STUDIES ON THE GROWTH EFFECT OF SOMATOMEDINS ON HUMAN BREAST CANCER CELLS

by

Yvonne Myal

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Submitted to the Faculty of Graduate Studies
as a Partial Requirement for the Degree of
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Department of Physiology
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ABSTRACT

The somatomedins (SM) are a family of serum peptides that are mitogens for a variety of normal and transformed cells in culture. The multiplication stimulating activity (MSA), similar to insulin-like growth factor-II (IGF-II), is believed to be important for fetal growth. The basic somatomedin (BSM), similar to insulin-like growth factor-I/somatomedin-C (IGF-I/SM-C), is believed to mediate the action, in vivo, of pituitary growth hormone. The proliferation in vitro of a human breast cancer cell line (T-47D) is dependent on factors in fetal bovine serum, and its growth in vivo in athymic nude mice is dependent on pituitary hormones. In addition, in this experimental model T-47D tumor size correlated with circulating levels of growth hormone (GH). These observations led us to examine the role that the SM play in the growth control of human breast cancer cells. It was found that serum-free conditioned medium of Buffalo rat liver (BRL) cells which produce MSA, stimulated the proliferation of T-47D cells 4-12-fold over control in a 9-day assay. However, purified MSA (1-1,000 ng/ml) did not affect cell growth in serum-free medium. If serum-free medium was supplemented with transferrin, hydrocortisone, epidermal growth factor (EGF), insulin and triiodothyronine (defined medium), the addition of MSA produced a 1.9-6.6 fold increase in T-47D cell number compared to defined medium alone. This suggests that MSA acts synergistically with other serum factors. In contrast, human BSM resulted in a small but reproducible 1.5-fold stimulation of cell growth either in serum-free or defined media. A larger

stimulation (2.5-fold) was achieved with BSM if the T-47D cells were grown on collagen gel instead of plastic substratum. This suggests that the nature of the substratum may influence the response of T-47D cells to growth factors. In addition, specific receptor binding of ^{125}I-BSM was demonstrated in the T-47D cells: 100 ng/ml of unlabelled BSM produced 50% inhibition of ^{125}I-BSM binding, whereas 1,000 ng/ml of unlabelled MSA did not displace ^{125}I-BSM binding. This result indicates that the binding sites for BSM and MSA are distinct in T-47D cells. Our results suggest that GH/BSM may play a role in the growth regulation of human breast cancer. The observation that human breast cancer cells responded to MSA, which is postulated to be a fetal growth factor, may indicate the fetal characteristics of these cells acquired as a result of de-differentiation associated with malignant transformation.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Hormones and growth factors

Prl	prolactin
GH	growth hormone
EGF	epidermal growth factor
FGF	fibroblast growth factor
PDGF	platelet-derived growth factor
NGF	nerve growth factor
MSF	mammary stimulating factor
SM	somatomedin
MSA	multiplication stimulating activity
NSILA	nonsuppressible insulin-like activity
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II

Units of measure

ng	nanogram
ug	microgram
mg	milligram
pg	picogram
g	gram
ul	microliter
ml	milliliter
l	liter
mm	millimeter
cm	centimeter
mM	millimolar
M	molar
MW	molecular weight
i.u.	international unit
uC_i	microcurie
C_i	curie
pI	isoelectric point
S.D.	standard deviation
v/v	volume per volume

w/v	weight per volume
^{o}C	degrees centigrade
rpm	revolutions per minute
cpm	counts per minute

Others

FBS	fetal bovine serum
DMEM	Dulbecco's Modified Eagle's Medium
BSA	bovine serum albumin
HBSS	Hank's balanced salt solution
PBS	phosphate buffer saline
EDTA	ethylenediamine tetraacetic acid
SDS	sodium dodecyl sulphate
RIA	radioimmunoassay
^{125}I	^{125}iodine
LKB	gamma counter
DMBA	7,12-dimethylbenz-(a)-anthracene
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
DNA	deoxyribonucleic acid
h	human

TABLE OF CONTENTS

GENERAL INTRODUCTION

A. Hormonal influences of breast cancer

The growth and differentiation of breast cancer tissue, as in normal mammary tissue, is influenced by a number of hormones. Hormones of ovarian origin such as estrogen and progesterone, pituitary hormones such as prolactin (Prl) and growth hormone (GH), and insulin are implicated in playing a role in the development of breast cancer. Growth factors derived from the pituitary, other endocrine glands and various other sources are also active in stimulating neoplastic mammary cells.

The following is a brief overview of some of the experimental findings that led to these conclusions.

1. Estrogens

A connection between estrogens and breast cancer has long been suspected for a number of reasons including the fact that (1) removal of the ovaries would lead to objective improvement in some patients with metastatic breast cancer (Beatson, 1896) and (2) many breast cancers in humans and animals were shown to be estrogen dependent. The mechanism by which the ovary acts on the breast has remained obscure for many years but it is only within the last decade that it has begun to be elucidated.

Early studies suggested that the binding of hormone to specific cytoplasmic receptor proteins was a critical first step in steroid hormone action (Jensen and De Sombre, 1972). After binding to the receptor, the steroid receptor complex is

translocated to the nucleus where it associates with a specific acceptor site in the chromatin and accelerates the rate of nuclear biosynthetic processes.

This observation led to the selection of an alternate course of endocrine therapy for those cancer patients whose tumors had identifiable hormone receptors. Synthetic estrogens and antiestrogens such as tamoxifen have been commonly used in the treatment of advanced breast cancer.

However the presence of an estrogen receptor did not always guarantee that ovariectomy and/or endocrine therapy would induce regression. About half of the tumors with estrogen receptors do not regress after ovariectomy (McGuire et al., 1975) and about one-third of the patients considered estrogen receptor positive do not respond to endocrine therapy.

The importance of estrogen in the growth of breast cancer has been repeated in vitro using several human breast cancer cell lines. Although the precise mechanism of action of estrogen is not known, breast cancer cells demonstrate a variety of growth responses to physiological concentrations of estrogen (Lippman, 1975) whereas antiestrogens have been shown to suppress growth (Lippman, 1976). A variety of specific products have also been shown to be under estrogen control in some breast cancer cell lines (Horwitz and McGuire, 1978; Westley and Rochefort, 1980; Mairesse et al., 1981). Other investigators have been unable to find a direct mode of action of estrogen in vitro, and this has led many workers to speculate about an indirect action. One of these workers, Sirbasku (1978), proposed that estrogen action may be mediated by the production

or secretion of polypeptide growth factors specific for various types of hormone-responsive tumor cells. These polypeptide growth factors were termed "estromedins" (Sirbasku, 1980).

Whether estrogen works directly or indirectly on human breast cancer cells, the fact remains that it is an important hormonal factor.

2. Prolactin and growth hormone

The important role of Prl in experimental tumorigenesis is well established (Welsch and Nagasawa, 1977), whereas its importance in human breast cancer is not. Prolactin receptors are present in variable quantities in carcinogen-induced rat mammary tumors (Costlow et al., 1976; De Sombre et al., 1976; Holdaway and Friesen, 1976; Horrobin, 1978). Rat mammary tumors which grow in response to Prl administration possess higher levels of Prl receptors than Prl unresponsive tumors after treatment with Prl (Holdaway and Friesen, 1976). Subsequently, suppression of Prl by bromoergocryptine has caused regression of those tumors with higher receptor content (Holdaway and Friesen, 1976).

The presence of Prl receptors in several human breast lines have been reported (Shiu, 1979) and have been shown to have high affinity for Prl. Human growth hormone can also compete with human (h) Prl for Prl receptor sites. The observation that hGH shares the same receptor site for Prl in human breast tumor cells (T-47D), raises the possibility that hGH may have Prl-like effects in this tumor (Shiu, 1979).

Clinical reports on Prl and GH status of women with breast cancer have been inconclusive. Higher plasma hPrl levels have

been reported in breast cancer patients than in healthy controls (Murray et al., 1972; Rolandi et al., 1974) but this could not be confirmed by other studies (Dicky et al., 1972; Boyns et al., 1973; Franks et al., 1974). Sarfaty et al. (1976) compared plasma Prl levels of normal and primary and metastatic cancer patients. They found that whereas hPrl levels were generally higher in normal premenopausal women or breast cancer patients than in respective postmenopausal subjects or women after ovariectomy, within each category breast cancer patients had significantly increased hPrl values. Following ovariectomy, hPrl levels dropped more sharply in responders than nonresponders to endocrine therapy. Higher levels of hPrl were also reported in daughters of women with breast cancer (Anderson, 1973; Kwa et al., 1974; Henderson et al., 1975).

Emerman et al. (1981) reported GH levels to be higher in some patients with breast cancer. In addition, Greenwood et al. (1968) had earlier reported an increase in GH levels in plasma of breast cancer patients before and after mastectomy, but Marlarkey et al. (1977) failed to observe such a change.

Recently, Leung and Shiu (1981) have reported that GH levels, but not Prl, correlated with the growth of human breast cancer transplanted in immunodeficient athymic mice (nude mice). Hobbs et al. (1974) found that in approximately 5-10% of breast cancers were Prl dependent to some degree. However purified hPrl and hGH failed to affect the growth rate of human breast tumor cells in vitro (Beeby et al., 1975; Shiu, 1981). These observations led some investigators to hypothesize that the mitogenic effect of Prl and GH must also be an indirect one.

The importance of Prl and GH in rodent breast cancer has provided the impetus to study the role of these hormones as well as their intermediates.

In vivo mediators of growth hormone and prolactin activity:

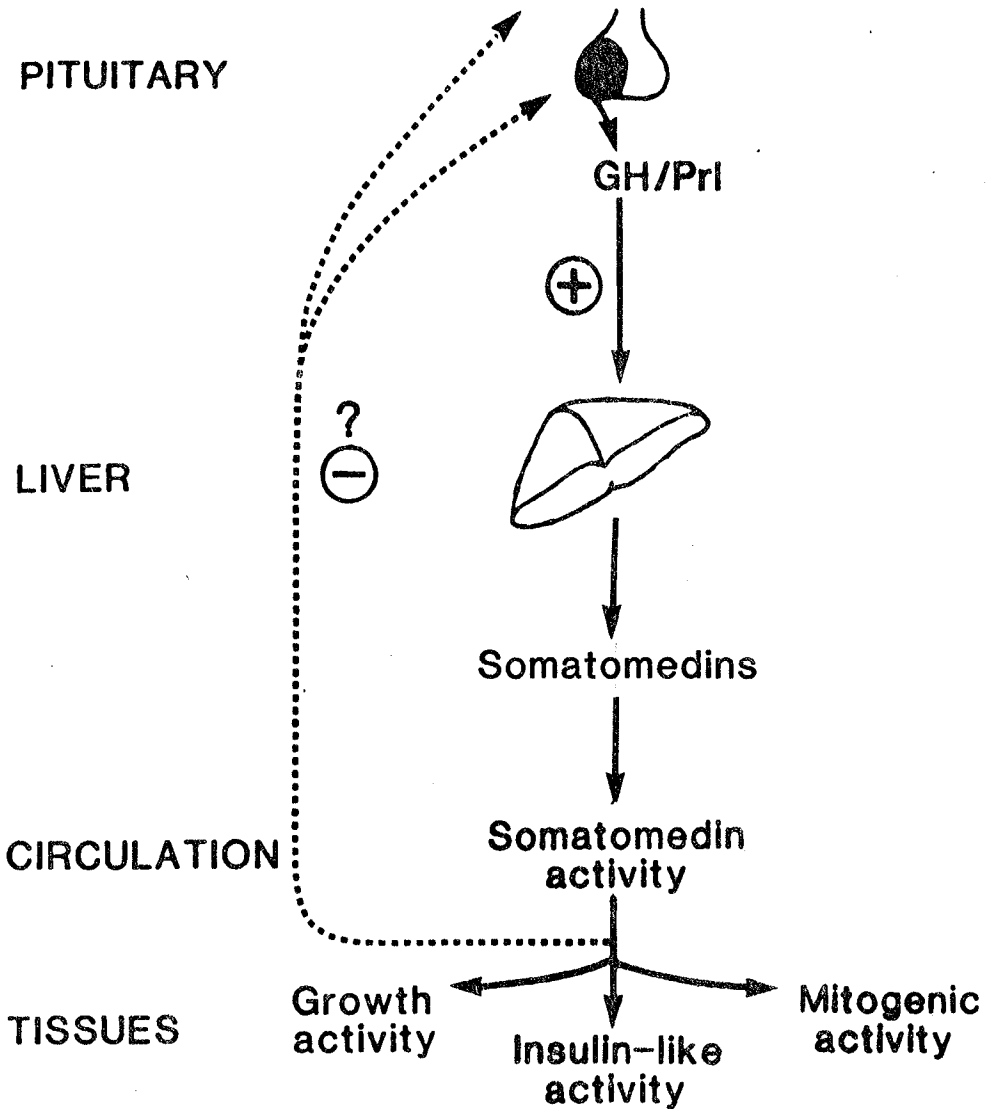
The somatomedin hypothesis

It has been long observed that GH stimulates linear growth by its action on cartilage. Originally, GH was thought to act directly on cartilage. In 1957, the classical experiment by Salmon and Daughaday examined the mechanism of action of GH. It was found that GH stimulated the formation of "sulphation factors" in serum which then directly stimulated the uptake of sulphate by cartilage. These sulphation factors therefore acted at the cellular level and promoted proliferation of cartilage cells.

These factors further exhibited a spectrum of biological actions which were not limited to cartilage. They were shown to display insulin-like actions on muscles and adipose tissue as well as growth promoting activity on Hela cells (Salmon and Hoss, 1971). Subsequently, a more general term "somatomedins" replaced the operational term sulphation factor activity. The term defines any GH-dependent substance in plasma which stimulates growth in responsive tissue. (See Fig. 1.)

The relationship between somatomedins (SM) and GH has been extensively reviewed (van Wyk and Underwood, 1975; Phillips and Vassilopoulou-Sellin, 1980a). It has been agreed that in order to qualify as a SM, a peptide should fulfill the following criteria: (1) production is dependent on GH, (2) enhances sulphate incorporation into cartilage, and (3) exerts insulin-

Fig. 1. A schematic illustration of the relationship between GH and somatomedin. After GH is secreted, it interacts with receptors on target tissue, triggering the secretion of small molecular weight (MW) peptides, somatomedins. Somatomedins, which have insulin-like properties, circulate to a large extent bound to a serum protein. After interacting with specific receptors, the free component of somatomedin causes cell multiplication and growth.


PITUITARY
GH/Prl
LIVER
?
Somatomedins
CIRCULATION
Somatomedin activity
TISSUES
Growth activity
Insulin-like activity
Mitogenic activity

like effects upon extraskeletal tissues. Despite the fact that it has been more than 20 years since SM activity was identified, purified preparations have become available only recently.

The difficulties in isolation and characterization of hSM have been formidable because there are no tissues for extraction. Therefore, the SM have been isolated from large volumes of plasma or serum.

The purified or partially purified SM isolated are listed in Table 1. Somatomedin-B (SM-B) has been excluded from the SM because (1) it does not qualify under the criteria listed above, and moreover, (2) its growth promotion of cultured cells is now known to stem from contamination with epidermal growth factor (EGF) (Heldin et al., 1981).

Ancestrally, the group probably springs from a proinsulin-like molecule (van Wyk and Underwood, 1978). It was observed that only 10% of insulin activity in serum could be blocked by antiinsulin antibodies. The activity that could not be blocked was termed nonsuppressible insulin-like activity (NSILA) (Froesch et al., 1963). Insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are both derived from NSILA.

Insulin-like growth factor-I shows 50% homology to human insulin and may be identical to somatomedin-C (SM-C) (Rinderkrecht and Humbel, 1978a). Insulin-like growth factor-II is more akin to insulin than to IGF-I/SM-C and the amount of IGF-II in serum is estimated 10 times as high as IGF-I (van Wyk et al., 1981). Interestingly enough, the synthesis of IGF-II does not seem to be dependent on GH as does IGF-I/

Table 1. Isolated somatomedin peptides.

Peptide	Source	MW[+]	pI	References
$SM\text{-}A_1$	H. plasma	7,000	Neutral	Fryklund et al., 1974
$SM\text{-}A_2$	plasma	7,000	Neutral	Fryklund et al., 1974
SM-C	plasma	7,600	Basic	van Wyk et al., 1975
IGF-I	plasma	7,649	Basic	Rinderknecht and Humbel, 1978a
IGF-II	plasma	7,491	Neutral	Rinderknecht and Humbel, 1978b
MSA	liver, calf serum	10,000	Neutral	Dulak and Temin, 1973 Rechler et al., 1977
BSM	plasma	7,600	Basic	Bala and Bhaumick, 1979a

[+] in daltons

SM-C. Thus IGF-II is not elevated in the serum of patients with acromegaly nor is it depressed when GH is (Zapf et al., 1978). Insulin-like growth factor-I/SM-C promotes sulphate incorporation into cartilage more vigorously than IGF-II.

From receptor studies it has been found that the receptors for the growth factors of the SM family differ from those normally occupied by insulin (Furlanetto et al., 1977; van Wyk and Underwood, 1978; van Wyk et al., 1980, 1981). By these assays IGF-I and SM-C again show virtual identity. They are distinct from multiplication stimulating activity (MSA) and IGF-II. As of 1979, it was the opinion of Froesch et al. that SM-C is identical with IGF-I and that somatomedin-A (SM-A) is a mixture of IGF-I and II. Bala and Bhaumick (1979a) have also recently purified a peptide from human plasma to homogeneity and called it basic somatomedin (BSM). This peptide appears to be the same as SM-C.

Evidence shows that Prl can also stimulate SM production by the liver (Francis and Hill, 1970). It is therefore possible that SM are not only mediators of GH but may somehow influence the action of Prl. However, this is yet to be demonstrated.

3. Insulin

Although insulin itself is not considered a primary hormone in mammary tumorigenesis, insulin is known to influence growth and development of the rodent mammary gland (Turkington, 1972) and the human mammary gland (Ceriani, 1972) in organ culture.

