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Alteration of oestradiol metabolism in *myc* oncogene-transfected mouse mammary epithelial cells

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Summary Targeted overexpression of the c-*myc* oncogene induces neoplastic transformation in immortalized, non-tumorigenic mouse mammary epithelial cells (MMEC). Experiments in the present study were conducted to examine whether cellular transformation induced by c-*myc* oncogene is associated with altered metabolism of 17β-oestradiol (E_2). The parental, MMEC and the stable c-*myc* transfectant (MMEC/myc_3) cell lines were compared for major oestrogen metabolic pathways, namely E_2 and E_1 interconversion, and C2- and C16α-hydroxylation by both high-pressure liquid chromatography (HPLC) analysis and the ^3H release assay using specifically labelled [C2-^{3}H]E_2 or [C16α-^{3}H]E_2. The reductive conversion of E_1 to E_2 was about 14-fold and 12-fold higher than the oxidative conversion of E_2 to E_1 in MMEC and MMEC/myc_3 cells respectively. However, in MMEC/myc_3 cells, both reductive and oxidative reactions were decreased by about 32% and 12% relative to those seen in the parental MMEC cells ($P = 0.0028$). The extent of C16α-hydroxylation was increased by 164.3% ($P < 0.001$), with a concomitant 48.4% decrease ($P < 0.001$) in C2-hydroxylation in MMEC/myc_3 cells; this resulted in a fourfold increase in the C16α/C2 hydroxylation ratio in this cell line. Thus, a persistent c-*myc* expression, leading to aberrant hyperproliferation in vitro and tumorigenesis in vivo, is associated with an altered oestrogen metabolism. However, it remains unclear whether this represents a result of oncogene expression/activation or is rather a consequence of phenotypic transformation of the cells.

Keywords: c-*myc* expression; oestradiol metabolism; mammary carcinogenesis

It is well recognized that oestrogens exert a profound influence on mammary epithelial cell growth, differentiation and neoplastic transformation (Fishman et al, 1980; Prudhomme et al, 1984; Mauvais-Jarvis et al, 1986; Siiteri et al, 1986). The molecular and biochemical mechanisms important for oestrogen responsiveness and the influence of altered oestrogen responsiveness on mammary cell carcinogenesis, however, are not fully understood. Our earlier studies on immortalized, non-tumorigenic mouse mammary epithelial cell lines have shown that transfection of the cell line with *myc* or *Ras* oncogenes results in neoplastic transformation. Before tumorigenesis in vivo, *myc* as well as *Ras* transfectants exhibit aberrant hyperproliferation in vitro (Telang et al, 1990, 1991; Suto et al, 1992). Thus, persistent oncogene expression and aberrant hyperproliferation may represent molecular and cellular biomarkers for neoplastic transformation.

The conventionally recognized markers for oestrogen responsiveness include (1) functional activity of oestrogen receptor as determined by receptor–ligand binding; (2) modulation of transcriptional activity, growth and induction of progesterone receptor (Prudhomme et al, 1984; Mauvais-Jarvis et al, 1986; Siiteri et al, 1986; Dubik and Shiu, 1992); (3) reversible suppression of growth by hormone antagonists (Clark et al, 1977; Mauvais-Jarvis et al, 1986; Siiteri et al, 1986; Dubik and Shiu, 1992). Our recent studies on *Ras*-initiated MMEC/pH06T cells as well as on *Ras*-transformed T1/Prl cells have shown that the oncogene-initiated and tumorigenically transformed cells are responsive to E_2, as also shown by their ability to metabolize the hormone and by reversible growth inhibition upon treatment with the non-steroidal anti-oestrogen tamoxifen (Telang et al, 1991; Suto et al, 1992).

Mammary epithelial cells initiated independently with chemical carcinogen 7,12-dimethylbenz(a) anthracene (DMBA) and *Ras* oncogene exhibit elevated oestrogen metabolism via the formation of C16α-hydroxylated metabolites (Telang et al, 1991, 1992; Suto et al, 1993). In addition, it has been proposed that the oestrogen-mediated stimulation of growth of breast tumour-derived MCF7 cells may involve transactivation in the c-*myc* promoter region (Dubik and Shiu, 1992). It is not clear whether these molecular and metabolic alterations characterize the initiated phenotype or represent a late-occurring, post-initiational event in a rapidly growing tumour cell phenotype.

The experiments in the present study were designed to (1) establish the validity of oestrogen metabolism as an endocrine biomarker for tumorigenic transformation in *myc* oncogene-transfected mammary epithelial cells; and (2) elucidate the relationship between *myc* expression, the extent of cellular metabolism of E_2 and tumorigenic transformation. To this end, we have compared the extent of E_2 metabolism in the spontaneously immortalized, non-tumorigenic mammary epithelial cell line MMEC and the stable transfectant MMEC/myc_3 that expresses activated c-*myc* proto-oncogene and is highly tumorigenic.

