Original Article

In Vitro and *In Vivo* Modulation of Growth Regulation in the Human Breast Cancer Cell Line MCF-7 by Estradiol Metabolites

Akihiko Suto*[1*2], Nitin T. Telang*[3], Hirokazu Tanino*[1], Toshio Takeshita*[2], Harumi Ohmiya*[2], Michael P. Osborne*[3], and Tetsuro Kubota*[1],

Background: The natural estrogen 17β-estradiol (E_2) functions as a potent tumor promoter during tumorigenic transformation of the mammary gland. From amongst the various pathways of E_2 metabolism upregulation of C16α-hydroxylation of E_2 has been associated with carcinogenesis. In the present study *in vitro* and *in vivo* experiments were performed on estrogen receptor positive human breast cancer MCF-7 cells to examine whether the natural estrogen E_2 and its metabolites 16α-hydroxyestrone (16α-OHE_1) and 2-hydroxyestrone (2-OHE_1) function as modulators of tumor cell growth.

Methods: An anchorage-independent growth assay was used for *in vitro* study by counting the number of tri-dimensional colonies formed by MCF-7 cells suspended in 0.33% agar. *In vivo* experiments examined the effect of implanting metabolite material pellets into female nude mice.

Results: In the anchorage-independent growth assay (AIG), continuous 14-day exposure to E_2 and to 16α-OHE_1 at 200 ng/ml induced a 59.4% and a 105.9% increase (P=0.001) respectively in the number of colonies of MCF-7 cells. Identical treatment with 2-OHE_1, however, failed to increase AIG relative to that seen in the solvent treated control cultures. In the *in vivo* tumorigenicity assay, treatment of nude mice with 1.5 mg E_2 or 16α-OHE_1 resulted in a 335.4% and a 384.1% increase ($P<0.0002$) in tumor growth, while identical treatment with 2-OHE_1 failed to exhibit any increase relative to the control group.

Conclusions: These results suggest that the 16α- and 2-hydroxylated metabolites of E_2 may directly affect *in vitro* growth of MCF-7 cells via an autocrine mechanism and *in vivo* growth via paracrine mechanisms. Thus, E_2-mediated growth regulation in MCF-7 cells may in part be due to distinct effects of specific E_2 metabolites on the breast cancer cells.

Breast Cancer 6:87-92, 1999.

Key words: Human breast cancer, Growth regulation, Estradiol metabolites

The natural estrogen 17β-estradiol (E_2) exerts modulatory influence on mammary epithelial cell proliferation, cytodifferentiated function and neoplastic transformation[1-3]. Several steroid and polypeptide hormones as well as mitogenic growth factors are implicated to act in concert with E_2 for its biological effects. These effects are manifested at the molecular level as upregulation of oncogenes and of specific E_2 responsive gene expression[4]. In one of our recent studies on c-Ha-*Ras* oncogene-transfected mouse mammary epithelial cells, it was observed that *Ras*-mediated tumorigenic transformation is accompanied by an increase in E_2 C16α-hydroxylation with a concomitant decrease in E_2 C2-hydroxylation[5]. Similarly, treatment of nontumorigenic mouse mammary epithelial cells with 16α-hydroxyestrone (16α-OHE_1), a product of the C16α-hydroxylation pathway of E_2 metabolism, results in induction of DNA damage and of aberrant hyperproliferation *in vitro*[6]. These *in vitro* studies suggest that 16α-OHE_1 may function as an initiator and/or promoter of mammary carcinogenesis. However, the mechanisms responsible for the direct effects of various metabolites of E_2 on fully transformed tumor cells are not fully understood. An *in vitro* model may allow for examination of the direct role of E_2 and its metabolites on tumor promotion and progression, to gain a better understanding of the role of E_2 in mam-

*[1]Department of Surgery, Keio University School of Medicine, *[2]Department of Surgery, Yamato Municipal Hospital, and *[3]Division of Carcinogenesis & Prevention, Strang-Cornell Cancer Research Laboratory, Cornell University Medical College, New York, USA.
Reprint requests to Akihiko Suto, Department of Surgery, Yamato Municipal Hospital, 8-3-6 Fukami-nishi, Yamato 242-8602, Japan.