Specific receptors for insulin have been characterized in the R3230AC (Harmon and Hilf, 1976) DMBA-induced (Shafie and

Hilf, 1978) and several continuous cultures of human mammary cell lines (Osborne et al., 1978). Specific binding of insulin to insulin receptors in neoplastic cells has been found to be similar to those of normal cells, but the number of receptors per neoplastic cell vary from their normal counterpart. Reports relating diabetes and breast cancer in humans are still inconclusive. Glicksman et al. (1956) reported that the incidence of diabetes was lower in breast cancer patients versus all other cancer patients. In contrast, Repert (1952) had found a higher percentage of diabetes in his breast cancer patients versus the general population.

In experimental animals, insulin may stimulate (Goranson and Tilser, 1955) or inhibit (Salter et al., 1958) rodent mammary tumor growth, depending on the animal model studied.

Heuson and Legros (1972) reported that induction of diabetes in rats, 3-4 weeks after carcinogen administration, completely prevented formation of tumors. A more recent demonstration of insulin dependence of breast cancer cells was reported by Shafie (1980). In diabetic nude mice, MCF7 cells did not form tumors; tumors were obtained with 100% frequency in diabetic mice treated with insulin.

The mitogenic effect of insulin has also been demonstrated for certain human breast cancer cell lines in long term culture. Osborne et al. (1976) reported that MCF7 cells were quite sensitive to insulin and grew in cell number in response to physiological concentration of insulin.

Actions, other than mitogenic, have been monitored for insulin. Data have been reported suggesting that insulin

may play a regulatory role for estrogen receptors (ER) (Hilf, 1981). Because many breast cancers are estrogen dependent or responsive, growth or inhibition of neoplasm may arise through the indirect effect of insulin on ER. Studies have also demonstrated that estrogens can down regulate insulin receptors in rodent mammary tumors thereby antagonizing the effects of insulin (Bertoli et al., 1980).

B. Somatomedin-like, insulin-like growth factors

1. Multiplication stimulating activity

Multiplication stimulating activity initially described a family of macromolecular factors present in serum which were responsible for the growth and maintenance of mammalian cells in culture. It was originally detected by Temin et al. (1972) and so called by Pierson and Temin (1972). Dulak and Temin (1973) successully purified and characterized MSA from serum-free conditioned Buffalo rat liver (BRL) cell medium which possessed NSILA, MSA and sulphation factor activity (Dulak and Temin, 1973). In addition, this rat liver MSA preparation possessed similar chemical properties to the MSA preparation purified from whole calf serum (Pierson and Temin, 1972), and the sulphation factor activity purified from human serum (van Wyk et al., 1971). It was also shown that the MSA activity resided in a family of four small polypeptides with similar molecular weights (MW) (approximately 10,000) but with different isoelectric points (pI) (7.1-5.7) (Dulak and Temin, 1973). One of them, MSA II-I has now been purified to homogeneity (Moses et al., 1978).

Although BRL cells are capable of synthesizing MSA in culture, it has been recently shown that the growth of BRL cells in serum-free medium does not depend upon the availability of MSA in the culture medium (Nissley et al., 1977).

Multiplication stimulating activity has been shown to be mitogenic to many cell lines. It is capable of stimulating DNA synthesis in a number of mammalian cell culture systems including mouse 3T3 cells (Nissley et al., 1976), chick embryo fibroblasts and rat fibroblasts (Dulak and Temin, 1973), a rat muscle cell line (Rechler et al., 1976) and human fibroblasts (Rechler et al., 1977). In addition, MSA receptors have been demonstrated in normal rat kidney cells, BRL cells, chick embryo fibroblasts and rat liver plasma membranes (Rechler et al., 1976). In most cases the binding of MSA to the cell receptor site was extremely specific, and was inhibited only by MSA, NSILA and SM-A (Rechler et al., 1977). Proinsulin and insulin also inhibited MSA receptor binding in chick embryo and human fibroblasts while EGF, fibroblast growth factor (FGF) and nerve growth factor (NGF) had no effect on MSA binding (Rechler et al., 1977). Rechler et al. (1980) compared the binding characteristics of MSA II-I to IGF-I and II. Three separate binding patterns were detected: (1) IGF-I/SM-C = IGF-II, 10-20 times greater affinity than MSA II (chick embryo fibroblasts), (2) IGF-II = 10-20 times greater affinity than IGF-I/SM-C or MSA II-I which are themselves equal (rat liver membranes, BRL-3A2 cells) and (3) IGF-I > IGF-II > MSA > insulin (human fibroblasts). The above results indicate that the three molecules, MSA II-I, IGF-I and II, are distinct from each other

as are the receptors on different membranes.

Multiplication stimulating activity is also capable of stimulating glucose, uridine and γ -aminoisobutyric acid uptake in chick embryo fibroblast (Smith and Temin, 1974). Recently, MSA was shown to be very mitogenic to F9 embryonal carcinoma cell line which suggests that MSA may have some role in growth regulation in cancer.

Another growth factor derived from serum was identified by Ptashne et al. (1979). He termed this factor "mammary stimulating factor" (MSF). This factor was a peptide of MW 10,000-10,400 with a pI of 5.5-6. Mammary stimulating factor stimulates growth of mouse mammary epithelial in vivo and to a greater extent than in mouse mammary tumor CZF cells. What was unique about this factor was that at high concentration it exhibits biological cross reactivity with MSA. Knauer et al. (1980) have identified several SM-like polypeptides (MW 7,000-20,000) produced by mammary tumors. A high MW SM-like substance has been reported to be produced by T-47D human breast cancer cell line in culture (Baxter et al., 1982). Rowe et al. (1980) have also observed that extracts of human breast tumor tissue contained growth-promoting peptide activity. It is therefore possible that breast tumor cells can produce several growth factors that may stimulate their own growth. Whether this factor is similar to other SM-like growth factors remains to be established.

2. Platelet-derived growth factor

An observation that serum but not plasma supports in vitro growth of mammalian fibroblast led to the discovery of a potent

factor that is contained in platelets and is mitogenic for mesenchymal cells (Balk, 1971; Kohler and Lipton, 1974; Ross et al., 1974). In 1979, Antoniades et al. and Heldin et al. purified platelet-derived growth factor (PDGF) independently. Platelet-derived growth factor from human platelets is a heat stable cationic peptide of MW 13,000. It has been shown to be mitogenic for a variety of connective tissue cells, vascular smooth muscle cells, glial cells, and synovial cells (Rutherford and Ross, 1976; Westermark and Wasteson, 1976; Castor et al., 1977). In addition, synthesis of both collagen and glycosoaminoglycan (two components of extracellular matrix (ECM)) by certain target cells is stimulated by PDGF (Castor et al., 1977; Burke and Ross, 1977). The most detailed studies have been done on clones of mouse 3T3 cells. The mode of action of PDGF is that of a "competence factor" (see Section C) in that it is necessary but not sufficient for movement of quiescent target cells in the G_0/G_1 phase of the cell cycle into the period of DNA synthesis, the S phase (Pledger et al., 1977). Cells exposed to PDGF become competent to synthesize DNA but require certain "progression factors" including either SM-A or SM-C, to actually progress into the S phase (Stiles et al., 1979). In addition, the PDGF induced competent state is stable for at least 13 hours after the mitogen is removed (Pledger et al., 1977). The site of synthesis of PDGF is still unknown although it is probably a megakaryocyte.

Recent reports have implicated PDGF in breast cancer. Eastman and Sirbasku (1978) reported that PDGF from both male and female rats were able to support growth in culture of

MTW9/PL, a rat mammary tumor cell line, 50-75% as well as whole serum. In addition, PDGF was shown to contain growth promoting activity for four well-established malignant cell lines, Neuro-2a, RAG, R2C, and a mouse mammary tumor line 060562 (Hara et al., 1980) and transformed fibroblasts (Kohler and Lipton, 1977).

The specific role of PDGF in breast cancer growth regulation is not yet established.

3. Epidermal growth factor

Epidermal growth factor is a polypeptide first isolated from the submaxillary gland of the adult male mouse (Cohen, 1962). It is a single chain 53 amino acid residue polypeptide with a MW of 6,045 (Taylor et al., 1970). Human EGF was later isolated from urine in small amounts. It differs from mouse EGF in that it has a different amino acid composition, a more neutral pI and a smaller MW (5,300-5,500), but both compete for the same site on the cell membrane, and antibodies to mouse EGF cross-react to some extent with hEGF (Cohen and Carpenter, 1975). Epidermal growth factor exhibits multiple effects in vivo and in vitro. In vivo effects include the proliferation of skin tissue, corneal, lung and tracheal epithelia, inhibition of gastric secretion, formation of fatty liver, hepatic hypertrophy and hyperplasia, and potentiation of cleft palate. In cell culture, EGF stimulates proliferation in a variety of cells such as mammary epithelia, mouse and human fibroblasts, glial cells, vascular endothelia, corneal endothelia and rabbit chondrocytes.

The role of EGF in the cell cycle has been studied most

extensively in fibroblasts. It is a "competence factor" (Scher et al., 1979). Its continuous presence in cell culture is necessary in order to elicit DNA replication (Carpenter and Cohen, 1976; Lembach, 1976). However, EGF was capable of inducing "competent" cells to progress to the S phase in serum-free medium (Wharton et al., 1981). Thus EGF appears to behave like a progression factor in serum-free culture of BALB/c 3T3 cells rendered competent by transient exposure to PDGF thereby demonstrating a kind of synergistic activity.

Epidermal growth factor has been shown to stimulate growth of cultured normal mammary epithelium in both rodents (Turkington, 1969; Tonelli and Sorof, 1980) and humans (Stoker et al., 1976; Taylor-Papadimitriou et al., 1977; Stampfer et al., 1980). The presence of high concentration of EGF in human milk was recently reported (Starkey and Orth, 1977). As a result, in recent years, attention has been focusing on what role it may have on breast cancer cells. Epidermal growth factor has been reported to be mitogenic in mouse mammary carcinoma cells (Turkington, 1969) and in one human breast cancer cell line (Osborne et al., 1980). In addition, EGF receptors have been studied in several breast cancer cells (Osborne et al., 1981). Most recently Imai et al. (1982) have shown that mammary cell lines maintained as monolayer have EGF receptors with high affinity for EGF while mammary cell lines grown in suspension had no detectable EGF receptors. Also, cell lines established from metastatic sites tend to have fewer EGF receptors. Furthermore, these workers reported that concentration of EGF was mitogenic to the human breast cancer

cell line T-47D while high concentration of EGF inhibited DNA synthesis of several breast cancer cell lines studied.

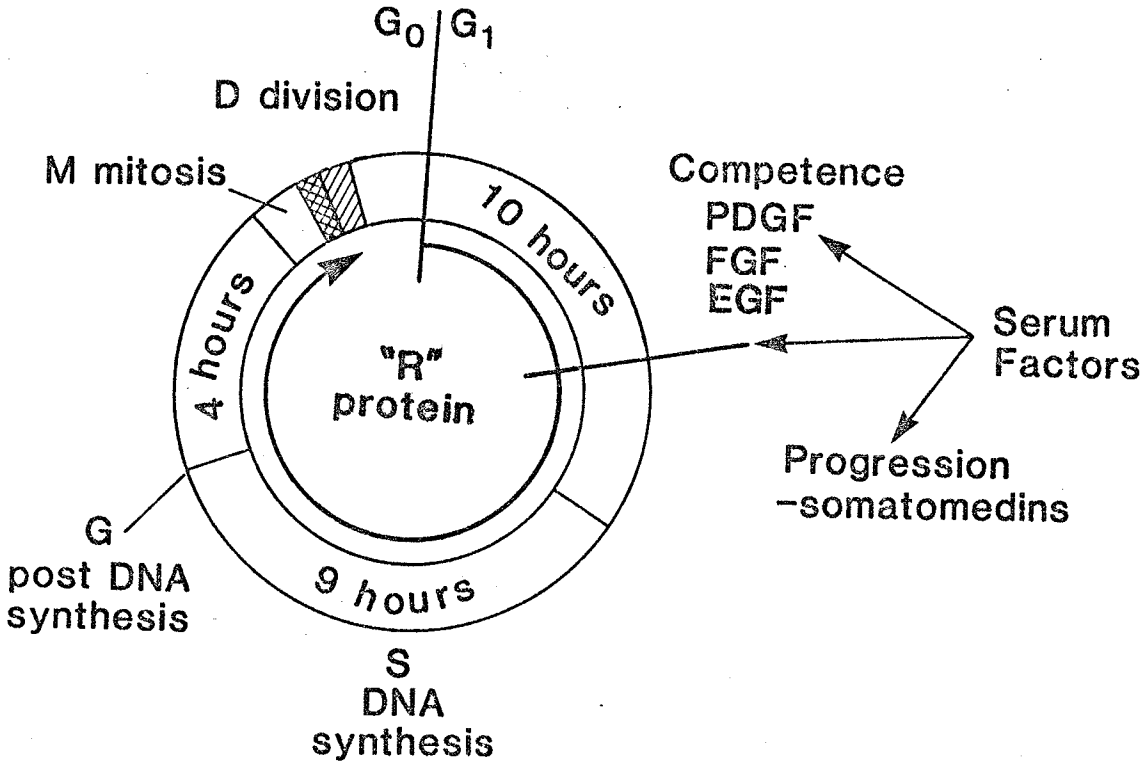
Whether EGF is intimately involved in the growth regulation of human breast cancer remains to be established.

C. Mode of action of the somatomedins and other growth factors

The cell cycle has been divided into four major phases, G_0/G_1, S, G_2 and M. In order for cells to proliferate there must be changes in cell permeability, RNA and protein synthesis and other phenomena mentioned in connection with the $G_0 \longrightarrow G_1$ transition. Therefore, how do SM and growth factors bring about these changes? For cells to transverse the cell cycle and replicate, initiators and promoters are required to move the cell from the G_0 phase past a critical part of the G_1 phase of the cycle (Rossow et al., 1979). One of the chief objects of these studies has been the BALB/c 3T3 cell, a murine fibroblast of embryonic origin. Quiescent cells must first be rendered "competent" to proliferate, for example, by exposure to PDGF or FGF, before "progression" factors such as SM-C can stimulate DNA synthesis (Stiles et al., 1979). (See Fig. 2.) In other cells such as the frog lens epithelium, Rothstein et al. (1980) have shown that SM-C either alone or in concert with other factors, causes the cells to transverse the entire cell cycle.

Several systems show similar dependency on a well-balanced and fairly extensive blend of hormones and growth factors (Weinstein et al., 1981). But this is not a universal feature as Weidman and Bala (1980) showed that purified SM-C could

Fig. 2. An outline of the action of serum and growth factors on the cell cycle in vitro. Quiescent cells reside in the G_1 or G_0 stage of the cycle. In the presence of serum, the synthesis of rapidly turning over initiator protein "R" is favored. After the protein has accumulated in a sufficient amount, DNA synthesis and the remainder of the cycle continue relatively unaffected. Human serum contains at least two sets of factors that function synergistically to promote growth of certain cells. Quiescent cells exposed briefly to PDGF become "competent" to replicate their DNA and divide; however, PDGF-treated competent cells do not "progress" efficiently into S phase unless they are incubated throughout G_0/G_1 with medium comtaining plasma. Progression factors in human plasma dictate the ultimate fraction of PDGF-treated competent cells that enter S phase.


G_0 / G_1
D division
M mitosis
10 hours
4 hours
"R" protein
Competence
PDGF
FGF
EGF
Serum Factors
Progression
-somatomedins
G
post DNA synthesis
9 hours
S
DNA synthesis

stimulate WI38 cells in serum-free medium. Just one round of division could be supported by the mitogen. The explanation for this has been provided by van Wyk et al. (1981). It is demonstrated that when human dermal fibroblasts are exposed to hGH they secrete immunoreactive SM-C themselves.

D. The use of tissue culture in the studies of hormonal action on cell growth

1. Problems and potentials

One of the alternatives in the approach of human breast cancer research has been the development of a number of cell lines derived from breast cancer specimens. Reports of attempts to culture breast cells appeared as early as 1937, but it was not until 1958 that Lasfargues and Ozzello reported the first successful long-term culture of a breast tumor.

One of the most obvious reasons for attempting to culture cells from primary breast cancer is to be able to directly assess the effect of drugs and hormones on individual tumors so that existing therapy can be used more effectively. A second reason, with less immediate practical advantage, is to determine the growth characteristics and metabolic properties of malignant cells as compared to normal cells with a view to developing new approaches to treatment and prevention of the disease. Thirdly, tissue culture of a fully viable cell line allows for repeated or sequential experiments on the same tissue. Fourthly, the environment of cells in monolayer or suspension may be easily controlled or manipulated. And

finally, in a more general way, a culture system for any human epithelial cells may be useful for basic studies on growth control in order to determine why the major solid tumors in man arise from this cell type. Therefore, established cell lines from breast tumors are useful for studies such as (1) the search for tumor specific antigens, (2) for investigating the mechanism of action of a particular hormone or growth factor, (3) drug action and (4) mechanism of oncogenesis, to name a few.

While cell lines may be useful for studying certain aspects of tumor cell biology, there are certain problems that exist. Successive passaging of cells which grow in vitro may select for cell types which are not representative of the original cancer. This may make quantitation of growth difficult and misleading observations may be drawn. Loss of integrity of cells may occur in culture due to disruption of organization and loss of interaction of the original cells with surrounding tissue in vivo. Culture conditions in which cells are maintained may not be physiological and many in vivo properties of the cells may be affected or lost and new properties may be acquired. Therefore, the researchers in the field have been trying to recreate an in vitro surrounding similar to the in vivo one.

2. Extracellular matrix and collagen

There is now plentiful evidence that the substrate upon which cells rest when maintained in tissue culture is important for their proliferation and differentiation. The pioneering work of Ehrmann and Gey (1956) has shown that various tissues

demonstrated enhanced growth and differentiation when cultured on collagen gels. Collagen is the major component of ECM which is the natural substrate upon which cells may rest *in vivo* in order to retain their normal orientation (Wessel, 1964). This substrate can be produced by mesenchyme which is closely associated with most epithelia or by epithelia themselves after interaction with ECM produced by other tissue (Hay, 1977). Recent experiments have shown that direct contact by epithelial cells with a collagen substrate is required if they are to produce their own ECM (Hay and Meier, 1976). Guzman *et al*. (1982) transplanted epithelial cells, grown on collagen gel, into the gland-free mammary fat pads of female mice. These workers reported that the phenotype of cells embedded in collagen gel matrix were not altered by culture conditions.

