorage-independent growth assay (Fig. 1a and 1b) clearly demonstrate that the tumor suppressing agents as well as E_2 metabolites generated via the C2-hydroxylation pathway are effective in suppressing the number of anchorage-independent colonies that have been induced by prior exposure to DMBA. It is of interest to note that treatment of DMBA-initiated cells with E_1 enhanced, while that with 2-OHE_1 suppressed, the number of anchorage-independent colonies. E_1 functions as a common precursor for the formation of 16α-OHE_1 and 2-OHE_1 [14, 15, 22, 26, 28, 38]. The enhancement of anchorage-independent growth in the E_1 treatment group may in part be due to formation of 16α-OHE_1. The suppression of anchorage-independent growth in the 2-OHE_1 treatment group may be a manifestation of the antiproliferative effect of the catechol estrogen [28, 39–41].

The induction of neoplastic transformation in C57/MG cells by DMBA, as evidenced by the *in vivo* mammary fat pad transplantation assay, is consistent with the documented mammary tumorigenicity of DMBA both *in vivo* [1, 11, 17, 18], as well as *in vitro* [16, 25, 35–37]. The results obtained by the anchorage-independent growth assay *in vitro* and by the mammary fat pad transplantation assay *in vivo*, taken together indicate that DMBA induces neoplastic transformation in immortalized, non-tumorigenic C57/MG cells. Prior to tumorigenesis *in vivo* the initiated cells exhibit enhanced anchorage-independent growth *in vitro*.

Distinct effects of the individual test compounds on the molecular marker and the endocrine marker, taken together with similar antiproliferative effects on the cellular marker, suggest that observed antiproliferative activity may be the manifestation of

different mechanism(s) of action of individual agents. The underlying mechanism(s), however, need to be elucidated. Enhanced anchorage-independent growth is suppressed by TAM and I3C in oncogene-transformed mouse mammary epithelial cells, as also in human mammary carcinoma-derived MCF-7 cells [28, 38]. Thus, the hyperproliferative activity of the fully transformed tumor cell phenotype represents a useful *in vitro* cellular marker. Results from the present study showing suppression of anchorage-independent growth in DMBA-initiated C57/MG cells by agents that are known to inhibit mammary tumors, indicate that the anti-tumor effect may in part be due to inhibition of preneoplastic transformation.

In conclusion, the present study on spontaneously immortalized but nontumorigenic C57/MG cells has shown that specific molecular, endocrine, and cellular biomarkers perturbed by DMBA can be downregulated by agents that are known to suppress mammary tumorigenesis *in vivo*. This experimental downregulation therefore provides quantitative parameters to assess the chemopreventive efficacy of synthetic and/or naturally occurring compounds.

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