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POLYAMINE INVOLVEMENT IN BASAL AND ESTRADIOL-STIMULATED INSULIN-LIKE GROWTH FACTOR I SECRETION AND ACTION IN BREAST CANCER CELLS IN CULTURE

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Summary—Recent evidence indicates that the polyamine pathway may play a significant role in the autocrine/paracrine control of breast cancer cell proliferation by hormones. To directly test this hypothesis, in the present experiments, we evaluated the polyamine involvement in immunoactive insulin-like growth factor I (IGF-I) secretion and IGF-I action using MCF-7 breast cancer cells cultured in serum-free medium in the presence and absence of estradiol (E_2). Administration of the polyamine biosynthetic inhibitor, α-difluoromethylornithine (DFMO) induced a marked suppression of cellular ornithine decarboxylase (ODC) activity and polyamine levels which was associated with significant, although partial, inhibition of E_2-stimulated growth. Exogenous putrescine administration repleted cellular polyamine pools and completely reversed the growth-inhibitory effect of DFMO. Despite these parallel changes in polyamine levels and proliferative activity, basal as well as E_2-stimulated levels of immunoactive IGF-I measured in the conditioned media were unaffected by DFMO with and without exogenous putrescine administration. On the other hand, induction of polyamine depletion and repletion by the same treatments significantly (although partially) affected the proliferative action of exogenously added IGF-I. These findings indicate that polyamines, while not involved in immunoactive IGF-I production, play an important role, at least in part, in IGF-I action in this experimental system. Furthermore, we observed that the administration of a monoclonal antibody directed against IGF-I was able to partially block basal as well as E_2-stimulated MCF-7 cell proliferation. We conclude that immunoactive IGF-I is an important but not sole mediator of MCF-7 breast cancer growth under our experimental conditions. The polyamine pathway plays an important role inthe expresion of its proliferative action.

INTRODUCTION

Insulin-like growth factor I (IGF-I) is one of multiple growth factors recently postulated to be involved in the control of breast cancer cell proliferation [1]. In the hormone-responsive MCF-7 human breast cancer cell line, the production of immunoactive IGF-I has been found to be hormonally regulated and tightly coupled to the endocrine control of cell proliferation [2]. In contrast, hormone-independent breast cancer cells constitutively secrete higher levels of immunoactive IGF-I, thus obviating the need for hormonal stimulation [3]. Additional evidence supporting an important role of IGF-I in tumor growth is provided by the finding that breast cancer cells have IGF-I receptors [4–6] and can be growth-stimulated by exogenous IGF-I administration [2–4, 7, 8]. Furthermore, the estrogen requirement for tumor formation in nude mice by MCF-7 cells can be partially replaced by IGF-I infusion [9]. Recent data, however, raise serious doubts on the authenticity of the IGF-I produced by breast cancer cells in culture [10]. Under these experimental conditions, it appears that IGF-I immunoactivity can be largely accounted for by IGF-I binding proteins [11–14] and possibly an "IGF-I-related peptide" closely related to authentic IGF-I [3, 14].

Using *N*-nitrosomethylurea (NMU)-induced rat mammary tumors grown in soft agar, we have initially shown that polyamines are important mediators of hormonal effects on colony formation [15, 16]. Subsequently, other laboratories have extended our observations to

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demonstrate a significant role of polyamines in the estradiol-induced proliferation of human breast cancer cell lines in liquid culture [17, 18]. Indirect evidence obtained by us in the soft agar clonogenic assay indicates that the polyamine pathway closely interacts with the autocrine/paracrine control of tumor growth, being possibly involved both in the synthesis [19, 20] and action [21, 22] of hormonally regulated growth factors. The present experiments were designed to directly test this hypothesis using the MCF-7 breast cancer cell line. The secretion of immunoactive IGF-I and the action of exogenously added genuine IGF-I were evaluated under conditions of cellular polyamine depletion and repletion. In addition, the biological significance of immunoactive IGF-I in this experimental system was further tested by evaluating the effect on basal and E_2-stimulated cell proliferation of a monoclonal antibody directed against this polypeptide.

EXPERIMENTAL

Chemicals

DFMO was kindly supplied by Merrell Dow Research Institute, Merrell Dow Pharmaceuticals Inc., Cincinnati, Ohio. Insulin-like growth factor I (IGF-I), threonine-59 was purchased from Amgen Biologicals, Thousand Oaks, Calif. (cat. No. 04010). L-[1-^{14}C]ornithine (54.3 mCi/mmol) was obtained from New England Nuclear Research Products, Boston, Mass. Richter's Improved Minimal Essential Medium without putrescine, with Phenol Red (cat. No. 87-5060 EC) and without Phenol Red (cat. No. 87-5059 EC) was specially ordered from Grand Island Biological Co., Grand Island, N.Y. Fetal bovine serum was purchased from Irvine Scientific, Santa Ana, Calif. The monoclonal antibody (IgG_1, Kappa) to IGF-I was a generous gift from Dr Judson J. Van Wyck (University of North Carolina, Chapel Hill, N.C.). The properties of this antibody have been recently reported in detail [23, 24]. E_2, putrescine dihydrochloride, L-ornithine hydrochloride, IgG_1, kappa methylbenzethonium hydroxide, pyridoxal-5′-phosphate, (−)-1,4-dithio-L-threitol and mouse IgG_1 Kappa (MOPC-21) were obtained from Sigma Chemical Co., St Louis, Mo.

