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Phenotypic Features of Breast Cancer Cells Overexpressing Ornithine-Decarboxylase

ANDREA MANNI,* RITA WECHTER, LISA WEI, DANIEL HEITJAN, AND LAURENCE DEMERS

Departments of Medicine (A.M., R.W., L.W., L.D.), Pathology (L.D.), and Center for Biostatistics and Epidemiology (D.H.) Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033

Polyamines (PA) have been shown to be critical mediators of estradiol-induced breast cancer cell proliferation. This finding suggests that constitutive activation of the PA pathway may promote tumor progression, possibly leading to hormone independence. To test this hypothesis, we transfected hormone-responsive MCF-7 breast cancer cells with a complementary DNA coding for ornithine-decarboxylase (ODC), the first rate-limiting enzyme in PA biosynthesis. Marked ODC overexpression observed in stably transfected clones was associated with a selective increase in cellular putrescine content, while spermidine and spermine levels were not altered. ODC-overexpressing MCF-7 cells were resistant to the antiproliferative effects of low but not high concentrations of the enzyme inhibitor, α-difluoromethylornithine. In agreement with our hypothesis, sensitivity to the growth-promoting action of estradiol was reduced by approximately one third ($P < 0.001$) in ODC-overexpressing MCF-7 cells compared with vector-only transfected clones. Basal growth under anchorage-dependent conditions was only marginally increased by ODC overexpression ($P = 0.048$), while clonogenicity in soft agar was actually reduced. These data suggest that activation of PA biosynthesis may contribute in part to the acquisition of estrogen independence by breast cancer cells. Since only putrescine content was increased as a result of ODC overexpression, these data may underestimate the overall influence of the PA pathway on breast cancer phenotype.

We and other investigators have shown that polyamines (putrescine, spermidine, and spermine) are important in the control of breast cancer cell proliferation, both in vitro and in vivo (Manni and Wright, 1984, 1985; Hoggard and Green, 1986; Kendra and Katzenellenbogen, 1987; Manni et al., 1986a) and that in hormone-responsive breast cancer cells polyamine biosynthesis is regulated by estrogen (Thomas and Thomas, 1993). Estradiol treatment of MCF-7 breast cancer cells stimulates ornithine-decarboxylase (ODC), the first rate-limiting enzyme in polyamine biosynthesis, at the transcriptional level and increases cellular polyamine levels (Thomas and Thomas, 1993). Administration of α-difluoromethylornithine (DFMO), a mechanism-based irreversible inhibitor of ODC activity, consistently inhibits estradiol-stimulated breast cancer cell growth in a variety of experimental systems (Manni and Wright, 1984, 1985; Thomas and Thomas, 1993; Glikman et al., 1990). Therefore, these data suggest that polyamines are critical distal effectors of hormonally stimulated mammary tumor growth.

In support of this concept, we have recently shown that manipulation of the polyamine pathway significantly enhances hormonally induced synchronization of breast cancer growth in rats bearing N-nitrosomethylurea–induced mammary tumors (Manni et al., 1992). Polyamines are likely to influence estrogen action at multiple steps. These include the association kinetics of the estrogen receptor to specific DNA sequences (Thomas, 1989), the synthesis of estradiol-regulated cell-cycle–specific genes (Thomas and Thomas, 1994), as well as the synthesis and/or action of estradiol-modulated growth factors (Glikman et al., 1990; Manni et al., 1986b, 1987). These findings led us to hypothesize that constitutive activation of the polyamine pathway could provide breast cancer cells with a growth advantage, possibly bypassing the need for estrogen stimulation. This concept is also supported by circumstantial evidence linking increased polyamine biosynthesis to an aggressive breast cancer phenotype (Glikman et al., 1987; Kingsnorth et al., 1984). At present, however, a direct cause-effect relationship between polyamine synthesis activation and breast cancer phenotype has not been definitively established.

Therefore, the primary objective of these experiments was to directly test the influence of increased polyamine biosynthetic activity on phenotypic features of breast cancer cells in culture, with particular focus

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*To whom reprint requests/correspondence should be addressed at the Department of Medicine, Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, P.O. Box 850, 500 University Dr., Hershey, PA 17033.

