THE ROLE OF POLYAMINES IN HORMONAL REGULATION OF HUMAN BREAST CANCER CELLS

by

Grace Lima

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ABSTRACT

Polyamines are essential for the differentiation and proliferation of many cell types. The role of polyamines in hormone-induced biological actions in human breast cancer cells has not been determined. The pituitary hormone, human growth hormone (hGH, 0.1-1 ug/ml), in the presence of hydrocortisone (H, 1 ug/ml), produced a maximum 3-fold stimulation in the activity of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine synthesis, in a human breast cancer cell line (T-47D). Of many pituitary hormones tested, only human prolactin can substitute for hGH in the induction of ODC. The observation confirms previous findings that these two "lactogenic" polypeptide hormones bind to the same cell surface receptors and produce the same biological responses in the human breast cancer cells. hGH and H have been previously demonstrated to induce the synthesis of a 14K glycoprotein and the alteration in cell shape of T-47D cancer cells. Inhibition of ODC by α-difluoromethylornithine (DFMO, 0.1 mM), a specific and irreversible inhibitor of the enzyme, abolished the hormone-induced synthesis of the 14K protein. This inhibition could be reversed by putrescine (0.1 mM), the natural product of ODC. However, DFMO was unable to reverse the hormone-induced shape alteration of the cells. These observations

suggest that hGH can induce several biological actions in human breast cancer cells, but not all of its actions are mediated by polyamines. The present study also explored the possible role of polyamines in estrogen-induced proliferation of an estrogen receptor-positive subline of T-47D human breast cancer cells (clone 11). 17β -estradiol (E_2, 10^{-10}M) stimulated cell proliferation 3-fold above control values in a 6-day assay. This estradiol-stimulated cell proliferation was associated with increased ODC levels. Twelve hours after E_2 stimulation, ODC activity was 2-fold higher than that in unstimulated controls. Estradiol-stimulated cell proliferation and ODC activity was noted only when 3-10% charcoal-treated, heat-inactivated fetal bovine serum was used in the support medium. This finding suggests that specific serum factors are required for estrogen action. DFMO (0.1 mM) blocked the estradiol- induced ODC activity and proliferation of clone 11 cells. The specificity of the DFMO effect was indicated by the recovery of cell growth with exogenous putrescine (0.1 mM). In the absence of DFMO, a combination of estradiol and putrescine, however, did not further stimulate growth above that seen with estradiol alone. Also, putrescine alone did not stimulate the growth of T-47D clone 11 cells. These results suggest that polyamines are essential, though not sufficient, in the mitogenic action of estrogen in human breast cancer cells.

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LIST OF ABBREVIATIONS

Hormones and growth factors

Prl	prolactin
GH	growth hormone
E_2	17β -estradiol
H	hydrocortisone
I	insulin
T_3	triiodothyronine
EGF	epidermal growth factor
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
NSILA	nonsuppressible insulin-like activity

Hormone prefixes

h	human
o	ovine
b	bovine

Units of measure

ng	nanogram
ug	microgram
mg	milligram
g	gram
ul	microlitre
ml	millilitre
l	litre

mm	millimetre
cm	centimetre
nM	nanomolar
uM	micromolar
mM	millimolar
M	molar
i.u.	international units
uCi	microcurie
mCi	millicurie
mA	milliamperes
MW	molecular weight
w/v	weight per volume
v/v	volume per volume

Others

AMP	adenosine monophosphate
BCNU	1,3-bis (2-chloroethyl)-1-nitrosourea
BSA	bovine serum albumin
cFBS	charcoal-treated fetal bovine serum
CM	complete medium
^{o}C	degrees centigrade
CPM	counts per minute
DFMO	α-difluoromethylornithine
DMBA	7, 12-dimethyl-1,2-benz-(a)-anthracene
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide

DNA	deoxyribonucleic acid
DPM	decompositions per minute
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
GMP	guanosine monophosphate
HBSS	Hank's balanced salt solution
mRNA	messenger ribonucleic acid
NMU	N-nitrosomethylurea
ODC	ornithine decarboxylase
PBS	phosphate buffer saline
PLP	5'-pyridoxal phosphate
RNA	ribonucleic acid
RPM	revolutions per minute
S.D.	standard deviation
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N' - -tetramethylethylenediamine

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INTRODUCTION

A. Hormonal influences on breast cancer

Breast cancer is considered a hormone-dependent malignancy and changes in hormonal milieu in which neoplasia is progressing have proved successful in stopping cancerous growth temporarily in some women or slowing it down. Polypeptide hormones such as prolactin (Prl), growth hormone (GH), and insulin, and steroid hormones such as estrogen and progesterone have been implicated in playing a role in breast cancer.

The following is a brief review of the hormonal influences on breast cancer.

1. Prolactin and growth hormone

The importance of prolactin (Prl) in influencing the growth behaviour of experimental rodent breast cancers has been well established (Holdaway and Friesen, 1976; Kim and Furth, 1976; Welsh and Nagasawa, 1977). However, the role of Prl in human breast cancer is still unclear (Nagasawa, 1977). The importance of prolactin in rodent turmorigenesis and the establishment of receptors for human Prl (hPrl) in several human breast cancer cell lines (Shiu, 1979) has led to postulations that this hormone may be involved in the etiology of human breast cancer (Pearson et al., 1969). Human growth hormone (hGH) has been reported to occupy the same receptors sites as hPrl in human breast cancer cells

(Shiu, 1979). Prolactin and growth hormone have similar high binding capacities (K_d= 3.4 x 10^{-10}) (Shiu and Friesen, 1974). The two hormones are equally effective in displacing radiolabeled hPRL from rabbit mammary glands (Shiu, 1973). These observations suggest that hGH may have prolactin-like effects.

Whereas the estrogen dependence of many breast cancers is well established (See section A 2), the role of Prl in human mammary malignancy is not understood. Clinical reports on Prl and GH status of breast cancer patients have generated inconclusive and often contradictory results.

An increase of pituitary growth hormone secretion in 5 to 40% of breast cancer patients has been deduced from the observation of hyperplastic changes of pituitary acidophils (Vorherr, 1980). Emerman et al. (1981) reported increased GH activity in some patients with breast cancer.

Although prolactin has diverse actions in more than 80 different target tissues (Nicoll, 1974), most of the information concerning the mechanism of action of prolactin has been obtained using cultured normal mammary tissues. Only a limited amount of information is available concerning the effects Prl exerts on neoplastic mammary cells.

The current understanding concerning the mechanism of action of prolactin is that it initiates its action at plasma membrane receptors (Rillema, 1982). The message from the

prolactin receptor is transmitted into the cell. For other hormones which work in this manner, various intracellular mediators of hormone action have been identified. These include cyclic nucleotides, prostaglandins, calcium ions, and polyamines (Rillema, 1982). Evidence suggests that several of these may be involved in the actions of prolactin on mammary cells (Rillema, 1980; Rillema, 1975; Russell and McVicker, 1972; Aisbaitt and Barry, 1973; Oka and Perry, 1976; Rillema, 1976 a).

Information regarding the involvement of polyamines in prolactin-stimulated breast cancer cells is limited. Frazier and Costlow (1982) reported elevated ornithine decarboxylase levels in prolactin-stimulated cultured DMBA-induced rat mammary tumor cells. It is, however, not known whether this response is related to prolactin's growth-stimulatory activity or whether it is related to some differentiation function which may be retained by the tumor cells.

2. Estrogens

The relationship of breast cancer and the ovary was first demonstrated by Beatson (1896) who observed beneficial responses in advanced breast cancer patients after bilateral ovariectomy. Whereas breast cancer in postmenopausal women has been related to diminished ovarian estrogen disturbance, the increase in breast cancer incidence after age 60 is thought

to be related to imbalance in adrenal estrogens (de Waard et al., 1964). Because of the long preclinical phase of breast cancer (8-10 years or more), clinical diagnosis may be possible only in the postmenopausal period, although malignant transformation may have occurred much earlier when ovaries were still functioning (Vorherr, 1980).

The mechanism by which ovarian secretory products influence the breast has remained obscure for many years but studies within the last two decades have provided some insight into the mechanism of action of estrogen and other steroid hormones. Although estrogen plays a role in carcinogen-induced tumorigenesis, as well as in growth and metabolic events of the tumor cells, the exact mode of action has not been established. It is presumed that it follows similar mechanisms as observed in normal target tissues. Low-capacity and high-affinity estrogen receptors (ER) are present in estrogen-responsive tumors of both humans and animals (Leung, 1982). Two phases in the mechanism are recognized: (1) after entry of estrogen into the cell binding of the steroid to cytoplasmic recptors and (2) energy-dependent activation and translocation of the cyto-plasmic steroid-receptor complex to the nucleus. Following ER interaction with the acceptor sites in the chromatin, new synthesis of specific mRNA and proteins is initiated. One is able to classify tumors into estrogen-dependent and

estrogen-independent tumors based on the presence or absence of cytoplasmic receptors. Selection of estrogen-receptor positive tumors has been the basis for newer approaches in the management of breast cancer using endocrine-ablative techniques as well as antiestrogenic drugs (McGuire et al., 1975 a; Moseky et al., 1974; Horwitz and McGuire, 1978 a; Katzenellenbogen et al., 1979; Sutherland and Jordan, 1981).

Patients with estrogen receptor-rich tumors have a remission rate of 60-70% after endocrine therapy compared to less than 10% in E receptor-poor tumors. However, 30-40% of the ER-positive patients fail to respond to treatment and about half of the tumors do not regress after ovariectomy (McGuire et al., 1975 b).

Estrogen-induced growth of breast cancer cells in vitro has been described. Lippman et al. (1976, 1977 b) have shown a variety of growth responses to physiological concentrations of estrogens and inhibition by antiestrogens in the MCF-7 human breast cancer cell line. A variety of specific products also have been shown to be under estrogenic control in these cells, including thymidine kinase (Bronzert et al., 1981), progesterone receptor (Horwitz and McGuire, 1978 b), lactic dehydrogenase isoenzymes (Burke et al., 1978), and a secreted protein of unknown function (Westley and Rochefort, 1980).

The direct effect of estrogens on breast cancer cells in culture could not be confirmed by some investigators. As a result, Sirbasku (1978) postulated that estrogens may stimulate the synthesis or secretion of various polypeptide growth factors, estromedins, that are specific for various types of estrogen-responsive tumors.

Several experimental findings favor the view that the mitogenic effects of estrogens on breast cancer cells are indirect. The MCF-7 human breast cancer cell line undergoes a drastic reduction in estrogen responsiveness (Jozan et al., 1979; Strobl and Lippman, 1979; Page et al., 1983). These results prompted an examination of the requirements of MCF-7 cells for serum. Page et al. (1983) have shown that a growth response to physiological concentrations of estradiol could be created if serum concentrations in the growth medium of the cells were raised to 15% (v/v). Thus, cells that were "estrogen-unresponsive" exhibited a full response to estrogen. These findings favor the hypothesis that estrogen requires specific serum factors to induce growth of breast cancer cells.

In vivo studies (i.e. in athymic nude mice) by Leung and Shiu (1981) showed that estrogen stimulated the growth of T-47D human breast cancer cells in the presence of pituitary factors. Injection of estrogen alone into female nude

mice resulted only in moderate growth of T-47D cells. This suggests that estrogen alone does not produce maximal growth of this human breast cancer cell and that other hormones, factors, or mediators from the pituitary are required. A similar conclusion had been drawn from clinical studies using breast cancer patients (Pearson and Ray, 1959; Lipsett and Bergenstal, 1960). It is not known whether the pituitary factor(s) required for estrogen stimulation *in vivo* or the serum factor(s) required for estrogen stimulation *in vitro* of human breast cancer cells are identical.

An alternative mechanism for an indirect action of estrogen mammary tumor growth is possible. Proponents of this hypothesis believe that in response to estrogens the tumor cells produce a mitogenic factor(s) which then stimulates cell growth, that is an autocrine mechanism. Vignon *et al*. (1983) and Danielpour and Sirbasku (1983) have reported that the conditioned medium of estrogen-stimulated MCF-7 human breast cancer cells is itself mitogenic to the cells, suggesting that the cells are synthesizing and secreting an estrogen-inducible mitogenic factor(s) into the medium. Whether or not this phenomenon is of physiological importance remains to be determined.

Finally, Sonnenschein and Soto (1979) have proposed that inhibition of cell multiplication may be central to the understanding of the mechanism of estrogen action. They

have reported that estrogen-sensitive tumor cells in rats and in culture are subject to the inhibitory control by serum factors and that these inhibitory factors are under estrogen control. Thus, this hypothesis proposes that estrogens do not act directly on cells to stimulate growth but rather act to remove or block the serum-inhibitory factors. This hypothesis has not received general acceptance.

3. Insulin and insulin-like growth factors

Insulin can act as a growth-stimulating hormone for experimental rat, mouse, and human mammary carcinomas. Heuson et al. (1972) showed that insulin played a role in stimulating growth of DMBA-induced tumors since insulin administration, together with prolactin, to hypophysectomized animals reactivated tumor growth. Prolactin alone was ineffective in stimulating tumor growth. Welsh et al. (1976) reported that in DMBA-induced mouse mammary tumors insulin dramatically increased thymidine incorporation into DNA. Prolactin had an additive effect on the insulin response in the rat but not in mouse adenocarcinomas.

The mitogenic effects of insulin have also been demonstrated for certain human breast cancer cell lines maintained in long-term tissue culture. Osborne et al. (1976) reported that MCF-7 cells were quite sensitive to physiological concentrations of insulin. Indirect or facilitative effects of

insulin in breast cancer have also been reported. Insulin may be involved in estrogen receptor (ER) regulation (Hilf, 1981). Because many breast cancers are estrogen-dependent or responsive, stimulation or inhibition of tumor growth may occur through the indirect effects of insulin on ER. Insulin has also been shown to alter the sensitivity to estrogen and antiestrogens in estrogen-responsive human breast cancer cells in culture (Butler et al., 1981).

Insulin represents only 10 percent of the insulin-like activity of the blood (Zapf et al., 1981). The activity of the factors responsible for the insulin-like actions on muscle and adipose tissue is not blocked by insulin antibodies and was therefore termed nonsuppressible insulin-like activity or NSILA (Froesch et al., 1963). NSILA consists of a large molecular weight protein (NSILAP) and two smaller molecular weight polypeptides now known as insulin-like growth factors (IGF I and IGF II). The concentrations of IGF I, but not that of IGF II, in blood are largely regulated by growth hormone (GH). This observation gave rise to the somatomedin hypothesis, according to which GH stimulates growth indirectly through somatomedins or IGF I (Salmon and Daughaday, 1957).

IGF I and II have somatomedins activity in vitro: they are potent stimulators of cell replication, DNA-, RNA-, and protein synthesis in fibroblasts (Froesch et al.,

1976), chondrocytes (Zapf et al., 1978 and 1981) and calvarian cells (Cannalis, 1980), and they stimulate incorporation into rat chick cartilage (Zapf et al., 1978 and Froesch et al., 1976).

Since growth hormone and prolactin do not stimulate breast cancer cell growth in vitro but have been implicated as factors influencing breast cancer growth in vivo studies have been undertaken to investigate the in vitro mitogenic effect of somatomedins on breast cancer cells. IGF I and II have been shown to be mitogenic to T-47D human breast cancer cells in culture (Myal, 1982).

4. Glucocorticoids and thyroid hormone

Glucocorticoids have long been known to play an important role in morphological and functional differentiation of the mammary gland (Lyons et al., 1958 and Nandi, 1959). It is also established that the regulation of mammary physiology by any single hormone involves the interaction of several hormones (Topper and Freeman, 1980). Therefore, glucocorticoids exert their effects on mammary tissue in conjunction with other hormones.