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MATERIALS AND METHODS

Cell lines

The non-tumorigenic mammary epithelial cell line MMEC was established from the mammary tissue of a 6- to 8-week-old virgin female BALB/c mouse. The stable *myc* transfectant MMEC/myc_3 was obtained by transfection of a recombinant *myc* construct comprising the second and third coding exons expressed from a MLV-LTR promoter in a NEO-derived vector. DM-*myc*, and expansion of a stable G-418-resistant clone in the presence of 400 μg ml^{-1} G-418, which is cytotoxic to the parental MMEC (Telang et al, 1990). Routinely, MMEC and MMEC/myc_3 cells were maintained in DME/F12 medium supplemented with heat-inactivated 10% fetal bovine serum, 4 mM L-glutamine and 5 μg ml^{-1} insulin. The stock MMEC/myc_3 cells were maintained in the presence of 400 μg ml^{-1} G-418 to eliminate the accumulation of spontaneous revertants. For the experiments measuring the cellular metabolism of E_2, the parental MMEC and the *myc* transfectant MMEC/myc_3 cells were cultured in the absence of G-418 for 72 h to exclude the possibility of interference of the antibiotic with E_2 metabolism.

Growth characteristics

The growth pattern of MMEC and MMEC/myc_3 cells was determined by a trypan blue exclusion test and haemocytometer counts for viability and growth. In addition, population doubling time (PDT), anchorage-independent growth (AIG) and mammary fat pad tumorigenicity assays were performed according to the published procedures (Ganguly et al, 1982; Telang et al, 1979, 1990, 1991). PDT was determined from the linear portions of the growth curves generated for at least 4 days after plating 5×10^3 cells cm^{-2}. AIG was evaluated by determining the number of anchorage-independent, tridimensional colonies formed in 0.33% agar after an initial seeding of 1.0×10^3 cells, and the data were expressed as colony-forming efficiency (CFE, %) at day 14. Tumorigenicity was determined by counting the number of palpable tumours in mammary fat pads after the injection of 1.0×10^5 cells as a single 20-μl bolus into parenchyma-free mammary fat pads of syngeneic recipients.

c-*myc* expression and oestrogen receptor content

The relative expression of transfected (exogenous) c-*myc* oncogene was determined by the Northern blot analysis of RNA from the parental MMEC and the stable transfectant MMEC/myc_3 cell lines essentially according to the method published previously (Telang et al, 1990, 1991). A [^{32}P]-labelled, nick-translated 1.8 kb *Sac*I fragment of human c-*myc* spanning the second exon was used as the probe. The blots were scanned and the hybridization signal was quantified by arbitrary scanning units (ASU) normalized to 20 μg of RNA loaded. The oestrogen receptor content of MMEC and MMEC/myc_3 cells was determined by the ligand binding assay (Castagnetta et al, 1992) and was expressed as fmol of oestrogen receptor protein (ERP) per 10 μg of DNA.

Table 1 Biomarker status of MMEC and MMEC/myc_3 cells

	Cell line	
Type of biomarker	**MMEC**	**MMEC/myc_3**
G418 resistance[a]	–	+
C-myc expression[b]	–	15.0 ± 2.6
Oestrogen receptor[c]	12.5 ± 3.9	5.0 ± 1.7
Population doubling	24.3 ± 0.5	18.3 ± 0.1
Anchorage independence[d]	0.01 ± 0.005	1.33 ± 0.075
Tumorigenicity[e]	–	+

[a]Growth in 400 μg ml^{-1} G418.[b]Arbitrary scanning units for 2.8 kb (exogenous) transcript hybridizing to [^{32}P]-labelled c-*myc* probe.[c] Fmol ER protein per 10 μg DNA.[d]percentage colony forming efficiency in 0.33% agar. [e]Tumour formation after mammary fat pad transplantation.

Table 2 Effect of c-*myc* oncogene expression in 17β-hydroxy steroid dehydrogenase (17β-HSD) activity in mammary epithelial cells

	17β-HSD activity[a]	
Cell line	**E_2 formed**	**E_1 formed**
	(*n* = 12)	(*n* = 11)
MMEC	22.14 ± 2.72	1.47 ± 0.13
MMEC/myc_3	15.14 ± 3.39	1.30 ± 0.13
	d.f. 11	d.f.10
	t 4.16	*t* 3.40
	P = 0.0028[b]	*P* = 0.05[b]

[a]Determined after 24 h incubation with [6,7-^{3}H(N)]E_1 or [6,7-^{3}H(N)]E_2 and HPLC analysis of conversion products. [b]Two-tailed Student *t*-test. Values represent mean ± s.d. pmol 10 μg^{-1} DNA