Cells and cell culture conditions

The MCF-7 human breast cancer cell line was kindly provided by Dr M. E. Lippman (NIH, Bethesda, Md) and was routinely grown in 75 cm^2 flasks in Richter's Improved Minimal Essential Medium without putrescine (IMEM) containing Phenol Red and 10% fetal bovine serum in a humidified atmosphere of 95% air : 5% CO_2 at 37°C. At the time of the experiment, cells were harvested by brief treatment with 0.05% trypsin in versene and plated at a density of 5×10^5 cells/100 mm Petri dish (6 dishes/experimental condition). Cells were allowed to attach for 30 h. The medium was then discarded and serum-free medium was added (4 mM glutamine, 0.2 mg% transferrin, 0.1 mg% fibronectin, 0.4 g% Fraction V BSA, 20 mM Hepes buffer in IMEM without Phenol Red). After a wash-out period of 18 h, the medium was discarded and fresh serum-free medium containing the experimental treatments or ethanol vehicle in 0.1% final concentration was added (day 0). The duration of treatment was 4 days with a medium change on day 2. Conditioned media from all 6 dishes/experimental group were collected at the time of the medium change and the cells from 2 dishes were harvested for determination of cell number and DNA content. Conditioned media from the remaining 4 dishes were again collected on day 4 and, after brief trypsinization, the cell suspensions were divided into 3 aliquots. Two were immediately placed on ice and processed for measurements of ODC activity and polyamine pools while the third was used for measurement of DNA and cell number. DNA content was measured by the diphenylamine assay [25] and cell number was determined using a hemacytometer. Cell viability (>90%) was assessed by trypan blue exclusion.

Processing of the cell suspensions for measurement of ODC activity and polyamines

All procedures were performed at 0–4°C. Cell suspensions were centrifuged at 800 *g* for 15 min and washed with ice-cold PBS. The cell pellets were then resuspended to give a final concentration of 2×10^7 cells/ml. For the polyamine assay, the cells were resuspended in 0.6 N $HClO_4$. After 30 min on ice, the suspension was centrifuged at 800 *g* for 15 min and the supernatant stored at −70°C until the time of the assay. For assessment of ODC activity, the cells were resuspended in buffer containing 0.1 mM EDTA, 2 mM dithiothreitol and 5 mM NaH_2PO_4, pH 7.4, and stored at −70°C until use.

Polyamine assay

Polyamines were determined by fluorometry following a modification of the method described by Seiler and Knodgen [26]. Following separation of putrescine, spermidine and spermine by high-pressure liquid chromatography, the polyamines were derivatized with the fluorochrome *O*-phtaldehyde and then subjected to fluorescence detection using an excitation wavelength of 340 nm and an emission wavelength of 455 nm. Calculations were performed using known pure standard preparations of putrescine, spermidine and spermine and a reference internal standard 1.7 diaminoheptane. Results are reported as nmol/10^6 cells. Interassay precision for all 3 polyamines averaged 13%.

Measurement of ODC activity

At the time of assay, the cell suspensions were thawed and sonicated for 20 s. The cell lysates were centrifuged at 100,000 *g* for 30 min. Enzyme activity was determined in the cytosolic fraction according to the method described by Pegg *et al.* [27]. Briefly, the reaction starts by adding 0.1 ml of cytosol to a vessel containing 0.1 ml of the incubation buffer (0.08 mM pyridoxal-5′-phosphate, 5 mM dithiothreitol, 0.8 mM L-ornithine and 0.2 μCi L-[^{14}C]ornithine in 100 mM Tris–HCl, pH 7.0). The vessels were capped with rubber stoppers containing center wells with 0.15 ml of methylbenzethonium hydroxide and the incubation was carried out at 37°C for 1 h. The reaction was stopped by injection of 0.3 ml of 40% trichloroacetic acid through the rubber septum directly into the reaction mixture. The incubation was continued for an additional 30 min period. The wells were then transferred to polystyrene vials and the radioactivity was counted in 2.5 ml of scintillation fluid [4 g Omnifluor (New England Nuclear Res. Products, Boston, Mass)/liter of toluene]. ODC activity was expressed as pmol CO_2/mg protein/h. Protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad Lab, Richmond, Calif.) using bovine plasma albumin as a standard.