Glucocorticoids influence the differentiation of breast cancer cells and/or inhibit their growth. In experimental mammary adenocarcinomas, cortisol can cause atrophy of tumors (Hilf et al., 1965 and Sparks et al., 1955). Prednisone will

cause some human breast cancers to regress, principally in those turmors which are estrogen receptor-positive (Pihl et al., 1975). Human mammary tumors, including those in culture, contain glucocorticoid receptors (Allegra et al., 1979; Horwitz et al., 1975, Lippman et al., 1977; Keydar et al., 1975 and Teulings and van Glix, 1977). Dexamethasone, when added to cultured human breast cancer cells, inhibits thymidine incorporation into cell DNA which, in turn, is accompanied by a marked reduction in cell division (Lippman et al., 1977).

The involvement of thyroid hormone in breast cancer has been based on contradictory and often unconvincing results. Some studies suggest that breast cancer is associated with hypothyroidism because the incidence of breast cancer parallels that of endemic goiter due to iodine insufficiency (Doll, 1969 and Stadel, 1976), while others have found the incidence of breast cancer to be greater in patients with hyperthyroidism (Finley and Bogardus, 1960 and Humphrey and Swerdlow, 1964).

In vitro studies using the MCF-7 human breast cancer cell line have demonstrated that the presence of classical high-affinity nuclear receptors for thyroid hormone and a growth response to physiological concentrations of thyroid hormones (Burke and McGuire, 1978). The ZR-75-1 human breast cancer cell line has an absolute requirement for

physiological concentrations of triiodothyronine (T_3) for growth (Allegra and Lippman, 1978).

Shiu and Paterson (1984) showed that human breast cancer cells (T-47D) in culture respond to prolactin or growth hormone in combination with hydrocortisone (H), insulin (I) and thyroid hormone (T_3) by exhibiting altered cell shape, decreased cell adhesion, and increased lipid production. Prolactin or growth hormone alone were incapable of inducing such changes. Similarly the combination of H, I, or T_3 alone was also ineffective. When each of the hormones was tested in combination with prolactin or growth hormone it was observed that hydrocortisone alone was needed to render cell responsiveness to prolactin. These findings suggest that prolactin and growth hormone, acting synergistically with glucocorticoids, may play an important role in the pathogenesis of human breast cancer.

B. Polyamines

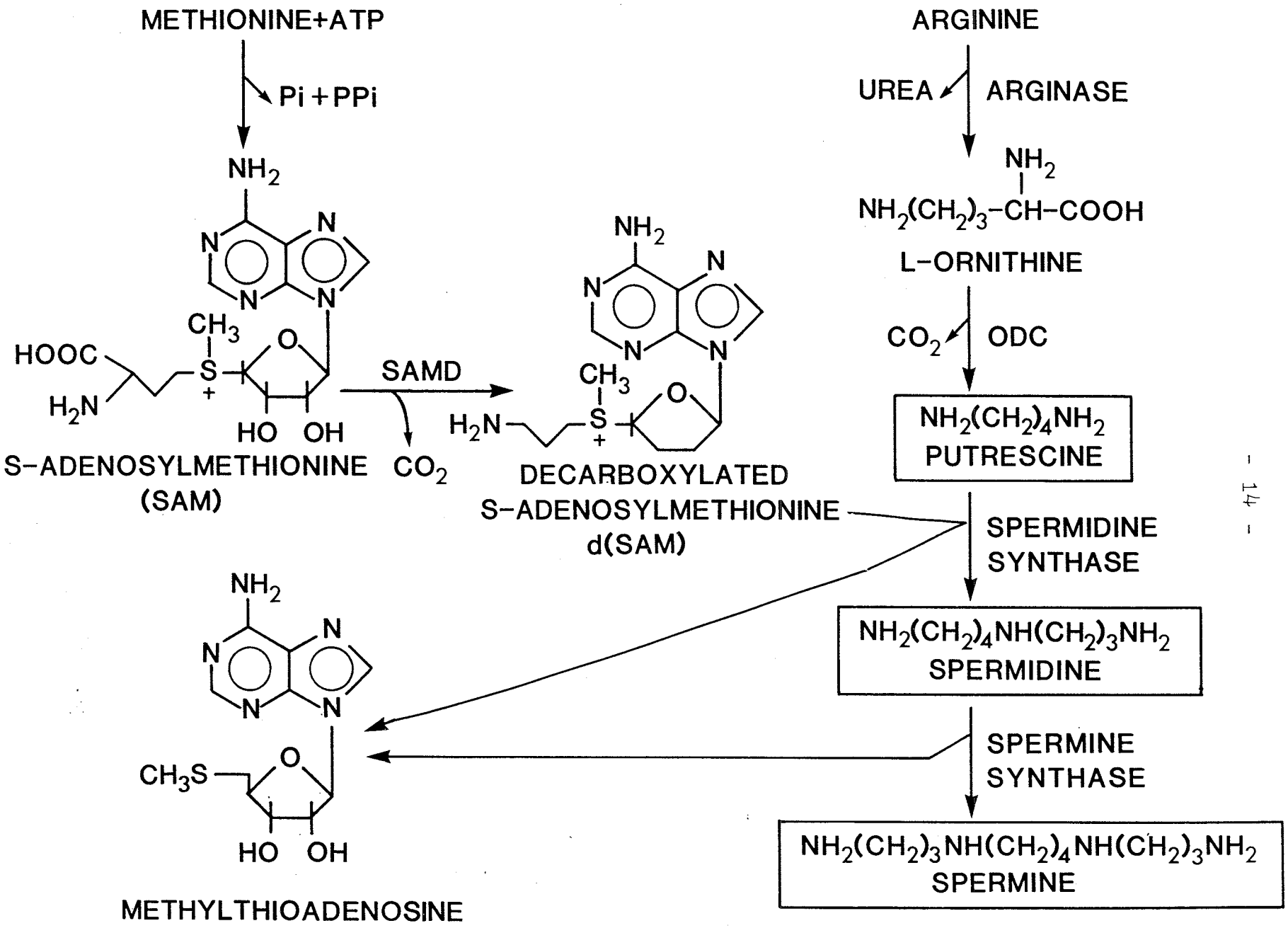
The polyamines, putrescine, spermidine, and spermine, are ubiquitous in mammalian cells, although the relative amounts of each vary markedly in different cells. Putrescine is usually found in lower levels than spermidine (Tabor and Tabor, 1976). For example, in the frontal lobe of the human brain the concentrations of putrescine, spermidine, and spermine are 0.02, 0.23, and 0.10 umole/g, respectively.

Polyamines are involved in the regulation of cell growth and tumor promotion (Mamont et al., 1978; Janne et al., 1978; Fozard and Prakash, 1982) and recent evidence suggests that they may play a role in the control of cell differentiation (Schindler et al., 1983; Chen et al, 1983; Verma and Sunkara, 1982). Of the enzymes involved in the polyamine biosynthetic pathway, ornithine decarboxylase (ODC, E.C.4.1.1.17) is of particular significance since it catalyzes the formation of putrescine from ornithine, which is the initial and rate-limiting step in polyamine biosynthesis and the only known route in mammals for the formation of putrescine (Tabor and Tabor, 1976; Williams-Ashman and Canellakis, 1979). The established pathway of polyamine synthesis is summarized in Fig. 1.

Ornithine decarboxylase is a pyridoxal phosphate-requiring enzyme (half-life 7-20 min) whose activity in resting cells is usually very low and is increased manyfold in response to a number of cellular effectors, including hormones and growth factors (Canellakis et al., 1979 and Bacharach, 1980).

Drugs that are capable of penetrating cells and selectively inhibiting polyamine biosynthesis can be exploited to alter tissue polyamines in a predictable manner and perhaps shed new light on the biological function of putrescine, spermidine, and spermine. The importance of polyamines in

Fig. 1 Biosynthetic pathway of polyamines in mammalian tissues. (From Williams-Ashman and Canellakis, 1979).


METHIONINE+ATP
Pi + PPi
S-ADENOSYLMETHIONINE
(SAM)
SAMD
CO2
DECARBOXYLATED
S-ADENOSYLMETHIONINE
d(SAM)
ARGININE
UREA
ARGINASE
L-ORNITHINE
CO2
ODC
NH2(CH2)4NH2
PUTRESCINE
SPERMIDINE
SYNTHASE
NH2(CH2)4NH(CH2)3NH2
SPERMIDINE
SPERMINE
SYNTHASE
NH2(CH2)3NH(CH2)4NH(CH2)3NH2
SPERMINE
METHYLTHIOADENOSINE

cell growth and differentiation has been demonstrated in studies with DL-∝-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC (Metcalfe et al., 1978 and Mamont et al., 1978).

The probable mode of action of DFMO is via formation of a Schiff base with the enzyme-bound pyridoxal phosphate, which after catalytic removal of CO_2 leads to a loss of a fluorine atom; the resulting very reactive intermediate then covalently combines with the ODC protein, rendering it inactive (Williams-Ashman and Canellakis, 1979).

Polyamines have been shown to be involved in the insulin-induced differentiation of L6 myoblast cells (Erwin et al., 1983) and parathyroid hormone-induction of differentiated chondrocytes (Takegawa et al., 1980). As well, polyamines have been implicated in the differentiation of various tumor cells, including Friend erythroleukemia cells (Gazitt and Friend, 1980), murine embryonal carcinoma cells (Schindler et al., 1983), mouse NB-15 neuroblastoma cells (Chen et al., 1982, 1983), and in non-tumor cell differentiation (Verma and Sunkara, 1982; Sepulveda et al., 1982).

Marked increases in ODC activity and polyamine biosynthesis accompany the onset of proliferative events in most cell types (Williams-Ashman and Canellakis, 1979; Russell and Durie, 1978; Tabor and Tabor, 1976; Janne et al.,

1978; Snyder and Russell, 1970). The role of polyamines in hormone-induced cell growth has also been examined. In the Nb_2 rat lymphona cell line the prolactin-induced growth is accompanied by an early increase in ODC activity (Elsholtz, 1984; Richards et al., 1982). Growth hormone induces ODC activity in the regenerating rat liver (Byus et al., 1977). The involvement of polyamines has also been implicated in the proliferation of a variety of tumor cells in animals and in culture, including EMT6 mouse mammary sarcoma cells (Prakash et al., 1980), L 1210 leukemia cells in mice (Prakash et al., 1978, human colon adenocarcinoma cells (Kingsworth et al., 1983 a), murine renal adenocarcinoma cells (Kingsworth et al., 1983 b), B 16 melanoma cells (Sunkara et al., 1983), and rat 9 L brain tumor cells (Seidenfeld et al., 1981).

C. Hormones and polyamines in mammary systems

A role for polyamines in the regulation of metabolic processes in mammary tissues has been suggested. Russell and McIver (1972) found that elevated spermidine levels accompanied rat mammary gland lactation. Others have found that the various enzymes involved in the polyamine biosynthic pathway (Fig. 1) are stimulated by one or more hormones (insulin, cortisol, and prolactin) that enhance the rate of synthesis of milk proteins in cultured mouse or rat mammary

tissues (Aisbitt and Barry, 1973; Oka and Perry, 1974 a, b, 1976; Oka et al., 1977; Rillema, 1976 a; and Rillema et al., 1977).

1. Prolactin and growth hormone

Rillema (1976 a), found that prolactin stimulated ornithine decarboxylase activity in mammary gland explants of mice. In subsequent studies (Rillema et al., 1981) it was found that prolactin-stimulated ODC activity occurred in a dose-response relationship whereas prolactin action on RNA and casein synthesis was an all-or-none type of response. Inhibition of ODC with α-DFMO did not inhibit prolactin-induced casein and lipid biosynthesis (Rillema and Cameron, 1983). This observation may be explained by the possibility that there is a supply of endogenous spermidine that is large enough to continue with polyamine synthesis, therefore an effect is still observed. However, inhibition of spermidine synthesis abolished the effect of Prl on casein and lipid biosynthesis. Our understanding of the role that polyamines play in hormone-dependent processes in the mammary gland thus remains incomplete.

The role of polyamines in breast cancer cell proliferation and differentiation is also unclear. α-DFMO has been observed to have an inhibitory effect on the growth and rate of appearance of DMBA-induced rat mammary tumors (Fozard and Prakash, 1982), as well as a growth-inhibition effect on murine

mammary tumor cells in culture (Prakash et al., 1980). Prolactin responsiveness in mammary tumors has been shown to be associated with increased levels of ODC (Frazier and Costlow, 1982). Although findings suggest that ODC activity is an early cellular response to prolactin stimulation, the function of the polyamines in prolactin and growth hormone action on breast cancer remains unclear.

2. Insulin and glucocorticoids

The parenchymal cell proliferation that is induced in mammary organ cultures by insulin is preceded by a transient increase in ODC activity (Aisbitt and Barry, 1973). This effect is seen even when RNA synthesis is inhibited. It has also been shown that the proliferative effect of insulin on mammary cells requires the presence of putrescine and spermidine (Oka et al., 1982).

It has been observed that spermidine can replace glucocorticoids in their differentiative function of milk protein synthesis (casein and α-lactalbumin)(Oka, 1974). This observation represented the first demonstration that spermidine can mediate the effects of glucocorticoid. The effect of spermidine in lactogenesis is specific for hydrocortisone since it did not replace the requirement for insulin or prolactin.

3. Estrogen

Estrogens have been found to stimulate ODC activity and consequently, polyamine formation on normal estrogen-responsive tissues (Cohen et al., 1970; Kay et al., 1971; Russell and Taylor, 1971; Morris and Fillingame, 1974). Certain human breast cancer cells in culture have been reported to be stimulated by physiological concentrations of estradiol (Lippman and Bolan, 1975; Lippman et al., 1976; Allegra and Lippman, 1978; Weichselbaum et al., 1978). The possibility that the mitogenic effect of estrogens may require the synthesis of polyamines in breast cancer cells is indicated by the report of Manni and Wright (1983) who showed that DFMO was effective in reducing the mitogenic effect of estrogen on nitrosomethylurea (NMU)-induced rat mammary tumors grown in soft agar culture. This system, however, may not be representative of human breast cancer cells. The precise role of polyamines in the estrogen-induced growth of human breast cancer cells has yet to be defined.

D. The use of cell culture in the study of hormonal regulation of breast cancer

Established human breast cancer cell lines constitute an important tool for immunological, genetic, virological and hormonal studies of human breast cancer. Cultivation of

long-term cell lines from solid tumors has proven to be quite difficult and few such human cultures have been reported (Lasfargues and Ozzello, 1958; Nordquist et al., 1975; Lasfarques, 1975; and Hacket et al., 1977). Attempts to culture breast cancer cells have been successful more often with cells from malignant effusions (Cailleau et al., 1974 a, b, and c; Engel et al., 1978; Vasquez, 1972; Keydar et al., 1979, and Whitehead et al., 1983). There are inherent advantages and disadvantages in using such an in vitro system for the study of hormonal regulation of human breast cancer.

1. Advantages

Despite the difficulties encountered when working with breast cancer cells in culture there are many advantages to the use of an in vitro system for the study of hormonal interactions with human breast cancer. First of all, by using a cell line of human origin one overcomes the difficulties associated with species differences. Secondly, the use of a single, cloned cell type allows greater confidence to be placed in what the primary cell type is, assuming an effect is seen (Lippman, 1981). Thirdly, the combination of ease of manipulation and continuous supply of cells avoids the problems encountered in obtaining biopsy specimens. Fourthly, the absence of tissue redistribution, plasma binding, and nontarget tissue sites of metabolism and

excretion offer advantages for the study of drug and hormone metabolism. Lastly, cell culture systems allow studies to be carried out under controlled environments, specifically by using defined media. In such cases one can be certain that the hormonal effect studied is not mediated by some additional unknown factor(s).