Abbreviations:
AIG, Anchorage-independent growth; E_1, Estrone; E_2, 17β-Estradiol; 2-OHE_1, 2-Hydroxyestrone; 16α-OHE_1, 16α-Hydroxyestrone; TGF, Transforming growth factor

Received October 19, 1998; accepted January 28, 1999

mary tumorigenesis.

It is known that E_2 is initially converted to estrone (E_1) by 17β-hydroxysteroid dehydrogenase. E_1 functions as a common precursor that is subsequently converted via p450-dependent, mutually competitive hydroxylation reactions to either 16α-OHE_1 or 2-hydroxyestrone (2-OHE_1). These two hydroxylated metabolites function *in vivo* as an estrogen agonist and antagonist *in vivo* respectively[7-9].

To examine whether the biological properties of the hydroxylated metabolites of E_2 are manifested at the cellular level as positive or negative growth factors, the present experiments were conducted on the well-characterized, estrogen receptor positive MCF-7 cell line, derived from human mammary carcinoma. The *in vitro* experiments measured the extent of modulation of anchorage-independent growth (AIG) of MCF-7 cells treated with E_2, 16α-OHE_1 or 2-OHE_1. The *in vivo* experiments measured the modulation of tumor growth in the presence of E_2 and/or its metabolite after xenotransplantation of MCF-7 cells into athymic nude mice.

Materials and Methods

In Vitro Experiments

Estrogen receptor positive MCF-7 cells, originally obtained from the Michigan Cancer Foundation (Detroit, MI, USA) were used in these experiments. The cells were maintained in Eagle's minimum essential medium containing 7% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml non-essential amino acids, 100 μg/ml sodium pyruvate, 50 μg/ml gentamycin and 0.4 μg/ml insulin (Eli Lilly, Indianapolis, IN, USA). This medium also contained 8.63 mg/l phenol red as the pH indicator.

Estradiol Metabolites: E_2 was obtained from Sigma Chemical Co, (St Louis, MO, USA). The metabolites 16α-OHE_1, and 2-OHE_1 were obtained from Steraloids (Wilton, NH, USA). The stock solutions (1000×) of E_2, 16α-OHE_1, and 2-OHE_1 were made up in 100% ethanol, and diluted with the culture medium to obtain a final concentration of 200 ng/ml. The culture medium concentration used in this *in vitro* experiment was 200 ng/ml, which is non-cytotoxic for a 14-day study. We have already assessed concentrations of 2, 20, 200 and 2000 ng/ml of estrogen metabolites for the AIG assay, and demonstrated poor colony forming ability at concentrations of 2 and 20 ng/ml, and that concentrations of 200 and 2000 ng/ml of the metabolites showed similar colony forming efficiency without any cytotoxicity. We therefore chose to use a concentration of 200 ng/ml for this study.

Anchorage-Independent Growth Assay: This assay was used to measure the relative extent of AIG as determined by the number of anchorage-independent, tri-dimensional colonies formed by MCF-7 cells suspended in 0.33% agar. Briefly, 2 ml culture solution containing 1.0×10^3 MCF-7 cells, 200 ng/ml of E_2, 16α-OHE_1 or 2-OHE_1 (the previously determined nontoxic dose) and 0.33% agar were obtained on a top of 2 ml matrix of 0.6% agar medium using six-well plates. The cultures were incubated in a humidified atmosphere of 5% CO_2: and 95% room air at 37℃ for 14 days. The cultures were fixed in 10% buffered formalin, stained with Giemsa, and the number of tri-dimensional, anchorage-independent colonies (>400 μm) were counted under 10× magnification.

The data was expressed as % colony forming efficiency (%CFE).