Preparation of conditioned media

Serum-free conditioned media obtained as described above were combined with 0.2% (v/v) aprotinin and centrifuged at 800 *g* at 4°C for 15 min. The supernatant was concentrated 5–8-fold in an Amicon ultrafiltration cell using an YM5 membrane, M_r 5000 cutoff (Amicon Corporation, Danvers, Mass), frozen at −70°C and then lyophilized.

IGF-I RIA

The lyophilized samples were reconstituted in appropriate buffer and IGF-I was determined using a somatomedin-C radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, Calif.) according to the manufacturer's instructions. In this experimental system, acid ethanol extraction has been shown to separate IGF-I from its binding protein [3]. However, this procedure has not been found to substantially alter IGF-I determinations under different experimental conditions [2]. Consequently, we performed our measurements in unextracted samples.

Experiments using monoclonal antibodies against IGF-I

8 × 10^4 MCF-7 cells were plated in triplicate 35 mm Petri dishes. Culture conditions were as specified before. All treatments were added on day 0 and on day 2 at the time of the medium change with the exception of the anti-IGF-I and irrelevant antibodies which were added daily. Cell number and DNA were determined after 4 days of treatment under serum-free conditions.

Statistical analysis

The effect of time on each treatment was estimated by 3-way analysis of variance (experiment as a block effect) followed by *t*-tests comparing day 2 vs day 4 by experimental groups. Within day 2 and 4, differences among treatments were evaluated by 2-way analysis of variance (experiment as a block effect) followed by the Duncan's multiple range test.

RESULTS

DFMO effects on growth, ODC activity, polyamine pools and immunoactive IGF-I production

E_2 administration significantly increased cellular DNA content to 136 ± 4% of control on day 2 and to 174 ± 8% of control on day 4 (Fig. 1). E_2-stimulated growth was not affected by increasing concentrations of DFMO on day 2 but a dose-dependent inhibition of proliferation was apparent on day 4 of treatment. However, even the highest concentration of DFMO tested (4 mM) was only able to partially

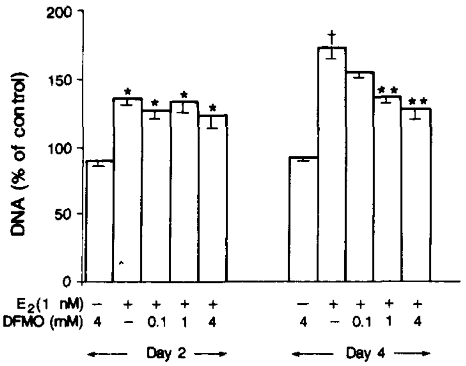

Fig. 1. Effect of E_2 and/or DFMO administration on MCF-7 breast cancer cell proliferation. MCF-7 cells grown in serum-free medium (see Materials and methods for details) were harvested after 2 or 4 days of treatment and the DNA content was determined. Data represent mean ± SEM of 3 replicate experiments and are expressed as percentage of control. DNA content in control groups was 33.3 ± 6.4 and 59.4 ± 20.5 μg/dish on day 2 and day 4, respectively. $^*P < 0.05$ vs control and 4 mM DFMO (day 2); $^{**}P < 0.05$ vs control, 4 mM DFMO and E_2 (day 4); $\dagger P < 0.05$ vs E_2 (day 2).

block E_2-stimulated growth (Fig. 1). Under our stepped-down culture conditions (i.e. absence of serum and Phenol Red), DFMO administration did not significantly inhibit MCF-7 cell growth either on day 2 or on day 4 of treatment (Fig. 1). Similar results were obtained when the data were expressed by cell number instead of DNA (not shown). Figure 2 depicts the cellular levels of ODC activity under our experimental conditions. Enzyme activity was similar in control and E_2-treated cells. Since, however, the measurement was performed 48 h after the last medium change, it is likely that we missed the E_2-stimulation of ODC activity which has been reported to occur 5–10 h after estrogen administration [18]. As can be seen in Fig. 2, DFMO administration exerted a profound dose-dependent inhibitory effect on ODC activity which was nearly complete at the 4 mM concentration both in the presence and in the absence of E_2. In agreement with this finding, DFMO markedly lowered the cellular levels of putrescine, spermidine and the spermidine:spermine ratio (Table 1) which has been reported to be positively correlated with proliferative activity [28, 29]. The DFMO effect on tumor polyamine pools was actually significantly greater in the presence than in the absence of E_2 (Table 1).

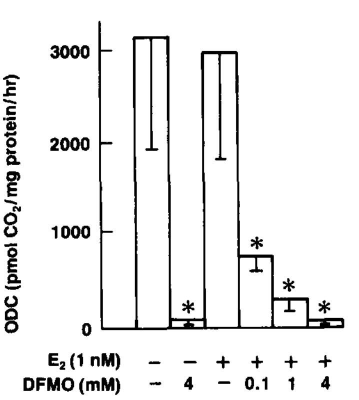

Fig. 2. Effect of DFMO on ODC activity of MCF-7 cells treated with or without E_2 for 4 days. Data represent mean ± SEM of the same 3 replicate experiments shown in Fig. 1. The asterisks denote significant differences ($P < 0.05$) from control and E_2.