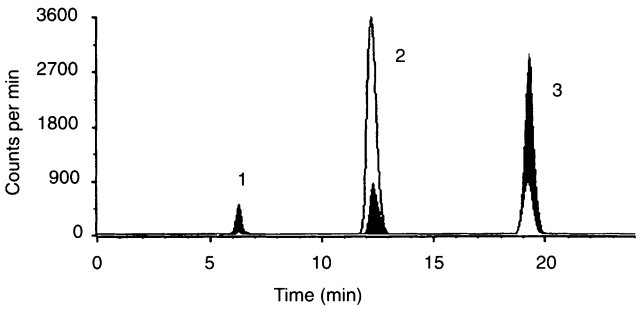
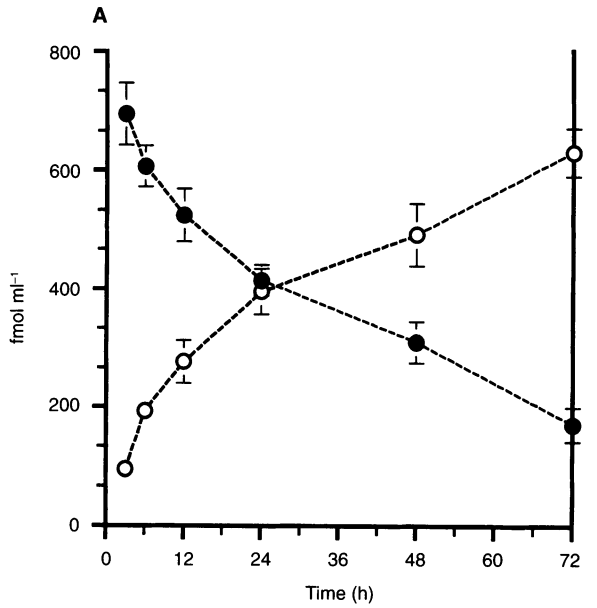


Figure 1 Typical high-pressure liquid chromatography (HPLC) profiles of 1 nM [6,7-^{3}H(N)]E_1 metabolites formed after 72 h incubation of 5×10^5 MMEC (white peaks) and MMEC myc_3 (dark peaks) cells. (1) 16αOHE_1; (2) E_2; (3) E_1

HPLC analysis of 17β-HSD activity

The relative extent of 17β-HSD activity was determined by measuring the interconversion of E_2 and E_1 in the two cell lines. T-25 flasks containing approximately 1.0×10^6 MMEC and MMEC/myc_3 cells were incubated for 24 h in serum-free, phenol red-free and G-418-free medium in the presence of 2 μCi ml^{-1} [6,7-^{3}H(N)]E_2 (specific activity 42.3 Ci $mmol^{-1}$, final concentration 4.6×10^{-8} M) or 2 μCi ml^{-1} [6,7-^{3}H(N)]E_1 (specific activity 41.9 Ci $mmol^{-1}$, final concentration 4.8×10^{-8} M). The incubation medium was collected, and 1-ml aliquots were extracted with 9:1 ethyl-ether:acetone. The extracts were analysed by reverse-phase HPLC (C18 column, 4.6 i.d. × 250 mm) under isocratic conditions (acetonitrile: 0.05 M citric acid, 40:60) at a flow rate of 1 ml min^{-1} using a computer-aided optimized mobile phase (D'Agostino et al, 1985; Castagnetta et al, 1986). The detection of E_2 metabolites was carried out using a UV detector and a three-channel radiometric detector, both on-line to HPLC as described previously



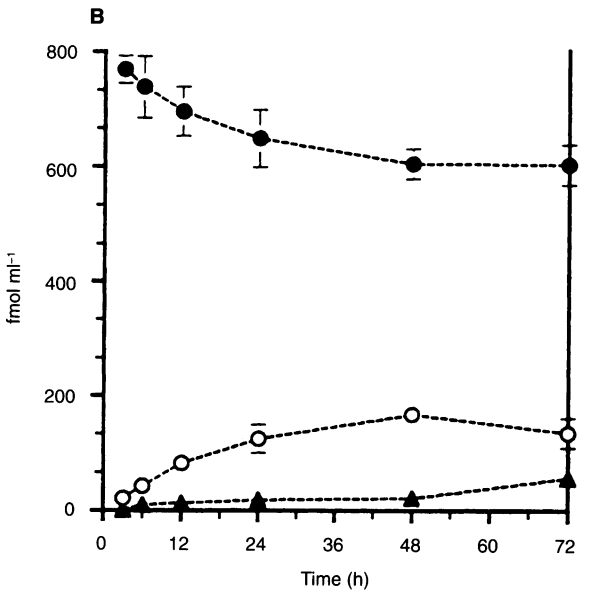


Figure 2 Time course of E_1 metabolism in MMEC (**A**) and MMEC/*myc*$_3$ (**B**) cells. Cells (5×10^5) were incubated in the presence of 1 nM [6,7-^{3}H(N)]E_1 for 3, 6, 12, 24, 48 and 72 h. Each data point represents the mean ± s.d. of duplicate experiments, performed in triplicate, after correction for equal cell numbers. (●) E_1; (○) E_2; (▲) 16αOHE_1