In Vivo Experiments

Mice: Female nude mice with the BALB/c genotype were purchased from CLEA Japan Inc, Tokyo, Japan. Mice were maintained under specific pathogen-free conditions using an Isorack, and were fed on sterile food and water *ad libium*. Six- to eight-week old mice weighing approximately 20 g were used for the experiments.

Tumor Xenografts: The MCF-7 cell line, provided by Dr Y. Nomura (National Kyushu Cancer Center, Japan) and successfully propagated in nude mice in our laboratory was used for the *in vivo* experiments. The stocks of MCF-7 xenografts were routinely maintained by subcutaneous transplantation of approximately 3×3×3 mm tumor fragments into athymic nude mice implanted with a 21-day sustained-release E_2 pellet as previously described[10].

Estradiol Metabolites: The 21-day sustained release pellets containing 1.5 mg E_2, 1.5 mg 16α-OHE_1 or 1.5 mg 2-OHE_1 (Innovative Research of America, Toledo, OH, USA) were implanted subcutaneously on the day of tumor inoculation. The steroid concentration used for this *in vivo* experiment, 1.5 mg per 21-day release pellet was considered to be the minimum exogenous dose of estradiol necessary for MCF-7 tumor growth.

One tumor tissue fragment (3×3×3 mm) in size was subcutaneously transplanted into the dorsum of ether-anesthetized nude mice using a trocar needle. Two fragments per mouse were inoculated separately into the dorsum to form two tumors. The tumors were measured (length and width) with sliding calipers three times weekly by the same observer, and the tumor weight was calculated from the linear measurements[11], using the formula[11]:

tumor weight (mg) = length (mm) × [width (mm)]²/2.

Four tumor-bearing mice from each group were used in the *in vivo* experiment. The growth curves were generated by plotting the mean tumor weight (mg) against the treatment duration (days). Mice were sacrificed on day 21 after tumor inoculation, and the effect of metabolites on tumor growth was evaluated by calculating a percent value with the numerator T as the actual tumor weight in the treated group and the denominator C as the actual tumor weight in the control group.

The statistical analysis was performed using Student's *t*-test.

Results

In Vitro Effects of E_2 Metabolites

The relative extent of growth regulation of MCF-7 cells by 16α-OHE_1 and 2-OHE_1 was examined using the *in vitro* AIG assay. CFE represented the quantitative end point. The data presented in Table 1 demonstrates that a continuous 14 day treatment of MCF-7 cells with 200 ng/ml E_2 or 16α-OHE_1 resulted in a 59.7% and a 106% increase (P=0.001) respectively in the CFE relative to the control group. In contrast, identical treatment with 2-OHE_1 failed to induce an in-

Table 1. Effect of 17β-Estradiol and Its Metabolites on Anchorade-Independent Growth of Human Mammary Carcinoma MCF-7 Cells

Agent	Concentration	Anchorage-independent colony forming efficiency (%CFE)[a]	Relative change (% of control)[b]
Ethanol (solvent control)	0.1%	14.9±2.1[c]	—
E_2	200 ng/ml	23.8±3.2[d]	+59.7
16α-OHE_1	200 ng/ml	30.8±3.2[e]	+106.7
2-OHE_1	200 ng/ml	14.1±2.7[f]	−5.4

[a](Number of colonies/initial seeding density)×100 (%).
[b][(Treated−control)/control]×100 (%).
[c-f]Mean±SD, n=12.
c-d, c-e, P=0.001; c-f, n.s.