Noticeable cell shape change occurred when cells were maintained on collagen as opposed to plastic (Gospodarowicz *et al*., 1978). Furthermore, Leung and Shiu (1982) reported changes in cell shape of the human breast cancer cell line T-47D on collagen as opposed to plastic, and that the cells grew slower and were more dependent on serum.

The physical substrate upon which epithelia rests can also modulate their response to growth factors (Gospodarowicz *et al*., 1978). For example, FGF added to corneal epithelial cells plated on collagen-coated dishes failed to stimulate cell proliferation whereas the addition of EGF resulted in a marked increase in cell number and an increased rate of keratinization (Gospodarowicz *et al*., 1977). On the other

hand, the cells grown on plastic now proliferated in response to FGF but no longer responded to EGF (Gospodarowicz et al., 1977).

3. Serum-containing and serum-free growth medium

Cells, once separated from their ECM and placed into culture, show a dependency on serum for growth. It is generally accepted that growth of virtually all types of cells in culture require the presence of serum in the medium. Serum is a complex mixture of hormones, growth factors, binding proteins and nutrients. It is a source of factors which may be necessary for the proper attachment and spreading of cells on the plastic culture substrate (Holmes, 1967; Hook et al., 1977; Yamada and Olden, 1978). Serum also acts as a pH buffer and provides protease inhibitors which protect cells from damage due to proteases released by cells (Ham and McKeehan, 1979). Nevertheless, many serum components are poorly characterized or completely unstudied. Many workers have long recognized the problems associated with the complexity and undefined nature of serum and have been looking for new ways to eliminate the requirement for a serum supplement in culture medium.

The elimination of serum from culture medium allows for the simple design and interpretation of experiments which would be difficult or impossible to carry out in serum-containing medium. In serum-free medium it would be possible to carry out hormone and drug studies on healthy cells in the absence of unwanted serum components which may bind, inactivate,

antagonize or mimic the action of the agent studied. It would also be possible to carry out nutritional studies in highly controlled environment in such a medium. Similarly, experiments examining the release into the medium of cellular products, such as growth factors or other secreted products, are much easier to perform in the absence of large amounts of serum proteins.

As of this date, several approaches have been taken to eliminate the requirement for a serum supplement in the culture medium, such as adaptation of cell lines to medium without serum. Attempts have also been made at purification of the active components from serum (Higuchi, 1973; Brooks, 1975) but these have been proven to be of limited utility to individuals using cell culture techniques. More useful methods have been developed for eliminating or reducing the amount of serum in the medium (McKeehan et al., 1977; Hamilton and Ham, 1977; Ham and McKeehan, 1979). This approach is based on the careful adjustment of medium components to provide cells with an optimum nutrient balance. Another method for the replacement of serum in culture medium has been developed by Barnes and Sato (1980) whereby growth stimulating activities of serum have been replaced by the proper choice of hormones and other factors provided by serum at the right concentration.

Clearly, the development of serum-free medium for cultured cells has a number of practical advantages. In addition to providing information on the functions of serum and the responses of cells to hormone and serum proteins, serum-free medium allows the simple design of many experiments which

would be difficult to carry out in serum-containing medium, including the growth of single cell types in primary culture.

E. Rationale and aims of investigation

Growth hormone and Prl do not stimulate breast cancer cells to grow *in vitro* but the two pituitary hormones have been implicated as factors influencing breast cancer growth *in vivo*. It is, therefore, necessary to study whether GH intermediates, that is SM, are responsible for modulating growth of breast cancer cells.

The aims of this study were to:

(1) investigate the mitogenic effect of SM on T-47D human breast cancer cells,

(2) observe if SM can act synergistically with other growth factors (competent factors) and hormones in stimulating growth of T-47D cells,

(3) compare the biological activities of the SM (IGF-I and II) with that of insulin,

(4) study the binding properties of SM and compare with their biological properties, and

(5) study whether substrate can modulate T-47D response to SM.

MATERIALS AND METHODS

A. Tissue culture supplies and chemicals

Culture dishes (35 x 10 mm), culture flasks (75 cm^2 and 150 cm^2) and sterile pipettes were purchased from Fisher Scientific Co. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, L-glutamine, and trypsin/EDTA (1 times) were supplied by GIBCO. HEPES and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Vitrogen 100^R (bovine dermal collagen mainly composed of Type 1 collagen) was purchased from Collagen Corporation and collagenase was obtained from Worthington.

B. Hormones and growth factors

Triiodothyronine, hydrocortisone and transferrin were purchased from Sigma Chemical Co. Unlabelled insulin was obtained from GIBCO and its biological activity was 25 i.u./mg of hormone. ^{125}I-insulin was a gift from Dr. Y. Imai (Dept. of Physiology, University of Manitoba). Epidermal growth factor was purchased from Collaborative Research Inc.

Multiplication stimulating activity, purified from conditioned medium of BRL-3A rat liver cells, was also purchased from Collaborative Research Inc. Its purity ranged from 80-98%.

Basic somatomedin (50% pure), isolated from human plasma (Bala and Bhaumick, 1979a), was a gift from Drs. R. M. Bala and B. Bhaumick. There were 4,000 SM bioactivity units per mg (hypophysectomized rat cartilage assay) and no detectable immunoreactive insulin activity. It was equipotent with

IGF-I and SM-C in the BSM radioimmunoassay (RIA) (Bala and Bhaumick, 1979b).

^{125}I-BSM was also kindly provided by Drs. R. M. Bala and B. Bhaumick. Highly purified BSM was iodinated using the chloramine-T method (Hunter and Greenwood, 1972) as previously described (Bala and Bhaumick, 1979b) resulting in specific activities of approximately 100-200 C_i/ug. The specific activity of the ^{125}I-BSM used in this study was 10,000 cpm/40 pg.

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, M.D.) and was derived from the pleural effusion of a 54-year old patient with disseminated carcinoma of the breast. These cells exhibit epithelial morphology and form monolayers in culture. The cytosol of these cells contain specific high affinity receptors for estradiol, progesterone, glucocorticoid and androgen (Keydar et al., 1979). Receptors for Prl and GH (Shiu, 1979), insulin (Engel and Young, 1978) and EGF (Imai et al., 1982) have also been reported from the membrane of these cells. The doubling time of T-47D cells is 32 hours.

Cells were maintained routinely in DMEM supplemented with L-glutamine (4 uM), glucose (4.5 g/l), penicillin (100 i.u./ml), streptomycin (100 ug/ml), bovine insulin (10 ug/ml) and 10% FBS (v/v). Cells were kept in a humidified atmosphere of 95% air, 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. BRL-3A cell line

This cell line was originated by Coon (1968) (Carnegie Institution, Baltimore, M.D.) as a clone from the liver of a normal 5-week old Buffalo rat. Some of the properties of these cells have been described by Perdue et al. (1971). These cells grow in monolayer culture with an epithelial and parenchyma-like appearance. As they multiply, the cells become packed in ridges, appearing almost like a thin section through liver. These cells had an interesting property in that following plating at low density in medium containing serum, BRL-3A cells would replicate at a nearly normal rate for several generations in serum-free medium without protein or hormonal supplements. This cell line was used by Dulak and Temin (1973) for the initial isolation of MSA. Since then, many workers have been using conditioned medium from these cells as a source of MSA.

3. WI38 cell line

This human embryonic lung fibroblast cell line was obtained from the American Type Culture Collection (Rockville, M.D.) and grown as monolayers in 10% FBS.

D. Collection of BRL-3A and WI38 conditioned medium

For production of conditioned medium, the BRL-3A and WI38 cells were plated in 75 ml plastic cell culture flasks in DMEM with 10% FBS, glucose (4.5 g/l), penicillin (100 i.u./ml), strepto-

mycine (100 ug/ml) and L-glutamine (4 uM). The cells were then incubated at 37°C in a humidified CO_2 incubator. After they had grown to confluency, the medium was discarded, the cell monolayer washed twice with serum-free DMEM and replaced by 30 ml of fresh serum-free DMEM. Twenty-four hours later, this medium was discarded and again 30 ml of fresh DMEM was added. On alternate days from then, the medium from the culture was collected, centrifuged at 1,000 rpm for 10 minutes and the supernatant pooled and stored at -20°C until required. Protein was determined by the method of Lowry et al. (1957).

E. Chemically defined serum-free medium

The chemically defined medium consisted of serum-free DMEM with a combination of essential hormones and growth factors which support a large variety of cells in culture (Barnes and Sato, 1979), namely triiodothyronine, insulin, hydrocortisone, EGF and transferrin (Table 2). Insulin and transferrin have been shown to be two essential co-factors for the growth of human breast cancer cells maintained on plastic substratum (Barnes and Sato, 1979). In addition, hydrocortisone has been shown to be important for epithelial cell differentiation.

All of the above mentioned components of defined medium are present in FBS which is known to support maximum proliferation of T-47D cells. Furthermore, these cells also contain receptors for insulin, EGF and glucocorticoids.

F. Determination of cell growth on plastic substratum

T-47D breast cancer cells (1×10^4 - 5×10^4 cells/dish)

Table 2. Components of the chemically defined medium.

Hormone	Concentration
transferrin	25 ug/ml
epidermal growth factor (EGF)	10 ng/ml
hydrocortisone	0.1 ug/ml
insulin	0.1 ug/ml
triiodothyronine	1 ng/ml

were plated in 2 ml DMEM supplemented with 10% FBS and incubated at 37°C for 48 hours to allow for cell attachment. This medium was then discarded, cells were washed twice with fresh serum-free DMEM and replaced with either serum-free DMEM, chemically defined medium or DMEM with 0.1% FBS. Test substances (for example MSA and BSM) were added to dishes at different concentrations. In the experiments in which the mitogenic effects of BRL conditioned medium were tested on these cells, 2 ml of undiluted BRL conditioned medium was added after the washing procedure. Medium was changed on days 3 and 6 and cell numbers were counted on days 4 and 8.

Cells were detached from dishes in the following manner. Medium was aspirated and cells were gently washed once with 1 ml trypsin/EDTA in HBSS. This trypsin was poured off and replaced with 1 ml of fresh trypsin. The cells were then incubated for approximately 5 minutes at 37°C, ample time to allow for cells to detach from the plastic substratum. Cells were then dispersed by pipetting, added to isoton solution and counted electronically by a coulter counter.

G. Determination of cell growth on collagen gel

Collagen solution was prepared by mixing 9 parts of Vitrogen 100^{R} (2.4 mg/ml) with 1 part of 10 times concentrated DMEM powder. One ml of this collagen solution was pipetted onto the bottom of each 35 mm culture dish. Gelation was completed after incubating the dish at 37°C for 1 hour. T-47D cells, 3×10^{4} cells/dish, were plated in 10% FBS on top of the collagen gel and incubated for 2 days. The 10%

FBS was then aspirated, the cells washed twice with serum-free DMEM, and replaced by 2 ml of fresh serum-free DMEM and incubated for another 24 hours. This medium was again discarded and replaced by either serum-free DMEM or chemically defined medium. The test substances (BSM and insulin) were added at this time. Cell growth was determined at various intervals after addition of hormones.

To determine the number of cells grown on collagen, the collagen gel was first digested with 0.1% collagenase (Worthington, Type III) in HBSS for 1 hour at 37°C. This medium, containing the cells, was then centrifuged at 3,000 rpm for 10 minutes, the supernatant discarded and replaced by 1 ml of trypsin/EDTA, followed by a 5 minute incubation period. Cells were dispersed by pipetting and counted in a coulter counter.

H. Determination of specific binding of ^{125}I-labelled BSM to T-47D cells

Subconfluent monolayers of T-47D cells on plastic culture dishes (35 mm) were washed 3 times with 25 nM HEPES buffered (pH 7.4) HBSS containing 0.1% (w/v) BSA. After aspiration of the washing medium, each dish received 1 ml of the same buffer containing 25,000 cpm of ^{125}I-labelled BSM with or without unlabelled BSM. The dishes were incubated at 18°C. At the end of incubation, the radioactive medium was aspirated, and the dishes washed 3 times with ice-cold phosphate buffered saline (PBS). The cells were then dissolved with 1 ml of 3% sodium dodecyl sulphate (SDS). The solutions were transferred to disposable glass tubes and radioactivity was deter-

mined in an LKB Wallac gamma counter with a counting efficiency of 70% of ^{125}I. Specific binding was taken as the total radioactivity bound (mean of duplicates) in the absence of unlabelled hormone minus that bound (mean of duplicates) in the presence of excess unlabelled hormone (2,000 ng). The latter represents nonspecific binding to cells and culture dish.

RESULTS

A. Growth promoting effects of BRL-3A conditioned medium and purified MSA

As a preliminary step in our investigation of the mitogenicity of MSA on T-47D cells, we observed cell growth in response to BRL conditioned medium, as it was felt that this would provide an indication of the type of response that might be obtained with purified MSA. The results are shown in Fig. 3. Buffalo rat liver conditioned medium caused a significant 12-fold increase in T-47D cell number compared to controls. The effect of cell density on the stimulation of T-47D cell growth is shown in Fig. 4. When plated at low cell density (1×10^4 and 3×10^4 cells/dish) cells grew, in the presence of BRL conditioned medium, at rates 50-75% that of cells growing in 10% FBS (condition for maximum cell growth). However, when cells were plated at a higher cell density (5×10^4 cells/dish) the growth rate of cells in BRL conditioned medium decreased to 37% that of the growth rate of cells in the positive control (10% FBS). Bovine serum albumin was used as a control to determine whether the growth stimulation of T-47D cells in response to BRL conditioned medium was due to the nonspecific action of proteins. Conditioned medium from a fibroblast-derived cell line, WI38, was used as another control medium. The results (Fig. 4) show that a 2-fold stimulation of T-47D cells in BRL conditioned medium was obtained over that of the BSA and the WI38 control groups.

When MSA (2 and 10 ng/ml) was added to these cells in serum-free DMEM, there was no apparent stimulation over that

Fig. 3. Effect of BRL conditioned medium and purified MSA on the growth of T-47D cells. Dulbecco's Modified Eagle's Medium containing 10% FBS was used for plating T-47D cells (5×10^4 cells/dish). The cells were washed twice with serum-free DMEM 2 days later and replaced with various media. Two hundred ug/ml of BSA was added to control dishes. The cell number was determined on days 2, 5 and 8. Each value represents the mean of triplicates $\pm$ S.D. ($*p < 0.01$).

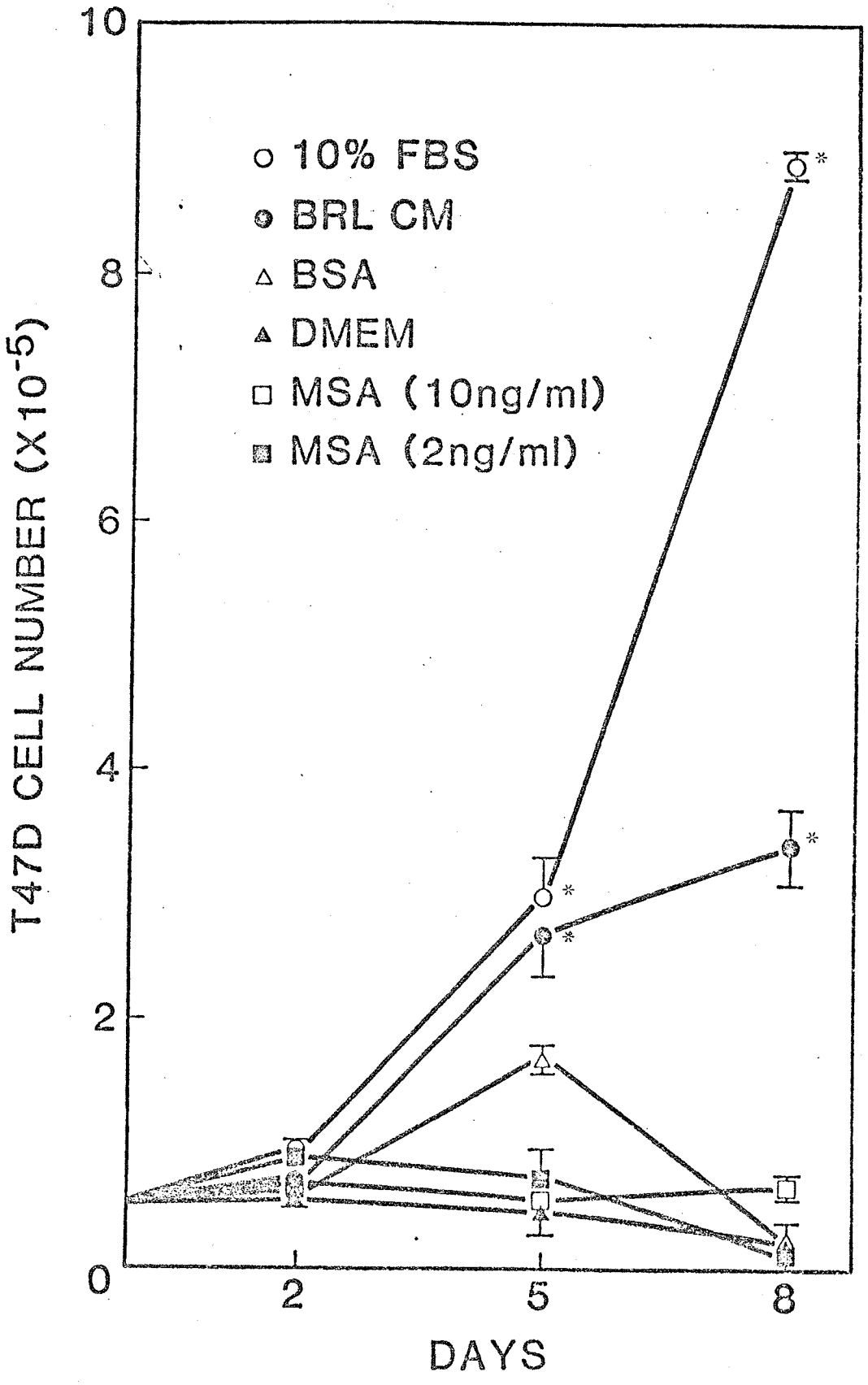
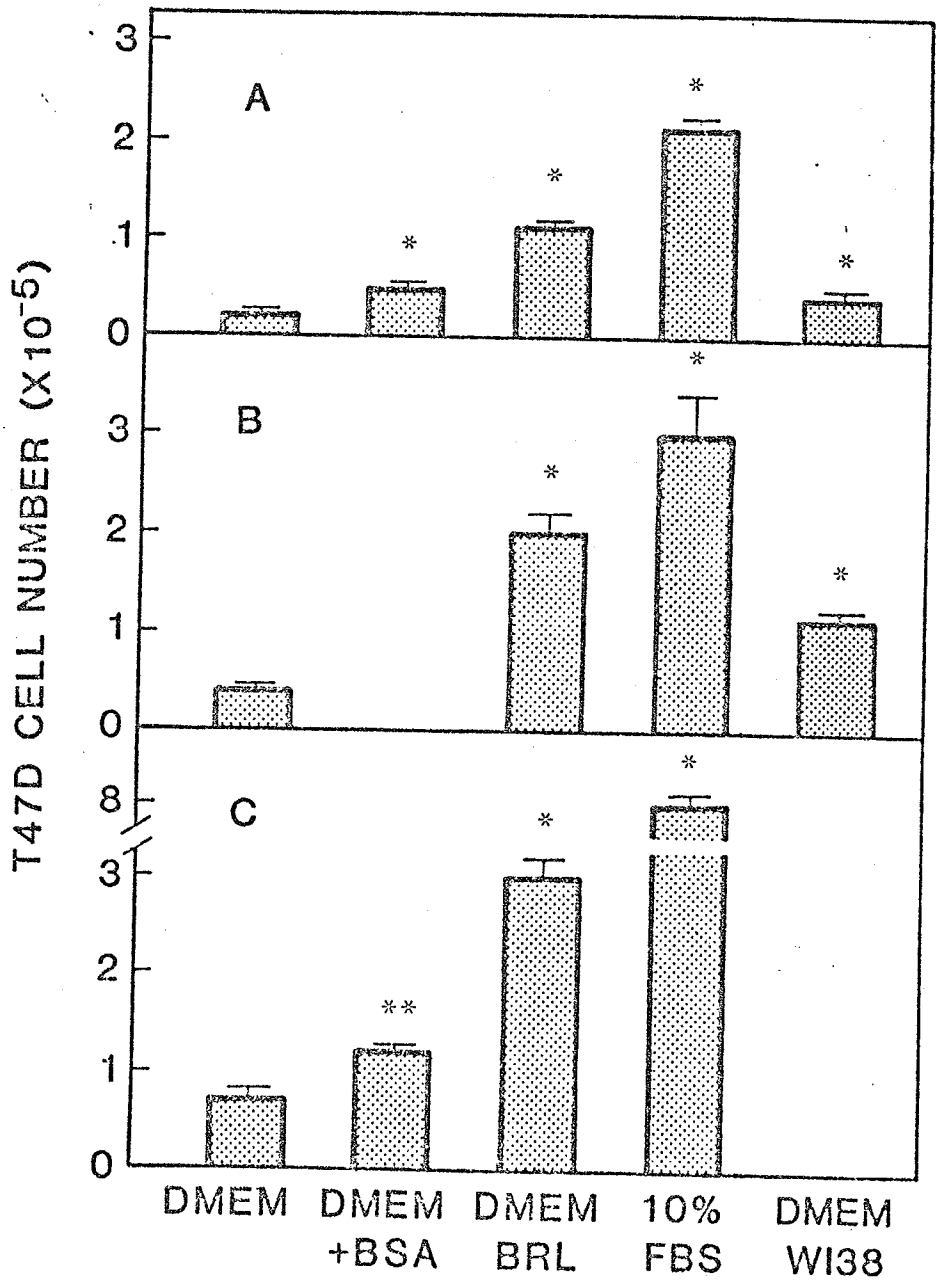