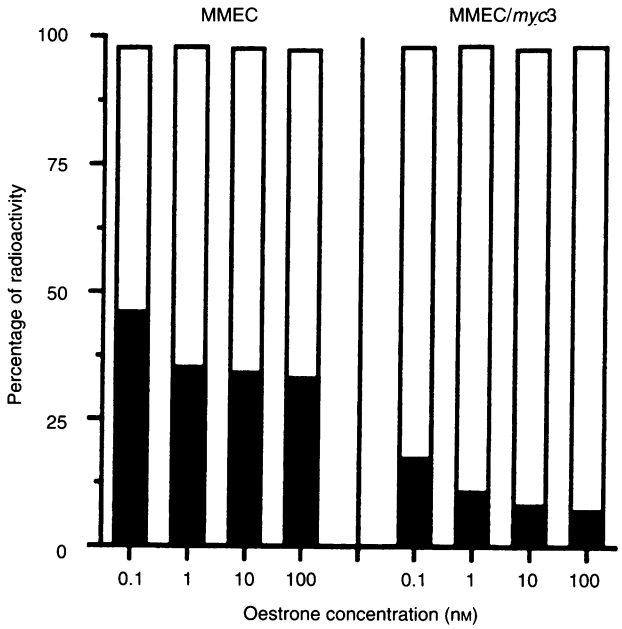


Figure 3 Dose-dependent E_1 conversion to E_2 in MMEC and MMEC/*myc*$_3$ cells. Cells were incubated for 24 h in the presence of increasing concentrations of [6,7-^{3}H(N)]E_1. Percentage values represent the mean of triplicate determinations corrected for total radioactivity and cell numbers. (□) unconverted E_1; (■) E_2 formed

(Castagnetta et al, 1986, 1991). The cells were lysed in 0.1% sodium dodecyl sulphate (SDS), and DNA content was determined (Carruba et al, 1994). The resulting data were normalized for total radioactivity and expressed as pmol 10 μg^{-1} cellular DNA or fmol ml^{-1} after correction for equal cell numbers.

Separate experiments were carried out to inspect the time and dose dependence of oestrogen metabolism in both MMEC and MMEC/*myc*$_3$ cells. To this end, 5×10^5 cells were incubated in the presence of 1 nM tritiated E_1 for 3, 6, 12, 24, 48 and 72 h or exposed to increasing concentrations (0.1, 1, 10 and 100 nM) of the same radioactive oestrogen for 24 h, using exactly the same experimental conditions and procedures described above.

Radiometric assay for E_2 metabolism

The relative extent of E_2 metabolism via the C2- and C16α-hydroxylation pathways was measured by determining ^3H_2O formation in cells incubated for 48 h in the presence of [C2-^{3}H]E_2 or [C16α-^{3}H]E_2 (final concentrations 5.6×10^4 d.p.m., 8.0×10^{-10} M) in a medium lacking serum, phenol red and G-418. Aliquots of 500 μl of incubation medium were diluted to 3.5 ml with water, and the lyophilized sublimate was counted for ^{3}H radioactivity in a liquid scintillation counter (Telang et al, 1991, 1992; Suto et al, 1992, 1993). The ^{3}H release from [C2-^{3}H]E_2 or [C16α-^{3}H]E_2 to form ^3H_2O provides an indirect measurement of regiospecific hydroxylation of the steroid leading to the stoichiometric formation of 2-hydroxyestrone (2-OHE_1) or 16α-hydroxyestrone (16α-OHE_1) (Fishman and Martucci, 1980; Fishman et al, 1980, 1995; Telang et al, 1991, 1992; Suto et al, 1992, 1993; Telang, 1996).

Statistical analysis

The data were analysed for statistical significance of the differences between cell types and treatment groups by unpaired two-tailed Student *t*-test, using the Statview 4.01 statistical software. Probability values of less than 0.05 were considered significant.

RESULTS

Growth characteristics of MMEC and MMEC/*myc*$_3$ cells

The proliferative status, including AIG and tumorigenic potential, of MMEC and MMEC/*myc*$_3$ cells, is presented in Table 1. The MMEC cell line exhibited toxicity to the aminoglycoside antibiotic

Table 3 Oncogene-mediated alteration of 17-β-oestradiol (E_2) metabolism in mouse mammary epithelial cells

Cell line	E_2 metabolism[a]		
	2-OHE_1 formed ($n = 12$)	16α-OHE_1 formed ($n = 12$)	C16α/C2 ratio
MMEC	44.29 ± 5.71	20.00 ± 2.86	0.45
MMEC/myc_3	22.86 ± 2.86	52.86 ± 7.14	2.31
	d.f.11	d.f.11	
	t 4.23	t 5.25	
	$P < 0.001$[b]	$P < 0.001$[b]	

[a]Determined by ^3H_2O formed after a 48 h incubation with [C2-^{3}H]E_2 or [C16α-^{3}H]E_2. [b]Two-tailed Student t-test; values are mean ± s.d. fmol 10 μg^{-1} DNA.