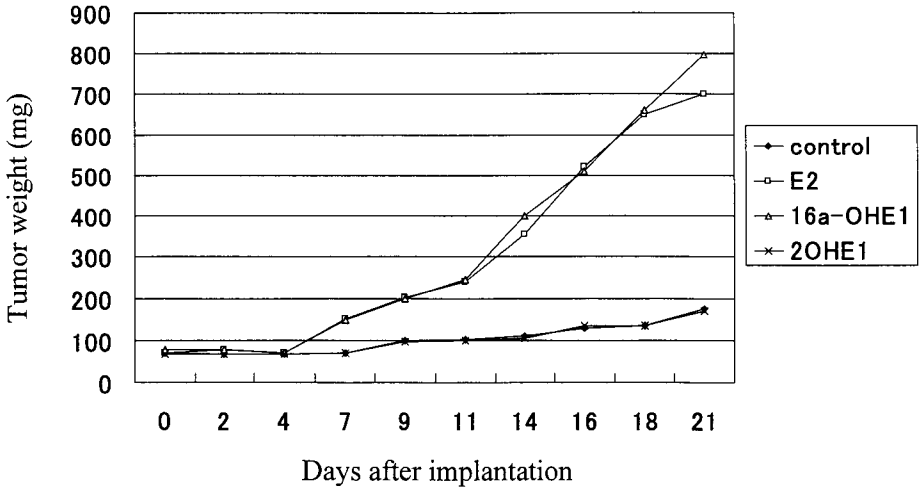

Fig 1. Effect of 17β-estradiol (E_2) and its metabolites on growth kinetics of tumors derived from xenotransplanted MCF-7 cells.

Table 2. Effect of 17β-Estradiol and Its Metabolites on the Growth of Xenotransplanted Human Mammary Carcinoma MCF-7 Cells

Treatment[a]	Tumor weight (mg) at day 21 post transplantation[b]	T/C (%)[c]
None	164±29[d]	100.0
E_2	714±225[e]	435.0
16α-OHE_1	794±124[f]	485.0
2-OHE_1	153±35[g]	93.2

[a]Subcutaneous implantation of sustained release pellet (21 days) containing 1.5 mg of the test compound.
[b]Tumor weight (mg)=[length (mm) × (width (mm)]^2]^2/2.
[c]T/C (%)=[tumor weight (treated group)/tumor weight (control group)]×100.
[d-g]Mean±SEM.
d-e, P=0.0002; d-f, P=0.0001; d-g, P=0.5 (n.s.).

crease in CFE.

In Vivo Effects of E_2 Metabolites

The growth kinetics of MCF-7 xenotransplants is presented in Fig 1. Subcutaneous implantation of 1.5 mg E_2 or of 1.5 mg 16α-OHE_1 resulted in substantial growth stimulation while implantation of 1.5 mg 2-OHE_1 demonstrated no detectable change in the growth of MCF-7 xenotransplants relative to the control group. The *in vivo* growth modulation of MCF-7-derived tumors was also assessed by determining the % T/C values in nude mice recipients implanted with 21-day sustained release pellets containing E_2, 16α-OHE_1 or 2-OHE_1 (Table 2). In the recipients receiving pellets containing E_2 or 16α-OHE_1 the T/C values increased by a factor of 4.35 (P=0.0002) and 4.85 (P=0.0001) respectively. In the recipients receiving 2-OHE_1, however, the T/C values were not significantly different from those of the control set (P=0.5). The total body weight gain of the recipients from the three treatment groups differed by less than 5% of that of the control group, indicating the lack of treatment-related toxicity.

Discussion

Estradiol is metabolized by 17α-oxidation to estrone (E_1) which is then hydroxylated by two competing pathways, 2-hydroxylation and 16α-hydroxylation, leading to the formation of 2-OHE_1 and 16α-OHE_1 respectively. Animal studies have shown that 16α-hydroxylation pathway elevation in mouse strains corresponds with their mammary cancer risk from the exogenously transmitted murine mammary tumor virus (MMTV)[7,8]. Similarly, in clinical investigations, increased 16α-hydroxylation of E_2 is observed in women with breast cancer, or in women at high risk for breast cancer[8,9]. Our previous studies on mammary explant cultures have shown that treatment of the cultures with chemical carcinogens upregulated the constitutive levels of E_2 16α-hydroxylation with a concomitant suppression of E_2 C2-hydroxylation in the epithelial component, the target site for mammary tumorigenesis[12-15].