As previously reported in MCF-7 cells cultured in the absence of Phenol Red [2], E_2 stimulated immunoactive IGF-I secretion (Fig. 3) even though, in these experiments, the E_2 effect was significant only on day 2. However, while having a suppressive effect on proliferation (Fig. 1), ODC activity (Fig. 2) and polyamine pools (Table 1), DFMO administration did not affect basal or E_2-stimulated immunoactive IGF-I secretion after either 2 or 4 days of treatment.

Reversal of DFMO effects with exogenous putrescine administration

In agreement with our previous experiment (Fig. 1), DFMO administration did not affect E_2-stimulated growth after 2 days of treatment (Fig. 4). Addition of putrescine to E_2 and DFMO-treated cells also did not modify cell proliferation at this time point. However, exogenous putrescine administration completely reversed in a dose-dependent fashion the partial inhibitory effect of DFMO on E_2-stimulated growth observed on day 4 (Fig. 4). Administration of putrescine alone did not affect cell proliferation on either day 2 or 4 (Fig. 4). Similar results were observed when the data were expressed in terms of cell number instead of DNA (not shown).

In parallel with the reversal of the inhibitory effect of DFMO on E_2-induced proliferation, exogenous putrescine administration repleted in a dose-dependent fashion the cellular level of this polyamine to values actually in excess of those observed in cells treated with E_2 alone (Table 2). Cellular levels of spermidine as well as spermidine:spermine ratios were also raised after exogenous putrescine administration (Table 2).

Figure 5 depicts the effect of manipulation of the polyamine pathway with DFMO and/or exogenous putrescine administration on immunoactive IGF-I secretion by MCF-7 cells. In

Table 1. Effect of DFMO and/or estradiol administration on polyamine pools of MCF-7 breast cancer cells

Treatment		Polyamines (nmol/10^6 cells)			
E_2	DFMO	Putrescine	Spermidine	Spermine	Spermidine/Spermine
0	0	0.48 ± 0.02	1.01 ± 0.08	1.50 ± 0.04	0.67 ± 0.04
0	4 mM	ND^a	0.31 ± 0.02^b	1.89 ± 0.07^b	0.16 ± 0.01^b
1 nM	0	0.46 ± 0.08	1.27 ± 0.07^b	1.38 ± 0.10	0.92 ± 0.01^b
1 nM	0.1 mM	ND^a	0.11 ± 0.06^c	1.69 ± 0.23^d	0.06 ± 0.03^c
1 nM	1 mM	ND^a	0.04 ± 0.04^c	1.77 ± 0.11^d	0.02 ± 0.02^c
1 nM	4 mM	ND^a	0.04 ± 0.04^c	1.66 ± 0.13	0.02 ± 0.02^c

^{a}ND = not detectable; $^bP < 0.05$ vs control; $^cP < 0.05$ vs the remaining groups; $^dP < 0.05$ vs E_2.

agreement with our previous experiment (Fig. 2), DFMO did not affect E_2-stimulated immunoactive IGF-I secretion at either day 2 or 4 (Fig. 5). Exogenous putrescine administration to either untreated cells or cells exposed to E_2 and DFMO also failed to influence immunoactive IGF-I levels measured in the conditioned media at both time points (Fig. 5).

Effects of DFMO in the presence and absence of exogenous putrescine administration on IGF-I regulation of proliferation, ODC activity and polyamine pools

Our results show that, at least under these culture conditions, polyamines are not involved in basal or E_2-stimulated immunoactive IGF-I secretion. However, we could not rule out a more distal effect of polyamines on IGF-I action. Therefore, we deemed it important to determine whether manipulation of the polyamine pathway would affect the action of exogenously added IGF-I. In these experiments, we used a concentration of IGF-I of 10 ng/ml which has been previously shown to have maximal proliferative activity in this system [3]. Figure 6 shows that on day 4 IGF-I stimulated cell proliferation to a greater extent than E_2, simultaneously tested for comparison. As we have previously shown for E_2, DFMO administration partially blocked the IGF-I stimulation of growth observed on day 4, while putrescine addition completely reversed the DFMO effect (Fig. 6). In these experiments, DFMO also significantly inhibited IGF-I-stimulated growth on day 2, while putrescine administration reversed its effects (Fig. 6). Similar results were obtained when data were expressed in terms of cell number (not shown). Concomitantly with its stimulatory effect on proliferation, IGF-I administration increased ODC activity (Fig. 7) and cellular polyamine pools (Table 3). Of interest, in these experiments, E_2 was as effective as IGF-I in increasing cellular polyamine levels (Table 3). In our previous experiments, however (Tables 1 and 2), the E_2 effect in this regard was less pronounced. The reason for the variability