G-418, lacked the expression of exogenous *myc*-specific 2.8 kb RNA transcript, exhibited a population doubling time of 24.3 ± 0.5 h, lacked anchorage-independent growth in vitro and lacked the ability to form tumours when transplanted into syngeneic recipients. These cells, however, exhibited a persistent ability for ductal morphogenesis at the transplant site (data not shown). In contrast, the MMEC/myc_3 cell line did not exhibit any G-418 cytotoxicity, expressed the exogenous 2.8 kb transcript (15.0 ± 2.6 ASU 20 μg^{-1} RNA) and showed a shorter population doubling time of 18.3 ± 0.1 h (d.f. 5, t = 3.40, P = 0.01). Furthermore, MMEC/myc_3 cells also showed a 132-fold increase in AIG relative to that observed in MMEC and were highly tumorigenic, exhibiting a 90% tumour incidence at 12 weeks after transplantation (data not shown). These results essentially confirm our earlier report (Telang et al, 1990), suggesting that the expression of activated c-*myc* confers neoplastic transformation to mammary epithelial cells. In addition to overexpression of exogenous c-*myc*, the MMEC/myc_3 cells exhibited a substantial reduction in oestrogen receptor levels. Thus, whereas the oestrogen receptor content of parental MMEC was 12.5 ± 3.9 fmol 10 μg^{-1} DNA, it was decreased to 5.7 ± 1.7 fmol 10 μg^{-1} DNA (d.f. 5, t = 4.07, P = 0.001) in the transfected MMEC/myc_3 cells, resulting in about a 60% reduction of ERP levels.

The experiment designed to establish the biological significance of altered E_2 metabolism examined whether treatment of MMEC/myc_3 cells with oestrogen metabolites 16α-OHE_1 or 2-OHE_1 affects aberrant proliferation as shown by anchorage-independent growth. Treatment of MMEC/myc_3 cells with 16α-OHE_1 resulted in about a 152% increase (d.f. 11, t = 4.07, P = 0.002) in anchorage-independent colony formation. In contrast, treatment with 2-OHE_1 resulted in a 12.6% decrease (d.f. 11, t = 3.41, P = 0.01) in anchorage-independent colony formation.

17β-HSD activity in MMEC and MMEC/myc_3 cells

The effect of c-*myc* oncogene on intrinsic 17β-HSD activity was evaluated by comparing the relative extent of interconversion of E_2 and E_1 in MMEC and MMEC/myc_3 cells. It is clear from the data presented in Table 2 that the reductive pathway of E_1 to E_2 dominates over the opposing oxidative pathway of E_2 to E_1 conversion, the reductive reaction being about 14-fold and 12-fold greater than the oxidative conversion in MMEC and MMEC/myc_3 cells respectively. However, in MMEC/myc_3 cells, both reductive and oxidative reactions are found to be significantly reduced, being about 32% (d.f. 11, t = 4.16, P = 0.0028) and 12% (d.f. 10, t = 3.40, P = 0.05) lower relative to those observed in MMEC cells.

Typical HPLC profiles of oestrogen metabolism in MMEC and MMEC/myc_3 cells are illustrated in Figure 1.

Time course experiments (3–72 h) were specifically designed to compare E_1 conversion to E_2 in parental MMEC and c-*myc*-transfected cells. As shown in Figure 2, the extent of the reductive pathway of 17β-HSD is significantly reduced (from three- up to 4.5-fold) in MMEC/myc_3 cells. It is of interest that maximum E_2 formation in the latter cell line was observed at 24 h (15.3%) or 48 h (20.4%), whereas it was steadily increasing with time in MMEC cells. Consistency in DNA values and cell counts was ensured for reproducibility of data.

Parallel experiments carried out on MMEC and MMEC/myc_3 cells using increasing precursor concentrations (from 0.1 up to 100 nM) showed that the proportion of E_2 formed remained relatively unchanged using either 1, 10 or 100 nM E_1 in both MMEC (33–35%) and MMEC/myc_3 cells (8–10%), whereas it was remarkably greater (46% in MMEC cells and 17% in MMEC/myc_3 cells) at the lowest E_1 concentration (0.1 nM) used (see Figure 3).

Nevertheless, the extent of E_2 formation was again significantly (from 2.6- up to 4.6-fold) lower in MMEC/myc_3 cells with respect to the parental MMEC cells.

E_2 hydroxylation in MMEC and MMEC/myc_3 cells

The oestrogen metabolism was compared by radiometric determination of the relative extent of E_2 conversion via the C2- and C16α-hydroxylation pathways (Table 3). The two cell lines exhibited persistent metabolic competence to convert E_2. In parental MMEC cells, the extent of conversion of [C2-^{3}H]E_2 and of [C16α-^{3}H]E_2 was 0.32 ± 0.04% and 0.14 ± 0.02% (per 10^4 cells) respectively (mean ± s.d., n = 12). In MMEC/myc_3 cells, the extent of conversion of [C2-^{3}H]E_2 was decreased to 0.16 ± 0.02%, while that of [C16α-^{3}H]E_2 was increased to 0.37 ± 0.05%. To maintain consistency with the data from the experiments on interconversion of E_2 and E_1, the data from E_2 metabolism are expressed as amounts of 2-OHE_1 and 16α-OHE_1 formed. The data presented in Table 3 demonstrate clearly that MMEC/myc_3 cells exhibit about a 164.5% increase (d.f. 11, t = 5.25, P = 0.001) in 16α-OHE_1 formation, with a concomitant 48.3% decrease (d.f. 11, t = 4.23, P = 0.001) in 2-OHE_1 formation. This results in a fourfold increase in the C16α/C2 hydroxylation ratio.