2. Disadvantages

Since most established cell lines are derived from pleural effusions they represent a subset of the original tumor population, which has the ability to metastasize and grow in a specific environment completely unlike that of the primary tumor. By the time cells are collected for culture the original tumor may have been exposed to a number of manipulations, including irradiation, cytotoxic drugs, and hormonal therapy, all of which may have affected the cells' ability to respond to a number of growth factors and hormones.

In long-term tissue culture human breast cancer cells may, acquire different properties (Lippman, 1981). Cells grown in culture are denied many physiological factors and conditions which may be important to the expression of a given response.

E. Rationale and aims of investigation

Increased ODC activity, thus polyamines, may play an important role in the initiation of both cellular differentiation and proliferation. To evaluate the process of

differentiation and proliferation separately has been a difficult task. Therefore, it has been unclear whether one or both cellular responses depend on polyamines.

The aims of this investigation were:

1) to determine if polyamine synthesis is required for the biological actions of prolactin and growth hormone in human breast cancer cells, and

2) to test the growth-promoting effect of physiological concentrations of estradiol on an estrogen receptor-positive clone of T-47D cells (clone 11), and to determine if polyamines are involved in the response.

MATERIALS AND METHODS

A. Tissue culture materials and chemicals

Culture dishes (35 x 10 mm and 100 x 20 mm), culture flasks (75 cm^2 and 150 cm^2), sterile pipettes, 15 ml centrifuge tubes, and active charcoal (Norit A) were purchased from Fisher Scientific Co. Fetal bovine serum (FBS), Dulbecco's Modified Eagles Medium (DMEM), RPMI 1640 medium, penicillin, streptomycin, L-glutamine, and trypsin/EDTA (1x) were supplied by GIBCO. Bovine serum albumin was obtained from Sigma Chemical Co.

B. Hormones and other chemicals

Human growth hormone (Lot 22-8-82) was generously provided by Dr. Ian Worsley. Hydrocortisone, 17-β-estradiol, and putrescine dihydrochloride were purchased from Sigma. Bovine insulin (25.7 U.S.P. units/mg) was obtained from GIBCO. α-difluoromethylornithine (DFMO) was kindly provided by Merrell Research, Cincinnati, Ohio. All reagents for SDS gel electrophoresis were obtained from BIO-Rad.

C. Cell Lines

1. T-47D cell line

The human breast cancer cell line was obtained from E.G. and G. Mason Research Institute (Rockville, Md.). It

was originally derived from the pleural effusion of a 54-year-old Caucasian patient with infiltrating ductal carcinoma. In culture these cells exhibit typical epithelioid morphology (polygonal in shape) and growth pattern in that they form cobblestone monolayers. The mammary epithelial characteristics are supported by immunohistologic detection of intracellular casein and the presence of cytoplasmic steroid receptors (Keydar et al., 1979). Membrane receptors for Prl and GH (Shiu, 1979), insulin (Engel and Young, 1978) and EGF (Imai et al., 1982) have also been reported. The doubling time of these cells is 32 hours (Engel and Young, 1978).

The cells were routinely maintained in DMEM supplemented with L-glutamine (4 mM), glucose (4.5 g/l), penicillin (100 i.u./ml), and 10% FBS (v/v). This medium will be referred to as complete medium (CM). Cells were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Trypsin/EDTA (0.05 and 0.02%, w/v, respectively) in Hank's Balanced Salt Solution (HBSS) was used for cell passages.

2. T-47D Clone 11 cells

An estrogen receptor-positive clone (clone 11) of the T-47D cell line was provided by Dr. I. Keydar, University of Tel Aviv. These cells were routinely maintained in RPMI 1640 medium supplemented with L-glutamine (4 mM), penicillin (100 i.u./ml), streptomycin (100 ug/ml), and 10% FBS (v/v). Cells were incubated and passaged as described previously for T-47D cells.

D. Ornithine decarboxylase studies

1. Ornithine decarboxylase assay materials and chemicals

Polypropylene assay tubes (17 x 100 mm) were purchased from Falcon. Rubber stoppers and plastic centre wells were obtained from Kontes Glass Co. Filter papers were Whatman GF/C 2.4 cm circles. L- 1 - ^{14}C -ornithine (50 mCi/mmol) and hyamine hydroxide were obtained from New England Nuclear. ACS scintillation fluid was obtained from Amersham. L-ornithine, pyridoxyal 5'-phosphate, and dithiothreitol were purchased from Sigma Chemical Co.

2. Preparation of cells for ornithine decarboxylase assay

T-47D cells to be used for ODC assays were passaged at confluency and plated at a concentration of 1 x 10^5 cells in 10 ml of CM per 100 x 20 mm dish and incubated at 37°C. Forty-eight hours later the medium was removed, the cells were washed twice and replaced with DMEM containing glutamine (4 mM), glucose (4.5 g/l), penicillin (100 i.u./ml), and streptomycin (100 ug/ml). Twenty-four hours later test substances were added. Medium was not changed since fresh medium reportedly increases ODC activity (Branca and Herbst, 1980). After incubation each dish was washed twice with ice-cold phosphate-buffered saline (PBS) and 5 ml of PBS replaced the medium. The dishes were placed on ice and the cells were scraped off with rubber policemen into 15 ml centrifuge

tubes. The cell suspensions were then centrifuged at 1500 rpm for 5-10 min. at 4°C. The buffer was aspirated and the cells were resuspended in 350 ul assay buffer that contained 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.1 mM EDTA, and 2 mM dithiothreitol, pH.7.4. This suspension was stored at -70°C until time of assay. For the assay cells were lysed by sonication using a megason sonicator for 30 seconds at a probe setting of 4. The cell homogenates were centrifuged at 90,000 x g for 30 min. at 4°C. 200 ul of the supernatant was used for ODC determination and 100 ul was reserved by protein determination by the method of Bradford (1976).

3. Ornithine decarboxylase assay

Enzyme activity was determined by measuring the amount of $^{14}CO_2$ liberated from 1- 1 - ^{14}C -ornithine (50 mCi/mmol). At assay 50 ul of 4 mM pyridoxal 5'-phosphate (PLP) was added to 200 ul of the cell extract to yield a final concentration of 0.8 mM PLP. 200 ul of the extract/co-factor mixture was added to 200 ul of assay buffer and pre-incubated for 10 minutes at 37°C in a shaking water bath. The reaction was started by the addition of 100 ul ornithine, including labelled ornithine (approximately 500,000 CPM per assay tube) for a final ornithine concentration of 0.5 mM. The tubes were capped with rubber stoppers containing centre

wells and incubated for 2 hours at 37°C in a shaking water bath. For cultured mammary tumor cells, the rate of $^{14}CO_2$ released from labelled ornithine is constant for at least 2 hours (Frazier and Costlow, 1982). The ODC activity is also linear with regard to protein concentration from 0 to 300 ug of cellular protein per assay tube (data not shown). In all ODC studies the protein concentration used was in the middle range. Blank tubes contained assay buffer instead of cell extract.

The reaction was stopped and the $^{14}CO_2$ driven out of the mixture by injecting 0.5 ml of 50% TCA into the reaction mixture. 200 ul of hyamine hydroxide was injected onto the GF.C filter paper inside the well to trap the radioactive CO_2 as described previously (Hogan, 1971). The tubes were allowed to incubate overnight at 37°C. The filters were then counted in 5 ml ACS. The amount of radioactivity was determined by a Beckman β-counter with a counting efficiency of 77-83%.

E. Growth studies

T-47D Clone 11 human breast cancer cells (2×10^4 cells per 35 mm dish) were plated in 2 ml of RPMI 1640 medium supplemented with L-glutamine (4 mM), penicillin (100 i.u./ml), streptomycin (100 ug/ml), and 10% (v/v) FBS and incubated for 2 days to allow for cell attachment. This medium was then removed by aspiration, cells were washed twice and replaced with RPMI 1640 medium supplemented with glutamine,

penicillin, streptomycin, and 10% (v/v) charcoal-treated heat-inactivated FBS. This was accomplished by incubating FBS containing 1% charcoal for 30 minutes at 55°C in a shaking water bath. The serum was then centrifuged at 10,000 RPM for 30 minutes to remove the charcoal and filter sterilized prior to use. Twenty-four hours later test substances were added to the dishes.

To detach cells from the dishes medium was aspirated and 1 ml trypsin/EDTA in HBSS was added. The cells were then incubated for 10-15 minutes. 1 ml CM was added and the cells were dispersed by pipetting, added to 8 ml isotonic solution and counted electronically by a coulter counter.

F. Protein synthesis studies

1. Protein labelling for electrophoresis

T-47D cells were plated, medium changed, and test substances added as described under growth studies (section E). 4-5 days after the addition of test substances 100 uCi ^{35}S-methionine was added to each of the dishes. Cells were incubated for an additional 12-24 hours. The medium was then harvested and placed in dialyzing membranes with a molecular weight cut off of 6,000-8,000 daltons. Dialysis was carried out against distilled water at 4°C for 24-48 hours. The medium was then lyophilized.

2. Discontinuous SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide slab gels were prepared according to the method described by Laemmli (1970). The stacking gel (4% and resolving gel (15%) were prepared from stock solutions of acrylamide (30% w/v) and N,N'-methylene bisacrylamide (0.8% w/v). TEMED and 10% ammonium persulphate were added to accelerate the polymerization. Gels (1.5 mm thickness) were cast using a Bio-Red Protean vertical slab gel unit.

Samples (20-50 ul) were prepared by dissolving in a cocktail containing Tris-HCl (0.0725 M, pH 7.0), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue (tracking dye). Samples were boiled 5 minutes prior to use.

Electrophoresis was carried out at a constant current of 30 mA/gel until the bromophenol blue migrated to within 1 cm of the bottom of the gel (approximately 6 hours).

Gels were fixed in 10% TCA for 1 hour and washed overnight in 7% acetic acid under gentle shaking.

3. Fluorography

Fluorography was performed by the method of Laskey and Mills, 1975. The gels were soaked in dimethyl-sulphoxide (DMSO) for 45 minutes followed by a second 45-minute wash in fresh DMSO. The gels were then transferred to 20% PPO in DMSO for 3 hours. This was followed by a distilled water rinse and finally a 1.5-2 hour wash in distilled water containing 1% glycerol.

The gels were dried on a piece of Bio-Rad filter paper backing in a vacuum drier (58°C) for 3 hours. Gels were exposed to X-ray film (Kodak X-Omat AR) at -70°C for 1 week. Films were developed in Kodak GBX developer and replenisher for 4 minutes followed by a 30-second water rinse and fixed in Kodak rapid fixer for 3 minutes, all at 20°C. Films were allowed to rinse in water for 30 minutes to one hour and air-dried at room temperature.

4. Determination of radioactivity associated with protein Bands in SDS gels

Autoradiographs were placed on top of the corresponding dried SDS gels and the desired areas for cutting were traced using a sharp pin. The SDS gel segments were cut and placed in 1 ml of distilled water for 1 hour. This allowed the gel to swell and become dissociated from the filter paper. The pieces of gel were placed in 1 ml 30% hydrogen peroxide and incubated at 60-65°C for 24 hours in counting vials. Once the gels had dissolved, 5 ml ACS scintillation fluid was added and the radioactivity counted in a β-counter.

RESULTS

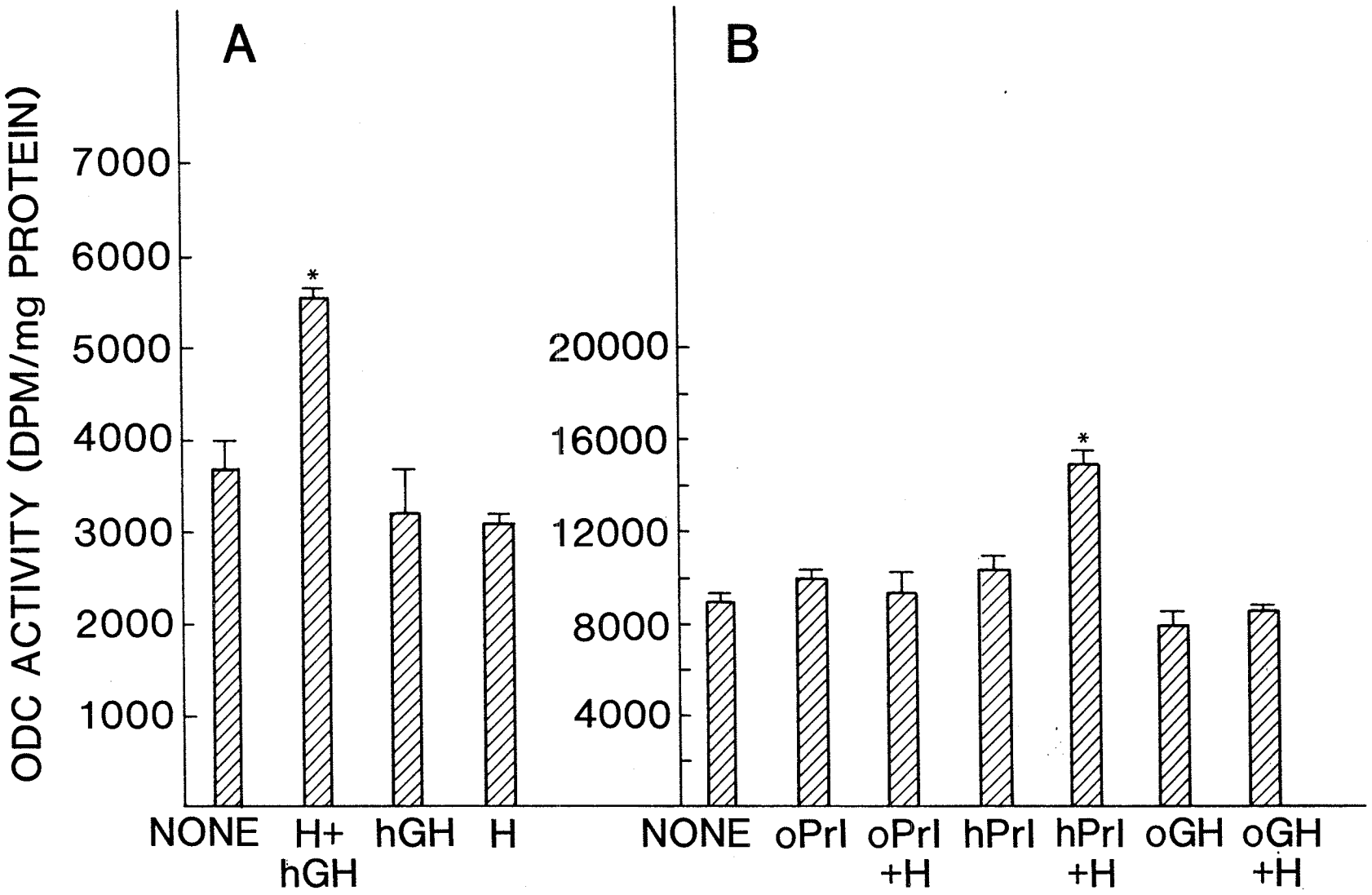
A. The role of polyamines in the action of growth hormone and prolactin in human breast cancer cells

1. Hormonal stimulation of ornithine decarboxylase (ODC) activity in T-47D cells

Since T-47D human breast cancer cells are responsive to human growth hormone (hGH) and prolactin (hPrl) (Shiu and Patterson, 1984; Shiu and Iwasiow, manuscript submitted), the role of polyamines in the action of these polypeptide hormones was examined. The activity of ODC, the first and rate-limiting enzyme in the polyamine biosynthetic pathway, under the influence of various hormone combinations was first studied. Fig. 2 A shows that ODC activity was stimulated approximately 50% in cells treated with hGH (1 ug/ml) and hydrocortisone (H, 1 ug/ml) for 48 hours. However, neither hGH nor H alone was effective in inducing enzyme activity.