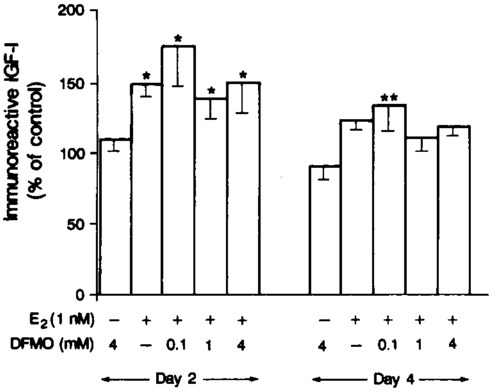

Fig. 3. IGF-I immunoactivity present in the conditioned media of MCF-7 cells treated for 2 or 4 days with DFMO and/or E_2. Data represent mean ± SEM of the same 3 replicate experiments shown in Fig. 1 and are expressed as percentage of control. Immunoreactive IGF-I levels in control groups were 113.3 ± 24.4 and 100.6 ± 2.4 ng/mg DNA on day 2 and day 4, respectively. *$P < 0.05$ vs control and 4 mM DFMO (day 2); **$P < 0.05$ vs control and 4 mM DFMO (day 4).

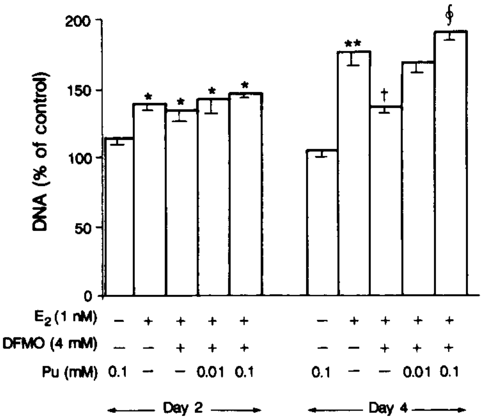

Fig. 4. Effect of DFMO with and without exogenous putrescine administration on E_2-stimulated MCF-7 cell proliferation. All treatments were added on day 0. Data represent mean ± SEM of 3 replicate experiments and are expressed as percentage of control. DNA content in the control groups was 33.6 ± 0.7 and 55.0 ± 4.8 µg/dish on day 2 and day 4, respectively. *$P < 0.05$ vs control and 0.1 mM Pu (day 2); **$P < 0.05$ vs control (day 4) and E_2 (day 2); †significantly different from each of the remaining groups (day 4); § $P < 0.05$ vs E_2 (day 4).

Table 2. Effect of DFMO with and without exogenous putrescine administration on polyamine pools of E_2-treated MCF-7 cells

Treatment			Polyamines (nmol/10⁶ cells)			
E_2	DFMO	Putrescine	Putrescine	Spermidine	Spermine	Spermidine/spermine
0	0	0	0.32 ± 0.03	0.84 ± 0.04	1.41 ± 0.04	0.59 ± 0.01
0	0	0.1 mM	1.08 ± 0.03^b	1.03 ± 0.01^b	1.39 ± 0.02	0.74 ± 0.01^b
1 nM	0	0	0.42 ± 0.06^b	1.27 ± 0.05^b	1.54 ± 0.02^b	0.82 ± 0.03^b
1 nM	4 mM	0	$ND^{a,c}$	0.06 ± 0.03^c	1.60 ± 0.02^b	0.03 ± 0.02^b
1 nM	4 mM	0.01 mM	0.45 ± 0.03	1.14 ± 0.02^d	1.41 ± 0.04^d	0.81 ± 0.01^d
1 nM	4 mM	0.1 mM	0.86 ± 0.04^c	1.12 ± 0.06^d	1.21 ± 0.02^d	0.93 ± 0.03^d

[a] ND: not detectable, $P < 0.05$ vs the remaining groups;
[b] $P < 0.05$ vs control;
[c] $P < 0.05$ vs E_2;
[d] $P < 0.05$ vs E_2 + 4 mM DFMO.

in the degree of E_2-induced activation of the polyamine pathway in our system is not apparent. In any event, as in the case of E_2, DFMO administration markedly suppressed ODC activity (Fig. 7) and polyamine pools (Table 3) in IGF-I treated cells, while exogenous putrescine administration restored polyamine levels (Table 3) but, as expected, not ODC activity (Fig. 7). These results indicate that polyamines are necessary, at least in part, for the expression of the stimulatory effect of IGF-I on MCF-7 cell growth under the present experimental conditions.