DISCUSSION

Altered endocrine status of the mammary tissue plays an important role in the expression of tumorigenic phenotype (Telang et al, 1979; Ganguly et al, 1982; McCormick et al, 1982; Mauvais-Jarvis et al, 1986; Siiteri et al, 1986; Welsch, 1987; Castagnetta et al, 1992; Fishman et al, 1995; Telang, 1996). The experiments designed in the present study have used the immortalized, non-tumorigenic MMEC and the tumorigenic *myc*-transfected MMEC/myc_3 cells to understand the relationship between oestrogen metabolic pathways and *myc*-mediated tumorigenic transformation better.

The MMEC/myc_3 cell line exhibits enhanced expression of the cellular markers for transformation, namely aberrant hyperproliferation in vitro before tumorigenicity in vivo. We have observed previously that (1) non-cancerous mammary tissue exhibits increased C16α-hydroxylation of E_2 to diverse carcinogenic agents

(Telang et al, 1991, 1997; Suto et al, 1992; Fishman et al, 1995; Telang 1996); (2) exposure to 16α-OHE_1 results in genotoxic DNA damage and aberrant proliferation in non-cancerous mammary epithelial cells (Telang et al, 1992); (3) specific E_2 metabolites modulate proliferation in cells pretreated with chemical carcinogens or those derived from mammary carcinoma (Schneider et al, 1984; Suto et al, 1993); and (4) mechanistically distinct classes of chemopreventive agents inhibit aberrant proliferation and induce C2-hydroxylation of E_2 (Suto et al, 1992, 1993; Telang et al, 1997). These observations taken together support the concept that E_2 metabolism may represent a biochemical/endocrine marker for mammary carcinogenesis and its prevention.

Interconversion of E_2 and E_1 has been reported to be altered in the neoplastic breast tissue owing to a change in intrinsic 17β-HSD activity (Pollow et al, 1977; Prudhomme et al, 1984; Gompel et al, 1986; Vermeulen et al, 1986; Tait et al, 1989; Poutanen et al, 1992; Pasqualini et al, 1996), which also appears to be different according to the hormone-responsive status of cancer cells (Castagnetta et al, 1995, 1996). This evidence is also relevant for other target cells of steroids (Carruba et al, 1997). The relative extent of 17β-HSD-mediated interconversion of E_2 and E_1 revealed interesting differences between MMEC and MMEC/*myc*$_3$ cells. Overall, the reductive conversion of E_1 to E_2 was remarkably greater than the opposing oxidative pathway in both MMEC and MMEC/*myc*$_3$ cells. However, both reactions were significantly lower in MMEC/*myc*$_3$ cells with respect to the parental MMEC cells. This could be, only partially, a reflection of the sustained increase of 16α-hydroxylation of E_1 seen in MMEC/*myc*$_3$ cells in association with the persistent expression of the *myc* oncogene.

Results from time course experiments and those obtained using increasing concentrations of precursor confirmed that the extent of E_1 reduction to E_2 is consistently and significantly lower in MMEC/*myc*$_3$ cells than that observed in MMEC cells, regardless of incubation time and dose of precursor used.

The alteration in 17β-HSD activity observed in the present study raises the possibility that deregulated *myc* expression may have preferentially suppressed the reductive isoform of 17β-HSD enzyme(s), resulting in an altered oestrogen substrate utilization by MMEC/*myc*$_3$ cells, as has been reported in other systems (Pollow et al, 1977; Strobl and Lippman, 1979; Tait et al, 1989; Poutanen et al, 1993). The oestrogen receptor status is critical for the genesis and/or evolution of a transformed cell phenotype and, as such, modulation of the receptor status may coincide with progression of hormone-dependent tumours to a hormone-independent status (Abul-Hajj, 1979; McCormick et al, 1982; Prudhomme et al, 1984; Welsch, 1985, 1987; Mauvais-Jarvis et al, 1986; Siiteri et al, 1986; Ball et al, 1988; Castagnetta et al, 1995; Nguyen et al, 1995). In this context, it is interesting to note that MMEC/*myc*$_3$ cells that express exogenous c-*myc* also exhibit about a 60% decrease in oestrogen receptor content relative to the parental MMEC cells.

The experiments in the present study (designed to inspect the metabolic pathways subsequent to the formation of E_1) demonstrated clearly that the two cell lines are able to metabolize E_2 via the mutually exclusive C2-hydroxylation and C16α-hydroxylation in a manner similar to that previously observed in mammary epithelial cells that are initiated with the *Ras* oncogene or with the chemical carcinogen DMBA (Suto et al, 1992; Telang et al, 1991, 1992). Furthermore, HPLC analysis confirmed that the incubation of MMEC/*myc*$_3$ cells with close to a physiological E_1 concentration also resulted in an appreciable 16α-OHE_1 formation. Consistent with the observed cellular effects of specific E_2 metabolites in carcinogen/oncogene-initiated or carcinoma-derived cells (Schneider et al, 1984; Suto et al, 1992, 1993; Telang et al, 1992), 16α-OHE_1 and 2-OHE_1 were also effective in modulating growth response of c-*myc* oncogene-transfected cells in the present model.