To determine the specificity of the ODC response in T-47D cells to other polypeptide hormones, hPrl (1 ug/ml), oGH (1 ug/ml), and oPrl (1 ug/ml) were also tested. Fig. 2 B shows that hPrl, oPrl, and oGH alone were ineffective in stimulating ODC activity in the T-47D cells. Since the previous study showed that hGH in combination with H induced ODC activity, the response of the cells to hPrl, oPrl, and

Fig. 2 Hormone specificity of ODC stimulation. T-47D cells were plated at 10^6 cells per dish in complete medium (CM). Two days later the cells were washed twice and the medium replaced with serum-free DMEM. 24 hours later the various hormones were added. Cells were harvested 48 hours after hormone additions and assayed for ODC activity. Control cultures (none) received only the BSA-containing diluent. The final concentration for all hormones is 1 ug/ml. Each value represents the mean of triplicates $\pm$ S.D. (* $p < .01$, compared with control). Panel A and panel B represent results of two separate experiments.


A
B
ODC ACTIVITY (DPM/mg PROTEIN)
7000
6000
5000
4000
3000
2000
1000
20000
16000
12000
8000
4000
*
*
NONE
H+ hGH
hGH
H
NONE
oPrl
oPrl +H
hPrl
hPrl +H
oGH
oGH +H

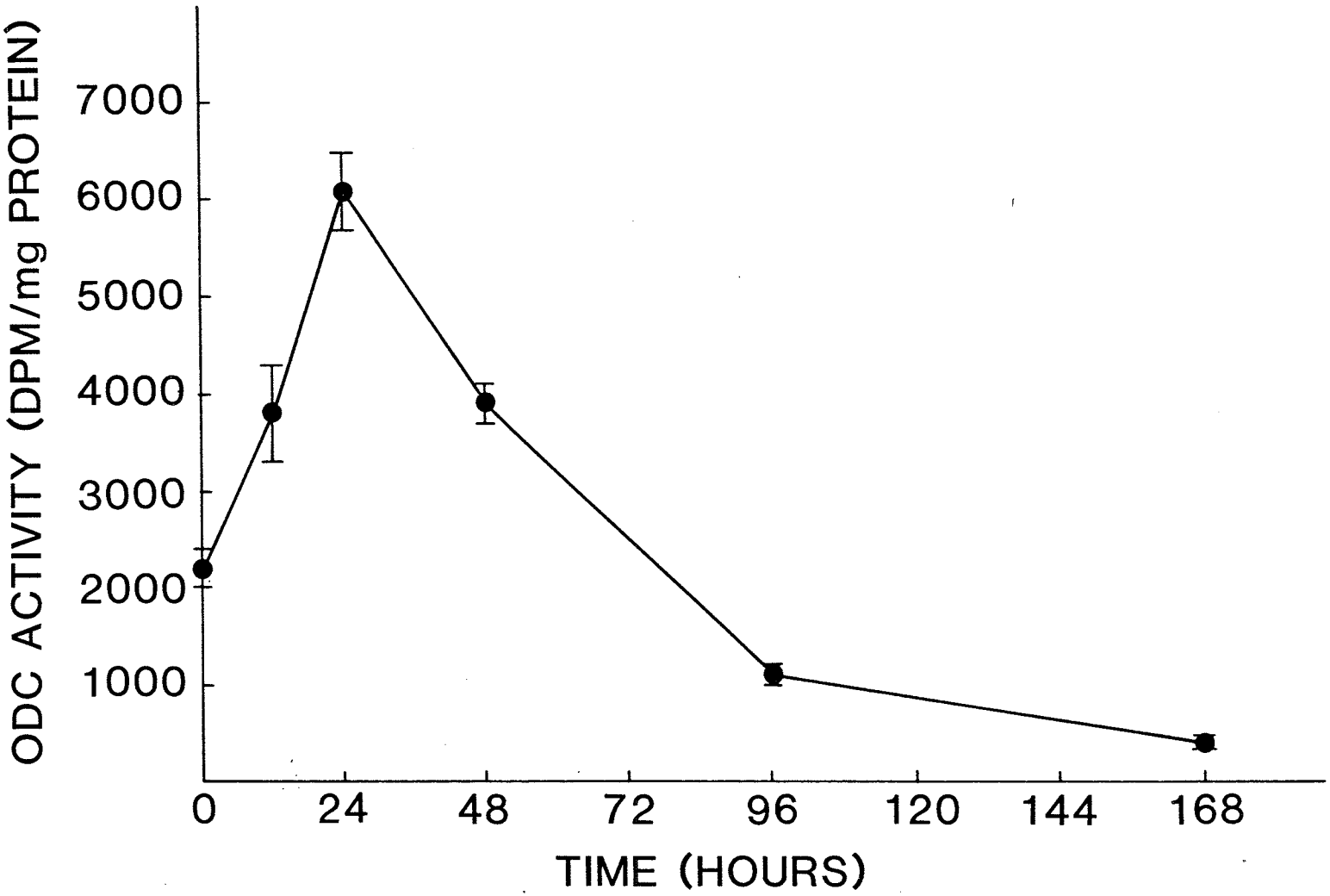
oGH together with H was compared. Only hPrl in the presence of H was effective in elevating enzyme levels 60-70% above that seen when no hormones are added. The sheep hormones in combination with hydrocortisone had no effect in stimulating ODC activity.

The results shown in Fig. 2 A and B represent two separate experiments. The difference in baseline levels of ODC activity (i.e. the "none" condition) seen between panels A and B can be attributed to variations observed from one experiment to another. Various factors may have contributed to this variation including the fact that cells from different passages were used.

In order to determine whether or not 48 hours was optimal for ODC induction in T-47D cells a time-course study of DOC activity in response to H + hGH stimulation was performed. Fig. 3 shows that enzyme activity peaked at 24 hours: a 300% stimulation was observed. By 48 hours, the stimulation had decreased to less than 100%, as shown in the previous studies. By 96 hours ODC activity had dropped to levels that were even lower than that seen at 0 hours. Based on these results subsequent enzyme assays were performed on cells that had been stimulated for 24 hours.

To determine which hormone concentration was optimal for inducing ODC activity in T-47D cells a dose-response

Fig. 3 Time course of ODC activity of T-47D cells stimulated with H + hGH. T-47D cells were plated as described in Fig. 2. After the addition of hormone cells were harvested at 0, 12, 24, 48, 96, and 168 hours. Each value represents the mean of triplicates ± S.D.


ODC ACTIVITY (DPM/mg PROTEIN)
7000
6000
5000
4000
3000
2000
1000
0
24
48
72
96
120
144
168
TIME (HOURS)

study was performed, the results of which are shown on Fig. 4. In the presence of 1 ug/ml hydrocortisone (H), hGH at final concentrations of 1 or 10 ng/ml was ineffective in stimulating ODC activity after 24 hours. However, 100 ng/ml and 1 ug/ml of hGH stimulated ODC activity by 50% and 235%, respectively.

2. Inhibition of ornithine decarboxylase by α-difluoromethylornithine (DFMO)

DFMO specifically and irreversibly inhibits ODC. This specific inhibition of polyamine synthesis can be used to determine the possible biological significance of the polyamines. The importance of the H + hGH induction of ODC in T-47D cells was studied using DFMO. Initially, it was of importance to determine the effective dose of DFMO that was capable of abolishing the H + hGH-induced ODC activity in human breast cancer cells. Fig. 5 shows the results of the dose-response study. Complete inhibition of the H + hGH-induced ODC activity was noted with DFMO concentrations ranging from 10 uM to 2 mM. Approximately 0.5 mM DFMO produced 50% inhibition of hormone-induced ODC activity. Based on these results, 100 uM DFMO was chosen as an effective concentration sufficient to inhibit ODC activity.

We also wanted to determine the stability of DFMO in culture as a reference for future experiments when cells would be treated with DFMO over a 4 to 7 day period. The

Fig. 4 ODC activity of T-47D cells: hGH dose-response study. Human growth hormone (hGH) was added to T-47D cells at concentrations of 1, 10, 100, and 1,000 ng/ml. Cells were harvested 24 hours after hormone stimulation and the ODC activity was determined. Hydrocortisone (H) concentrations were constant at 1 ug/ml. Each value represents the mean of triplicates ± S.D.

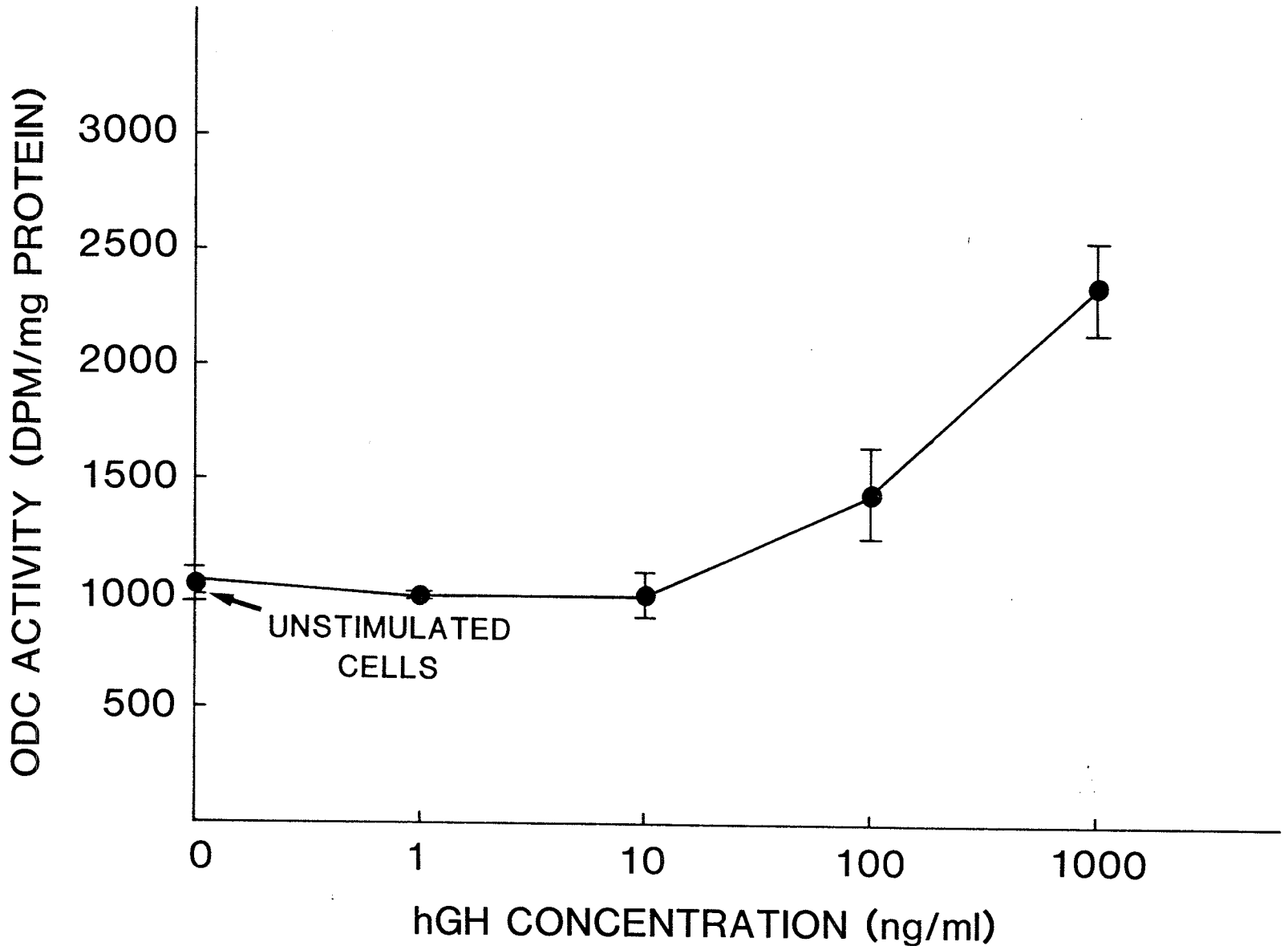
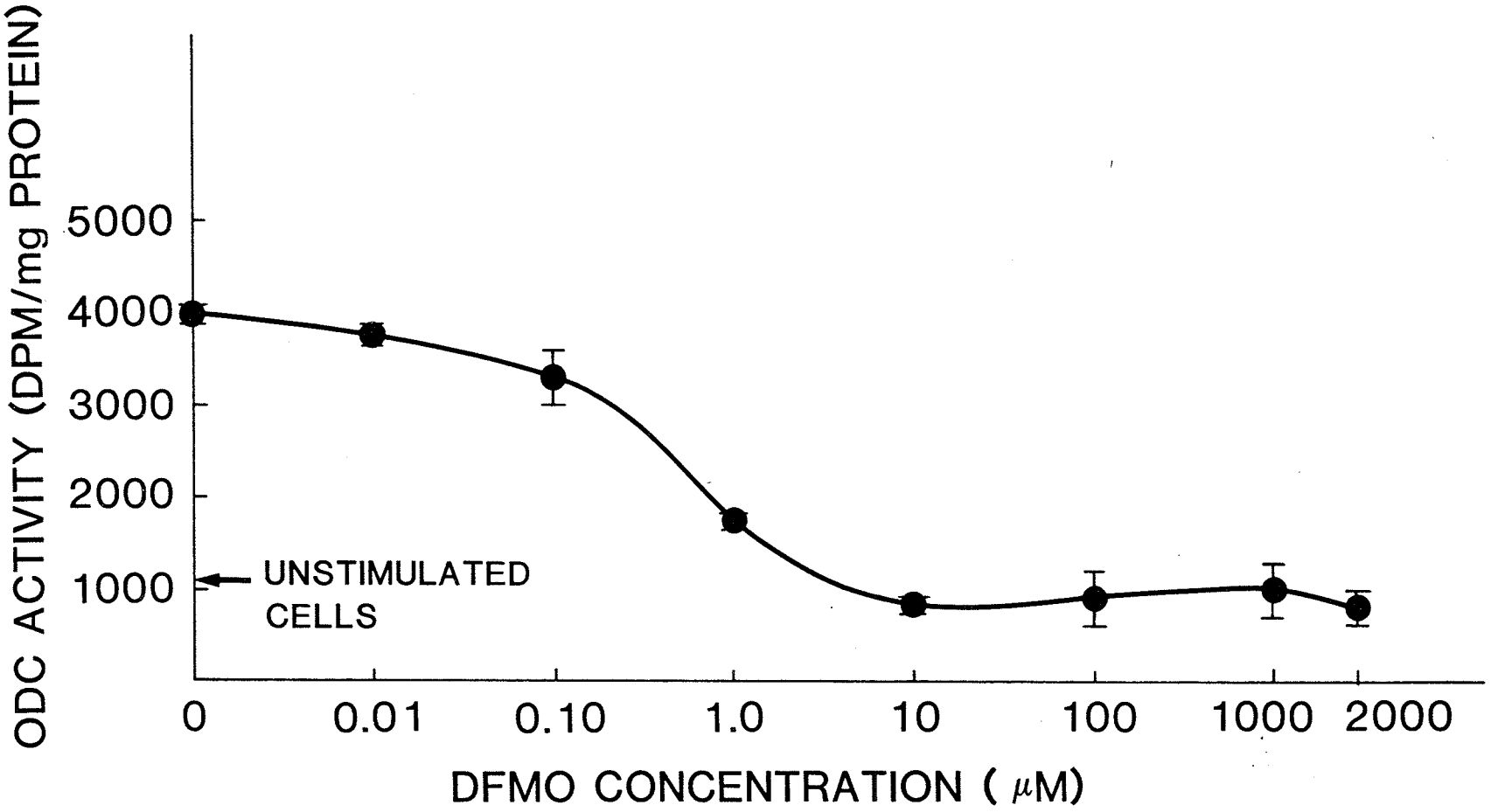

ODC ACTIVITY (DPM/mg PROTEIN)
3000
2500
2000
1500
1000
500
UNSTIMULATED
CELLS
0
1
10
100
1000
hGH CONCENTRATION (ng/ml)

Fig. 5 Effect of DFMO on H + hGH-stimulated ODC activity in T-47D cells. T-47D cells were plated and medium changed as described previously (Fig. 2). DFMO (final concentrations indicated in figure) was added simultaneously with the hormones, H + hGH. Diluent was added to control dishes. Cells were harvested 24 hours later and assayed for ODC activity. Each value represents the mean of triplicates ± S.D.