Effect of anti-IGF-I monoclonal antibody on MCF-7 breast cancer growth

In these experiments, we utilized a monoclonal antibody directed against IGF-I to probe the biological importance of the endogenously produced polypeptide in MCF-7 breast cancer cell proliferation. In preliminary experiments (not shown) we observed that 6 μg/ml concentration of this antibody completely blocked the growth-stimulating effect of IGF-I (10 ng/ml) in our system. In agreement with our previous experiments, DFMO administration partially blocked E_2-stimulated proliferation, while exogenous putrescine addition completely reversed the DFMO effect (Table 4). Administration of the anti-IGF-I monoclonal antibody significantly inhibited proliferation in control cells as well as cells treated with E_2 and those rescued with putrescine (Table 4). In these two latter groups, 3 μg/ml concentration of the antibody exerted maximum antiproliferative effect which was similar to that observed with the 6 μg/ml dose in control cells. In contrast, the irrelevant antibody did not affect growth under any experimental condition tested (Table 4).

DISCUSSION

Polyamines are well known to be involved in normal and neoplastic cell proliferation in

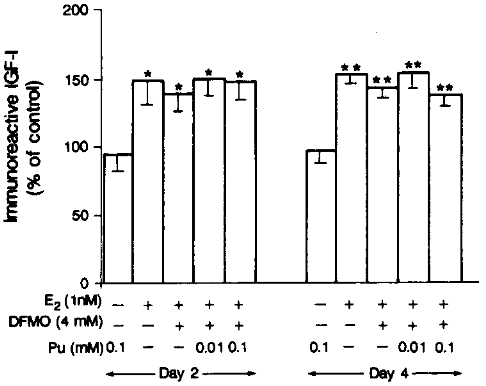

Fig. 5. IGF-I immunoactivity present in the conditioned media of MCF-7 cells treated with E_2 with and without DFMO and/or putrescine. Data represent mean ± SEM of the same 3 replicate experiments shown in Fig. 4 and are expressed as percentage of control. Immunoreactive IGF-I in the control groups was 98.8 ± 10.1 and 81.4 ± 2.9 ng/mg DNA on day 2 and day 4, respectively. *$P < 0.05$ vs control and 0.1 mM Pu (day 2); **$P < 0.05$ vs control and 0.1 mM Pu (day 4).

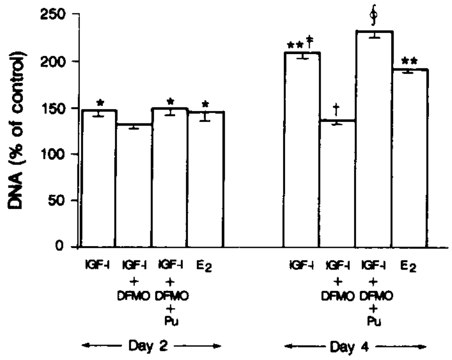

Fig. 6. Effect of DFMO (4 mM) in the presence or absence of exogenous putrescine administration (0.1 mM) on IGF-I (10 ng/ml) stimulated proliferation of MCF-7 cells. E_2 (1 nM) was simultaneously tested for comparison. Growth conditions were as specified in Materials and Methods. Treatment was continued for 2 or 4 days. Data represent mean ± SEM of 3 replicate experiments. DNA content in control groups was 31.6 ± 2.7 and 48.9 ± 3.1 on days 2 and 4, respectively. *$P < 0.05$ vs control and IGF-I + DFMO (day 2); **$P < 0.05$ vs each of the remaining groups (day 4) and the same treatment groups (day 2); †$P < 0.05$ vs control (day 4); ‡ $P < 0.05$ vs E_2 (day 4); § $P < 0.05$ vs IGF-I (day 4).

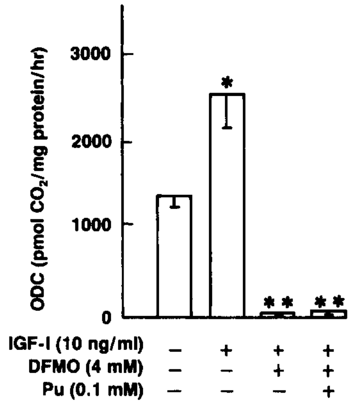

Fig. 7. Effect of DFMO in the presence and absence of exogenous putrescine administration on ODC activity of MCF-7 cells treated with IGF-I for 4 days. Data represent mean ± SEM of the same 3 replicate experiments shown in Fig. 6. $*P < 0.05$ vs all other groups; $**P < 0.05$ vs the remaining 2 groups.

numerous experimental systems [30]. Our own, as well as other laboratories, have now clearly shown that these compounds are important mediators of hormonal proliferative effects in experimental and human breast cancer cells in culture [15–18]. However, the specific mechanism(s) by which polyamines mediate the mitogenic action of hormones in these systems is not known. Our interest has recently focused on the interaction between the polyamine pathway and the autocrine/paracrine mechanisms of growth control using the NMU mammary tumor in the soft agar clonogenic assay. Our results have suggested that both the synthesis [19, 20] as well as the action [21, 22] of hormonally regulated growth factors may be influenced by polyamines. Due, however, to the inability to measure cellular polyamine levels and quantitate growth factor production in the soft agar system, our conclusions have only been supported by indirect data.