10% FBS
BRL CM
BSA
DMEM
MSA (10ng/ml)
MSA (2ng/ml)
T47D CELL NUMBER (X10-5)
DAYS
0
2
4
6
8
2
5
8
*

Fig. 4. Effect of cell density on the proliferation of T-47D in BRL-3A conditioned medium. Cells were plated at 1×10^4 - 5×10^4 cells/dish in DMEM plus 10% FBS. Two days later, cells were washed twice with serum-free DMEM and fresh BRL-3A conditioned added. The medium was changed on day 3 and cells were counted on day 6. Each value represents the mean of triplicates $\pm$ S.D. ($*p < 0.01$, $**p < 0.05$).

Panel A: cells plated at 1×10^4 cells/dish

Panel B: cells plated at 3×10^4 cells/dish

Panel C: cells plated at 5×10^4 cells/dish


T47D CELL NUMBER (X10⁻⁵)
A
B
C
DMEM
DMEM +BSA
DMEM BRL
10% FBS
DMEM WI38

of the control (Fig. 3). These results indicate that, either the MSA present in the BRL conditioned medium acted synergistically with some component produced by the liver, such as transferrin, in stimulating T-47D cells to grow, or that possibly some factor(s) other than MSA present in the medium was capable of stimulating T-47D cell growth. In order to test the first possibility, that is, whether MSA could act synergistically with other growth factors and/or hormones in stimulating T-47D cell growth, we developed a chemically defined serum-free medium. (See Materials and Methods.)

B. Pattern of growth of T-47D cells in chemically defined medium

The effect of the defined medium on the growth of T-47D cells is shown in Fig. 5. This defined medium in its undiluted form was as effective as 10% FBS in stimulating maximum cell growth. However, its effectiveness was decreased by dilution with DMEM; the most diluted (1:20) fraction being the least effective in promoting cell growth. Nevertheless the cells remained healthy even at that dilution. As a result, it was decided that a 1:20 or 1:25 dilution of the defined medium was to be used as a basal medium in future experiments.

C. Growth promoting effect of MSA on T-47D cells

The growth promoting effect of MSA on T-47D cells under three conditions (serum-free DMEM, 0.1% FBS, and defined medium (1:25)) were compared (Fig. 6). Low concentrations of MSA (1,5 and 10 ng/ml) were effective in stimulating these cells 2-3 fold in 0.1% DMEM containing FBS, and a maximum of

Fig. 5. Growth of T-47D cells in chemically defined serum-free medium. Various dilutions of the chemically defined-serum free medium were made, and their ability to promote T-47D cell growth was observed. The undiluted defined medium was composed of serum-free DMEM with insulin (100 ng/ml), transferrin (25 ug/ml), EGF (10 ng/ml), triiodothyronine (1 ng/ml) and hydrocortisone (100 ng/ml). Cells were plated at 5×10^4 cells/dish. The values shown represent a mean of triplicates $\pm$ S.D. ($*p < 0.01$).

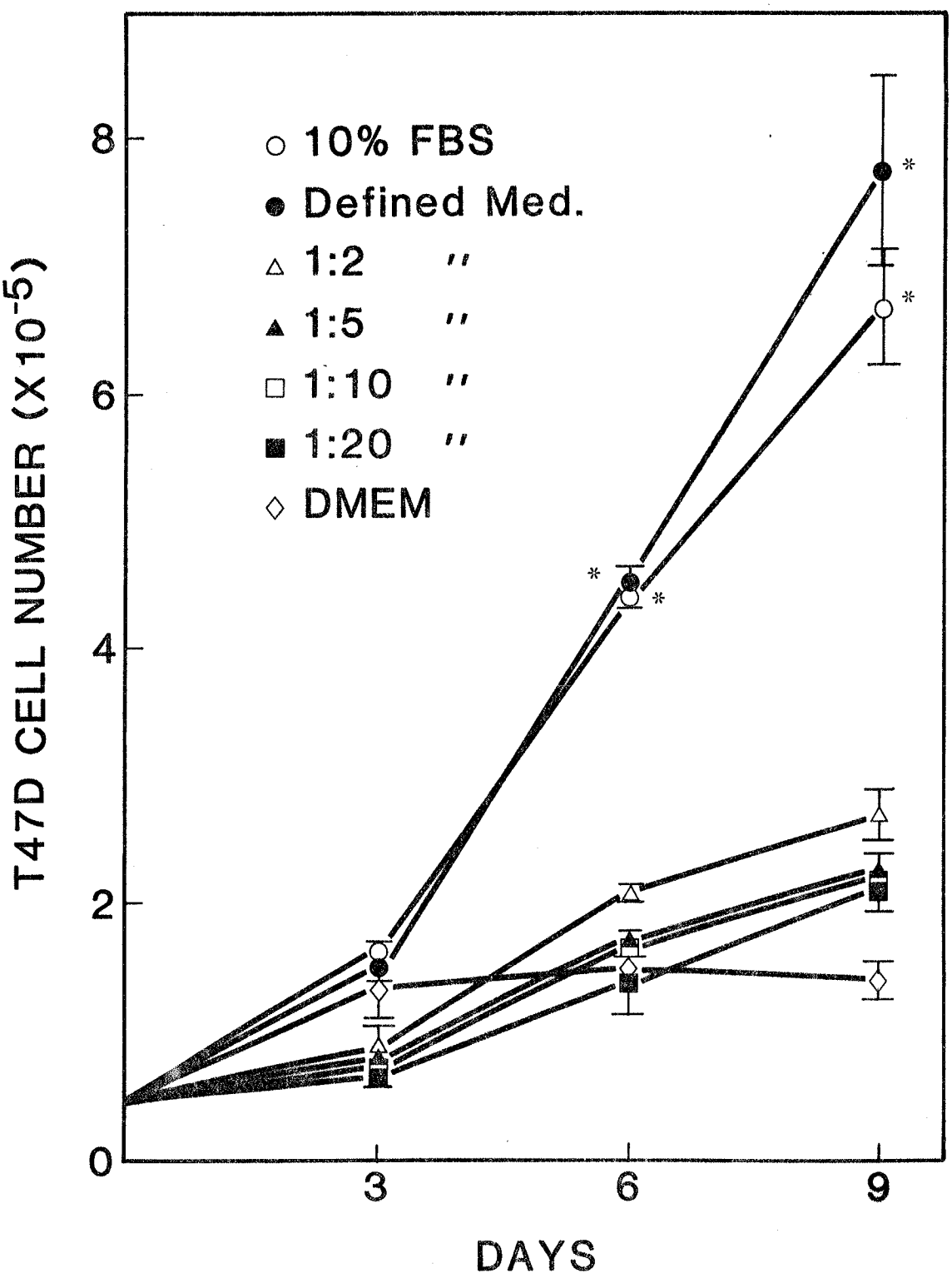
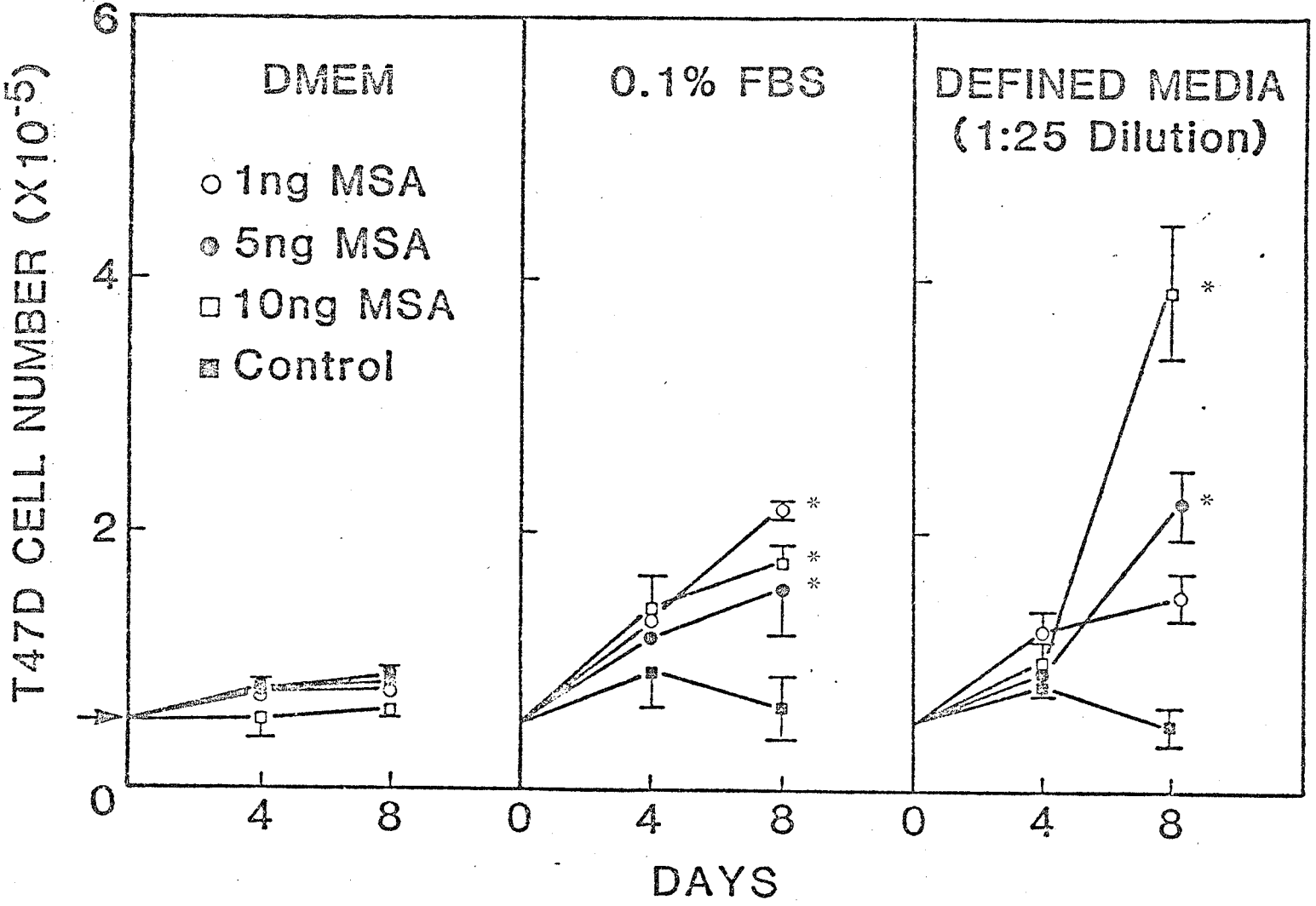

○ 10% FBS
● Defined Med.
△ 1:2 "
▲ 1:5 "
□ 1:10 "
■ 1:20 "
◇ DMEM
T47D CELL NUMBER (X10-5)
0
2
4
6
8
3
6
9
DAYS
*
*
*
*

Fig. 6. Effect of MSA on growth of T-47D cells on plastic substratum. The effect of MSA on the growth of T-47D cells (plated at 5×10^4 cells/dish) under three different conditions are compared. Multiplication stimulating activity in chemically defined medium was most effective in causing T-47D cells to proliferate. Multiplication stimulating activity in DMEM was the least mitogenic. Each value represents the mean of triplicates $\pm$ S.D. ($*p < 0.01$).


DMEM
0.1% FBS
DEFINED MEDIA
(1:25 Dilution)
1ng MSA
5ng MSA
10ng MSA
Control
T47D CELL NUMBER (X10⁻⁵)
DAYS
0
2
4
6
0
4
8
*

6.6-fold in chemically defined medium. Again, no stimulation of cells in DMEM was observed after 8 days. Since MSA did not stimulate these cells in serum-free DMEM, but did so in the presence of 0.1% FBS and also in defined medium, it seemed reasonable to assume that some hormone(s) and/or growth factor(s) present in serum and chemically defined medium may have acted synergistically with MSA in promoting cell growth. Also, the same phenomenon may have occurred in medium containing 0.1% FBS since many growth factors of serum still exist in a very diluted form.

In three separate experiments it was observed that once again maximum stimulation produced by MSA occurred in the presence of defined medium (Table 3). A smaller growth response was observed with cells in 0.1% FBS and the least stimulation was seen with cells growing in the serum-free DMEM medium. Since such low concentrations of MSA (1, 5 and 10 ng/ml) were effective in stimulating T-47D cell growth, the effect of higher concentrations of MSA were subsequently tested. It was found that 50, 100, 500, 1,000 and 2,000 ng/ml were not more stimulatory to T-47D cells than lower MSA concentrations.

Having established that MSA, a rat SM, was mitogenic to T-47D cells, we decided to study the effect of BSM (SM-C or IGF-I), a hSM, on this human breast cancer cell line.