ODC ACTIVITY (DPM/mg PROTEIN)
5000
4000
3000
2000
1000
UNSTIMULATED CELLS
0
0.01
0.10
1.0
10
100
1000
2000
DFMO CONCENTRATION (μM)

time course study of the DFMO inhibitory effect on ODC is shown on Fig. 6. At 24 hours 0.1 mM DFMO inhibits the H + hGH-induced ODC peak. Enzyme levels are reduced to that seen in unstimulated cells. DFMO alone does not further reduce ODC levels in non-hormonally treated cells. At 48 hours H + hGH-induced ODC activity is reduced 2.5 fold to a level that is about 75% lower than that in unstimulated cells (basal ODC activity). DFMO alone further reduces the basal ODC activity. At 4 and 6 days DFMO is still effective in inhibiting ODC activity in both the hormone-treated and the unstimulated cells. These results indicate that DFMO is stable even after 6 days in culture.

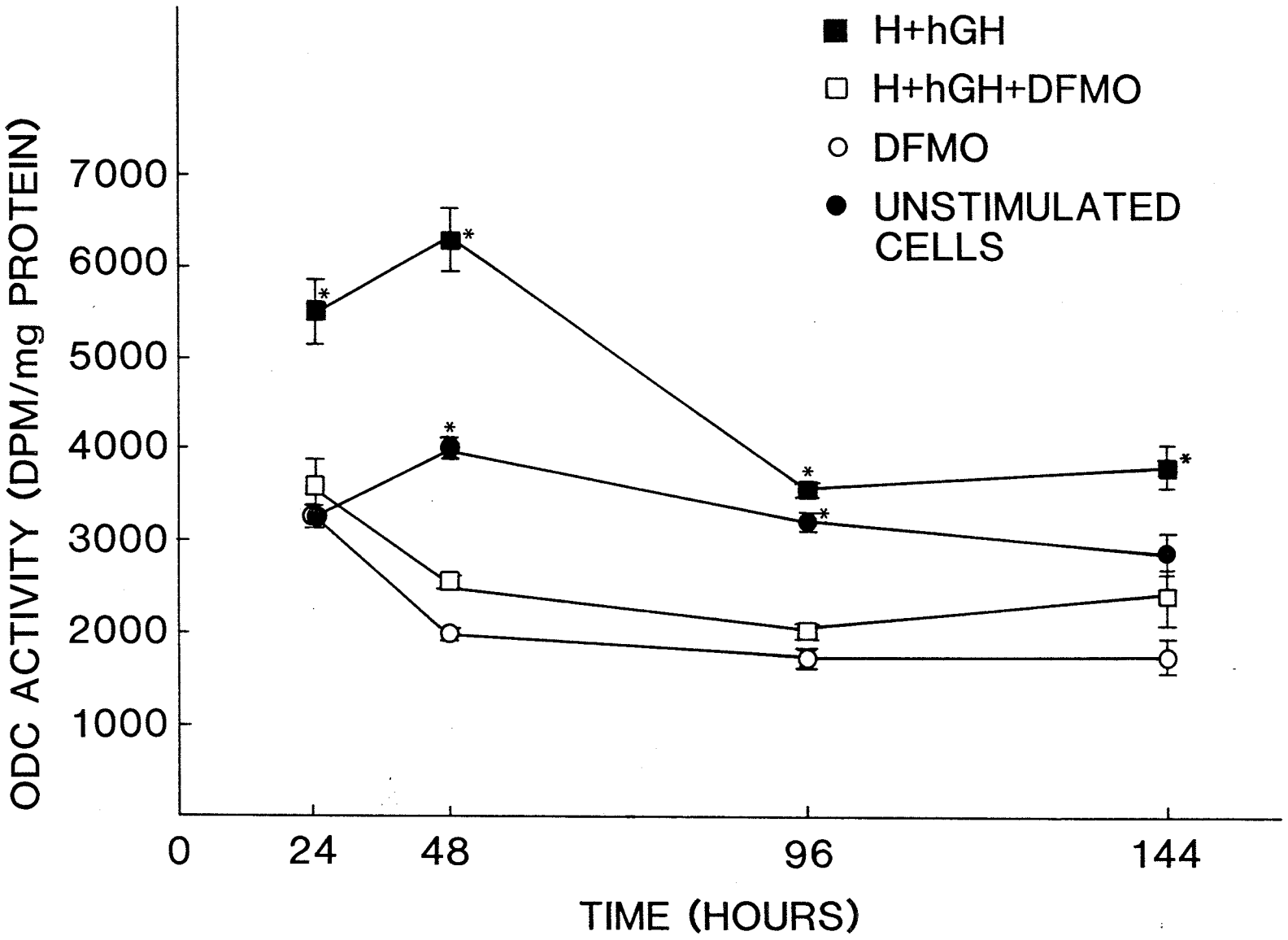
The peak of enzyme activity was continuous for 48 hours. However, the ODC levels at 48 hours were not statistically significant from those at 24 hours.

3. Biological consequences of ornithine decarboxylase inhibition on T-47D cells

a. Study on hormone-induced cell shape

It has been observed in this laboratory (Shiu and Paterson, 1984) that the combination of H and hGH, which causes an elevation of ODC activity in T-47D cells, reported in this study, also causes the cells to undergo a shape change. T-47D cells normally display typical epithelioid characteristics; cells are flat and polygonal in shape and are very adhesive to the plastic substratum.

Fig. 6 Time course of DFMO effect on ODC levels of H + hGH--stimulated and unstimulated T-47D cells. T-47D cells were plated and medium changed as described previously (Fig. 2). DFMO (0.1 mM) and hormones (H and hGH, each at final concentrations of 1 ug/ml) were added simultaneously. Cells not treated with hormones contain BSA-containing diluent. Cells were harvested at various times and assayed for ODC. Each value represents the mean of duplicates $\pm$ S.D. (* $p < .05$ compared to DFMO-treated cells in the same time point).


H+hGH
H+hGH+DFMO
DFMO
UNSTIMULATED CELLS
ODC ACTIVITY (DPM/mg PROTEIN)
7000
6000
5000
4000
3000
2000
1000
0
24
48
96
144
TIME (HOURS)

Under H + hGH stimulation the cells become round and adhere less strongly to the plastic substratum. To determine whether polyamines are involved in this phenomenon, ornithine decarboxylase was inhibited with DFMO and cell shape observed.

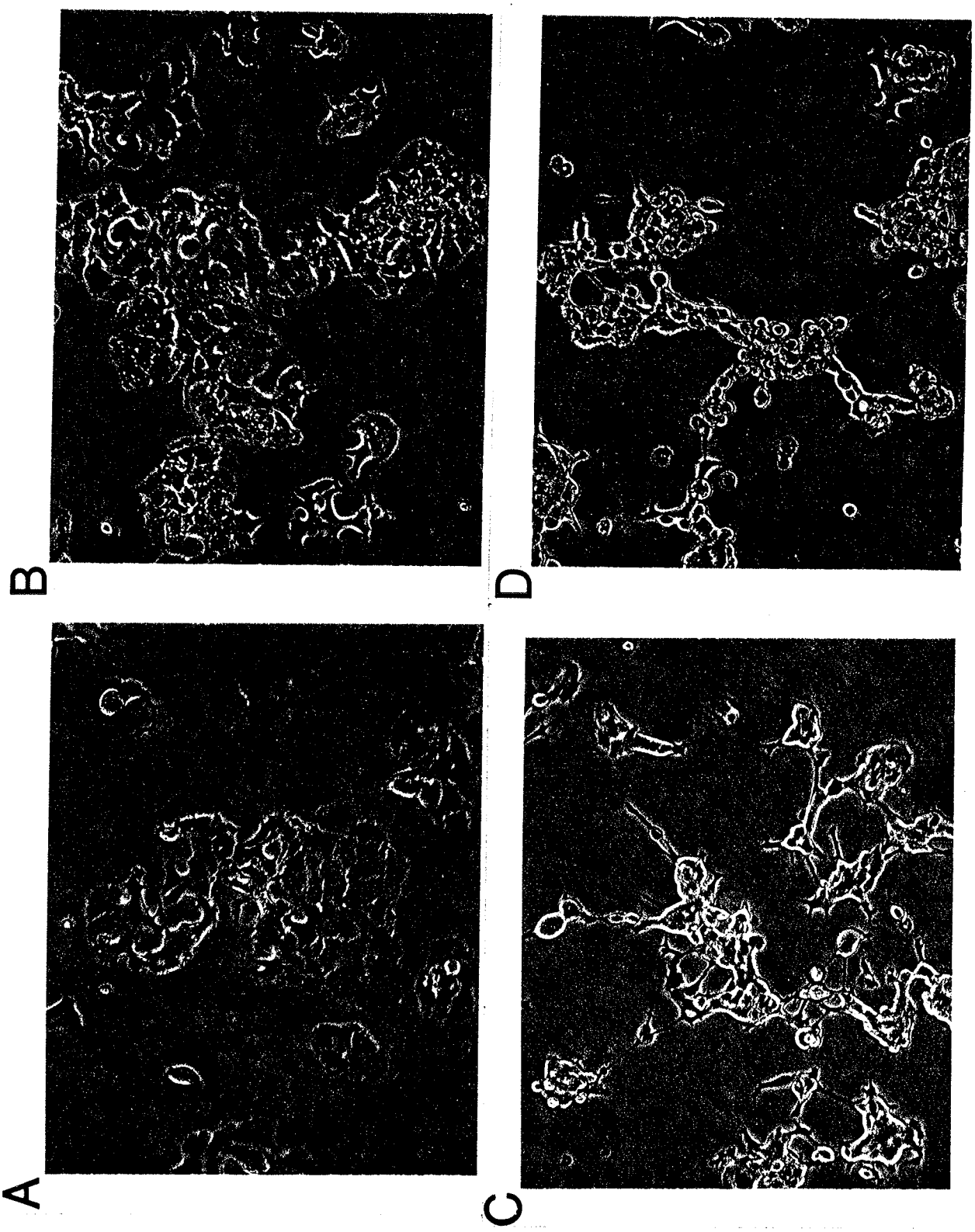
The hormone-induced shape change is most noticeable by the sixth day after hormone addition, thus all pictures were taken at six days. Fig. 7 A shows the normally flat T-47D cells when they have not been exposed to hormones or DFMO. Fig. 7 B shows that DFMO alone has no effect on the shape of these cells. When cells are stimulated with both H and hGH (Fig. 7C) they undergo a dramatic shape change by rounding up into clusters and becoming refractile. This morphological alteration of T-47D cells is not reversed by 0.1 mM DFMO (Fig. 7D).

b. Study on hormone-induced protein synthesis

To determine whether polyamines play a role in other hormone-mediated effects in T-47D human breast cancer cells, hormone-induced protein synthesis of these cells was studied. Shiu and Iwasiow (manuscript submitted) have shown that T-47D cells stimulated with hydrocortisone and human prolactin or growth hormone synthesize and secrete into the medium proteins of molecular weights 11,000 (11K), 14,000 (14K), and 16,000 (16K) daltons, with 14K being the most prominent. The biological function and significance of these

Fig. 7 Effects of hormones and DFMO on T-47D cell morphology. T-47D cells (1×10^5) were plated on 35 mm culture dishes DMEM containing 10% (v/v) FBS. Two days later medium was changed to serum-free DMEM. Each hormone was added to a final concentration of 1 ug/ml. DFMO (0.1 mM) was added simultaneously. Phase contrast microscopic pictures were taken 6 days later (magnification = 128 X).

Panel A No hormones, no DFMO
Panel B No hormones, 0.1 mM DFMO
Panel C H + hGH without DFMO
Panel D H + hGH with 0.1 mM DFMO


A
B
C
D

proteins has not been established. The T-47D cells that were exposed to hydrocortisone (H) alone (Fig. 8 C) and human growth hormone (hGH) alone (Fig. 8 D) showed essentially the same protein pattern as those of untreated cells (Fig. 8 B). In the presence of both H and hGH, 14K is most prominently induced (Fig. 8 F).

When DFMO (0.1 mM) was added to cells stimulated with H + hGH to inhibit the hormone-induced ODC activity there was a modest inhibition of the 14K protein (Fig. 8 E). When putrescine (0.1 mM) was added to the T-47D cells cultured in the presence of hormones and DFMO the inhibition of the synthesis of 14K by DFMO was reversed but putrescine alone had no effect beyond that seen with the hormones alone (Fig. 8 H).

To quantitate the degree of inhibition of 14K synthesis by DFMO in H + hGH-stimulated cells, the 15% SDS gels were cut and the radioactivity associated with each band was determined. Fig. 9 A shows that there is no difference in the synthesis of 14K in untreated T-47D cells and in those stimulated with H or hGH alone. H + hGH induces 14K protein synthesis 3 to 4-fold above untreated cells. DFMO inhibits the H + hGH induction of 14K and this inhibition is completely reversed by 0.1 mM putrescine to levels seen with H + hGH alone.

To determine whether the DFMO effect was specific for 14K hormone or whether DFMO inhibited protein synthesis

Fig. 8 Sodium dodecyl sulphate gel electrophoresis of T-47D cells secreted proteins. T-47D cells were plated at 1 x 10^5 cells per 35 mm dish in CM. Two days later medium was changed to serum-free DMEM. Hormones were added to a final concentration of 1 ug/ml. DFMO and putrescine were each added to a final concentration of 0.1 mM. Five days later 100 uCi ^{35}S-methionine was added to the medium for 24 hours. The dialyzed and lyophilized medium was run on 15% SDS PAGE, and the labelled proteins visualized by fluorography, as described in the methods. Each lane contained 30,000 CPM of proteins.