Furthermore, 16α-OHE_1, the product of E_2 C16α-hydroxylation, functions as an initiator and promoter of preneoplastic transformation in non-tumorigenic C57/MG cells, while 2-OHE_1, the product of E_2 C2-hydroxylation, inhibits carcinogen-induced genotoxicity and aberrant hyperproliferation[6,15,16].

Zhu *et al* recently reviewed the catechol-*O*-methyltransferase pathway and its metabolite 2-methoxyestradiol, and suggested that it exerts a protective effect on estrogen-induced cancer cells[17]. Taken together these observations provide evidence that mammary carcinogenesis is modulated by various E_2 metabolites.

The experiments in the present study were conducted on MCF-7 cells to examine the effects of E_2 and its metabolites and focused on biotransformation of E_2 and its effects on regulation of tumor growth and cell growth. The results of the *in vitro* experiments demonstrate a clear positive growth regulation by E_2 and 16α-OHE_1 but not by 2-OHE_1, as evidenced by increased AIG by the parent compound and the 16α-hydroxylated metabolite but not by the 2-hydroxylated metabolite. Since MCF-7 cell cultures were exposed directly to the test compounds, it is possible that modulation of AIG is a manifestation of an autocrine cellular effect. Consistent with our AIG results, a similar modulation of anchorage-dependent growth of MCF-7 cells has been demonstrated by E_2, 16α-OHE_1 and 2-OHE_1 [18]. The mechanism(s) responsible for the observed growth regulation are not fully understood. The cellular effects of these agents may be due to their influence on c-*fos* and c-*jun* or c-*myc* expression, which are recognized molecular targets for early mitogenic signal transduction[19-21]. Alternatively, E_2 and/or its metabolites may indirectly affect growth regulation via elaboration of such peptide growth factors as transforming growth factors α or β (TGF-α or TGF-β) that function as positive or negative growth regulators, respectively. In this context it

is noteworthy that E_2 induces secretion of TGF-α, while the synthetic antiestrogen tamoxifen as well as 2-OHE_1 induce TGF-β secretion[22], and that the growth of c-*myc* transfected mammary epithelial cells is positively regulated by TGF-α and negatively regulated by TGF-β[23].

It is interesting to note that modulation of AIG *in vitro* strongly correlated with a similar modulation of tumor growth after xenotransplantation *in vivo*. Unlike *in vitro* experiments, xenotransplanted MCF-7 cells are subject to paracrine regulatory effects exerted by surrounding tissue as well as autocrine regulatory effects from *in vivo* MCF-7 cells. Koh *et al* reported the existence of aromatase activity in fibroblast cells in breast cancer xenograft tissue, suggesting an *in vivo* paracrine regulatory system[24]. It is therefore conceivable that growth regulation of xenotransplanted MCF-7 by E_2 and its metabolites may be a manifestation of combined autocrine and paracrine effects. The binding affinity of estrogen receptor plays a critical role in the biological potency of agonists or antagonists that potentiate or inhibit the effect of natural estrogen E_2 on the target cell. In the present study, differential growth regulation by E_2 metabolites both *in vitro* as well as *in vivo* may also in part be due to the differential receptor binding ability of the two metabolites, as has been reported in other studies[25-27]. Additional definitive experiments focusing on each of the possible mechanisms discussed above should further elucidate the role of individual E_2 metabolites in estrogen-mediated growth regulation and mammary carcinogenesis.

In conclusion, the results from the present study suggest that *in vitro* and *in vivo* growth modulation of estrogen receptor positive MCF-7 may represent a useful cellular marker for potential modulators of estrogen-responsive cell proliferation.

Acknowledgments

The authors acknowledge the expert technical assistance by Milan Zvanovec for the in *vitro* experiments and excellent editorial assistance by Lana Winter. This study was supported in part by the NIH Grant #P01 CA 29502, the Department of Defense Grant #DAMD-17-94-J-4208, and by the philanthropic support to the Strang Cancer Prevention Center.

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