D. Growth promoting effect of BSM on T-47D cells on plastic substratum

The effects of BSM on the growth rate of T-47D cells are shown in Fig. 7. Two conditions were studied, T-47D cells in serum-free DMEM and T-47D cells in the presence of defined

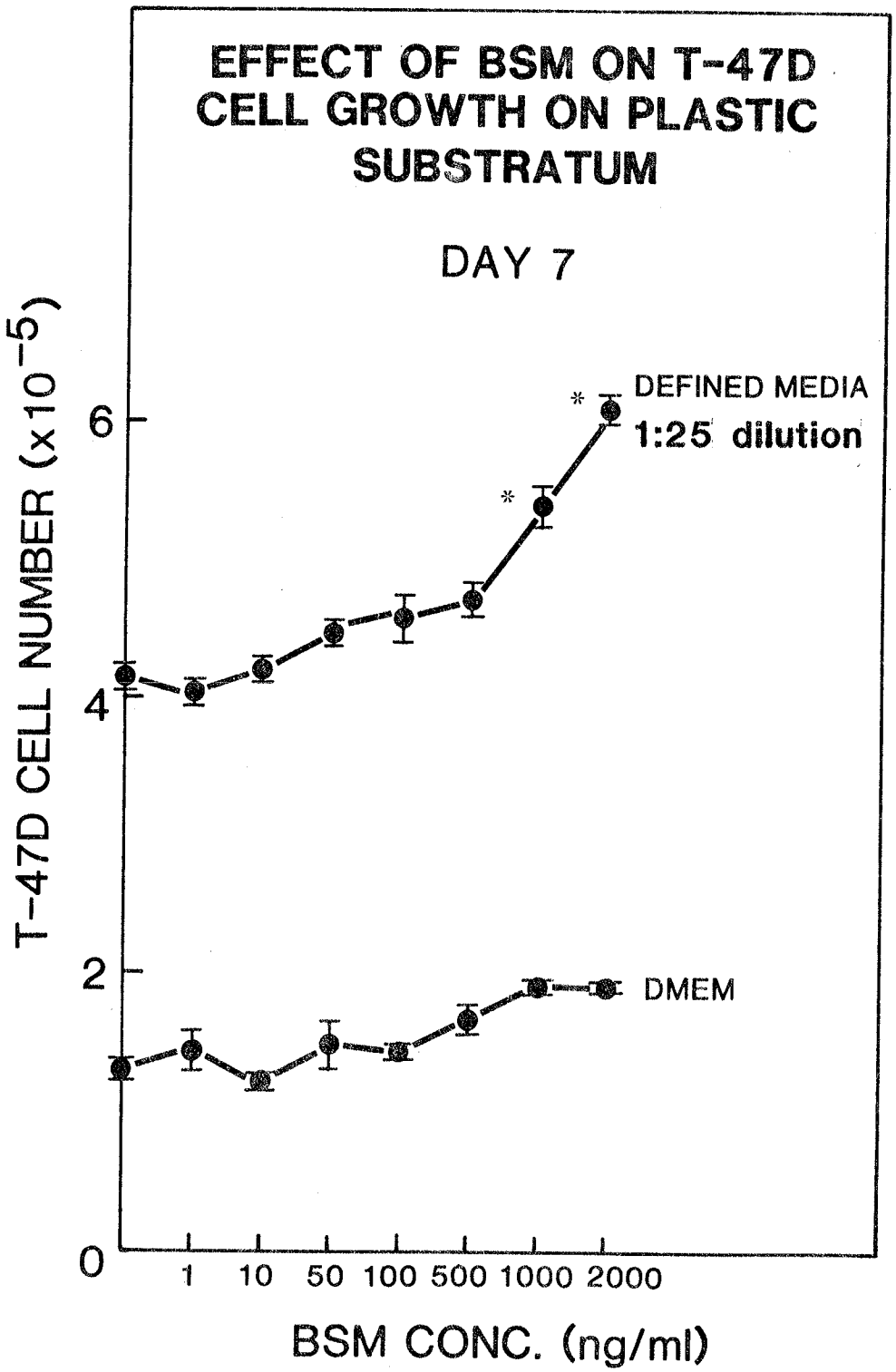
Table 3. Summary of the growth response of T-47D cells to MSA. The mitogenic effect of MSA on T-47D cells, from three separate experiments, are summarized. Cells were plated at 5×10^4 cells/dish in 10% FBS and incubated for 48 hours. At the end of this period, FBS was poured off and the cells washed twice with serum-free DMEM and replaced by either serum-free DMEM, 0.1% FBS or chemically defined medium (1:25 dilution). Three concentrations (1, 5 and 10 ng/ml) were tested. Medium was changed on days 3 and 6, and cells were counted on day 8.

Medium	MSA concentration (ng/ml)	Mean fold stimulation ± S.D.
DMEM	0	1.00
	1	1.00 ± 0.00
	5	1.08 ± 0.14
	10	1.20 ± 0.18
0.1% FBS	0	1.00
	1	** 2.23 ± 0.93
	5	1.40 ± 0.53
	10	** 1.90 ± 0.66
Defined medium	0	1.00
	1	* 1.93 ± 0.31
	5	** 2.33 ± 0.81
	10	3.57 ± 2.67

* $P < 0.01$

** $P < 0.05$

Fig. 7. Effect of BSM on the growth of T-47D cells on plastic substratum. T-47D cells were plated (3×10^4 cells/dish) in 10% FBS on day -2. On day 0, this medium was discarded and cells were washed twice with serum-free DMEM and replaced by fresh media (either serum-free DMEM or chemically defined medium (1:25 dilution)). Cells were counted on day 7. Each value represents the mean $\pm$ S.D. of triplicate dishes (*$p < 0.01$).


EFFECT OF BSM ON T-47D CELL GROWTH ON PLASTIC SUBSTRATUM
DAY 7
T-47D CELL NUMBER (x10^{-5})
DEFINED MEDIA
1:25 dilution
DMEM
0
2
4
6
1
10
50
100
500
1000
2000
BSM CONC. (ng/ml)

medium (1:25 dilution). Under both conditions maximum stimulation (1.41-1.46) was observed at 2,000 mg/ml BSM. This result was found to be reproducible (Table 4). However, the overall stimulation of T-47D cell growth observed in response to BSM was much lower than the stimulation seen with MSA. It was possible that BSM required different conditions in order to stimulate T-47D cell growth. We therefore examined the effect of BSM on T-47D cells grown on collagen gel, since collagen is the major component of ECM, which is the natural substrate upon which cells may rest in vivo. In addition, since SM are known to demonstrate insulin-like activity, we compared the abilities of BSM and insulin to stimulate T-47D cell growth.

E. Effect of BSM and Insulin on the growth of T-47D cells on collagen gel

The effects of BSM and insulin on T-47D cells on collagen gel were studied and compared. The results are shown in Fig. 8. Basic somatomedin produced a 2-fold growth stimulation in the serum-free DMEM condition and a 2.5-fold stimulation in the chemically defined medium (1:25 dilution). Similar observations were made with insulin. This experiment was repeated and the same degree of stimulation was obtained in subsequent experiments.

F. Hormone binding studies

Somatomedins, like many other hormones, are believed to initiate their biological effects by binding to specific receptor sites on target cell membranes. Since BSM and MSA both stimulated the growth of T-47D cells, it was of interest to

Table 4. Summary of the growth response of T-47D cells to BSM. Cells were plated at 3×10^4 cells/dish in 10% FBS and incubated for 48 hours. At the end of this period FBS was aspirated and the cells then washed twice with serum-free DMEM. This was then replaced with either fresh serum-free DMEM or 0.1% FBS or chemically defined medium. Concentration of BSM tested was 1 ug/ml. Cells were counted 7 days later. The results shown are the mean of 3 separate experiments.

Medium	Mean fold stimulation[a] ± S.D.
DMEM	1.00
DMEM + BSM[†]	* 1.47 ± 0.13
0.1% FBS	1.00
0.1% FBS + BSM[†]	** 1.50 ± 0.28
Defined medium	1.00
Defined medium + BSM[†]	** 1.57 ± 0.21

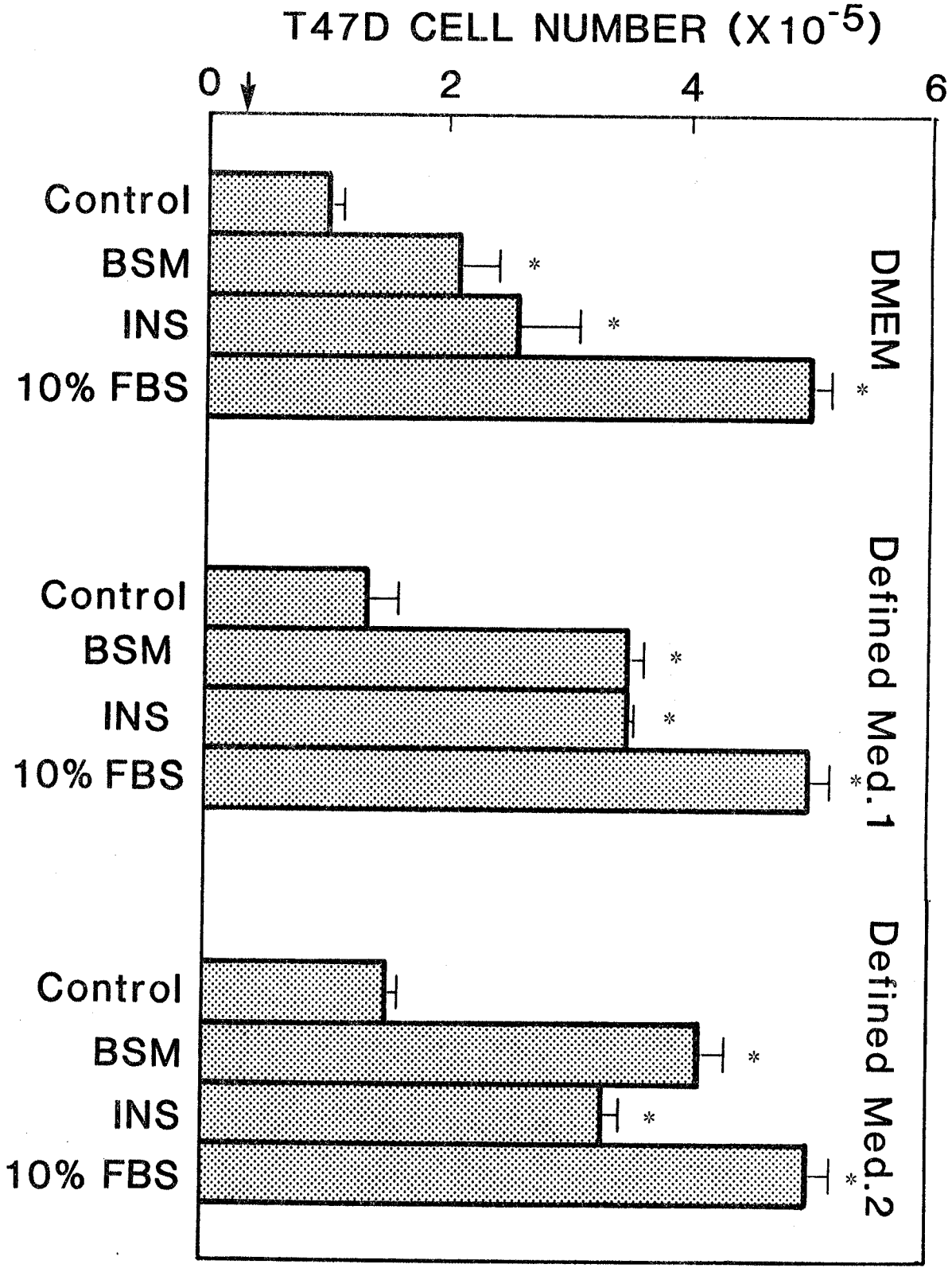
[a] stimulation observed as compared to control (no BSM added)

[†]BSM concentration (0.5-2 ug/ml)

* $P < 0.001$

** $P < 0.01$

Fig. 8. The effects of BSM and insulin (INS) on the growth of T-47D cells on collagen gel. Cells were plated at 3×10^4 cells/dish. Concentration of BSM tested was 1 ug/ml. Cells were counted 7 days after addition of the BSM. The results shown are a mean of triplicates $\pm$ S.D. ($*p < 0.01$). Undiluted Defined Medium 1 contained transferrin (25 ug/ml), hydrocortisone (100 ng/ml), EGF (10 ng/ml) and triiodothyronine (1 ng/ml). Undiluted Defined Medium 2 contained transferrin (25 ug/ml), hydrocortisone (100 ng/ml) and triiodothyronine. A 1:25 dilution of Defined Media 1 and 2 was used in these experiments.


T47D CELL NUMBER (X10-5)
0
2
4
6
Control
BSM
INS
10% FBS
DMEM
Control
BSM
INS
10% FBS
Defined Med.1
Control
BSM
INS
10% FBS
Defined Med.2
*

compare the binding of BSM and MSA to the receptor(s) on T-47D cells.

G. Optimization of conditions for the binding of ^{125}I-BSM by intact T-47D cells

In order to determine the optimal conditions for studying the binding of ^{125}I-BSM to the human breast cancer cell line T-47D, the effect of temperature (Table 5) and time (Fig. 9) on the binding of ^{125}I-BSM on T-47D were tested. Table 5 shows the result of temperature on specific binding to T-47D cells. The incubation period was 90 minutes. At 15°C maximum binding of ^{125}I-BSM was observed. At 4°C the rate of association of ^{125}I-BSM was slow. At higher temperatures, 18°-37°C, there was a gradual decrease of ^{125}I-BSM binding. Fig. 9 shows the time course of BSM binding to T-47D cells at 18°C. ^{125}I-BSM binding to T-47D cells was maximal at 1 hour. This was followed by a gradual decline upon further incubation.

H. Competitive binding studies of BSM, insulin and MSA

Insulin-like growth factor-I and IGF-II isolated from human plasma have been shown to interact with MSA and insulin receptors in a number of cell types. To determine if MSA, insulin and BSM share the same receptor sites on T-47D cells, the ability of these peptides to compete for ^{125}I-BSM binding was investigated. In Fig. 10 the ability of unlabelled MSA and unlabelled insulin to compete for ^{125}I-BSM is shown. Multiplication stimulating activity was not effective in displacing ^{125}I-BSM, even though low concentrations of MSA (1-10 ng/ml) were effective in promoting the growth of T-47D cells

Table 5. Effect of temperature on the specific binding of ^{125}I-labelled BSM to T-47D cells. Each dish contained 1.1 x 10^6 cells. To each dish was added 26,000 cpm of ^{125}I-labelled BSM ± unlabelled BSM (2 ug/ml). The incubation period was 1 hour. Specific binding was determined by substracting the mean value of duplicates for nonspecific binding from the mean value of duplicates for total binding.

Temperature (°C)	% Specific Binding
4	0
15	6.2
18	5.2
21	2.7
37	1.6

Fig. 9. Effect of time on the specific binding of ^{125}I-labelled BSM to T-47D cells. There were 1 x 10^6 cells/dish. ^{125}I-labelled BSM (25,000 cpm) was added to each dish ± unlabelled BSM (2 ug/ml). Each point represents the specific binding of ^{125}I-labelled BSM to T-47D cells.

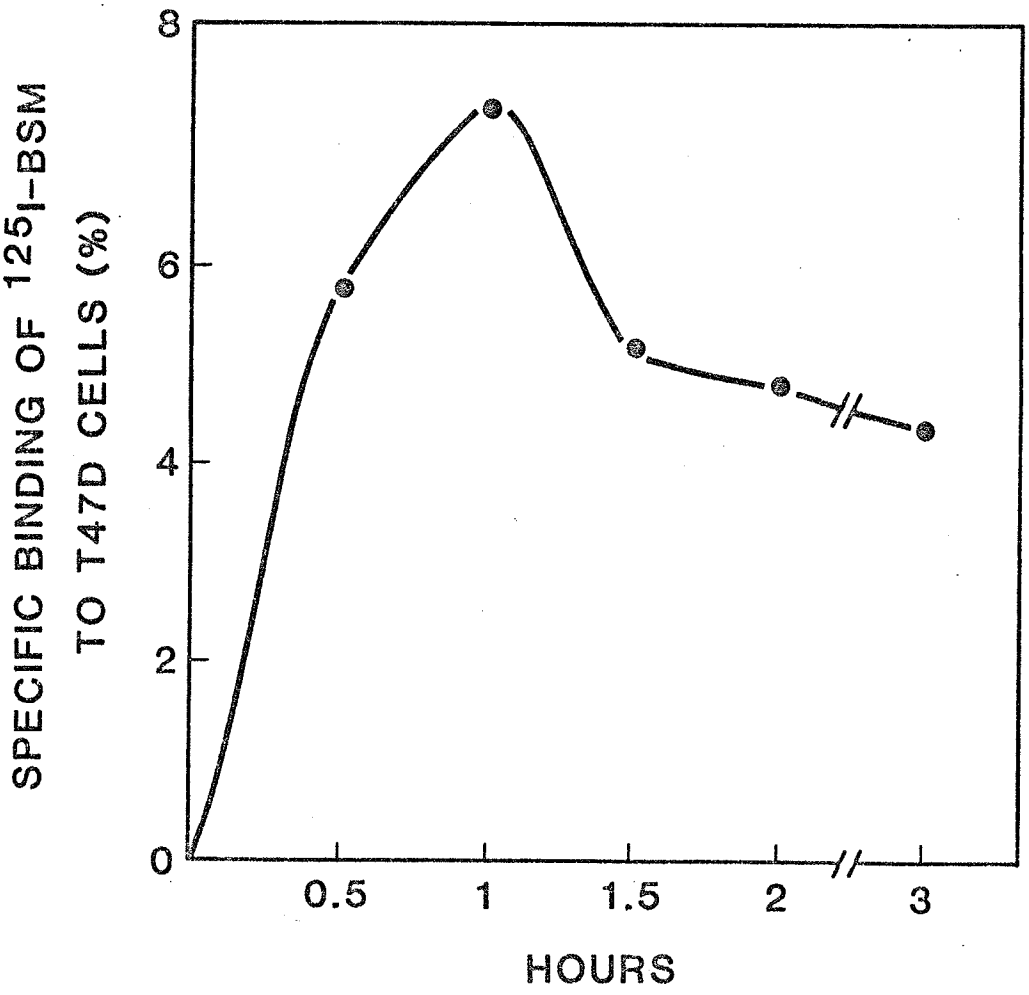
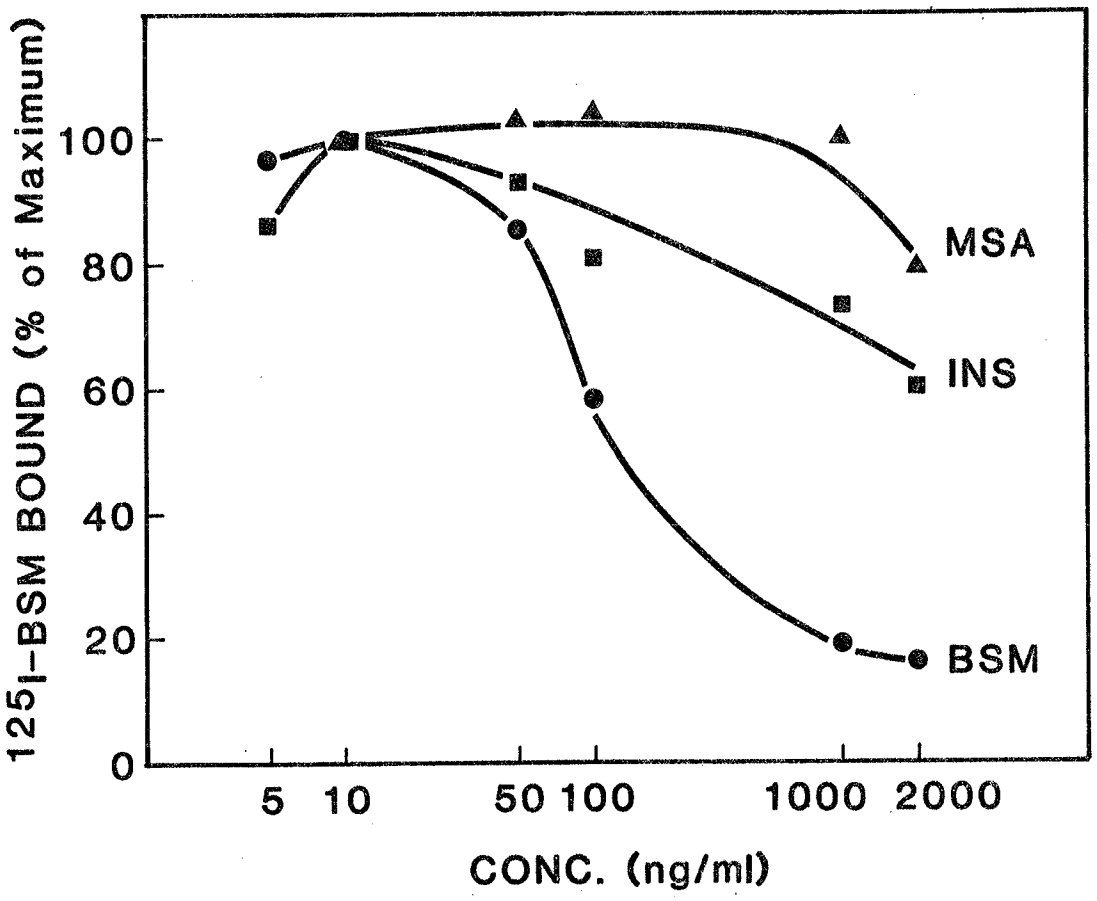

SPECIFIC BINDING OF ^{125}I-BSM TO T47D CELLS (%)
8
6
4
2
0
0.5
1
1.5
2
3
HOURS

Fig. 10. Competitive binding by BSM, insulin (INS) and MSA for ^{125}I-BSM binding sites on T-47D cells. T-47D cells (1.4 x 10^6 cells/dish) were incubated with 25,000 cpm of ^{125}I-BSM for 1 hour in a final volume of 1 ml. Various amounts of unlabelled BSM (1-2,000 ng/ml), insulin (1-2,000 ng/ml), or MSA (1-2,000 ng/ml) were added to displace bound ^{125}I-BSM. The results of the measurements of the binding of ^{125}I-BSM and its displacement by BSM, insulin and MSA are presented. Basic somatomedin was more effective in displacing ^{125}I-BSM than insulin. Multiplication stimulating activity was ineffective in displacing ^{125}I-BSM from T-47D binding sites.