Lane A DFMO alone
B Untreated cells
C H alone
D hGH alone
E H + hGH + DFMO
F H + hGH
G H + hGH + DFMO + putrescine
H H + hGH + putrescine

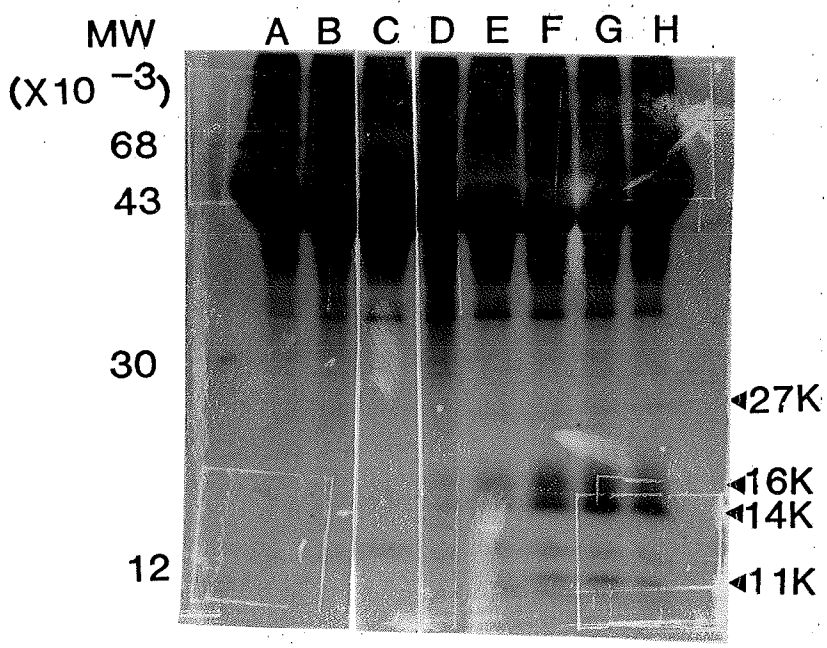
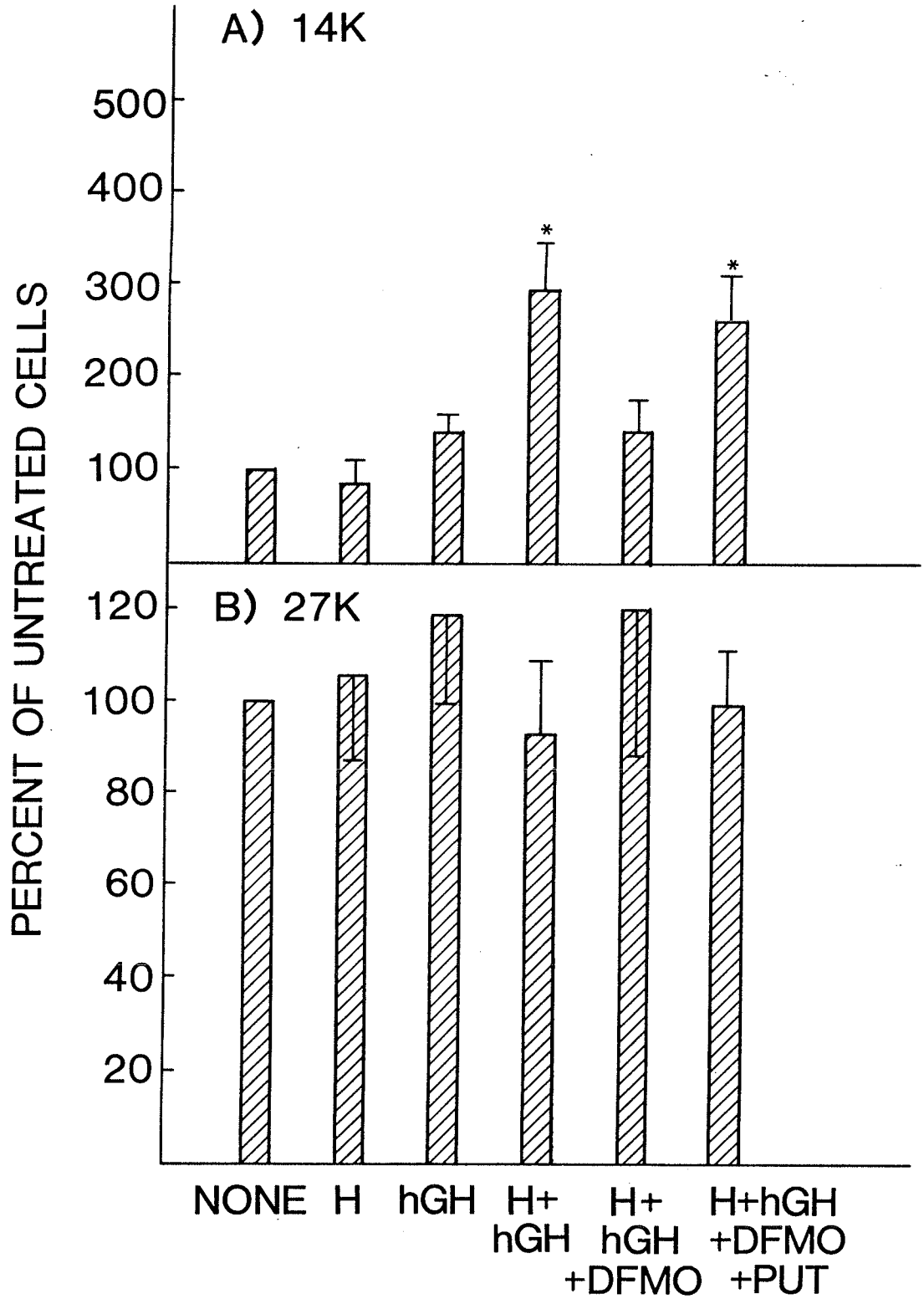

MW
(X10 -3)
A B C D E F G H
68
43
30
12
27K
16K
14K
11K

Fig. 9 Quantitative analysis of protein synthesis of T-47D cells. Protein bands from 15% SDS gels were cut and radioactivity determined as described in the methods. The counts per minute (CPM) associated with the 14K and 27K bands for each incubation condition were compared to the CPM in the same protein bands of untreated cells; the CPM in each of the protein bands of untreated cells was taken as 100%. Each value shown represents the mean of 5 experiments $\pm$ S.D. (* $p < .01$).


A) 14K
B) 27K
PERCENT OF UNTREATED CELLS
500
400
300
200
100
120
100
80
60
40
20
*
*
NONE
H
hGH
H+ hGH
H+ hGH +DFMO
H+hGH +DFMO +PUT

in general, a similar quantitative analysis was performed on a protein of molecular weight 27K. Fig. 9 B shows that this protein is not induced by either H or hGH alone. Nor is it induced by H + hGH. Addition of DFMO to the H + hGH-treated cells did not alter the appearance of the 27K hormone. Further treatment of the H + hGH-stimulated cells with putrescine also was ineffective. Thus, DFMO appears to inhibit the synthesis of only hormone-inducible proteins.

B. The role of polyamines in the growth-promoting activity of estrogens in T-47D cells

1. Growth studies

The important role of estrogens in the growth of mammary tumors has been well established. In vitro models for the study of estrogen-responsive human breast cancer have been established with cell lines such as the MCF-7 and ZR-75-1. The availability of a new T-47D subline (clone 11) that is positive for estrogen receptors has made it possible to study the factors involved in estrogen responsiveness in this human breast cancer cell line. We first studied the effect of physiological concentrations (10^{-10} M) of 17β-estradiol (E_2) under various serum conditions. Estradiol concentrations fluctuate from prepubertal levels of 3×10^{-11}M to 1×10^{-9}M at mid-menstrual cycle. Of the total E_2 approximately 2% is free, 38% is bound to

steroid hormone binding globulin, and the remainder is non-specifically bound (Wu et al., 1976). Thus, the E_2 concentrations we have used are in the physiological range. Fig. 10 A shows the estrogen responsiveness of cells maintained in fetal bovine serum depleted of steroid hormones by treatment with charcoal (cFBS). When cells are maintained in serum-free medium or 3% cFBS, a mitogenic effect of estrogen is not seen. The growth of cells maintained in 10% cFBS is slower than that of cells in 3% cFBS. But in 10% cFBS the growth of cells is stimulated 2 to 3-fold by 10^{-10} M E_2.

Cells maintained in 3% or 10% untreated FBS proliferate at a similar rate, and this growth rate is not affected by estrogen (Fig. 10 B).

2. Ornithine decarboxylase studies

a. ODC activity of E_2-stimulated T-47D clone 11 cells

Since the previous study showed that the growth of T-47D clone 11 cells was stimulated by estrogen, it would be important to determine if polyamines were involved in this estrogen-induced growth of T-47D clone 11 cells. The enzymatic activity of ODC was first determined. Fig. 11 A shows that a 24-hour exposure to E_2 resulted in an approximately 50% increase of ODC activity in cells maintained in 3% or 10% cFBS and failed to stimulate the enzyme in cells

Fig. 10 Effect of estrogen on T-47D clone 11 cell growth under various serum conditions. T-47D clone 11 cells were plated at 5×10^4 cells per 35 mm dish in RPMI 1640 medium supplemented with 10% (v/v) FBS. Two days later cells were washed twice and medium changed to the designated serum conditions. 24 hours later 17β-estradiol (E_2) was added to a final concentration of 10^{-10} M. Cells were counted 6 days later. Each value represents the mean of triplicates $\pm$ S.D. (* $p < .01$ compared to the corresponding serum-only situation).

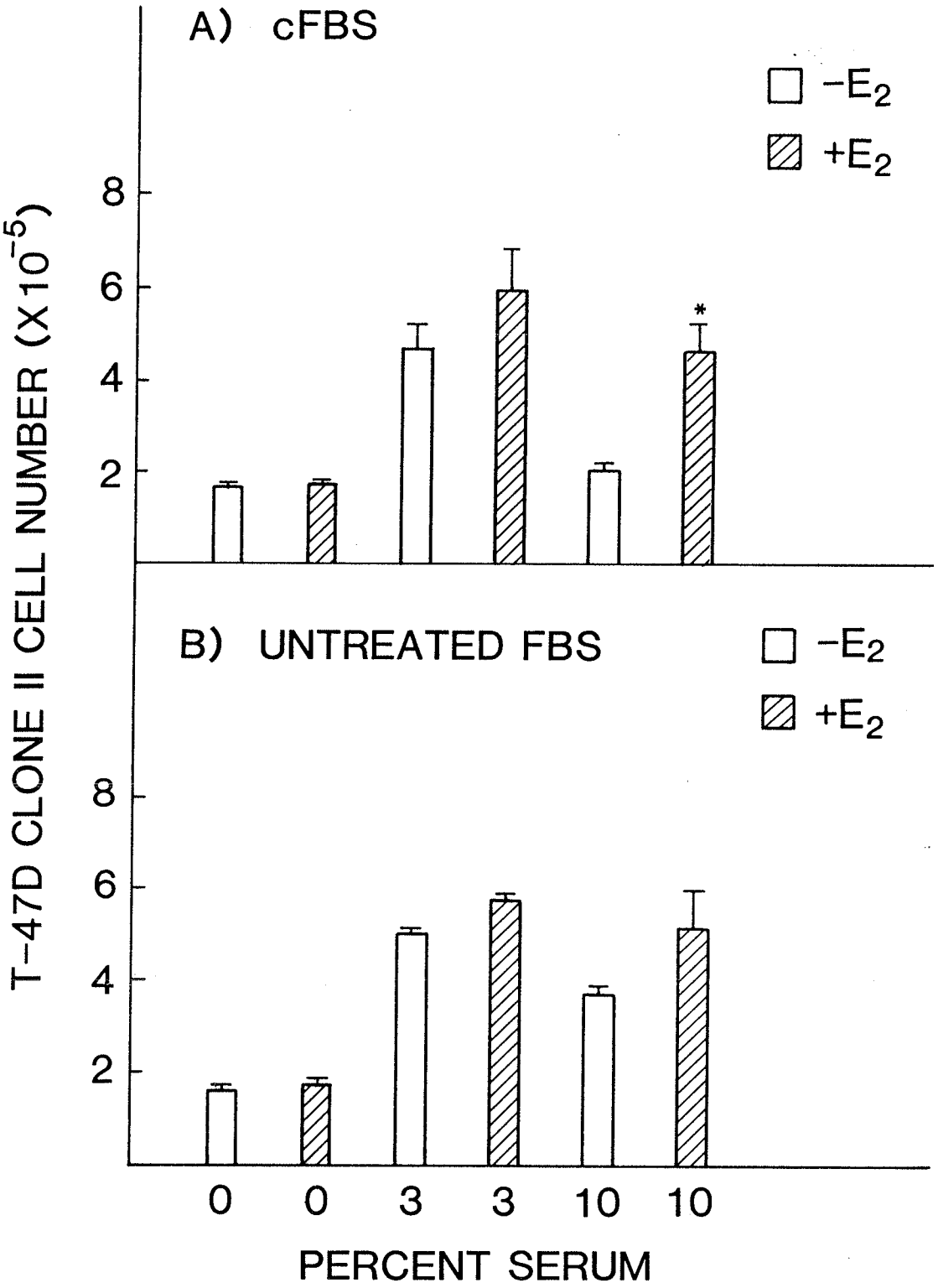
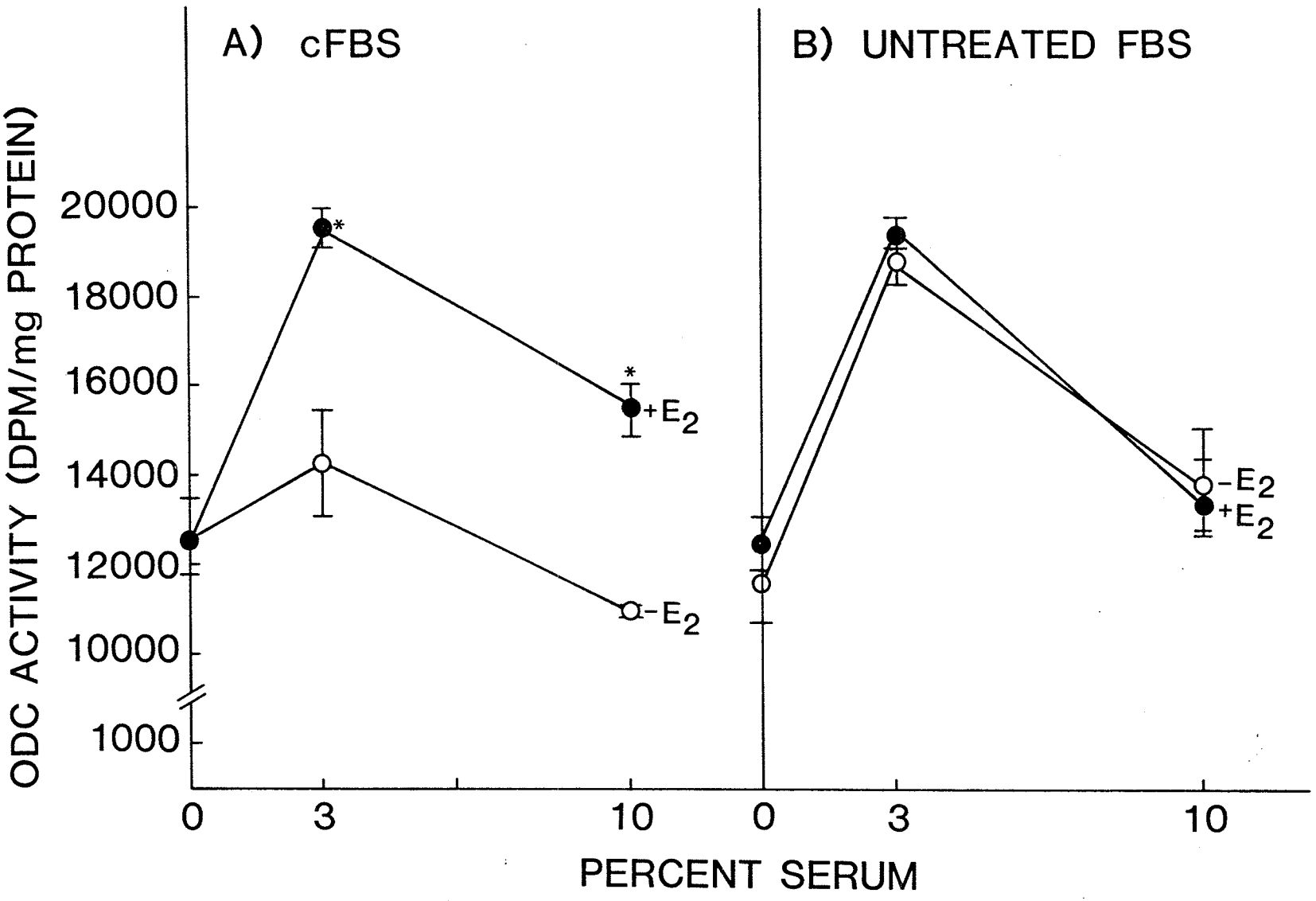

A) cFBS
$-E_2$
$+E_2$
8
6
4
2
*
B) UNTREATED FBS
$-E_2$
$+E_2$
8
6
4
2
T-47D CLONE II CELL NUMBER ($X10^{-5}$)
0 0 3 3 10 10
PERCENT SERUM

Fig. 11 ODC activity of E_2-stimulated T-47D clone 11 cells under various serum conditions. T-47D clone 11 cells were plated at 1 x 10^6 cells per 100 mm dish in RPMI 1640 medium supplemented with 10% (v/v) FBS. Two days later cells were washed twice and medium changed to the designated serum conditions. 24 hours later 17β-estradiol (E_2, 10^{-10} M) was added. Cells were harvested 24 hours later and assayed for ODC. Each value represents the mean of triplicates $\pm$ S.D. (* $p < .01$ compared to unstimulated cells in same serum condition).