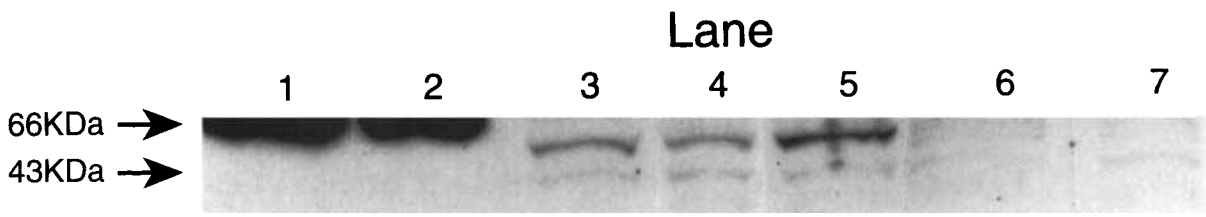

Fig. 1. Western blot analysis of cell extracts of wild-type and transfected clones of MCF-7 cells using an anti-ODC antiserum as described in Materials and Methods. Lane 1: Recombinant intact ODC (100 ng); lane 2: recombinant intact ODC (50 ng); lane 3: clone T8 maintained in the presence of DFMO until 12 days prior to its use in this experiment; lane 4: clone T8 grown in the absence of DFMO; lane 5: clone T35 maintained in the presence of DFMO; lane 6: wild-type MCF-7 cells; lane 7: clone pCD-1, vector only transfected. Note the expected lower MW (47 kDa vs. 51 kDa) of the truncated exogenous ODC expressed by our transfected clones (lanes 3–5) whether or not maintained in the presence of DFMO. The ODC antiserum was not sufficiently sensitive to detect the much lower levels of endogenous ODC expressed by wild-type (lane 6) or vector-only transfected cells (lane 7).

on cell proliferation and estrogen sensitivity. To this end, we employed a transfection approach to induce overexpression of ODC in the hormone-responsive MCF-7 breast cancer cell line.

MATERIALS AND METHODS

Cell lines and culture conditions

Wild-type MCF-7 breast cancer cells (kindly provided by Dr. V.C. Jordan, University of Wisconsin, Madison, Wisconsin) and the transfected clones were propagated in 75 cm^2 flasks in Richter's improved minimal essential medium (IMEM; Grand Island Biological, Grand Island, NY) containing phenol red and 5% fetal bovine serum (FBS) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. In selected experiments (see Results and figure legends), the culture conditions were stepped down to phenol red–free IMEM containing dextran-coated charcoal (DCC)–stripped 5% FBS or to serum-free media. Clonogenicity in soft agar was assessed as previously published (Manni et al., 1990).

Plasmid and transfection technique

A 1582-bp mouse ODC cDNA subcloned into the *Eco*RI-*Xba*I restriction sites of the SV40-driven pCD expression vector was kindly provided by Dr. A.E. Pegg (M.S. Hershey Medical Center, Hershey, PA) (Lu et al., 1991; Li, 1992). This cDNA codes for a truncated ODC that lacks the PEST region (the extreme 36 C-terminus amino acids), which is important in regulating the half-life of the enzyme (Ghodo et al., 1989). The truncation of ODC at position 426 through 461 has been found by Dr. A.E. Pegg and his collaborators to be more stable and to retain enzymatic activity (Lu et al., 1991).

MCF-7 breast cancer cells that were 60–70% confluent were transfected with this plasmid and an RSV-neo cDNA (ratio 5:1) using a modified calcium phosphate technique (Chen and Okayama, 1987). The major differences between this method and the conventional calcium phosphate precipitation technique include the lower pH of the buffer (6.95 vs. 7.10), the CO_2 level (3%) during the overnight incubation of DNA with the cells, and the amount of DNA (20 μg). Parallel dishes were also transfected with the vector only and RSV-neo. Forty-eight hours after transfection, the cells were grown in the presence of G-418 (400 μg/ml) to allow selection of cells that had incorporated the exogenous DNA. Three control clones (pCD1, pCD4, and pCD7) and three ODC-overexpressing clones (T8, T27, and T35) were isolated, expanded, and used in our experiments. ODC expression tended to slowly decline (over a few months) in T8 and T27 cells, wheras the decline was rather abrupt (over a few weeks) in T35 cells. We empirically observed that chronic treatment with DFMO was able to maintain ODC overexpression in all three clones, which persisted despite discontinuation of the drug.

In several biological systems, chronic DFMO treatment has been shown to result in ODC overexpression predominantly on the basis of gene amplification (McConlogue and Coffino, 1983; Leinonen et al., 1987; Pegg et al., 1988). In our clones, however, endogenous ODC did not appear to be affected, since ODC overexpression was accounted for by the exogenously transfected truncated gene (Fig. 1). Since no differences in biochemical and biological features were observed between DFMO-treated transfected clones and untreated transfected cells, the results of our experiments are combined. In the case of DFMO treatment, the cells were washed off DFMO for 1 week prior to being used for the experiments.