^{125}I-BSM BOUND (% of Maximum)
100
80
60
40
20
0
5
10
50
100
1000
2000
CONC. (ng/ml)
MSA
INS
BSM

(Fig. 6). Insulin was more effective than MSA but not as effective as unlabelled BSM in displacing ^{125}I-BSM on T-47D cells.

I. Kinetic analysis

Theoretical considerations to the analysis of binding data by the method of Scatchard (1949) has been detailed in the legend of Fig. 11. ^{125}I-BSM binds to T-47D cells with high affinity; the dissociation constant being 1.2×10^{-10} M. There are 410,000 receptor sites per cell.

J. Displacement of ^{125}I-insulin by BSM and insulin

In order to determine whether BSM was binding to insulin receptor sites on T-47D cells or whether BSM had its own binding sites, the ability of unlabelled BSM and unlabelled insulin to displace ^{125}I-insulin was compared. The results are shown in Fig. 12. Unlabelled BSM failed to displace ^{125}I-insulin even at very high concentrations (4,000 ng/ml). Unlabelled insulin displaced ^{125}I-insulin to a small degree.

Fig. 11. Scatchard analysis of the binding of ^{125}I-BSM to T-47D cells. Scatchard plot of data was obtained by using the formula,

$$B/F = K - B/K_d$$

where B=^{125}I-BSM specifically bound, pg; F=free ^{125}I-BSM; K_d=dissociation constant; and K=constant. Slope of the plot yields $-1/K_d$ (K_d=1.2 x 10^{-10} M). The number of receptors was determined to be 4.1 x 10^5 receptor sites/cell.

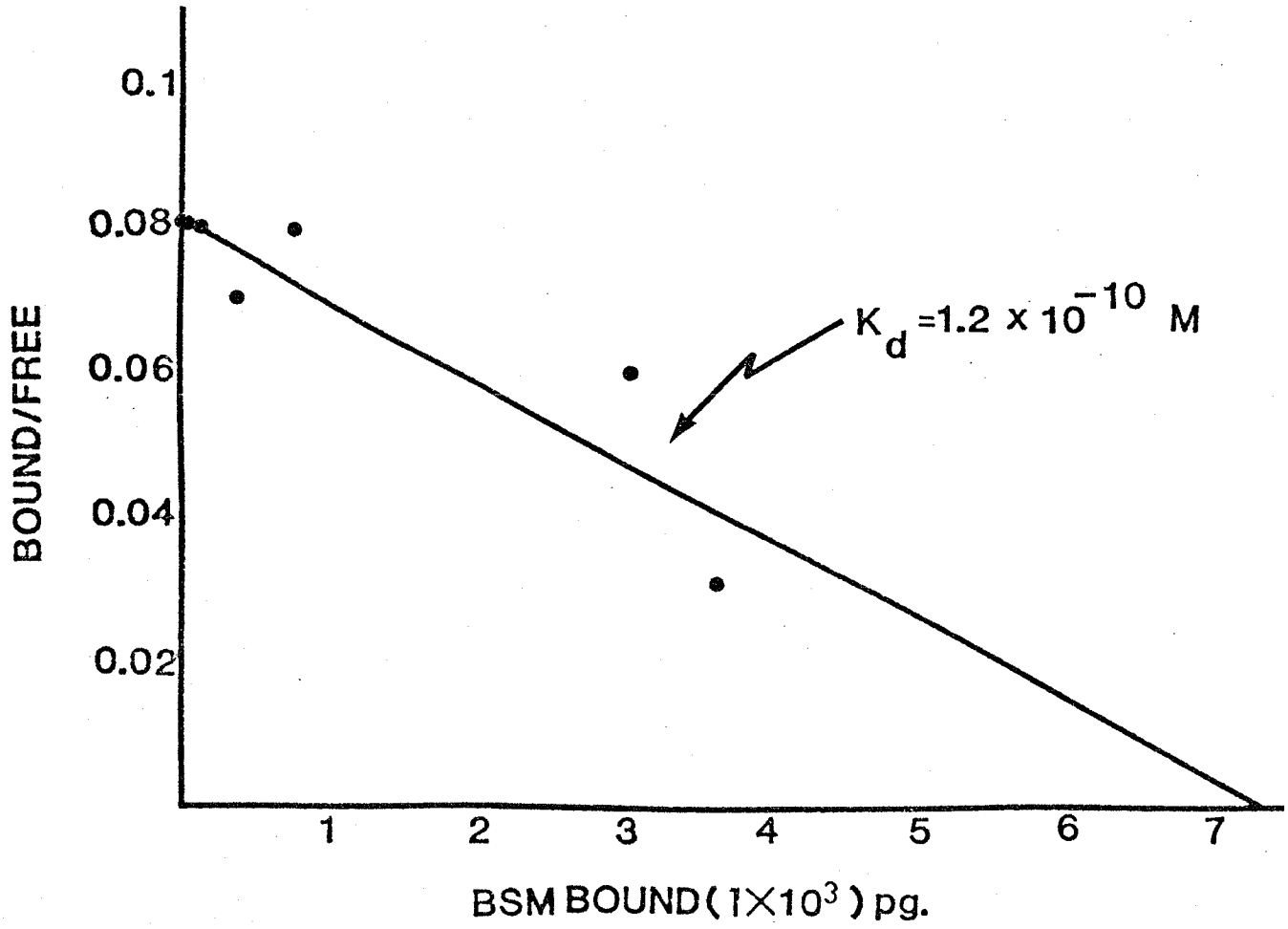
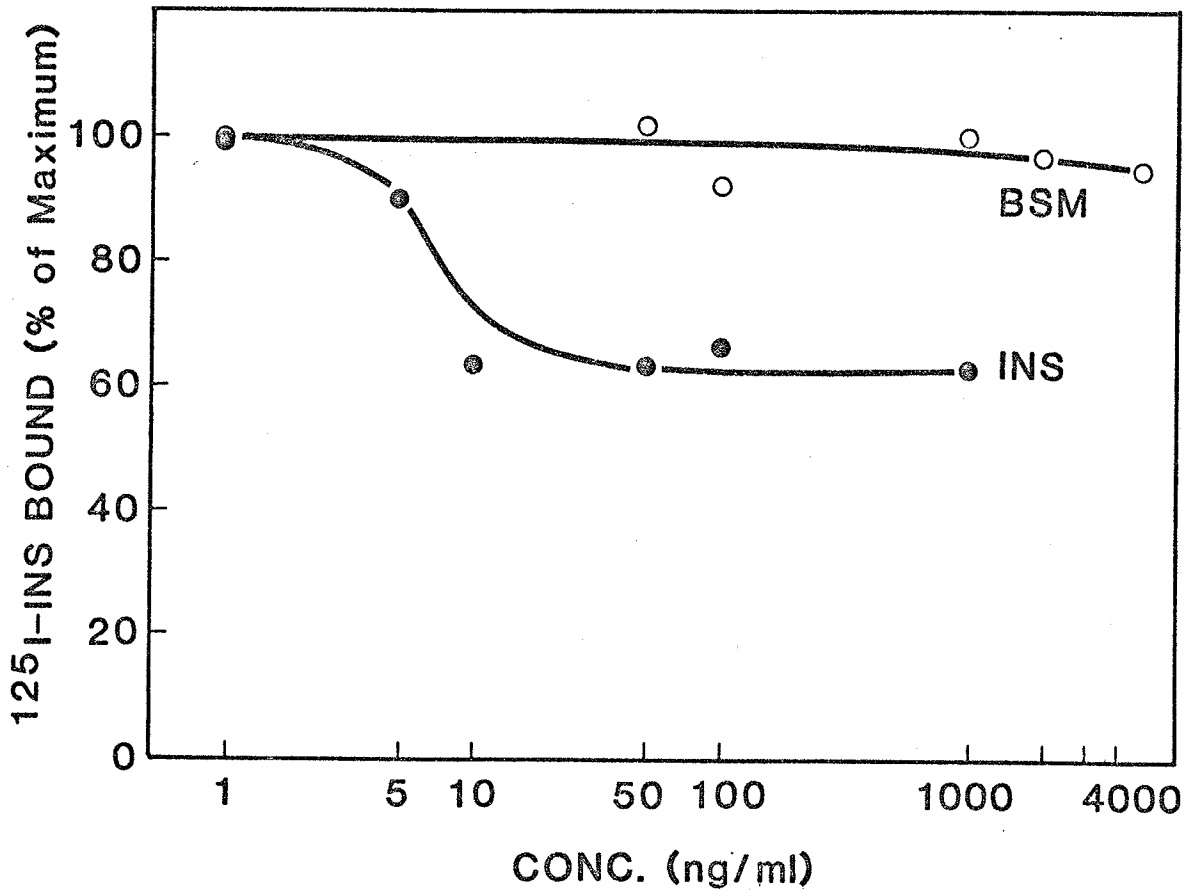

0.1
0.08
0.06
0.04
0.02
BOUND/FREE
$K_d = 1.2 \times 10^{-10}$ M
1
2
3
4
5
6
7
BSM BOUND (1×10^3) pg.

Fig. 12. Competitive binding by insulin (INS) and BSM for ^{125}I-insulin binding sites on T-47D cells. There were 0.9 x 10^6 cells/dish. ^{125}I-insulin (200,000 cpm/dish) was added ± unlabelled insulin or unlabelled BSM. The incubation period was 1 hour at 18°C.


125I-INS BOUND (% of Maximum)
100
80
60
40
20
0
1
5
10
50
100
1000
4000
CONC. (ng/ml)
BSM
INS

DISCUSSION

To determine whether SM are by themselves mitogenic to human breast cancer, we have studied the ability of partially and highly purified SM to stimulate T-47D human breast cancer cells *in vitro*. As a preliminary step in this study, the mitogenic effect of BRL conditioned medium on T-47D cells was investigated, since these cells secrete MSA into their medium. The results (Figs. 3 and 4) show that BRL conditioned medium stimulated the growth of these cells 2-12 fold over that of control groups (BSA and WI38). Since BRL medium was observed to be a better mitogen than BSA, this result indicates that the stimulation of T-47D cell growth was not due to a non-specific effect produced by proteins in the medium. On the other hand, WI38 cells are known to secret a SM-like factor into their medium (van Wyk *et al.*, 1981). Therefore, cell growth stimulated by WI38 conditioned medium could have been due to this SM-like factor.

The wide range of T-47D cell growth rate observed in response to BRL medium may have been as a result of (1) variation between batches of collected BRL conditioned medium and (2) differences in the number of T-47D cells plated in experimental dishes. It was observed that if a low baseline level of cells was maintained, the presence of BRL conditioned medium resulted in a greater stimulation of cell growth. Therefore, cell density seemed to play an important role in determining cell responses to the conditioned medium.

Since the major growth promoting component of BRL conditioned medium is MSA, the effect of purified MSA (commercially

available) on T-47D cells was subsequently examined. Purified MSA (2 and 10 ng) failed to stimulate the growth of T-47D cells in serum-free medium (Fig. 3). However, BRL conditioned medium stimulated cell growth. Thus, it was possible that (1) the MSA secreted by BRL cells may have acted synergistically with some other component(s) produced by these liver cells to stimulate T-47D cell growth or (2) that some growth factor(s) present in the BRL conditioned medium, other than MSA, was mitogenic to T-47D cells. Verification of the latter hypothesis could be brought about by the use of an antibody to MSA in the collected conditioned medium to block any MSA activity. In support of the first hypothesis, it was observed that, in the presence of DMEM containing 0.1% FBS, and in chemically defined serum-free medium, low concentrations (1-10 ng/ml) of purified MSA were capable of stimulating the proliferation of T-47D cells. This implied a possible synergistic interaction between MSA and another growth factor(s) and/or hormone(s).

Fetal bovine serum was chosen for this experiment as it is known to contain a mixture of hormones, nutrients and various other factors required for cell growth. By using FBS at a dilution of 0.1%, cells could remain in a healthy but nonproliferative state.

Chemically defined medium was a combination of transferrin, triiodothyronine, hydrocortisone, insulin and EGF. In its undiluted form, this defined medium was capable of stimulating cell growth to the same extent as 10% FBS (which is used for maximum stimulation of cell growth). Therefore, it was necessary to dilute the defined medium in order to obtain

minimal cell growth and also to avoid masking the effect of the test substance, MSA.

In subsequent experiments, MSA (1-10 ng/ml) resulted in stimulation of T-47D cells ranging from 1.25-6.6 fold in the 0.1% FBS and the defined medium conditions. There may be many reasons for these variable responses: (1) the purity of MSA varied from batch to batch (80-98% pure) and (2) the baseline level of cells in control groups has an important bearing on the observed stimulation produced by MSA. Since in some experiments we were unable to obtain a low baseline level in our negative control and since the stimulation of cell growth observed was seldom more than 2-fold in experimental groups, we felt that the effect of the substance (in this case, MSA) may have been masked.

Nevertheless, since these results showed that maximum stimulation, due to MSA, occurred in the presence of chemically defined medium, it once again raised the possibility that indeed some synergistic interaction between MSA and the components of the chemically defined medium had occurred. In a related study, Nagarajan et al. (1982), using serum-free conditions, demonstrated that MSA in the presence of transferrin and fibronectin, was an effective mitogen of F9 embryonal carcinoma cells. He found that at 100 ng/ml MSA completely replaced the growth requirements for fetal calf serum. Since MSA has been implicated as an important fetal growth factor (Moses et al., 1980), these results were not at all surprising.

The concentrations of MSA/IGF-II (1-10 ng/ml) used in our

experiments up to this point were below physiological values which are 150-200 ng/ml. The presence of low MSA concentration resulted in an average of a 2.5-fold increase in cell number over control groups. Higher concentrations of MSA (50-2,000 ng/ml) failed to stimulate cell growth more than 1.5-fold in each case. It is thus possible that these cells may have receptors for MSA which "down regulate" in the presence of excess MSA.

Since MSA, a rat SM, was capable of stimulating T-47D human breast cancer cells to grow in vitro, we proceeded to investigate the mitogenicity of a hSM on this human breast cancer cell line. A wide range of BSM concentrations were tested on T-47D cells. Fig. 7 shows the effect of BSM on the growth of T-47D, both in serum-free DMEM and in chemically defined medium (1:25 dilution). The results showed that in each condition, maximum stimulation (1.45-fold) was brought about by the highest concentration tested, 2,000 ng/ml.

When compared to the results obtained with low concentrations of MSA, the growth stimulation due to BSM was comparatively much lower. Some possible reasons for this observed phenomenon could be that (1) different conditions may be necessary to demonstrate a better response of these cells to BSM and (2) lower basal growth rate has to be achieved to see an increased growth stimulation due to BSM.

We then subsequently examined the mitogenic effect of BSM on T-47D cells grown on collagen coated dishes, since, as previously mentioned, the physical substrate upon which epithelial cells rest, both in vivo and in vitro, can modulate their

response to growth factors. Recent experiments by Gospodarowicz and Tauber (1980) demonstrated that corneal epithelial cells maintained on plastic dishes failed to respond to EGF although EGF is known to stimulate the proliferation of corneal epithelial cells in vivo or in organ cultures (Savage and Cohen, 1972; Gospodarowicz et al., 1978). However, addition of EGF to these cells grown on collagen coated dishes resulted in both a marked increase in cell number and a noticeable change in the shape of the cells. Therefore, it seemed possible that although the response of T-47D to BSM on plastic was not always significant the response of these cells on collagen gel might be.

In these experiments, the defined medium used consisted of transferrin, triiodothyronine and hydrocortisone, as insulin and EGF were recently reported to be mitogenic to T-47D cells (Shiu, 1981; Imai et al., 1982). As a result, they were not included as components of the chemically defined medium so as not to contribute to the basal growth rate of T-47D.

Our results (Fig. 8) showed that in all conditions, cell growth rate was slower on collagen gel than on plastic substratum. The retarded growth rate of T-47D cells on collagen gel confirms a previous report by Leung and Shiu (1982).