A) cFBS
B) UNTREATED FBS
ODC ACTIVITY (DPM/mg PROTEIN)
20000
18000
16000
14000
12000
10000
1000
*
*
+E2
-E2
-E2
+E2
0
3
10
0
3
10
PERCENT SERUM

maintained in serum-free medium. When the cells were maintained in untreated FBS (Fig. 11 B) E_2 failed to stimulate ODC activity.

Based on the growth and ODC studies subsequent experiments were performed on cells maintained in 10% cFBS.

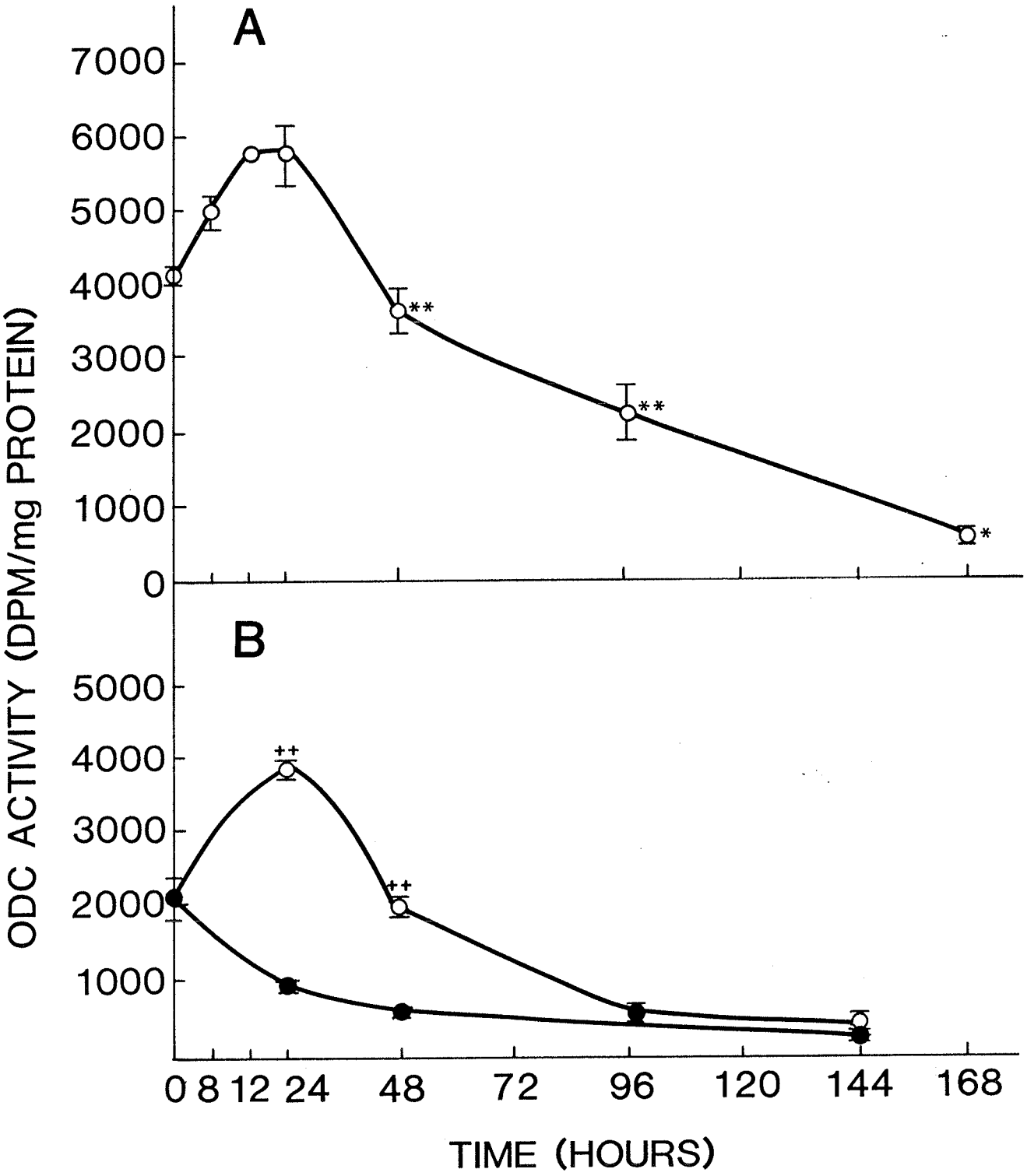
b. Time course study of E_2 stimulation

In order to get some idea as to how rapid the response of ODC to estrogen was, a time course study was performed. A significant (25%) increase in ODC activity was observed 8 hours after E_2 treatment. Maximal activity was seen at 12 hours after 17β-estradiol (10^{-10} M) stimulation, and this elevated level was maintained at 24 hours (Fig. 12 A). By 48 hours the enzyme activity had dropped to baseline levels and condinued to decrease until almost undetectable levels by 7 days. Based on the results of this study, subsequent enzyme assays were performed on cells that had been stimulated with estradiol for 24 hours.

c. Time course study of ODC inhibition by DFMO

In order to determine the possible involvement of polyamines in estrogen-induced growth of T-47D cells DFMO was used to block ODC activity, thus polyamine synthesis. Since the growth assay for estrogen response was performed with cells that do not undergo a medium change for 6 days it was also important to determine if DFMO was stable after

Fig. 12 Time course study of ODC activity of T-47D cells: Estrogen stimulation and DFMO effect. T-47D clone 11 cells were plated at 1 x 10^6 cells per 100 mm dish in RPMI 1640 medium supplemented with 10% (v/v) FBS. Two days later cells were washed twice and medium replaced with RPMI supplemented with 10% (v/v) cFBS. Panel A. 24 hours later E_2 (10^{-10} M) was added. Cells were harvested at the times indicated and assayed for ODC activity. Panel B. 24 hours later E_2 (10^{-10} M) and DFMO (0.1 mM) were added. Cells were harvested at various times and assayed for ODC. Each value represents the mean of triplicates $\pm$ S.D. (* $p < .01$ compared to 24 hr. cells. ** $p < .05$ compared to 24 hr. cells. ** $p < .01$ compared to E_2 + DFMO cells at the same time point).


A
B
7000
6000
5000
4000
3000
2000
1000
0
5000
4000
3000
2000
1000
**
**
*
++
++
ODC ACTIVITY (DPM/mg PROTEIN)
0 8 12 24 48 72 96 120 144 168
TIME (HOURS)

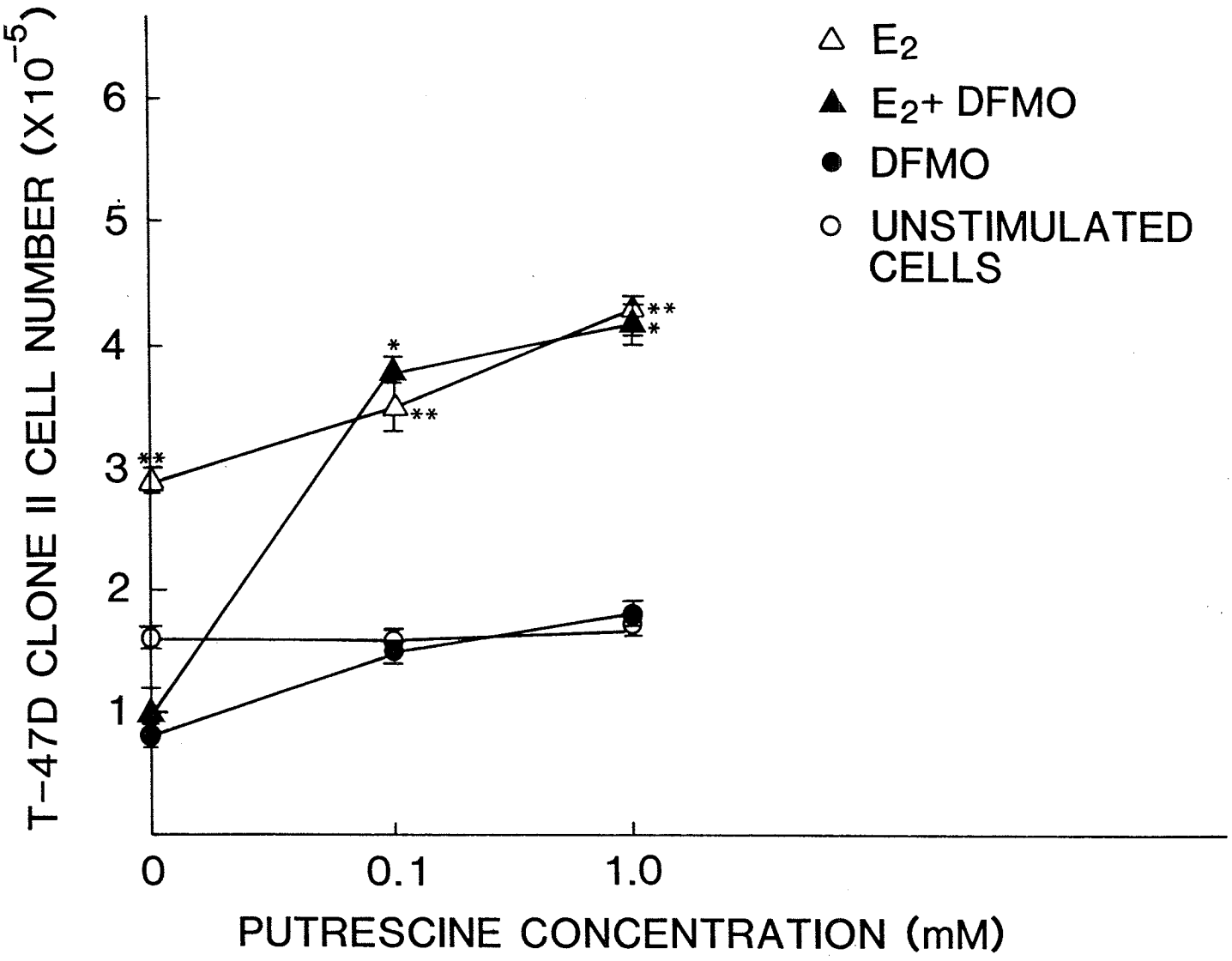
6 days in culture. Fig. 12 B shows that 0.1 mM DFMO effectively inhibited the E_2-induced ODC activity throughout.

3. DFMO effect on growth of estrogen-stimulated T-47D cells

Having established that (1) 17β-estradiol stimulated ODC activity in T-47D clone 11 cells; (2) that the hormone also stimulates growth of this cell line; and (3) that the enzyme levels are effectively reduced by DFMO, we next determined whether or not polyamines were involved in the estrogen-stimulated growth of T-47D cells. DFMO (0.1 mM) was added to the cells simultaneously with E_2 in 10% cFBS and cells were counted after 6 days. Fig. 13 shows that E_2 stimulated cell growth approximately 2-fold over unstimulated controls. DFMO prevented the estradiol stimulatory effect and also decreased growth of unstimulated cells by approximately 30%.

Exogenously added putrescine (0.1 mM), the natural product of ODC, was able to rescue the E_2-induced growth of the cells grown in the presence of the inhibitor, DFMO (Fig. 13). However, putrescine alone was not mitogenic to the cells not exposed to estradiol, and in combination with estradiol, it did not further stimulate cell growth above levels seen with E_2 alone.

Fig. 13 Effect of DFMO and putrescine on E_2-stimulated T-47D clone 11 cell growth. T-47D clone 11 cells were plated at 2×10^4 cells per 35 mm dish in RPMI 1640 medium supplemented with 10% (v/v) FBS. Two days later medium was changed to RPMI 1640 supplemented with 10% cFBS. 24 hours later E_2 (10^{-10} M), DFMO (0.1 mM), and putrescine (0.1 and 1.0 mM) were added. Cells were counted 6 days later. Each value represents the mean of triplicates $\pm$ S.D. (* $p < .01$ compared to E_2 + DFMO in the absence of putrescine; ** $p < .01$ compared to cells in the absence of E_2 and DFMO).


T-47D CLONE II CELL NUMBER (X10^{-5})
6
5
4
3
2
1
0
0.1
1.0
PUTRESCINE CONCENTRATION (mM)
E_2
E_2+ DFMO
DFMO
UNSTIMULATED CELLS
*
**

DISCUSSION

Although an unambiguous biological role for polyamines has yet to be clearly defined, they have been implicated to be essential for the proliferation and differentiation of a variety of cell types. The present studies were carried out to determine whether polyamines are involved in the hormonal regulation of human breast cancer cells.

The role of growth hormone and prolactin in human breast cancer and the mechanism of action of these hormones on breast cancer cells are poorly understood. The inability of prolactin and growth hormone to stimulate growth of T-47D human breast cancer cells in culture prompted investigations seeking other biological effects of these hormones.

We first examined the ability of a variety of polypeptide hormones to induce ornithine decarboxylase (ODC) activity in the human breast cancer cell line T-47D. Of all the polypeptide hormones tested only hGH and hPrl at concentrations higher than 100 ng/ml were capable of producing a 2 to 3-fold elevation of ODC activity but only in the presence of hydrocortisone. While the effective concentrations of hGH and H are higher than physiological levels (5-20 ng/ml and 10-100 ng/ml, respectively), it must be kept in mind that these studies were performed under <u>in vitro</u> conditions

where hormone degradation and inactivation occurs (Shiu, 1980) and this necessitates the use of higher than physiological concentrations of the hormones. Ovine Prl and GH were incapable of stimulating ODC activity, thus this response in T-47D cells is specific for human lactogenic hormones. It has been shown that hPrl and hGH occupy the same receptor sites in T-47D cells (Shiu, 1979), supporting the finding that these two hormones have the same biological effect in these breast cancer cells.