Overall, MMEC/*myc*$_3$ cells exhibit strikingly enhanced proliferative activity and persistence of the transformed phenotype that appear to be associated with (1) altered equilibrium of E_1 to E_2 conversion and consequent reduction in E_2 production; and (2) increased ratio of C16α/C2 hydroxylation with consequent possible overstimulation of cell proliferation induced by both increased 16αOHE_1 level and decreased $2OHE_1$. However, concerning the relationship between aberrant hyperproliferation, altered oestrogen metabolism and c-*myc*-deregulated expression, it remains unclear whether this represents a result of oncogene expression/activation or is rather a consequence of phenotypic transformation of the cells.

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REFERENCES

Abul-Hajj YJ (1979) Relationship between estrogen receptors, 17β-hydroxysteroid dehydrogenase and estrogen content in human breast cancer. *Steroids* **34**: 217–225

Ball RK, Ziemiecki A, Schonenberger CA, Reichmann E, Redmond SMS and Groner B (1988) V-myc alters the response of a cloned mouse mammary epithelial cell line to lactogenic hormones. *Mol Endocrinol* **2**: 133–142

Carruba G, Leake RE, Rinaldi F, Chalmers D, Comito L, Sorci C, Pavone-Macaluso M and Castagnetta L (1994) Steroid-growth factor interaction in human prostate cancer. 1. Short-term effects of transforming growth factors on growth of human prostate cancer cells. *Steroids* **59**: 412–420

Carruba G, Adamsky J, Calabro M, Miceli MD, Cataliotti A, Bellavia V, Lo Bue A, Polito L and Castagnetta LA (1997) Molecular expression of 17β-hydroxysteroid dehydrogenase types in relation to their activity in intact human prostate cancer cells. *Mol Cell Endocrinol* **131**: 51–57

Castagnetta L, Granata OM, Lo Casto M, D'Agostino G, Mitchell F and O'Hare MJ (1986) Steroid profiles and optimization of high performance liquid chromatographic analytic procedure. *Ann NY Acad Sci* **464**: 316–330

Castagnetta L, Granata OM, Lo Casto M, Calabro M, Arcuri F and Carruba G (1991) Simple approach to measure metabolic pathways of steroids in living cells. *J Chromatogr* **572**: 25–39

Castagnetta L, Traina A, Carruba G, Fecarotta E, Palazzotto G and Leake RE (1992). The prognosis of breast cancer patients in relation to the estrogen receptor status of both primary disease and involved nodes. *Br J Cancer* **65**: 167–170

Castagnetta L, Granata OM, Farruggio R, Cannella S, Montesanti A, Oliveri G, Sorci C, Mesiti M and Carruba G (1995) Oxidative and reductive pathways of estrogens in hormone responsive and non-responsive human breast cancer cells in vitro. *J Steroid Biochem Mol Biol* **53**: 367–374

Castagnetta LA, Granata OM, Taibi G, Lo Casto M, Comito L, Oliveri G, Di Falco M and Carruba G (1996) 17β-hydroxysteroid oxidoreductase activity in intact cells significantly differs from classical enzymology analysis. *J Endocrinol* **150**: S73–S78

Clark JH, Pasko Z and Peck EJ (1977) Nuclear binding and estrogen receptor complex: relation to the agonistic and antagonistic properties of estriol. *Endocrinology* **100**: 91–96

D'Agostino G, Castagnetta L, Mitchell F and O'Hare MJ (1985) Computer-aided mobile phase optimization and chromatogram simulation in HPLC: a review. *J Chromatogr* **388**: 1–23

Dubik D and Shiu RPC (1992) Mechanism of estrogen activation of c-*myc* oncogene expression. *Oncogene* **7**: 1587–1594

Fishman J and Martucci CP (1980) Differential hydroxylations of estrone and estradiol in man. *J Clin Endocrinol Metab* **51**: 611–615

Fishman J, Bradlow HL, Schneider J, Anderson KE and Kappas A (1980) Radiometric analysis of oxidation in man: sex differences in estradiol metabolism. *Proc Natl Acad Sci USA* **77**: 4957–4960

Fishman J, Osborne MP and Telang NT (1995) The role of estrogen in mammary carcinogenesis. *Ann N Y Acad Sci* **768**: 91–100

Ganguly N, Ganguly R, Mehta NM and Banerjee MR (1982) Growth and differentiation of hyperplastic outgrowths derived from mouse mammary epithelial cells transformed in organ culture. *J Natl Cancer Inst* **58**: 453–463

Gompel A, Malet C, Spritzer P, Lalardrie JP, Kutten F and Mauvais-Jarvis P (1986) Progestin effect on cell proliferation and 17β-hydroxysteroid dehydrogenase activity in normal human breast cells in culture. *J Clin Endocrinol Metab* **63**: 1174–1180

McCormick DL, Mehta RG, Thompson CA, Dinger N, Caldwell JA and Moon RC (1982) Enhanced inhibition of mammary carcinogenesis by combination N-(4-hydroxyphenil) retinamide and ovariectomy. *Cancer Res* **42**: 509–512