The BSM, at 1,000 ng/ml stimulated cell growth rate on collagen 2- and 2.5-fold over controls in serum-free DMEM and chemically defined medium respectively. Comparable results were also observed with high concentrations (1,000 ng/ml) of insulin. We have speculated that some of the following factors may be responsible for the observed lack of a more marked response. (1) "Down regulation" of receptors may have ensued

following addition of such a high concentration of our mitogen. (2) The BSM used in this study was 50% pure, compared to MSA which was 80-98% pure. The presence of impurities could have caused the retardation of the T-47D response. There was no known contamination with insulin (Bala and Bhaumick, 1979). (3) The stimulation observed may be part of a more general phenomena. We have so far examined the mitogenicity of one component of a complex system. In the body the cells are exposed to many growth factors and hormones. Therefore, the growth rate of breast cancer cells in the body may reflect a summation of the effects produced by many trophic substances.

Since insulin was as effective as BSM in promoting T-47D cell proliferation on collagen, it was possible that insulin and BSM were mediating their actions through insulin receptors for the simple reasons that (1) T-47D cells contain insulin receptors and (2) SM demonstrate insulin-like activities. In addition, receptors for insulin and BSM may be different from the binding sites for MSA which has been shown to be a more potent mitogen for T-47D cells, than these two hormones. In chick embryo fibroblast, MSA, IGF-I and II, appear to bind to the same receptor, whereas in rat liver plasma membranes, human fibroblast, and BRL-3A cells, there is evidence for more than one receptor type (Rechler et al., 1980). We therefore wanted to establish if indeed these peptides were both acting through an insulin receptor or if BSM and MSA were acting via their own receptor sites.

Earlier studies have suggested that the common metabolic effects of insulin and IGF are mediated through the insulin

receptor, whereas the common growth promoting effects are mediated by the receptors for the IGF (Megyesi et al., 1974; Rechler et al., 1976; Rechler et al., 1978; Zapf et al., 1978). Therefore, the binding characteristics of BSM on T-47D cells was investigated. The effect of temperature on the specific binding of ^{125}I-labelled BSM to T-47D cells (Table 4) revealed that maximum binding of ^{125}I-BSM occurred at 15°C and a gradual decrease in binding occurred at higher temperature. This decrease at higher temperatures was probably due to more rapid internalization of receptors. Over the entire incubation period (1.5 hours) no binding was observed at 4°C. However, when the effect of time on the specific binding of ^{125}I-BSM (at 18°C) was studied, it was found that maximum specific binding of 7.6% was observed after a 1 hour incubation period followed thereafter by a gradual decrease.

Since BSM demonstrated a lower capacity to stimulate T-47D cell growth on plastic than on collagen, it is of interest in the future to investigate whether cells cultured on collagen contain more BSM receptors than cells grown on plastic substratum.

We have also investigated the specificity of the BSM binding sites on the T-47D cells. The results of this study (Fig. 10) clearly show that MSA/IGF-II does not compete with ^{125}I-BSM for binding sites. Insulin was found to displace some ^{125}I-BSM although it was not as effective as unlabelled BSM. This again may explain why insulin at high concentrations (1,000-10,000 ng/ml) is mitogenic in most human breast cancer cell lines, as well as other cell lines (Heuson and Legros, 1970; Osborne et

al., 1976; Osborne et al., 1978). Since insulin and MSA were effective mitogens of T-47D cells, the latter findings suggest that MSA and BSM may exert their mitogenic effect through binding sites different from each other and different from insulin. In an attempt to determine if BSM binds to another site different from the insulin receptor site, we investigated whether BSM could displace ^{125}I-insulin for binding sites on T-47D cells. The results (Fig. 12) showed that (1) insulin demonstrated low specific binding to T-47D cells, verifying earlier reports by Imai (unpublished observations) and (2) that BSM failed to compete with ^{125}I-insulin for T-47D binding sites. Due to the unavailability of MSA and ^{125}I-MSA at this time, we were unable to do further binding studies with MSA.

In summary, from this study, it has been demonstrated that

(1) SM (MSA/IGF-II and BSM/IGF-I) are mitogenic to T-47D human breast cancer cells,

(2) MSA can act synergistically with other factors and hormones in promoting cell growth,

(3) BSM and insulin displayed similar mitogenicity on T-47D cells,

(4) T-47D cells are more responsive to BSM on collagen than on plastic substratum,

(5) BSM showed high specific binding to T-47D cells, and

(6) the binding sites for BSM may be different from MSA.

Braun (1975) referred to the neoplastic state as a composite state of differentiation and dedifferentiation. Neoplastic cells can demonstrate differentiated characteristics (for example, hormone secreting pituitary tumors) and at the same

time maintain properties characteristic of undifferentiated (embryonal) cells, such as expression of fetal antigens like carcinoembryonic antigen and α-fetoprotein (made by fetal, but not adult, liver). Neoplastic cells also demonstrate the inherent ability to multiply, undergoing frequent mitotic division.

Recently, Moses et al. (1980) have reported that MSA levels are enormously elevated in fetal blood but are reduced to much lower levels in the postnatal period. Sara et al. (1981), using a modified SM-A assay, also reported that human fetal serum levels of SM are 5-6 fold greater than levels after birth. D'Ercole et al. (1980) have shown that SM-C is produced by many fetal tissues. Therefore, MSA/IGF-II is believed to be important in fetal growth. The observation that human breast cancer cells respond to MSA, believed to be a fetal growth factor, may reflect the embryonic characteristics of these cells that were acquired as a result of cellular dedifferentiation during malignant transformation.

In another study, Ptashne et al. (1979) reported that there was a similarity between MSA and a serum mitogen that stimulated mouse breast cancer cells in culture. On further investigation, it was concluded that this mammary stimulating factor (MSF) had SM-like characteristics. Since this factor stimulated mammary cell growth, it is not surprising that MSA, a SM, could also stimulate human breast cancer cell proliferation.

There are of course a variety of other tissue growth factors reported, for example NGF, EGF, and FGF, and therefore, the separate categorization of actions of the SM from other

tissue growth factors may not be entirely appropriate. Imai et al. (1980) reported that hGH increases serum EGF levels. Also, EGF has been reported to increase SM secretion by fibroblast cells in culture (Weidman and Bala, 1980). Other hormones, for example estrogen and glucocorticoids, have also been shown to be intimately involved in SM secretion (Phillips and Vassilopoulou-Sellin, 1980b). Therefore, MSA may only play a small part in the overall in vivo growth regulation of breast cancer. Further work pertaining to this type of interaction needs to be done.

It is well known that SM levels in human subjects vary considerably (Friesen, 1980) and that the growth rate of breast cancer cells in cancer patients also varies. For example, it has been well established that in breast cancer patients, tumor doubling times may vary from 25 days to over 2 years (the average is 108 days) (Ariel, 1978; Bassler, 1978). If indeed SM were active in promoting tumor growth in vivo then the presence or absence of SM in the patient would be important. The results of our present investigation may indicate future studies correlating the growth rate of breast cancer and SM levels in patients may yield new insights into the roles that SM play in breast tumor growth.

These studies presented have been performed on the T-47D human breast cancer cell line. However, a mixed population of cell types usually exists within a breast tumor. Since SM were stimulatory to this cell line, conclusions drawn may be limited to one subpopulation of cancer cells. In order to verify that these results may represent a more general phe-

nomena, further investigations on the role of SM in the growth regulation of several other breast cancer cell lines as well as primary cells isolated from tumor specimens will have to be studied.

REFERENCES

Anderson, D. E. A high-risk group for breast cancer. Cancer Bull. 25:23, 1973.

Antoniades, H., Scher, C. and Stiles, G. Purification of human platelet-derived growth factor. Proc. Natl. Acad. Sci. USA 86:1809, 1979.

Ariel, I. M. The treatment of breast cancer by radical and super radical mastectomy. Resident and Staff Physician 24(9):57, 1978.

Bala, R. M. and Bhaumick, B. Purification of a basic somatomedin from human plasma Cohn fraction IV-I, with physiochemical and radioimmunoassay similarity to Somatomedin-C and insulin-like growth factor. Can. J. Biochem. 57:1289, 1979a.

Bala, R. M. and Bhaumick, B. Radioimmunoassay of basic somatomedin. Comparison of various assay techniques and somatomedin levels in various sera. J. Clin. Endocrinol. Metab. 49:770, 1979b.

Balk, S. D. Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblast in a plasma-containing medium. Proc. Natl. Acad. Sci. USA 68:271, 1971.

Barnes, D. and Sato, G. Growth of a human mammary tumor cell line in a serum-free medium. Nature 281:388, 1979.

Barnes, D. and Sato, G. Methods for growth of cultured cells in serum-free medium. A review. Analytical Biochem. 102:255, 1980.

Bassler, R. Pathologie der Brustdruse. Springer-Verlag, Berlin-Heidelber, 1978.

Baxter, R. C., Zaltman, Z., Raison, R. L., Reddel, R. R. and Sutherland, R. L. Somatomedin C immunoreactivity from T-47D human breast cancer cells detected using monoclonal antibody. Presented at the 64th Endocrinology Symposium, California. Abstract No. 316, 1982.

Beatson, G. T. On the treatment of inoperable cases of carcinoma of the mamma: Suggestion for a new method of treatment with illustrative cases. Lancet 2:104, 1896.

Beeby, D. I., Easty, G. C., Gazet, J. C., Grigor, K. and Neville, A. M. An assessment of the effects of hormones on short term organ culture of human breast carcinomata. Br. J. Cancer 31:317, 1975.

Bertoli, A., Pirro, R., Fusco, A., Greco, A. V., Magnatta, R., and Lauro, R. Differences in insulin receptors between men and menstruating women and influences of sex hormones on insulin binding during the menstrual cycle. J. Clin. Endocrinol. Metab. 50:246, 1980.

Boyns, A. R., Cole, E. N., Griffiths, K., Roberts, M. M., Buchan, R., Wilson, R. G. and Forrest, A. P. M. Plasma prolactin in breast cancer. Europ. J. Cancer 9:99, 1973.

Braun, A. C. Differentiation and dedifferentiation. In: Cancer. A comprehensive treatise. Becker, F. F. (ed.). New York: Plenum Press, 1975.

Brooks, R. F. In: Structure and function of plasma proteins. Allison, A. C. (ed.). New York: Plenum Press, 1975.

Burke, J. M. and Ross, R. Collagen synthesis by monkey arterial smooth muscle during proliferation and quiescence in culture. Exp. Cell Res. 107:387, 1977.

Carpenter, G. and Cohen, S. Human epidermal growth factor and proliferation of human fibroblasts. J. Cell Physiol. 88: 227, 1976.

Castor, C. W., Ritchie, J. C., Scott, M. E. and Whitney, S. L. Connective tissue activation: XI. Stimulation of glycosaminoglycan and DNA formation by a platelet factor. Arithritis Rheum. 20:859, 1977.

Ceriani, R. L., Contess, G. P. and Natof, B. M. S. Hormone requirement for growth and differentiation of the human mammary gland in organ culture. Cancer Res. 32:2190, 1972.

Cohen, S. Isolation of a mouse submixillary gland protein accelerating incisor eruption and eyelid opening in new born animals. J. Biol. Chem. 237:1555, 1962.

Cohen, S. and Carpenter, G. Human epidermal growth factor: Isolation and chemical and biological properties. Proc. Natl. Acad. Sci. USA 72:1317, 1975.

Coon, H. G. Clonal culture of differentiated rat liver cells. J. Cell Biol. 39:29a, 1968.

Costlow, M. E., Buschow, R. A. and McGuire, W. L. Prolactin receptors in 7,12-dimethylbenz-(a)-anthracene-induced mammary tumors and response to endocrine ablation. Cancer Res. 36:3941, 1976.

D'Ercole, A. J., Applewhite, G. T. and Underwood, L. E. Evidence that somatomedin is synthesized by multiple tissues in the fetus. Dev. Biol. 75:315, 1980.

De Sombre, E. R., Kledzik, G., Marshall, S. and Meites, J. Estrogen and prolactin receptor concentration in rat mammary tumors and responses to endocrine ablation. Cancer Res. 36:354, 1976.

Dicky, R. P. and Minton, P. L-Dopa effect on prolactin, follicle-stimulating hormone, and lutenizing hormone in women with advanced breast cancer: A preliminary report. Amer. J. Obstet. Gynec. 114:267, 1972.

Dulak, N. C. and Temin, H. M. Multiplication-stimulating activity for chicken embryo fibroblasts from rat liver cell conditioned medium: A family of small polypeptides. J. Cell Physiol. 81:161, 1973.

Eastman, C. T. and Sirbasku, D. A. Platelet-derived growth factor(s) for a hormone-responsive rat mammary tumor cell line. J. Cell Physiol. 97:17, 1978.

Ehrmann, R. L. and Gey, G. O. The growth of cells on a transparent gel of reconstituted rat tail collagen. J. Natl. Cancer Inst. 16:1375, 1956.

Emerman, J. T., Leaky, M., Gout, P. W. and Bruchovsky, N. A possible role for growth hormone in the human breast cancer. Clin. Invest. Med. 4:Abstract No. 24B, 1981.

Engel, L. W. and Young, N. A. Human breast carcinoma cells in continuous culture: A review. Cancer Res. 38:4327, 1978.

Francis, M. J. O. and Hill, D. J. Prolactin stimulated production of somatomedin by rat liver. Nature 255:167, 1970.

Franks, S., Ralphs, D. N. L., Seagroath, V. and Jacobs, H. S. Prolactin concentrations in patients with breast cancer. Brit. Med. J. 4:320, 1974.

Friesen, H. G. Raben Lecture 1980: A tale of stature. Endo. Rev. 1:309, 1980.

Froesch, E. R., Burgi, H., Ramseier, E. B., Bally, P. and Labhart, A. Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiological significance. An insulin assay with adipose tissue of increased precision and specificity. J. Clin. Invest. 42:1816, 1963.

Froesch, E. R., Zapf, J., Rinderknecht, E., Morell, B., Schoenle, E. and Humbel, R. E. Insulin-like growth factor (IGF-NSILA): Structure, function, and physiology. In: Hormones and cell culture. Sato, H. G. and Ross, R. (eds.) Cold Spring Harbor Laboratory 61, 1979.

Fryklund, L., Uthne, K. and Sievertsson, H. Identification of two somatomedin A active polypeptide and in vivo effects of a somatomedin A concentrate. Biochem. Biophy. Res. Commun. 61:957, 1974.

Furlanetto, R. N., Underwood, L. E., van Wyk, J. J. and D'Ercole, A. J. Estimation of somatomedin-C levels in normals and patients with pituitary diseases by radio-immunoassay. J. Clin. Invest. 60:648, 1977.

Glicksman, A. S., Meyers, W. P. L. and Rawson, R. W. Diabetes mellitus and carbohydrate metabolism in patients with cancer. Med. Clin. N.A. 40:887, 1956.

Goranson, E. S. and Tilser, G. J. Studies on the relationship of alloxan-diabetes and tumor growth. Cancer Res. 15:626, 1955.

Gospodarowicz, D., Greenberg, C. and Birdwell, C. R. Cellular shape is determined by the extracellular matrix and is correlated with the control of cell growth. Cancer Res. 38:4155, 1978.

Gospodarowicz, D., Mescher, A. L., Brown, K. and Birdwell, C. R. The role of fibroblastic growth factor and epidermal growth factor in the proliferative response of the cornea and lens epithelium. Exp. Eye Res. 25:631, 1977.

Gospodarowicz, D. and Tauber, J. P. Growth factors and the extracellular matrix. Endocrine Reviews 1:201, 1980.

Greenwood, F. C., James, V. H. T., Meggitt, B. F., Miller, J. D. and Taylor, P. H. Pituitary function in breast cancer. In: Prognostic factors in breast cancer. Proceedings of First Tenovus Symposium, Cardiff, April 12-14, 1967. Forrest, A. M. P. and Klinkler, P. B. (eds.). The Williams and Wilkins Company, Baltimore, Maryland, 1968.

Guzman, R. C., Osborne, R. C., Yang, J., De Ome, K. B. and Nandi, S. Transplantation of mouse mammary epithelial cells grown in primary collagen gel cultures. Cancer Res. 42:2376, 1982.

Ham, R. G. and McKeehan, W. L. In: Methods in Enzymology, 43:44. Jacoby, W. B. and Pastan, I. H. (eds.). New York: Academic Press, 1979.

Hamilton, W. G. and Ham, R. G. Clonal growth of chinese hamster cell lines in protein-free media. In vitro 13: 537, 1977.

Hara, Y., Steiner, M. and Baldini, M. G. Platelets as a source of growth-promoting factor(s) for tumor cells. Cancer Res. 40:1212, 1980.

Harmon, J. T. and Hilf, R. Identification and characterization of the insulin receptors in the R3230AC mammary adenocarcinoma of the rat. Cancer Res. 36:3993, 1976.

Hay, E. D. Interaction between the cell surface and extracellular matrix in corneal development. In: Cell and tissue interactions. Society and general physiologist series. 32:115, Lash, J. W. and Burger, M. M. (eds.). New York: Raven Press, 1977.

Hay, E. D. and Meier, S. Stimulation of corneal differentiation by interaction between cell surface and the extracellular matrix. II. Further studies on the nature and site of transfilter "induction". Dev. Biol. 52:141, 1976.

Heldin, C. H., Wasteson, A., Fryklund, L. and Westermark, B. Somatomedin B: Mitogenic activity derived from contaminant epidermal growth factor. Science 213:1122, 1981.

Heldin, C. H., Westermark, B. and Wasteson, A. Platelet-derived growth factor, purification and partial characterization. Proc. Natl. Acad. Sci. USA 76:3722, 1979.

Henderson, B. E., Gerkins, V., Rosario, I. and Casagrande, J. and Pike, M. C. Elevated serum levels of estrogen and prolactin in daughters of patients with breast cancer. N. Engl. J. Med. 293:790, 1975.

Heuson, J. C. and Legros, N. Effect of insulin and of alloxan diabetes on the growth of the rat mammary carcinoma *in vivo*. Eur. J. Cancer 6:349, 1970.

Heuson, J. C. and Legros, N. Influence of insulin deprivation on growth of the 7,12-dimethylbenz-(a)-anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction. Cancer Res. 32:226, 1972.

Higuchi, K. Cultivation of animal cells in chemically defined media: A review. Adv. App. Microb. 16:111, 1973.

Hilf, R. The actions of insulin as a hormonal factor in breast cancer. In: Hormones and breast cancer. Pike, M. C., Siiteri, P. K. and Welsch, C. W. (eds.). Cold Spring Harvor Laboratory, 317, 1981.