Consequently, we recently focused our efforts on human breast cancer cell lines in liquid culture where the relationship between polyamines and growth factor secretion/action can be more directly and quantitatively assessed. In this manuscript, we concentrated our attention on polyamines and immunoactive IGF-I, a polypeptide growth factor recently postulated to play a major role in breast cancer growth [2, 3]. It should be noted that the precise nature of the IGF-I immunoactivity measured in the conditioned media is not yet fully defined. Although previous experiments showed the presence of IGF-I mRNA by Northern analysis [3], more recent studies using a specific ribonuclease protection assay have failed to show such message in human breast cancer cell lines [10]. Both we [11] as well as other investigators [12–14] have shown that, following chromatography, a significant fraction of IGF-I immunoactivity elutes in the high molecular weight region. This activity appears to be accounted for by IGF-I binding proteins [12–14], which are known to interfere in the IGF-I radioimmunoassay [31]. Of interest, recent evidence indicates that these proteins may not simply serve a transport function but may play a key role in the control of cell proliferation [32].

In addition to the high molecular weight IGF-I immunoactivity, a low molecular weight fraction comigrating with authentic IGF-I has also been observed in conditioned media obtained from breast cancer cells in culture [3, 14]. The biochemical nature of such "IGF-I-related peptide" is presently unknown but could be closely related to authentic IGF-I and account for the transcripts detected on IGF-I Northern analysis of breast cancer cells [3]. The present studies, however, were not aimed at the characterization of immunoactive IGF-I but rather at the regulation of its secretion by polyamines and evaluation of its role in the control of MCF-7 breast cancer cell proliferation.

Our results clearly show that DFMO administration was able to optimally suppress in a dose-dependent fashion cellular ODC activity as

Table 3. Effect of DFMO (4 mM) with and without Putrescine (0.1 mM) administration on polyamine pools of IGF-I (10 ng/ml) treated MCF-7 cells in culture

	Polyamines (nmol/10^6 cells)			
Treatment	Putrescine	Spermidine	Spermine	Spermidine/spermine
Control	0.15 ± 0.08	0.59 ± 0.03	0.87 ± 0.11	0.69 ± 0.04
IGF-I	0.32 ± 0.06[e]	1.02 ± 0.09[d]	0.94 ± 0.09	1.09 ± 0.02[d]
IGF-I + DFMO	ND[b]	ND[b]	0.94 ± 0.02	ND[b]
IGF-I + DFMO + Pu	0.71 ± 0.10[c]	1.08 ± 0.04[d]	0.95 ± 0.09	1.15 ± 0.08[d]
E_2^a	0.32 ± 0.05[e]	1.11 ± 0.08[d]	0.81 ± 0.15	1.44 ± 0.24[d]

[a] E_2 (10^{-9} M) was simultaneously added for comparison;
[b] ND = not detectable, $P < 0.05$ vs IGF-I;
[c] $P < 0.05$ vs all the other groups;
[d] $P < 0.05$ vs control;
[e] $0.1 > P > 0.05$ vs control.

Table 4. Effects of the anti-IGF-I monoclonal antibody as well as irrelevant antibody on MCF-7 cell proliferation[a]

Treatment	No antibody	Monoclonal anti-IGF-I IgG_1, kappa (3 μg/ml)	Monoclonal anti-IGF-I IgG_1, kappa (6 μg/ml)	Mouse IgG_1, kappa (6 μg/ml)
Control	$(3.64 \pm 0.24) \cdot 10^5$	$(3.11 \pm 0.56) \cdot 10^5$	$(2.68 \pm 0.34) \cdot 10^{5*}$	$(3.57 \pm 0.24) \cdot 10^5$
E_2 (1 nM)	$(6.52 \pm 0.35) \cdot 10^5$	$(4.60 \pm 0.43) \cdot 10^{5*}$	$(4.57 \pm 0.50) \cdot 10^{5*}$	$(6.26 \pm 0.30) \cdot 10^5$
E_2 + DFMO (4 mM)	$(4.94 \pm 0.16) \cdot 10^5$	—	—	—
E_2 + DFMO + Pu (0.1 mM)	$(6.82 \pm 0.46) \cdot 10^5$	$(5.36 \pm 0.46) \cdot 10^{5*}$	$(5.13 \pm 0.43) \cdot 10^{5*}$	$(6.53 \pm 0.23) \cdot 10^5$

[a] MCF-7 cells were plated under serum free media conditions (see Experimental for details) in the presence of the indicated treatments. Data represent cell number after 4 days in culture.