Mauvais-Jarvis P, Kutten F and Gompel A (1986) Estradiol/progesterone interaction in normal and pathologic breast cells. *Ann N Y Acad Sci* **464**: 152–167

Nguyen BL, Chetrite G and Pasqualini JR (1995) Transformation of estrone and estradiol in hormone-dependent and hormone-independent human breast cancer cells. *Breast Cancer Res Treat* **34**: 139–146

Pasqualini JR, Chetrite G, Blacker C, Feinstein M-C, Delalonde L, Talbi M and Maloche C (1996) Concentration of estrone, estradiol, estrone sulfate, and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *J Clin Endocrinol Metab* **81**: 1460–1464

Pollow K, Bouquoi E, Baumann J, Schmidt-Gollwitzer M and Pollow B (1977) Comparison of the in vitro conversion of estradiol-17beta to estrone in normal and neoplastic human breast tissue. *Mol Cell Endocrinol* **6**: 333–348

Poutanen M, Montcharmont B and Vihko R (1992) 17β-hydroxysteroid dehydrogenase gene expression in human breast cancer cells: regulation of expression by a progestin. *Cancer Res* **52**: 290–294

Poutanen M, Meittinen M and Vihko R (1993) Differential estrogen substrate specificities for transiently expressed human placental 17beta-hydroxysteroid dehydrogenase and an endogeneous enzyme expressed in cultured COS-m6 cells. *Endocrinology* **133**: 2639–2644

Prudhomme JF, Malet C, Gompel A, Lalardrie JP, Ochoa P, Mauvais-Jarvis P and Kutten F (1984) 17β-hydroxysteroid dehydrogenase activity in human breast epithelial cell and fibroblast cultures. *Endocrinology* **114**: 1483–1489

Schneider J, Huh MM, Bradlow HL and Fishman J (1984) Antiestrogen action of 2-hydroxyestrone on MCF-7 human breast cancer cells. *J Biol Chem* **159**: 4840–4845

Siiteri PK, Simberg N and Murai J (1986) Estrogens and breast cancer. *Ann NY Acad Sci* **464**: 100–105

Strobl JS and Lippman ME (1979) Prolonged retention of estradiol by human breast cancer cells in tissue culture. *Cancer Res* **39**: 3319–3327

Suto A, Bradlow HL, Wong GYC, Osborne MP and Telang NT (1992) Persistent estrogen responsiveness of Ras oncogene-transformed mouse mammary epithelial cells. *Steroids* **57**: 262–268

Suto A, Bradlow HL, Wong GYC, Osborne MP and Telang NT (1993) Experimental down-regulation of intermediate biomarkers of carcinogenesis in mouse mammary epithelial cells. *Breast Cancer Res Treat* **27**: 193–202

Tait G, Newton CJ, Reed MJ and James VHT (1989) Multiple forms of 17beta-hydroxysteroid oxidoreductase in human breast tissue. *Mol Cell Endocrinol* **2**: 71–80

Telang NT (1996) Oncogenes, estradiol biotransformation and mammary carcinogenesis. *Ann N Y Acad Sci* **784**: 277–287

Telang NT, Banerjee MR, Iyer AP and Kundu AB (1979) Neoplastic transformation of epithelial cells in whole mammary gland in vitro. *Proc Natl Acad Sci USA* **76**: 5886–5890

Telang NT, Osborne MP, Sweterlitsch L and Narayanan R (1990) Neoplastic transformation of mouse mammary epithelial cells by deregulated *myc* expression. *Cell Regul* **1**: 863–872

Telang NT, Narayanan R, Bradlow HL and Osborne MP (1991) Coordinated expression of intermediate biomarkers for tumorigenic transformation in Ras-transfected mouse mammary epithelial cells. *Breast Cancer Res Treat* **18**: 155–163

Telang NT, Suto A, Wong GYC, Osborne MP and Bradlow HL (1992) Induction by estrogen metabolite 16alpha-hydroxy estrone of genotoxic damage and aberrant proliferation in mouse mammary epithelial cells. *J Natl Cancer Inst* **84**: 634–638

Telang NT, Katdare M, Bradlow HL and Osborne MP (1997) Estradiol metabolism: an endocrine biomarker for modulation of human mammary carcinogenesis. *Environ Health Perspect* **105**: 559–564

Vermeulen A, Deslypere JP, Paridaens R, Leclercq G, Roy F and Heuson JC (1986) Aromatase, 17β-hydroxysteroid dehydrogenase and intratissular sex hormone concentrations in cancerous and normal glandular breast tissue in postmenopausal women. *Eur J Cancer Clin Oncol* **22**: 515–525

Welsch CW (1985) Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. *Cancer Res* **45**: 3415–3443

Welsch CW (1987) Dietary retinoids and chemoprevention of mammary gland tumorigenesis. In *Cellular and Molecular Biology of Breast Cancer*. Medina D, Kidwell W, Heppner G and Anderson E (eds), pp. 495–508. Plenum Press: New York