Influence of Extracellular Matrix on Hormonal Responsiveness of Human Breast Cancer Cells

by

Martin Bryan Levy

A Thesis

Presented to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements for
the Degree of Master of Science

Department of Physiology
Faculty of Medicine
University of Manitoba

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"Men think,

God laughs."

- Yiddish proverb

To my parents, my guidance, my beginnings;

To Pamela, my inspiration, my life.

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This manuscript is dedicated to Pamela Glass, who through her warmth, concern and guidance allowed this study to be completed. Her gentle support, sent from overseas during the preparatory stages of this manuscript, was appreciated.

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List of Abbreviations:

Hormones Used

GH	Growth Hormone
PRL	Prolactin
E_2	17β-estradiol
h	human (hormone prefix)
o	ovine (hormone prefix)
b	bovine (hormone prefix)
FCS	Fetal Calf Serum

Units of Measure

g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
ml	milliliter
µl	microliter
cm	centimeter
mm	millimeter

nm	nanometer
hr	hour
min	minute
sec	second
M	molar
mM	millimolar
μM	micromolar
nM	nanomolar
rpm	revolutions per minute
^{o}C	degrees Celsius
S	Svedberg units
K	times 1000
$\overset{o}{A}$	Angstrom units
IU	international units
cc	cubic centimeters
xg	times force of gravity

Miscellaneous

BSA	bovine serum albumin
matrix	extracellular matrix or basement membrane

ABSTRACT

The extracellular matrix, the collagenous boundary between epithelial cells, is essential for proper cellular migration, differentiation, proliferation and orientation (Hay, 1981, Gospodarowicz, 1981). In the present study, the influence of the extracellular matrix on the proliferative response of human breast cancer cells (T47D) to human plasma, serum and mammotrophic hormones was studied. The natural extracellular matrix produced by bovine corneal endothelial cells was used in these studies.

In the human plasma and serum study, fresh human plasma and serum were obtained from whole blood of healthy male or female volunteers. Ten percent (v/v) human plasma or serum was added to the growth medium. The T47D cells cultured on plastic dishes proliferated logarithmically in response to human plasma or serum. However, the T47D cells on extracellular matrix were stimulated to grow only by human plasma; human serum resulted in inhibition of cell growth. The effects of mammotrophic hormones on the proliferation of T47D cells cultured in the presence or absence of the extracellular matrix were then examined. Estradiol (17β) at 10^{-10}M had no effect on T47D cells cultured on extracellular matrix, although it had a slight

stimulation on cells cultured on plastic. When T47D cells were cultured on an extracellular matrix, human growth hormone consistently stimulated cell growth from 2 to 6 fold. This stimulation was blunted by the presence of 10^{-10}M estradiol. Other hormones that include human prolactin, sheep prolactin, ovine growth hormone have no effect on the proliferation of T47D cells. In contrast, when cultured on plastic substratum, the T47D cells were not responsive to the addition of any one of the above hormones. It is a natural environment, an environment supplemented with an extracellular matrix, which controls the effectiveness of various mammotrophic hormones on T47D proliferation.

These findings demonstrate a vital role of basement membranes in the functioning of breast cancer cells. Mammotrophic substances on breast cancer cells proliferation was investigated. Indeed, these findings are useful to shed light on possible new aspects of hormonal stimulation of breast cancer cells, and the mechanism of action of these hormones.

RATIONALE

The extracellular matrix has many essential roles to epithelial cells. Epithelial-matrix interactions occur *in vivo*, during embryogenesis, wound repair, differentiation and proliferation (Hay, 1969, 1978, 1981, Gospodarowicz, 1979, 1982). Since the growth and differentiation of human breast cancer cells has only been investigated *in vitro* on artificial plastic substrata, we attempted to identify the proliferative patterns of human breast cancer cells cultivated on a physiological carpet of extracellular matrix.

Human plasma and serum has many important agents that stimulate or inhibit cellular proliferation (Gospodariwocz, 1982). In addition, recent work by various investigators demonstrated the presence of a human specific serum tumor inhibitor (Frendriech *et al.*, 1930, Green *et al.*, 1978, 1979, 1981, Gaffney *et al.*, 1979). We therefore were interested in studying the effects of human plasma and serum on the proliferation of human breast cancer cells maintained on extracellular matrix and plastic.

Numerous steroid and polypeptide hormones are believed to be important in influencing the growth of

human breast cancer. Yet the identity of many of these hormones, and their mechanism of action are largely unknown. Human breast cancer cells are largely unresponsive to hormones when they are cultivated *in vitro* on plastic dishes. It is therefore important to examine whether or not the extracellular matrix restores the hormonal responsiveness of human breast cancer cells *in vitro*. In this manner, it is hoped that we can identify those hormones which are important in influencing the growth of human breast cancer cells.

INTRODUCTION

All multi-cellular organisms, from the simplest mycobacteria to the most complex primate subscribes to the principles of homeostasis. This principle is crucial to the understanding of cell-cell, cell-substrata interactions.

Homeostasis was introduced to the North American scientific community in 1926 by Cannon. He asserted, "biologists have long been impressed with the ability of living things to maintain their own stability" (Cannon, 1929). Bernard, in 1878, described a similar phenomenon, (translated): "It is the fixity of the 'milieu interieur' which is the condition of free independent life. All the vital mechanisms, however varied they may be, have only one object: that of preserving the condition of life in the internal environment." Bernard and then Cannon both agreed on basically the same process; namely, organisms must control their internal milieu and in doing so control themselves. Cannon later supplemented his original thesis for unhomeostitically regular variables such as 1) plasma concentrations of free fatty acids, urea and creatinine, 2) insulin and other hormones, and 3) fat deposits.

It is to these unregulated variables (specifically hormones) and to certain regulated ones (substrata) which will be explored in later sections.

It is firmly established, that de facto homeostasis is vital to the understanding of cellular interaction in vivo and in vitro. Tissue culture cells must react as if they were in vivo, and at all costs regulate their "milieu interieur." In contrast, cells are plated artificially, without any regard to maintenance of a status quo. The following sections of the introduction serve as a foundation to the understanding of how the "milieu interieur" is regulated.

I. Hormones for the Development of the Mammary Gland

Numerous steroid and polypeptide hormones including insulin (Topper, 1970, and Turkington et al., 1971), prolactin (Dilley, 1971), growth hormone (Nandi, 1958) and estradiol (Chen et al., 1970) are known to interact with the primate breast. The interaction of the above listed hormones with breast epithelium has been elucidated in both rodent and human systems. The basic mechanism of action has also been identified.

Mechanism of action of polypeptide hormones

Polypeptide hormones, classically, bind to membrane receptors (Hechter, 1955, Pastan et al., 1966, Crofford, 1968), causing a cascade of cytoplasmic reactions in a hormone specific response. For many hormones, these reactions are mediated by adenylate cyclase (Sutherland, 1970). According to this process, cyclic AMP stimulates a specific protein kinase which phosphoralates other macromolecules. Once phosphoralated, and thereby activated, these substances (usually proteins) initiate gene activation, followed by new RNA and protein synthesis. There are a number of hormones that do not activate adenylate cyclase. For example, growth hormone and prolactin utilize alternate pathways. Growth hormone stimulates the production of small insulin-like growth factors called somatomedins in the liver as well as other tissues. Sometomedin then carries out responses which are growth hormone specific. Prolactin on the other hand, appears to cause production of an intracellular peptide that is believed to be the second messenger (Teyssot et al., 1981).

Mechanism of action of steroid hormones

Steroid hormone action has been eluciated by Gorski (1968) and Jensen et al. (1968). The first step of their hypothesis involves the binding of the hormone molecule to high affinity cytoplasmic receptor protein. The second step is the activation of the steroid-receptor complex, followed by translocation of the activated complex to the nucleus and binding to specific sites on the chromatin. This in turn causes gene activation, terminating in the production of hormone specific RNA which are translated into proteins.

II. Hormones and Malignant Breast Cancer

Many hormones are known to be important in promoting breast neoplasia such as insulin, estrogen, corticosteroids, prolactin, growth hormone and growth factors (for reviews, Hilf, 1976, Kirschner, 1977, McGuire, 1978). Three hormones, namely estrogen, growth hormone and prolactin have been shown to be of primary importance in the etiology of breast cancer.

Estrogen

Rodent systems have been very useful for elucidating

the role of estrogen in breast neoplasia. Various investigators (Lyons et al., 1958, Ahren and Jacobsohn, 1956, Norgren, 1967, Nagasawa and Yani, 1971) have demonstrated estrogen's trophic role on the normal breast.

Recently, Doa and co-workers (1982) demonstrated that physiological concentrations of estrogen and progesterone cause rapid proliferation of rat mammary glands and rat mammary tumor explants. Sirbasku et al. (1978), utilizing a rat mammary tumor cell line, MTW9/PL, identified estrogen dependant growth factors.

Furthermore, estrogen alone is unable to support tumor proliferation in the absence of a pituitary (Sterental et al., 1963) binding support for its secondary role.

In the area of estrogenic hormones influencing the risk of development of human breast cancer, Kirschner (1977) compiled a list of experimental, epidemiological and clinical factors which denote estrogen as a potent breast carcinogen. Numerous other investigators [specifically Jensen et al. (1968)] have proposed a plausible role of estrogens in human breast cancer.

It is now fully accepted that estradiol has a component in human breast cancer growth. Estrogen responsive

tumors, identified by estrogen receptor analysis (McGuire, 1973) respond positively to hormonal deprivation.

Furthermore, estrogen in pharmacologic doses causes regression of mammary tumors (Pearson and Nasr, 1971), while at physiological doses acts synergistically on the human breast (McGuire et al., 1975).

In tissue culture, Lippman (1979) has demonstrated a definite trophic role for estrogen. Shafie (1980) and many others (Sirbasku et al., 1978, Sonnenschein et al., 1980, and Butler et al., 1982) could not reproduce the mitogenic effect of estrogen on human breast cancer cells maintained in culture.

Perhaps, indeed, the role of estrogens has not yet been reproduced in vitro due to the environment of the cells.

Growth hormone

Growth hormone interaction with rodent or human breast tissue has not been well documented (Li and Yang, 1974).

Nagasawa (1970) documented that bovine growth hormone and bovine prolactin were stimulatory for dimethyl-benzanthracene (DMBA) induced rodent tumors. Work by

Pearson et al. (1969) indicated that the active hormone is growth hormone, while prolactin might have a minor role.

Other workers (Li and Yang, 1974; Ghosh, Ghosh and das Grupta, 1978) noted a two-fold increase in rat tumor growth following the administration of bovine growth hormone into tumor-bearing rats. Earlier work by Smith (1952, 1954) and Smith et al. (1954b) noted a similar effect.

Furthermore, CB154 (α - ergo-bromocryptine), a potent inhibitor of prolactin secretion, failed to induce complete regression of rodent mammary tumor growth (Eur. Breast Cancer Group, 1972). Yanai and Nagasawa (1970) demonstrated that prolonged treatment of mice with CB154 did not influence the growth hormone content of the pituitary. Therefore growth hormone could have a vital role in the etiology of rodent breast cancer.

Investigation into the role of human growth hormone in human breast cancer has been scarce. Nevertheless, the in vitro studies of Souza et al. (1974) utilizing histochemical techniques showed that human growth hormone

is active in cultured human breast tumor tissue. Ghosh, Ghosh and das Grupta (1978) localized human growth hormone within human mammary carcinoma cells in culture, utilizing immunology.

Further, it was observed by Emerman and coworkers (1981) that many breast cancer patients have elevated levels of serum growth hormone-like activity. The same authors noted that immunoreactive human growth hormone was elevated in half of their patient samples (usually 8 to 10 fold) while only 9% had elevated prolactin levels. When the samples were tested for bioactivity (using the Nb2 rat lymphoma cell line as originally described by Noble and Coworkers, 1980), the reported values were six times higher. Singh et al. (1974) reported a variant of human growth hormone with greatly increased mitogenicity. It can be speculated that the cleaved form of hGH could be the circulating GH which Emerman identified in breast cancer patients. Hubbard and Liberti (1980) also documented that cleaved fragments of hGH possess somatomedin-like activity.

Prolactin

The role of prolactin in the development of rodent

breast cancer has been well documented (Welsch and Nagasawa, 1977). Briefly summarized, Welsch and Nagasawa note that many mammary tumors are dependant on hormones (either prolactin or estrogen or both) for growth. The administration of prolactin decreases the latent period of the appearance of carcinogen induced mammary tumors in rats. After hyperphysectomy, prolactin responsive tumors regress, but will recur after the administration of prolactin.

Studies on the role of prolactin in the development of mammary tumors in rodents have provided, in part, the impetus to explore the hypothesis that this hormone may be similarly involved in human breast tumorigenesis. Clinical studies revealed that a significant proportion of all human breast cancer is hormone responsive. While major progress has been achieved in elucidating the importance of estrogen in human breast cancer, the role that pituitary hormones play in the human disease is poorly understood. Because of its great potency in supporting the growth of rodent breast cancer, prolactin has been singled out for studies on its role in the tumorigenesis of the human breast. So far, limited clinical

and epidemiological studies have failed to define a definitive role for prolactin in human breast cancer. An early first full term pregnancy has been repeatedly shown to have a protective effect against subsequent breast cancer development (Shapiro et al., 1968).

Evidence to suggest an important role of prolactin in the tumorigenesis of the human breast also came from studies in vitro. Thus it has been reported that human breast tumor biopsies maintained in organ culture (Welsch et al., 1976, Salih et al., 1972) or transplanted into athymic nude mice (McManus et al., 1978) respond to prolactin.

Reports by Barry (1970), Oka and Topper (1972) and Muhherji et al. (1973) all indicate PRL to be stimulatory in human systems. Ceriani et al. (1972) as well as Flaxman and Lasfargues (1973) reported bovine PRL was mitogenic to explants of normal human breast tissue in vitro. Dilley and Kester (1975) published that hPRL but not oPRL was stimulatory to human tissue. Furthermore, DiCarlo and Muccisli (1979) noted that 32.5% of primary human breast cancers contain significant amounts of PRL sites. In addition, Shiu (1982) noted 20,000 binding sites for PRL

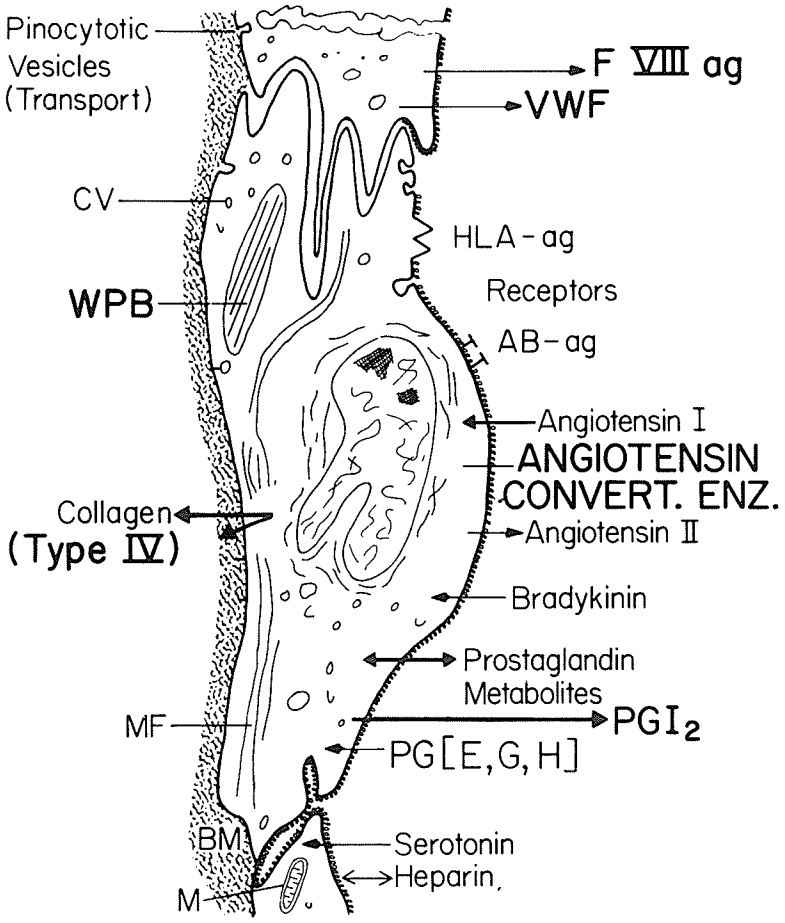
in the human breast cancer established cell line, T47D.

It is therefore clearly evident that prolactin exerts an influence in rodent and human neoplastic proliferation. The effect is mediated through the PRL receptor.

In addition, prolactin was shown by Shiu (1982) to induce morphological changes (cell shape, cell aggregation) in a human breast tumor cell line.

III. Biological Function of ECM

Although this role of matrix is rather outdated, it still serves as an example of a primitive function of matrix. All cells need support, for structures are in three dimensions. Matrix, it was thought, is the foundation that glues the cells to each other and to a common surface, akin to a potato tuber. It was quickly realized that this was not the case. Work by Pitelka and coworkers, Gospodarowicz and associates and numerous others (Bernfield, 1981, Reid, 1980) illustrated the multifaceted role of the matrix.



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Extracellular Matrix and Cell Function

a) What is extracellular matrix?

What is the extracellular matrix? An eloquent definition is supplied by Ashton (1974): "Acellular hyaline sheets of gel-like plasticity, which are eosinophilic, argyrophelic and intensely PAS positive and lie extracellularly in intimate relation to epithelial cells, smooth muscle cells, endothelial cells, pericytes and nerve sheaths, characteristically occurring at the interface between cells and connective tissue, but often, as in the case of vascular walls, between the cellular components." Hay (1981) continues: ". . . the basal (basement) lamina or basement membrane is a zone about 100 nm wide under epithelia and around muscle cells, it consists of a central compact sheet of collagen . . . called lamina densa. . . . The term basement membrane was originally used by eight microscopists to refer to the whole condensation of connective tissue." An excellent recent review expanding these definitions is supplied by Heathcote and Grant (1978). In addition, figure 1 pictorially represents endothelial and their characteristic biology.

The extracellular matrix is a highly organized layer of proteinaceous and carbohydrate moieties. Ultrastructurally, it is composed of fine fibrils about 40 Å in diameter, arranged randomly in a granular matrix (Farquar, 1960; Jakus, 1964). The thickness of the matrix has been reported to be from 200 to 50,000 Å (Jakus, 1964).

Briefly, the matrix is composed of collagen fibrils (making up 50% of constituents) as well as proetoglycans and glycoproteins. The interaction of the various components is essential for management of cell growth.

The distribution of matrix is ubiquitous. In matrix tissue, basement membranes (a highly specialized form of extracellular matrix) are found at the base of epithelium lining urinary, reproductive, respiratory and digestive tracts, at the base of endothelium lining vascular tree, at basal luminal cells of endocrine and exocrine glands and surrounding adipocytes, Schwann cells and skeletal and smooth muscle cells. In most instances, the basement membranes separate the epithelium or endothelium facing the lumen from the stroma or connective tissue layer. In other words, the matrix divides the parenchymal cells. It is this effective boundary which aids in the segmenta-

tion of cellular layers, necessary for proper functioning.

b) Parenchymal-matrix interactions

The ability of a cell to differentiate and proliferate as well as migrate, is determined rather closely by the matrix it resides upon. Work by Liotta (1980) and others (review: Bernfield, 1981) have noted this relationship.

As stated at the outset of this introduction, it is the control of the "milieu interieur" which imparts a certain independence. In most cases, the external environment demanded an EXTRA-cellular matrix.

Grobstein (1953) first noted the essential need for a matrix for parenchymal differentiation. This claim was later supported by numerous workers (Gospodarowicz, 1981). In view of this, tissue culture work has evolved around increasingly higher concentrations to fetal bovine serum and plastics (a non-physiological platform for growth). As Gospodarowicz reports: "The last 50 years of cell culture is the history of the inadequacies of substrata." This statement more than any other is the benchmark of cell culture.

c) Cell proliferation

Normal cells plated onto agar do not differentiate, and fail to proliferate (Overton, 1977) until a basement membrane is produced. On bacteriologic plastic, there is poor adhesion and the proliferation rates are reduced. If the cells are cultured on tissue culture plastic, the cells will proliferate until no further free substratum is available. On collagen or basement membrane, there would be both proliferation and differentiation. Therefore, the crucial role of the substrata is to influence cell shape (Folkman and Moscona, 1978), and in doing so affect proliferation rates.

Embryologically speaking, the breast is an outshoot of the ectodermal layer of the developing blastula. From the origins of this layer, a compact and regulatory basal lamina forms to direct and maintain correct development alterations. Ductal formation occurs through the degradation of matrix at one segment of epithelia but not at another (Wicha, 1979). The final product is quiescence at the lamina poor areas and proliferation at the lamina rich areas, forming an invagination. This process contin-

ues to form the primitive alveolus. The primitive breast is formed. Interaction with lactogenic hormones serves to mature and further differentiate the various mammary components leading ultimately to the adult lactating breast.

d) Endothelial Cells

Endothelial cells form an integrated role in the entire reticuloendothelial system. These cells can be isolated from the entire vascular tree, lymphatics, serosal membranes and cornea.

Corneal endothelium produce a highly fibrillar matrix, the Descemet's membrane, which is one of two matrix sites which can be visualized without the aid of light microscopy. Kefalides and Denduchis (1969) isolated and analyzed corneal endothelial matrix, and noted stratification. They described this separation thusly: just below the base of the cell one sees an electron lucient zone. Below this zone, lies an electron dense layer of finely fibrillar material identified as the basement membrane. Adjacent to this is a layer of fibrils resembling collagen fibers. Furthermore, Kefalides (1969) noted basement

membranes from various tissues differ in their amino acid, carbohydrate composition and tissue specificity which ultimately determines structural organization. Watzha (1935), Neifakh (1952), Krause (1934) and Dohlman and Balazs (1955) independently reported consistencies to the chemical composition as described by Kefalides (1969).

Endothelial cell culture has undergone a number of modifications since its conception in 1921 (Lewis, 1921). Advances in enzymatic digestion of the underlying stroma, propagation and growth medium environment flourished. The advances of Maruyama (1913) paved the way for modern isolation techniques. Further advances of Jaffe (1972) came with the advent of collagenous proteinases, short incubation times and less manipulation of the tissue.

Endothelial cells have a number of specific markers, including endothelial AT.AM antigens (motility antigens, probably actin), factor VIII antigen, blood group and histo-compatibility antigens, Weibel-Palade bodies as well as extracellular matrix components such as fibronectin, laminin and type IV collagen.

METHODS AND MATERIALS

I. Endothelial Cell Culture

The method employed was adapted from the procedure of Gospodarowicz and co-workers (1976). Briefly, adult steer eyes were obtained from recently killed animals at a local slaughterhouse. The eyes were transported in a solution of Delbecco Modified Eagles Medium (DMEM), 100 IU/ml penicillin and 100 μg/ml streptomycin on ice. All eyes were utilized within 1 hour or discarded. All procedures hereafter were performed aseptically. The eye was washed four times in 50% ethanol and rewashed five times in sterile Delbecco's phosphate buffered saline (DPBS) (without calcium or magnesium). The eye was then placed in a sterile 150 mm^3 Falcon brand dish and the cornea was identified. Thereafter, it was clipped away from the sclera and the posterior surface was scraped with a grooved director (Ingram and Bell). The cells liberated were washed into a 50 ml conical tube containing DMEM, 10% Fetal Bovine Serum (Gibco), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were pelleted and rewashed. The pellet was re-suspended in the

above solution supplemented with 4mM glutamine and 4.5 gm/l glucose and plated into 25 mm^3 flasks (Falcon brand). After initial passaging, the cells were plated into larger 75 mm^3 flasks with each eye corresponding to a specific lot number. Approximately 10 aliquots were frozen and stored in liquid nitrogen for each lot.

II. Characterization of Endothelia

1. Light Microscopy

Bovine corneal endothelial cells were observed utilizing an inverted phase contrast Nikon microscope (model S-KE). All photographs were taken using the photography attachment and Polaroid type 667 black and white film.

2. Electron Microscopy

Bovine corneal endothelia cells were grown as previously described. At one week post-confluency, the cells were washed with cold PBS, and fixed in 2% glutaldehyde (prepared fresh) for one hour. Thereafter, the cells were washed in phosphate buffered saline, 0.01M phosphate buffer, 0.9% (w/v) saline pH 7.4 (PBS), and stained with 2% osmium tetraoxide. After dehydration in graded alcohol, the cells were embedded in Epon 812. Thin sections (700 Å) were cut and collected on a nickel grid. Post-

staining was with uranyl acetate and lead citrate. Specimens were observed on a Phillips 300 transmission electron microscope.

3. Indirect Immunofluorescence for Fibronectin

Bovine corneal endothelial cells grown on sterile glass coverslips, one week post-confluency, were either subjected to 0.5% Triton to denude the matrix (see next section) or washed extensively in PBS (pH 7.4). After one hour, both the Triton X-100 and PBS were removed from the cells. Both the cells and matrix were then subjected to the same conditions. Unless noted, all reagents were kept at 4°C on ice. Fixation occurred in 2% paraformaldehyde (prepared fresh) for 20 minutes at 25°C. A positive control (human primary culture fibroblasts) was added to the processing. The immunostain consisted of a 1:40 primary antibody (goat, anti-fibronectin) which was allowed to react for 30 minutes at 25°C. After vigorous washing with PBS (over a period of 1 hour), a second antibody (fluorescene conjugated rabbit anti-goat IgG) was added at a dilution of 1:20. The antibody was allowed to react for 30 minutes, in

the dark. After four washes with PBS, the glass coverslip (nonfluorescening, Corning) was placed on a nonfluorescening glass slide (Corning) containing a drop of immunofluorescent grade glycerol buffered saline. The slide was washed twice by dipping in double distilled water, pat dried and mounted using an acrylic finish. The slide was then observed on a Zeiss fluorescent microscope. Photo reproductions were done on Tripan X film (ASA 400). The shutter speed was manually set, allowing equal time of exposure for all samples.

Preparation of Extracellular Matrix

Bovine corneal endothelial cells were plated onto $35mm^3$ dishes (Falcon) at a density of approximately 5×10^4 cells/dish in growth medium consisting of Delbecco's Modified Eagle's Medium (with 4 mM glutamine and 4.5 g/l glucose, 10% fetal bovine serum, and penicillin/streptomycin (100 IU/ml and 100 µg/ml respectively). The dishes were allowed to attain confluency in 98% humidity, 5% CO_2, 95% air, with media changes every three days. One week post-confluency the dishes were used.

The preparation of the matrix follows the procedure

of Gospodarowicz (1979) with the modification of Brown (1976). Briefly, the endothelial cells were removed by exposing them to 0.5% Triton X-100 in PBS for 1 hour with gentle shaking. Thereafter the detergent was removed and the matrix that was left coating the dish washed thrice in PBS.

Preparation of Human Plasma and Human Serum

Human whole blood was obtained from healthy male and female (pre-menopausal) volunteers by venipuncture.

The blood was sterilely passed into a conical 50 ml tube and centrifuged for ten minutes at 2,500 rpm at 4°C. The plasma fraction (which usually composed 45-55% of entire sample) was removed and frozen at -70°C.

Serum was prepared after clotting of whole blood (no anti-coagulant). The clot was expressed and the supernatant was removed and frozen at -70°C.

Preparation of Hormones

All hormones except human prolactin (Drs. I. J. Worsley and H. G. Friesen, lot 81-9-10) and 17-β estradiol (Sigma, lot 19C-0519), were obtained as gifts from the National Pituitary Agency, Baltimore, Maryland.

Biological activities in IU /mg are as follows:

hPRL	30.1	
OPRL	35	(NIH-P-S-12)
OGH	0.56	(NIH-GH-S-11)
hGH	2	(NIH-GH-HS2160E)

Hormones were dissolved in 0.1M $NaHCO_3$, diluted in DMEM and sterilized using 0.45 Mm millipore filters (Millex-HA).

Breast cancer cells used in study

An established human breast cancer cell line (T47D) was employed in these studies. The cell line was obtained from a pleural effusion of an infiltrating ductal carcinoma of a 54 year old female Caucasian (Keydar et al., 1979). The morphology is epitheloid. Doubling time is 32 hours. The cells grow in soft agar. They have membrane receptors for human prolactin and human growth hormone (Shiu, 1979), epidermal growth factor (Imai et al., 1982) and insulin as well as cytoplasmic receptors for estrogen, progesterone, hydrocortisone (Keydar, 1979, Engel et al., 1978).

T-47D were maintained in growth medium (DMEM supplemented with glucose (4.5g m/l), glutamine (4mM) and antibiotics (penicillin (100 I.U/ml) and streptomycin (100 µg/ml, insulin (1 Mg/ml) and 10% Fetal bovine serum. Upon 50% confluency, the cells were trypsinized (trypsin-EDTA solution, Gibso), and plated (see next section).

Gell growth assay: effects of human plasma and serum

1. Extracellular matrix

Extracellular matrix was prepared from bovine corneal endothelium as described (see preparation of matrix). On day 0, T47D cells in 10% FBS was plated onto dishes of the extracellular matrix. Twenty-four hours later, the medium was changed to serum-free DMEM containing human plasma or human serum with 4IU/ml heparin (Balk, 1980). All counts were performed every three days electronically with media changes to the unused dishes. Cells were detached with Trypsin-EDTA; resuspended; diluted in Isoton and counted electrically by using a Coulter Counter.

2. Plastic

All experiments utilizing plastic substrata followed the extracellular matrix series, with the exception that the extracellular matrix isolation step was not performed.

Cell growth assay: effects of mammotrophic hormones

The experimental protocols for testing the effects of hormones on the growth of T-47D cells were similar to that described above. Unless otherwise noted, the following procedure was employed in all experiments performed. T47D, breast carcinoma cells were plated at various concentrations onto plastic or extracellular matrix at day 0. The medium contained 10% fetal bovine serum. Twenty-four hours later, the medium was removed and the dishes washed twice with serum-free medium. Serum-free medium was then added with the proper hormonal additions. In these experiments, all counts were performed on the 7th day (media change occurred on the 3rd day). Hormones were added to the incubation to a final concentration of 1 μg/ml, with the exception of estradiol, which was used at a final concentration of 10^{-10} M.

RESULTS

I. Characterization of bovine endothelial cells and their extracellular matrix

The characteristic morphology of endothelial cells is illustrated in figure 2A. Endothelial cells are flat and polygonal in shape, similar to that of epithelioid cells. At confluency, a monolayer of cells has the characteristics of a cobblestone formation (figure 2B). The electron micrograph (figure 3) illustrates the flatness of the endothelial cell. The endothelial cells extrude a carpet of electron dense, amorphous extracellular matrix.

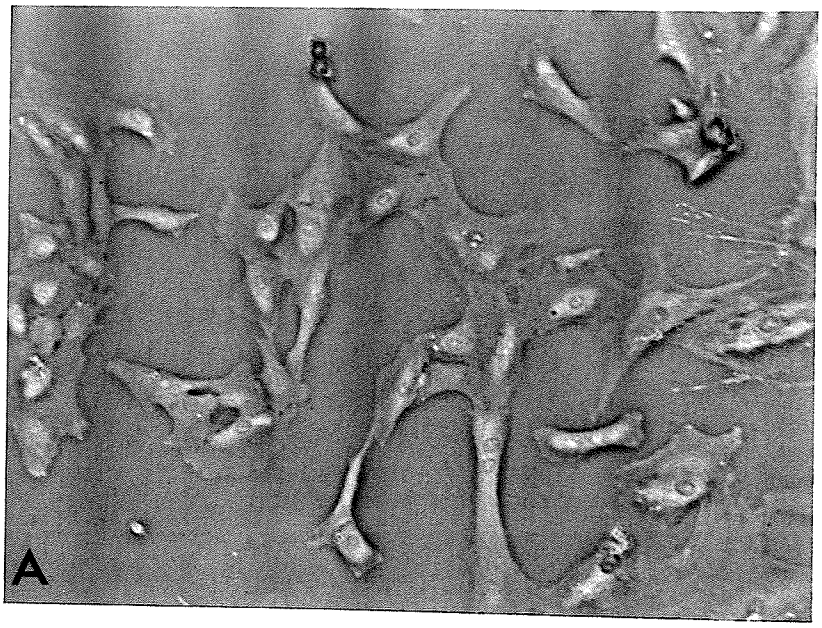
Presence of fibronectin (Jaffe, 1979) and laminin (Kidwell, 1980, Albrectsen, 1981 and Gospodarowicz, 1979) have been used as extracellular matrix markers. Figures 4 and 5 offer two different views of fibronectin immunofluorescence staining on bovine corneal endothelial cells. Figure 4 shows that fibronectin is only secreted on the basal surface of the cells. Furthermore, clumps of fibronectin are visible at the lower portion of this photograph. It is at this region that endothelial cells were removed during the fixation process, exposing the

Figure 2. Appearance of bovine corneal endothelial cell culture.

Panel A depicts phase contrast photo-micrographs of bovine corneal endothelial cells. The cells were grown in DMEM, supplemented with 20% fetal bovine serum, high glucose and glutamine.

Panel B illustrates a confluent culture grown under similar conditions to Panel A. Note the characteristic cobblestone appearance of confluent cultures.

Magnification: 240 times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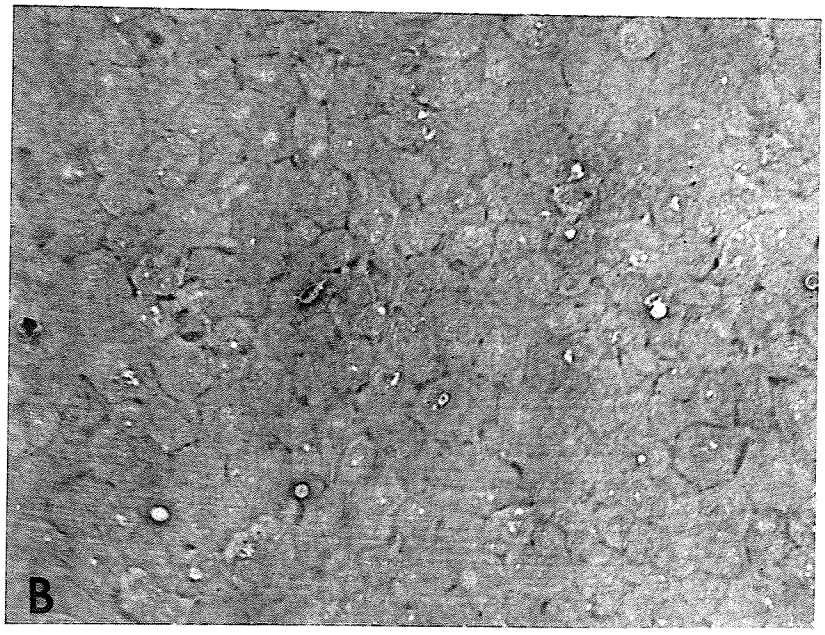

B

Figure 3. Electron micrograph of bovine corneal endothelial cells.

Bovine corneal endothelial cells were grown to one week postconfluency and prepared for electron microscopy. The section was cut perpendicular to the tissue culture plate bottom. Extracellular matrix is evident on the basal surface of the cell. Magnification: 22,000 times.

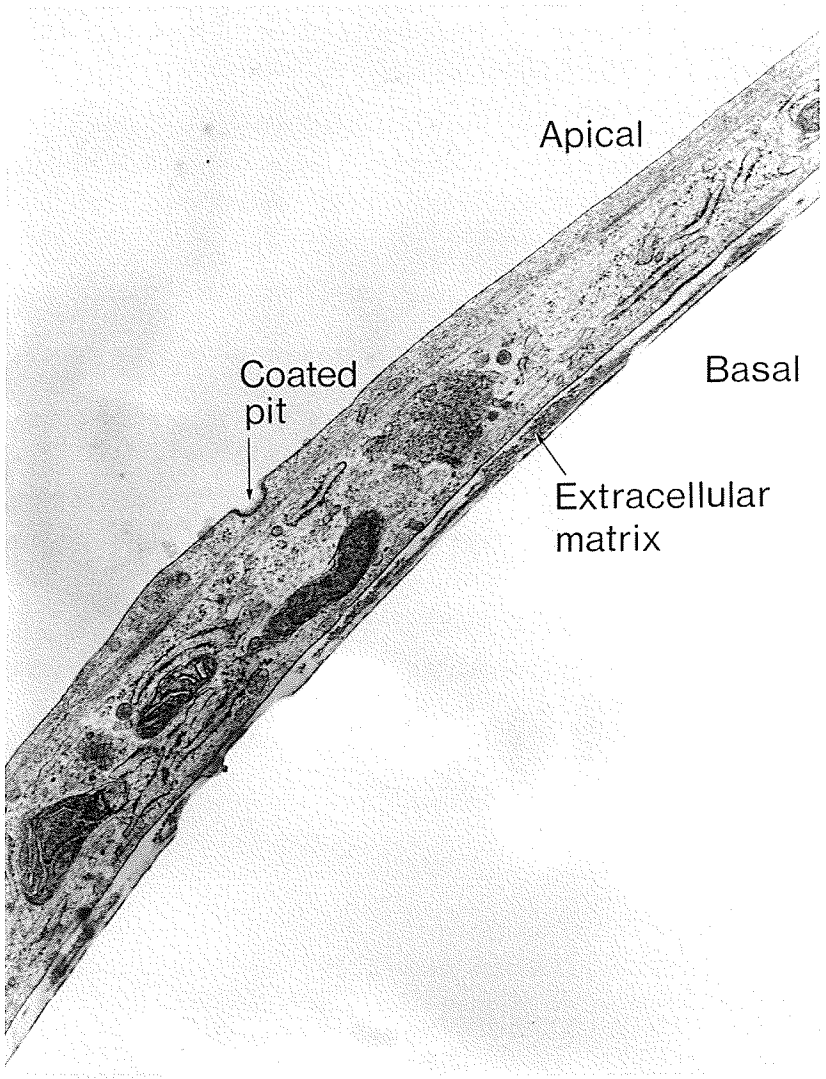
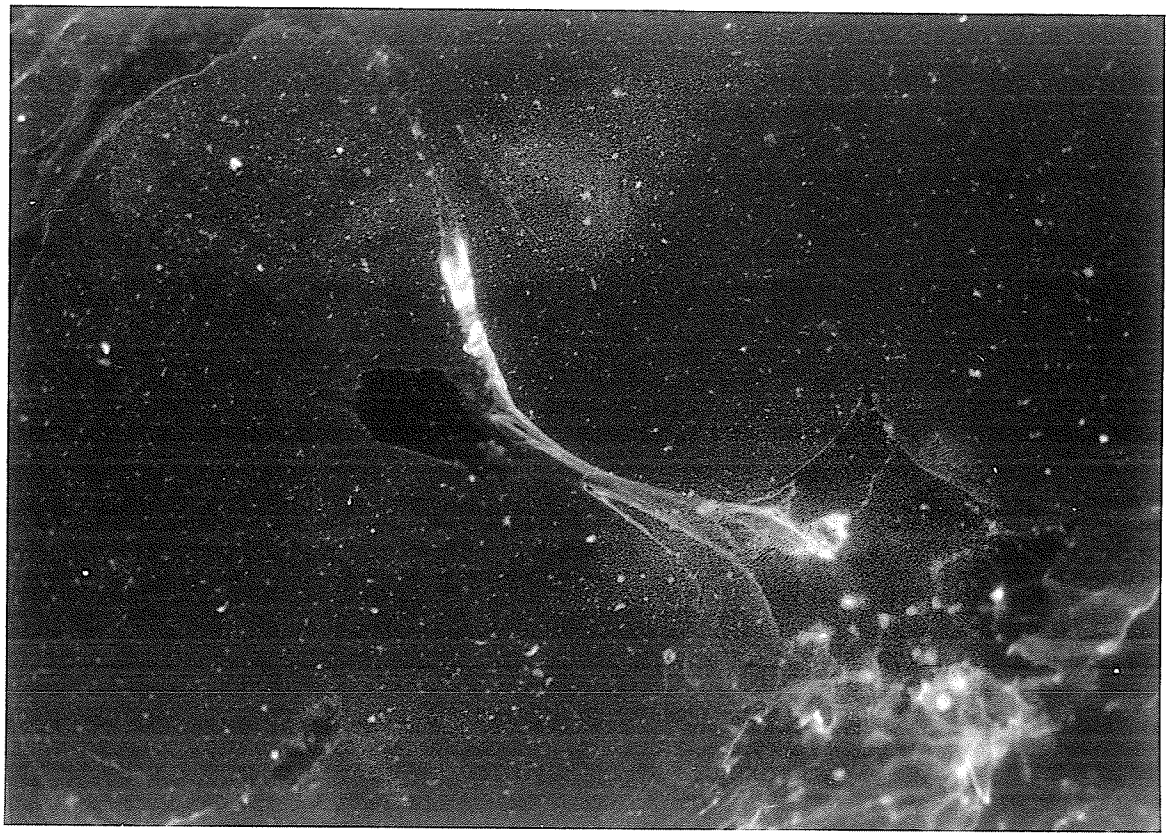

Apical
Coated pit
Basal
Extracellular matrix

Figure 4. Localization of fibronectin between two endothelial cellular islands.

Monolayer culture of cells was fixed with glutaraldehyde and used for the localization of fibronectin. Triton X-100 was not added. Fibronectin was only visualized on the basal, not apical, surface. Fibronectin is found between two endothelial islands. Magnification: 400 times.



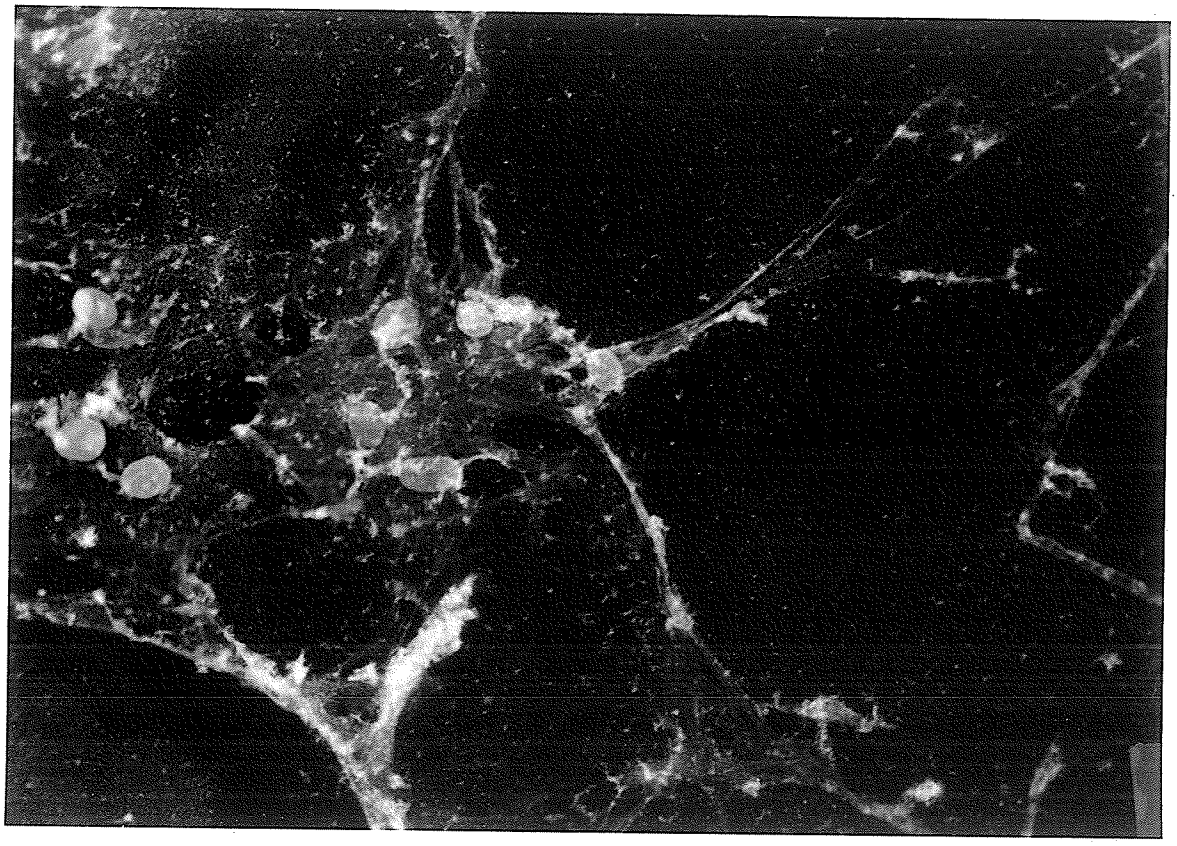
extracellular matrix to the fibronectin antisera. Figure 5 illustrates the fibrillar network of matrix after removal of the cellular monolayer with 0.5% Triton X-100. The framework of fibronectin containing extracellular matrix is clearly evident.

Having established that the cells utilized were indeed corneal endothelial and that an extracellular matrix was generated, we attempted a series of experiments to study the growth behavior of human breast carcinoma cells (T47D) maintained on extracellular matrix.

The T47D human breast carcinoma cells were developed and characterized by Keydar et al. (1979). These cells were obtained from the pleural effusion of a patient suffering from disseminating carcinoma of the breast. The cells have receptors for both polypeptide hormones such as prolactin and growth hormone (Shiu, 1981) as well as for steroid hormones (estrogen, progesterone and hydrocortisone) (Keydar et al., 1979). Imai et al. (1982) has identified epidermal growth factor receptors in these cells.

Figure 5. Localization of fibronectin in the extracellular matrix.

Post-confluent corneal bovine endothelia was allowed to produce extracellular matrix for one week. Thereafter the endothelial cells were removed with 0.5% Triton X-100. Immunofluorescence localization of fibronectin was carried out on the extracellular matrix. The fibrillar network of fibronectin is apparent. The dot-like structures are the nuclear ghosts which were not removed with Triton X-100. The nuclear ghosts have some non-specific staining. Magnification: 220 times.



Extracellular Matrix and Human Breast Cancer Growth

I.

A) Effect of Fetal Bovine Serum

Figure 6 illustrates the first series of experiments performed utilizing the corneal endothelial extracellular matrix. It is well established that fetal bovine serum was mitogenic on T47D cells grown on a plastic substratum as well as on collagen, one extracellular matrix component (Leung and Shiu, 1982). On a complete extracellular matrix, 10% FBS (fetal bovine serum) supports growth of T47D cells to the same extent as cells maintained on plastic (figure 6). On extracellular matrix the T47D cell doubling time was 24 hours while on plastic there was an increase to 36 hours. In the absence of FBS, T47D failed to proliferate either on plastic or on extracellular matrix (figure 7).

B) Effects of Human Plasma and Human Serum

In the body, cells normally are not exposed to serum, rather they are bathed by an ultrafiltrate of 100% plasma. Only in situations of wounding or organic pathology are

Figure 6. T47D, cellular growth with fetal bovine serum on extracellular matrix or plastic.

The T47D, human breast cancer cells were seeded at 2 x 10^4 cells/dish onto plastic or extracellular matrix. Ten percent fetal bovine was supplemented to DMEM. Cells were detached from the substrata by trypsin and counted electronically. Vertical bars represent standard deviation (n=3).

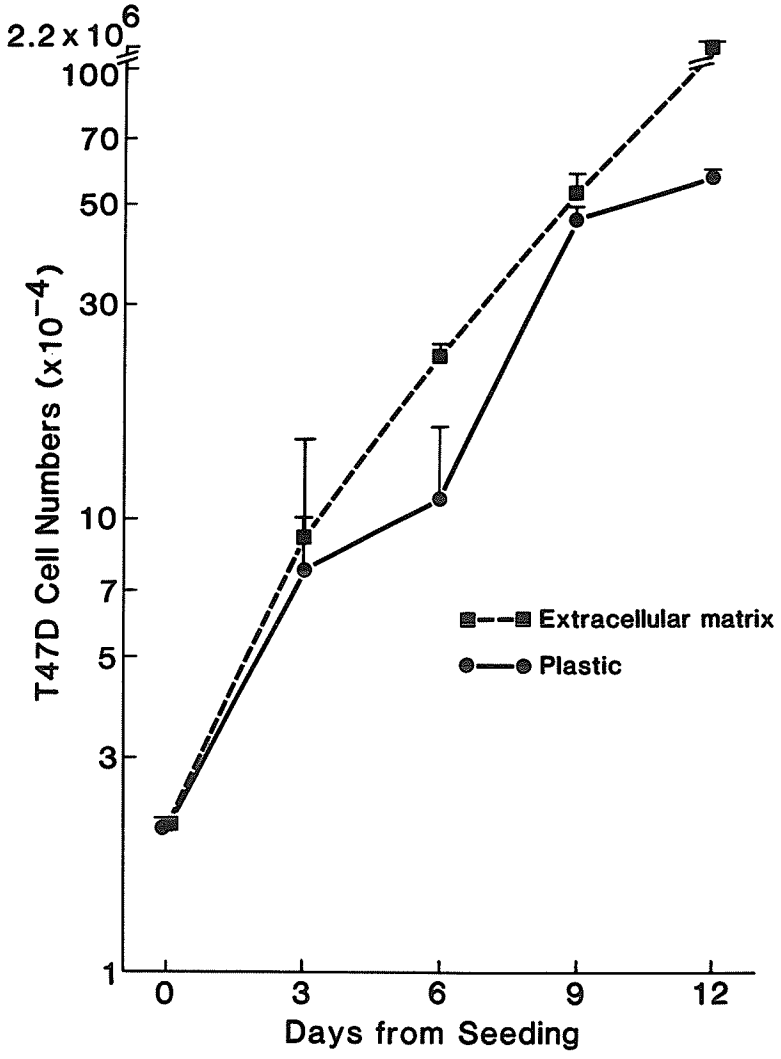
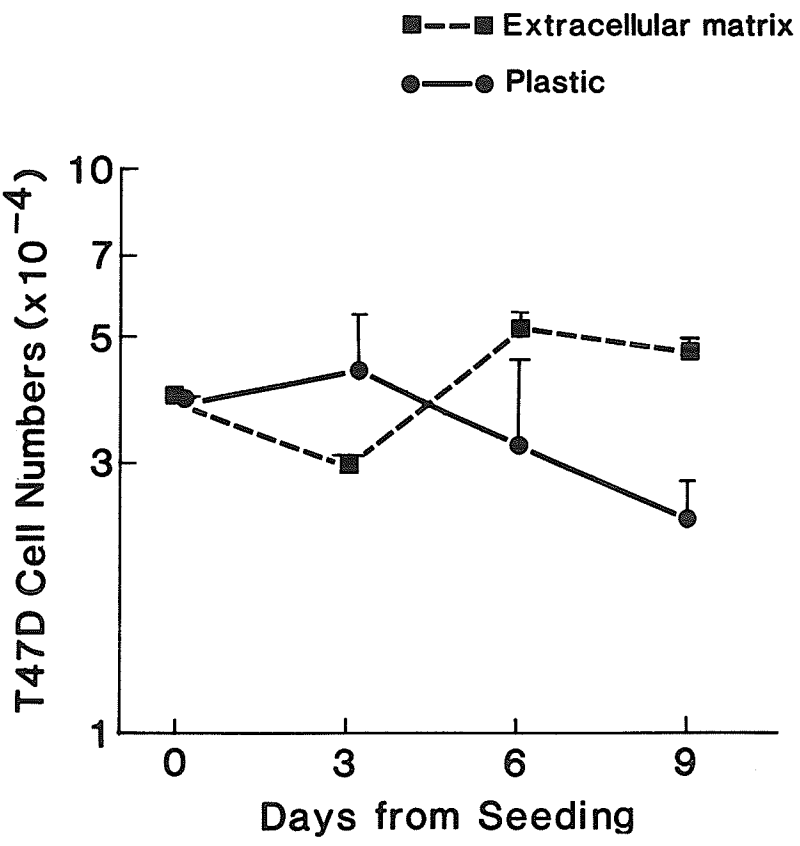

2.2 x 10^6
100
70
50
30
10
7
5
3
1
T47D Cell Numbers (x10^-4)
0
3
6
9
12
Days from Seeding
Extracellular matrix
Plastic

Figure 7. T47D cells grown in serum-free conditions on various substrata.

The T47D cells were seeded at 4×10^4 cells/dish onto extracellular matrix or plastic in serum-free conditions. Cells were counted electronically.


Extracellular matrix
Plastic
T47D Cell Numbers (x10^{-4})
10
7
5
3
1
0
3
6
9
Days from Seeding

the somatic cells exposed to serum. In addition, it has been documented that human serum has cytotoxic potential toward tumor cells (Friedenreich et al., 1930, Gaffney et al., 1979, Green, 1978, 1980). We were therefore interested in comparing the effects of human plasma and serum on the proliferation of human breast carcinoma cells.

Human male plasma and serum promote the growth of T47D cells maintained on plastic, with a doubling time of 24 hours (figure 8). However, on extracellular matrix, only plasma could support cell growth. The T47D cells appear to become quiescent (figure 9) in the presence of human serum.

On plastic, the proliferation of T47D cells produced by female plasma and serum mirrored that induced by male plasma and serum (figure 10). However, female serum caused widespread cellular death of T47D cells after 9 days in culture on extracellular matrix (figure 11). This effect was not observed for the male serum (figure 9).

C) Effects of Mammotrophic Hormones

Various polypeptide and steroid hormones are known to influence the growth, development and function of normal and neoplastic mammary glands. However, there has

Figure 8. Cell growth on plastic with human plasma or serum.

Plasma and sera from blood of healthy donors were prepared using the procedures described in Methods. Approximately 1 x 10^4 cells were seeded per dish. Cells were grown on tissue culture plastic in the presence of DMEM supplemented with either 10% plasma or serum. Heparin was added to the culture dishes to a final concentration of 4 I.U./ml. Electronic counting was employed to determine cell numbers.

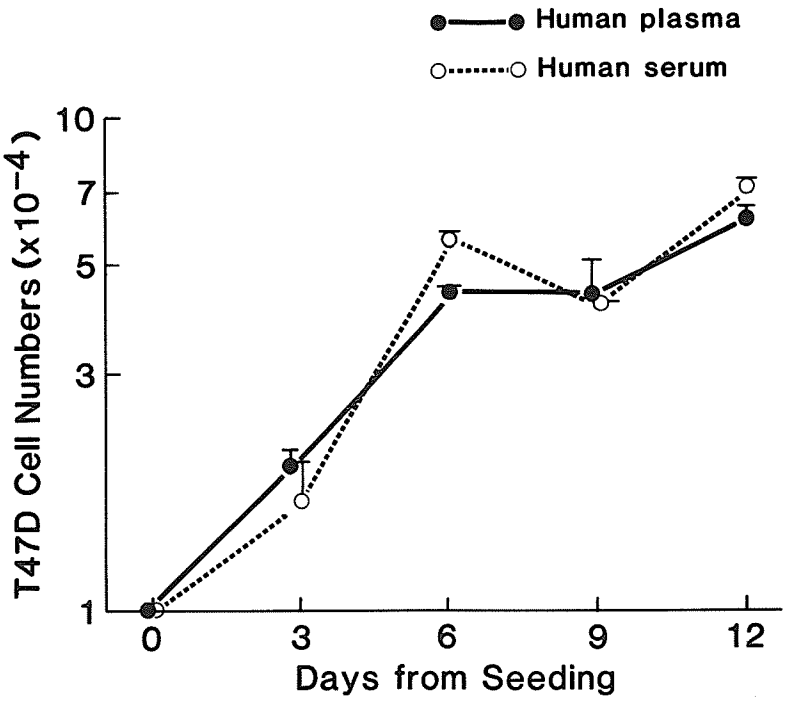

Human plasma
Human serum
T47D Cell Numbers (x10⁻⁴)
10
7
5
3
1
0
3
6
9
12
Days from Seeding

Figure 9. T47D cell growth on extracellular matrix with human male plasma and serum.

Human male plasma and serum was obtained as discussed in Figure 8. Extracellular matrix was obtained from corneal bovine endothelial cells. Initial cell density was 3×10^4 cells/dish. Cells were trypsinized and counted electronically. The differential effect of plasma and serum is evident on extracellular matrix.

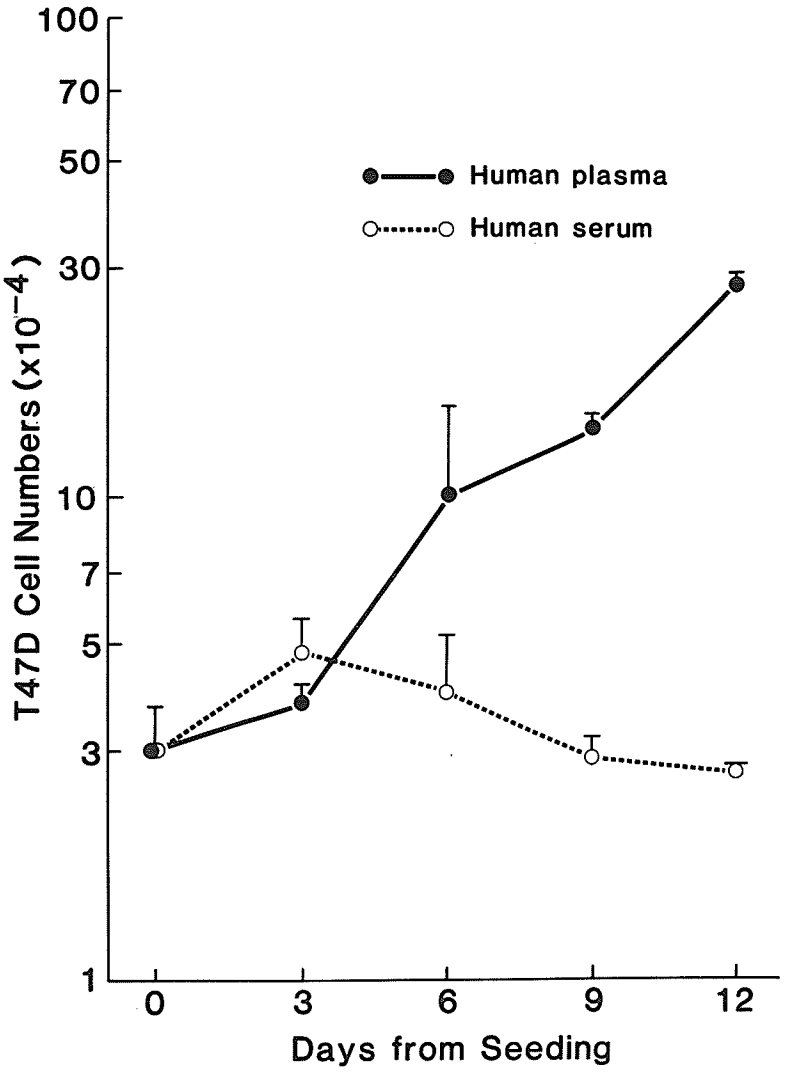

100
70
50
30
10
7
5
3
1
T47D Cell Numbers (x10⁻⁴)
Human plasma
Human serum
0
3
6
9
12
Days from Seeding

Figure 10 Cell growth on plastic with female human plasma or serum.

Human female plasma was collected from healthy premenopausal volunteers. Preparation of the plasma and serum is similar to that presented in figure 8.

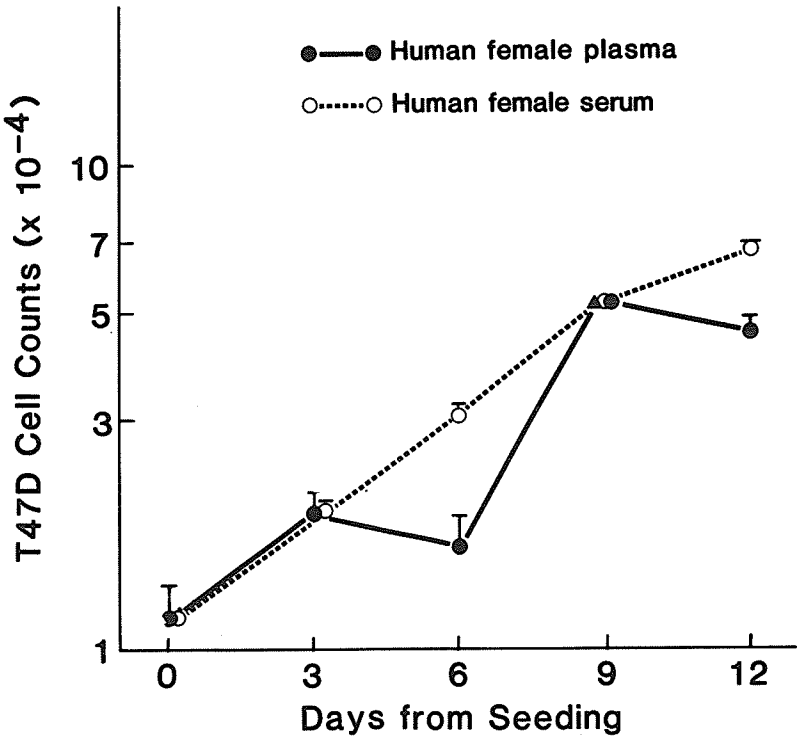
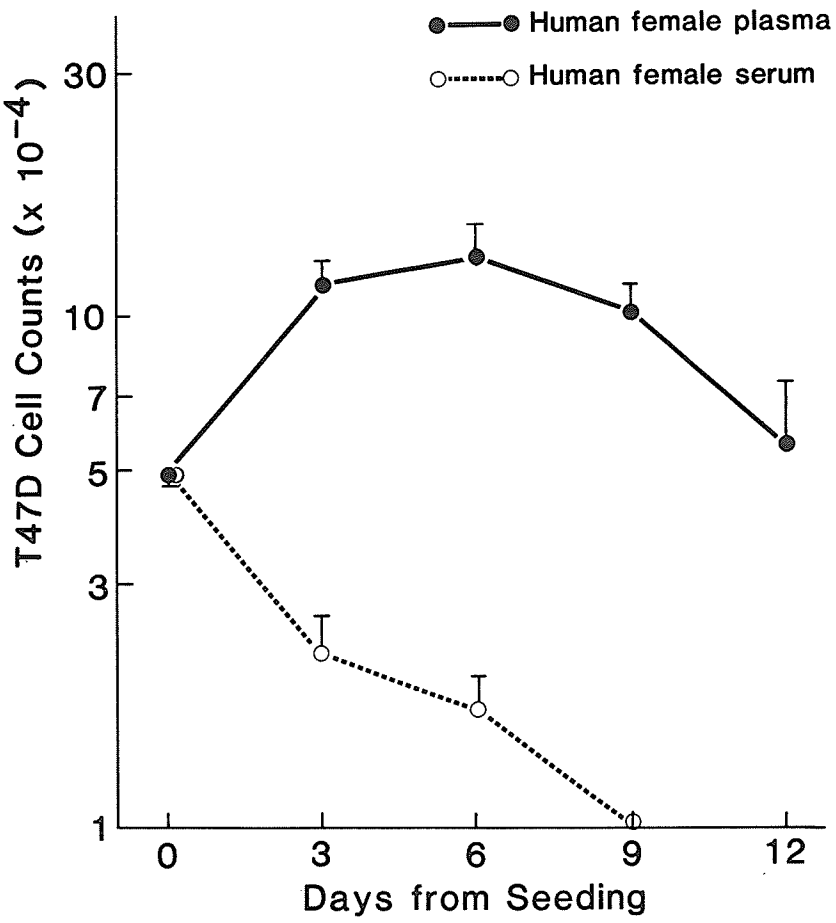

Human female plasma
Human female serum
T47D Cell Counts (x 10⁻⁴)
10
7
5
3
1
0
3
6
9
12
Days from Seeding

Figure 11 Cell growth on matrix with female human plasma or serum.

All procedures are similar to Figure 9, except human female plasma and serum were utilized.


Human female plasma
Human female serum
T47D Cell Counts (x 10^{-4})
30
10
7
5
3
1
0
3
6
9
12
Days from Seeding

been little success in demonstrating direct effects of hormones on cultured mammary cells, especially on human mammary cancer cells. It has been demonstrated that the extracellular matrix restores hormone responsiveness to normal epithelial cells *in vitro* (Gospodarowicz *et al.*, 1979). Therefore, the next series of experiments were carried out to examine the effectiveness of various polypeptide and steroid hormones on T47D cells cultured on extracellular matrix. It is hoped that the extracellular matrix will restore the ability of human breast cancer cells to respond to hormones. In this manner, we may be able to identify some of the physiologically important hormones that might be risk factors in human breast cancer.

It has previously been documented that various polypeptide and steroid hormones are essential for proper development of the breast *in vivo*. Amongst these are estrogen, prolactin, and growth hormone. These same hormones are thought to be major factors in influencing the growth of neoplastic breast epithelium. The actions of these hormones on human breast cancer cells maintained on extracellular matrix were therefore investigated.

Figure 12 illustrates the first experiment in the series of mammotrophic hormone studies. The data depicts the cell numbers of T47D cells 7 days after exposure to various hormones in serum-free medium. All hormones with the exception of 17-β estradiol (10^{-10}M) were added at a concentration of 1 μg/ml. Initial cell seeding was 8 x 10^3 cells/dish. Human growth hormone (hGH) alone caused a 6.5 fold increase in cell number. In combination with 17-β estradiol, the effect of hGH was blunted, with only a 3.6 fold increase in cell numbers (45% decrease compared with growth hormone alone). Other hormones tested, (ovine growth hormone, bovine prolaction and human prolaction, and 17-β estradiol), had no significant effect on the proliferation of the T47D breast cancer cells grown on extracellular matrix.

Next, T47D cells were seeded at a higher initial density (4.4 x 10^4 cells/dish) in serum-free medium (figure 13). On the left is the cell growth on extracellular matrix. On the right, a parallel experiment in which cells were cultured on ordinary plastic. Again on extracellular matrix, hGH produced a 2.2 fold increase in cell number; estrogen again diminishes the effect of

Figure 12. Growth of T47D in serum-free DMEM on extracellular matrix.

T47D cells were seeded at an initial density of 8 x 10^3 cells/dish onto extracellular matrix. Mammotrophic hormones alone (at a concentration of 1 μg/ml) or in combination with 17-β estradiol (concentration of 10^{-10} M) were added to various dishes. One week later, cell numbers were determined electronically.

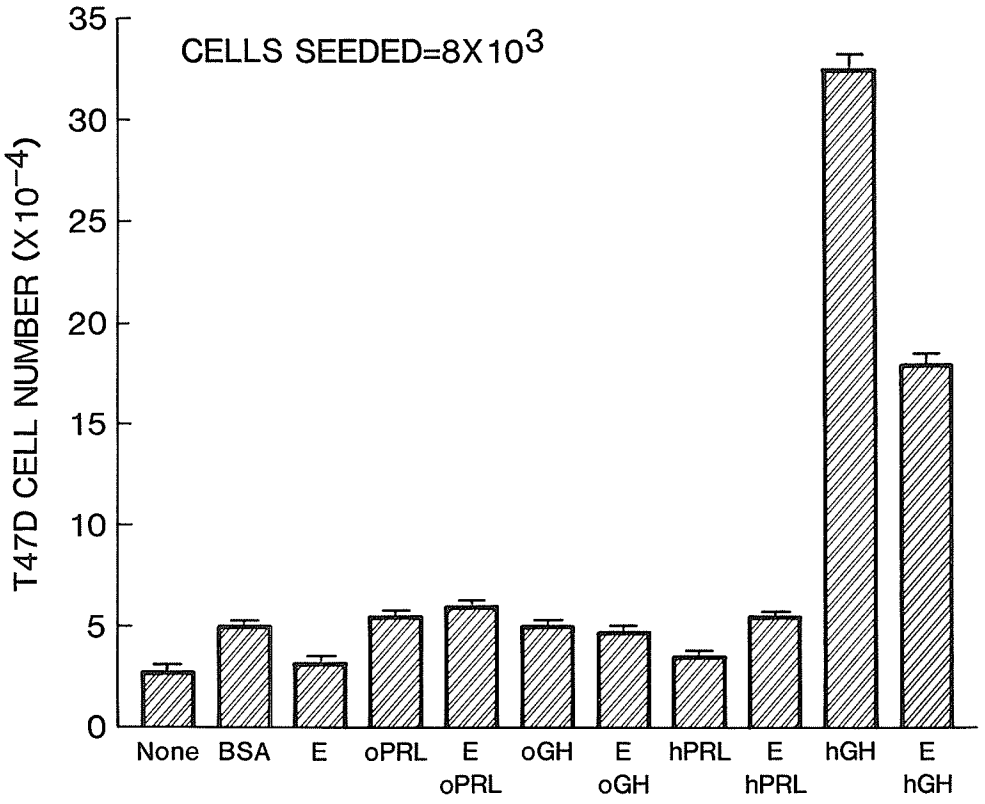
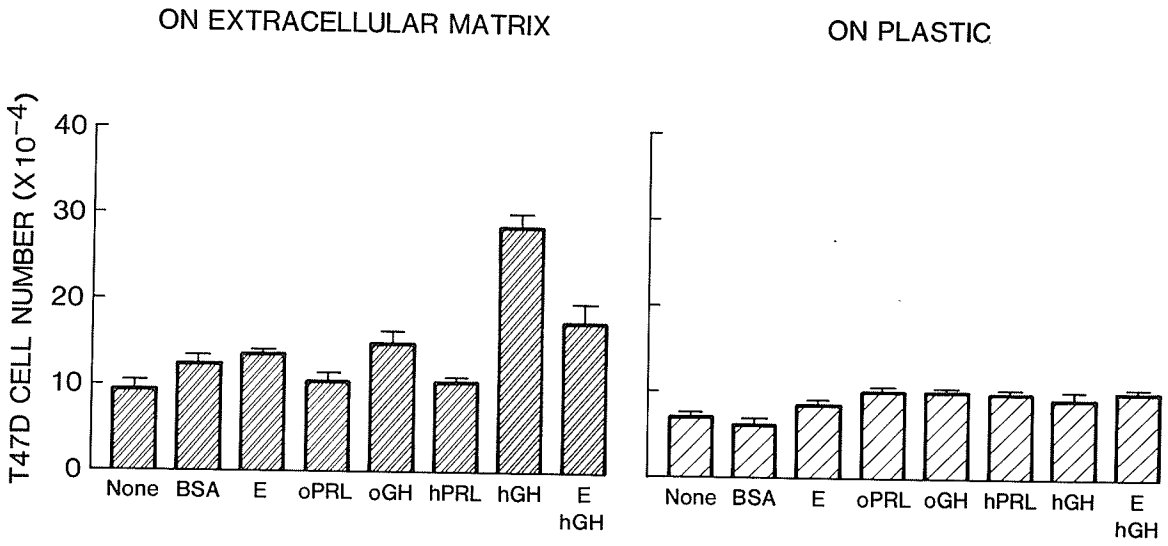

CELLS SEEDED=8X10^3
T47D CELL NUMBER (X10^{-4})
35
30
25
20
15
10
5
0
None
BSA
E
oPRL
E oPRL
oGH
E oGH
hPRL
E hPRL
hGH
E hGH

Figure 13. Growth of T47D in plain serum-free medium.

Human breast carcinoma cells, T47D, were seeded onto extracellular matrix or on plastic tissue culture dishes at an initial innoculum of 4.4×10^4 cells/dish. Mammotrophic hormones were then added as described previously. Cell numbers were determined electronically after 7 days.

CELLS PLATED $4.4X10^4$



hGH (see figure 12) by 41%. It is interesting to note that, when T47D cells were maintained on a plastic substratum (figure 13, right), they failed to respond to hGH.

In an attempt to investigate whether or not other hormones modify the response of T47D cancer cells to hGH, a synthetic, serum-free medium was used in the next experiment. The synthetic medium contained insulin (20 ng/ml), epidermal growth factor (2 ng/ml), hydrocortisone (20 ng/ml) and transferrin (5 μg/ml). The result of this experiment is shown in figure 14; the results were similar to that of the previous experiment (figure 13). Only hGH caused a 3.5-fold increase in T47D cell number on extracellular matrix. The various added hormones in the synthetic medium did not influence the response of T47D cells, grown on extracellular matrix or on plastic, to the action of hGH.

When cells were seeded at even higher density (8 x 10^4 cells/dish), the effect of hGH on T47D proliferation was small (2.5-fold) (figure 15).

When all the data was analyzed for significance by SPSS computer compilation, the hGH significantly stimu-

Figure 14. Growth of T47D in serum-free synthetic medium. The T47D cells were plated at 4.4×10^4 cells onto extracellular matrix or plastic with synthetic medium composed of transferrin, insulin, hydrocortisone and epidermal growth factor. Cell counts were performed electronically after 7 days.

CELLS SEEDED=4.4×10^4

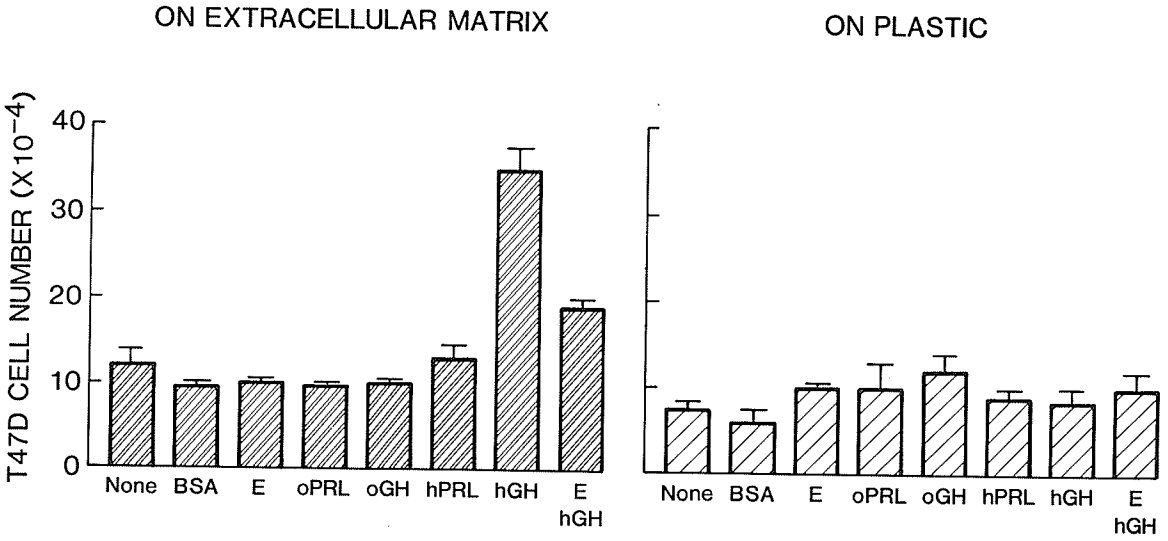
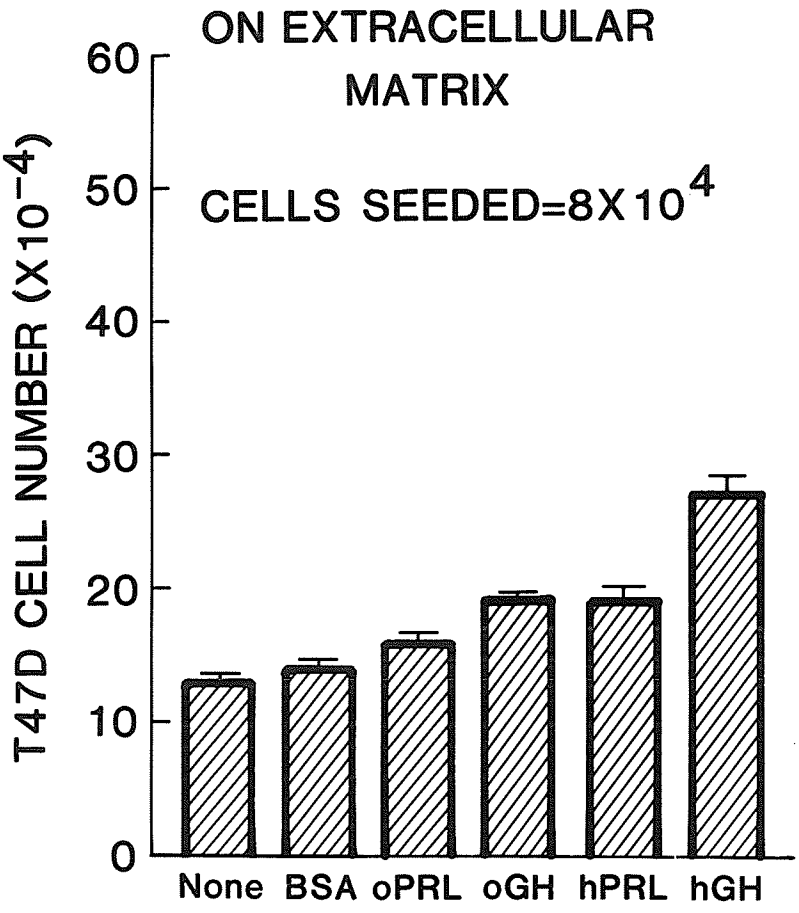


Figure 15. T47D growth in serum free medium on extracellular matrix.

T47D cells were seeded at 8 x 10^4 cells/dish onto extracellular matrix as prepared in Methods. Cell counts were performed one week later. Serum free DMEM, supplemented with various hormones, was utilized throughout the experiment.


ON EXTRACELLULAR
MATRIX
CELLS SEEDED=8X10^4
T47D CELL NUMBER (X10^-4)
60
50
40
30
20
10
0
None
BSA
oPRL
oGH
hPRL
hGH

lated the proliferation of T47D cancer cells maintained on extracellular matrix ($P < 0.001$). The inhibitory effect of estrogen on hGH action is also significant ($P < 0.01$). There is also a significant negative correlation between the degree of stimulation by hGH and the initial cell density (correlation coefficient $r = -0.789$, $P < 0.01$). This is taken to mean that the effect of hGH would be maximally observed when seeding cell density is low.

DISCUSSION

The epithelium that forms the breast normally resides on an extracellular matrix which is similar to the endothelial matrix (Gospodarowicz, 1982). The importance of an extracellular matrix for normal cellular functioning cannot be underplayed (Wicha, 1980, Bethea, 1981, Gospodarowicz, 1979, 1980, 1981). Removal of the matrix, or any component of the extracellular matrix, removes the selectivity of the cells to its environment. Cellular division, migration and differentiation are noticeably impeded in the absence of an extracellular matrix. Two experiments serve to illustrate this point. Folkman (1969) demonstrated that anchorage dependant V2 carcinoma cells fail to grow in the rabbit vitreous humor, but grow logarithmically on extracellular matrix. Liotta et al. (1979) illustrated that cis-hydroxyproline, an inhibitor of collagen synthesis, prevented the differentiation of rat mammary epithelial cells. However, cultures that were provided with an existing extra-cellular matrix expressed differentiation even in the presence of the inhibitor. These experiments illustrate the importance of an environment containing an

extracellular matrix which determines cellular growth and function.

In cell culture, cells grown on plastic are therefore disadvantaged. Cells either fail to proliferate or they will grow out enormously in response to unknown factors in fetal calf serum. Furthermore, cells in culture on plastic frequently lose their responsiveness to factors that control cellular functions in vivo. This is adequately demonstrated by Cohen (1965) and Gospodarowicz (1979). When corneal epitheloid cells were grown on plastic they lost their EGF responsiveness. However, when corneal epitheloid cells were grown on a carpet of extracellular matrix, they responded maximally to the mitogen actions of EGF.

The responsiveness of T47D to human plasma and serum was presented. The lack of a differential response was clearly evident on plastic. However, human plasma caused logarithmic growth of T47D, while human serum caused inhibition of cell growth while the cells were maintained on matrix. The human serum effects could be explained by the work performed by Green et al. (1979, 1981). These investigators isolated to near homogeneity

a serum tumor cytotoxic agent from normal healthy volunteers. The substance is active on a wide range of tumors especially breast, cervix and gut. Indeed, the cytolysis obtained in the experiments indicates this factor is present and active.

Mammotrophic hormones

Many hormones are responsible for breast epithelial proliferation *in vivo*. However, *in vitro*, demonstration of hormonal effects has met with limited success.

The experiments performed in this study demonstrated that human growth hormone is trophic to T47D human breast cancer cells cultured in the presence of an extracellular matrix. Furthermore, the addition of 10^{-10}M 17-β estradiol antagonizes the response to hGH. One could speculate the reasons for this effect. Perhaps the concentration of 17- estradiol used in these experiments was too high, approaching the pharmacological range. In the body, estradiol concentration (bound and free) is below 10^{-12}M. Furthermore, it is known that high concentrations of estradiol are inhibitory for breast tumor cells (Pearson and Nasr, 1971). Indeed, a common endocrinological

therapeutic measure for breast cancer is the administration of pharmacological doses of estradiol. At present, it is not clear if the inhibitory effect of estradiol on hGH action is a specific one or not. Future studies utilizing various steroids and their analogs may clarify this problem.

It was also demonstrated that prolactin and estradiol alone or in concert had no effect on the proliferation of T47D cells. Why is estrogen and prolactin ineffective? It is known that T47D cells contain estrogen and prolactin receptors (Shiu, 1981). However, these hormones fail to cause an increase in the rate of cell growth. Several speculations for the lack of cellular response with prolactin and estradiol are possible. First, prolactin may not be a mitogenic hormone in humans; it may only be responsible for initiation of lactation. Second, perhaps there is a lack of specific competence factors needed for cell cycling. If prolactin can only supply the initial push as a progression factor, without additional competence factors the cells would remain quiescent. Third, estrogen may exert an indirect effect through estromedins (Sirbasku, 1978). Fourth, the doses of prolactin and

estrogen used in these experiments might have been inappropriate. Future experiments to examine the dose response of these hormones should be warranted.

The observation that hGH stimulates breast cancer cell growth *in vitro* not only raises the possibility that hGH may be a risk factor for the disease, but also suggests that hGH has a direct effect on target cells. The dogma of hGH action is that the hormone acts through its intermediates, the somatomedins. One of the main sites of somatomedin production is the liver. The hGH stimulates the liver to produce somatomedins. However, the direct growth promoting effect of hGH has recently been demonstrated in several systems. For example, hGH has a direct effect on Nb2 rat lymphoma cells (Noble, Beer and Gout, 1980). Human growth hormone stimulates tibia and chrondocyte growth *in vitro* directly (Isaksson *et al.*, 1982). Finally, hGH stimulates the proliferation of preadipocytes and to promote the formation of adipocytes (Hayashi *et al.*, 1981).

Another possibility is that the hGH action is of an autocrine nature: hGH may stimulate the production of somatomedins in the target T47D breast cancer cells.

The somatomedins thus produced will in turn stimulate cellular growth. Indeed, Baxter et al. (1982) have demonstrated that T47D cells contain somatomedin C (Sm-C) receptors and produce immunoreactive Sm-C into the medium. Thus, Sm-C could be stimulated by hGH in T47D cells. The newly synthesized Sm-C could then act mitogenically on the T47D cells in an autocrine fashion. It would be of interest, therefore, to determine if hGH stimulates the production of Sm-C in T47D cells.

We have also demonstrated that T47D cells responded to hGH only when they were maintained on a natural extracellular matrix. What then is the role of the extracellular matrix? It has recently been demonstrated that the subendothelium is very sticky to a wide array of proteins (Scott, 1981, Smith, 1982). Platelet-derived growth factor and factor VIII-related antigen are two such proteins shown to stick to the matrix. It is possible that proteinases, secreted by the endothelial cells and/or human breast cancer cells, are tightly associated with the extracellular matrix. Proteinases can be activated by detergents such as Triton X-100 (Bloxham, 1980, Wiegand, 1979) which was used to prepare

the extracellular matrix. When human growth hormone (22K) is then added to the culture media, it is cleaved to the active form that is mitogenic for breast cancer cells. Lewis et al. (1974) have identified two enzymatically-cleaved forms of human growth hormone (hGH-E and hGH-F) which have high mitogenic activity in the rat tibial assay. These forms of hGH seem to be variants of the 22K form. The existence of a proteinase which would cleave hGH into its mitogenic form for T47D is essential to this hypothesis.

Furthermore, work by Emerman (1981) has shown an increase in bioactive, but not immunoactive forms of hGH in sera of breast cancer patients. Indeed, coupled with this is the study of Pearson (1959) demonstrating that in hypophysectomized breast cancer patients, administration of hGH caused an exacerbation of a quiescent breast tumor.

Expansion of the present experiments may lead to a better insight into the hormonal treatment of breast cancer patients. Hormonally responsive breast tumors need not be solely estrogen dependent; perhaps the iden-

tification of GH receptors in breast cancer would be useful. Inhibition of secretion of growth hormone as well as estrogen and prolactin as a combined regimen of endocrine therapy may be worthy of consideration.

In summary: a) An established human breast cancer cell line, T47D, responded to human plasma but not human serum when the cells were maintained on matrix.

b) Human growth hormone stimulated the proliferation of T47D cells on extracellular matrix. Furthermore, 17-β estradiol acted antagonistically to the action of hGH.

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