CELLULAR AND MOLECULAR BIOLOGY OF MAMMARY CANCER

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PREFACE

The idea for this book arose during the 1985 Gordon Conference on "Mammary Gland Biology". New developments in the methodology of cell biology and the explosive growth of molecular biology had begun to impact upon our understanding of mammary gland growth and function. It seemed a propitious time for summarizing the current status of knowledge of the cell and molecular biology of mammary cancer and for attempting to outline future areas of concern and interest. The reviews presented here were completed by the Fall of 1986. Although new insights will surely continue to emerge, it is hoped that the material in this volume will form not only a current update but a basic core of information for future experiments.

We have not attempted to cover all areas of mammary gland transformation. Those areas where recent detailed reviews are already available have been omitted. Also, the areas of normal gland development, cell ultrastructure, hormone responsiveness, chemotherapy and clinical aspects of mammary cancer have not been included. Instead, we have selected those areas where the development of new methodology, reagents and results have led to new ideas about mammary gland function and development as they are related to neoplasia.

The first section presents topics on cellular aspects of the mammary gland, particularly those aimed at defining the cell lineages important in neoplastic development and progression. The availability of a wide range of polyclonal and monoclonal antibodies to cell proteins has led to new concepts in this area. The second section includes topics in cell-cell and cell-extracellular matrix interactions in mammary gland development and function. This is an area that has benefited growth from new cell culture methodology. As these chapters illustrate, cellular interactions play a major role in the fetal development of the gland, in normal functional differentiation, and in neoplastic progression.

The third section addresses topics in hormonal aspects of mammary gland tumorigenesis. Historically, this is one of the most intensively investigated areas of mammary gland biology; however, it is evident that even after 60 years of investigation, new issues are still emerging. The fourth section contains reviews of the viral aspects of mammary gland tumorigenesis. Like the area of hormones and mammary tumorigenesis, viral aspects have a long history. Recently there has been renewed interest in this area after a nearly 10 year doldrum. The application of molecular approaches has yielded an abundance of new data and concepts. Indeed, MMTV has come to be the model of choice both for "enhancer-insertion" carcinogenesis as well as for hormonal regulation of gene expression.

The last section of this book offers topics on several aspects of mammary gland transformation. Multiple approaches to the analysis of mammary gland neoplastic transformation have led to improved model systems in which diverse questions can be examined. Such questions span the areas of mutagenic activation of oncogenes, the significance of differentiation susceptibility to neoplastic development and the role of dietary constituents in the prevention of neoplasia.

Although a major aim of this volume was to illustrate the multiple approaches that have been used to analyze mammary gland function and transformation, the area of mammary gland transformation elicits more than an academic interest. Not only does the system excite the experimental scientist because of its inherent beauty, experimental applicability, complex biological interactions, and fundamental cellular properties, it also stimulates a compelling desire to try to understand and solve the problem of breast cancer. Human breast cancer strikes over 100,000 women the United States annually and leads to over 40,000 deaths. Many of these deaths occur in the prime years of life. This is a great emotional as well as an economic tragedy. Similar statistics occur in the Western European nations. Breast cancer is a problem which, at one time or another, strikes practically every family in the United States. Thus, for both humanitarian and scientific reasons, research on mammary gland function and tumorigenesis assumes a high priority. It is our hope that these chapters may serve to elicit new insight into mammary neoplasia, insights which eventually lead to its control. In addition, the mammary gland (rodent and human) is one of the best systems for investigating and eventually understanding the basic concepts and processes in the initiation and progression of neoplasia.

A book like this one requires the commitment and work of many contributors. However, the Editors wish especially to extend our heartfelt and enormous gratitude to Dr. Clement Ip, Roswell Park Memorial Institute, and the staff of the Organ Systems Coordinating Center who helped translate this volume into reality. