THE INFLUENCE OF HORMONAL FACTORS IN HUMAN BREAST CANCER

A Thesis

Presented to the Faculty of Graduate Studies

University of Manitoba

In partial fulfillment

of the

requirements for the degree of

Doctor of Philosophy

by

Clement K. H. Leung, B.Sc., M.Sc.

Department of Physiology

Faculty of Medicine

February 1982

THE INFLUENCE OF HORMONAL FACTORS IN HUMAN BREAST CANCER

BY

CLEMENT K.H. LEUNG

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

CONTENTS

ABSTRACT

Breast cancer is known to be influenced by hormones. Although numerous studies using experimental animal models have been reported, relatively few studies have been done with breast cancer cells from humans. It has become increasingly evident that murine mammary tumor may not be a suitable model for its counterpart in humans. We have therefore investigated some of the hormonal influences in human breast cancer.

Recently epidermal growth factor (EGF) was found in human milk and was suggested to influence the development of the mammary epithelium. We therefore examined the receptors and mitogenic activity of EGF in nine human mammary cell lines. EGF binding was present in all monolayer forming cell lines. Both high affinity (10^{-10}M) and low affinity (10^{-9}M) sites were detected. In contrast, no EGF binding can be detected in floating cell lines. Only T-47D cells were stimulated by EGF (0.1 ng/ml) and there was no apparent correlation between EGF binding and its mitogenic activity.

The behavior of breast cancer cells are known to be influenced by their substrates. Thus, the growth characteristics of breast cancer cells cultured in collagen and on plastic were compared. In collagen, tumor cells displayed a spherical shape and formed multilayered aggregates and became

more serum-dependent for growth. In contrast, these cells grew as monolayers on plastic. The doubling time was longer for cells cultured in collagen than on plastic. Both human breast cancer cell lines MCF-7 and T-47D proliferate rapidly in a serum-free medium containing insulin, epidermal growth factor, estrogen and transferrin, suggesting that these cells can respond to the above hormones. Since several mammotrophic hormones such as growth hormone and prolactin, as well as steroid hormones, did not stimulate the growth of T-47D cells in vitro, we then studied the growth of these cells in athymic nude mice. Initially, four groups of mice were used: T, subcutaneous injections of T-47D cells only; TE, injected with T-47D cells and estrogen; TG, injected with T-47D cells and rat pituitary tumor GH_3 cells; and TEG, injected with T-47D cells, estrogen and GH_3 cells. The T-47D cells did not proliferate in T and TG groups despite the presence of high cirulating levels of prolactin and growth hormone produced by the GH_3 tumors in the TG group. This suggested that PRL and GH were not sufficient to stimulate the growth of human breast cancer cells. T-47D cells grew moderately in TE group, but proliferated rapidly in TEG group. The T-47D tumors of the TEG group were eight times larger than those in the TE group after 42 days of growth. These results demonstrated that the simultaneous presence of estrogen and pituitary factors are required for maximal growth of T-47D tumors in nude mice. The

results also suggested that the pituitary cells might produce a growth factor which stimulated the growth *in vivo* of human breast cancer cells. The next step was to identify this pituitary factor(s). The possibility that estrogen and classical pituitary hormones (e.g. prolactin and growth hormone) can regulate the growth of human breast cancer *in vivo* was also examined. Three other pituitary tumor cell lines were used in estrogenized nude mice. The GH_1 cells (secrete growth hormone predominantly), AtT-20 cells (secrete adrenocorticotropic hormone) and 235-1 cells (secrete only prolactin) were used as exogenous sources of hormones. T-47D tumors grew rapidly in estrogenized animals co-transplanted with GH_1 cells. In contrast, the T-47D tumors in mice transplanted with AtT-20 or 235-1 cells grew at the same rate as the control. Highly purified human growth hormone (hGH) and ovine prolactin were infused at a constant rate of 1.25 ug/hr into the athymic nude mice by Alzet osmotic mini pumps. The T-47D tumors were not stimulated by these hormones at this concentration. When the infusion rate of hGH was increased to 15 ug/hr, however, the growth of the T-47D tumors in two surviving animals was stimulated. This result suggests that growth hormone and its intermediates may promote the growth of human breast cancer cells.

Preliminary results indicate that conditioned medium of GH_3 cells and GH_3 tumor extract can both stimulate the

growth of T-47D cells *in vitro*. Secreted proteins from normal rat pituitaries and GH_3 pituitary tumors were analyzed by SDS polyacrylamide gel electrophoresis. Protein bands (14,000 to 60,000) were present in abundance. Gel exclusion chromatography of GH_3 tumor extract using Sephadex G-100 also showed a peak of mitogenic activity around the 40,000-50,000 region. The exact nature of this growth factor(s) remains to be elucidated.

Hence, besides growth hormone, other proteins secreted by pituitary cells may be mitogenic in the human breast cancer cells. The identification of the pituitary factor(s) which are involved in human breast cancer is extremely complex and will undoubtedly offer some exciting challenges for future studies.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Robert P. C. Shiu, for his direction and support throughout this investigation. Being Bob's first student is a privilege as well as a challenge to me.

Thanks are also due to Doctors Henry Friesen, Robert Matusik and F. William Orr for their helpful advice and constructive criticism.

I also want to thank Dr. Joanne Emerman (University of British Columbia) for acting as my external thesis examiner.

The collaboration with Doctors Jean Paterson, Yasuo Imai and John Rowe on some of the studies is acknowledged. They have shared a lot of their expertise and wisdom with me.

I am especially indebted to Marilyn Lavallee for her enthusiasm and invaluable help in preparing this thesis.

Finally, I am grateful to the University of Manitoba for a graduate fellowhip which was awarded to me during the tenure of this research.

LIST OF ABBREVIATIONS

Units of Measurement

ul	microliter
ml	milliliter
l	liter
ng	nanogram
ug	microgram
mg	milligram
g	gram
nm	nanometer
mm	millimeter
cm^2	$centimeter^2$
M	Molar
nM	nanomole
iu	international unit
uCi	microcurie
min	minute
v/v	volume per volume
w/v	weight per volume
fmol	femtomole

Others

BSA	bovine serum albumin
FBS	fetal bovine serum
DME	Dulbecco's modified Eagle's medium
HBSS	Hank's balanced salt solution
I^{125}	^{125}-iodine
CPM	counts per minute
^oC	degree centigrade
MW	molecular weight

PBS	phosphate buffer saline
RIA	radioimmunoassay
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
EDTA	ethylenediamine tetraacetic acid
DMSO	dimethyl sulfoxide
TCA	trichloroacetic acid
OCS	organic counting scintillator solution
SC	subcutaneous
DMBA	7,12-dimethylbenzanthracene
TEMED	N,N,N',N'-tetramethylenediamide

Prefix to Hormones and Growth Factors

b	bovine
h	human
m	mouse
r	rat
o	ovine

Hormones and Growth Factors

GH	growth hormone
PRL	prolactin
E_2	estrogen (estradiol)
EGF	epidermal growth factor
FGF	fibroblast growth factor
IGF	insulin-like growth factor
NGF	nerve growth factor
PDGF	platelet-derived growth factor
SM	somatomedins
NSILA	non-suppressible insulin-like activity
MSA	multiplication stimulating activity
OGF	ovarian growth factor

LIST OF FIGURES

LIST OF TABLES

SECTION 1: General Introduction

The normal development and expression of differentiated functions of the mammary glands are under the influence of a number of hormones. Some of the major hormones which are involved in mammogenesis include a number of pituitary-derived polypeptide hormones such as growth hormone and prolactin and other hormones such as thyroxine, cortisol, insulin and other steroid hormones. Other growth factors (eg. epidermal growth factor) and many yet unidentified growth factors are also thought to play a role in this regard. These various hormonal factors, by themselves or in combination with each other, are essential for the regulation of growth and function of the mammary epithelial cells.

In this review of literature, the etiology and incidence of human breast cancer will be briefly described. This is followed by an overview of the major hormones and growth factors which are known to influence human breast cancer. Finally, some of the advantages and disadvantages of using *in vitro* cell culture system (especially the roles of collagen and

extracellular matrix) and *in vivo* models such as the athymic nude mice for studying the hormonal requirements of human breast cancer will be discussed.

Etiology and Incidence of Breast Cancer

The most common site of malignancy in women is the breast. Although the disease has been under investigation for many years, the etiology and pathogenesis of breast cancer is still largely unknown.

Many factors are likely to be involved in the etiology of breast cancer. For instance, genetic predisposition, oncogenic factors (viruses, diet, obesity), environmental conditions (estrogen intake, smoking, chemical carcinogens in food, water and air), and endogenous hormone imbalance (estrogens, progesterone, androgens, prolactin and other yet unidentified factors) may all play a role in the etiology and incidence of breast cancer. Many of these risk factors are interdependent. In North America, breast cancer is the most common cause of death from cancer for all women. The breast cancer rate is about 6.6 times higher in the United States and Canada than in Japan. One of every thirteen women in North America will eventually develop the disease. A significantly higher risk of developing breast cancer has been found among women with an earlier age at menarche. Epidemiological studies show that

breast cancer risk decreased with increased parity while the risk increased in single women. The protective effect of parity was suggested to be due to the early age at first full-term pregnancy. The number of subsequent births had no influence on the risk of developing breast cancer (MacMahon et al., 1970). Thus, early menarche, late age at first full-term pregnancy and late age at menopause all appear to increase the risk of breast cancer. In light of the high incidence of breast cancer, it is imperative to search for a better understanding of the disease. For futher readings, the epidemiology and etiology of human breast cancer was reviewed by MacMahon et al. (1973).

Current Approaches to Breast Cancer Treatment

Until recently, the traditional treatment for the patient with primary breast cancer has been radical mastectomy with or without postoperative radiation therapy. However, less than 50% of the patients subjected to this treatment survived ten years. This aggressive local therapy failed to control the distant micrometastases.

To compensate for the limited effectiveness of surgical treatment, various forms of systemic adjuvant therapy were developed. For instance, chemotherapy, immunotherapy and endocrine manipulation are given after local treatment to

improve survival. Clinical trials of cytotoxic chemotherapy have been initiated because it was rationalized that micrometastases have a higher growth fraction and thus may be more susceptible to cytotoxic drugs such as cyclophosphamide, methotrexate, fluorauracil, adriamycin and prednisolone.

The actions of estrogen have been shown to be mediated by estrogen receptors (Jensen et al., 1968, Jensen and DeSombre, 1972). Estrogen receptors can be detected in approximately 60% of human breast cancer specimens. Tumors lacking estrogen receptors (ER-) rarely regress with endocrine therapy. In contrast, about two-thirds of those containing estrogen receptors (ER+) will respond to similar treatment. It was reported that ER- tumors tend to have a higher growth fraction and more cytologic atypia (Meyer et al., 1978). Thus, estrogen receptor together with the status of axillary lymph nodes can be used to give an indication of the prognosis of the patients. The subject of estrogen receptors in human breast cancer has been thoroughly discussed by McGuire et al. (1975).

In recent years, the measurement of progesterone receptors in breast tumors has also been used to predict hormone dependency of breast cancer (Horwitz and McGuire, 1978). The discovery of the presence of estrogen receptors in human breast cancer specimens also lead to the potential therapeutic usefulness of antiestrogens such as tamoxifen. The results from clinical studies with antiestrogens are still inconclusive.

Because some human breast cancers depend on steroid sex hormones to proliferate, endocrine ablation such as hypophysectomy and adrenalectomy have become a common therapy for advanced breast cancer. In addition, combination chemotherapy has been used to treat patients with advanced breast cancer. However, the results from these treatments are far from adequate. Further research to acquire a better understanding of breast cancer biology and innovative treatments are urgently required in order to gain control of the disease.

Hormonal Influences in Human Breast Cancer

The current interest in the hormonal influence on breast cancer derives from increased understanding of the mechanism of action of hormones. The concept that hormones are necessary for growth and maintenance of function of human breast cancer was first demonstrated by the remarkable clinical experiment of Beatson in 1896. He achieved regression of metastatic breast cancer by oophorectomy. That hormones might be involved in the development of cancer was suggested by Lacassagne (1936) who was able to induce mammary carcinoma by estrone in mice with low natural incidence of cancer.

There are several difficulties in assessing the role of a single hormone in neoplasia. First, the tissue undergoing

neoplastic transformation may be responsive to many hormones. Second, the interactions of the hormones (synergism or antagonism) depend on the target cell examined, the level of hormone studied, or the presence of other as yet undescribed factors. Since neoplastic transformation may be taking place over long periods of time, it becomes extremely difficult to identify those critical events that occur in an internal milieu that is under constant change. Thus, although breast cancer is clinically more apparent in post-menopausal women, the tumor may be present and remain undetected for many years. Despite these difficulties, many hormones have now been shown to influence either growth or differentiated functions of human breast cancer cells. These hormones include both steroid and polypeptide hormones and recently other growth factors are also added to the list.

Estrogen

Among the sex steroid hormones, estrone and estradiol have been related to an increased risk of breast cancer, whereas ertriol, 2-hydroxyestrone, and progesterone are thought to be protective against the disease.

Many breast cancers in both humans and experimental animals are estrogen dependent. Estrogen receptors are demonstrated in many human breast cancer cell lines (Engel and Young, 1978). Various specific products of breast cancer cells have been shown to be regulated by estrogen. For instance, thymidine

kinase (Bronzert et al., 1981), plasminogen activator (Butler et al., 1979), progesterone receptor (Horwitz and McGuire, 1978), a secreted 46,000 daltons protein of unknown function (Westley and Rochefort, 1980) and a 26,000 daltons cytoplasmic protein (Edwards et al., 1980) are all stimulated by estrogen.

Estrogens were thought to exert their effects by first binding to specific cytoplasmic receptors. The presence of these specific hormone receptors in tumors suggested their hormone dependence. Thus, hormonal control of tumor cell depends not only on plasma concentrations of hormones, but also on the presence of hormone receptors. The measurement of estrogen receptors in breast cancer tissue has proven to be of predictive value for prognosis and choice of treatment. Synthetic estrogens and antiestrogens have been commonly used in the treatment of advanced breast cancer. The nature of mammary tumor response to endocrine manipulation may depend on the type of tumor (receptor positive or negative) and the number of receptors involved.

Progesterone

Progesterone has both positive and negative effects on mammary development. Progesterone stimulates lobuloalveolar proliferation in prepared tissue and acts as the main inhibitor of lactation during pregnancy. Progesterone also inhibits the action of estrogen by suppressing its receptors. Receptors for progesterone have been detected in several human breast cancer

cell lines including the MCF-7 and ZR-75-1 cells (Lippman et al., 1976; Engel et al., 1978). These receptors have been shown to be regulated by estrogens (Horwitz and McGuire, 1978). However, no direct effect of progesterone on human breast cancer cells has been reported yet.

Prolactin

The role of prolactin in experimental mammary tumorigenesis is fairly well established. DMBA-induced rat mammary tumor was shown to be highly dependent on prolactin (Pearson et al., 1969). Furth (1972) also showed that prolactin can act as a cocarcinogen in the development of experimental mammary tumors in mice and rats. In contrast, there is no convincing or direct evidence that prolactin plays a role in human breast cancer.

Holdaway and Friesen (1977) examined the binding of prolactin, insulin, growth hormone and estrogen in a number of human mammary tumors. Approximately 20% of the tumor specimens studied exhibited significant binding of prolactin. None of the nonmalignant tissues bound either prolactin or growth hormone. Prolactin receptors have also been identified in human breast cancer cells in long term tissue culture (Shiu, 1979).

At least three molecular weight forms of prolactin are recognized. All three can be detected by conventional radioimmunoassays. Prolactin secretion is influenced by a

number of physiological factors. Sassin et al. (1973) demonstrated a circadian variation in prolactin secretion. It is also well known that serum prolactin levels can be increased due to stress and during estrogen treatment. However, it is generally agreed upon that oral contraceptives and estrogen replacement therapy do not increase the risk of breast cancer. Pituitary prolactin secretion is presumably regulated by a prolactin inhibiting factor (PIF) from the hypothalamus.

Kwa et al. (1974) have shown that the serum prolactin levels were elevated in a number of family members but not in patients with breast cancer. Subsequently, Henderson et al. (1975) showed that there is a significantly higher concentration of serum prolactin in the daughters of patients with breast cancer than in control subjects. An abnormal peak of plasma prolactin in women with a family history of breast cancer also was reported (Kwa and Wang, 1977). L-dopa and CB-154, two substances that suppress prolactin secretion by the pituitary, have been used to treat patients with breast cancer (Frantz et al., 1973). The results from these studies were not conclusive.

It is well known that Japanese women have a much lower risk of breast cancer than Caucasian women. However, the prolactin levels were found to be similar in both races (Kumaoka et al., 1976). Thus it appears that the low incidence of breast cancer in Japan compared to that in Western countries is not due to

basic endocinologic differences. The lack of correlation between prolactin and breast cancer risk is further strengthened by the fact that despite the high prolactin levels of pregnancy and lactation, breast cancer risk is actually decreased (MacMahon et al., 1973). One explanation may be that the role of prolactin is to enhance the mitogenic effect of estrogen rather than to be a primary mitogen itself.

Growth Hormone

Shiu (1979) reported the presence of prolactin receptors in several human breast cancer cell lines. It was found that human growth hormone can compete with human prolactin for these receptor sites, suggesting that human growth hormone may influence these cells. These results may explain why treatment using ergot derivatives such as CB-154 to lower prolactin may not be adequate to control the growth of the mammary tumors. It may be essential to lower or remove growth hormone as well. In addition, Leung and Shiu (1981) have demonstrated that the growth hormone level *in vivo* has a good correlation with human breast tumor size. The role of growth hormone in human breast cancer is further supported by the recent findings of Emerman et al. (1981). These workers measure growth hormone like substances in the serum samples from breast cancer patients using RIA and a newly developed Nb_2 rat lymphoma cell bioassay (Tanaka et al., 1980). They observed that the level of immunoreactive growth hormone in 50% of the 34 patients was

consistently higher than in normal females. Whether growth hormone acts directly on the breast cancer cells or indirectly via somatomedins is unknown at present.

Thyroid Hormones

The relationship between thyroid hormone and breast cancer is largely unknown. Both hypothyroidism (Bogardus and Finley, 1961) and administration of thyroid hormones (Kapdi and Wolfe, 1976) have been claimed to enhance risk of breast cancer development. Thyroid hormone receptors are present in MCF-7 cells and these cells respond to physiological concentrations of thyroid hormones (Burke and McGuire, 1978). The dependency of MCF-7 cells on thyroid hormone when cultured in defined medium has been demonstrated (Barnes and Sato, 1979). In addition, Allegra and Lippman (1978) have shown that physiologic concentrations of triiodothyronine are essential for culturing the ZR-75-1 cells in defined medium. The exact function of thyroid hormone in human breast cancer is still unknown.

Insulin

Insulin is required for the normal differentiation of the mammary gland as well as for lactogenesis in rodents. Because patients with advanced breast cancer often have a delayed but prolonged increase in the secretion of insulin, it has been suggested that insulin may play a role in breast cancer. Physiologic concentrations of insulin stimulate the growth of

human mammary cancer cells (Osborne et al., 1976). Further, insulin increased the incorporation of ^{3}H-thymidine into DNA of mammary carcinoma slices from rodents and humans (Welsch et al., 1976). High-affinity, specific receptors for insulin have been found in human breast cancer cell lines, and binding and biological effects were well correlated (Osborne et al., 1978).

Vitamin D

The mechanism of action of vitamin D is very similar to that of the steroid hormones. Recently, vitamin D receptors in human breast tumor samples and the MCF-7 cells have been described (Eisman et al., 1980). These receptors resemble those in intestinal mucosa in both physical characteristics and binding properties. It is well known that human breast cancer has a high tendency to metastasize to bone and to induce hypercalcemia. Calcitonin receptors have recently been detected in T-47D cells (Findlay et al., 1980). Thus, vitamin D and other hormones (such as calcitonin and parathyroid hormone) which are involved in regulation of calcium may influence the behavior of breast cancer cells.

Retinoids

Vitamin A derivatives are required for normal epithelial differentiation. Administration of vitamin A derivatives to rats can significantly decrease the appearance of DMBA-induced breast tumors (Moon et al., 1976). Specific binding sites for vitamin A derivatives have been shown in some human breast

tumor cytosols (Ong et al., 1975). The effects of retinoids on human breast cancer cell lines in tissue culture have also been studied (Lacroix and Lippman, 1980). In general, vitamin A derivatives inhibit the growth of the breast cancer cells. These effects can be reversed by removal of retinoids. Bilateral ovariectomy also was found to enhance the inhibitory effect of N-(4-hydroxyphenyl) retinamide on breast tumors in rats (McCormick et al., 1982).

Growth Factors

The importance of pituitary-secreted growth hormone and hormones secreted by the thyroid, gonads and pancreas in regulation of growth has long been recognized. In addition, other growth-promoting substances have been identified. Growth factors may be defined as any substance which acts to increase cell size and rate of proliferation as well as matrix production and to prolong cell survival. Undoubtedly, some of these growth factors may take part in the regulation of normal and cancerous cell growth. Some of the better characterized growth factors are briefly reviewed below.

Somatomedins (SM)

Somatomedins are a family of polypeptides which exhibit growth-promoting activity both *in vivo* and *in vitro*. Somatomedins were discovered incidentally during the search for

an improved bioassay for growth hormone (Salmon and Daughaday, 1957). It was found that serum from normal rats stimulated sulfate incorporation into proteoglycans in the cartilage of hypophysectomized rats, whereas that from hypophysectomized rats failed to do so even when growth hormone was added to the incubation mixture. Subsequently, a series of low molecular weight peptides with growth-promoting activity were identified. There are three main types of somatomedins: SM-A (PI 7.1-7.5, M.W. 7600), SM-B (PI 8.4-9.2, M.W. 7600) and SM-C (PI 5.9-6.4, M.W. 5000). Somatomedins are present in cartilage, muscle, liver, kidney, brain, pancreas, heart and pituitary gland, as well as in body fluids including lymph and amniotic fluid. The liver and the kidneys are the two major sites of control of serum somatomedins, the former being the centre of synthesis and the latter being the main site of catabolism.

Nonsuppressible insulin-like activity (NSILA) is a family of two or more peptides that has the the property of not being suppressed by insulin antibodies and that possesses approximately 90% of the total insulin-like activity of serum (Froesch et al, 1963). The two insulin-like growth factors (IGF-I and IGF-II) which are structurally related to insulin are a part of the NSILA. Somatomedin-like polypeptides have been reported to be produced by mammary tumors from Balb/c mice (Knauer et al., 1980).

Muliplication-Stimulating Activity (MSA)

Muliplication-stimulating activity was isolated from calf serum, which is the most commonly used biological fluid for promoting cell growth in culture (Pierson and Temin, 1972). Muliplication-stimulating activity is slightly different from NSILA and SMS, and has a molecular weight of 4,000 to 5,000 daltons. It is believed that MSA also is regulated by growth hormone (McKeehan et al, 1978). Recently, molecules similar to MSA but with higher molecular weights (around 10,000 daltons) have been isolated from Buffalo rat liver tumor cells (Dulak and Temin, 1973).

Nerve Growth Factor (NGF)

Nerve growth factor is a protein molecule so-named because of its potent growth-promoting effect on embryonic sensory nerve cells as well as on fully differentiated sympathetic nerve cells (Levi-Montalcini, 1965). Nerve growth factor has been isolated from the mouse submaxillary gland (Cohen, 1960). Nerve growth factor elicits a variety of pleiotypic responses in cultured neurons including stimulation of RNA synthesis, protein synthesis, lipid synthesis and enhanced transport of glucose and nucleotides (Levi-Montalcini, 1976). The insulin-like properties of NGF can be explained by a 21% homology in the amino acid sequences of NGF and pro-insulin, and similarities in the secondary and tertiary structure between NGF and the insulin monomer.

Epidermal Growth Factor (EGF)

Epidermal growth factor is a single-chain polypeptide composed of 53 amino acids (Cohen, 1962; Taylor et al, 1970) and has a molecular weight of 6,045 daltons. Epidermal growth factor was first isolated from submaxillary glands of adult male mice. Subsequently, a similar molecule with a lower molecular weight (5,300 to 5,500 daltons) that competes with m-EGF cell membrane receptors and antibodies has been discovered in human urine (Cohen and Carpenter, 1975). It was also found that human B-urogastrone and m-EGF share common receptors and have a homology of about 80% in their amino acid sequences (Cohen and Savage, 1974). The ability of m-EGF to inhibit gastric secretion and urogastrone to prematurely open the eyelids of newborn mice has confirmed their high degree of biological similarity (Gregory, 1975). Cell surface receptors for EGF have been detected in a number of cells and crude membrane preparations (Carpenter and Cohen, 1979). Epidermal growth factor can stimulate cell proliferation in a variety of cell types including fibroblasts, glial cells, corneal endothelial cells, rabbit chondrocytes, bovine granulosa cells and mammary epithelial cells. Receptors for EGF and mitogenic activity of EGF in human breast cancer cells will be discussed in detail in Section 4.

Fibroblast Growth Factor (FGF)

Holley and Kiernan (1968) reported the presence of mitogens in partially purified preparations of thyroid stimulating hormone. Gospodarowicz (1974) subsequently purified the mitogen from bovine pituitary glands and called it fibroblast growth factor because it stimulated DNA synthesis in serum-starved cultures of Balb/c 3T3 cells. Pituitary FGF has an isoelectric point of 9.5 and a molecular weight of 13,000 daltons (Gospodarowicz, 1975). Fibroblast growth factor was later purified from bovine brain (Gospodarowicz et al., 1978). It has been shown that FGF can stimulate a variety of mesodermal cells including corneal and vascular endothelial cells, granulosa cells, chondrocytes and human glial cells, but endodermal and ectodermal derived cells are not affected. Recently, it was found that vascular endothelial cells of bovine and human origin will not proliferate in plasma unless FGF is added when grown on plastic dishes. In contrast, when endothelial cells are maintained on extracellular matrix, these cells proliferate equally well in plasma or serum and no longer require FGF. Thus, FGF may be required by certain cells for the production of extracellular matrix and in turn render the cells responsive to mitogenic factors in plasma. Human breast cancer cells also grow better on extracellular matrix and Type 4 collagen (Wicha et al., 1980). It is possible growth of the breast cancer cells is indirectly influenced by FGF, by its

actions on extracellular matrix and vascular endothelium which is vital for supplying oxygen and nutrition to the tumor.

Platelet Derived Growth Factor (PDGF)

In 1971, Balk showed that chicken plasma was less effective than serum in stimulating cell division. He postulated that growth promoting activity was either derived from inactive plasma precursors or from platelets during the clotting process. Subsequently, the mitogenic activity lacking in plasma was shown to be restored by the addition of a platelet extract (Ross et al., 1974). The platelet derived growth factor was later purified by Antoniades et al. (1979) and Heldin et al. (1979) independently. The PDGF has an isoelectric point of 9.8 and a molecular weight of 25,000-32,000 daltons. The PDGF is mitogenic for a variety of cells *in vitro* such as 3T3 cells (Kohler and Lipton, 1974), human glial cells and fibroblasts. It was suggested that PDGF may promote tissue repair and wound healing. However, the effect of PDGF on epithelial cells is not well understood.

Pituitary Derived Growth Factors

Besides the classical hormones and FGF, several pituitary-derived growth factors have been identified. For instance, an ovarian growth factor (OGF) (M.W. 10,000-13,000) which can stimulate proliferation of ovarian cells has been isolated from the bovine pituitary (Gospodarowicz et al., 1974). A glial growth factor (M.W. 30,000) was also isolated

from bovine pituitary and was shown to stimulate the growth of rat Schwann cells (Brockes et al., 1980). In addition, human pituitary extract was shown to contain a factor (M.W. 12,000-24,000) which is mitogenic to Balb/c 3T3 cells (Antoniades and Scher, 1978).

Mammary Growth Factors

Mitogens for mammary epithelial cells have been identified from a number of sources including platelets, serum, kidney, uterus, brain and pituitary. A pituitary mammary growth factor (PMGF) was found to be present in certain fractions of bovine pituitary from a CM-cellulose column (Rudland et al., 1979). Ovarian growth factor was also present in the same fraction. Growth factors from the kidney, uterus, platelet and brain extracts for a rat mammary tumor cell line has been reported by Sirbasku and Benson (1979). Another pituitary-derived growth factor for mammary tumor cells was isolated by Kano-Sueoka, who has subsequently identified the mitogen to be phosphoethanolamine (Kano-Sueoka et al., 1979). A unique serum mitogen for mammary epithelial cells was recently identified and partially purified (Ptashne et al., 1979). At high concentrations, this factor exhibits biological cross-reactivity with multiplication stimulating activity.

The above review by no means represents a complete list of all the growth factors. However, it does reflect the important

roles of hormones and growth factors in human breast cancer. Future identification of other novel mammary growth factors and elucidation of the hormonal actions on breast cancer will undoubtedly provide better understanding of the disease.

In Vitro Studies of Human Breast Cancer

Clinical responses in breast cancer patients to ablative and additive hormone therapies suggest that several hormones are important growth regulators of breast cancer. However, clinical and animal studies do not provide a complete answer to the mechanisms whereby hormones influence the growth and metabolism of human breast cancer. This deficiency may be due to the difficulty in interpreting the results from *in vivo* studies, since secondary effects of the hormone on the activities or concentrations of other factors, or hormonal effects on the adjacent stroma or immune system rather than the malignant epithelial component, cannot be excluded.

In order to circumvent these problems, many investigators have turned to *in vitro* systems such as short-term organ culture of breast tumors and long-term tissue culture of established human breast cancer cell lines. The mechanisms of hormone action on breast cancer cells can be studied in a defined medium and controlled environment using these tissue culture systems.

Organ Culture of Human Breast Cancer

The technique of organ culture developed by Fell (1929) has become a very useful biological tool. Human breast carcinoma explants were first maintained in organ culture in 1937 (Cameron and Chambers, 1937). The potential advantage of the organ culture method is that the architecture of the tumor cells and their surrounding stroma is maintained. Studies with human breast tumor biopsies maintained in organ culture suggested that some tumors responded to prolactin treatment with an increase in pentose phosphate pathway enzyme activity (Salih et al., 1972), in the production of alpha-lactalbumin (Kleinberg, 1975) and in the uptake of ^{3}H-thymidine (Welsch et al., 1976).

Long Term Tissue Culture of Human Breast Cancer Cells

One of the disadvantages of long term tissue culture of human breast cancer cells is that these cells are devoid of supporting stroma present *in vivo* or in organ culture, a feature that may be an important prerequisite for hormone responsiveness. Furthermore, long term culture might select out strains or cell types present in only small numbers in the original tumor.

On the other hand, this technique offers many advantages over *in vivo* or organ culture systems for the study of hormone action in human breast cancer. First, the continuous culture of established cell lines allows for sequential experiments on

the same cells. Second, the ambiguity of whether an observed hormone effect is on tumor cells or adjacent stroma can be eliminated. Third, the cell culture conditions can be rigorously controlled. Nearly all the cells are exposed to the defined medium, whereas in organ culture, the central portion of a tissue may be deprived of certain essential factors. Fourth, mutant hormone-independent cell lines can be developed to facilitate genetic studies. Finally, the in vivo situation can be re-enacted stepwise by adding back in vitro individual stromal components or their diffusible products. Thus, long term tissue culture of human breast cancer cells is an extremely useful tool for furthering our understanding of the disease.

The first continuous culture of a human breast cancer cell line (BT-20) was described by Lasfargues and Ozzello in 1958. Subsequently, many other human breast cancer cell lines were established (Engel and Young, 1978). It is generally very difficult to establish a cell line from primary or metastatic solid tumors. The success rate of isolating cells from pleural effusion is much higher (Cailleau et al, 1974).

There have been several attempts to culture normal human mammary epithelial cells from lacteal secretions (Taylor-Papadimitriou et al., 1977). However, the growth rates of these cells are slow and they can usually be subcultured one to three times. The only report of an epithelial cell line

developed from non-malignant breast tissue is the HBL-100 cell line. These cells were derived from breast fluids of an asymptomatic woman; the cells may not be normal since they have an abnormal karyotype and will grow in soft agar (Polanowski et al., 1976).

Human breast cancer cell lines have been used as models for studying mechanisms of hormone action in breast tumors. Many human breast cancer cell lines were shown to possess specific high affinity receptors for estrogen, progesterone, androgens, glucortocoids, vitamin D, thyroxine, prolactin and growth hormone (Lippman et al., 1976; Engel and Young, 1978; Shiu, 1979).

One of the major obstacles in tissue culture is the presence of serum in the medium. Recently, attempts have been made to develop serum free media for various lines (Barnes and Sato, 1979; Allegra and Lippman, 1978). The factors required for ZR-75-1 cell line include insulin, estradiol, transferrin, dexamethasone and triiodothyronine. On the other hand the MCF-7 cells required insulin, transferrin, EGF, prostaglandin F2-alpha and fibronectin for serum-free growth. Thus, different cell lines may have different growth requirements. An excellent review on serum-free culture was reported by Barnes and Sato (1980).

Although human breast cancer cells maintained as monolayers on plastic dishes were commonly used in in vitro studies, it is suggested that the natural basement membrane or extracellular matrix may influence the behavior of the human breast cancer cells as well as their hormonal responsiveness.

Extracellular Matrix and Collagen

The extracellular matrix (ECM) produced by cells is the natural substrate upon which they migrate, proliferate and differentiate in vitro. Extracellular matrix is mainly composed of collagen, glycosaminoglycans and glycoproteins. Three types of interstitial collagen (I, II, III) and two types of basement membrane collagens (IV and V) are currently recognized. Glycosaminoglycans (GAGs) are sugar polymers of high molecular weight composed of repeating dimers of amino sugars that tend to be linked to proteins to form proteoglycans. Variations in type and distribution of GAGs and proteins, leads to formation of many types of proteoglycans. Glycoproteins such as fibronectin (Hedman et al, 1978) and laminin (Timpl et al, 1979) are also associated with the ECM and are considered to be involved mainly in cell-substrate adhesion. Direct contact by epithelial cells with a collagen substrate is required if they are to produce their own ECM. This newly produced ECM could in turn regulate the growth of

the basal epithelial cell layer, possibly by affecting the cell shape (Hay, 1978). Erhmann and Gey (1956) have shown that various tissues enhance growth and differentiation when cultured on collagen gels.

Collagen is a family of closely related proteins, produced by different gene products. Collagens contain a triple-helical segment composed of three chains with 33% glycine, about 10% proline, 10% hydroxyproline and variable amounts of hydroxylysine. No other proteins, except collagens, are degraded by bacterial collagenases, which recognize and cleave the sequence gly-X-pro-gly.

At least five isotypes of collagen molecules have so far been characterized but others may exist as well. Type I collagen is found mostly in skin, bone and tendon. Type II collagen is found mainly in cartilage while Type III collagen is most prominent in blood vessels, skin, and the parenchyma of internal organs. On the other hand, Type IV collagen is found in basement membranes while Type V collagen can be found in blood vessels and smooth muscle.

With the exception of lymphocytes, reticulocytes, and other blood-borne cells, most cells in culture synthesize collagen. Most fibroblasts produce both Type I and III collagen. Smooth muscle cells, however, can synthesize Types I, III, IV and V collagens. Epithelial and endothelial cells, which are important in the formation of basement membrane, synthesize Type IV collagen.

Tumors in general synthesize the collagen type characteristic of their tissue of origin. For instance, osteosarcomas contain Type I collagen, whereas chondrosarcomas contain Type III collagen.

Erhmann and Gey in 1956 were the first to show that various tissues enhanced growth and differentiation when cultured on collagen substrates. Michaelopoulos and Pitot (1975) successfully cultured parenchymal liver cells on rat tail collagen membrane. This floating collagen gel culture system was subsequently used to culture mouse mammary epithelial cells (Emerman et al, 1977; Emerman and Pitelka, 1977).

Recently Liotta and his colleagues (1978) demonstrated that fibroblasts cultured on plastic deposit a collagen substrate that is required for proliferation. Cells maintained on plastic substratum and exposed to cis-hydroxyproline (an inhibitor of collagen synthesis) did not produce collagen and did not proliferate, whereas cells exposed to cis-hydroxyproline and provided with an artificial collagen substrate did proliferate. It is not known whether collagen promotes cell attachment, thereby indirectly facilitating the growth of cells, or whether it has a direct effect on both cell attachment and cell proliferation. Fibronectin, like collagen, also promotes cell attachment, migration and proliferation. It is now believed that fibronectin is responsible for the attachment of mesenchymal cells to their substrate, whereas

laminin subserves the same function for epithelial cells (Terranova et al, 1980).

Although *in vitro* systems are convenient and provide controlled environments for studying hormonal influences on human breast cancer cells, ultimately it is necessary to test the hypotheses in *in vivo* models. Rodents are commonly used as experimental models for breast cancer. One of the most direct approaches is to transplant human breast cancer into immunodeficient animals such as the athymic nude mice.

The Use of Athymic Nude Mice for Tumor Transplantation

In 1966 Flanagan first reported mice with "nude" mutation. Nude is a mutant allele of the nude locus of the VII linkage group (chromosome II). Mice homozygous for this mutation (nu/nu) are essentially hairless. Heterozygous mice (nu/+) appear to be normal in all aspects. Attention was first drawn to the nude mice by the observation of Pantelouris in 1968 that these animals lack a normal thymus. Because of their thymic deficiency, the nude mouse is deficient in its immune responsiveness. There are several mutant genes in mice that produce a hairless phenotype (eg. nude, naked, hairless, rhino). However, these genes are located at separate loci. Only the nude is known to produce a thymus-deficient phonotype. As a result, nude mice are deficient in the

thymus-dependent (T-cell) immunological functions. Since immunological rejection of heterogeneous tissue grafts in an animal depends mainly on the T-cell-mediated immunity to the host, the nude mouse is unable to reject implanted cells or tissues from a genetically nonidentical donor. Thus, the nude mouse is useful for determination of cellular tumorigenicity and for mass propagation of animal cells that can form tumors in this host.

Rygaad and Povlsen (1969) reported the successful transplantation of a human carcinoma of the colon in athymic nude mice. The transplanted tumor grew and retained its original morphology. This report generated a lot of interest amongst oncologists, and the nude mice became the most suitable experimental model for heterotransplantation of human tumors.

The nude mice require special care to ensure long survival. Animals are kept under pathogen-limited conditions inside a laminar filtration system.

McManus et al (1978) showed that administration of human placental lactogen increased DNA synthesis in 10 transplanted specimens of mammary dysplasia and fibroadenomas. Hormonal dependency of some mammary tumors were demonstrated by Hirohashi et al (1977) on a serially transplantable human breast cancer Br-10. The investigators report arrest of tumor growth by oophorectomy and stimulation by weekly injections of 0.1 mg. of estradiol in male mice. Inoculation of cultured

human breast cancer cells into nude mice usually results in the formation of tumors. Engel and Young (1978) reviewing the experience of several investigators indicated that of 17 human breast cell lines, 14 produced tumors when transplanted into nude mice. The tumorigenicity of cultured mammary carcinoma cells is variable and the number of cells necessary to produce tumors varies from line to line.

Following subcutaneous injections of the cell suspensions, many of the cells necrose. Those that survive begin to divide. The latency period between the injection of cells and the first appearance of a palpable nodule at the site of injection varies greatly, depending on the malignancy of the cells and the inoculum used. Simultaneously, small blood vessels proliferate around the graft and eventually penetrate into the developing tumor. Thus, the tumors are composed of human carcinoma cells supported by a vascularized stroma of murine origin. Tumors induced in nude mice by heterologous malignant cells grow as well encapsulated masses and metastases occur infrequently, although metastasis of human breast cancer cells (MCF-7) has been observed in estrogen-treated athymic nude mice (Shafie and Liotta, 1980). The factors that determine the final tumor size in nude mice are not well understood. It is possible the residual tumor immunity of the nude mouse against specific cellular antigens may play a role.

SECTION 2: Human Breast Cancer Cell Lines

Human breast cancer cell lines are very useful for research in breast cancer. Attempts to culture breast cells were first reported in 1937 (Cameron and Chambers, 1937). It was not until 1958 that Lasfargues and Ozzello reported the first successful long term culture of a breast tumor which they termed BT-20.

There are several technical difficulties in establishing continuous cultures of breast tumors. The number of malignant cells in tumor samples is usually small and the viability of these cells is often low. In addition, fibroblasts tend to outgrow less prolific epithelial cells. The success rate appears to be higher when breast cancer cells from malignant effusions are cultured than when solid tumors are used (Cailleau et al., 1974a; Cailleau et al., 1974b; Engel et al., 1978). This is due to the larger numbers of dissociated, viable tumor cells and fewer fibroblasts in effusions.

The usefulness of any purported breast cancer cell line as an <u>in vitro</u> model depends on its pedigree and its

characterization. There are close to 50 existing human breast cancer cell lines reported to date. In the studies presented in this thesis, eight human breast cancer cell lines and one non-tumorous mammary epithelial cell line were used. The light microscopic appearance of these cells are shown in Fig. 1. Some of the characteristics, tissue source and origins of these nine cell lines are summarized in Table 1.

Undoubtedly, human breast cancer cells maintained in continuous tissue culture offer many advantages for studying hormone action on these cells. However, it is important to understand some of the problems and pitfalls of using in vitro models to study the hormonal dependency of human breast cancer. First, since most breast cell lines were established from malignant effusions, these cells represent a subset of the original tumor population which has the ability to metastasize. This subset of cells may be present in only small numbers in the original tumor. In addition, by the time the tumor sample is collected for culture, various therapies, such as irradiation, cytotoxic drugs and hormonal manipulations, may have selected out only certain kinds of cells.

Second, tissue culture conditions almost always select against slower growing populations of cells. Since differentiation very often is related inversely to growth rate, many populations of cells that may express those hormonal responses of greatest interest may be overgrown by faster

Fig. 1 Morphology of nine established human mammary epithelial cell lines

The cells were plated in 35 mm dishes in DME with 10% FBS; T-47D, MCF-7, SK-Br-3, AlAb496, BT-20, BT-474 and HBL-100 were maintained as monolayer cultures.

DU4475 and Lev III were maintained in suspension cultures.

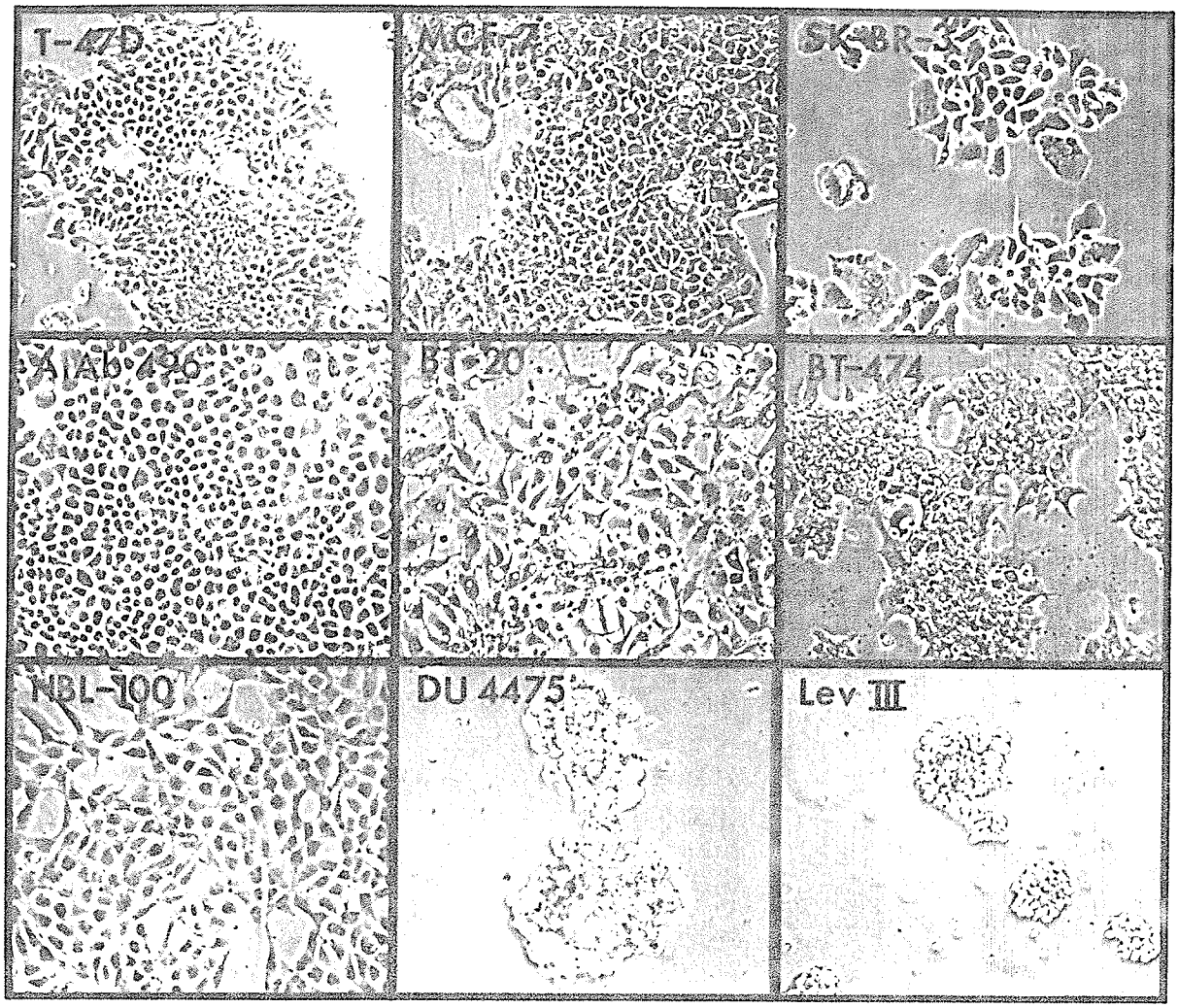

T-47D
MCF-7
SK-BR-3
AIAb 496
BT-20
BT-474
NBL-100
DU 4475
Lev III

Table 1: Tissue source and growth pattern of human mammary cell lines

Cell Lines	Tissue Source	Growth Pattern	References
T-47D	pleural effusion	monolayer	Keydar et al., 1979
MCF-7	pleural effusion	monolayer	Soule et al., 1973
BT-20	original tumor	monolayer	Lasfargues & Ozzello, 1958
BT-474	original tumor	monolayer	Lasfargues et al., 1978
SK-Br-3	pleural effusion	monolayer	Fogh & Trempe, 1975
AlAb 496	lung metastasis	monolayer	Reed & Gey, 1962
HBL-100	normal milk	monolayer	Polanowski et al., 1976
DU4475	cutaneous metastasis	suspension	Langlois et al., 1979
Lev III	pleural effusion	suspension	Feller et al., 1970

growing hormone-independent cells. Third, contamination of cultures by viruses or mycoplasma may substantially alter the expression of some phenotypic effects. Fourth, cells maintained in culture are deprived of many factors which may be crucial to the expression of a given response. For instance, attachment to a basement membrane or extracellular matrice, other adjacent supporting cell types, a polarized orientation or presence of certain growth factors may all be necessary for a cell to behave "normally". Finally, a cell line may be thought to be unresponsive to a given factor because of the incorrect assumption that this factor has been removed from the medium. Serum supplements, which are frequently used in tissue culture, contain high concentrations of many growth factors and steroids (Esber et al., 1973). Recently, the use of serum-free chemically defined media by many investigators to maintain human breast cancer cells has virtually eliminated this problem (Barnes and Sato, 1979; Allegra and Lippman, 1978).

Despite these problems, the cell culture technique offers many advantages over in vivo or organ culture systems for the study of hormonal influences in human breast cancer. First, by using tumor cells derived from humans, many of the problems associated with species differences are eliminated. Second, the use of a single cell type leads to more readily interpretable results. Third, direct effects of trophic substances can be studied. Fourth, the hormonal milieu can be

accurately regulated especially in serum-free chemically defined media. Thus one can be certain that the hormonal effect observed is not mediated by some additional unknown serum factors. Fifth, the convenience of tissue culture drastically reduces the time and cost which are involved in *in vivo* studies. Sixth, the continuous culture of established cell lines allows for sequential experiments on the same tissue. Finally, the *in vivo* situation can be re-enacted stepwise by adding back *in vitro* individual stromal components or their diffusible products. Thus, long-term tissue culture of human breast cancer cell lines is an extremely useful tool for furthering our understanding of the disease.

Cell Lines

Human breast cancer cell line MCF-7 was generously provided by Dr. M. Rich, Michigan Cancer Foundation; BT-474 was a gift of Dr. E. Y. Lasfargues, Institute for Medical Research, Camden, N.J. The T-47D and HBL-100 cell lines were obtained from EG&G/Mason Research Institute, Rockville, Md. The AlAb 496, DU4475, Lev III, BT-20 and SK-Br-3 cell lines were provided by J. F. Weaver, Naval Biosciences Laboratory, Oakland, California. All of these cell lines are characterized with respect to human origin (Engel and Young, 1978).

Growth Conditions

All cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DME) supplemented with L-glutamine

(4 mM), glucose (4.5 g/l), penicillin (100 IU/ml), streptomycin (100 ug/ml), bovine insulin (10 ug/ml) and 10% (v/v) fetal bovine serum (FBS). This will be referred to as complete medium (CM) in this thesis.

Trypsin/EDTA in Hanks' balanced salt solution (HBSS) was used for cell passages. Cells were maintained in a humidified atmosphere of 95% air - 5% CO_2 at 37°C. Culture flasks and dishes were obtained from Corning (Fisher Scientific Co., Winnipeg, Manitoba, Canada); culture medium, trypsin, antibiotics, and fetal bovine serum were purchased from Grand Island Biological Co. (Burlington, Ontario, Canada).

SECTION 3: Aims of Investigation

The objectives of this investigation were several:

1. To study the receptor and growth promoting effect of epidermal growth factor on various human mammary cell lines.
2. To develop a collagen gel culture system using Vitrogen 100 for culturing mammary epithelial cells.
3. To compare the morphological and proliferative characteristics of human breast cancer cells on plastic and in collagen matrix.
4. To study the effect of estrogen and pituitary factors on the growth of human breast cancer in athymic nude mice.
5. To identify and characterize the pituitary factors which are responsible for stimulation of growth of human breast cancer.

SECTION 4: Receptors and Growth Promoting Activity of Epidermal Growth Factor in Human Breast Cancer Cell Lines.

INTRODUCTION

Human breast cancers are known to be influenced by steroids and polypeptide hormones (McGuire et al., 1978). Recently, the use of in vitro long-term tissue culture of human breast cancer cells has provided an excellent model for studying the mechanisms of hormone action in breast cancer (Lippman et al., 1977). Receptors for insulin (Osborne et al., 1978), steroid hormones (Engel and Young, 1978; Lippman et al., 1977) and prolactin (Shiu, 1979) in several established human breast cancer cell lines have been reported.

In addition to the major steroid and peptide hormones, epidermal growth factor (EGF) regulates the development of normal mammary epithelium both in rodents (Tonelli and Sorof, 1980; Turkington, 1969) and humans (Stampfer et al., 1980; Stoker et al., 1976; Taylor-Papadimitriou et al., 1977). The

presence of high concentrations of EGF in human milk was recently reported (Starkey and Orth, 1977). This suggests that EGF may have a role in the human breast. Epidermal growth factor was also reported to be mitogenic in mouse mammary carcinoma cells (Turkington, 1969) and in one human breast cancer cell line (Osborne et al., 1980). Furthermore, EGF was shown to act as a tumor promoter in an in vivo study involving application of methylcholanthrene to mouse skin (Rose et al., 1976). On the basis of these observations, it seemed worthwhile to determine the biologic effect of EGF in human breast cancer cells. In the present study, we examined the binding of EGF to nine human mammary cell lines established from various sources and compared the receptors and mitogenic activity of EGF in these cells.

MATERIALS AND METHODS

Cell Lines

Nine human mammary cancer cell lines (T-47D, MCF-7, BT-20, BT-474, SK-Br-3, AlAb496, HBL-100, DU4475, Lev III) are used in this study. The source, growth pattern and culture conditions of these cell lines are summarized in Section 3.

Preparation and Iodination of EGF

Epidermal growth factor was purified from male mouse submaxillary glands according to the method of Savage and Cohen (1972), and was iodinated by a modification of the chloramine-T

method (Greenwood and Hunter, 1963), using $Na^{125}I$ (Amersham). The specific activity of the ^{125}I-labeled EGF was 200-250uCi/ug.

Determination of Specific Binding of ^{125}I-labeled EGF to cultured cells

Four dishes (35 mm) of subconfluent monolayer cells were washed once with 25 mM Hepes buffered (pH 7.4) Hanks' balanced salt solution (HBSS) containing 0.1% (w/v) bovine serum albumin (BSA). After aspiration of the washing medium, each dish received 1.5 ml of the same buffer containing about 0.05 ng of ^{125}I-labeled EGF with or without various doses of unlabeled EGF (0.01 ng - 100 ng). The dishes were incubated at 24°C (room temperature) for 4 hours when equilibrium was achieved. At the end of this incubation period, the radioactive medium was aspirated, and the dishes were washed twice with 2 ml portions of ice-cold buffer. The cells were then dissolved with 2 ml of 0.1 N NaOH containing 0.5% Triton X-100. The solutions were transferred to disposable glass tubes, and radioactivity was determined in a LKB Wallac gamma counter with a counting efficiency of 70% for ^{125}I. Specific binding was taken as the total radioactivity bound (mean of duplicates) in the absence of unlabeled hormone minus that bound (mean of duplicates) in the presence of excess unlabeled hormone (100 ng). The latter represents nonspecific binding to cells and culture dish.

In the case of cells cultured in suspension (DU4475 and Lev III), the cells were incubated in plastic tubes. Free and bound hormones were separated by centrifugation of the cell suspension at 250xg for 5 minutes. The pellets were resuspended and washed twice with cold buffer. The cell pellets were counted in the gamma counter.

Determination of Growth Promoting Effect of EGF in Cultured Cells

Human breast cancer cells (5 - 8 X 10^4 cells per dish) were plated in DME supplemented with 10% FBS and incubated for 24 hours. The cells were then washed with fresh medium without serum and incubated in 2 ml of DME containing 0.1% FBS for 24 hours. Twenty microlitres of EGF dissolved in the above culture medium was added. Twenty hours later, the cells were labeled with ^{3}H-thymidine (1 uCi/dish) for 4 hours. The medium was aspirated and the cells were washed once with phosphate buffered saline (PBS), followed by one washing with 3 ml of cold 10% trichloroacetic acid (TCA) and two washings with cold 5% TCA. Acid insoluble radioactivity was dissolved in 1 ml of 0.1 N NaOH containing 0.5% Triton X-100. The solution was mixed with Aquasol-2 (New England Nuclear) and counted in a liquid scintillation counter. In the case of floating cells, cells were cultured in plastic culture tubes (12 x 75 mm) in the same volume as monolayer cells. Trichloroacetic acid insoluble materials were collected by centrifugation (1500 xg, 30 minutes).

RESULTS

Optimization of Conditions for the Binding of ^{125}I-EGF by Intact Human Breast Cancer Cells

In order to determine the optimal conditions for studying the binding of ^{125}I-EGF to the human mammary cell lines, the effects of time and temperature on the binding of ^{125}I-EGF to several cell lines were tested. Figure 2 shows the results of EGF binding to T-47D cells. At 37°C, maximum binding of ^{125}I-EGF was observed at about 90 minutes, and the binding decreased gradually with time. At 4°C, the rate of association of ^{125}I-EGF was slow. At 24°C, the binding increased gradually and reached a plateau after 4 hours without a decrease thereafter. The time course of BT-20 and HBL-100 showed a similar pattern to the T-47 cells (data not shown). In all subsequent experiments of binding, cells were incubated at 24°C for 4 hours in an equilibrium condition. There was no apparent change in the morphology of the cells as judged by phase-contrast microscopy after 4 hours of incubation at any of the above temperatures.

Equilibrium Analysis of Binding of ^{125}I-EGF to Human Breast Cancer Cell Lines

For all the cell lines tested, specific binding of ^{125}I-EGF was a direct function of the concentration of hormone in the medium. For clarity, the binding of ^{125}I-EGF

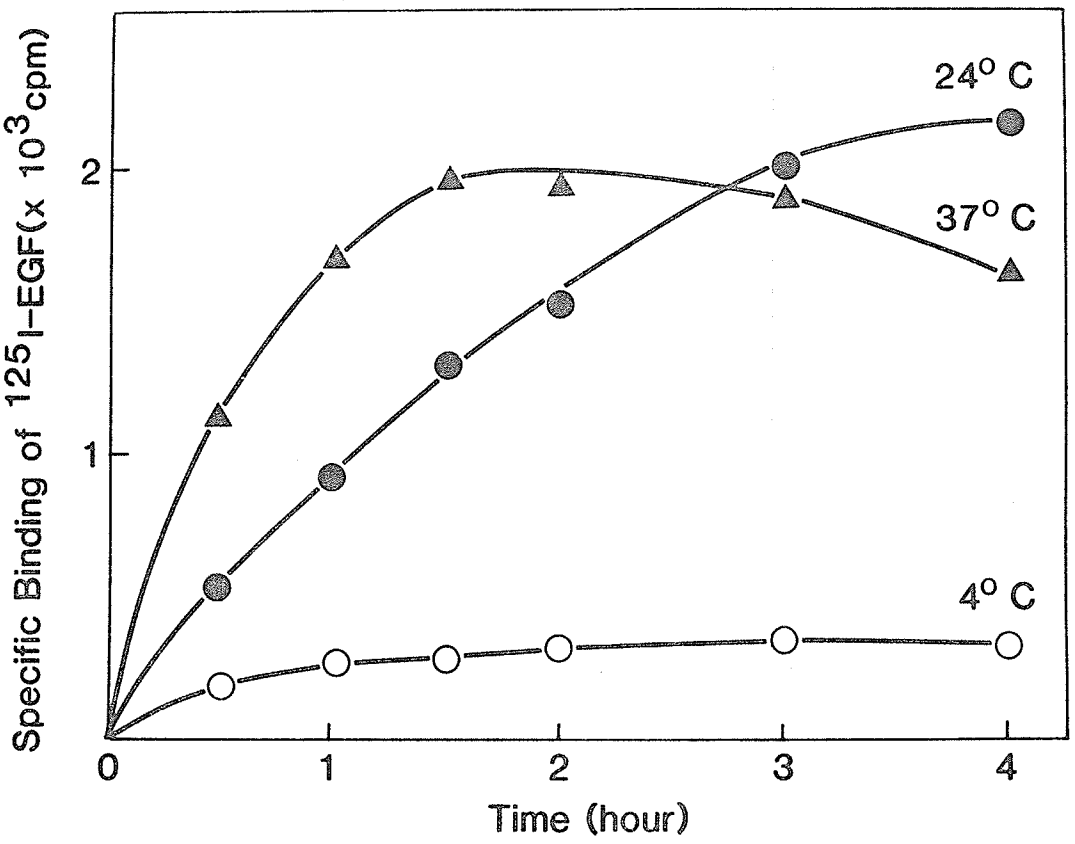


Fig. 2 Effect of time and temperature on the specific binding of ^{125}I-labelled EGF to T-47D cells

Each dish contained 1.2 x 10^6 cells and 12,500 cpm of ^{125}I-labelled EGF. Each point represents the specific binding of ^{125}I-labelled EGF to the T-47D cells. Specific binding was determined by subtracting the mean value of duplicates for non-specific binding from the mean value of duplicates for total binding.

to T-47D and HBL-100 cells as a function of hormone concentration are illustrated in Figs. 3a and 3b.

The amount of hormone bound gradually increased as the hormone concentrations increased, until a plateau was reached. Scatchard analysis of the binding data (Fig. 3, inset) exhibited a curvilinear pattern. Scatchard plots of the other cell lines also showed a curvilinear pattern. The apparent affinity constant and total binding sites were determined from these data by the method of Kahn et al. (1974).

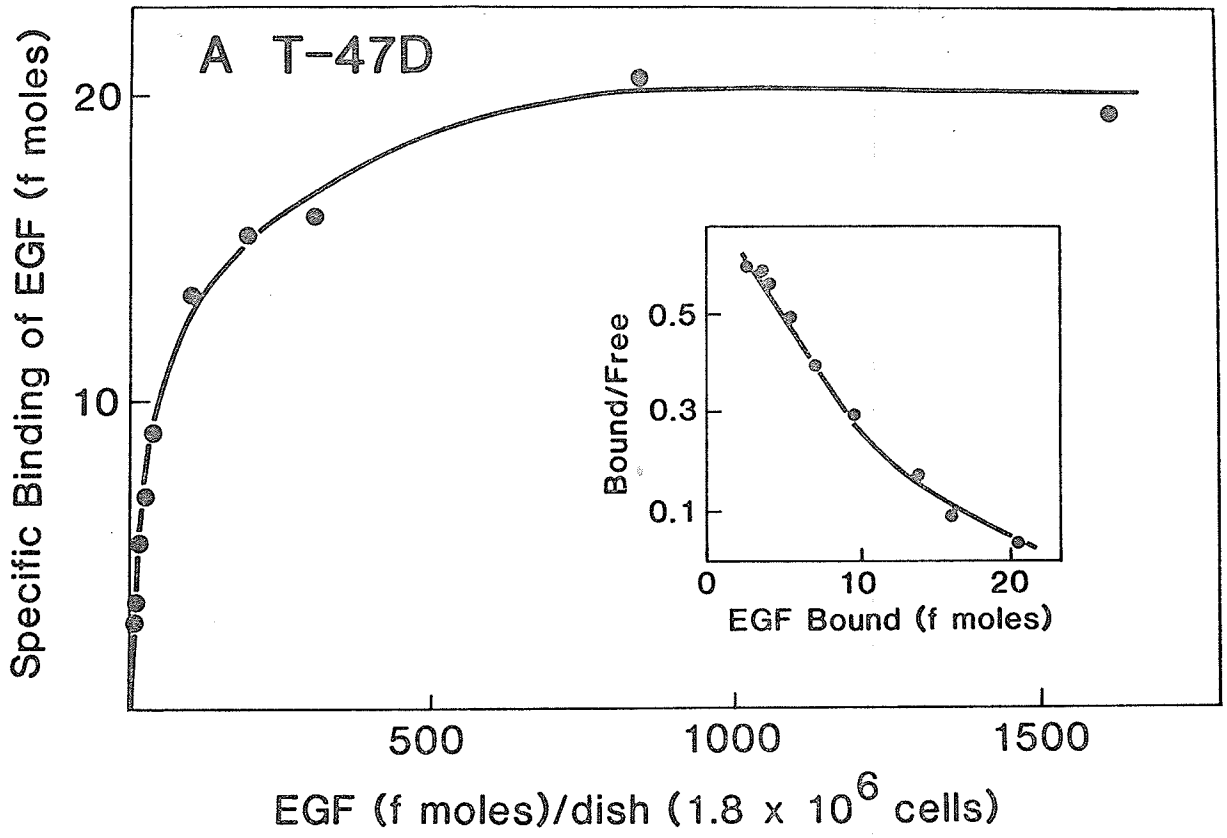
Summary of EGF Receptor Content in Human Breast Cancer Cells

The optimal conditions illustrated in Fig. 2 were used to quantitate EGF receptors by Scatchard Analysis. Table 2 shows that 6 human breast cancer cell lines (T-47D, MCF-7, SK-Br-3, AlAb 496, BT-20, BT-474) and one non-tumorigenic cell line (HBL-100) which grew in monolayer had EGF receptors with both high and low affinities. The association constant for the high affinity sites was approximately $10^{10}{}_{M}{}^{-1}$ and that of low affinity sites was approximately $10^{9}{}_{M}{}^{-1}$.

The two solid tumor derived cell lines, BT-20 and BT-474, showed very high specific binding of EGF (1.5×10^{6} and 3.0×10^{4} sites per cell, respectively). The MCF-7 and T-47D, both of which were derived from pleural effusions, had a lower number of EGF receptors (2.8×10^{3} and 7.6×10^{3} sites per cell, respectively). The AlAb 496 cells which were derived from lung metastases, had 1.6×10^{3} sites per cell. The

Fig. 3 Analysis of EGF binding in T-47D and HBL-100 cells

Cultures of T-47D (A) and HBL-100 (B) cells were grown to a density of 1.8×10^6 and 0.9×10^6 cells per dish (35 mm), respectively. After washing the cells with binding buffer, 0.05 ng of ^{125}I-labelled EGF (1.8×10^4 cpm) mixed with various known amounts (a range from 0.01 ng to 100 ng) of unlabeled EGF were added to the dishes in 1.5 ml of binding buffer. After a four hour incubation at room temperature ($24^{\circ}C$), the dishes were washed and the cells were lysed as described. Inset showed the Scatchard analysis of the binding data.


A T-47D
Specific Binding of EGF (f moles)
20
10
500
1000
1500
EGF (f moles)/dish (1.8 x 10^6 cells)
Bound/Free
0.5
0.3
0.1
0
10
20
EGF Bound (f moles)

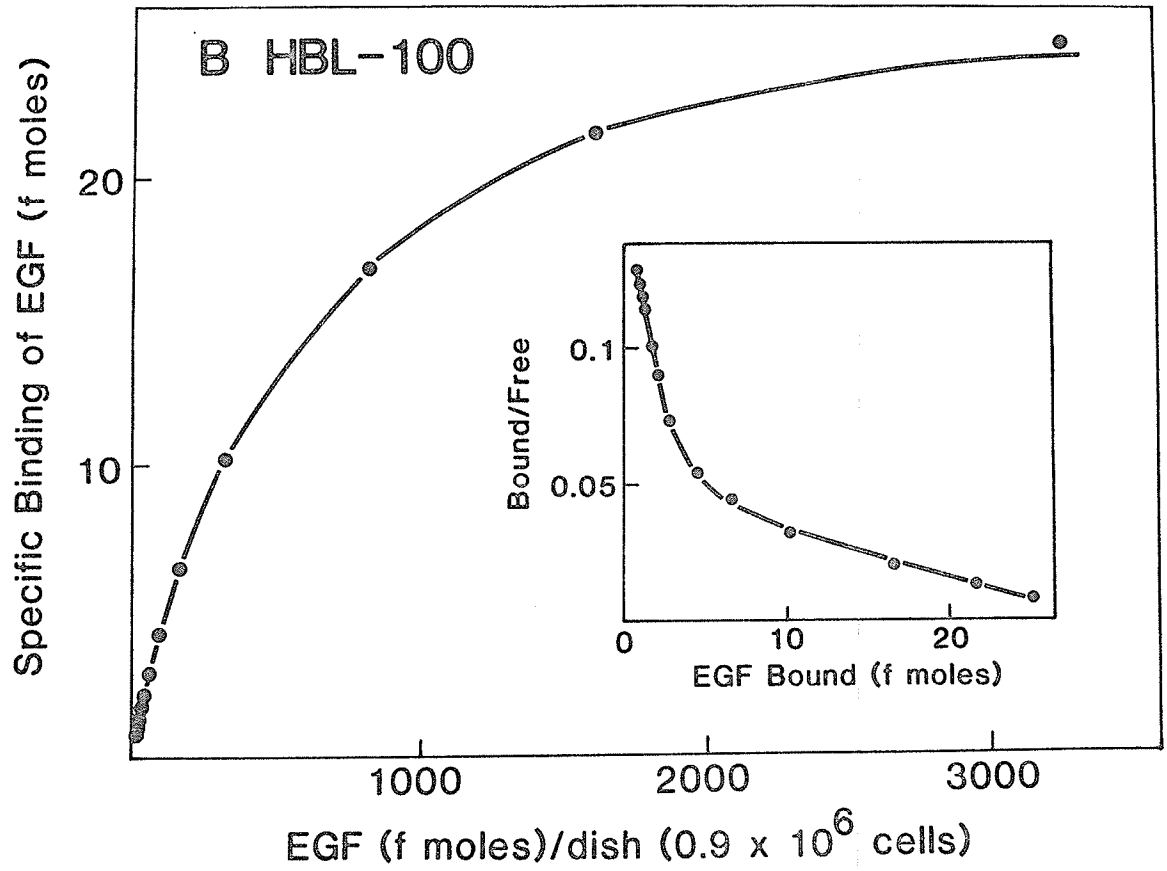

B HBL-100
Specific Binding of EGF (f moles)
20
10
1000
2000
3000
EGF (f moles)/dish (0.9 x 10^6 cells)
Bound/Free
0.1
0.05
0
10
20
EGF Bound (f moles)

Table 2: EGF receptors in human mammary cell lines

Cell Lines	Ka (M^{-1}) High	Ka (M^{-1}) Low	Total Binding Sites/Cell
AlAb 496	6.2×10^{10}	8.2×10^{9}	1.6×10^{3}
MCF-7	8.4×10^{10}	9.7×10^{9}	2.8×10^{3}
T-47D	6.7×10^{10}	3.1×10^{10}	7.6×10^{3}
HBL-100	3.2×10^{10}	2.8×10^{9}	1.9×10^{4}
BT-474	12.0×10^{10}	1.2×10^{10}	3.0×10^{4}
SK-Br-3	8.0×10^{10}	1.6×10^{9}	9.0×10^{4}
BT-20	1.2×10^{10}	0.2×10^{9}	1.5×10^{6}
DU4475	Non Detectable		
Lev III	Non Detectable		

The binding assays were performed as described in Materials and Methods. Affinity constant (Ka) and total binding sites were determined by Scatchard analyses.

HBL-100 cells, which were derived from normal human milk, had 1.9×10^4 EGF receptors per cell. Interestingly, the two floating cell lines, DU4475 and Lev III, did not exhibit any detectable binding of ^{125}I-EGF.

Growth Promoting Effect of EGF in Human Breast Cancer Cells

The effects of EGF on incorporation of ^{3}H-thymidine into DNA of nine human mammary cell lines are shown in Fig. 4. The DNA synthesis of T-47D cell lines was stimulated by EGF at 0.1 ng/ml and maximal stimulation occurred at 1 ng/ml. The DNA synthesis of T-47D cells was inhibited slightly at higher EGF concentrations (10 ng/ml and 100 ng/ml respectively). In contrast, EGF did not stimulate DNA synthesis in MCF-7, SK-Br-3, BT-20 and BT-474. High concentrations of EGF inhibited DNA synthesis in these cells. The AlAb 496 cells, which were derived from lung metastases, did not respond to any of the EGF concentrations tested. Inhibition by higher concentrations of EGF was also observed in the HBL-100 cell line which was derived from normal human milk. The two floating cell lines DU4475 and Lev III, which had no detectable EGF receptors, did not show any response to EGF.

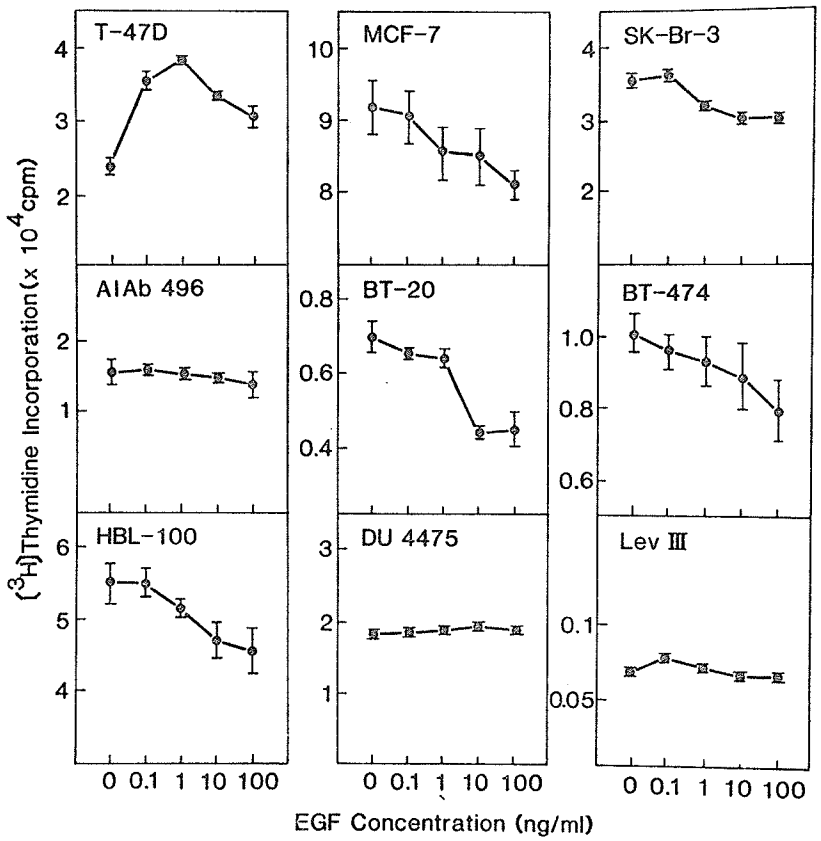


Fig. 4 Effect of EGF on incorporation of ^{3}H-thymidine in nine human mammary cell lines

Triplicate dishes (5 x 10^4 cells/dish) were plated in DME with 10% FCS. Twenty-four hours later, the media were changed to DME with 0.1% FCS. Twenty hours later, the cells were pulse labelled with ^{3}H-thymidine (1 uCi/dish) for four hours. Bars, S.D.

DISCUSSION

Our results show that the mammary cell lines maintained as monolayers have EGF receptors while two cell lines grown in suspension had no detectable EGF receptors. Scatchard analysis of the binding data revealed a curvilinear pattern suggesting heterogeneity of the receptors. High affinity constants determined from the analysis were in the order of $10^{10}_{M}{}^{-1}$ which were similar to those reported for the other cells (Adamson and Anthony, 1981). In addition, cell lines established from metastatic sites (AlAb 496, MCF-7, T-47D, DU4475 and Lev III) tend to have fewer EGF receptors than cell lines established from human milk (HBL-100) and primary breast tumor (BT-474, BT-20). The significance of this finding is not clear. Yuhas et al. (1978) suggested that breast cancer cells derived from pleural effusions were genetically different from the bulk of the tumor cells in solid breast cancer samples. Cells lines from effusions were unable to grow as organized spheroid aggregates while cell lines derived from primary cancers could. Therefore it is possible that metastatic mammary cancer cells, reflecting such a difference, may possess fewer EGF receptors than primary tumors. Alternatively, the differences in EGF binding of these cell lines may be due to the production of endogenous EGF-like substances, which may

bind to the EGF receptors, thus limiting the binding of exogenously applied EGF. We have tested this possibility by looking for EGF-like activity in serum-free conditioned medium of these cell lines using radioreceptor assay described previously (Imai et al., 1979). No EGF-like activity was detected in all the cell lines tested (Imai and Leung, unpublished observation).

It is interesting to observe that the two floating cell lines, DU4475 and Lev III, had no detectable binding of EGF. It has been shown that the induction of increased adhesion of 3T3 cells to culture dishes is mediated by specific EGF receptors (Berliner et al., 1981). Epidermal growth factor also stimulates the production of extracellular matrix which is necessary for cell adhesion (Chen and Gudor, 1977; Lembach, 1976). Thus, a correlation between the capability of these cell lines to grow in suspension cultures and loss of EGF receptor may exist.

Since all the cell lines grown in monolayer cultures had EGF receptors with high affinity, we then tested whether EGF had mitogenic activity in these cells. Under our experimental conditions, EGF stimulates the incorporation of ^{3}H-thymidine into DNA only in the T-47D cells. The stimulatory effects of EGF on T-47D cells were confirmed by monitoring the increase in cell number (data not shown). Epidermal growth factor inhibited the DNA synthesis of all the other monolayer cell

lines except AlAb 496. Recent reports indicate that EGF also inhibits the growth of the human epidermoid A431 cells (Gill and Lazar, 1981) and a pituitary tumor cell line GH_4C_1 (Schonbrunn et al., 1980) at concentrations which are stimulatory in other cells.

Osborne et al. (1980) reported that EGF stimulated the incorporation of ^{3}H-thymidine into MCF-7 cells. However, our results are at variance with these findings. These authors studied the effect of EGF (obtained from Collaborative Research) on the incorporation of ^{3}H-thymidine into MCF-7 cells which were plated to subconfluent density and cultured in serum free medium. We have also tested the effect of commercially available EGF on MCF-7 cells using the protocol of Osborne et al. However, we could not observe mitogenic activity of EGF on MCF-7 cells. The discrepancy in the finding could be due to difference in the maintenance of this cell line. Also, serial passages of the cells may affect responsiveness of MCF-7 cells to EGF, and the passage number of the cells used in our study may be different from that of cells employed by Osborne et al.

It has been shown that EGF binding sites and responsiveness to EGF change during the differentiation of granulosa cells (Vlodavsky et al., 1978), pheochromocytoma cells (Hufi and Guroff, 1979) and mouse teratocarcinoma cells (Rees et al., 1979). Therefore the variation in EGF binding sites and

responses to EGF observed in nine human breast cancer cell lines may reflect the diverse origins and different stages of differentiation of these cells.

It is possible that EGF may have other biological roles in mammary cells besides regulation of cell proliferation. For instance, EGF stimulates human chorionic gonadotropin secretion by cultured choriocarcinoma cells (Benveniste et al., 1978) and inhibits histamine-mediated gastric acid secretion from gastric chief cells (Bower et al., 1974). Further, EGF modulates the production of growth hormone and prolactin from GH_4C_1 pituitary tumor cells (Schonbrunn et al., 1980). Whether or not EGF plays a role in influencing the differentiated functions of human breast cancer cells remained to be elucidated.

SECTION 5: Morphological and Proliferative Characteristics of Human Breast Cancer Cells Cultured on Plastic and in Collagen Matrix.

INTRODUCTION

The importance of extracellular matrix, which is mainly composed of collagen, proteoglycans, glycosaminoglycans, and glycoproteins, for growth and development *in vivo* of many cell types has long been recognized (Wessels, 1964). When cells are maintained *in vitro* in the presence of extracellular matrix, their proliferative or functional responses to trophic factors resemble those responses observed *in vivo*. For example, epidermal growth factor markedly stimulated the rate of proliferation and keratinization of corneal epithelial cells *in vivo* as well as of cells cultured on collagen matrix, but the same cells were no longer responsive to this growth factor when they were grown on plastic substratum (Gospodarowicz et al, 1978; Savage and Cohen, 1973). Furthermore, mouse mammary epithelial cells when cultured in collagen, responded to the

addition of lactogenic hormones by secreting milk protein (Emerman et al, 1977; Emerman et al, 1979). In addition, primary cultures of human breast epithelial cells grown on rat tail collagen proliferated in response to hydrocortisone and epidermal growth factor (EGF) (Yang et al, 1979; Yang et al, 1980 a, b). Thus, collagen, a component of the extracellular matrix, is important in regulating the proliferation as well as in enabling the mammary epithelial cells to respond to trophic factors. We are interested in studying the role of hormonal factors in the control of proliferation and other functions of human breast tumor cells maintained in long term culture. Therefore we explored the possibility of using these cells cultured in collagen matrix as models for studying the effects of trophic factors on human breast cancer cells. In the present study, we compared the morphological and growth characteristics of two human breast tumor cell lines cultured on plastic substratum with those maintained in collagen matrix.

MATERIALS AND METHODS

Routine Maintenance of Cell Lines

Two human breast tumor cell lines MCF-7 and T-47D and one cell line derived from normal milk, HBL-100, were used. The culture conditions for these cell lines were described in Section 3. Trypsin/EDTA in Hank's balanced salt solution (HBSS) was used for cell passages.

Preparation of Collagen Gel

Vitrogen 100^{R} (bovine dermal collagen mainly composed of Type 1 collagen) was obtained from Collagen Corporation (Palo Alto, California). Collagen solution was prepared by mixing 8.5 parts of Vitrogen 100^{R} (2.4 mg/ml) with 1.5 parts of 10 times concentrated DME. The 10 X DME is composed of: 1 packet of DME powder, 3.7 g $NaHCO_3$, 5 ml gentamicin, 10 ml glucose (35%) and 85 ml distilled water. The mixture was stirred and then filtered through a millipore filter (0.45 micron) to remove small amounts of undissolved residues. The collagen solution was adjusted to pH 7.4.

Cell Culture in Collagen Gel

In experiments using collagen, either Falcon plastic multiwell plates (well diameter 16 mm) or Flow Lab Linbro multiwell plates were used. The bottom of each well was first coated with 0.3 ml of collagen solution and the plates were incubated at 37°C for 15 minutes to allow for collagen to gel (Chart 1).

Dispersed cells were pelleted by centrifugation and resuspended in collagen solution. Approximately 5 x 10^4 cells in 0.3 ml of collagen solution was layered on top of the collagen gel and the dishes were incubated at 37°C. Fresh growth medium was added to individual wells after gelation occurred. The medium was changed every three days. At the end of each culture period, the collagen was digested with 0.1%

collagenase (Worthington, Type III) in HBSS for two hours at 37°C. After dispersing the cells by pipetting, the cell number was determined using a Coulter counter.

For monolayer cultures, approximately 5 x 10^4 cells were plated in 2 ml of medium in 35 mm plastic culture dishes. Cell number was counted after trypsinization.

Transmission and Scanning Electron Microscopy

The morphology of the cells cultured on plastic or in collagen were compared by phase contrast light microscopy. Some of the cultures were fixed with 3% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated and embedded in epon plastic. Thin sections were stained with uranyl acetate and lead citrate and were examined in a Phillips 300 electron microscope. Some samples were fixed with 3% glutaraldehyde in 0.05 M cocadylate buffer at 37°C for 30 minutes. This was followed by washing in distilled water for five minutes and postfixed with osmium tetroxide in 0.2 M cocadylate buffer for 15 minutes and washed with distilled water. The samples were dehydrated in 50%, 70%, 90% and 100% acetone. They were then critical-point dried and sputter coated with gold. The specimens were viewed in a Cambridge Steroscan scanning electron microscope.

RESULTS

Morphology

The light microscopic appearance of the two tumor cell lines (T-47D and MCF-7) and one non-tumorigenic cell line (HBL-100) cultured on plastic or in collagen are shown in Fig. 5. Both cell lines when grown on plastic display the familiar cobblestone monolayers. The nuclei of these cells are prominent and the cells are polygonal in shape. The T-47D and MCF-7 breast tumor cells become rounded and aggregate into multilayer clusters when grown in the collagen matrix. On the other hand, the HBL-100 cells are spindle shaped and exhibit a polarized interlacing pattern when grown in collagen.

By scanning electron microscopy, the breast tumor cells (T-47D) assume a rounded shape when cultured in collagen matrix and they form tumor-like aggregates (Fig. 6). The ultrastructure of these tumor cells also were examined by transmission electron microscopy (Figs. 7, 8). Typical cell junctions (desmosomes) are observed between neighboring cells cultured in collagen. The cells possess many micro-villi on the cell surface; numerous microfilaments are observed in the cytoplasm. Thus, these human breast tumor cells retain their epithelial cell characteristics when grown in collagen.

Fig. 5 Light microscopic appearance of human mammary epithelial cells cultured on plastic and in collagen

1) T-47D cells, 2) MCF-7 cells, 3) MBL-100 cells

a) cells grown as monolayer in plastic 10% fetal bovine serum

b) cells grown in collagen gel 10% fetal bovine serum

c) cells grown in collagen gel 0.1% fetal bovine serum

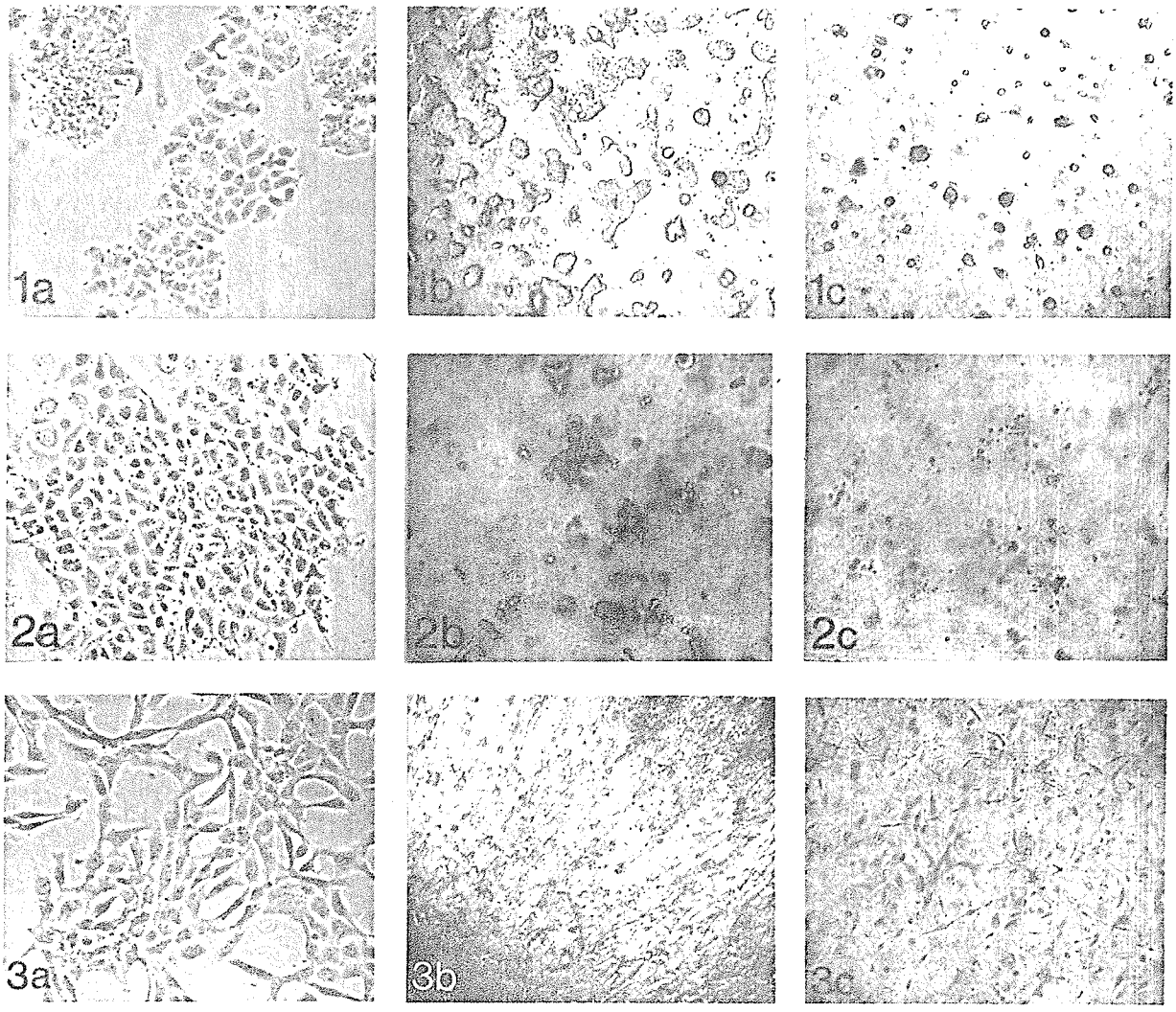
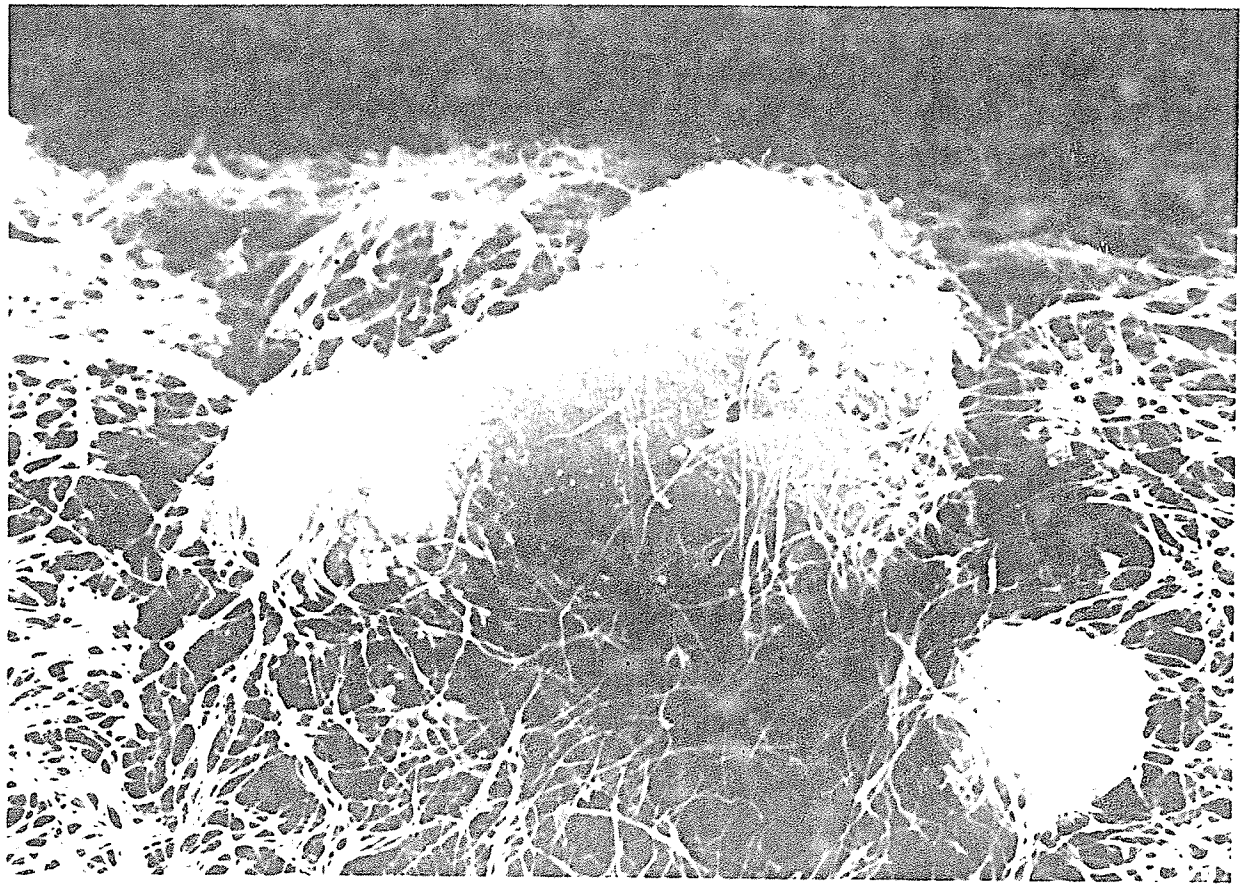

1a
1b
1c
2a
2b
2c
3a
3b
3c

Fig. 6: Scanning electron micrograph of human breast tumor cells (T-47D) in collagen gel

The fixed collagen gel was broken manually to reveal the cell aggregates which were embedded inside the collagen matrix. x 3000.

Fig. 7: Transmission electron micrograph of an aggregate of T-47D cells in collagen gel

Desmosomes (D), microvilli (M). x 6500. Inset shows higher magnification of desmosome between two epithelial cells. x 25000.



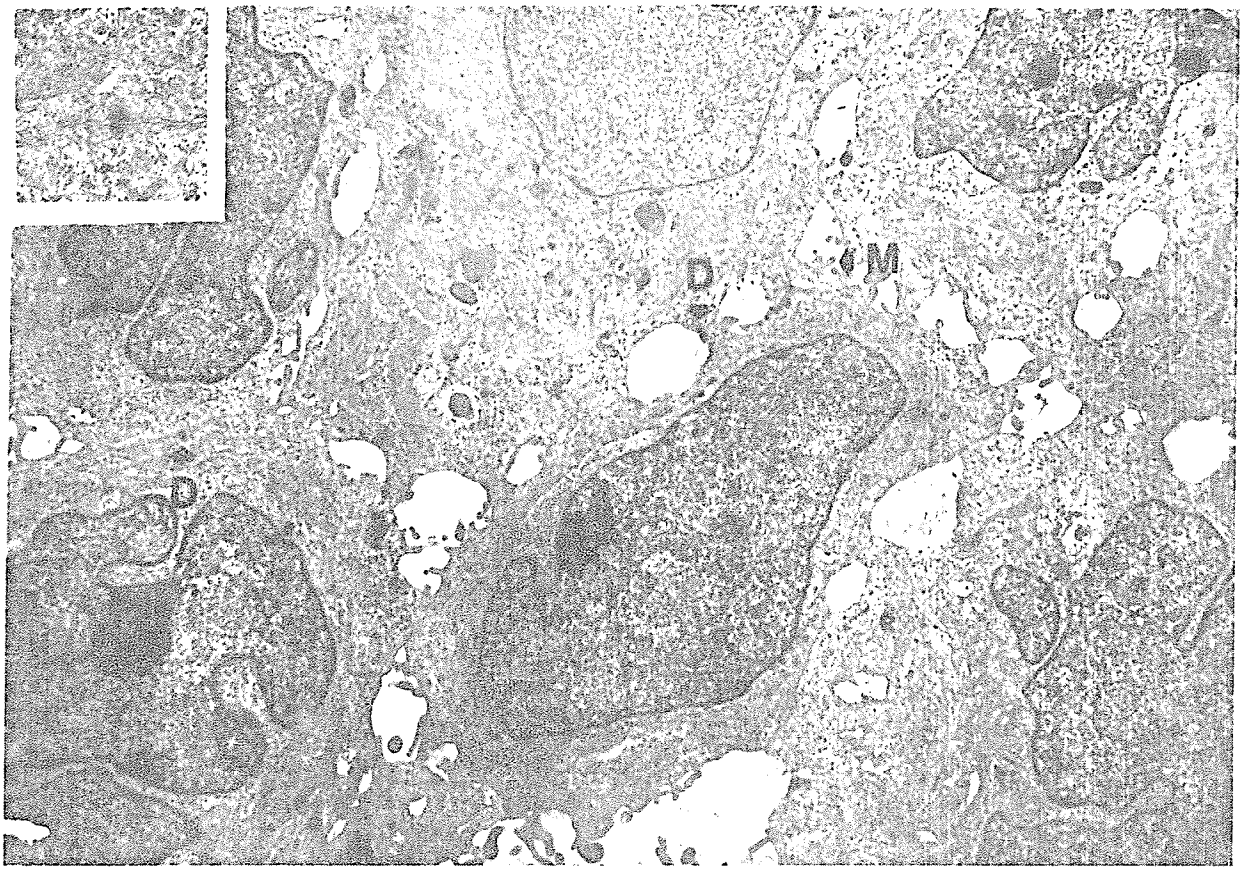
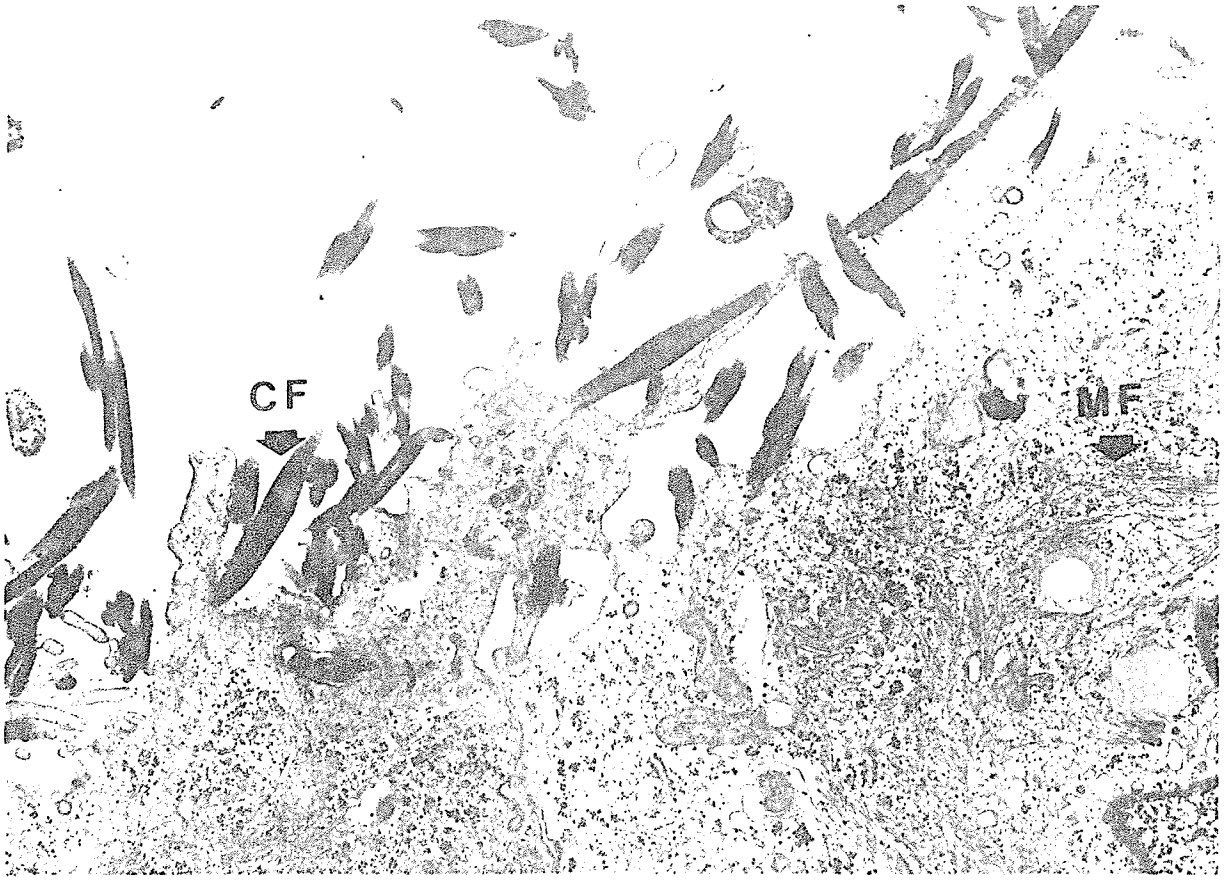

P
M
P

Fig. 8: Electronmicrograph of T-47D cells cultured in collagen gel

Collagen fibers (CF) and microfilaments (MF). x 17000.


CF
MF

Growth Characteristics

The growth rate of breast tumor cells in media supplemented with 10%, 1%, and 0.1% fetal bovine serum (FCS) and in serum-free media were measured. When grown on plastic, both cell lines proliferate rapidly in the presence of 1% and 10% FCS but none of them proliferate in the absence of FCS (Figs. 9, 10, 11). It is noteworthy that when grown on plastic, the growth rate of these cells produced by 1% serum is comparable to that achieved with 10% serum.

The human breast cancer cell lines grow slower in collagen (Figs. 12, 13, 14). A lag time of about 48-72 hours is required before growth occurs. Unlike in the monolayer culture on plastic, these cells apparently become more serum-dependent when cultured in collagen where they proliferate in the presence of 10% serum but fail to grow in 1% serum concentrations or lower. Cell death usually occurs after five to seven days in serum free medium. In addition, the doubling time of cells cultured in collagen is longer. The doubling time for T-47D in collagen is 2.25 days as compared to 2.00 days on plastic. For MCF-7, the difference is even greater; being 2.40 in collagen and 1.50 days on plastic (Table 3).

Both T-47D and MCF-7 tumor cells were able to grow in collagen gel in a serum-free, chemically-defined medium that contained insulin, transferrin, dihydrotestosterone, estradiol and epidermal growth factor (Fig. 15). The chemically-defined

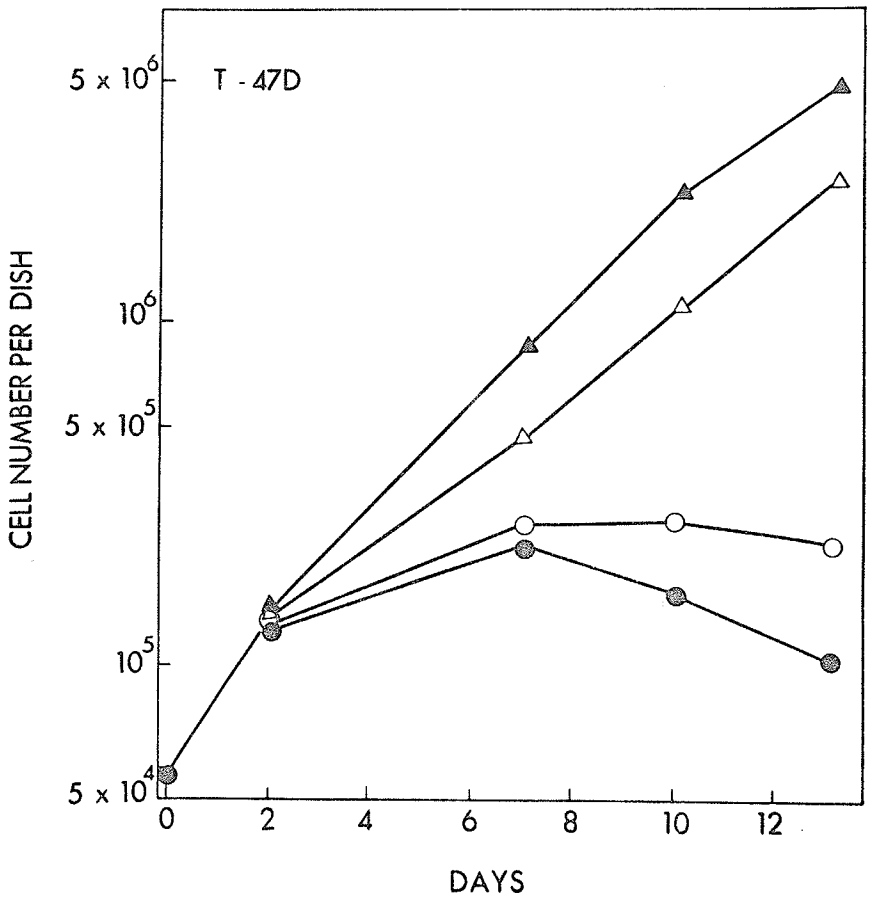


Fig. 9 Effect of fetal bovine serum on growth of T-47D cells cultured as monolayer on plastic dishes

(▲) 10% fetal bovine serum, (△) 1% serum, (○) 0.1% serum

(●) no serum

Each value represents the mean of triplicate. The standard deviation is always less than 10% of the mean.

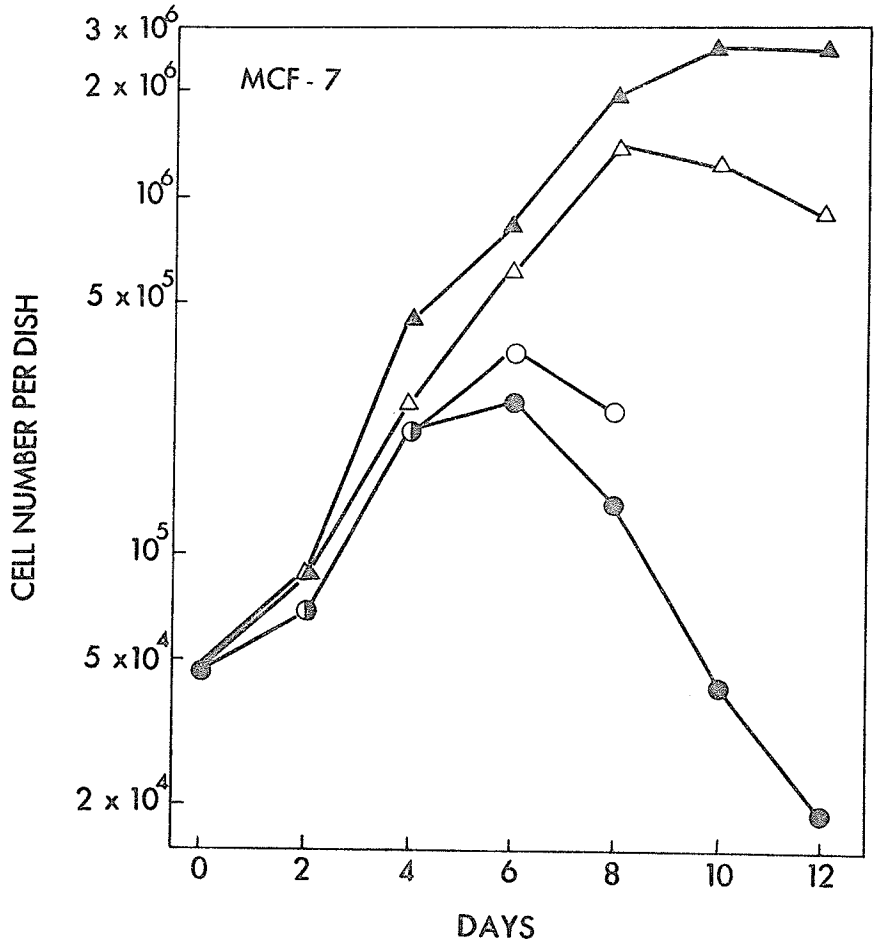


Fig. 10 Effect of fetal bovine serum on growth of MCF-7 cells cultured as monolayer on plastic dishes

(▲) 10% fetal bovine serum, (△) 1% serum, (o) 0.1% serum

(●) no serum

Each value represents the mean of triplicate. The standard deviation is always less than 10% of the mean.

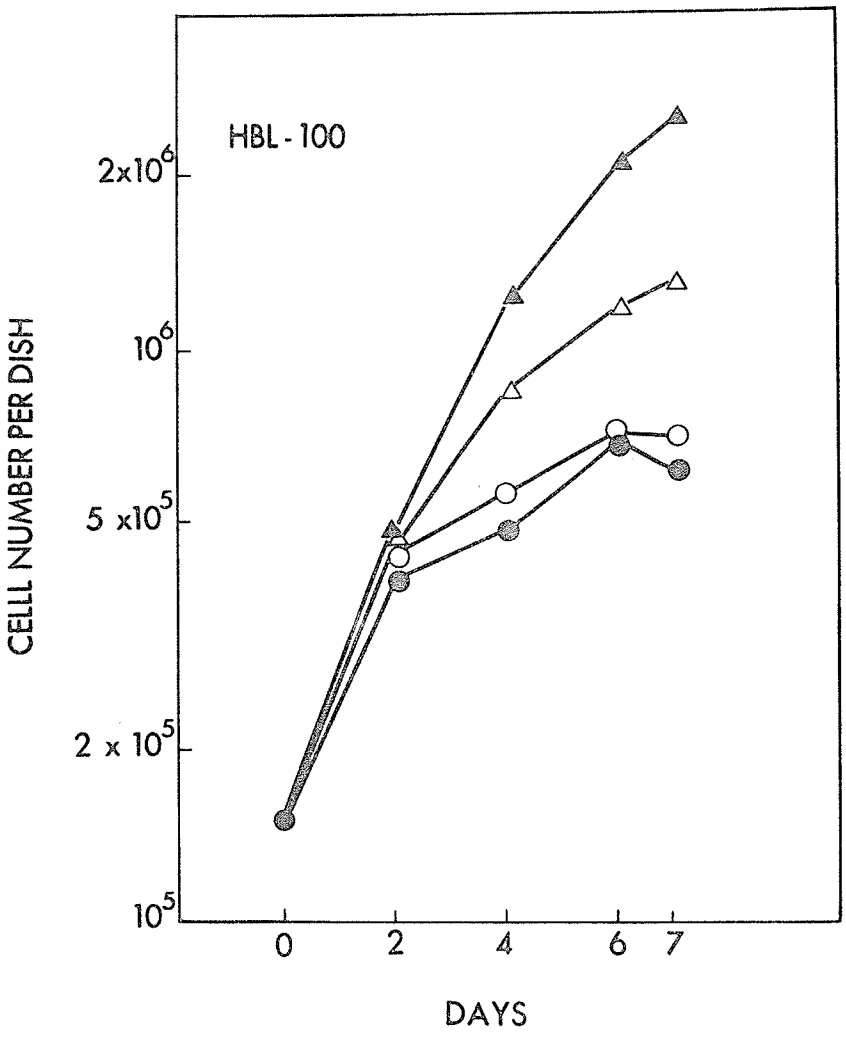


Fig. 11 Effect of fetal bovine serum on growth of HBL-100 cells cultured as monolayer on plastic dishes

(▲) 10% fetal bovine serum, (△) 1% serum, (○) 0.1% serum

(●) no serum

Each value represents the mean of triplicate. The standard deviation is always less than 10% of the mean.

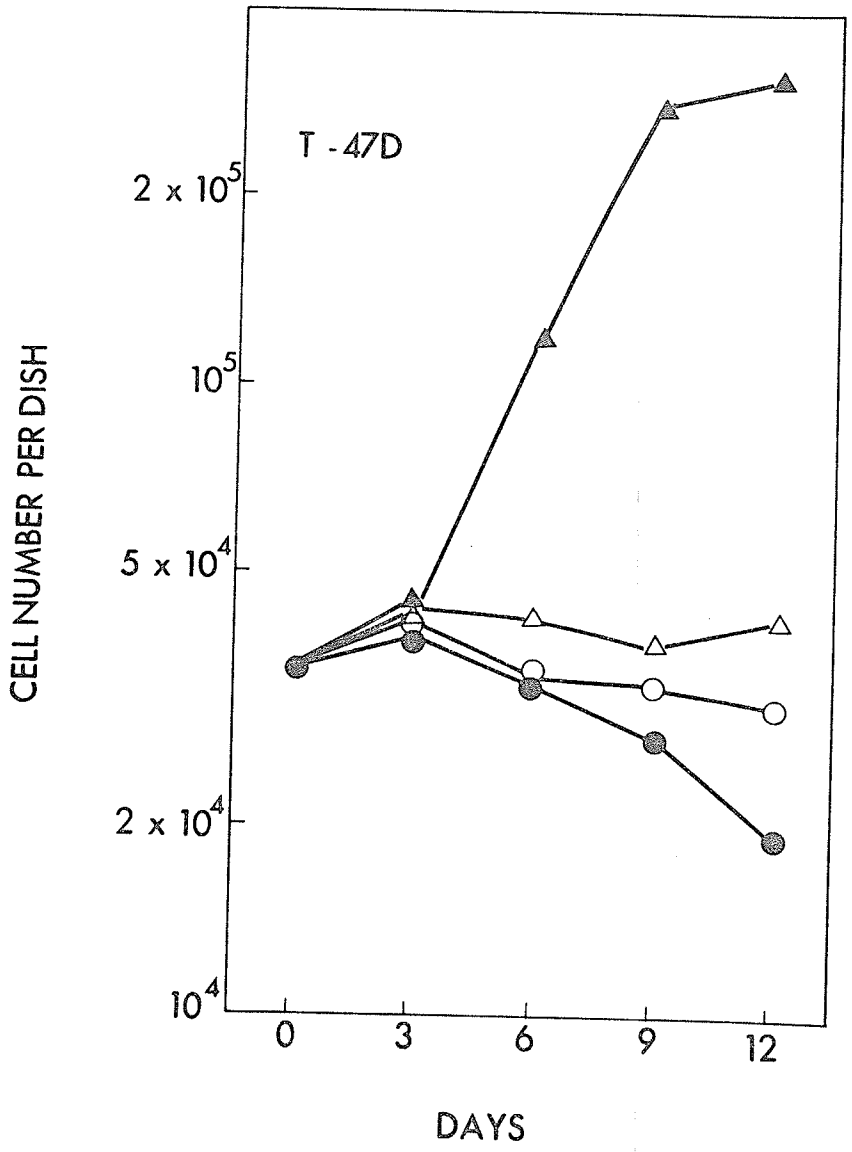


Fig. 12 Effect of fetal bovine serum on growth of T-47D cells cultured in collagen gel

(▲) 10% fetal bovine serum, (△) 1% serum, (○) 0.1% serum

(●) no serum

Each value represents the mean of triplicate. The standard deviation is always less than 10% of the mean.

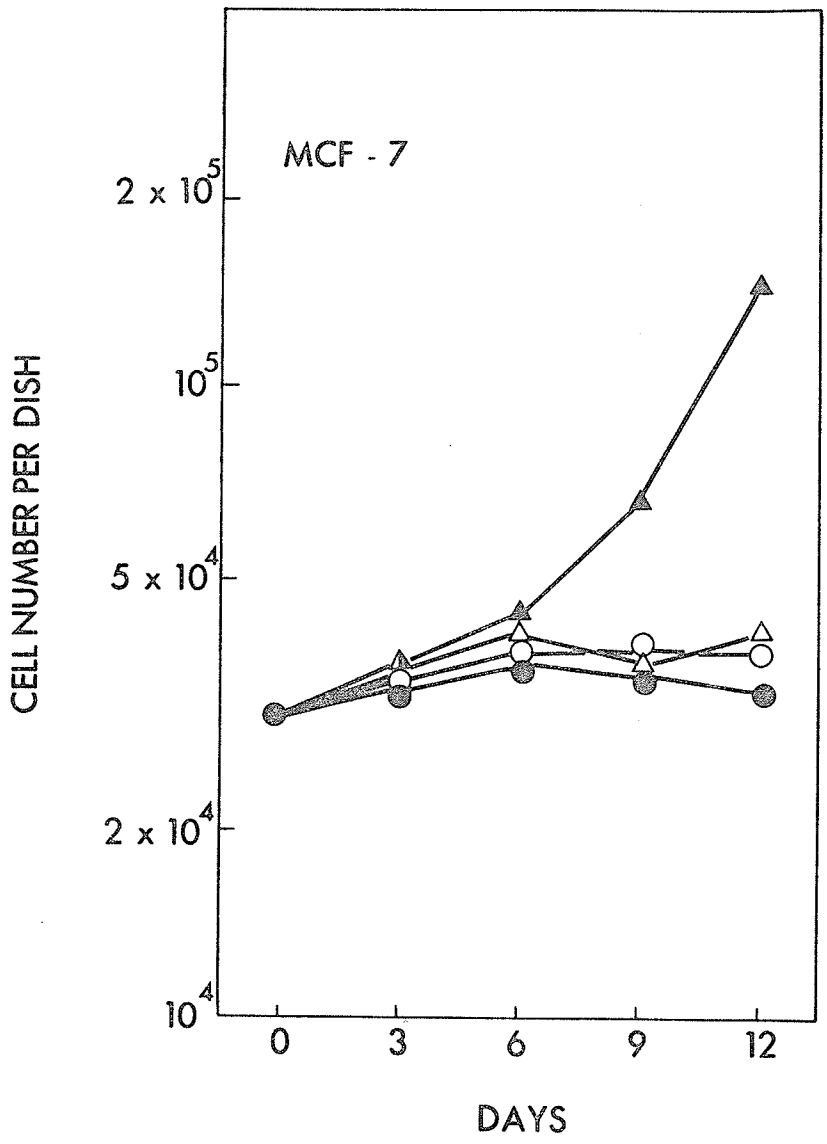


Fig. 13 Effect of fetal bovine serum on growth of MCF-7 cells cultured in collagen gel

(▲) 10% fetal bovine serum, (△) 1% serum, (o) 0.1% serum

(●) no serum

Each value represents the mean of triplicate. The standard deviation is always less than 10% of the mean.

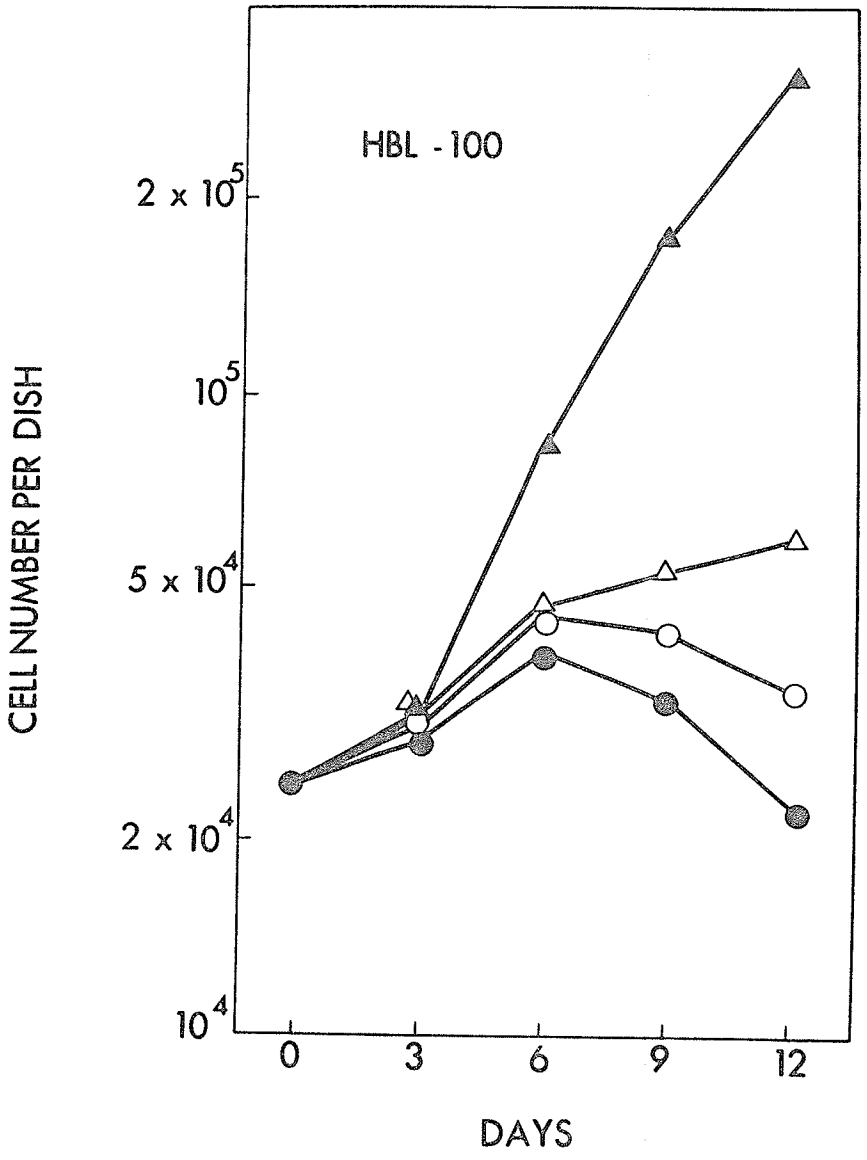


Fig. 14 Effect of fetal bovine serum on growth of HBL-100 cells cultured in collagen gel

(▲) 10% fetal bovine serum, (△) 1% serum, (o) 0.1% serum

(●) no serum

Each value represents the mean of triplicate. The standard deviation is always less than 10% of the mean.

Table 3: Population doubling time of human mammary epithelial cells

Cell Lines	Collagen Doubling Time	Plastic (days)
T-47D	2.25	2.00
MCF-7	2.40	1.50
HBL-100	2.25	1.25

Each value represents the mean obtained from three separate experiments. The cells were maintained in Dulbecco's modified Eagle's medium (DME) containing 4 mm glutamine, 4.5 G/L glucose, 100 IU/ml penicillin, 100 ug/ml streptomycin, 10 ug/ml bovine insulin and 10% (V/V) fetal bovine serum.

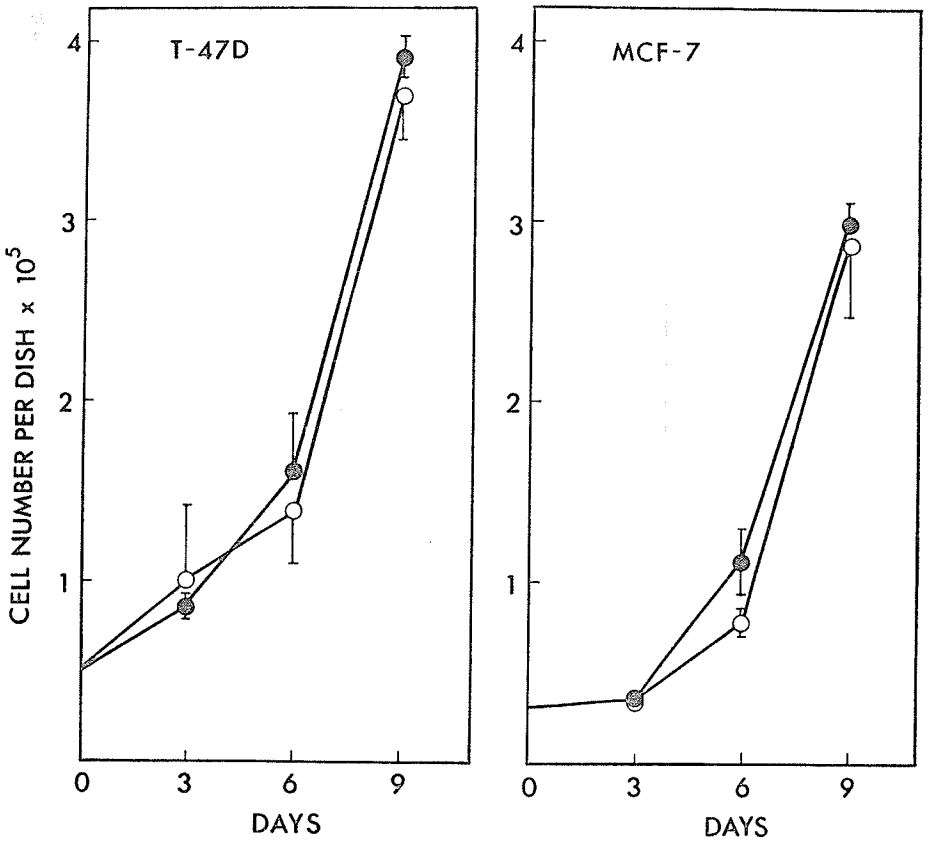


Fig. 15 Growth of T-47D and MCF-7 cells in collagen gel in chemically defined serum-free media

Cells are maintained in Dulbecco's modified Eagle's medium (DME) with 10% fetal bovine serum (●) and in chemically defined serum-free media (o). The defined medium consists of DME with insulin (2 ug/ml), transferrin (4 ug/ml), Dihydrotestosterone (4×10^{-7}M), estradiol (10^{-8}M) and epidermal growth factor (40 ng/ml).

medium is essentially the same as the one reported by Barnes and Sato (1979). There is no significant difference in the growth rate of these cells in either chemically-defined medium or in medium supplemented with 10% serum, suggesting that these human breast cells can respond to the above mentioned hormones when cultured in collagen.

Effects of Hormones and Tissue Extracts on the Growth of T-47D Cells in Collagen Matrix

The effects of several hormones and growth factors on the growth of T-47D cells cultured in collagen gel are shown in Fig. 16. Despite the healthy appearance of the cells, none of the hormones stimulated the growth of T-47D cells in collagen gel as compared to the control (DME supplemented with 1% charcoal treated fetal bovine serum). The growth promoting effect of extracts from various tissues was then tested on the T-47D cells in collagen gel. Although extracts from uterus and kidney stimulated the cells slightly, the tissue extracts in general are not mitogenic.

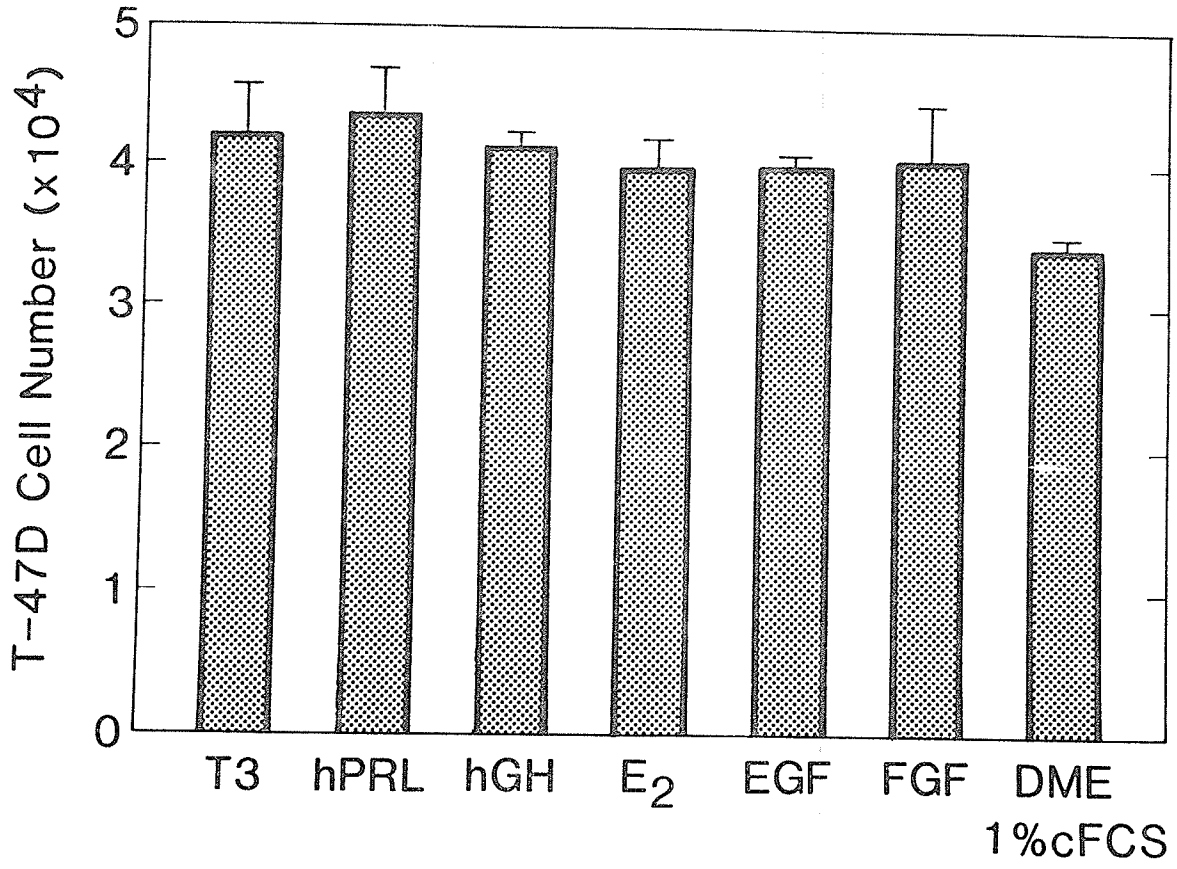
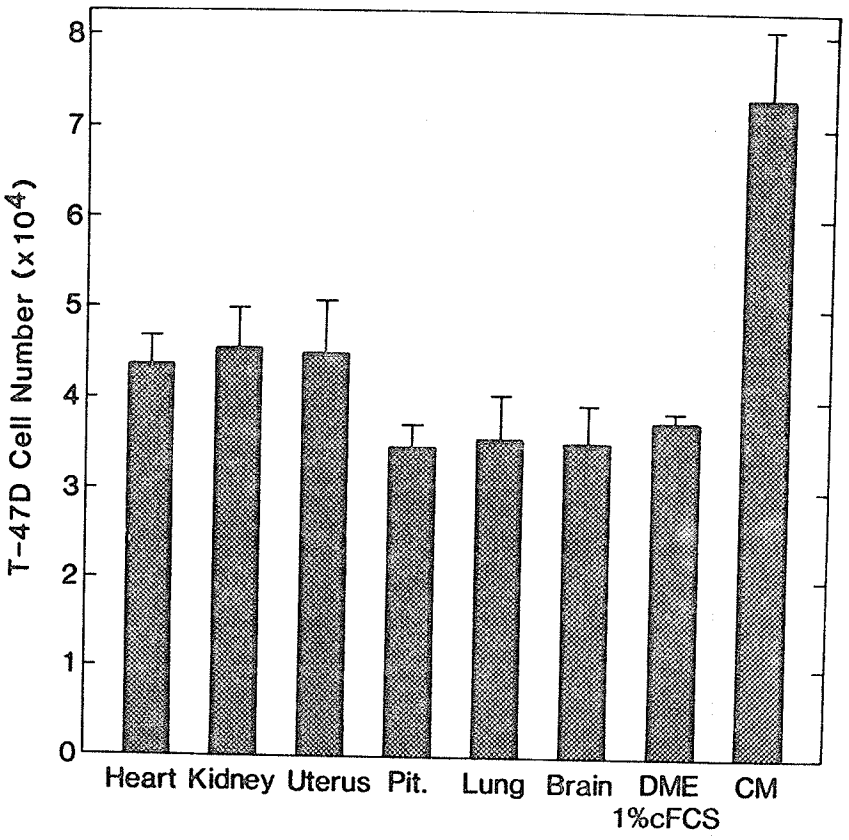


Fig. 16 Effects of hormones and growth factors on the growth of T-47D in collagen matrix

Using multiwell plates, T-47D cells (5×10^4 per well) were plated in DME supplemented with 1% charcoal-treated FBS. Charcoal-treated FBS was prepared by stirring activated charcoal, 100 mg/ml serum, at 55°C for 30 minutes. Hormones were added to the dishes 24 hours later at the following final concentrations: Triiodothyronine (T_3, 10^{-6}M); human prolactin (hPRL, 1 ug/ml); human growth hormone (hGH, 1 ug/ml); 17β-estradiol (E_2, 10^{-8}M); epidermal growth factor (EGF, 10 ng/ml). On day 7, the cells were harvested and counted as described in Materials and Methods. Each value represents mean ± S.D. of triplicate wells.

Fig. 17 Effects of tissue extracts on the growth of T-47D cells in collagen gel

Using multiwell plates, T-47D cells (5 x 10^4 cells per well) were plated in DME supplemented with 1% charcoal-treated FBS. Crude extracts of various organs from female Sprague-Dawley rats were added to each well 24 hours later. Tissue extracts were prepared as follows: The tissue is rinsed with cold distilled water, trimmed and weighed. The tissue is then homogenized in 3 ml of PBS per gram of tissue and sonicated for 20 seconds. The homogenate is centrifuged at 20,000 RPM for 90 minutes. The supernatant is collected and filtered to sterilize. Ten ug of tissue extract is added to each well. The number of cells were determined on day 7 as described in Materials and Methods. Each value represents the mean $\pm$ S.D. of triplicate wells.


T-47D Cell Number (x10⁴)
0
1
2
3
4
5
6
7
8
Heart
Kidney
Uterus
Pit.
Lung
Brain
DME 1%cFCS
CM

DISCUSSION

When human breast cancer cell lines were cultured in collagen matrix, many properties of these cells were altered. The human breast cancer cells were flat and polygonal and formed monolayers on plastic. While in collagen gels the cells became rounded and formed three-dimensional aggregates. Scanning and transmission electron microscopy revealed that the human breast tumor cells (T-47D) were closely associated with the collagen fibrils which seem to serve as anchorage for the growing aggregates of tumor cells. Typical epithelial cell junctions (desmosomes) were found between cells. Similar observations for MCF-7 cells grown on collagen-coated cellulose sponge have been reported (Russo et al, 1977). Interestingly, HBL-100 cells, which were derived from normal human milk, did not form large clusters when maintained in collagen. Instead, they lined up in a polarized fashion to form an interlacing pattern. This is in contrast to the tumor cell lines, T-47D and MCF-7, which formed tumor-like structures in collagen.

When the T-47D and MCF-7 cells were cultured in collagen, there was a lag time of 48-72 hours before cell division occurred. It is possible that the cells require this length of time to produce substances which interacted with the collagen matrix. Some of the substances synthesized by these cells

could be type IV basement membrane collagen, laminin or fibronectin. Wicha et al (1979) have shown that normal ductal and alveolar cells from rat mammary gland, when cultured on type I collagen, required 24 hours before cell growth commenced. However, if the cells were grown on type IV collagen, this 24 hour lag time was not observed. The attachment of human carcinoma and sarcoma cells onto collagen coated dishes also has been shown to be mediated by laminin and fibronectin, respectively (Vlodavsky and Gospodarowicz, 1981).

The doubling time of the human breast cells maintained in collagen was longer as compared with that of cells on plastic. Folkman and Moscona (1978) demonstrated that cell shape was coupled to DNA synthesis and growth of bovine aortic endothelial cells and WI-38 lung fibroblasts. By varying the adhesiveness of plastic tissue culture dishes with various concentrations of poly (2-hydroxyethyl methacrylate), they have shown that the rate of incorporation of tritiated thymidine by the cells was inversely proportional to the height of the cells. Thus, the slower growth rate of the human breast cells in collagen may be related to the change of cells shape. It also has been suggested that cell shape may be related to the expression of cellular differentiation (Bennet, 1980; Burwen and Pitelka, 1980; Emerman et al, 1979). Hence, cells cultured in collagen gel may be in a more differentiated state than those grown on plastic.

In addition to alteration of cell shape and growth rate, the human breast cells became more serum dependent when cultured in collagen. The human breast cells failed to proliferate at low serum concentrations (≤1%). Proliferation of these cells required the presence of 10% fetal bovine serum. In contrast, the growth rate of these cells maintained on plastic substratum in the presence of 1% serum was comparable to that produced by 10% serum. The mechanism for this increase in serum dependency of cells grown in collagen is not known. It is possible that some serum factors may be required for the cells to interact with the collagen fibrils. The observation that human breast tumor cells cultured in collagen proliferated in chemically-defined medium suggested that these cells are responsive to hormones and growth factors. The chemically-defined medium contains insulin and transferrin which have been shown to be the two essential factors for the growth of human breast cancer cells maintained on plastic substratum (Barnes and Sato, 1979).

Our results show that human breast tumor cell lines can be cultured in collagen, a principal component of the extracellular matrix. Many cells cultured in the presence of extracellular matrix have been shown to retain their in vivo characteristics (Gospodarowicz and Tauber, 1980). Corneal epithelial cells maintained as monolayers on plastic dishes responded to the addition of fibroblast growth factor (FGF) but

not to epidermal growth factor (EGF), although EGF is known to stimulate the proliferation of corneal epithelial cells in vivo or in organ cultures (Gospodarowicz et al, 1978; Savage and Cohen, 1973). However, when the same cells were cultured in collagen, they were stimulated by EGF but not by FGF. Therefore, the use of cells maintained in collagen matrix may lead to identification of those trophic factors which are also active in vivo.

The effects of various hormones and growth factor on DNA synthesis of human breast cancer cells cultured in collagen matrix were then studied (Figs. 16, 17). We were unable to demonstrate any significant growth promoting effect of the hormones on the breast cancer cells despite the healthy appearance of these cells cultured in collagen matrix. It is possible that type 1 collagen may not be the most suitable substrate for culturing epithelial cells. Recently, mammary epithelial cells were shown to grow preferentially on type 4 collagen (Wicha et al., 1980). Alternatively, since type 1 collagen is only a very small component of the extremely complex extracellular matrix (ECM), it is conceivable that the presence of other components of the ECM may be necessary for the breast cancer cells to respond to the trophic factors. Thus, from these studies we concluded that human breast cancer cells can be successfully cultured in type 1 collagen. However, in order for the human breast cancer cells to be

hormonally responsive in terms of growth, ECM may be a better substrate for culturing these cells.

There are several alternate explanations for the lack of response of the human breast tumor cells to prolactin and growth hormone *in vitro*. First, permissive factors, which are absent in *in vitro* situations, are needed to render the cells responsive to prolactin. Second, prolactin and growth hormone may act *in vivo* through an intermediate pathway which is missing in the *in vitro* experiments. Third, a pituitary factor, other than prolactin or growth hormone, is responsible for growth regulation of human breast cancer. Future experiments using *in vivo* models such as the athymic nude mice may provide answers to the hypotheses proposed.

SECTION 6: Required Presence of Both Estrogen and Pituitary Factors in the Growth of Human Breast Cancer Cells Transplanted in Athymic Nude Mice.

INTRODUCTION

Ovarian and pituitary hormones play important roles in the etiology of breast cancers (Jensen and DeSombre, 1972; McGuire et al, 1978; Welsch and Nagasawa, 1977). The most extensively studied of these hormones are estrogen and prolactin, both of which are able to promote the growth rate of experimental breast tumors in rodents (Holdaway and Friesen, 1976; Kim and Furth, 1976; Leung and Sasaki, 1975; Meites et al, 1972; Pearson et al, 1969). The involvement of prolactin in the tumorigenesis of the human breast is still unclear (Nagasawa, 1979). Whether or not trophic factors other than these two hormones are essential for growth of breast cancers remains to be elucidated.

In an attempt to study the mechanism of action of steroid and polypeptide hormones in the control of proliferation of

breast cancers, many investigators have turned to in vitro models, breast cancer cells maintained in tissue culture (Cailleau et al, 1978; Engel and Young, 1978; Lasfargues and Ozzello, 1958; Lippman et al, 1977). This approach is particularly useful in the study of human breast cancers. For example, a large number of trophic factors derived from many organs were found to stimulate the proliferation of breast cancer cells. Some of these factors include insulin (Barnes and Sato, 1979; Cohen and Hilf, 1974; Osborne et al, 1976), epidermal growth factor (Taylor-Papadimitriou et al, 1977), mammary growth factor (Kano-Sueska et al, 1977; Ptashne et al, 1979; Rudland et al, 1977; Rudland et al, 1979), and many tissue-derived factors (Eastment and Sirbasku, 1978; Sirbasku, 1978; Sirbasku and Benson, 1979). Moreover, a critical and thorough review of the literature describing in vitro studies using cultured cells reveals a number of puzzling and paradoxical findings. For instance, whether estrogen has a direct mitogenic effect on breast cancer cells is still unresolved (Sonnenschein and Soto, 1980), although many of these cells contain estrogen receptors (Engel and Young, 1978). Likewise, prolactin has not been shown to be active in stimulating human breast cancer growth in vitro (Lippman et al, 1977; Shafie and Brooks, 1977; Shiu, 1981; also see Section 5), despite the fact that many of the breast cancer cells possess receptors for prolactin (Shiu, 1979). These in vitro studies

indicate that hormonal influences on breast cancer cells *in situ* is a complex phenomenon involving the interplay of many trophic factors derived from many organs.

Athymic nude mice have been used as a model to study behavior of human tumors *in vivo* (Fogh et al, 1977; Giovanella et al, 1974; Shimosato et al, 1976). The growth of one human breast cancer cell line, MCF-7, transplanted into nude mice was stimulated by pituitary explants in the kidney capsule (Russo et al, 1976), and by exogenous estrogen and insulin (Shafie et al, 1980). McManus et al (1978) reported that the incorporation of ^{3}H-thymidine into benign human breast tumor transplanted into nude mice was stimulated by injection of human placental lactogen. These studies indicate that the hormonal influences on human breast tumor cells can be studied *in vivo* in nude mice.

In this section, we present evidence to suggest that *in vivo* growth of human breast tumor cell line, T-47D, in athymic nude mice requires the simultaneous presence of estrogen and pituitary factor(s).

MATERIALS AND METHODS

Tumor Cell Lines

Two tumor cell lines were used in this study. A pituitary tumor derived clonal cell line GH_3 obtained from American Type Culture, Rockville, Md. (Tashjian et al, 1970) was

injected into nude mice to serve as an exogenous source of pituitary factors. The human breast cancer cell line, T-47D, obtained from EG & G Mason Research Institute, Rockville, Maryland, was derived from the pleural effusion of a patient with disseminated carcinoma of the breast (Keydar et al, 1979).

Athymic Nude Mice

Four to five week old female Balb/c athymic nude mice were obtained from A.R.S. Sprague Dawley, Madison, Wisc. Animals were kept under standard conditions for a 5-7 day period prior to use. The animals were kept inside a laminar-flow air filtration system and food and water were supplied ad libitum.

Inoculation of Cells in Nude Mice

The tumor cells (T-47D and GH_3) were routinely maintained in T-75 flasks with Dulbecco's Modified Eagle's Medium supplemented with 10 ug/ml insulin, 4 mM L-glutamine, 4.5 g/l glucose, 50 ug/ml gentamicin and 10% fetal bovine serum. Cells were detached with trypsin-EDTA and resuspended in a small volume of medium. The cells were injected subcutaneously in the flanks of the animals: T-47D on the left and GH_3 on the right.

In the first experiment, T-47D was injected at a dose of 10^7 cells and GH_3 was injected at 10^6 cells per animal. In the second experiment each animal received doses of $2x10^7$ T-47D cells and $4x10^5$ GH_3 cells. Some animals also

received 500 ug estradiol valerate once every two weeks. Estrogen was injected s.c. at the dorsal midline caudal to the neck.

Growth and Histology of the Tumors

The tumors were measured by calipers in three dimensions and their sizes were expressed as the product of the values obtained.

At the completion of the experiments, the tumors were dissected and weighed. Some of the tumors were fixed in glutaraldehyde and prepared for histological examination. The sections were stained by hematoxylin and eosin.

Determination of Hormones in Sera by Radioimmunoassays

The concentrations of growth hormone (GH) and prolactin (PRL) in sera from nude mice were measured by radioimmunoassays using kits for rat GH and PRL from The Endocrine Study Section, National Institute of Arthritis, Metabolism and Digestive Diseases, N.I.H., Bethesda, Md.

RESULTS

The presence of receptors for prolactin (Shiu, 1979) and estrogen (Keydar et al, 1979) in the human breast cancer cells, T-47D, prompted us to examine whether or not these two hormones and possibly other pituitary factors affect the growth of T-47D cells transplanted in athymic nude mice. A rat pituitary tumor

clonal cell line, GH_3, was used since these cells are known to secrete growth hormone and prolactin (Tashjian et al, 1970). Four groups of nude mice were used in the first experiment: mice in group I (T) were injected with T-47D cells; group II mice (TE) received both estrogen and T-47D cells; group III mice (TG) were injected with GH_3 and T-47D cells (one cell type on each flank); and mice in group IV (TEG) were injected with T-47D, GH_3 cells and estrogen. The growth of T-47D human breast cancer cells, T-47D, in these four groups of nude mice are shown in Fig. 18.

Growth of T-47D Human Breast Tumors in Nude Mice

In the control group (T), T-47D tumor was visible up to 18 days after injection, but regressed completely after day 25. The T-47D tumor in the TG group, similar to the T group, also regressed completely despite the fact that the GH_3 tumor was growing (see Fig. 22) and the serum levels of GH and PRL were high (See Table 4). The T-47D tumors of the TE group showed slow but sustained growth, reaching a size of 25 ± 14 (SD) mm^3 on day 32. Simultaneous injection of E_2 and GH_3 further enhanced the growth of T-47D tumor in the TEG group reaching a tumor size of 150 ± 42 mm^3. This represented a six-fold increase in T-47D tumor size as compared to those in the TE group. The appearance of the four groups of mice is shown in Fig. 24.

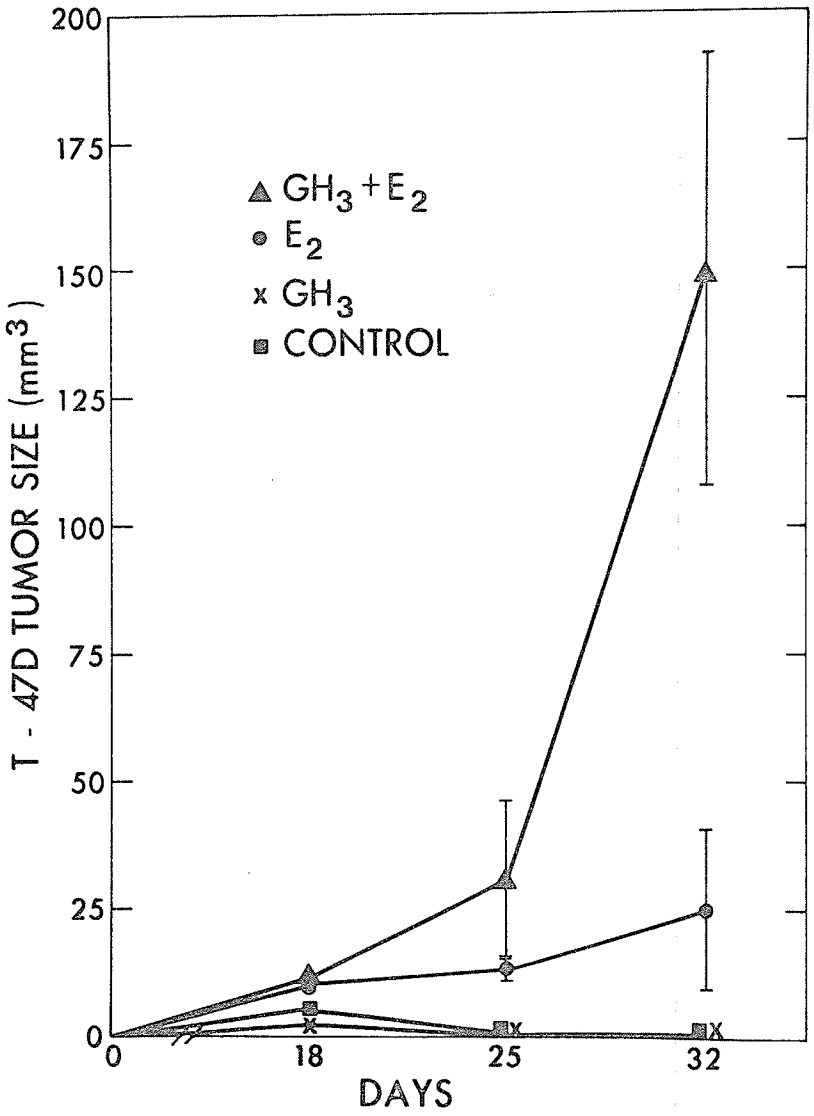


Fig. 18 Growth of T-47D tumors in nude mice (Experiment 1)

Each point represents the mean ± standard deviations from 4 animals. Procedures for determining growth of tumors were described in Methods. Injection of 10^7 of T-47D cells and 10^6 of GH_3 cells per animal was made.

In the first experiment we observed that GH_3 tumors grew at a greater rate than T-47D tumors. The large size of the GH_3 tumors resulted in the death of some animals after 32 days. In order to substantiate our findings of the first experiment and to observe the growth of the human tumor for a longer period of time, we repeated the experiment in which more T-47D and fewer GH_3 cells were injected as compared with the first experiment (see Methods). Only the TE and TEG groups were included in this experiment (Fig. 19). The growth pattern of the T-47D tumor was very similar to that of the first experiment. The T-47D tumor in estrogen-treated mice (TE) showed sustained growth up to 63 ± 52 mm^3 by day 42. In contrast, the T-47D tumor in the TEG group grew to 528 ± 113 mm^3 which was about an eight-fold increase in size compared to the TE group.

All animals were sacrificed on day 42 and the tumors were dissected and weighed. Figure 20 shows that the T-47D tumor in the TE group averaged 34 ± 11 mg, whereas that of TEG group averaged 175 ± 26 mg. This represents a 5.2-fold difference in tumor weight and was in good agreement with the values determined by measuring tumor size. Histologically, the T-47D tumor consisted of a homogeneous mass of epithelial cells, surrounded by a fibrous capsule.

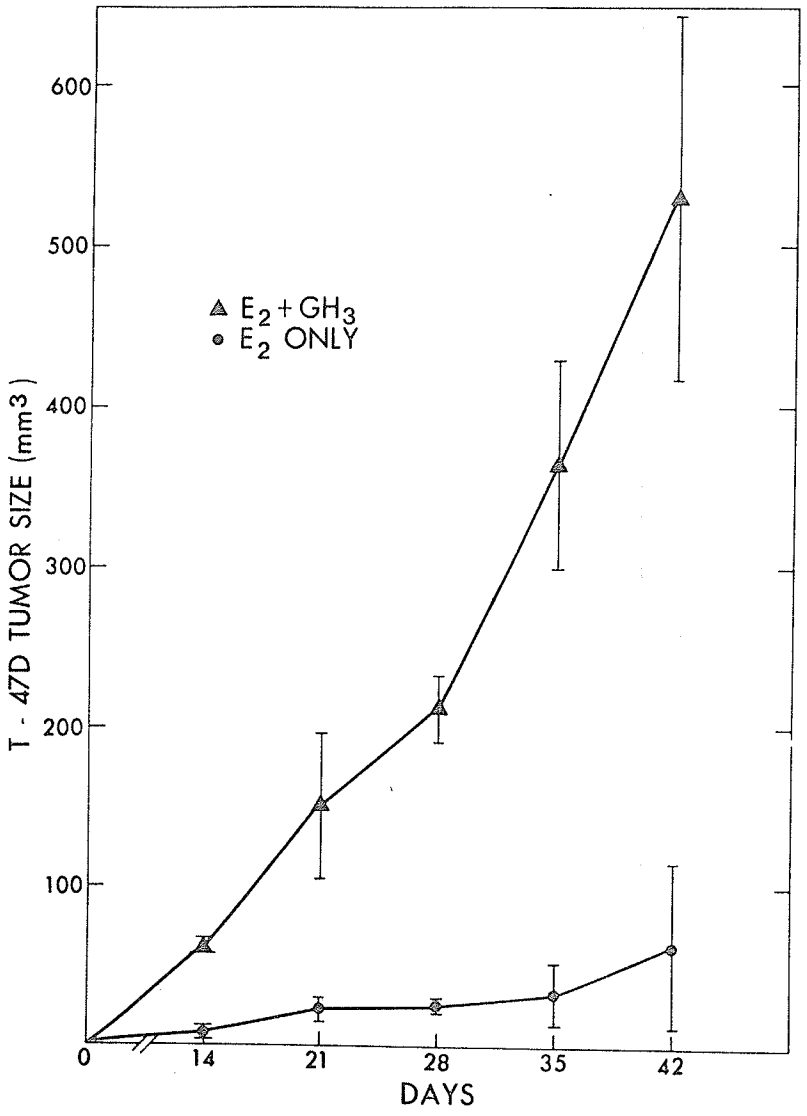


Fig. 19 Effects of estrogen and/or GH_3 cells on growth of T-47D tumor in nude mice (Experiment 2)

The protocol of this experiment is essentially the same as that in Fig. 18 except that only two conditions, T-47D and estrogen (TE) and T-47D + Estrogen + GH_3 (TEG), were tested in this experiment. Injection of T-47D ($2x10^7$) cells and GH_3 cells ($4x10^5$) per animal was made.

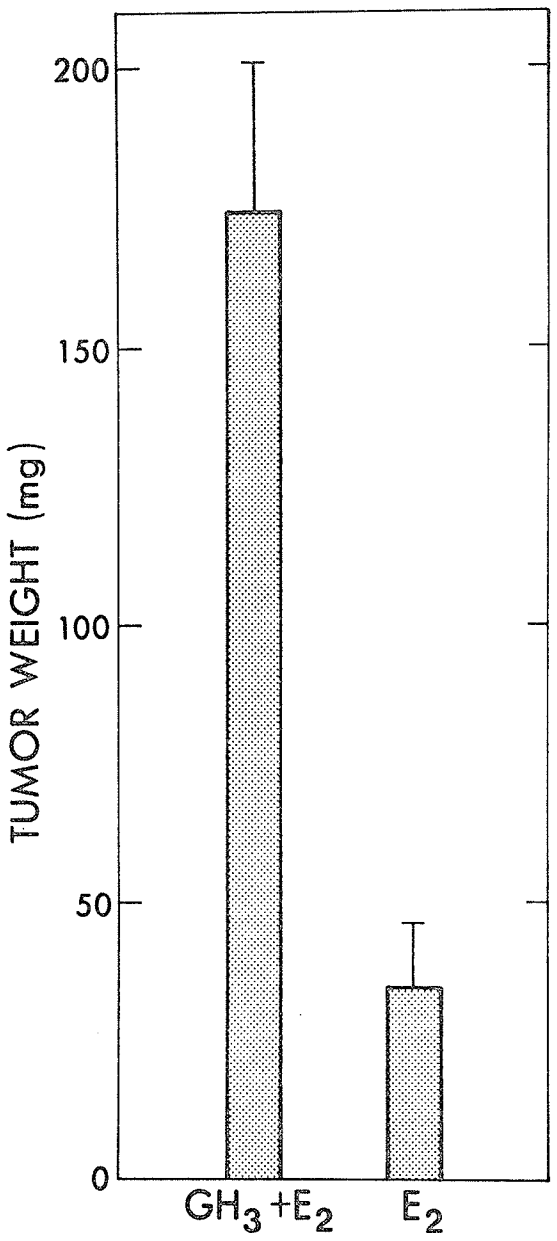


Fig. 20 Effects of estrogen and GH_3 cells on T-47D tumor weight

Each value represents the mean ± standard deviation from 4 animals. The T-47D tumors in GH_3 + Estrogen (TEG) treated mice were significantly heavier than those in estrogen-treated mice (TE) ($P < 0.001$).

Growth of GH_3 Rat Pituitary Tumors in Nude Mice

We further examined whether or not the patterns of growth and hormone secretion of the pituitary tumor cells GH_3 were correlated with the growth of T-47D human tumors. The growth of GH_3 tumors in the TG and TEG groups is illustrated in Fig. 22. The GH_3 tumors grew in the absence of exogenous estrogen to a size of approximately 1 cm^3 whereas estrogen stimulated the growth of GH_3 tumors to approximately 4 cm^3 by day 32. This result was confirmed by measuring the GH_3 tumor weight (Fig. 21). This finding is in agreement with that reported by Sorrentino et al (1976) using GH_3 cells transplanted in Wistar-Furth rats. In the second experiment, where few GH_3 cells were injected, administration of estrogen stimulated the growth of GH_3 tumor to approximately 2.5 cm^3 at 32 days and reaching 5 cm^3 on day 42.

Serum Concentration of GH and PRL

Since T-47D tumor did not grow in the presence of GH_3 tumor alone (i.e. TG), we therefore determined the serum concentration of GH and PRL (Table 4). All the animals in the TEG and TG groups had higher serum GH and PRL levels than the T and TE groups; the hormones in the latter 2 groups were apparently of pituitary origin. The trend of serum GH and PRL levels in these nude mice was TEG > TG > TE > T. It was interesting to note that in the TG group the levels of PRL and GH achieved already were elevated, being 437 ng/ml and 1008

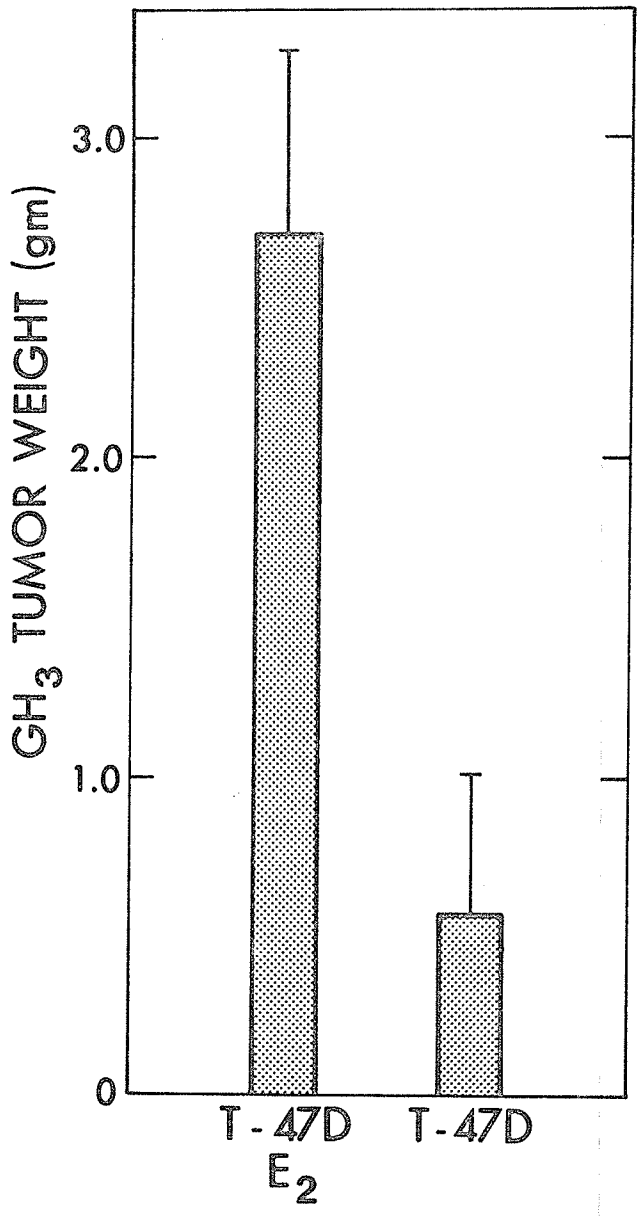


Fig. 21 Effects of estrogen and T-47D cells on GH_3 tumor weight

Each value represents the mean ± standard deviation from 4 animals. The GH_3 tumors in TEG group were significantly heavier than those in the TG group ($P < 0.001$).

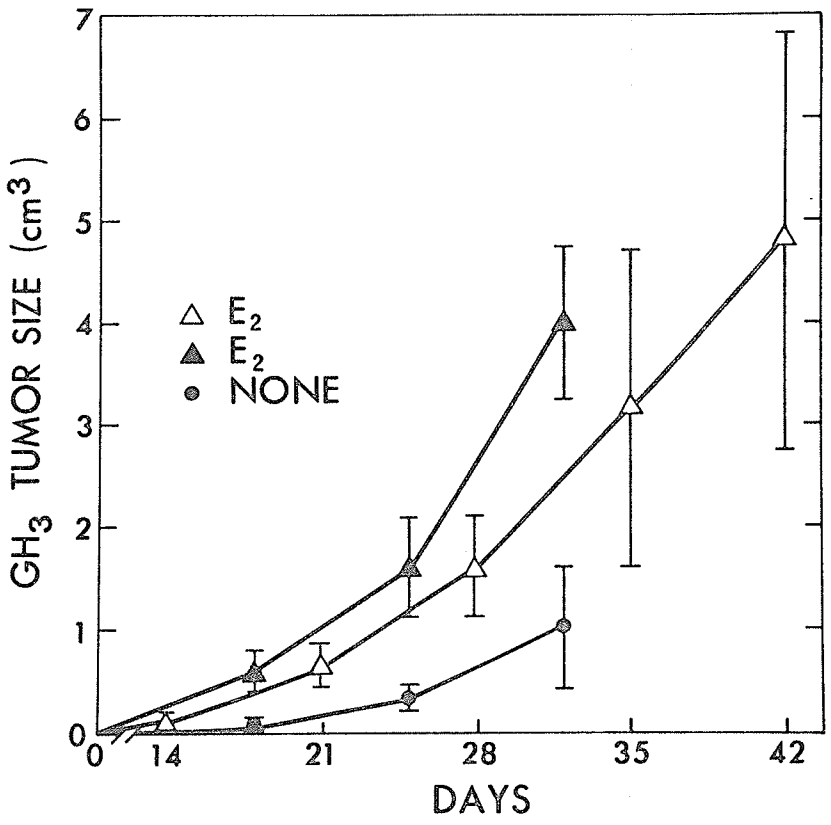


Fig. 22 Effects of estrogen on growth of GH_3 tumor in nude mice

(▲) and (●) were values obtained from the first experiment. (△) were values obtained from the second experiment. Each value represents the mean ± standard deviations from 4 animals.

Table 4: Serum levels of rat growth hormone and rat prolactin in athymic nude mice

Animal Group	Growth Hormone ng/ml		Prolactin ng/ml	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
T	58 ± 43	----	8 ± 5	----
TE	181 ± 91	142 ± 60	26 ± 12	9 ± 2
TG	1008 ± 204	----	437 ± 376	----
TEG	8900*	4114 ± 4702	7300*	4560 + 3769

The serum samples were collected at the completion of each of the experiments. Rat growth hormone and prolactin were determined by radioimmunoassays.

* In the first experiment, only one mouse of the TEG group survived after 32 days. This animal was bled on the 36th day.

ng/ml respectively. This suggested that the failure of T-47D tumor to grow in this group was apparently not due to a lack of circulating GH and PRL. It is apparent from Table 4 that there was a great variation in the concentrations of GH and PRL in the sera of the TEG group. This prompted us to exmaine whether or not the growth of T-47D and GH_3 tumors was correlated with the concentrations of the two hormones. Table 5 shows the correlation coefficient (r) of serum hormone concentrations and tumor weights. There was very little correlation between prolactin concentrations and the T-47D tumor weight ($r = 0.37$), while a good correlation between growth hormone concentration and T-47D tumor weight was noted ($r = 0.95$). In contrast, the serum concentrations of the two hormones correlated well with the weights of the GH_3 tumors, the correlation coefficient being 0.91 for PRL and 0.75 for GH.

Body Weights of Nude Mice

The body weights of all animals were recorded at the completion of the experiment after the tumors were removed (Fig. 23). The estrogen-treated mice weighed slightly less (not significant) than the corresponding groups that received no steroid (compare T vs TE and TG vs TEG). This was probably due to the general growth inhibitory effect of estrogen. The body weights of nude mice injected with GH_3 cells (TG) were the highest of all groups, presumably due to the effect of high concentration of GH in the blood. Despite the presence of

Table 5: Correlation between serum hormone concentrations and tumor weights*

Serum Concentrations	Tumor Weight	
	T-47D	GH_3
Rat Prolactin	0.37	0.91
Rat Growth Hormone	0.95	0.75

* data derived from the TEG group of the 2nd experiment. Values represent correlation coefficient obtained by polynomial regression analysis.

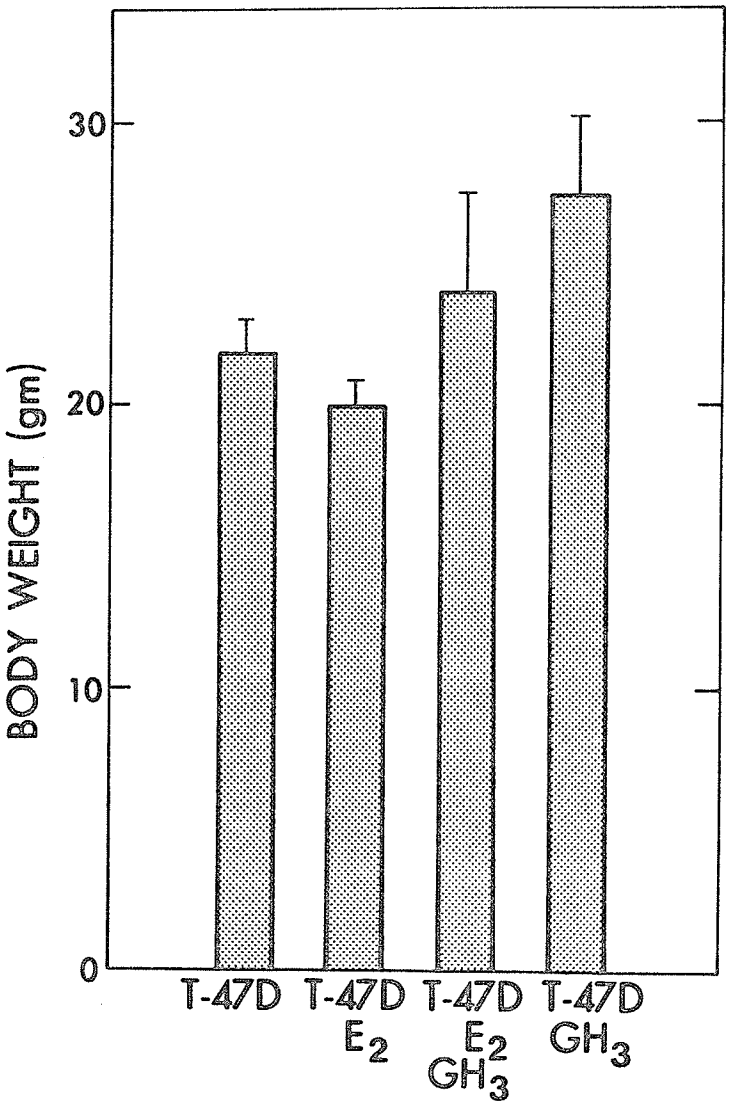


Fig. 23 Effects of estrogen and/or GH_3 tumors on body weight of nude mice

Each value represents the mean ± standard deviations from 4 animals after the tumors were removed. The measurements were recorded 32 days after treatment (Experiment 1).

varying sizes of tumors in the four groups of animals, the body weights in the treatment groups remained comparable (ranging from 20 to 27 gm).

Fig. 24 Morphology of athymic nude mice with T-47D tumor and/or GH_3 tumor

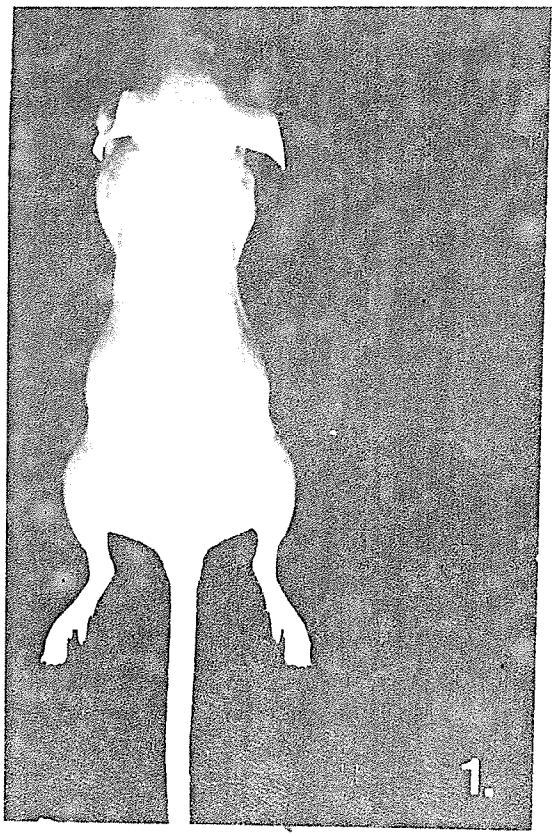
1) Control (T). Injected with T-47D cells only. No tumor present.

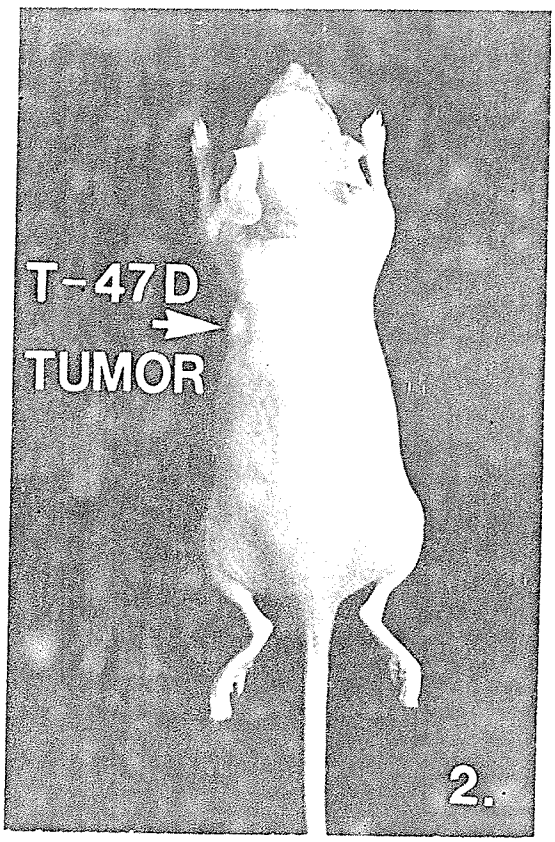
2) T-47D + E_2 (TE). Small but definite growth of T-47D tumor.

3) T-47D + GH_3 (TG). No T-47D tumor present.

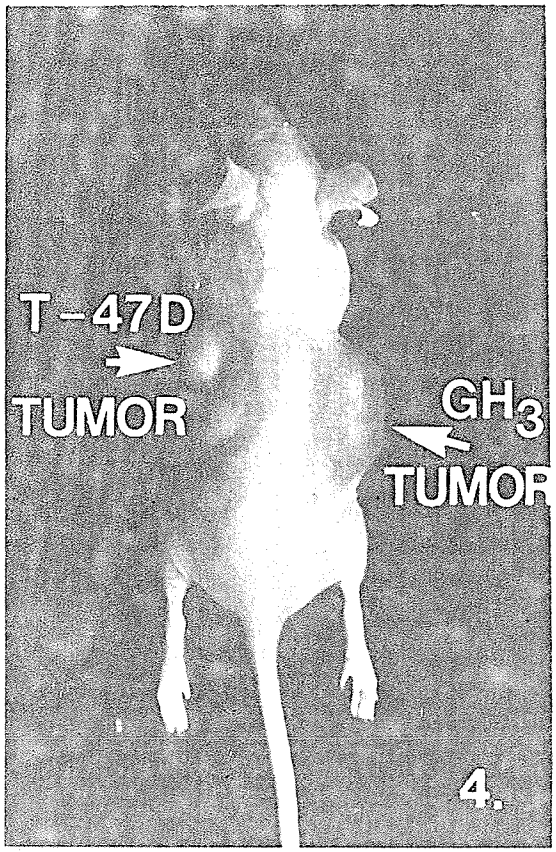
4) T-47D + E_2 + GH_3 (TEG). Both T-47D and GH_3 tumor are present.

The pictures were taken 32 days after treatment.


1.


T-47D
TUMOR
2.


GH3
TUMOR
3.


T-47D
TUMOR
GH3
TUMOR
4.

DISCUSSION

The results of our study indicate that the growth of human breast cancer cells (T-47D) transplanted into athymic nude mice is stimulated by the simultaneous presence of estrogen and GH_3 rat pituitary tumor cells. We chose to use GH_3 cells for our study since 1) they are known to secrete prolactin and growth hormone and the secretion of these hormones is influenced by estrogen; 2) there is increasing evidence to suggest that the pituitary gland produces many growth factors other than the traditional hormones. The presence of fibroblast growth factor, ovarian growth factor and mammary growth factor in the pituitary has been reported (Gospodarowicz and Moran, 1976; Kano-Sueska et al, 1977; Ptashne et al, 1979; Rudland et al, 1979). Several of these growth factors have been shown to affect the growth of breast cancer cells in vitro. In view of the lack of convincing evidence for a role of prolactin in the etiology of human breast cancers (Nagasawa, 1979), there is more reason to search for and identify other factors which might regulate the proliferation of human breast cancer cells.

The T-47D human breast cancer cells injected into female nude mice (T) did not proliferate. This suggests that the hormonal milieu in the athymic nude mouse is not optimal for

the growth of the human cancer cells. From their study using a different human breast cancer cell line (MCF-7), Russo et al (1976) also came to a similar conclusion. The fact that T-47D cells proliferated in estrogen-treated hosts (TE and TEG) supports the current view that estrogen is involved in the growth regulation of some human breast cancers. However, injection of estrogen alone (TE) resulted only in a very moderate growth of T-47D tumor. This suggests that estrogen alone cannot produce maximal growth of the T-47D tumor. Other hormones, factors or mediators are required.

It is somewhat surprising to observe that in mice bearing GH_3 tumors (TG), no apparent growth of the human breast cancer cells was observed in the absence of estrogen despite the high prolactin and growth hormone concentrations in the blood of the host. Thus, prolactin and/or growth hormone alone are not sufficient to stimulate the proliferation of T-47D tumor in nude mice. The simultaneous presence of estrogen and GH_3 cells (TEG) induced rapid and sustained growth of T-47D tumors. There are several possible mechanisms to explain the apparent synergism of estrogen and GH_3 cells on the growth of human breast cancer cells. Firstly, it is plausible that the extreme high level of PRL/GH obtained in the TEG group may be responsible for the rapid growth of the T-47D tumor. However, this explanation seems improbable because the serum concentration achieved in the TG group was already

unphysiologically high and yet no growth of T-47D tumor was observed. Secondly, an inital action of estrogen on T-47D cells is required in order that prolactin and/or growth hormone can exert their direct effects; alternatively, PRL/GH may potentiate the effect of estrogen. We feel that this mechanism is unlikely because prolactin and growth hormone, by themselves or in combination with estrogen, were not able to stimulate the growth of T-47D cells in vitro (Shiu and Leung, unpublished observation). In fact, no convincing direct mitogenic effect of prolactin and growth hormone on human breast cancer cells has been reported (Lippman et al, 1977; Shafie and Brooks, 1977). Since animal growth hormones are not active in man and are unable to bind to growth hormone receptors in human tissues (Carr and Friesen, 1976), a direct effect of rat growth hormone on T-47D cells can be ruled out. Rat prolactin may be able to stimulate human cells because animal prolactin can compete with human prolactin for prolactin receptor sites in T-47D cells (Shiu, 1979).

Thirdly, the stimulatory effect of estrogen or PRL/GH or both could be mediated by growth factors derived from organs of the host animals. In the case of estrogen, Sirbasku and Benson (1979) suggested that the growth-stimulating effect of estrogen in many estrogen-responsive cell types is mediated by tissue-derived intermediates which they termed "estromedins". As for PRL/GH, it is possible that tissue-derived factors such

as the somatomedin-like substances (Phillips and Vassilopoulou-Sellin, 1980 a, b) are responsible for stimulating the growth of the human cells in the nude mice. Our observation that the growth of T-47D human tumors in nude mice correlated well with the serum concentration of rat growth hormone, but not with rat prolactin, suggest that growth hormone intermediates might have been partly responsible for the growth of the human cells. This hypothesis requires that the "estromedins" and "somatomedins" produced by mouse tissues are active in human cells and they have to be present simultaneously.

The fourth and the simplest mechanism which can account for our findings is that estrogen stimulates GH_3 pituitary cells to produce a novel growth factor (not PRL or GH) which in turn stimulates the growth of T-47D human tumor cells. The secretion of this factor by GH_3 cells is dependent on estrogen. Perhaps this factor can be termed pituitary-derived "estromedin". Whether or not this growth factor is unique to the GH_3 pituitary tumor cells or whether it is also produced by normal pituitary glands cannot be ascertained in this study. However, Russo et al (1976) demonstrated that MCF-7 human breast cancer cells formed tumors in nude mice that received transplants of normal rat pituitary glands and ovarian grafts. This study indicates that normal pituitary gland is capable of stimulating the growth of human breast cancer cells,

suggesting that growth factors can be produced by normal pituitary glands. This will explain the slow but sustained growth of T-47D tumors in nude mice treated only with estrogen (TE) because the nude mice used in our study possessed intact pituitary glands. Estrogen could have induced the production of small amounts of growth factor by the pituitary glands of nude mice. This fourth hypothesis implies that prolactin and growth hormone probably do not play major roles in the growth regulation of human breast cancers, but it supports the traditional concept that both the ovary and pituitary gland have pivotal roles in the etiology of human breast cancers.

The above hypotheses await further investigation in order to elucidate the mechanism of interplay between ovarian steroids, pituitary factors and tissue-derived growth factors in the control of proliferation of human breast cancers.

SECTION 7: Effects of Tumor-Derived Pituitary Factors and Purified Pituitary Hormones on Human Breast Cancer Cells Transplanted into Estrogenized Athymic Nude Mice

INTRODUCTION

In vitro studies with cell and organ culture of human breast cancer have provided us with some insights into the neoplasia process. However, the limitation of the in vitro system and the conflicting results, especially regarding the role of estrogen in human breast cancer, is still unresolved.

We were the first to demonstrate that the growth of T-47D human breast cancer cells in athymic nude mice was greatly accelerated by the simultaneous presence of both estrogen and pituitary factors from GH_3 pituitary tumors (Leung and Shiu, 1981). McManus and Welsch (1981) have subsequently confirmed our findings and showed that benign human breast tumor biopsies as well as another human breast cancer cell line (MCF-7) (Welsch et al., 1981) also were stimulated by the simultaneous

presence of estrogen and pituitary factors. However, whether this pituitary factor(s) is one of the known pituitary hormones or is a novel pituitary-derived factor has not been examined. In addition, it is not known whether the normal pituitary glands also secrete this growth factor. In the present study, the roles of prolactin, growth hormones and other pituitary factors on the growth of human breast cancer are investigated.

MATERIALS AND METHODS

Pituitary Tumor Cell Lines

Rat pituitary tumor-derived clonal cell lines, GH_3 and GH_1 were used in the present studies. Whereas GH_3 cells secrete prolactin and growth hormone to the same extent, GH_1 cells secrete predominantly growth hormone. The AtT-20 cells were derived from an adrenocorticotropin (ACTH) secreting mouse pituitary tumor (Buonassisi et al, 1962). The culture conditions for these cell lines were described in Section 3.

Animals

Four to five week old female Balb/c athymic nude mice were obtained from ARS/Sprague-Dawley Division, Madison, Wisconsin. Normal female Sprague-Dawley rats were used as donors of pituitary transplants. Two pituitaries were transplanted subcutaneously to each nude mouse. Each mouse received two fresh pituitaries every two weeks.

In the first experiment, seven groups of animals were used (Table A). The growth promoting effect (if any) of normal pituitary transplants, rat and mouse pituitary tumor cells and ovine prolactin on the human breast cancer cells were monitored.

In the second experiment, four groups of animals were used to evaluate the effects of prolactin and growth hormone on the human breast cancer cells (Table B).

Table A

Animal Group	Treatment
TE	T-47D + Estrogen
TP	T-47D + Pituitary Transplant
TEP	T-47D + Estrogen + Pituitary Transplant
TEoPRL	T-47D + Estrogen + Ovine Prolactin
TEatT	T-47D + Estrogen + AtT-20
TEG_3	T-47D + Estrogen + GH_3
TEG_1	T-47D + Estrogen + GH_1

Table B

Animal Group	Treatment
TE	T-47D + Estrogen
TEG	T-47D + Estrogen + GH_3
TEoPRL	T-47D + Estrogen + Ovine Prolactin
TEhGH	T-47D + Estrogen + Human Growth Hormone

Both prolactin and growth hormone were supplied by mini osmotic pump.

Hormone Treatment and Osmotic Mini Pump

Some athymic nude mice received 500 ug estradiol valerate once every two weeks. Estrogen were injected subcutaneously at the dorsal midline caudal to the neck. Ovine prolactin (2.5 mg/ml) and human growth hormone (2.5 mg/ml) were delivered by Alza mini osmotic pump implanted subcutaneously in the nude mice. Purified human growth hormone was obtained from Dr. Henry Friesen, Department of Physiology, University of Manitoba. Purified ovine prolactin was obtained from the Endocrine Study Section of the N.I.H. Alzet osmotic mini pumps (model 2002 - reservoir volume 200 ul) were used to deliver hormones. The pumps were filled with various hormones and implanted subcutaneously in the athymic nude mice. The pumps were replaced every two weeks (0.5 ul/hr mean pumping rate).

Inoculation of Cells in Nude Mice

Cultures of tumor cells (T-47D, GH_3, GH_1 and AtT-20) were detached with trypsin-EDTA and resuspended in a small volume of medium. The cells were injected subcutaneously in the flanks of the animals. In experiment 1, $6x10^6$ T-47D cells were injected into all animals. Some animals were injected with $5x10^5$ GH_3 cells, $5x10^5$ GH_1 cells or 10^6 AtT-20 cells, respectively. In experiment 2, all animals were injected with $2x10^7$ T-47D cells. Some animals were injected with $5x10^5$ GH_3 cells. The various treatment groups are summarized in Tables A and B.

Growth of the Tumors

The tumors were measured by calipers in 3 dimensions and their sizes were expressed as the products of the values obtained. At the completion of the experiments, all the tumors were dissected and weighed.

Determination of Hormones in Sera

The concentration of growth hormone and prolactin in sera from the nude mice were measured by RIA using kits for rat growth hormone and prolactin from the NIH. Human growth hormone and ovine prolactin levels also were determined by RIA in some of the animals.

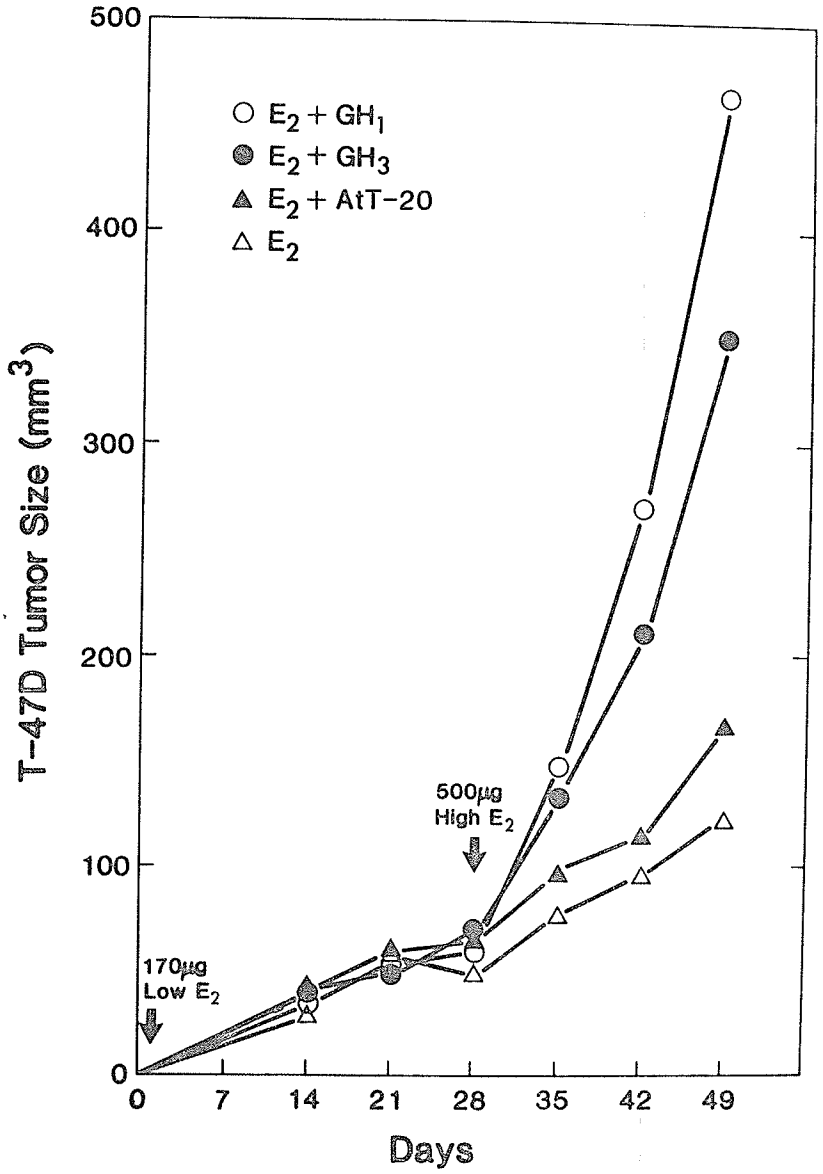
RESULTS

Effects of Estrogen and Pituitary Factors on Growth of T-47D Tumors

In the first experiment, rat pituitary tumor cell lines GH_3 and GH_1, mouse pituitary tumor cells AtT-20 and normal rat pituitaries were transplanted into estrogenized athymic nude mice (Figs. 25, 26). Normal pituitary transplanted into nude mice without estrogen treatment (TP), did not stimulate the growth of the T-47D tumor. However, the pituitaries transplanted into estrogenized nude mice enhanced the growth of the T-47D cells (Fig. 27). The T-47D tumor in nude mice treated with estrogen only (TE) increased in size reaching a tumor size of 136 $\pm$ 34 mm^3 on day 49. In contrast, both T-47D tumors in TEG_1 and TEG_3 group grew rapidly and were 467 $\pm$ 104 mm^3 and 351 $\pm$ 66 mm^3 on day 49, respectively. The T-47D tumors in the TEAtT group grew slightly faster than those of the TE group. However, the differences were not statistically significant. The growth rate of the T-47D tumors in all animals were slow when the low dose of estradiol valerate (170 ug/animal/two weeks) was injected. In contrast, when the concentration of estrogen injected was raised to 500 ug/animal/two weeks on day 28, the T-47D tumors grew at a faster rate thereafter.

Fig. 25 Effect of estrogen and transplanted pituitary tumor cells on the growth of T-47D tumors in athymic nude mice

Procedures for determining growth of tumors were described in Materials and Methods. All the animals were injected with 6×10^6 T-47D cells. Some of the animals received 5×10^5 GH_3 cells, 5×10^5 GH_1 cells and 10^6 AtT-20 cells, respectively. Initially, each animal was injected with 170 ug of estradiol valerate. On day 28, the dose of estrogen injected was increased to 500 ug per animal. E_2, estradiol valerate. Each value represents the mean of measurements obtained from five animals. For clarity, only the mean values are shown.


500
400
300
200
100
0
T-47D Tumor Size (mm3)
○ E2 + GH1
● E2 + GH3
▲ E2 + AtT-20
△ E2
500µg
High E2
170µg
Low E2
0
7
14
21
28
35
42
49
Days

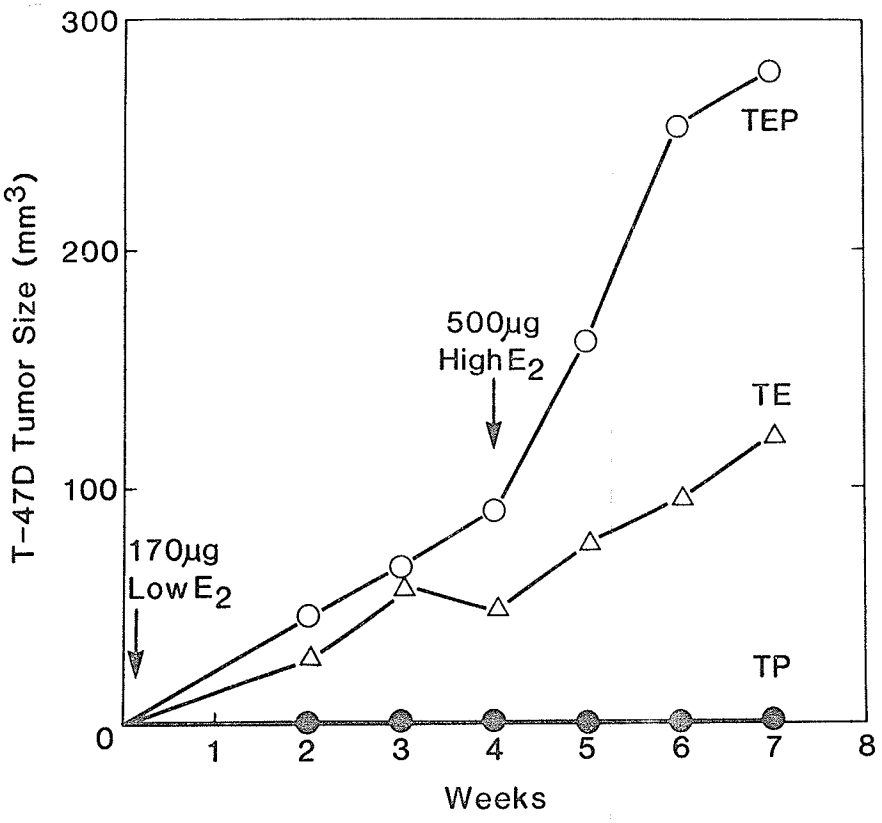


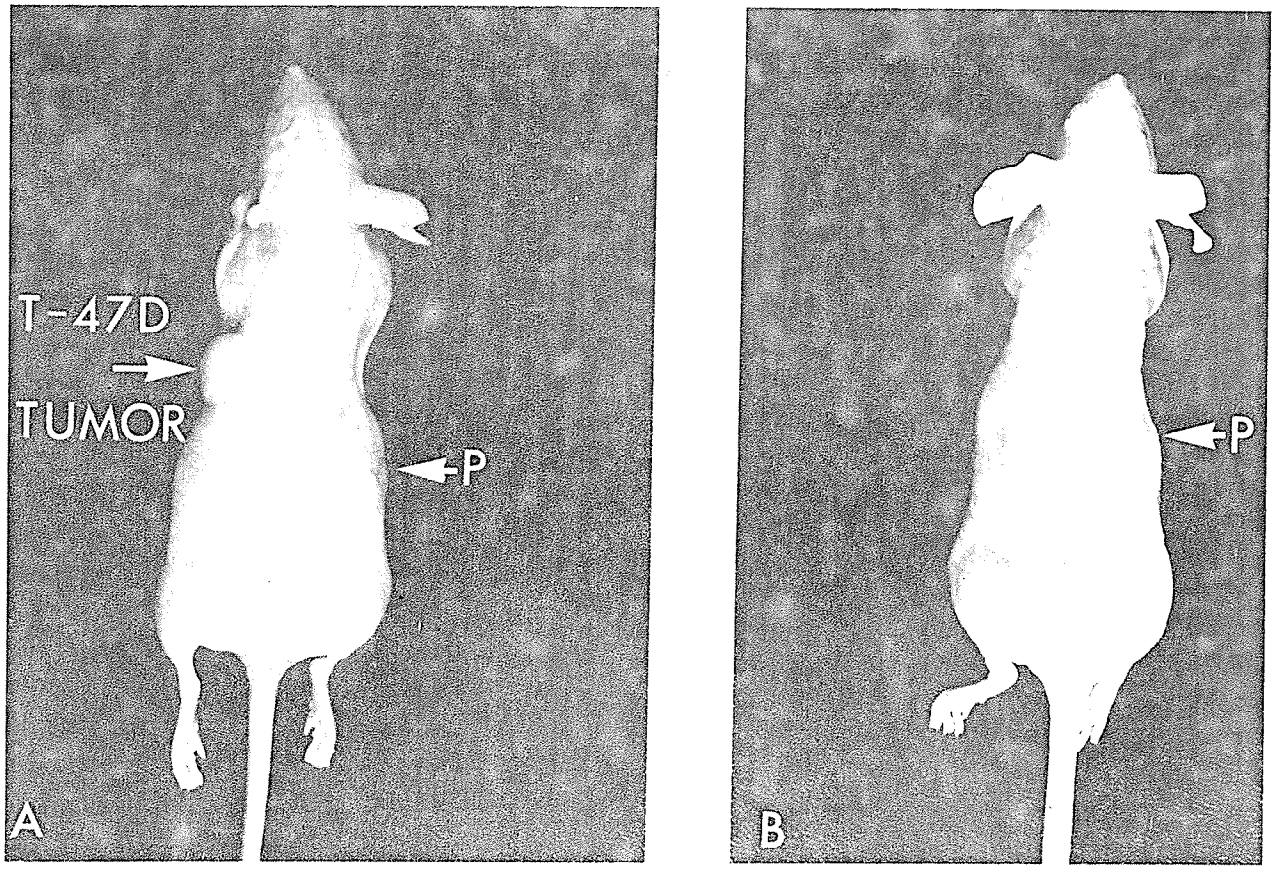
Fig. 26 Effect of estrogen and normal pituitary transplants on the growth of T-47D tumors in athymic nude mice

Each animal was injected with 6 x 10^6 T-47D cells. Two pituitaries per animal were transplanted into some nude mice. These nude mice received fresh pituitary transplants every two weeks. TE, T-47D cells plus estradiol valerate; TEP, T-47D cells plus estrogen plus pituitary transplants; TP, T-47D cells plus pituitary transplants. Each value represents the mean of measurements obtained from five animals. For clarity, only the mean values are shown.

Fig. 27 Morphology of athymic nude mice with T-47D cells and normal pituitary transplants

A) TEP, T-47D cells + estrogen + pituitary transplants

B) TP, T-47D cells + pituitary transplants


T-47D
TUMOR
P
A
P
B

Effects of Estrogen and Pituitary Factors on the Body Weights of Athymic Nude Mice

The body weights of the nude mice were measured at the completion of experiment 1 on day 56 (Table 6). The animals in TP, TEP, TEoPRL groups (23.4 $\pm$ 2.1 g; 23.5 $\pm$ 2.0 g; 21.2 $\pm$ 2.0 g, respectively) did not show any differences when compared to the TE group (22.3 $\pm$ 1.5 g). In contrast, both TEG_1 and TEG_3 groups increase significantly in their body weights (27.0 $\pm$ 2.2 g and 28.6 $\pm$ 4.7 g, respectively). This increase in body weights is probably due to the growth hormone secreted by the transplanted rat pituitary tumors. It is interesting that the body weight of the TEatT animals increases significantly from 19.7 $\pm$ 0.7 g to 32.4 $\pm$ 3.1 g. This dramatic increase in body weight in the nude mice was due to an accumulation of body fat as a result of excess cortisol production by the adrenal glands. Animals bearing the ACTH-secreting AtT-20 tumors closely resemble the Cushing's syndrome in humans. We have further investigated these observations (Leung et al., 1982). The results of these studies are not included in this thesis.

Serum Levels of Hormones

The serum concentrations of growth hormone and prolactin of the athymic mice were measured (Table 6). All the animals in TEG_1 and TEG_3 groups have higher serum growth hormone and prolactin levels than those in the TE and TP groups. In the

Table 6: Effect of estrogen and pituitary factors on the body weight and hormone levels in athymic nude mice

Animal Group (n=5)	Body Weight (g) Initial	Final	Rat Growth Hormone (ng/ml)	Rat Prolactin (ng/ml)
TE	19.5±0.5	22.3±1.5	25±7	113±58
TP	19.6±1.3	23.4±2.1	26±11	153±41
TEP	21.3±1.4	23.5±2.0	33±25	1177±334
TEoPRL	21.0±1.6	21.2±2.0	41±20	97±9
TEatT	19.7±0.7	32.4±3.1	235±115	210±40
TEG_1	19.3±1.0	27.0±2.2	>10,000	> 10,000
TEG_3	19.6±1.0	28.6±4.7	>10,000	8233±5432

Each value represents the mean ± S.D.

The measurements were performed 56 days after initial treatment.

Ovine prolactin levels were not measured.

TEP group, the growth hormone level (33 ± 25 ng/ml) is similar to those of the TE and TP groups (25 ± 7 and 26 ± 11 ng/ml, respectively). However, the prolactin level of the TEP group (1177 ± 334 ng/ml) is significantly higher than the TE and TP groups (113 ± 58, 153 ± 41 ng/ml, respectively).

Effect of Estrogen, Growth Hormone and Prolactin on Growth of T-47D Tumors

In experiment 2, the effects of growth hormone and prolactin on the growth of T-47D tumors in athymic nude mice were studied. Human growth hormone (2.5 mg/ml) and ovine prolactin (2.5 mg/ml) were used for this experiment. The concentrations of hormones used were arbitrarily chosen. The hormones were delivered to the animals by implanted mini osmotic pumps. With an infusion rate of 0.5 ul/hr, each animal will therefore receive 1.25 ug hormone/hr. The TE and TEG groups were included in this study as controls. The growth rate of T-47D tumors in the TEhGH and TEoPRL groups did not differ from that of the TE group over a period of 49 days (Fig. 28). In contrast, the T-47D tumors in TEG group as reported by us, grew rapidly during the same time. The tumor weights were recorded at completion of the experiment (Table 7). The tumor weights of TE, TEhGH and TEoPRL were 235 ± 94 mg, 238 ± 160 mg and 275 ± 85 mg, respectively. In contrast, the T-47D tumors of TEG group was 1097 ± 405 mg. The increase in tumor weights is in agreement with the results of the tumor size measurements.

Fig. 28 Effect of growth hormone and prolactin on the growth of T-47D tumors in estrogenized athymic nude mice

All the animals were injected with 2×10^7 T-47D cells and estradiol valerate (500 ug per animal per two weeks). Some nude mice were also injected with 5×10^5 GH_3 cells. Human growth hormone (hGH, 2.5 mg/ml) and ovine prolactin (oPRL, 2.5 mg/ml) were delivered to some animals via implanted mini osmotic pumps. The pumps were changed every two weeks. Each value represents the mean of measurements obtained from five animals. For clarity, only the mean values are shown.

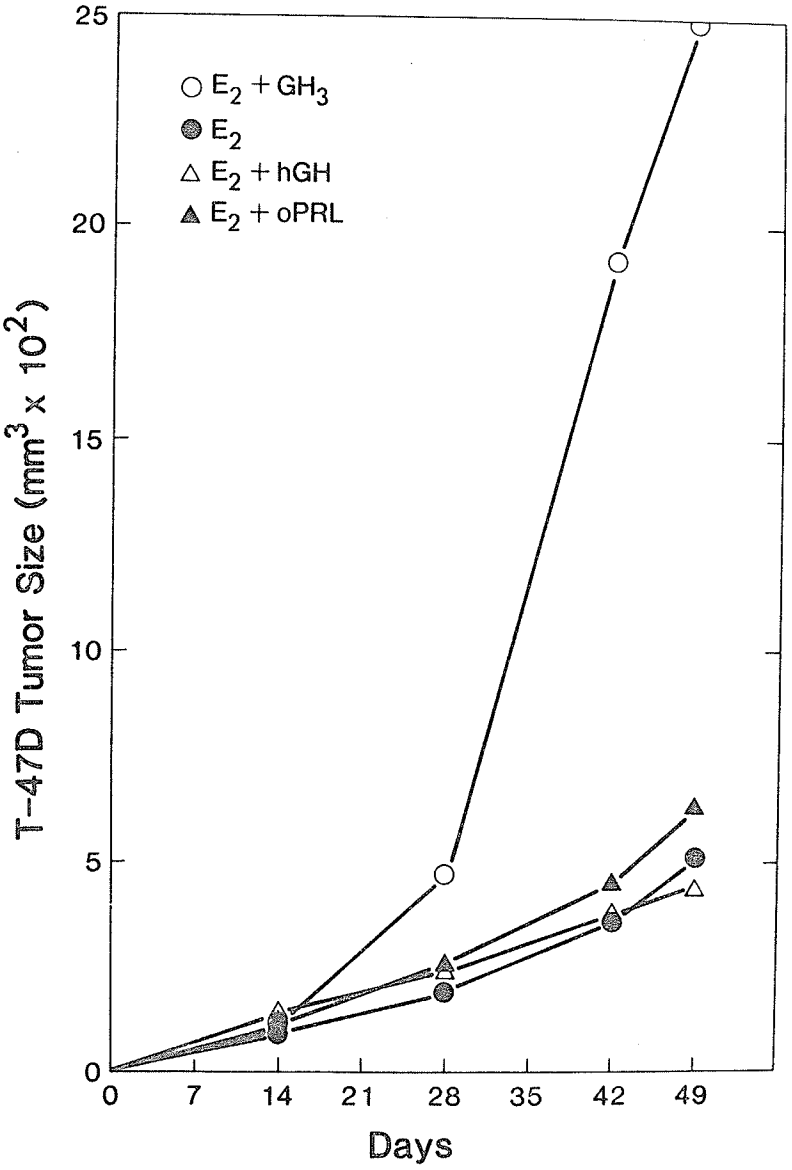

25
20
15
10
5
0
○ E_2 + GH_3
● E_2
△ E_2 + hGH
▲ E_2 + oPRL
T-47D Tumor Size (mm^3 x 10^2)
0
7
14
21
28
35
42
49
Days

Table 7: Effect of purified pituitary hormones on T-47D tumors in athymic nude mice

Animal Group (n=5)	Body Weight (g) initial	Body Weight (g) final	T-47D tumor weight (mg)	Rat Growth Hormone (ng/ml)	Rat Prolactin (ng/ml)	Human Growth Hormone (ng/ml)
TE	18.9±0.7	20.0±0.9	235±94	29.1±13.6	25.5±6.7	< 1
TEG	18.6±1.7	23.1±1.8	1097±405	15780±3420	6060±580	--
TEhGH	18.9±1.2	19.0±1.6	238±160	33.4±11.4	27.3±8.5	1-20
TEoPRL	18.1±2.0	17.5±2.6	275±85	59.3±24.8	30.8±2.4	--

Each value represents the mean ± S.D.

hGH (2.5 mg/ml) and oPRL (2.5 mg/ml) were delivered to the animals by mini osmotic pumps.

Ovine prolactin levels were not measured.

Serum Concentrations of Growth Hormone and Prolactin

Since the T-47D tumors of the TE, TEhGH and TEoPRL were growing at the same rate, we therefore determined the serum concentration of growth hormone and prolactin (Table 7) to see whether there are any differences in the hormone levels. The rat growth hormone and prolactin levels of all three groups were not significantly different from each other. Low level of hGH (1 - 20 ng/ml) can be detected in the serum of the TEhGH animals. This result suggested that the amount of hormones delivered by the mini osmotic pump may not be high enough.

Morphology of T-47D Cells in Athymic Nude Mice and in Cell Culture

Some of the T-47D tumors were dissected from the athymic nude mice and examined histologically (Fig. 29A). The tumor is hard and is composed mainly of epithelial cells surrounded by a collagen capsule. The T-47D tumors were cut into small pieces and incubated with collagenase. The epithelial cells isolated by this procedure were maintained in tissue culture . The morphology of the re-established cells is identical to the parent T-47D cell line (Fig. 29B). These results demonstrated that the tumor is indeed made up of T-47D human breast cancer cells.

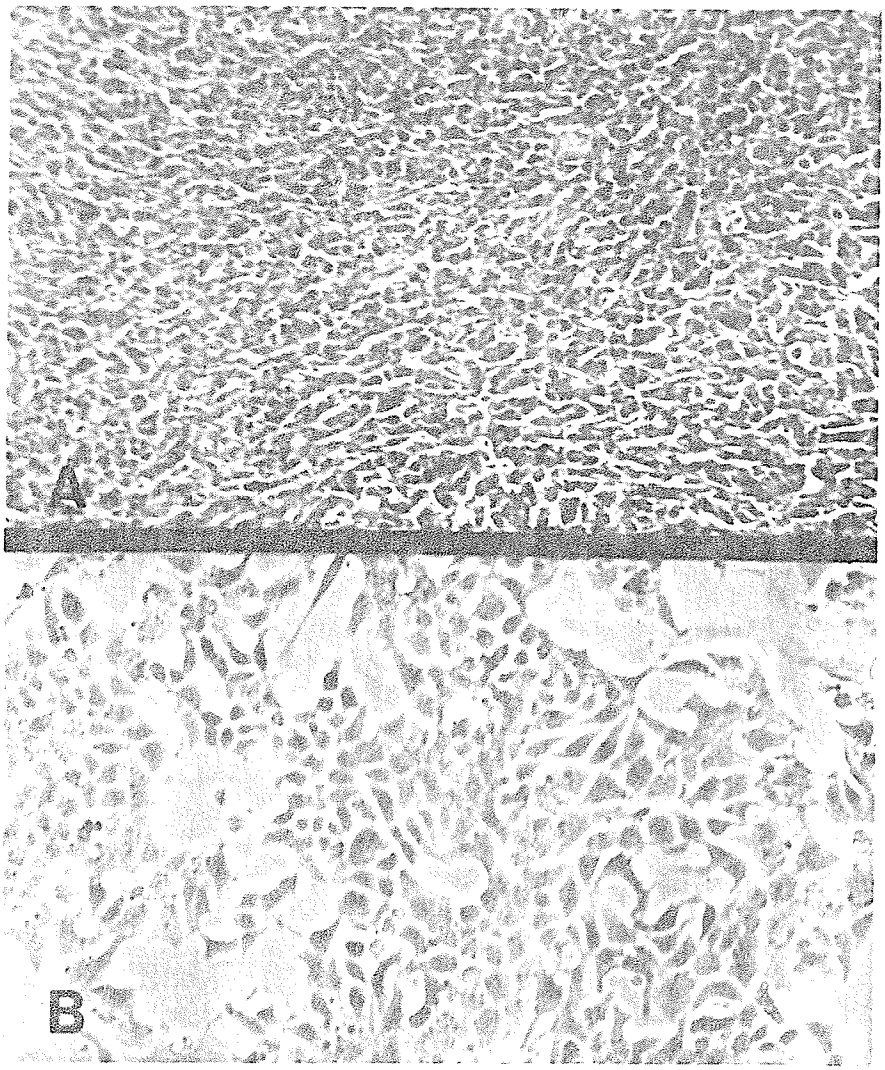


Fig. 29 Morphology of T-47D cells in athymic nude mice and in cell culture

A) Histology of T-47D tumor transplanted in athymic nude mice

B) The same T-47D tumor dissected from athymic nude mice was treated with collagenase and re-establish in tissue culture. The morphology of these cells are identical to the parent cell line (see Section 2).

DISCUSSION

Crude bovine and ovine pituitary preparations were reported to stimulate the growth of a transplantable mammary fibroadenoma in intact female Sprague-Dawley rats (Millar and Noble, 1954). However, a growth hormone preparation did not have any effect on the tumor. Huggins et al. (1956) transplanted mammary fibroadenomas into hypophysectomized Sprague-Dawley rats. They observed that the transplanted tumor was not affected by estrone, progesterone, prolactin, or growth hormone when administered separately. The simultaneous administration of progesterone and estrone stimulated the growth of the tumor moderately. Interestingly, when growth hormone was added together with these steroids, the growth rate of the tumors was restored to that of intact female rats. It was also found that prolactin was not effective as a substitute for growth hormone in the steroid-peptide hormonal combination.

The synergistic effect of anterior pituitary and steroid hormones for growth of transplantable rat mammary tumors was reported by MacLeod et al. (1964). Using a transplantable rat pituitary tumor, MtTW5, or pituitary homograft as sources of pituitary hormones, these investigators observed that the transplanted mammary tumor in ovariectomized pituitary tumor bearing Wistar Furth rats did not proliferate unless estrogen

and progesterone were simultaneously administered. Pituitary transplants to intact or hypophysectomized rats also enhanced the growth of the mammary tumor. In contrast, ovariectomy of rats with pituitary transplants inhibited the growth of the mammary tumor, whereas the administration of estrogen and progesterone to ovariectomized pituitary-homograft bearing rats stimulated the growth of the mammary tumor.

Thus, development of mammary neoplasia in rodents is undoubtedly influenced by estrogen and pituitary-derived hormones such as prolactin and growth hormone. However, the role of prolactin and growth hormone in human breast cancer is still unknown (Nagasawa, 1979).

Our previous results demonstrated that estrogen and pituitary factors are both required for the growth of human breast cancer cells in athymic nude mice (Leung and Shiu, 1981). These results also suggested that there is a high correlation between human breast tumor size and serum level of growth hormone. In order to further investigate these pituitary factors, we decided to transplant the rat pituitary tumor GH_1 cells as well as the mouse pituitary tumor AtT-20 cells into the nude mice. These two cell lines were chosen because GH_1 cells were reported to secrete large quantities of growth hormone (Tashjian et al., 1970) while AtT-20 cells secrete adrenocorticotropin (ACTH) (Eippers and Main, 1980). Thus, tumors formed from these cells can act as an exogenous source of the respective pituitary hormones.

In the first experiment, seven groups of animals were used (Table A). When the animals were injected with low dose of estrogen (170 ug/animal/two weeks), all the T-47D tumors grew slowly (Fig. 25). In contrast, when high dose of estrogen (500 ug/animal/two weeks) was injected on day 28, the growth rate of the T-47D tumors was accelerated. Interestingly, the T-47D tumors of the TEG_1 group grew to a larger size as compared to the TEG_3 group. This may be due to the higher amount of growth hormone being secreted by the GH_1 tumor. The T-47D tumors of the TE group grew moderately to the same extent as those of the TEA group.

The sera levels of growth hormone and prolactin of these animals were measured (Table 6). Both TEG_1 and TEG_3 animals have very high levels of growth hormone and prolactin. These high concentrations of hormones were likely due to growth of the pituitary tumors. One explanation for the high level of prolactin in the TEG_1 animals is that the GH_1 cells may alter their secretory functions once they were transplanted in vivo. The hormone levels of the TEA group was higher than the TE group. Whether these slight differences may be responsible for the differences in the tumor sizes is not known.

Since factors from GH_3 and GH_1 pituitary tumors can stimulate the growth of T-47D tumors in estrogenized athymic nude mice, it is interesting to see whether normal pituitary transplants can also enhance the growth of the T-47D tumors.

The pituitaries remain functional after transplantation into the athymic nude mice as indicated by the increase in hormone level in the sera in both estrogen treated (TEP) and untreated (TP) nude mice (Table 6). The T-47D tumor cells did not proliferate in the TP group despite the presence of the transplanted pituitaries. In contrast, the T-47D cells in the TEP group formed palpable tumors two weeks after innoculation and continue to increase in size (Fig. 26). These results suggest that breast tumors in humans and rodents have different properties since grafting of pituitaries to female Sprague-Dawley rats with estrogen treatment can sharply increase the incidence of spontaneous mammary tumors (Welsch et al., 1970). In estrogen treated nude mice (TE) the T-47D cells grew to a mean tumor weight of 48 mg after 56 days as compared to the TEP group (125 mg). The body weight of the animals were recorded at the beginning and at the completion of the experiments. As shown in Table 6 there were no significant differences between the body weights of animals from the TP and TEP groups. The range of tumor size is quite wide and may be attributable to the relative viability of the human breast tumor and/or pituitary transplants in the hosts. It is interesting to note that despite the elevation of prolactin level in the TP group as compared to TE group (153 ng/ml vs. 113 ng/ml), no T-47D tumors were observed in the TP group. Hence, prolactin alone may not be sufficient to promote the

growth of the human breast cancer cells. Both the prolactin and growth hormone levels were higher in the TEP animals suggesting that the pituitary transplants may be stimulated by estrogen. Estrogen may also stimulate the T-47D cells indirectly through intermediates such as estromedins. A direct effect of rat growth hormone on T-47D cells can be ruled out because animal growth hormone cannot bind to human growth hormone receptors. It is possible that the high level of prolactin may stimulate T-47D cells. However, this explanation is unlikely because the prolactin level achieved in the TP group is higher than the TE group and yet no T-47D tumors were observed in the TP group.

Since the pituitary tumors and normal pituitaries secrete a combination of many pituitary factors, it is difficult to identify this factor(s).

We attempted to circumvent this problem by delivering highly purified hormones into athymic nude mice by means of implanted osmotic mini pumps. The growth rates of T-47D tumors in nude mice with osmotic mini pumps were shown in Fig. 28. TEG and TE groups were included as controls. The doses of prolactin and growth hormone delivered was arbitrarily chosen to be 1.25 ug/hr. Under these conditions, no significant differences in growth rates of the T-47D tumors were observed between the TEhGH, TEoPRL and TE groups 49 days after treatment. The hormone levels of these animals were then

determined (Table 7). The serum level of rat growth hormone of the TE group (29.1 ± 13.6 ng/ml) was not significantly different from those of the TEhGH and TEoPRL groups (33.4 ± 11.4 ng/ml and 59.3 ± 24.8 ng/ml, respectively). Similarly, the prolactin level of the TE group (25.5 ± 6.7 ng/ml) was also not significantly different from the TEhGH and TEoPRL groups (27.3 ± 8.5 ng/ml and 30.8 ± 2.4 ng/ml, respectively). Because purified human growth hormone was used in the experiment, we therefore determined how much hGH is in the sera of the TEhGH. Very low concentrations of hGH (1-20 ng/ml) can be detected in the TEhGH group. Although the serum levels of oPRL were not determined, it is expected that they will be similar to that of hGH. Thus, the concentrations of oPRL and hGH used in this experiment seem to be too low.

The experiment was subsequently repeated with a higher dose (15 ug/hr) of hGH delivered by the osmotic mini pump (data not shown). Out of six animals receiving both estrogen and hGH (i.e. TEhGH), four of them died before the completion of the experiment. This may be due to the presence of pyrogens in the particular batch of growth hormone preparations. However, the remaining two animals both have larger T-47D tumors as compared to the TE group. These observations will be confirmed by future studies. In addition, a rat pituitary tumor cell line, 235-1, which secretes only prolactin (Reymond et al., 1981) will be transplanted into athymic nude mice to act as an

exogenous source of prolactin. It is hoped that from these studies, the relationship between prolactin, growth hormone and other pituitary factors and breast cancer will be better defined.

Section 8: Preliminary Studies on a Putative Pituitary-Derived Growth Factor for Human Breast Cancer Cells.

INTRODUCTION

Mammary growth factors from a number of sources which include platelets, serum, pituitary, kidney, uterus and brain have been reported. Sirbasku and Benson (1979) have identified growth factors from the kidney, brain, uterus and platelets which stimulate the growth of a rat mammary tumor cell line in culture. Kano-Sueoka et al (1979) reported the presence of a factor in the pituitary which can stimulate the growth of a rat mammary tumor cell line in the presence of prolactin, insulin and hydrocortisone. This factor, subsequently identified as phosphoethanolamine, enhanced the growth of the rat mammary tumor cells. However, phosphoethanolamine by itself had no stimulatory effect and the apparent synergism between these hormones and phosphoethanolamine remains to be elucidated. A unique serum mitogen which stimulates cultured mammary epithelial cells was also reported (Ptashne et al, 1979). At

high concentrations, this mitogen exhibits biological cross activity but lacks immuno cross-reactivity with another serum mitogen, multiplication stimulating activity (MSA). Based on a variety of physiological, biological or immunological properties, this mitogen was found to be different from other mitogens such as EGF, FGF, NGF, MSA, somatomedins A and C, and PDGF. Rudland et al. (1979) have also identified a mammary growth factor activity from bovine pituitary gland, although the nature of this factor has not been established.

From results of the previous sections, we postulated that GH_3 rat pituitary tumors may produce a growth factor which stimulates the growth of human breast cancer cells in vivo. This growth factor is tentatively termed "pituitary-derived estromedin" (Leung and Shiu, 1981). We have also demonstrated that normal pituitary transplanted into estrogenized athymic nude mice also enhance the growth of human breast cancer cells (see Section 6). Leland and Sirbasku (1981) have also identified factors from the extracts of GH_3/C14 rat pituitary tumors which stimulate the growth of the MTW9/PL rat mammary tumor cells in culture.

In the present study, we attempt to partially purify and characterize the growth factors from GH_3 cells using T-47D, a human breast cancer cell line, as a bioassay.

MATERIALS AND METHODS

Conditioned Medium of GH_3 Pituitary Tumor Cells

Rat pituitary GH_3 tumor cells were plated and maintained in DME with 10% FCS. The cells were allowed to grow to approximately 70% confluency. The GH_3 cells were then washed twice with serum free DME and fresh serum free DME was added to each flask. The conditioned media from these GH_3 cultures were collected two days later and stored at -20°C until use.

Preparation of GH_3 Tumor Extract

Rat pituitary GH_3 tumors were grown in nude mice from experiments described in Sections 6 and 7. After dissection, the tumors were trimmed, rinsed in cold distilled water and weighed. Three ml of PBS per gram of tissue was added and homogenized with a polytron homogenizer. The homogenate was sonicated for 20 seconds and then spun at 20,000 RPM for 90 minutes. The supernatant was collected and sterilized by filtering. Protein concentration was determined by the Lowry method (1955).

Organ Culture of Normal Rat Pituitary and GH_3 Rat Pituitary Tumors

The tissues were cut into small pieces approximately 1 mm^3. Five explants were put on a piece of silconized lens paper. The papers were allowed to float in 2 ml of DMEM. Estrogen was added to some of the dishes. Labelled ^{35}S-

methionine (100 uCi) was added to each dish and incubated at 37°C overnight. The labeled media were collected, dialyzed against distilled water, and lyophilized. The explants were pooled and sonicated in 2 ml of PBS, followed by centrifugation at 20,000 RPM for 60 minutes. The supernatant was dialyzed and lyophilized. The samples were analyzed by SDS polyacrylamide gel electrophoresis.

Discontinuous SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gels were prepared according to the method described by Weber and Osborne (1975). The stacking gel (5 x 140 mm) and resolving gel (95 x 140 mm) were prepared from stock solutions of acrylamide (30% w/v) and N,N'-methylene bisacrylamide (0.8% w/v). Ammonium persulphate (10%) and TEMED were added to accelerate the polymerization. Gels were casted using a Bio-Rad vertical slab gel unit. Samples (10-20 ul) were prepared by dissolving in a cocktail contaning Tris-Hcl (0.0725M, pH 7.0), 2% SDS, 10% glycerol and 5% B-mercaptoethanol and 0.01% bromophenol blue. Samples were boiled for 10 minutes prior to use.

Electrophoresis was carried out at a constant power of 10 mA/gel (LKB powerpac) until the bromophenol blue migrated to within 5 mm of the bottom of the gel.

Gels were fixed in 50% TCA for 30 minutes and washed overnight in 7% acetic acid on a shaker.

Fluorography

The fluorographic procedure is similar to the one described by Laskey and Mills (1975). The gel was soaked in DMSO, with one change, for 30 minutes each. It is then soaked in DMSO/PPO (4:1) for 3 hours. This is followed by one hour washing in distilled water. The gel is then dried in a vacuum heater for two hours. The dried gel was allowed to cool down and an X-ray film was pressed against the gel. This was left in a $-70^{o}C$ freezer for exposure.

Column Chromatography

Sephadex G-100 chromatography was performed at $0-5^{0}C$. Homogenate of GH_3 tumors (70 mg protein) was applied to the column equilibrated with PBS (pH 7.4). Protein content of the fractions was estimated by the absorbance at 280 nM. The column was eluted with PBS and the fractions were pooled and added to T-47D cells in culture to determine the mitogenic activity. I^{125}hGH, I^{125}EGF and ovalbumin were used as molecular weight markers.

RESULTS

Effects of GH_3 Conditioned Media and Tumor Extract on the Growth of T-47D Cells

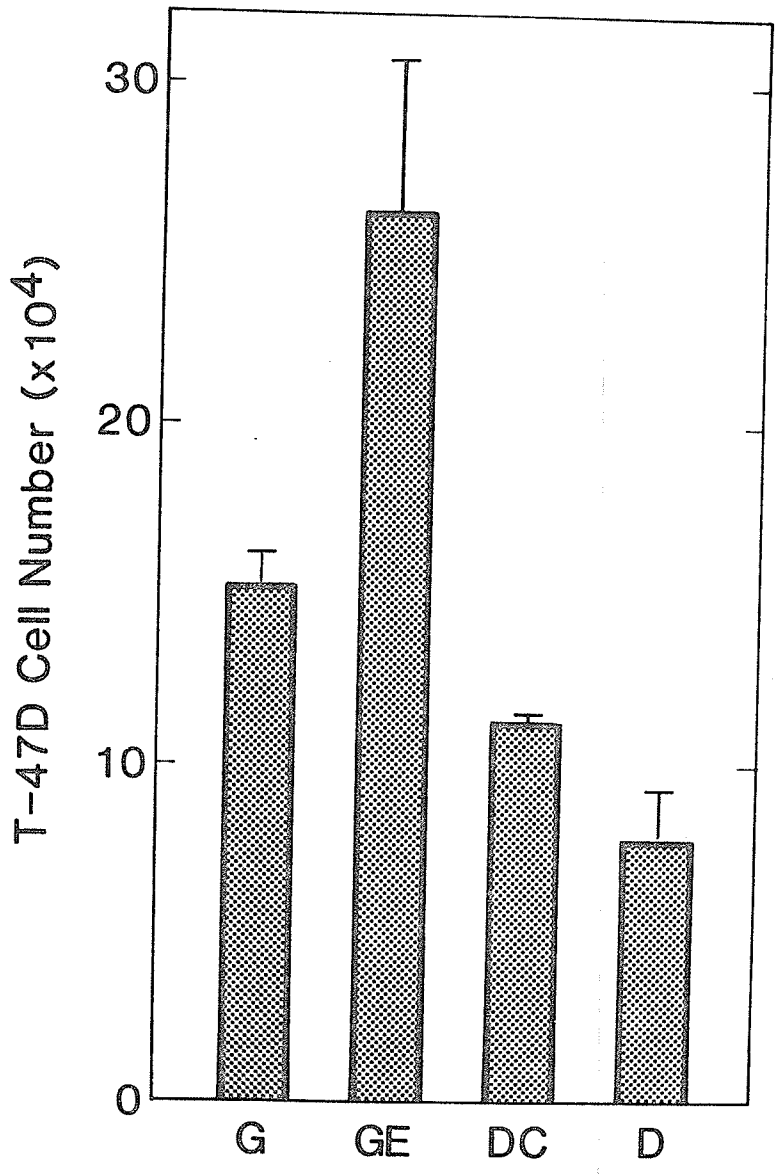
The effect of conditioned media of GH_3 pituitary tumor cells on the growth of T-47D cells is shown in Fig. 30. Dulbecco's modified Eagle's medium (D) or DME from a culture flask preincubated with complete medium (DC) were used as controls. The T-47D cells proliferate faster in GH_3 conditioned medium (G) as compared to the controls (DC and D). Interestingly, the conditioned medium (GE) of GH_3 cells cultured in the presence of 17β-estradiol (10^8M) stimulated the T-47D cells even more.

Tissue extract of GH_3 tumors were prepared as described in the Materials and Methods. The GH_3 tumor extract stimulated the T-47D cells in a dose-response fashion (Fig. 31). The T-47D cell number in dishes with tumor extract from TEG group was 8.6 ± 0.2 ($\times 10^5$) after 12 days in culture. In contrast, the T-47D cell number in DME supplemented with 0.1% FCS was 5.6 ± 0.2 ($\times 10^5$).

The extract of GH_3 tumors from TG group also exhibits mitogenic activity on the T-47D cells. Addition of estrogen to GH_3 tumor extract from TG group (TG + E) did not alter the effect on T-47D cells significantly.

Fig. 30 Effect of Conditioned Medium of GH_3 Cells on the Growth of T-47D Cells

Complete medium was used for plating T-47D cells (5×10^4 cells per dish). The cells were washed once with DME twenty-four hours later and replaced with various media. The cell number was determined on day 9. The media were changed every three days. G = conditioned medium of GH_3 cells; GE = conditioned medium of GH_3 cells treated with estrogen; DC = DME collected from flasks pre-incubated with complete medium; D = DME only. Each value represents the mean $\pm$ S.D.


T-47D Cell Number (x10⁴)
30
20
10
0
G
GE
DC
D

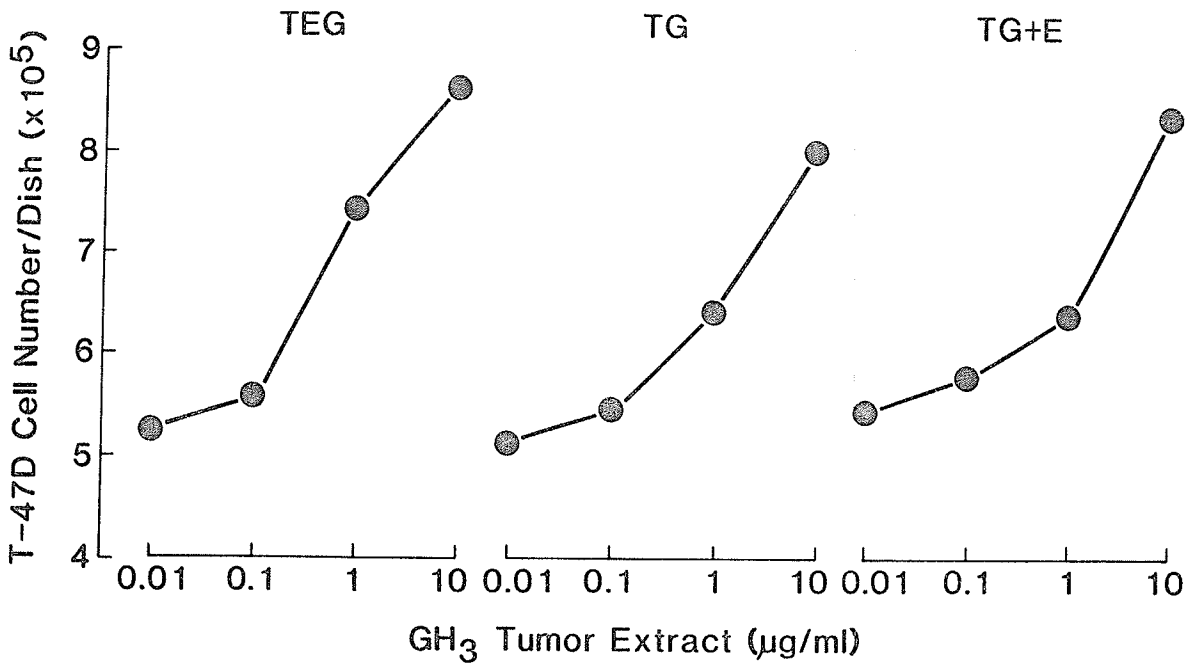


Fig. 31 Mitogenic Effect of GH_3 Tumor Extract on T-47D Cells

Complete medium was used for plating of T-47D cells. Twenty-four hours later, the cells were washed once with DME and fresh DME + 0.1% FCS was added to each dish. Extract of GH_3 tumor and estrogen (in some dishes) were also added at the same time. The cell number was determined by using a Coulter counter on day 12. The medium was changed every three days and fresh extract and estrogen was added at time of medium change. Each value represents the mean of triplicates. The S.D. is less than 10% of the mean.

Secreted Proteins from Pituitaries of Normal and Estrogen Treated Rats and Pituitary Tumors

Explants of pituitaries from rats treated with or without estrogen for four weeks were maintained in organ culture in the presence of ^{35}S methionine. The secreted proteins from these cultures were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography (Fig. 32). Proteins of molecular weight of 30,000-60,000 daltons and 14,000-16,000 daltons, in addition to prolactin and growth hormone, were in abundance in the estrogen treated pituitary organ cultures.

The GH_3 tumors from TEG group of athymic nude mice were also maintained in organ culture and labeled with ^{35}S methionine. The explants were either cultured in the presence of estrogen (E), nafoxidine (N), or estrogen plus nafoxidine (EN). The SDS-PAGE autoradiograph of the secreted proteins of these explants is shown in Fig. 33. As compared to the control (c), the GH_3 tumor explants cultured in the presence of estrogen have larger amounts of secreted proteins. In contrast, the amounts of secreted proteins is decreased in the nafoxidine treated cultures. This inhibition of secreted proteins of the nafoxidine treated cultures is apparently prevented when estrogen was added simultaneously. These results suggested that estrogen may in some ways regulate these secreted proteins.

Fig. 32 SDS-PAGE of proteins from pituitaries of normal and estrogen treated rats (top)

Female Sprague-Dawley rats injected with or without estradiol valerate (2 mg/rat) were sacrificed after 30 days. The pituitaries from these animals were maintained in organ culture and labeled overnight with ^{35}S-methionine (100 uCI/dish). The explants were homogenized, sonicated and centrifuged at 20,000 RPM for 90 minutes. The supernatant is dialyzed against distilled water and lyophilized. The spent media was also dialyzed and lyophilized. Lyophilized samples are reconstituted with a cocktail containing 2% SDS, 10% glycerol and 5% mercaptoethanol. The samples were analyzed on a 15% resolving gel with a 4% stacking gel. Reduced or non-reduced ^{125}I-rat prolactin standards were run in the same gel. (Fluorograph is shown.)

Lanes: 1) Intracellular proteins of control pituitary
2) Intracellular proteins of estrogen treated pituitary
3) Secreted proteins of control pituitary
4) Secreted proteins of estrogen treated pituitary
5) Non-reduced rat prolactin
6) Reduced rat prolactin

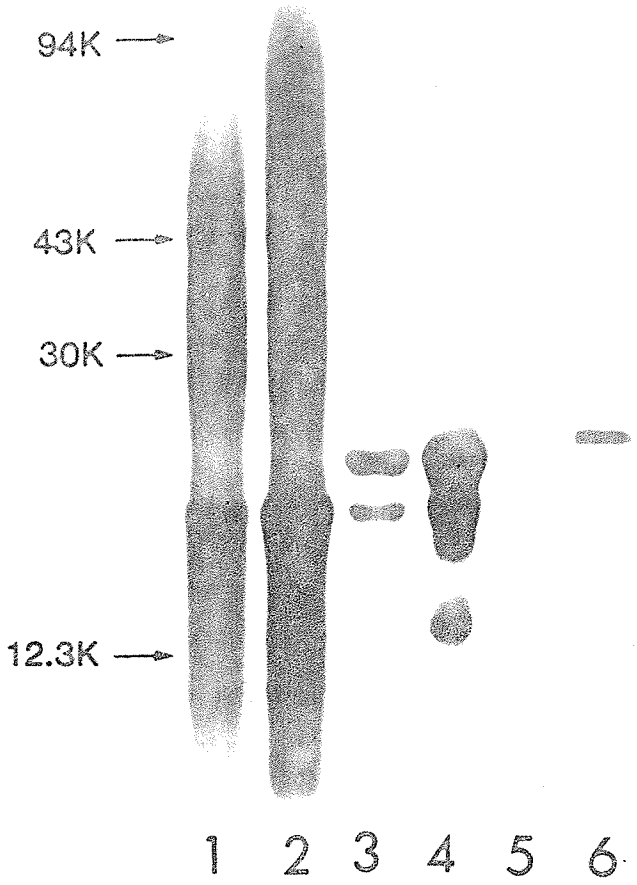
Fig. 33 SDS-PAGE of secreted proteins from organ cultures of GH_3 tumors (bottom)

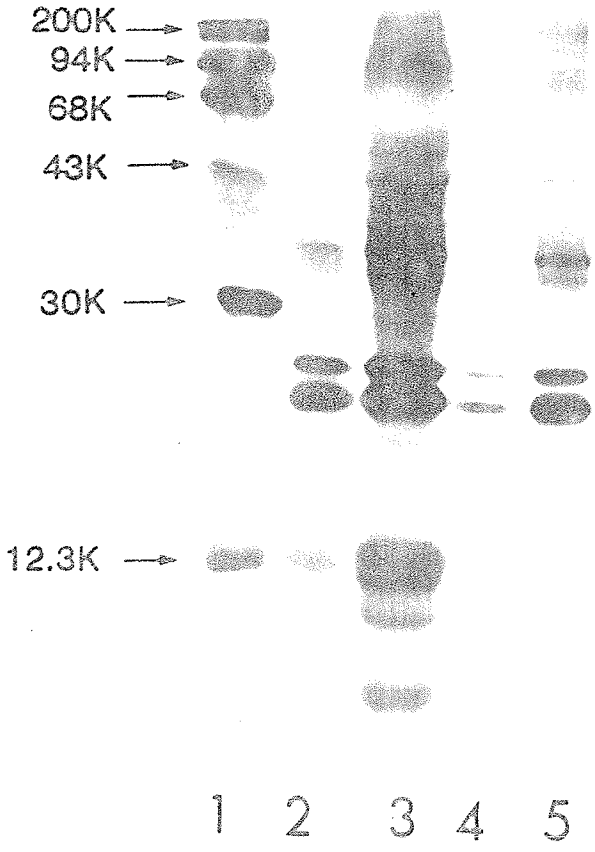
Rat pituitary GH_3 tumors were dissected from athymic nude mice and maintained in organ culture in the presence of estrogen, nafoxidine, or both. The cultures were labeled with ^{35}S-methionine (50 uCi/dish) overnight. Spent media were dialyzed, lyophilized and then analyzed on 15% resolving gel with a 4% stacking gel. (Fluorograph is shown.)

Lanes: 1) Molecular weight markers*
2) Control, no hormone additions
3) Plus estrogen
4) Plus nafoxidine
5) Plus estrogen and nafoxidine

* Molecular weight markers

myosin	200,000
phosphorylase B	94,000
bovine serum albumin	68,000
ovalbumin	43,000
carbonic anhydrase	30,000
cytochrome C	12,300


94K
43K
30K
12.3K
1 2 3 4 5 6


200K
94K
68K
43K
30K
12.3K
1 2 3 4 5

Macroprolactinomas were induced in female Sprague-Dawley rats by treating the animals with estradiol valerate for nine months. These macroprolactinomas were obtained from Dr. F.F. Casanueva, University of Manitoba. The pattern of secreted proteins of these tumors and normal pituitaries in organ culture were compared (Fig. 34). A very dark band around a molecular weight of 22,000 daltons (presumably prolactin) was detected in the control and the tumor cultures. Large amounts of secreted proteins were found in the tumor cultures as compared to the controls. These proteins have molecular weights of 30,000-60,000 and 12,000-16,000 daltons.

Gel Exclusion Chromatography of GH_3 Tumor Extract

The GH_3 tumor extract from TEG athymic nude mice was fractionated by Sephadex G-100 (Fig. 35). Most of the proteins were eluted in the void volume. The fractions were pooled and the mitogenic activity was monitored by using the T-47D cells as a bioassay. A peak of mitogenic activity was detected just before the ovalbumin marker suggesting that the molecular weight of the growth factor to be between 45,000-60,000 daltons. Small amounts of growth promoting activity was also present just after the ovalbumin marker.

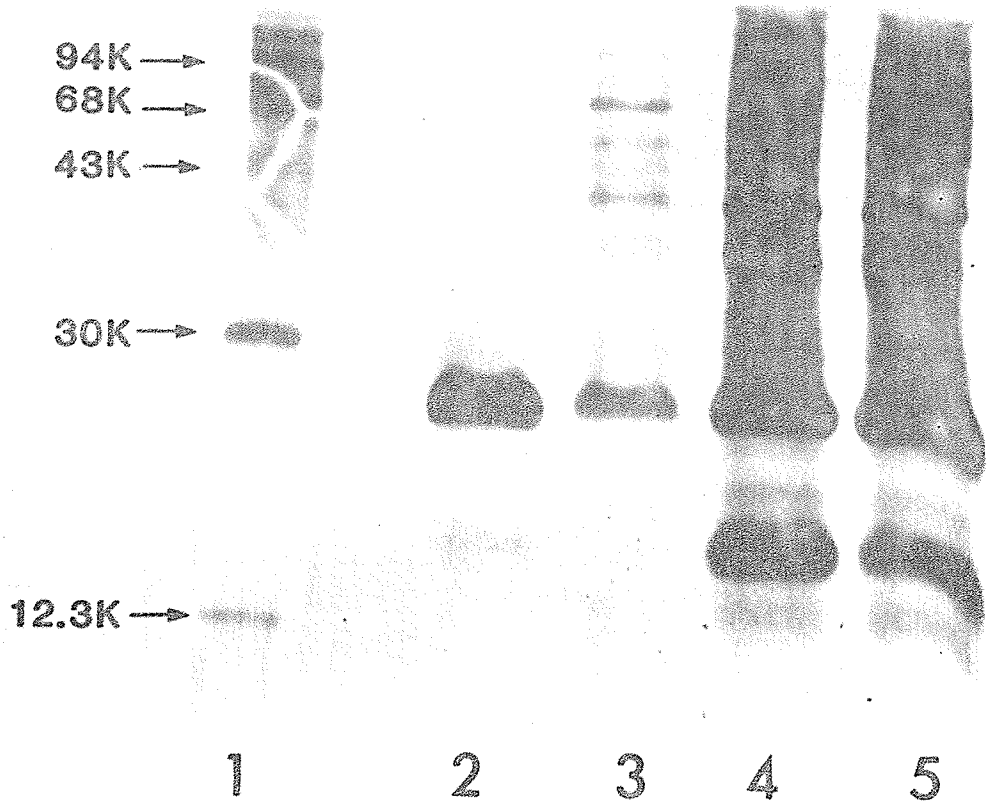


Fig. 34 SDS-PAGE of secreted proteins from normal pituitary and estrogen induced macroprolactinomas

Organ cultures or normal pituitary and tumors were labeled with ^{35}S-methionine (50 uCi/dish). The samples were analyzed on 15% resolving gel with a 4% stacking gel. (Fluorograph is shown.)

Lanes: 1) Molecular weight markers*

2) Normal pituitary

3) Pituitary from estrogen treated, ovariectomized rat

4) Prolactinoma I from estrogen treated, intact rat

5) Prolactinoma II from estrogen treated, intact rat

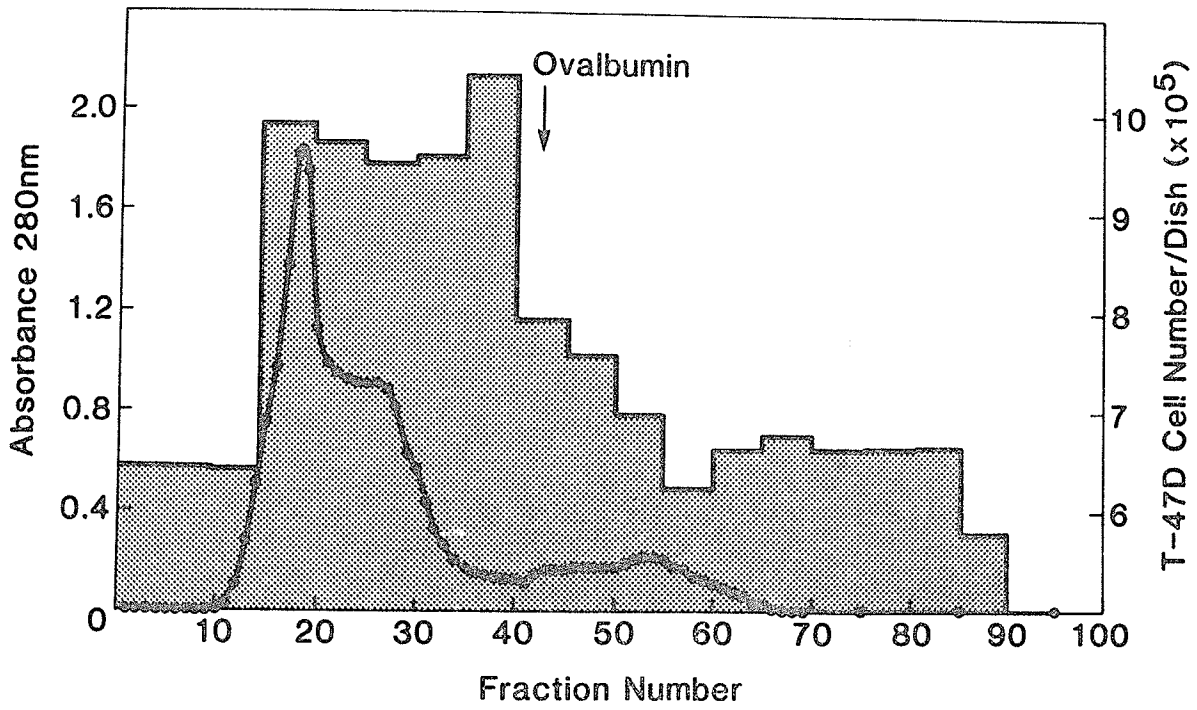


Fig. 35 Elution Profile of GH_3 Tumor Extract on Sephadex G-100

Rat pituitary GH_3 tumors from TEG athymic nude mice were homogenized and sonicated. The extract was applied to the column and eluted with PBS (pH 7.4). The fractions were pooled and growth promoting activity of the fractions was determined by using the T-47D cells as a bioassay and monitoring the increase in cell number (shaded area). The protein content of each fraction was estimated by absorbance at 280 nM (⊶).

DISCUSSION

The pituitary gland is well known to be one of the major sources of polypeptide hormones in the body. Recent studies suggested that the pituitary gland may produce many other hormonal factors other than the classical hormones. For instance, a new peptide growth factor, fibroblast growth factor (FGF) has been isolated from bovine pituitaries (Gospodarowicz, 1975). In addition, a new chondrocyte growth factor, which can stimulate the proliferation of chondrocytes in vitro, has also been recently isolated from human pituitary glands (Kasper et al., 1982).

From our previous nude mice experiments (see Sections 6 and 7), we postulated the existence of an estrogen dependent pituitary derived growth factor. In the present study, conditioned media from GH_3 rat pituitary tumor cell line were found to stimulate the growth of T-47D human breast cancer cells in vitro (Fig. 30). Interestingly, pretreatment of GH_3 cells with estrogen enhanced the mitogenic activity observed. In addition, soluble extracts prepared from the GH_3 tumors dissected from the nude mice also stimulated the proliferation of T-47D cells in vitro (Fig. 31). It is likely that the mitogenic activity observed was not due to known mammotropic hormones since prolactin and growth hormone did not affect the

in vitro growth of T-47D cells (Shiu, 1981). Thus, it appears that the pituitary may produce a mammary growth factor(s) which is also under the influence of estrogen.

The characteristics of this factor(s) was further studied by analysis of the secreted proteins from the pituitary tumors. Pituitaries from rats treated with estrogen for four weeks showed larger amounts of secreted proteins than the control. It was therefore of interest to see whether chronic estrogen treatment also exert similar effects on the pituitary gland. Pituitary tumors were induced in female Sprague Dawley rats by chronic treatment with estradiol valerate for six months. These tumors and normal pituitary glands from control rats were organ cultured in the presence of ^{35}S-methionine, and the secreted proteins in the media were analyzed by SDS-PAGE and autoradiography. Several proteins of M.W. between 30,000-60,000 daltons and one protein (M.W. 14,000) were observed in abundance in the pituitary tumors as compared with the controls.

In addition, the pattern of secreted proteins of GH_3 tumors from TEG athymic nude mice cultured in the presence of estrogen, nafoxidine and estrogen plus nafoxidine was examined. Nafoxidine functions as an antiestrogen. Our results showed that estrogen increased the secreted proteins by these tumors. The production of these proteins was inhibited by nafoxidine. However, when estrogen was added simultaneously

with nafoxidine, this decrease in secreted proteins was prevented. These results suggested that estrogen may regulate the secretion of these proteins. It is possible that one or a combination of these pituitary proteins may be mitogenic to human breast cancer cells.

Recently, Mittra (1980) reported that a 16,000 fragment of prolactin can be generated in pituitaries of estrogen-treated rats. This 16K fragment was shown to increase the mitotic indexes of normal rat mammary epithelial cells (Mittra, 1980b). Whether the 14K protein observed in our experiment is related to this 16K prolactin fragment has not been determined. Preliminary results (Leung and Owens, unpublished observations) showed that our 14K protein failed to stimulate the growth of Nb_2 lymphoma cells which is specifically stimulated by prolactin-like substances (Tanaka et al., 1980). These results suggest that the 14K protein may not be related to prolactin. A growth factor which stimulates the proliferation of ovarian cells was isolated from bovine pituitaries (Gospodarowicz et al., 1974). This ovarian growth factor (OGF) is a basic protein with a molecular weight in the range of 10,000 and 13,000 daltons. Whether the secreted proteins observed in our experiment correspond to the OGF is not known. Interestingly, the bovine pituitary-derived mammary growth factor reported by Rudland (1979) was present in the same CM-cellulose fraction as OGF. More intensive

characterization will be needed to determine the identity of this 14K protein.

The soluble extracts of the GH_3 tumors were also analyzed by gel exclusion chromatography using Sephadex G-100 (Fig. 35). Most of the proteins were eluted in the void volume. The fractions were pooled and their mitogenic activity were monitored using the T-47D cells as a bioassay. A peak of mitogenic activity was detected just before the ovalbumin marker suggesting that the growth factor(s) has a molecular weight of between 45,000 to 60,000 daltons. It is tempting to speculate that this peak of mitogenic activity may correspond to the 30,000-60,000 protein bands observed in the SDS-PAGE experiment. However, the identity of this factor(s) remains to be determined.

Section 9: Concluding Remarks

While reviewing the literature on breast cancer, it became clear that despite the enormous volume of work that has been done using experimental animal models, the amount of information regarding the mechanisms of hormonal influence in human breast cancer is relatively scanty. Thus, the objective of this thesis was to study some of the hormonal factors which regulate the growth of human breast cancer and hopefully from these studies, some new information and hypotheses will be generated. One of the problems facing the biomedical researchers in this field is the difficulty in obtaining large quantities of fresh human breast tumor biopsies to work on. The recent establishment of a number of human breast cancer cell lines has provided an excellent alternative to study this disease. By using these cell lines as models, the culture conditions and hormonal milieu can be precisely controlled.

Epidermal growth factor is a single chain polypeptide first isolated from mouse submaxillary gland. Many epithelial cells and fibroblasts were stimulated to proliferate by EGF.

Recently, EGF was found in human milk and was suggested to play a role in the development of the mammary epithelium. Therefore, we decided to examine the binding of EGF to nine human mammary cell lines (T-47D, MCF-7, SK-Br-3, AlAb 496, BT-20, BT-474, HBL-100, DU4475 and Lev III) and studied the growth promoting activity of EGF on these cells. Receptors for EGF was found in all human breast cancer cell lines which can be grown as monolayers. Both high affinity (10^{-10} M) and low affinity (10^{-9} M) sites were detected. In contrast, no EGF binding can be detected in cells maintained as suspension cultures. It was found that only T-47D cells were stimulated by EGF (0.1 ng/ml) while the other cell lines either were inhibited by EGF or did not show any responses. Thus, there was no apparent correlation between EGF binding and its growth promoting activity. Whether EGF can act synergistically with estrogen to stimulate breast cancer development is unknown. These results indicate that breast cancer cells from various sources respond to EGF differently, perhaps a reflection of the differences in the state of differentiation. It is quite possible that EGF may have other biological roles in human breast cancer cells besides growth regulation.

Besides EGF, the effects of various hormones and growth factors on the growth of human breast cancer cells cultured on plastic were also tested. Although the human breast cancer cells were stimulated by insulin, triiodothyronine and

fibroblast growth factor, other mammotropic hormones (such as growth hormone and prolactin) and estrogen did not exert any significant influence on the growth of these cells in vitro. In view of the recently reported importance of extracellular matrix (ECM) on the growth of epithelial cells, it is possible that breast cancer cells cultured on plastic dishes are "unphysiological" and therefore cannot respond to the same hormonal signals as their in vivo counterparts.

One of the major components of ECM is collagen. Therefore, in order to improve the culture conditions of the human breast cancer cells, we decided to use commercially available cowhide type 1 collagen (vitrogen 100) as a substrate for maintaining these cells. Using the artificial collagen gel, the morphological and proliferative characteristics of two breast cancer cell lines (T-47D and MCF-7) and one non-tumorigenic cell line (HBL-100) cultured in collagen were compared with those of cells grown on plastic substratum. The human breast cancer cells grew as monolayer on plastic. In contrast, the tumor cells displayed a spherical shape and formed multilayered aggregates when cultured in collagen. Interestingly, the human mammary epithelial cells cultured in collagen matrix became more serum dependent for growth. In addition, the doubling time was longer for cells cultured in collagen than on plastic. These studies illustrate that the substrate can indeed alter the behavior of the breast tumor cells. Cell-cell

contact, cell-substrate interactions, and cell shape as related to its differentiated function are some of the areas which warrant further research. The T-47D and MCF-7 cells were also shown to proliferate rapidly in a serum free medium containing insulin, epidermal growth factor, estrogen and transferrin, suggesting that these cells can respond to the above hormones. Thus, the collagen gel system can be successfully used for maintaining human breast cancer cells. We then ask the question: "Can prolactin, growth hormone and other hormonal factors by themselves or in combination with each other stimulate the growth of human breast cancer cells cultured in collagen?" Despite the healthy appearance of the cells, they did not show any significant growth response when the hormones were added. Similar observations of mouse mammary epithelial cells cultured in collagen gel have been reported (Yang et al., 1980).

In retrospect, these results may not appear to be so surprising. Since ECM is made up of many other components such as type 4 collagen, glycosaminoglycans, laminin and fibronectin besides type 1 collagen, it is possible that cells cultured on type 1 collagen may still require other factors in order to respond to hormones. Future experiments can be designed to study hormone effect on human breast cancer cells maintained on ECM. Extracellular matrix can readily be generated from endothelial cell cultures using the procedures described by Gospodarowicz et al (1978).

Due to the lack of response of human breast cancer cells to prolactin and growth hormone *in vitro*, we then turned to animal models (athymic nude mice) to see whether hormones are capable of influencing the proliferation of human breast cancer cells *in vivo*.

The athymic nude mouse, which has a congenital thymic deficiency, allows one to transplant tissue from another species without worrying about rejection. Human breast tumor cells, T-47D, possessing both prolactin and estrogen receptors were transplanted subcutaneously in athymic nude mice. It was rationalized that since rat pituitary tumor cells (GH_3) secrete both prolactin, growth hormone and other pituitary factors (Tashjian et al., 1970), these tumors may be useful as exogenous sources of these hormones. Therefore, we subcutaneously transplanted either rat pituitary glands or GH_3 cells into the nude mice. Initially, five groups of female nude mice were used: The first group (T) received T-47D cells only; the second group (TE) was injected with T-47D cells and estradiol valerate; the third group (TG) was injected with T-47D and GH_3 cells; the fourth group (TEG) received T-47D cells, estradiol valerate and GH_3 cell; and the fifth group (TEP) received T-47D cells, estradiol valerate and normal rat pituitary transplants. The T-47D cells formed solid tumors in the nude mice and the tumor sizes were monitored. It was found

that the T-47D cells did not grow in female nude mice (T group), suggesting that the hormonal milieu in these animals is not optimal for the growth of the human breast tumor cells. Interestingly, no apparent growth of T-47D tumors in mice bearing GH_3 pituitary tumors (TG group) was observed, despite very high concentrations of prolactin and growth hormone in the blood of these animals. Thus, it seems that prolactin and growth hormone alone are not sufficient to stimulate the growth of T-47D tumor *in vivo*. On the other hand, injection of estrogen alone (TE group) resulted in a moderate growth of T-47D tumor. In contrast, the simultaneous presence of both estrogen and GH_3 tumor (TEG groups) induced a rapid and sustained growth of T-47D tumor, resulting in an eight-fold increase in volume over that of TE group, indicating that estrogen alone cannot produce maximal growth of human breast cancer cells. Implantation of normal rat pituitaries in estrogen-treated nude mice (TEP) also induced very rapid growth of T-47D tumors to a size comparable to that seen in the TEG group. Whether the factors from the normal pituitary are the same as those from the GH_3 tumors is unknown.

The *in vivo* studies show that human breast tumor cells are responsive to estrogen and pituitary hormones, even to pituitary factors from a non-human species. What then is (are) the pituitary factor(s) that stimulate the growth *in vivo* of human breast cancer cells? Could the principal active factor be prolactin or growth hormone; or is it another unknown factor?

To answer these questions, we decided to take two approaches. First, to transplant other pituitary tumors besides GH_3 pituitary tumors into the athymic nude mice to see whether factors secreted from these pituitary tumors can also stimulate the growth of T-47D tumors. We have chosen to transplant AtT-20 cells (secrete ACTH only), GH_1 (secrete primarily growth hormone) and 235-1 cells (secrete prolactin only). Second, highly purified human growth hormone and ovine prolactin were delivered to the athymic nude mice by implanted osmotic mini pumps. The reason for using ovine prolactin rather than human prolactin in these experiments is because of the scarcity of the latter.

All the transplanted pituitary tumor cells formed tumors in the athymic nude mice. Both the GH_1 and GH_3 tumors enhanced the growth of T-47D tumors in the estrogenized nude mice. In contrast the transplanted AtT-20 tumor, which secrete ACTH only, did not have any significant effects on the T-47D tumors in the estrogenized animals. This observation suggests that ACTH may not have a direct influence on human breast cancer cells. Preliminary results indicate that the 235-1 cells also form tumors rapidly after transplantation into athymic nude mice. Again no significant effect of the transplanted 235-1 tumors on the T-47D tumors was observed. Since 235-1 cells produce only prolactin, it would imply that perhaps prolactin may not be directly involved in breast cancer

development. It follows that growth hormone or its intermediate may play a more important role in the etiology of breast cancer. To further study these problems, we implanted osmotic mini pumps into the athymic nude mice.

We arbitrarily chose the infusion rate of growth hormone and prolactin to be 1.25 ug/hr in the mini pump. It turned out that the dosage was too low and there was no significant effect on the growth of the T-47D tumors. The dose of human growth hormone was then raised to 15 ug/hr in the mini pump. Some of the animals died, probably due to intolerance of the high dosage or perhaps due to the presence of pyrogens in the growth hormone preparation used. However, in the surviving animals, the T-47D tumors were significantly larger than the TE controls. These findings remain to be confirmed. Nevertheless the results suggest that growth hormone may be involved in the growth of human breast cancer. To determine whether human growth hormone acts directly on human breast cancer cells, or through some intermediate pathways, the following approach can be taken in the future. Ovine growth hormone can be used instead of human growth hormone. Ovine growth hormone, being a non-primate hormone, does not bind to receptors on the human breast cancer cells (Shiu, 1979). Therefore, if ovine growth hormone can also stimulate T-47D tumor growth in nude mice, it would imply that growth hormone probably acts through an intermediate pathway, a pathway that involves somatomedins.

Therefore, the levels of somatomedins in the blood of the nude mice treated or not treated with growth hormone can be determined and correlated with the growth of the T-47D human breast tumors. Recently, somotomedin-like polypeptides have been reported to be produced by mammary tumors (Knauer et al., 1980). It is conceivable that growth hormone may act on the breast cancer cells which in turn produce their own somatomedin-like growth factors. These factors can then stimulate the growth of the breast cancer cells. This hypothesis can be tested by culturing human breast cancer cells in the presence or absence of growth hormone and by monitoring the cell growth and somatomedin levels in the culture medium.

Alternatively, the pituitary factor(s) that stimulated the in vivo growth of human breast cancer is not one of the classical hormones. It is known that the pituitary gland is a rich source of many other factors besides the classical hormones. For instance, Gospodarowicz (1975) had purified fibroblast growth factor from bovine pituitary. Kasper et al (1982) have also identified and partially purified a novel peptide from human pituitary glands which they termed chondrocyte growth factor. Both fibroblast growth factor and chondrocyte growth factor are mitogenic in many cell types but have very little effect in human breast cancer cells. In addition, Rudland et al. (1979) provided evidence for a pituitary-derived factor that stimulates the growth of rat

mammary tumor cells. Therefore, efforts were made to identify a pituitary-derived growth factor for human breast cancer. Preliminary steps have been taken towards the identification and isolation of this factor (see Section 7).

If GH_3 pituitary tumor cells secreted a growth factor in nude mice, they may also secrete it into the cultured medium in vitro. Indeed, preliminary results suggest conditioned media of GH_3 cells stimulated the proliferation of T-47D cells in vitro. In addition, extract of GH_3 tumors dissected from nude mice also stimulated the growth of T-47D cells in vitro. The protein synthesized and secreted by normal rat pituitary and GH_3 tumors were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. It was found that pituitary glands from estrogen treated rats or GH_3 pituitary tumors of estrogen-treated nude mice secreted more proteins than the corresponding tissues from animals that received no estrogen. There are protein bands around the 40-60,000 region in the estrogen treated group which are absent in the controls. Preliminary gel exclusion chromatography of the GH_3 tumor extract using sephadex G100 reveals an active peak around the 40-60,000 region. Thus, results from these studies suggest that GH_3 pituitary tumor cells secrete a factor(s) which stimulates the growth of T-47D cells.

For future studies, two sources can be used as starting materials for purification of the putative pituitary-derived

growth factor: GH_3 pituitary tumors dissected from nude mice, or conditioned medium of GH_3 cells. The choice of starting material would depend on which source contains higher growth factor activity. If both sources contain equal amounts of growth promoting activity, then the conditioned medium of GH_3 cells will be used as starting material. This is because there are less "contaminating" proteins in the conditioned medium. The factor will then be purified using standard protein purification techniques such as chromatography techniques (gel exclusion, ion exchange, absorption, and affinity chromatographies), preparative isoelectric focusing and high performance liquid chromatography. The purification steps will be developed empirically. The partially purified factor can then be characterized to see whether it resembles any known pituitary hormones.

Once this pituitary-derived growth factor has been purified, antiserum to this factor can be generated by immunizing rabbits with this material. Studies such as identification of the specific cell type in the pituitary which produce this factor using immunochemical methods can be performed. If indeed a pituitary-derived growth factor is purified from rat pituitary tumor cells, it will be worthwhile to search for a similar factor in the human pituitary.

Although the link between hormones and breast cancer was observed several decades ago, advances in breast cancer

treatment have produced only limited improvement. In fact, the mortality rate for breast cancer has remained the same for the last 50 years despite extensive research done on this disease. Some of the recent advances in breast cancer include early diagnosis, mammographic screening, combination radiation and chemotherapy, and most recently, the detection of hormone receptors (such as estrogen and progesterone receptors) in the breast tumor. Although these new developments have undoubtedly proven useful in some cases, a large population of patients with breast cancer remain unsuccessfully treated.

These obviously unsatisfactory results should alert us to how little one actually understands the disease. Could it be that we are asking the wrong questions? Assuming we are asking the right questions, what then are the exact mechanisms which allow hormones to influence the breast tumors? To answer these questions, one must be prepared to discard the old dogmas and hypotheses and search for new ones.

For instance, mastectomy has been used extensively in the past as a primary treatment and cure for advanced breast cancer. It was simplistically thought that if the tumors were in the breast, then the best way to cure the disease was by surgically removing the mammary tissue. However, studies by Peaker and Maule Walker (1980) showed that the reproductive cycles in mastectomized goats were greatly disturbed. These results suggest that maybe the mammary gland is not just a

passive target organ for hormones to act on. It is possible that the mammary gland may secrete its own hormones to communicate with the pituitaries and other endocrine organs, which in turn regulate the growth and function of the mammary gland.

An alternate explanation for the etiology of breast cancer was proposed by us in Section 6. It is possible that the pituitary gland may produce another factor(s) besides the classical hormones which can regulate the growth of breast cancer. These factors may act directly on the tumor cells, or they can act via some intermediate pathways.

Many external stimuli can affect the secretion of the pituitaries. For example, stress, pregnancy, aging, diet and hormonal fluctuations can all influence the way the pituitary functions. It is well documented that classical pituitary hormones such as prolactin, growth hormone and oxytocin definitely play an important role in the normal development and function of the breast. It is therefore conceivable that production of another unidentified pituitary factor(s) may be triggered by various insults to the body and thus may set the stage for mammary carcinogenesis.

The role of hormones in human breast cancer is extremely complex. But it is because of this complexity that the problem becomes so much more intriguing and challenging. It was said that "ambiguous ideas are usually the most fertile ones". This

is definitely true for the hormone-breast cancer field. Future research in this area will undoubtedly provide fascinating insights into the process of neoplasia and other exciting aspects of basic cell biology.

Section 10: References

Adamson, E.D. and Anthony, R.R. Epidermal growth factor receptors. Mol. Cell. Biochem., 34:129-152, 1981.

Allegra, J.C. and Lippman, M.E. Growth of a human breast cancer cell line in serum-free hormone-supplemented medium. Cancer Res., 38:3823, 1978.

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

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

Barnes, D. and Sato, G. Growth of a human mammary tumor cell line in a serum-free medium. Nature, 281:388-389, 1979.

Barnes, D. and Sato, G.H. Serum-free cell culture: A unifying approach. Cell, 22:649-655, 1980.

Beatson, G.T. On the treatment of inoperable cases of carcinoma of the mamma: Suggestions for a new method of treatment, with illustrative cases. Lancet, 2:104-107, 1896.

Bennet, D.C. Morphogenesis of branching tubules in cultures of cloned mammary epithelial cells. Nature, 285:657-659, 1980.

Benveniste, R., Speeg, K.V., Jr., Carpenter, G., Cohen, S., Lidner, J. and Rabinowitz, D. Epidermal growth factor stimulates secretion of human chorionic gonadotropin by cultured human carcinoma cells. J. Clin. Endocr. Metab., 46:169-172, 1978.

Berliner, J.A., Aharanov, A. and Prusso, R. Cell surface changes associated with EGF and insulin-induced cell adhesion. Exp. Cell Res., 133:227-235, 1981.

Bogardus, G.M. and Finley, J.W. Breast cancer and thyroid disease. Surgery, 49:491, 1961.

Bower, J.M., Camble, R., Gregory, H., Gerring, E.L. and Willshire, I.R. The inhibition of gastric acid secretion by epidermal growth factor. Experimentia, 30:22-23, 1974.

Brockes, J.P., Lemke, G.E. and Balzer, D.R. Purification and preliminary characterization of a glial growth factor from the bovine pituitary. J. Biol. Chem., 255:8374-8377, 1980.

Bronzert, D.A., Monaco, M.E., Pinkus, L., Aitken, S. and Lippman, M.E. Purification and properties of estrogen responsive of thymidine kinase from human breast cancer. Cancer Res., 41:604, 1981.

Buonassisi, V., Sato, G. and Cohen, A.I. Hormone producing cultures of adrenal and pituitary origin. Proc. Natl. Acad. Sci. U.S.A., 48:1184-1190, 1962.

Burke, R.E. and McGuire, W.L. Nuclear thyroid hormone receptors in a human breast cancer cell line. Cancer Res., 38:3769, 1978.

Burwen, S.J. and Pitelka, D.R. Secretory function of lactating mouse mammary epithelial cells cultured on collagen gels. Exp. Cell Res., 126:249-262, 1980.

Butler, W.B., Kirkland, W.L. and Jorgenson, T.I. Induction of plasminogen activation by estrogen in a human breast cancer cell line (MCF-7). Biochem. Biophys. Res. Commun., 90:1328, 1979.

Cailleau, R., Young, R., Olive, M. and Reeves, W.J., Jr. Breast tumor cell lines from pleural effusions. J. Natl. Cancer Inst., 53:661-674, 1974.

Cailleau, R., Olive, M. and Cruciger, Q.V.J. Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. In Vitro, 14:911-915, 1978.

Cameron, G. and Chambers, R. Neoplasm studies: Organization of cells of human tumors in tissue culture. Am. J. Cancer, 30:115-129, 1937.

Carpenter, G. and Cohen, S. Epidermal growth factor. Ann. Rev. Biochem., 48:193, 1979.

Carr, D. and Friesen, H.G. Growtn hormone and insulin binding to human liver. J. Clin. Endocrinol. Metab., 42:484-493, 1976.

Chen, L.B. and Gudor, R.C. Control of a cell surface major glycoprotein by epidermal growth factor. Science, 197:776-778, 1977.

Cohen, N.D. and Hilf, R. Influence of insulin on growth and metabolism of 7,12-Dimethylbenz(A)anthracene-induced mammary tumors. Cancer Res., 34:3245-3252, 1974.

Cohen, S. Purification of a nerve growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. Proc. Natl. Acad. Sci. U.S.A., 46:302-311, 1960.

Cohen, S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem., 237:1555-1562, 1962.

Cohen, S. and Savage, C.R. Recent studies on the chemistry and biology of epidermal growth factor. Prog. Horm. Res., 30:551-574, 1974.

Cohen, S. and Carpenter, G. Human epidermal growth factor: Isolation and chemical and biological properties. Proc. Natl. Acad. Sci. U.S.A., 72:1317-1321, 1975.

Dulak, N.C. and Temin, H.M. A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication stimulating activity for embryo fibroblasts. J. Cell Physiol., 81:153-160, 1973.

Eastment, C.T. and Sirbasku, D.A. Platelet derived growth factor(s) for a hormone-responsive rat mammary tumor cell line. J. Cell Physiol., 97:17-28, 1978.

Edwards, D.P., Adams, D.J., Squage, N. and McGuire, W.L. Estrogen induced synthesis of specific proteins in human breast cancer cells. Biochem. Biophys. Res. Commun., 93:804, 1980.

Ehrmann, R.L. and Gey, G.O. The growth of cells on a transparent gel of reconstituted rat tail collagen. J. Natl. Cancer Inst., 16:1375-1403, 1956.

Eippers, B.A. and Main, R.E. Structure and biosynthesis of proadrenocorticotropin/endorphin and related peptides. Endocr. Rev., 1:1-27, 1980.

Eisman, J.A., Martin, T.J., MacIntyre, I., Framptin, R.J., Moseley, J.M. and Whitehead, R. 1,25-Dihydroxy vitamin D_3 receptor in a cultured human breast cancer cell line (MCF-7 cells). Biochem. Biophys. Res. Commun., 93:9, 1980.

Emerman, J.T., Enami, J., Pitelka, D.R. and Nandi, S. Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes. Proc. Natl. Acad. Sci. U.S.A., 74:4466-4470, 1977.

Emerman, J.T., Burwen, S.J. and Pitelka, D.R. Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue and Cell, 11:109-119, 1979.

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

Engel, L.W., Young, N.A., Tralka, T.S., Lippman, M.E., O'Brien, S. and Joyce, M.J. Breast carcinoma cells in continuous culture: Establishment and characterization of three new cell lines. Cancer Res., 38:3352, 1978.

Engel, L.W. and Young, N.A. Human breast carcinoma cells in continuous culture: A review. Cancer Res., 38:4327-4339, 1978.

Esber, H., Payne, I. and Bogden, A. Variability of hormone concentrations and ratios in commercial sera used for tissue culture. J. Natl. Cancer Inst., 50:559, 1973.

Fell, H. and Robinson, R. The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro. Biochem. J., 23:767-784, 1929.

Feller, W.F., Old, L. and Beth, E. Type C particles in an established cell line of human origin. Proc. Am. Assoc. Cancer Res., 11:25A, 1970.

Findlay, D.M., DeLuise, M., Michelangeli, V.P., Ellison, M. and Martin, T.J. Properties of a calcitonin receptor and adenylate cyclase in BEN cells, a human cancer cell line. Cancer Res., 40:1311-1317, 1980.

Flanagan, S.P. Nude, a new hairless gene with pleiotropic effects in the mouse. Genet. Res., 8:295-309, 1966.

Fogh, J., Fogh, J.M. and Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst., 59:221-225, 1977.

Fogh, J. and Trempe, G. New human tumor cell lines. In: J. Fogh (ed.), Human tumor cells in vitro. Pp. 115-145. New York: Plenum Press, 1975.

Folkman, J. and Moscona, A. Role of cell shape in growth control. Nature, 273:345-349, 1978.

Frantz, A.G., Hobif, D.V., Hyman, G.A., Sub, H.K., Sassin, J.F., Zimmerman, E.A., Noel, G.L. and Kleinberg, D.L. In: Human Prolactin. Pasteels, J.L., Robyn, C. (eds.), Amsterdam: Excerpta Medica, 273-290, 1973.

Froesch, E.R., Burgi, H. and Ramseier, E.B. Antibody suppressible and non-suppressible insulin-like activities in human serum and their physiologic significance: An insulin assay with adipose tissue of increased precision and specificity. J. Clin. Invest., 42:1816-1834, 1963.

Furth, J. Prolactin and carcinogenesis. In: 4th Tenovus workshop on prolactin and carcinogenesis. Boyns, A.R., Griffiths, K. (eds.), 137-142, 1972.

Gill, G.N. and Lazar, C.S. Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. Nature, 239:305-307, 1981.

Giovanella, B.C., Stehlin, J.S. and Williams, L.J., Jr. Heterotransplantation of human malignant tumors in "nude" thymusless mice. II. Malignant tumors induced by injection of cell cultures derived from human solid tumors. J. Natl. Cancer Inst., 52:921-930, 1974.

Gospodarowicz, D. Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature, 249:123-127, 1974.

Gospodarowicz, D., Jones, K.L. and Sato, G. Biological activity of a growth factor for ovarian cells. Proc. Natl. Acad. Sci. U.S.A., 71:3372-3376, 1974.

Gospodarowicz, D. Purification of a fibroblast growth factor from bovine pituitary. J. Biol. Chem., 250:2515-2520, 1975.

Gospodarowicz, D. and Moran, J.S. Growth factors in mammalian cell culture. Ann. Rev. Biochem., :531-557, 1976.

Gospodarowicz, D., Bialecki, H. and Greenburg, G. Purification of the fibroblast growth factor from bovine brain. J. Biol. Chem., 253:3736, 1978.

Gospodarowicz, D., Greenburg, G. and Birdwell, C.R. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. Cancer Res., 38:4155-4171, 1978.

Gospodarowicz, D. and Tauber, J.P. Growth factors and the extracellular matrix. Endocr. Rev., 1:201-227, 1980.

Greenwood, F.C. and Hunter, W.M. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J., 89:114-123, 1963.

Gregory, H. Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature, 257:325-327, 1975.

Haigler, H., Ash, J.F., Singer, S.J. and Cohen, S. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A431. Proc. Natl. Acad. Sci. U.S.A., 75:3317-3321, 1978.

Hay, E.D. Embryonic induction and tissue interaction during morphogenesis. In: Littlefield, J.W., de Grouchy, J. (eds.), Birth Defects, Excerpta Medica: Amsterdam, Oxford, 126-140, 1978.

Hedman, K., Vaheri, A. and Wartiovaara, J. External fibronectin of cultured human fibroblasts is predominantly a matrix protein. J. Cell Biol., 76:748, 1978.

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

Henderson, B.E., Gerkins, V., Rosario, I., Casagrande, J. and Pike, M.C. Elevated serum levels of estrogen and prolactin in daughters of patients with breast cancer. N. Engl. J. Med., 293:790-795, 1975.

Hirohashi, S., Shimosato, Y., Kameya, T., Nagai, K. and Tsunematsu, R. Hormone dependency of a serially transplantable human breast cancer (Br-10) in nude mice. Cancer Res., 37:3184-3189, 1977.

Holdaway, I.M. and Friesen, H.G. Correlation between hormone binding and growth response of rat mammary tumor. Cancer Res., 36:1562-1567, 1976.

Holdaway, I.M. and Friesen, H.G. Hormone binding by human mammary carcinoma. Cancer Res., 37:1946-1952, 1977.

Hollenberg, M.D. and Gregory, H. Human urogastrone and mouse epidermal growth factor share a common receptor site in cultured human fibroblasts. Life Sci., 20:267-274, 1976.

Holley, R.W. and Kiernan, J.A. "Contact inhibition" of cell division in 3T3 cells. Biochem., 60:300-304, 1968.

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

Huff, K.R. and Guroff, G. Nerve growth factor-induced reduction in epidermal growth factor receptors in PC 12 cells: An aspect of cell differentiation. Biochem. Biophys. Res. Commun., 89:175-180, 1979.

Huggins, C., Torralba, Y. and Charr, A. Endocrine influences on growth of a benign transplantable mammary tumor. Science, 123:674, 1956.

Imai, Y., Tsushima, T., Sasaki, N. and Matsuzaki, F. Radioreceptor assay for epidermal growth factor. In: I. Shizume (ed.), Growth and growth factors. Pp. 275-288. Tokyo: University of Tokyo Press, 1980.

Imai, Y., Leung, C.K.H., Shiu, R.P.C. and Friesen, H.G. Receptors and growth promoting activity of epidermal growth factor in human breast cancer cell lines. J. Cell Biol., 91:225, 1981.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and DeSombre, E.R. A two step mechanism for the interaction of estradiol with rat uterus. Proc Natl. Acad. Sci., 59:632-638, 1968.

Jensen, E.V. and DeSombre, E.R. Mechanism of action of the female sex hormones. Ann. Rev. Biochem., 41:203-230, 1972.

Kahn, C.R., Freychet, P. and Roth, J. Quantitative aspects of the insulin-receptor interaction in liver plasma membrane. J. Biol. Chem., 249:2249-2257, 1974.

Kano-Sueoka, T., Campbell, G.R. and Gerber, M. Growth stimulating activity in bovine pituitary extract specific for a rat mammary carcinoma cell line. J. Cell Physiol., 93:417-424, 1977.

Kano-Sueoka, T., Cohen, D.M., Yamaizumi, Z., Nishimura, S., Mori, M. and Fujiki, H. Phosphoethanolamine as a growth factor of a mammary carcinoma cell line of rat. Proc. Natl. Acad. Sci. U.S.A., 76:5741-5744, 1979.

Kapdi, C.C. and Wolfe, J.N. Breast cancer. Relation to thyroid supplements for hypothyroidism. J. Am. Med. Assoc., 236:1124, 1976.

Kasper, S., Worsley, I.G., Rowe, J.M., Shiu, R.P.C. and Friesen, H.G. Chondrocyte growth factor from the human pituitary gland. J. Biol. Chem., 1982 (in press).

Keydar, I., Chen, L., Karby, S., Weiss, F.R., Delarea, J., Radu, M , Chaitcik, S. and Brenner, H.J. Establishment and characterization of a cell line of human breast carcinoma origin. Eur. J. Cancer, 15:659-670, 1979.

Kim, V. and Furth, J. The role of prolactin in carcinogenesis. Vit. Horm., 34:107-136, 1976.

Kleinberg, D.L. Human alpha-lactalbumin: Measurement in serum and in breast cancer organ cultures by radioimmunoassays. Science, 190:276, 1975.

Knauer, D.J., Iyer, A.P., Banerjee, M.R. and Smith, G.L. Identification of somatomedin-like polypeptides produced by mammary tumors of Balb/c mice. Cancer Res., 40:4368-4372, 1980.

Kohler, N. and Lipton, A. Platelets as a source of fibroblast growth-promoting activity. Exp. Cell Res., 87:297-301, 1974.

Kumaoka, S., Takatani, O., Abe, O., Utsunomiya, J., Wang, D.Y., Bulbrook, R.D., Hayward, J.L. and Greenwood, F.C. Plasma prolactin, thyroid stimulating hormone, follicle stimulating hormone and luteinizing hormone in normal British and Japanese women. Eur. J. Cancer, 12:767-774, 1976.

Kwa, H.G., Engelsman, E., DeJong-Bakker, M. and Cleton, E.J. Plasma prolactin in human breast cancer. Lancet, 1:433-434, 1974.

Kwa, H.G. and Wang, D.Y. An abnormal luteal-phase evening peak of plasma prolactin in women with a family history of breast cancer. Int. J. Cancer, 20:12-14, 1977.

Lacassagne, A. A comparative study of the carcinogenic action of certain oestrogenic hormones. Am. J. Cancer, 28:735-740, 1936.

Lacroix, A. and Lippman, M.E. Binding of retinoids to human breast cancer cell lines and their effects on cell growth. J. Clin. Invest., 65:586, 1980.

Langlois, A.J., Holder, W.D., Jr., Iglehart, J.D., Nelson-Rees, W.A., Wells, S.A., Jr. and Bolognesi, D.P. Morphological and biochemical properties of a new human breast cancer cell line. Cancer Res., 39:2604-2613, 1979.

Lasfargues, E.Y. and Ozzello, L. Cultivation of human breast carcinomas. J. Natl. Cancer Inst., 21:1131-1147, 1958.

Lasfargues, E.Y., Coutinho, W.G. and Redfield, E.S. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. J. Natl. Cancer Inst., 64:967-978, 1978.

Laskey, R.A. and Mills, A.D. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem., 56:335-341, 1975.

Leland, F.E. and Sirbasku, D.A. Estrogen-responsive pituitary tumors and normal rat pituitary tissue are sources of mammary tumor cell growth factors. Proc. Am. Assoc. Cancer Res., 22:30 (abstract), 1981.

Lembach, K.J. Enhanced synthesis and extracellular accumulation of hyaluronic acid during stimulation of quiescent human fibroblasts by mouse epidermal growth factor. J. Cell Physiol., 89:277-288, 1976.

Leung, B.S. and Sasaki, G.H. On the mechanism of prolactin and estrogen action in 7,12-dimethylbenz(A)anthracene- induced mammary carcinoma in the rat. II. In vivo tumor responses and estrogen receptors. Endocrinol., 97:564-572, 1975.

Leung, C.K.H. and Shiu, R.P.C. Growth characteristics of human breast cancer cells in collagen gel. J. Cell Biol., 87:115, 1980.

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., 41:546-551, 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, 1982 (in press).

Leung, C.K.H., Paterson, J.A., Imai, Y. and Shiu, R.P.C. Transplantation of ACTH-secreting pituitary tumor cells in athymic nude mice. Vichows Archiv A. (Path. Anat.), 1982 (in press).

Levi-Montalcini, R. The nerve growth factor: Its mode of action on sensory and sympathetic nerve cells. Harvey Lec., 60:217-259, 1965.

Levi-Montalcini, R. and Angeletti, P.U. Nerve growth factor. Physiol. Rev., 48:534-569, 1968.

Liotta, L.A., Vembu, D., Kleinman, H.K., Martin, G.R. and Boone, C. Collagen required for proliferation of cultured connective tissue cells but not their transformed counterparts. Nature (Lond.), 272:622-624, 1978.

Lippman, M.E., Bolan, G. and Huff, K. The effects of glucocorticoids and progesterone on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res., 36:4602, 1976.

Lippman, M.E., Osborne, C.K., Knazek, R. and Young, N. In vitro model systems for the study of hormone-dependent human breast cancer. N. Engl. J. Med., 296:154-159, 1977.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193:265-275, 1951.

MacLeod, R.M., Allen, M.S. and Hollander, V.P. Hormonal requirements for the growth of mammary adenocarcinoma (MTW9) in rats. Endocrinol., 75:249, 1964.

MacMahon, B., Cole, P., Lin, T.M., Lowe, C.R., Mirra, A.P, Ravnihar, B., Salber, E.J., Valaoras, V.G. and Yuasa, S. Age at first birth and breast cancer risk. Bull. W.H.O., 43:209, 1970.

MacMahon, B., Cole, P. and Brown, J. Etiology of human breast cancer: A review. J. Natl. Cancer Inst., 50:21-42, 1973.

McCormick, D.L., Mehta, R.G., Thompson, C.A., Dinger, N., Caldwell, J.A. and Moon, R.C. Enhanced inhibition of mammary carcinogenesis by combined treatment with N-(4-hydroxyphenyl) retinamide and ovariectomy. Cancer Res., 42:508-512. 1982.

McGuire, W.L., Carbone, P.P. and Vollmer, E.P., Eds. Estrogen receptors in human breast cancer. New York: Raven Press, 1975.

McGuire, W.L., Horwitz, K.B., Zava, D.T., Garola, R.E. and Chamness, G.C. Hormones in breast cancer: Update 1978. Metabolism, 27:487-501, 1978.

McKeehan, W.L., Genereux, D.P. and Ham, R.G. Assay and partial purification of factors from serum that control multiplication of human diploid fibroblasts. Biochem. Biophys. Res. Commun., 80:1013-1021, 1978.

McManus, M.J., Dembroske, S.E., Pienkowski, M.M., Anderson, T.J., Mann, L.C., Schuster, J.S., Vollwiler, L.L. and Welsch, C.W. Successful transplantation of human benign breast tumors into the athymic nude mouse and demonstration of enhanced DNA synthesis by human placental lactogen. Cancer Res., 38:2343-2349, 1978.

McManus, M.J. and Welsch, C.W. Hormone-induced ductal DNA synthesis of human breast tissues maintained in the athymic nude mouse. Cancer Res., 41:3300-3305, 1981.

Meites, J., Lee, K.H., Wuttke, W., Welsch, C.W., Nagasawa, H. and Quadri, S.K. Recent studies on functions and control of prolactin secretion in rats. Recent Prog. Horm. Res., 28:471-526, 1972.

Meyer, J.S., Bauer, W.C. and Rao, B.R. Subpopulation of breast carcinoma defined by S-phase fraction, morphology, and estrogen receptor content. Lab. Invest., 39:225, 1978.

Michaelopoulos, G. and Pitot, H.C. Primary culture of parenchymal liver cells on collagen membranes. Exp. Cell Res., 94:70-78, 1975.

Millar, M.J. and Noble, R.L. Effects of exogenous hormones on growth characteristics and morphology of transplanted mammary fibroadenoma of the rat. Br. J. Cancer, 8:495, 1954.

Mittra, I. A novel "cleaved prolactin" in the rat pituitary: Part I - Biosynthesis, characterization and regulatory control. Biochem. Biophys. Res. Comm., 95:1750-1759, 1980.

Mittra, I. A novel "cleaved prolactin" in the rat pituitary: Part II - In vivo mammary mitogenic activity of its N-terminal 16K moiety. Biochem. Biophys. Res. Comm., 95:1760-1767, 1980.

Monaco, M.E., Lippman, M.E., Knacek, R. and Kidwell, W.R. Vasopressin stimulation of acetate incorporation into lipids in a dimethylbenz(a)-anthracene-induced rat mammary tumor cell line. Cancer Res., 38:4101, 1978.

Moon, R.C., Grubbs, C.J. and Sporn, M.B. Inhibition of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis by retinyl acetate. Cancer Res., 36:2626, 1976.

Nagasawa, H. Prolactin and human breast cancer: A review. Europ. J. Cancer, 15:267-279, 1979.

Ong, D.E., Page, D.L. and Chytil, F. Retinoic acid binding protein: Occurrence in human tumors. Science, 190:60, 1975.

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. U.S.A., 73:4536-4540, 1976.

Osborne, C.K., Monaco, M.E., Lipmann, M.E., and Kahn, C.R. Correlation among insulin binding, degradation and biological activity in human cancer cells in long-term tissue culture. Cancer Res., 38:94-102, 1978.

Osborne, C.K., Hamilton, B., Titus, G. and Livingston, R.B. Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res., 40:2361-2366, 1980.

Pantalouris, E.M. Absence of thymus in a mouse mutant. Nature (Lond.), 217:370-371, 1968.

Peaker, M. and Maule Walker, F.M. Mastectomy and mammary glands in reproductive control in the goat. Nature (Lond.), 284:165-166, 1980.

Pearson, O.H., Llerena, O., Llerena, L., Molina, A. and Butler, T. Prolactin-dependent rat mammary cancer: A model for man? Trans. Assoc. Am. Physicians, 82:225-238, 1969.

Phillips, L.S. and Vassilopoulou-Sellin, R. Somatomedins part 1. N. Engl. J. Med., 302:371-380, 1980.

Phillips, L.S. and Vassilopoulou-Sellin, R. Somatomedins part 2. N. Engl. J. Med., 302:438-446, 1980.

Pierson, R.W., Jr. and Temin, H.M. The partial purification from calf serum of a fraction with multiplication stimulating activity for chicken fibroblasts in cell culture and with non-suppressible insulin-like activity. J. Cell Physiol., 79:319-330, 1972.

Polanowski, F.P., Gaffney, E.V. and Burke, R.E. HBL-100, A cell line established from human breast milk. In Vitro, 12:328 (abstract), 1976.

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, 18:3533-3539, 1979.

Reed, M.V. and Gey, G.O. Cultivation of normal and malignant human lung tissue. 1. The establishment of three adeno-carcinoma cell strains. Lab. Invest., 11:638-652, 1962.

Rees, A.R., Adamson, E.D. and Graham, C.F. Epidermal growth factor receptors increase during the differentiation of embryonal carcinoma cells. Nature, 281:309-311, 1979.

Reymond, M.J., Nansel, D.D., Neaves, W.B., Burrows, G.H. and Porter, J.C. Regulation of prolactin secretion by a new clonal strain of rat pituitary tumor cells. Program of The Endocrine Society 63rd Annual Meeting, Cincinnati, Abstract No. 529, 1981.

Rose, S.P., Stahn, R., Passovoy, D.S. and Herschman, H. Epidermal growth factor enhancement of skin tumor induction in mice. Experimentia, 32:913-915, 1976.

Ross, R.J., Glomset, J.A., 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. U.S.A., 71:1207-1210, 1974.

Rudland, P.S., Hallowes, R.C., Durbin, H. and Lewis, D. Mitogenic activity of pituitary hormones on cell cultures of normal and carcinogen-induced tumor epithelium from rat mammary glands. J. Cell Biol., 73:561-577, 1977.

Rudland, P.S., Bennett, D.C. and Warburton, M.J. Hormonal control of growth and differentiation of cultured rat mammary gland epithelial cells. In: Sato, G., Ross, R. (eds.), Hormones and Cell Culture, 6:677-699, 1979.

Russo, J., McGrath, C., Russo, I.H. and Rich, M.A. Tumoral growth of human breast cancer cell line (MCF-7) in athymic mice. International Symposium on Detection and Prevention of Cancer, New York 1:617-626, 1976.

Russo, J., Bradley, R.H., McGrath, C. and Russo, I.H. Scanning and transmission electron microscopy study of a human breast carcinoma cell line (MCF-7) cultured in collagen-coated cellulose sponge. Cancer Res., 37:2004-2014, 1977.

Rygaad, J. and Povlsen, C.O. Heterotransplantation of human malignant tumor to nude mice. Acta Pathol. Microbiol. Scand., 77:758-760, 1969.

Salih, H., Flax, H., Brander, W. and Hobbs, J.R. Prolactin dependence in human breast cancers. Lancet, 11:1103, 1972.

Salmon, W.D., Jr. and Daughaday, W.H. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J. Lab. Clin. Med., 49:825-836, 1957.

Savage, C.R., Jr. and Cohen, S. Epidermal growth factor and a new derivative: Rapid isolation procedures and biological and chemical characterization. J. Biol. Chem., 247:7609-7611, 1972.

Savage, C.R. and Cohen, S. Proliferation of corneal epithelium induced by epidermal growth factor. Exp. Eye Res., 15:361-366, 1973.

Schonbrunn, A., Krasnoff, M., Westendorf, J.M. and Tashjian, A.H., Jr. Epidermal growth factor and thyrotropin-releasing hormone act similarly on a clonal pituitary cell strain. J. Cell Biol., 85:786-797, 1980.

Shafie, S.M. and Brooks, S.C. Effect of prolactin on growth and the estrogen receptor level of human breast cancer cells (MCF-7). Cancer Res., 37:792-799, 1977.

Shafie, S.M., Grantham, F.H. and Gullino, P.M. Role of hormones in the growth or regression of a human mammary carcinoma line (MCF-7) transplanted in athymic nude mice. Program of The Endocrine Society 62nd Annual Meeting, Washington, D.C. Abstract No. 323, 1980.

Shafie, S.M. and Liotta, L.A. Formation of metastasis by human breast carcinoma cells (MCF-7) in nude mice. Cancer Lett., 11:81-87, 1980.

Shimosato, Y., Kameya, T., Nagai, K., Hirohashi, S., Koide, T., Hayashi, H. and Normura, T. Transplantation of human tumors in nude mice. J. Natl. Cancer Inst., 56:1251-1260, 1976.

Shiu, R.P.C. Prolactin receptors in human breast cancer cells in long-term tissue culture. Cancer Res., 39:4381-4386, 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, 185-196, 1981.

Sirbasku, D.A. Estrogen induction of growth factors specific for hormone-responsive mammary, pituitary and kidney tumor cells. Proc. Natl. Acad. Sci. U.S.A., 75:3786-3790, 1978.

Sirbasku, D.A. and Benson, R.H. Estrogen-inducible growth factors that may act as mediators (estromedins) of estrogen-promoted tumor cell growth. In: Sato, G., Ross, R. (eds.), Hormones and Cell Culture, 6:477-497, 1979.

Sonnenschein, C. and Soto, A.M. But are estrogens per se growth-promoting hormones? J. Natl. Cancer Inst., 61:211-215, 1980.

Sorrentino, J.M., Kirkland, W.L. and Sirbasku, D.A. Control of cell growth. I. Estrogen-dependent growth in vivo of a rat pituitary tumor cell line. J. Natl. Cancer Inst., 56:1149-1154, 1976.

Soule, H.D., Vazquez, J., Long, A., Albert, S. and Brennan, M.J. Human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Cancer Inst., 51:1409-1416, 1973.

Stampfer, H., Hallowers, R.C. and Hackett, A.J. Growth of normal human mammary cells in culture. In Vitro, 16:415-425, 1980.

Starkey, R. and Orth, D.N. Radioimmunoassay of human epidermal growth factor. J. Clin. Endocrinol. Metab., 45:1144-1153, 1977.

Stoker, M.G.P., Pigott, D. and Taylor-Papadimitriou, J. Response to epidermal growth factors of cultured human mammary epithelial cells from benign tumors. Nature, 264:764-767, 1976.

Tanaka, T., Shiu, R.P.C., Gout, P.W., Beer, C.T., Noble, R.L. and Friesen, H.G. A new sensitive and specific bioassay for lactogenic hormones: Measurement of prolactin and growth hormone in human serum. J. Clin. Endocrinol. Metab., 51:1058, 1980.

Tashjian, A.H., Jr., Bancroft, F.C. and Levine, L. Production of both prolactin and growth hormone by clonal strains of rat pituitary tumor cells. J. Cell Biol., 47:61-70, 1970.

Taylor, J.M., Cohen, S. and Mitchell, W.M. Epidermal growth factor: High and low molecular weight forms. Proc. Natl. Acad Sci. U.S.A., 67:164-171, 1970.

Taylor-Papadimitriou, J., Shearer, M. and Stoker, M.G.P. Growth requirements of human mammary epithelial cells in culture. Int. J. Cancer, 20:903-908, 1977.

Terranova, V.P., Rohrbach, D.H. and Martin, G.R. Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. Cell, 22:719-726, 1980.

Timpl, R., Rohde, H., Gehron-Robey, P., Rennard, S.I., Foidart, J.M. and Martin, G.R. Laminin - a glycoprotein from basement membrane. J. Biol. Chem., 254:9933-9937.

Tonelli, Q.J. and Sorof, S. Epidermal growth factor requirement for development of cultured mammary gland. Nature, 285:250-252, 1980.

Turkington, R.W. Stimulation of mammary carcinoma cell proliferation by epithelial growth factor in vitro. Cancer Res., 29:1457-1458, 1969.

Turkington, R.W. The role of epithelial growth factor in mammary gland development in vitro. Exp. Cell Res., 57:79-85, 1969.

Vlodavsky, I., Brown, K.D. and Gospodarowicz, D. A comparison of the binding of epidermal growth factor to cultured granulosa and luteal cells. J. Biol. Chem., 253:3744-3750, 1978.

Vlodavsky, I. and Gospodadowicz, D. Respective roles of laminin and fibronectin in adhesion of human carcinoma and sarcoma cells. Nature, 289:304-306, 1981.

Weber, K. and Osborn, M. The reliability of molecular weight determinations by Dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem., 244:4406, 1969.

Welsch, C.W., de Iturri, G.C., and Brennan, M.J. DNA synthesis of human, mouse and rat mammary carcinomas in vitro: Influence of insulin and prolactin. Cancer, 38:1272, 1976.

Welsch, C.W. and Nagasawa, H. Prolactin and murine mammary tumorigenesis: A review. Cancer Res., 37:951-963, 1977.

Welsch, C.W., Swim, E.L., McManus, M.J., White, A.C. and McGrath, C.M. Estrogen induced growth of human breast cancer cells (MCF-7) in athymic nude mice is enhanced by secretions from a transplantable pituitary tumor. Cancer Lett., 14:309-316, 1981.

Wessels, N.D. Substrate and nutrient effect upon epidermal cell orientation and proliferation. Proc. Natl. Acad. Sci. U.S.A., 52:252-259, 1964.

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

Wicha, M.S., Liotta, L.A., Garbisa, S. and Kidwell, W.R. Basement membrane collagen requirements for attachment and growth of mammary epithelium. Exp. Cell Res., 124:181-190, 1979.

Wicha, M.S., Liotta, L.A., Vonderhaar, B.K. and Kidwell, W.R. Effects of inhibition of basement membrane collagen deposition on rat mammary gland development. Dev. Biol., 80:253-266, 1980.

Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D. and Nandi, S. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. U.S.A., 76:3401-3405, 1979.

Yang, J., Guzman, R., Richards, J., Jentoft, V., DeVault, M.R., Wellings, S.R. and Nandi, S. Primary culture of human mammary epithelial cells embedded in collagen gels. J. Natl. Cancer Inst., 65:337-343, 1980.

Yang, J., Richards, J., Guzman, R., Imagawa, W. and Nandi, S. Sustained growth in primary culture of normal mammary epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. U.S.A., 77:2088-2092, 1980.

Yuhas, J.M., Tarleton, A.E. and Molzen, K.B. Multicellular tumor spheroid formation by breast cancer cells isolated from different sites. Cancer Res., 38:2486-2491, 1978.