Experimental protocols

Control and ODC-overexpressing MCF-7 cells were compared with regard to the following parameters: (1) Basal proliferative activity in liquid culture. In these experiments, the cells were plated in duplicate at a density of 6×10^4 cells/35 mm dish in regular growth medium. Twenty-four hours later, the medium was replaced with either the same or phenol red–free IMEM containing DCC-treated 5% FBS or serum free IMEM supplemented with 5 mM glutamine, 0.2 mg% transferrin, 0.1 mg% fibronectin, 0.4 g% fraction V BSA, and 20 mM Hepes buffer. Medium changes occurred every 48 hours. Duplicate dishes per cell line were harvested on days 2, 4, and 6, and the number of cells was counted using a Coulter counter. (2) Clonogenicity in soft agar. Plating in soft agar was done as previously described (Manni et al., 1990). Cells were plated in triplicate at a density of 1×10^4 cells/35 mm dish. The number of colonies (aggregates greater than 50 cells) was scored after 15–18 days.

(3) Sensitivity to DFMO. To assess growth, cells were plated in triplicate at a density of 6×10^4 cells/35 mm dish in regular growth medium in the absence and presence of increasing concentrations of DFMO (.01, .1, and 1 mM). The media were changed every 48 hours, and the cells were harvested and counted on day 6. For measurement of ODC activity and polyamine levels (only tested in the absence and presence of 1 mM DFMO), parallel sets of duplicate cultures/cell line were plated at a density of 4×10^5 cells/100 mm dish. (4) Estrogen sensitivity. In these experiments, the cells were grown for 7 days in phenol red–containing IMEM supplemented with DCC-treated 5% FBS prior to plating in the experimental dishes. For assessment of growth, cells were plated in triplicate at a density of 6×10^4 cells/35 mm dish in phenol red–free IMEM containing DCC-treated 5% FBS. The treatment consisted of either vehicle alone (0.1% ethanol) or increasing concentrations of estradiol (10^{-13}M, 10^{-11}M, and 10^{-9}M). The media with the treatments were replaced every 2 days, and the cells were harvested and counted on day 6. For assessment of ODC activity and polyamine levels, parallel triplicate cultures/cell line were set up (4×10^5 cells/100 mm dish) in the absence and presence of 10^{-9}M estradiol. The cells were again harvested on day 6 of culture.

(5) Estrogen and progesterone receptor levels. For measurement of estrogen receptors, the cells were cultured under stepped-down conditions to deplete the receptor of endogenously bound steroid. Logarithmically growing cells were plated in 150 cm^2 flasks containing 5% DCC-treated fetal calf serum in IMEM without phenol red. After 48 hours, the medium was changed to serum-free IMEM. The cells were harvested 4 days later with a medium change at 48 hours. For determination of progesterone receptors, parallel flasks were treated identically, except for the presence of 10^{-9}M estradiol during the 6-day culture period.

Analytical techniques

ODC activity. At the time of harvesting, the cells were washed three times with ice-cold PBS and then resuspended in buffer containing 50 mM Tris, 2.5 mM DTT, and .1 mM EDTA, pH7.5, and stored at −70°C until use. At the time of the assays, the cells were frozen and thawed twice. The cell lysates were centrifuged at 13,600*g* for 20 minutes. Enzyme activity was determined in the cytosolic fraction according to the method described by Pegg et al. (1970) with minor modifications. Briefly, reaction tubes were placed on ice and filled with 200 μl of sample extract. Fifty microliters of reaction mixture (0.04 mM pyridoxal phosphate, 2.5 mM dithiotreitol [DTT], 50 mM Tris-Hcl pH 7.5, 0.4 mM L-ornithine, and 0.1 μCi ^{14}C ornithine) were added to each tube. The reaction was started by capping each tube with a Kontes well containing 250 μl hyamine hydroxide and incubating at 37°C for 30 minutes. The reaction was stopped by injection of 0.3 ml 5 NH_2SO_4 through the rubber septum directly into the reaction mixture. The tubes were incubated for an additional 30 minutes at 37°C. The wells were then transferred to glass scintillation vials containing 10 ml of Econofluor-2 (Dupont NEN, Boston, MA). ODC activity was expressed as nmol CO_2/mg protein/30 minutes. Protein concentration was measured with the Bio-Rad dye protein assay dye (BioRad Labs, Melville, NY) using bovine serum albumin as a standard.

Polyamine levels. After washing with ice-cold PBS, the cells were resuspended in 0.2 N perchloric acid and kept at 4°C overnight. The suspension was then centrifuged at 800 × g for 15 minutes, and the supernatant was stored until the time of the assay. Polyamines were determined by high-performance liquid chromatography (HPLC) with fluorometry following a modification of the method described by Seiler and Knodgen (1980), as recently reported by Glikman et al. (1989, 1990).

Western blot analysis. Proteins in cell extracts (20 μg/lane) were separated by SDS/PAGE on a 12% acrylamide gel (mini-PROTEAN 11, 0.37 m Tris-HCl ready gels; Biorad). The proteins were elctrophoretically transferred to a 0.45 μm nitrocellulose membrane (Transblot transfer medium, Biorad). The membrane was incubated with Tris-buffered saline, 0.1% Tween 20 (TBST; 20 mM Tris HCl, pH 7.4, and 0.5 m NaCl) containing 10% skim milk (Carnation) and then washed with TBST. The membrane was then incubated with 20 ml of TBS and 20 μl of anti-ODC antiserum (Lu et al., 1991) for 1 hour. Although the anti-ODC antibody has not been purified, this antiserum has been shown to be valid in visualizing ODC by Western analysis (Lu et al., 1991). After washing with TBST, the membrane was incubated with 20 ml of TBST and 4 μl of goat anti-rabbit IgG-horse radish peroxidase conjugate (BioRad) for 1 hour. The membrane was washed five times with Tris-buffered saline, 0.3% Tween 20, and three times with TBST. The membrane was incubated for 1 minute with ECL reagent (enhanced chemiluminescence Western blotting, Amersham, Arlington Heights, IL), and light-emitting protein was detected by short exposure to Kodak XARS autoradiography film.

Estrogen and progesterone receptor measurement. After being harvested by brief trypsinization and washed once with phosphate buffered saline (PBS), the cells were resuspended in Tris buffer, sonicated for 20 seconds, and ultracentrifuged for 30 minutes at 100,000*g*. The estrogen and progesterone receptors were measured in the supernatant fraction using previously published techniques (Feil et al., 1979; McGuire et al., 1977).

Statistical methods

We first analyzed the data to determine an appropriate scale for modeling. For cell growth data, our analyses suggested that the log of cell count was an appropriate scale. For data on clonogenicity in soft agar, the square root of the colony count was the best scale. All subsequent analyses were by linear models, including a random clone effect. In each experiment, we modeled the effects of treatment (vector vs. ODC transfected), experiment (each study consisted of two to five experiments), any other factors in the design, and higher order interactions of the factors. Specifically, when testing E_2 sensitivity we fit an analysis of variance model including fixed effects and interactions of transfection status, estrogen concentration, and experiment, with clone entering as a random effect. We simultaneously

TABLE 1. ODC activity and polyamine levels of wild-type and transfected MCF-7 breast cancer cells[1]

Cell line	ODC (nmol/mg/30 min)	Polyamines (nmol/mg)		
		Putrescine	Spermidine	Spermine
Wild type	0.224	1.58	11.84	8.33
pCD-1	0.578	1.98	11.07	8.59
pCD-4	0.867	2.23	11.97	7.75
T8	30.91	20.18	14.10	7.45
T27	12.33	9.33	14.01	8.48
T35	87.72	43.12	14.20	8.78

[1]Clones T8, T27, and T35 were maintained in the presence of DFMO (0.05 mM) to preserve ODC overexpression, as discussed in Materials and Methods. The drug was removed 7 days prior to plating the cells in the experimental dishes. The cells were harvested 5 days later for measurement of ODC activity and polyamines. Each value represents the mean of two replicate dishes.

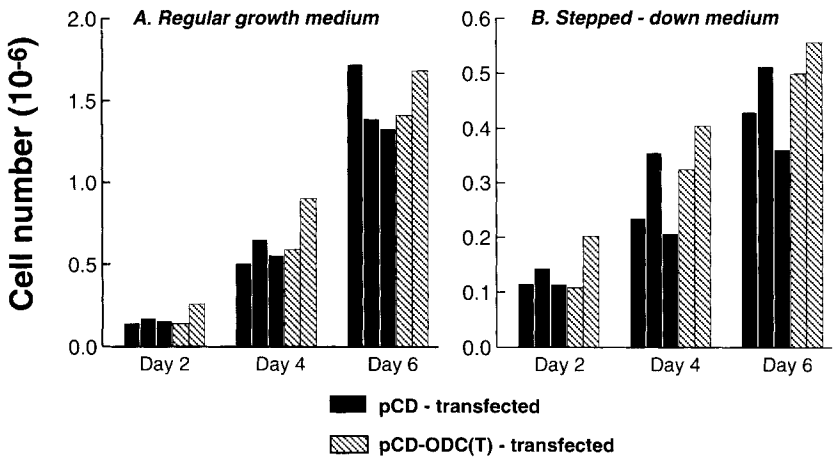

Fig. 2. Basal growth of control and ODC-overexpressing MCF-7 breast cancer cells in liquid culture. Control (pCD-1, pCD-4, and pCD-7) and ODC-transfected (T-8 and T-27) cells were grown in the indicated media (plus serum-free IMEM, not shown) as specified in Materials and Methods. The T8 and T27 clones used in this experiment had not been previously treated with DFMO. The bars represent mean cell numbers of duplicate dishes. Similar results were obtained in an identical replicate experiment (not shown).

tested whether the difference between lack of E_2 and increasing concentrations of E_2 was the same for ODC- and vector-transfected cells, simultaneously for all doses of E_2. Because we found that these contrasts were not significantly different between experiments ($P = 0.97$), we aggregated across experiments. We assessed statistical significance by likelihood-ratio chi-square tests. All computations were executed in S Plus (Version 3.1, Statistical Sciences, Seattle, WA).

RESULTS

ODC activity and polyamine profiles of transfected clones

Table 1 illustrates a typical ODC and polyamine profile of our wild-type and transfected MCF-7 cells. As can be seen, transfection with the truncated ODC cDNA resulted in marked ODC overexpression, which was associated with a selective increase in cellular putrescine content. Spermidine and spermine levels, on the other hand, were not significantly altered. Using a specific ODC antiserum, we were able to show that the transfected cells (whether or not treated with DFMO) expressed the exogenous truncated ODC protein (Fig. 1).

Biologic properties of ODC-overexpressing MCF-7 breast cancer cells

As can be seen in Figure 2, proliferative activities of control and ODC-overexpressing MCF-7 cells were similar when tested in regular growth medium (Fig. 2A), stepped-down conditions (Fig. 2B) or in the absence of serum (data not shown). Statistical analysis of two replicate experiments (one of which is shown in Fig. 2) showed that the effect of transfection on growth was only marginally significant ($P = 0.048$), with ODC-overexpressing cells growing somewhat better than vector-transfected cells, both in regular and stripped medium. ODC activity was not simultaneously measured in these experiments. However, the degree of ODC overexpression ranged between 30-fold and 18-fold over control for the T8 clone when tested approximately 2 weeks before and 2 weeks following these experiments, respectively. For the T27 cell line, the degree of ODC overexpression at the same time points ranged between 14-fold and eightfold.

We then assessed the clonogenicity in soft agar of control and ODC-transfected clones. As can be seen in Figure 3, clonogenicity was significantly reduced ($P = 0.006$) by an average of 35% (range 11–77%) in ODC-overexpressing MCF-7 cells. Of interest, the colony number was restored to near-control levels when these cells were plated in the presence of DFMO (0.05 mM) to reduce ODC activity (data not shown).

Figure 4 illustrates the sensitivity of control and ODC-overexpressing MCF-7 cells to increasing concentrations of DFMO. As can be seen, ODC-transfected clones manifested resistance to the antiproliferative action of low (0.01 mM) but not high concentrations of

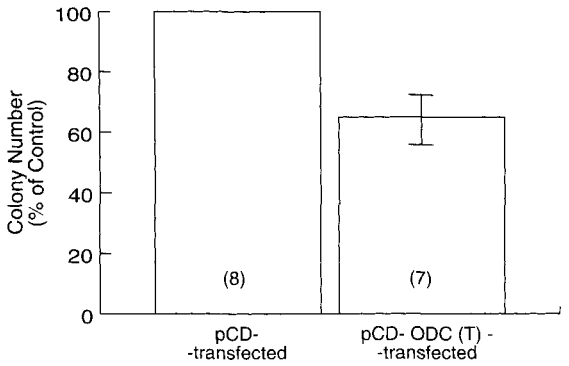

Fig. 3. Clonogenicity in soft agar of control and ODC-overexpressing MCF-7 breast cancer cells. The data (mean ± SEM) represent the combined results of five replicate experiments and are expressed as the percent of control. The number of determinations is shown in parentheses. ODC-overexpressing clones manifested similar growth in soft agar whether or not they had been pretreated with DFMO; therefore, the results were combined. In the case of DFMO treatment, the drug was removed 7 days prior to plating in soft agar.

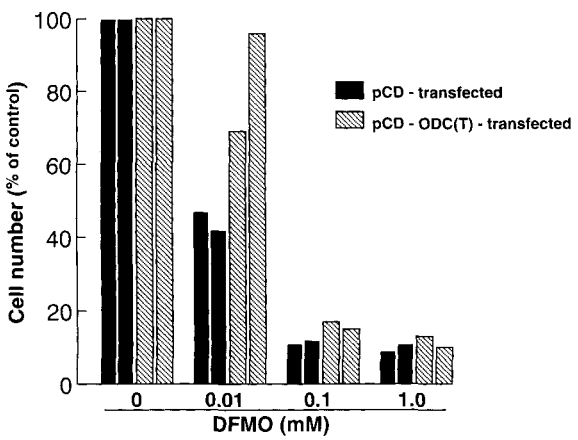

Fig. 4. Sensitivity to DFMO of control and ODC-overexpressing MCF-7 breast cancer cells in culture. Control (pCD-1 and pCD-4) and ODC-transfected (T8 and T27) MCF-7 cells were plated in the absence and presence of increasing concentrations of DFMO as specified in Materials and Methods. Bars represent mean cell numbers (expressed as the percent of control) of triplicate dishes after 6 days in culture. Clones T8 and T27 used in this experiment had not been maintained in DFMO. Similar results were obtained in a similar replicate experiment using clones T8, T27, and T35, which had been kept in 0.05 mM DFMO until 7 days prior to plating in the experimental dishes (data not shown). Statistical analysis revealed that ODC overexpression induced a significant reduction ($P < .0001$) in DFMO sensitivity at low concentrations of the drug (01 mM). This effect was roughly the same $P = 0.23$) in both experiments.

DFMO. Similar results were obtained in an identical replicate experiment where the ODC-transfected clones used had been kept in 0.05 mM DFMO until 7 days prior to plating in the experimental dishes (data not shown).

Table 2 reports the ODC activity and polyamine levels in the cells used in both experiments in the absence and presence of 1 mM DFMO. These measurements were not performed in the cells treated with lower concentrations of the drug. As can be seen, significant overexpression of ODC activity in untreated cells was associated with a selective increase in putrescine content, while cellular levels of spermidine and spermine were not altered. It should be noted that the degree of ODC overexpression was higher in experiment 2 as a result of chronic exposure to DFMO, which we found effective in preserving high levels of enzyme activity in our ODC-transfected cells. Administration of 1 mM DFMO similarly suppressed ODC activity, putrescine, and spermidine to undetectable levels in control and ODC-transfected clones. This result is in agreement with the antiproliferative effect exerted on these cells by 1 mM concentration of the drug (Fig. 4).

We were interested in determining whether induction of ODC overexpression would alter the estrogen sensitivity of breast cancer cells. We did observe that ODC-transfected MCF-7 clones manifested a reduced proliferative response to increasing concentrations of estradiol (Fig. 5). This effect, however, while consistent and highly significant ($P < 0.0001$), was rather modest. The degree of ODC overexpression detected in these cells ranged between 14- and 59-fold over control (average, 41-fold). This was associated with a six-fold mean increase in putrescine levels (range 5.0- to 8.2-fold), while spermidine and spermine were again unchanged. Estradiol administration did not produce any consistent effect on the activity of the exogenously transfected ODC (data not shown).

Estrogen and progesterone receptor levels

Estrogen receptor levels (mean ± SD) were similar in vector-only transfected (275 ± 46 fmol/mg protein; n = 2) and ODC-overexpressing MCF-7 cells (239 ± 180 fmol/mg protein; n = 3). The corresponding progesterone receptor levels in the same sets of cells were 422 ± 126 fm/mg protein and 301 ± 89 fmol/ml protein, respectively.

DISCUSSION

ODC and polyamines have been postulated to play a critical role in both cell proliferation and carcinogenesis (Heby and Persson, 1990; Pegg, 1988; Tabor and Tabor, 1984). ODC activity, which is transiently increased upon growth factor exposure, becomes constitutively activated during cell transformation by a variety of agents, such as chemical carcinogens (Gilmour et al., 1987), viruses (Gazdar et al., 1976; Haddox et al., 1980), and oncogenes (Sistonen et al., 1989a,b). Recent studies have indicated that the ornithine decarboxylase gene is a transcriptional target for c-*myc* (Wagner et al., 1993), *fos* (Wrighton and Busslinger, 1993), and *ras* (Pakala et al., 1988). An important role for ODC in tumorigenesis is further supported by the recent demonstration that transfection of NIH/3T3 fibroblasts with an ODC cDNA induces a transformed phenotype (Moshier et al., 1993) or at least enhances oncogene-induced transformation (Hibshoosh et al., 1991). A direct role of polyamines in mammary carcinogenesis has not yet been demonstrated. However, the finding that breast cancer specimens have considerably higher levels of polyamines than the surrounding normal breast tissue (Persson and Rosengren, 1989; Chanda and Ganguly, 1988) suggests the possible involvement of these compounds in the development of mammary tumors.

In the present experiments, we attempted to address the potential role of polyamines in breast cancer pro-

TABLE 2. ODC activity and polyamine levels of control and ODC-overexpressing MCF-7 cells in the absence and presence of DFMO (ImM)

Clone	ODC activity (nmol/mg/30')		Polyamines (nmol/mg)					
			Putrescine		Spermidine		Spermine	
	−DFMO	+DFMO	−DFMO	+DFMO	−DFMO	+DFMO	−DFMO	+DFMO
Experiment 1								
pCD-1	1.06	0.041	1.82	<0.1	9.67	<0.1	6.67	3.56
pCD-4	0.94	0.052	2.86	<0.1	11.36	<0.1	7.87	3.04
T8	15.75	0.105	6.39	<0.1	11.52	<0.1	6.10	4.19
T27	10.57	0.151	5.91	<0.1	11.71	<0.1	6.20	3.30
Experiment 2[1]								
pCD-1	1.45	<0.1	1.62	<0.1	8.27	<0.1	5.49	4.83
T8	39.07	0.251	8.86	<0.1	9.81	<0.1	4.98	4.21
T27	25.8	0.199	6.40	<0.1	10.89	<0.1	5.55	4.63
T35	86.82	0.525	20.02	<0.1	9.8	<0.1	4.85	6.33

[1]In this experiment, clones T8, T27, and T35 had been treated with 0.05 mM DFMO until 7 days prior to plating in the experimental dishes.

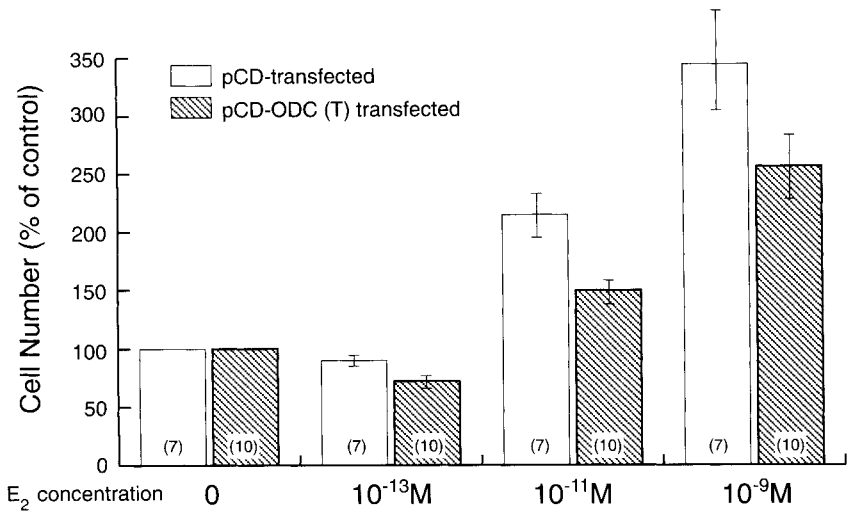

Fig. 5. Estrogen sensitivity of control and ODC-overexpressing MCF-7 breast cancer cells in culture. Control (pCD-1 and pCD-4) and ODC-transfected (T8, T27, and T35) MCF-7 cells were plated in the absence and in the presence of increasing concentrations of E_2 under stepped-down conditions, as described in Materials and Methods. The data (mean ± SEM) represent the combined results of five replicate experiments and are expressed as the percent of control. The number of determinations is shown in parentheses. pCD1 was tested in all five experiments, while pCD4 was tested in two (n = 7). Clones T8, T27, and T35 were tested in four, four, and two experiments, respectively (n = 10). ODC-overexpressing clones manifested a similar proliferative response to E_2 administration whether or not they had been pretreated with DFMO (three and two experiments, respectively). Therefore, the results were combined. In the case of DFMO treatment, the drug was removed 7 days prior to stepping down the culture conditions. Statistical analysis, averaging across experiments, revealed a significant reduction in the estrogen sensitivity of the ODC-overexpressing clones. The significance level was $P < 0.0001$ in a likelihood ratio chi-square test with three degrees of freedom (one degree of freedom for each dose of E_2). The size of the effect increased with increasing E_2 concentration.

gression, with particular focus on the acquisition of hormone independence. The recently demonstrated involvement of the polyamine pathway in estrogen-induced breast cancer cell proliferation suggests a possible mechanism for the development of hormones unresponsiveness. It is conceivable that during transition to hormone independence, the ODC gene could become constitutively activated, thus bypassing the need for estrogen stimulation. Alternatively, control of ODC could be shifted from estrogens to other growth factors or oncogenes that are activated during the acquisition of hormone resistance.

In either dase, one would anticipate that induction of ODC overexpression in hormone-responsive breast cancer cells could lead to a hormone-independent phenotype. To test this hypothesis, we successfully transfected the hormone-dependent MCF-7 breast cancer cell line with an ODC cDNA coding for a stable enzyme. Of interest, in order to maintain a high degree of ODC overexpression in our transfected clones (particularly the T35 cell line), we needed to maintain the cells in the presence of DFMO. Chronic administration of escalating doses of DFMO over periods of months has indeed been shown to induce ODC overexpression in a variety of biological systems, which in some cases persists despite discontinuation of the inhibitor (McConlogue and Coffino, 1983; Leinonen et al., 1987; Pegg et al., 1988). In our cells, removal of DFMO after chronic exposure (≥1 month) was associated with maintenance of high levels of ODC activity for at least 2 months (the longest period tested).

DFMO-induced ODC overexpression has been found to be due predominantly to gene amplification (McConlogue and Coffino, 1983; Leinonen et al., 1987; Pegg et

al., 1988). In our case, however, we found no evidence of any effect of this treatment on the endogenous gene, since ODC activity in our DFMO-treated clones appeared to be due to the expression of the transfected cDNA (Fig. 1). Therefore, it seems that administration of DFMO exerted its effect on the expression of the exogenous ODC by unknown mechanisms. In any event, we observed that overexpressing ODC MCF-7 cells manifested reduced sensitivity to the proliferative effect of estradiol.

The biological significance of this finding remains uncertain, since the magnitude of reduction in estrogen sensitivity was modest. We do not believe, however, that this finding represents a nonspecific consequence of cellular toxicity associated with ODC overexpression. Firstly, progesterone receptor levels (an additional endpoint of estrogen action) were similar in control and ODC-overexpressing MCF-7 cells. Secondly, basal proliferative activity was actually marginally increased ($P = 0.048$) by ODC overexpression. Clonogenicity in soft agar, on the other hand, was actually reduced. One would have anticipated an increase in this parameter (as well as a more profound increase in basal anchorage-dependent growth) if activation of ODC is causally linked to the development of a more aggressive hormone-independent breast cancer phenotype.

These results are somewhat at variance with recent findings in our laboratory showing an increase in tritiated thymidine incorporation in MCF-7 breast cancer cells transiently transfected with the same ODC cDNA (Manni et al., 1994). The observed generally modest biological consequences induced by ODC overexpression in our system are probably due to the multiple compensatory mechanisms present in the polyamine metabolic pathway, which are aimed at preserving cellular polyamine homeostasis (Pegg, 1988). For instance, we observed that our transfected clones manifested a selective increase in putrescine, while spermidine and spermine levels were not altered. Similar polyamine profiles have been reported in other ODC-overexpressing systems such as DFMO-resistant L1210 leukemia cells (Pegg et al., 1988) and transgenic mice (Hakovirta et al., 1993). Accumulation of putrescine may be responsible for cellular toxic effects (as recently described in rat hepatoma cells; Tome et al., 1994), which may explain the observed decreased clonogenicity in soft agar.

Comparison of human breast cancer specimens to normal breast tissue reveals more profound alteration in the polyamine pathway than a selective increase in putrescine (Persson and Rosengren, 1989). They include elevations in cellular contents of spermidine, spermine, acetylated polyamines, and spermidine/spermine N-1-acetyltransferase (Persson and Rosengren, 1989). These results indicate that multiple alterations in the polyamine metabolic pathway (as opposed to a simple increase in ODC) take place during mammary carcinogenesis and possibly tumor progression. Therefore, it is likely that our results may have underestimated the influence of polyamines rather than negating a major effect of these compounds in breast cancer phenotype. Additional studies aimed at evaluating the biological importance of other enzymes (particularly S-adenosylmethionine decarboxylase and spermidine/spermine N-1-acetyltransferase) and the possible interactions between the polyamine pathway and other oncogenic signals are necessary to fully assess the importance of polyamines in the biologic behavior of breast cancer.

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