*$P < 0.05$ vs growth in the absence of the anti-IGF-I antibody or in the presence of the irrelevant antibody

well as putrescine and spermidine levels. In parallel with these effects on the polyamine pathway, DFMO partially blocked in a dose-dependent manner E_2-stimulated growth. Exogenous putrescine administration repleted cellular polyamine pools and reversed the DFMO effect, thus totally restoring E_2-stimulated growth. Despite, however, these parallel changes in polyamine pools and proliferative activity, neither basal nor E_2-stimulated immunoactive IGF-I secretion was affected by DFMO with and without exogenous putrescine administration. Taken together, our data do not support a significant role of polyamines in the production of IGF-I immunoactivity. In our experiments, however, the increase in immunoactive IGF-I measured in the conditioned media following E_2 administration was less than previously reported by other investigators [2]. Thus, our findings may not necessarily apply to conditions of more optimal stimulation of immunoactive IGF-I production by E_2. Our results, however, indicate that the polyamines are involved in IGF-I action since the proliferative effect of exogenous IGF-I administration was partially blocked by DFMO and restored by putrescine addition. Clearly, however, the role of polyamines in this regard may not be unique and may simply reflect the general need of these cells for polyamines to grow following exposure to any mitogens.

Our findings provide support for an autocrine role of immunoactive IGF-I in MCF-7 breast cancer growth. We observed, in fact, that, under serum-free media conditions, the administration of a monoclonal antibody directed against IGF-I was able to partially block basal as well as E_2-stimulated proliferation. Our data are in agreement with the recent report by Rohlik *et al.* [33] who obtained similar results using a monoclonal antibody to the IGF-I receptor. Since, however, their experiments were conducted in the presence of serum which contain small amounts of IGF-I, an autocrine role for IGF-I could not definitively be established in their study.

Overall, our data emphasize a significant role of polyamines in E_2-stimulated growth of MCF-7 breast cancer cells as also reported by Kendra and Katzenellenbogen [18]. A partial blockade of E_2-promoted proliferation with DFMO administration was also observed by these investigators [18] who, however, did not measure cellular polyamine pools under their experimental conditions. Under our serum-free media conditions, cellular levels of putrescine and spermidine were nearly completely suppressed by DFMO administration in E_2-treated cells. Spermine levels, on the other hand, were slightly increased as already reported in other experimental systems in the presence of DFMO treatment [34–36]. It is conceivable that the preserved spermine pools may account for the only partial blockade of E_2 effect by DFMO. It would be of interest to determine whether a more complete blockade may be achievable with the newly introduced polyamine analogs which have been found to suppress spermine levels [37]. It is conceivable that under conditions of more optimal suppression of cellular polyamine levels and E_2-stimulated growth by such analogs IGF-I production may also be affected. Alternatively, E_2 effects in this experimental system may be partially independent of the polyamine pathway. Of interest, no inhibition of E_2-stimulated growth was observed after only 2 days of treatment. Since we did not measure polyamine pools at this early time point, our data do not allow us to establish whether this finding was due to a delay in suppression of polyamine biosynthesis, by DFMO or, more likely, a lag time between lowering of polyamine pools and inhibition of proliferation.

DFMO administration did not significantly inhibit basal MCF-7 cell proliferation (Fig. 1). In agreement with this finding, the suppression of spermidine and spermidine:spermine ratio by 4 mM DFMO was also significantly less compared to that observed in E_2-treated cells (Table 1). A much more dramatic antiproliferative effect of DFMO on basal MCF-7 cell growth has recently been reported by us under serum-repleted conditons [38]. A similar marked influence of serum on DFMO sensitivity of breast cancer cells has also been observed by us under conditions of anchorage-independent growth using the NMU-rat mammary tumor [19, 39].

Finally, our data indicate that both E_2 and IGF-I stimulate polyamine biosynthesis in our system, even though the magnitude of the E_2 effect was variable from experiment to experiment. Whether, however, the E_2/IGF-I-induced rise in polyamine pools is instrumental in the stimulation of proliferation by these compounds or whether basal polyamine levels simply play a permissive role in these mitogenic effects cannot be established by our data. We observed, in fact, that even the lowest concentration of DFMO tested (0.1 mM) suppressed the levels of putrescine and spermidine and the spermidine:spermine ratio to values well below control (Table 1). A more selective inhibition of just the E_2/IGF-I-induced alterations in the polyamine milieu is necessary before the individual roles of basal vs stimulated polyamine pools can be established.

In summary, our data indicate that E_2 stimulates immunoactive IGF-I production by MCF-7 cells grown in the absence of Phenol Red as already reported by other investigators [2]. The polyamine pathway does not appear to be involved in immunoactive IGF-I synthesis but seems to play an important role, at least in part, in IGF-I action in our experimental system. The partial inhibition of basal and E_2-stimulated MCF-7 cell growth observed with the addition of a monoclonal antibody to IGF-I indicates that this polypeptide is an important but not sole mediator of growth in this system. The role of other growth factors as well as their interaction with the polyamine pathway is currently under investigation.

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