Hobbs, J. R., De Souza, I., Salih, H. and Raggat, P. Selection of hormone-dependent breast cancers. Br. J. Surg. 61:785, 1974.

Holdaway, I. M. and Friesen, H. G. Correlation between hormone binding and growth response of rat mammary tumor. Cancer Res. 36:1562, 1976.

Holmes, R. J. Preparation from human serum of an alpha-one protein which induces the immediate growth of unadapted cells *in vitro*. J. Cell Bio. 32:297, 1967.

Hook, M., Rubin, K., Oldberg, A., Obrink, B. and Vaheri, A. Cold-insoluble globulin mediates the adhesion of rat liver cells to plastic dishes. Biochem. Biophys. Res. Comm. 79:726, 1977.

Horrobin, D. F. Prolactin 6. Montreal: Eden Press, 1978.

Horwitz, K. B. and McGuire, W. L. Estrogen control of progesterone receptor in human breast cancer. J. Biol. Chem. 253:2223, 1978.

Hunter, W. M. and Greenwood, F. C. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194:495, 1962.

Imai, Y., Leung, C. K. H., Friesen, H. G. and Shiu, R. P. C. Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long term tissue culture. Cancer Res. 42:5472, 1982.

Imai, Y., Sasaki, N., Matsuzaki, F. and Tsushima, T. Growth hormone and thyroxine increase the production and urinary excretion of epidermal growth factor. 6th International Congress of Endocrinology, Melbourne, 1980 (Abstract 11).

Jensen, E. V. and De Sombre, E. R. Mechanism of action of the female sex hormones. Annu. Rev. Biochem. 41:203, 1972.

Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitchik, S. and Brenner, H. J. Establishment and characterization of a cell line of human breast carcinoma origin. Europ. J. Cancer 15:659, 1979.

Knauer, D. J., Lyer, A. P., Banejee, M. R. and Smith, G. L. Identification of somatomedin-like polypeptides produced by mammary tumors of Balb/c mice. Cancer Res. 40:4368, 1980.

Kohler, N. and Lipton, A. Platelets as a source of fibroblast growth-promoting activity. Exp. Cell Res. 87:297, 1974.

Kohler, N. and Lipton, A. Release and characterization of a growth factor for SV40 virus-transformed cells from human platelets. Proc. Am. Assoc. Cancer Res. 18:244, 1977.

Kwa, H. G., Cleton, F., de Jong-Bakker, M., Bulbrook, R. D., Hayward, J. L. and Wang, D. Y. Plasma prolactin and its relationship to risk factors in human breast cancer. Int. J. Cancer 17:441, 1976.

Lasfargues, E. Y. and Ozello, L. Cultivation of human breast carcinomas. J. Natl. Cancer Inst. 61:967, 1958.

Lembach, K. I. Induction of human fibroblast proliferation by epidermal growth factor (EGF): Enhancement by an EGF-binding arginine esterase and by ascorbate. Proc. Natl. Acad. Sci. USA 73:183, 1976.

Leung, C. K. H. and Shiu, R. P. C. Required presence of both estrogen and pituitary factors for the growth of human breast cancer cells in athymic nude mice. Cancer Res. 41:546, 1981.

Leung, C. K. H. and Shiu, R. P. C. Morphological and proliferative characteristics of human breast tumor cells cultured on plastic and in collagen matrix. In vitro 18:476, 1982.

Lippman, M. E. and Bolan, G. Oestrogen-responsive human breast cancer in long-term tissue culture. Nature 256:592, 1975.

Lippman, M. E., Bolan, G. and Huff, K. The effect of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res. 36:4595, 1976.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenor reagent. J. Biol. Chem. 193:265, 1951.

Mairesse, N., Derleeschouwer, W., Leclercq, G. and Galand, P. Estrogen-induced synthesis and secretions of proteins in the human breast cancer cell line MCF7. J. Steroid. Biochem. 15:375, 1981.

Malarkey, W. B., Schroeder, L. L., Stevens, V. C., Jones, A. G. and Lanese, R. R. Twenty-four hour preoperative endocrine profiles in women with benign and malignant breast disease. Cancer Res. 37:4655, 1977.

McGuire, W. L., Carbone, P. P., Sears, M. E. and Escher, G. C. Estrogen receptors in human breast cancer: An overview. In: Estrogen receptors in human breast cancer. McGuire, W. L., Carbone, P. P. and Volmer, E. P. (eds.). New York: Raven Press, 1975.

McKeehan, W. L., McKeehan, K. A., Hammond, S. L. and Ham, R. G. Improved medium for clonal growth of human diploid fibroblast at low concentrations of serum protein. In vitro 13:399, 1977.

Megyesi, K., Kahn, C. R., Roth, J., Froesch, E. R., Humbel, R. E., Zapf, J. and Neville, D. M. Insulin and non-suppressible insulin-like activity (NSILAs): Evidence for separate plasma membrane receptor sites. Biochem. Biophys. Res. Comm. 52:307, 1974.

Moses, A. C., Cohen, F. L., Johnsonbaugh, R. and Nissley, S. P. Contribution of human somatomedin activity to the serum growth requirement of human skin fibroblast and chick embryo fibroblast in culture. J. Clin. Endocrinol. Metab. 46:937, 1978.

Moses, A. C., Nissley, S. P., Short, P. A., Rechler, M. M., White, R. M., Knight, A. B. and Higa, O. Z. Increased levels of multiplication-stimulating activity, an insulin-like growth factor, in fetal rat serum. Proc. Natl. Acad. Sci. USA 77:3649, 1980.

Murray, R. M. L., Mozaffarian, G. and Pearson, O. H. Prolactin levels with L-dopa treatment in metastatic breast carcinoma. In: Prolactin and carcinogenesis. Boyn, A. R. and Griffiths, K. (eds.). Alpha Omega Alpha Cardiff, 1972.

Nagarajan, L., Nissley, S. P., Rechler, M. M. and Anderson, W. B. Multiplication-stimulating activity stimulates the multiplication of F9 embryonal carcinoma cells. Endocrinolog 110:1231, 1982.

Nissley, S. P., Passamani, J. and Short, P. Stimulation of DNA synthesis, cell multiplication and ornithine decarboxylase in 3T3 cells by multiplication stimulating activity (MSA). J. Cell Physiol. 89:393, 1076.

Nissley, S. P., Short, P. A., Rechler, M. M. et al. Proliferation of Buffalo rat liver cells in serum-free medium does not depend upon multiplication-stimulating activity (MSA). Cell 11:441, 1977.

Osborne, C. K., Bolan, G., Monaco, M. E. and Lippman, M. E. Hormone responsive human breast cancer in long-term tissue culture: Effect of insulin. Proc. Natl. Acad. Sci. 73:4536, 1976.

Osborne, C. K., Hamilton, B. and Nover, M. Receptor binding and processing of epidermal growth factor (EGF) by human breast cancer cells. Proc. Am. Assoc. Cancer Res. 22:4, 1981.

Osborne, C. K., Hamilton, B., Titus, G. and Livingston, R. B. Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res. 40:2361, 1980.

Osborne, C. K. and Lippman, M. E. Human breast cancer in tissue culture. The effect of hormones. In: Breast cancer. McGuire, W. K. (ed.). New York: Plenum Press, 1978.

Osborne, C. K., Monaco, M. E., Lippman, M. E. and Kahn, C. R. Correlations among insulin binding, degradation and biologic activity in human breast cancer cells on long-term tissue culture. Cancer Res. 38:94, 1978.

Perdue, J. F., Kletzien, R., Miller, K., Pridmore, G. and Lee Wray, V. The isolation and characterization of plasma membranes from cultured cells. II. The chemical composition of membrane isolated from unifected and oncogenic RNA virus-converted parenchyma-like cells. Biochem. Biophys. Acta. 249:435, 1971.

Phillips, L. S. and Vassilopoulou-Sellin, R. Somatomedins. N. Engl. J. Med. 302:371, 1980a.

Phillips, L. S. and Vassilopoulou-Sellin, R. Somatomedins. N. Engl. J. Med. 302:438, 1980b.

Pierson, R. W. (Jr.) and Temin, H. M. The partial purification from calf serum of a fraction with multiplication-stimulating activity for chicken fibroblast in cell culture and non-suppressible insulin-like activity. J. Cell Physiol. 79:319, 1972.

Pledger, W. J., Stile, C. O., Antoniades, H. N. and Scher, C. D. Induction of DNA synthesis in Balb/c-3T3 cells by serum components: Reevaluation of the commitment process. Proc. Natl. Acad. Sci. USA 74:4481, 1977.

Ptashne, K., Hsueh, H. W. and Stockdale, F. E. Partial purification and characterization of mammary stimulating factor, a protein which promotes proliferation of mammary epithelium. Biochemistry 18:3533, 1979.

Rechler, M. M., Fryklund, L., Nissley, S., Hall, K., Podskalny, S. M., Skottner, A. and Moses, A. C. Purified human somatomedin A and rat multiplication stimulating activity mitogens for cultured fibroblast that cross-react with the same group peptide receptors. Eur. J. Biochem. 82:5, 1978.

Rechler, M. M., Nissley, S. P., Podskalny, J. M., Moses, A. C. and Fryklund, L. Identification of a receptor for somatomedin-like polypeptides in human fibroblasts. J. Clin. Endocrinol. Metab. 44:820, 1977.

Rechler, M. M., Podskalny, J. M. and Nissley, S. P. Interaction of multiplication-stimulating activity with chick embryo fibroblasts demonstrates a growth receptor. Nature 259:134, 1976.

Rechler, M. M., Zapf, J., Nissley, S. P., Froesch, E. R., Moses, A. C., Podskalny, J. M., Schilling, E. S. and Humbel, R. E. Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. Endocrinolog. 107: 1451, 1980.

Repert, R. W. Breast carcinoma study: Relation to thyroid disease and diabetes. J. Mich. State Med. Soc. 51:315, 1952.

Rinderknecht, E. and Humbel, R. E. The amino acid sequence of human insulin-like growth factor I and its structural homology to proinsulin. J. Biol. Chem. 253:2769, 1978a.

Rinderknecht, E. and Humbel, R. E. Primary structure of human insulin-like growth factor II.

Rolandi, E., Barreca, T., Masturzo, P., Polleri, A., Indiver, F. and Barabino, A. Plasma prolactin in breast cancer. Lancet ii:845, 1974.

Ross, R., Glomset, J., Kariya, B. and Harker, L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. USA 71:1207, 1974.

Rossow, P. W., Riddle, V. G. H. and Pardee, A. B. Synthesis of labile serum dependent protein in early G-1 controls animal cell growth. Proc. Natl. Acad. Sci. USA 76:4446, 1979.

Rothstein, H., van Wyk, J. J., Hayden, J. H., Gorden, S. R. and Weinsieder, A. Somatomedin C: Restoration in vivo of cycle traverse in G_0/G_1 blocked cells of hypophysectomized animals. Science 208:410, 1980.

Rowe, J. and Kasper, S. Partial purification and characterization of putative growth factors in extracts of human breast cancer. Program of the 62nd Annual Meeting of the Endocrine Society, Washington, D. C., 1980 (Abstract 280).

Rutherford, R. B. and Ross, R. Platelet factors stimulate fibroblast and smooth muscle cells quiescent in plasma serum to proliferate. J. Cell Biol. 69:196, 1976.

Salmon, W. D. and Daughaday, W. H. A hormonally controlled serum factor which stimulates sulphate incorporation by cartilage in vitro. J. Lab. Clin. Med. 49:825, 1957.

Salmon, W. D. and Hoss, B. R. Stimulation of Hela cell growth by a serum fraction with sulphation factor activity. Proc. Soc. Exp. Biol. Med. 136:805, 1971.

Salter, J. M., Meyer, R. and Best, C. H. Effect of insulin and glucagon on tumor growth. Br. Med. J. 2:5, 1958.

Sara, V. R., Hall, K., Rodeck, C. H. and Wetherberg, L. Human embryonic somatomedin. Proc. Natl. Acad. Sci. USA 78:3175, 1981.

Sarfaty, G., Tallis, G. M., Murray, R. M. L., Pitt, P., Gardner, H. and Leppard, P. Hormonal commitants of breast cancer: Urinary androgen metabolites, plasma testosterone and prolactin. In: Endocrinology. James, T. (ed.). International Congress Series 2, 1976.

Savage, C. R. and Cohen, S. Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. J. Biol. Chem. 247:7609, 1972.

Scatchard, G. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51:660, 1949.

Scher, C. D., Shepard, R. C., Antoniades, H. N. and Stiles, C. D. Platelet-derived growth factor and the regulation of the mammalian fibroblast cell cycle. Biochem. Biophys. Acta. 560:217, 1979.

Shafie, S. M. and Hilf, R. Relationship between insulin and estrogen binding to growth response in 7,12-dimethylbenz-(a)-anthracene-induced rat mammary tumors. Cancer Res. 38:759, 1978.

Shiu, R. P. C. Prolactin receptors in human breast cancer cells in long-term tissue culture. Cancer Res. 39:4381, 1979.

Shiu, R. P. C. Prolactin, pituitary hormones and breast cancer. In: Hormones and breast cancer. Pike, M. C., Siiteri, P. K. and Welsch, C. W. (eds.). Cold Spring Harbor Laboratory, 185, 1981.

Sirbasku, D. A. Estrogen induction of growth factors specific for hormone responsive mammary, pituitary and kidney tumor cells. Cell Biol. 75:3786, 1978.

Sirbasku, D. A. Estromedins: Uterine-derived growth factors for estrogen-responsive tumor cells. In: Control mechanisms in animal cells. Jimenez de Asua, L. et al. (eds.). New York: Raven Press, 1980.

Smith, G. L. and Temin, H. M. Purified multiplication-stimulating activity from rat liver cell conditioned medium. Comparison of biological activities with calf serum, insulin, and somatomedin. J. Cell Physiol. 84:181, 1974.

Stampfer, H., Hallowers, R. C. and Hackett, A. J. Growth of normal human mammary cells in culture. In vitro 16:415, 1980.

Starkey, R. and Orth, D. N. Radioimmunoassay of human epidermal growth factor. J. Clin. Endocrinol. Metab. 45: 1144, 1977.

Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., van Wyk, J. J. and Pledger, W. J. Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc. Natl. Acad. Sci. USA 76:1279, 1979.

Stoker, M. G. P., Pigott, D. and Taylor-Papadimitrious, J. Response to epidermal growth factors of cultured human mammary epithelial cells from benign tumors. Nature 264: 764, 1976.

Taylor, J. M., Cohen, S. and Mitchell, W. M. Epidermal growth factor: High and low molecular weight forms. Proc. Natl. Acad. Sci. 67:164, 1970.

Taylor-Papadimitrious, J., Shearer, M. and Stoker, M. G. P. Growth requirements of human mammary epithelial cells in culture. Int. J. Cancer 20:903, 1977.

Temin, H. M., Pierson, R. W. and Dulak, N. C. In: Growth, nutrition and metabolism of cells in culture. Rothblat, G. H. and Cristofalo, V. J. (eds). New York: Academic Press, 1972.

Tonelli, Q. J. and Sorof, S. Epidermal growth factor requirement for development of cultured mammary gland. Nature 285:250, 1980.

Turkington, R. W. Stimulation of mammary carcinoma cell proliferation by epithelial growth factor in vitro. Cancer Res. 29:1457, 1969.

Turkington, R. W. Multiple hormonal interactions: The mammary gland. In: Biochemical actions of hormones. Litwack, G. (ed.). New York: Academic Press, 1972.

Weidman, E. R. and Bala, R. M. Direct mitogenic effects of human somatomedin on human embryonic lung fibroblasts. Biochem. Biophys. Res. Comm. 92:577, 1980.

Weinstein, R., Stemerman, M. B. and Meriag, T. Hormonal requirements for growth of arterial smooth muscle cells in vitro: An endocrine approach to artherosclerosis. Science 212:818, 1981.

Welsch, C. W. and Nagasawa, H. Prolactin and murine mammary tumorigenesis: A review. Cancer Res. 37:951, 1977.

Wessel, N. K. Substrate and nutrient effects upon epidermal basal cell orientation and proliferation. Proc. Natl. Acad. Sci. USA 52:252, 1964.

Westermark, B. and Wasteson, A. A platelet factor stimulating human normal glial cells. Exp. Cell Res. 98:170, 1976.

Westley, B. and Rochefort, H. A secreted glycoprotein induced by estrogen in human breast cancer cell lines. Cell 20: 353, 1980.

Wharton, W., Leof, E. B., Gillespie, G. Y., van Wyk, J. J. and Pledger, W. J. Synergistic action of multiple growth factors. In: Plasma and cellular modulatory proteins. Bing, D. (ed.). Boston. Ctr. Blood Res., 1, 1981.

van Wyk, J. J., Hall, K., van den Brande, J. L. and Weaver, R. P. Further purification and characterization of sulphation factor and thymidine factor from acromegalic plasma. J. Clin. Endocrinol. Metab. 32:389, 1971.

van Wyk, J. J., Svoboda, H. E. and Underwood, L. E. Evidence from radioligand assays that somatomedin-C and insulin-like growth factor I are similar to each other and different from other somatomedins. J. Clin. Endocrinol. Metab. 50:206, 1980.

van Wyk, J. J., Underwood, L. E., Baseman, J. B., Hintz, R. L., Clemmons, D. R. and Marshall, R. N. Explorations of the insulin-like and growth-promoting properties of somatomedin by membrane receptor assays. Adv. Metab. Disord. 8:127, 1975.

van Wyk, J. J. and Underwood, L. E. In: Biochemical actions of hormones. Litwak, G. (ed.). New York: Academic Press, 5:101, 1978.

van Wyk, J. J., Underwood, L. E., D'Ercole, A. J., Clemmons, D. R., Pledger, W. J., Wharton, W. R. and Leof, E. B. In: Biology of normal human growth. Ritzen, E. M. (ed.). New York: Raven Press, 1981.

Yamada, K. M. and Olden, K. Fibronectins-adhesive glycoproteins of cell surface and blood. Nature 275:179, 1978.

Zapf, J., Rinderknecht, E., Humbel, R. E. and Froesch, E. R. Non-suppressible insulin-like activity (NSILA) for human serum: Recent accomplishments and their physiological implications. Metab. Clin. Exp. 27:1803, 1978.