The elevation in ODC activity is an early cellular response to growth hormone and hydrocortisone stimulation. Time course studies of enzyme induction by H + hGH showed that the enzyme activity peaked at 24 hours to levels that were 3-fold higher than controls. At 48 hours hormone-stimulated ODC activity had decreased and by 96 hours enzyme levels were no longer elevated. This pattern of ODC induction in response to hormonal stimulation is commonly seen (Cohen et al., 1970; Russell et al., Russel and Taylor, 1971). Perhaps the initial elevation in ODC activity is sufficient to raise polyamine concentrations to levels that are required for the hormone response.

When T-47D cells are grown on plastic substratum they display typical epithelioid characteristics. The cells are flat and polygonal in shape and adhere strongly to the plastic. In response to stimulation by H + hGH

the T-47D cells undergo a dramatic morphological alteration. Cells become round and refractile, adhering less firmly to the plastic substratum. As a result, they grow in clusters containing many of these rounded cells. This observation represents the first time such a biological effect has been demonstrated in T-47D human breast cancer cells in response to hGH + H (Shiu and Patterson, 1984).

We wanted to determine if the increase in ODC activity of the H + hGH-stimulated cells was at all related to the hormone-induced cell shape change. α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, and thus polyamine synthesis, was used to inhibit the hormone-induced enzyme activity. DFMO at 0.1 mM maximally inhibited ODC activity and was effective even after 7 days in culture. However, DFMO was unable to reverse the hormone-induced T-47D cell shape change. This initial observation suggested that polyamines are not required for this particular biological effect of hGH and H action. It is not known at this time what role the altered morphology of T-47D human breast cancer cells, under the synergistic action of growth hormone and hydrocortisone, plays in the pathogenesis of human breast cancer. For this reason it is difficult to attribute a specific role for polyamines in the hGH + H-induced cell shape change.

The observation that hGH synergizes with H to induce the synthesis of unique secretory proteins by T-47D cells (Shiu and Iwasiow, manuscript submitted) opened another avenue to aid in elucidating the mechanism of growth hormone action on breast cancer cells. Treatment of cells with hGH and H for 3 to 4 days results in the maximal induction of three proteins of molecular weights 11,000 (11K), 14,000 (14K), and 16,000 (16K) daltons, the latter two being glycoproteins. Neither hGH nor H alone is capable of inducing the synthesis of these proteins. Of the three hormone-inducible proteins, 14K was the most prominent.

The involvement of polyamines in the hormone-induced protein induction was studied with the use of DFMO. When DFMO (0.1 mM) was added simultaneously with hGH and H it was observed that the synthesis of 14K protein was significantly reduced. However, the synthesis of other proteins did not appear to be affected. Quantitation of the degree of inhibition of protein synthesis by DFMO (Fig. 9) showed that 14K was suppressed to an extremely low level, comparable to that seen in cells not treated with hormone. This inhibition appears to be specific for the hormonally-regulated 14K protein since other proteins not regulated by hormones are not affected. Further, 0.1 mM putrescine effectively rescued the DFMO-induced inhibition of the 14K protein.

The identity of the growth hormone-inducible 14K protein and its significance to human breast cancer, though presently unkown, are under active investigation in this laboratory. It does not appear that the 14K protein is

responsible for the hGH + H-induced cell shape change of the T-47D breast cancer cells since inhibition of ODC with DFMO does not reverse the hormone-induced shape change, yet it inhibits the appearance of the hormone-induced 14K protein. It is possible that the appearance of the 14K protein in hGH + H-treated cells may reflect a differentiated function that is induced by these hormones, but at the present time there is no evidence for this.

The induction of new proteins by hGH suggests that the mechanism of action of the hormone in T-47D human breast cancer cells may involve the activation of gene expression and that polyamines are part of the pathway required for the induction of the gene. This is suggested by the observation that putrescine, the natural product of ODC activity, was unable to augment the hormone effect.

The mechanism of action of polypeptide hormones in mammary glands has been proposed to involve a complex interplay of polyamines, prostaglandins, cyclic nucleotides, phospholipids, and calcium ions (Rillema, 1982). In normal mammary cells synthesizing casein it has been proposed that after prolactin binding to its plasma membrane receptor, membrane-bound phospholipases A_1 and A_2 are stimulated; causing the release of polyunsatured fatty acids, including arachidonic acid, to be released from membrane phospholipids. The arachidonic acid is then converted to prostaglandins via

the membrane-associated prostaglandin synthetase complex. The prostaglandins may then stimulate guanylate cyclase activity and increase cyclic GMP synthesis. The cyclic GMP in turn stimulates cyclic AMP-phosphodiesterase and reduces tissue content of cyclic AMP. The altered cyclic nucleotide levels may then stimulate RNA synthesis which is required for the enhanced rate of casein synthesis. Polyamines also appear to be required since inhibition of spermine decreases casein synthesis (Rillema, 1976 b).

Whether prolactin and growth hormone exert their actions in mammary tumors via the same mechanism is not clear, since polyamines have been shown to be required for at least one of the biological effects of hGH in T-47D cells. Further, the recent finding that growth hormone phosphorylation by an epidermal growth factor-stimulated protein kinase is required for the insulin-like action of growth hormone on human epidermoid carcinoma cells (Baldwin et al., 1983) suggests that the above-mentioned pathway may not be complete in describing the action of polypeptide hormones at their target cells. Also, in Nb2 rat lymphoma cells, protein phosphorylation occurs within minutes of Prl stimulation and this is followed by a peak in ODC activity in 6 to 8 hours after Prl stimulation (Elsholtz, 1984).

The exact role that ODC, and therefore polyamines, plays in the hGH + H-inducible 14K glycoprotein in T-47D human breast cancer cells is uncertain at this point in time.

Previous findings of hormone-inducible proteins (Mariesse et al., 1981; Edwards et al., 1980; Veith et al., 1983), specific hormone-regulated genes (Masiakowski et al., 1982) and nuclear-associated ODC (Emanuelson and Heby, 1983) lends credence to the hypothesis that the hGH-induced proteins are regulated by gene expression and that polyamines may be involved in the process of gene expression.

The role of polyamines in hormone-induced cell proliferation is not clear. This led us to investigate the possible role of polyamines in estrogen-stimulated proliferation of human breast cancer cells. For these studies we used an estrogen receptor-positive subline of T-47D cells (clone 11) that has been shown to be estrogen-responsive (Chalbos et al., 1982).

The clone 11 cells were most estrogen-responsive when they were maintained in charcoal-treated, heat-inactivated fetal bovine serum (cFBS). Cells grown in either serum-free medium, 3%, or 10% untreated FBS were not responsive to 17β-estradiol (E_2, 10^{-10} M). These findings suggest that it is first necessary to deplete the serum of endogenous steroids, including estrogens, in order to see an estrogen response.

In 10% cFBS, E_2 consistently stimulated cell growth 2 to 3-fold above controls. Cells maintained in 3% cFBS were less responsive to E_2 than those maintained in 10% cFBS because the growth rate of cells in the absence of E_2 is already high.

There is an apparent inhibition of cell growth at high serum levels and this inhibition is reversed by estrogen. A clear explanation has not been given for the observation that cells grow slower in 10% serum than in 3% serum. The same observation has been reported by Soto and Sonneschein (1983). Their explanation was that estrogen removes the inhibitory factors found at high serum levels. An alternative explanation is that E_2 can act directly on the cell and reverse the inhibitory effect of the serum factors.

The high serum requirement for estrogen responsiveness is supported by the findings of Page et al. (1983) who showed that growth stimulation by estrogen of MCF-7 breast tumor cells could only be observed when cells were maintained in medium supplemented with 15% newborn calf serum but not in 0.5% serum. This observation suggests that serum contains a specific factor or factors that can influence the expression of the growth response to estradiol. The identity of this factor(s) is presently under investigation but several substances have been shown to regulate the estrogen-sensitive growth of breast cancer cells. These include the tripeptide glycyl-histidyl-lysine (Green and Dembinski, 1984), and phosphoethanolamine (Kano-Sueoka et al., 1979).

After 12 to 24 hours of E_2 (10^{-10} M) stimulation, an approximate 2-fold increase in ODC activity of the T-47D clone 11 cells was noted in cells maintained in medium containing 3% or 10% cFBS. The same elevation of ODC in response to E_2 was not observed in cells maintained in serum-free medium or medium supplemented with untreated FBS. The peak of enzyme activity occurred at 12 to 24 hours after hormone stimulation. At 48 hours enzyme activity had returned to control levels. It appears that E_2 induces the appearance of ornithine decarboxylase more rapidly than do the polypeptide hormones, hGH and hPrl, since ODC activity of T-47D cells had not yet peaked at 12 hours when cells were treated with hGH + H. The more rapid induction of enzyme by E_2 in the T-47D clone 11 cells may reflect their increased estrogen-responsiveness.

To determine if polyamines were involved in the estrogen-induced growth of T-47D human breast cancer cells DFMO was again used to inhibit ornithine decarboxylase activity. The stability of 0.1 mM DFMO over the 6-day growth period was once again determined to verify that the ODC inhibitor was equally effective in suppressing enzyme activity in E_2- stimulated cells as well as in cells that had been stimulated with hydrocortisone and growth hormone. DFMO (0.1 mM) effectively removed the E_2-induced ODC peak at 12 to 24 hr and continued to suppress enzyme activity up to 7 days. Thus it was determined that DFMO was still active at the end of the 6-day growth assay.

DFMO (0.1 mM) produced a 2 to 3-fold inhibition of growth in estradiol-stimulated T-47D clone 11 cells. This DFMO-induced inhibition of growth was abolished by exogenous putrescine at concentrations of 0.1 mM or 1 mM. However, putrescine alone was not stimulatory to T-47D cells and did not produce an additive or synergistic effect with estrogen. These findings suggest that polyamines are essential in the action of estrogen stimulation of human breast cancer cells but alone are not sufficient to mimic the effect of the hormone.

The mechanism by which polyamines are involved in the estrogen-induced cell growth is unclear. We found that estradiol stimulated ODC activity of T-47D cells maintained in 3% and 10% cFBS but an estradiol effect on cell growth was observable only in cells maintained in 10% cFBS. We suggest that since the cells are already growing at a maximal rate in 3% cFBS an increase in ODC activity is not going to push the growth of these cells any further. However, cells maintained in 10% cFBS are not maximally growing, therefore stimulation of ODC enhances growth.

The fact that estradiol acts as an inducer of ornithine decarboxylase suggests that the induction of the enzyme is an early, but not necessarily primary, event in the action of estrogens. The rapid induction of ornithine decarboxylase and the subsequent accumulation of putrescine or polyamines may provide a mechanism by which the cell may rapidly alter

its internal environment to optimize conditions for a variety of biosynthetic reactions (Cohen et al., 1970). The mechanism(s) by which estrogens regulate ornithine decarboxylase levels in T-47D human breast cancer cells is not known. The possibility exists that the ODC gene is an estrogen-inducible gene. Whether estrogens induce the expression of the ODC gene directly or via some mediator(s) of hormone action remains to be determined. Other factors may first be induced by estrogens in breast cancer cells prior to ODC induction. The net result is a finely controlled system that regulates the growth of these cells.

The exact mode of action of estrogen-induced growth of human breast cancer cells has not been well established. The mechanisms observed in normal estrogen target tissues are assumed to apply to breast cancer. Estrogens passively enter the target cell and bind to high-affinity cytoplasmic estrogen receptors (ER). This is followed by the energy-dependent transformation and translocation of the cytoplasmic steroid-receptor complex into the nucleus where the ER complex is believed to interact with acceptor sites in the chromatin to initiate synthesis of specific mRNA and new proteins. Several specific estrogen-induced proteins have been identified (Mairesse et al., 1981; Edwards et al., 1980; Veith et al., 1983). Whether these proteins are mitogenic factors induced by estrogens to stimulate

cell growth via an autocrine mechanism has not been clearly demonstrated. It cannot be ruled out that these proteins are also synthesized by the cells in the absence of estrogen and that they are required by estrogen in order to induce growth.

Recent work on the regulation of gene expression by estrogen (Masiskowski et al., 1982) supports the potential importance of the ornithine decarboxylase gene as possibly being estrogen-induced or to undergo amplification in response to estrogen. It will be important to obtain the genetic clones corresponding to ODC mRNA whose levels are rapidly increased by estrogens in T-47D breast cancer cells and to determine whether expression of the corresponding gene (ODC) is in any way related to the estrogen-responsiveness of the cells. Thus, the ODC gene can be used as a marker of estrogen-responsiveness.

The present studies also raise the possibility that DFMO may have therapeutic potential in the treatment of breast cancer patients with increased ODC activity in estrogen-responsive tumors. In vivo studies have shown that DMFO is able to reduce the rate of growth and pulmonary metastatic spread of renal adenocarcinomas in mice (Kingsworth et al., 1983 b). Polyamines also have an apparent role in the stabilization of DNA by inducing the condensation of DNA (Bloomfield and Wilson, 1981). It has been shown that polyamine depletion appears to cause an alteration in

mammalian cell DNA (Hung et al., 1983). These effects of polyamines on the structural integrity of DNA, the probable target of cytotoxic actions of many anticancer drugs, suggests a possible role for polyamine depletion in cancer chemotherapy. Reports of increased or decreased cross-linking of cytotoxic drugs (1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU) and cis platinum, respectively) to DNA by DFMO suggests that polyamine depletion can be either beneficial or detrimental in the action of chemotherapeutic agents (Tofilon et al., 1983). Since DFMO is currently undergoing clinical trials (Warrell et al., 1983) for the treatment of various types of advanced cancer, including colon, prostate, and bladder cancers, it is important that the effects of DFMO treatment in conjunction with a given agent be studied in the laboratory before any combination is used in a clinical setting.

The possible clinical effectiveness of DFMO as a chemotherapeutic agent and its ability to block hormone-induced ODC activity in human breast cancer cells maintained in culture makes it a powerful tool for the clinical oncologist as well as endocrinologist in the elucidation of the mechanism of polypeptide hormone and estrogen action.

The present studies represent the first time that ODC has been shown to be regulated by hormones in a human breast cancer cell line, T-47D. It is possible, then, that ODC

can be used as a marker for hormone-responsive cancers and that treatment protocols can be based on ODC activity. Further, polyamines have been shown to be required for specific hormone responses.

These studies were performed on the T-47D human breast cancer cell line derived from the pleural effusion of an infiltrating carcinoma of the breast. Thus, it represents a subpopulation of human mammary tumors and in itself is a mixed population of cell types. Conclusions drawn from these studies are therefore limited to one subpopulation of cancer cells. In order to verify the results as generally occurring in breast cancer further studies must be performed using other responsive cell lines and cells obtained from primary cultures of human breast cancer.

The findings of this study can be used to direct further studies, especially in the area of gene regulation. It is possible that the role of polyamines in estrogen-induced T-47D cell growth is to act as modifiers of hormonal responsive gene activity. New avenues in breast tumor regulation by hormones can be opened via studies of estrogen-induced gene regulation.

In summary, this study has shown that:

(1) ODC enzyme levels are increased in T-47D human breast cancer cells stimulated by human growth hormone (hGH) and hydrocortisone (H);

(2) Polyamines are not required for the hGH + H-induced shape change of T-47D cells;

(3) Polyamines are required for the hGH + H-induced synthesis of a 14K glycoprotein;

(4) Estradiol (E_2) stimulates ODC levels and growth of T-47D clone 11 cells; and

(5) Polyamines are required for the E_2-induced growth of T-47D clone 11 cells.

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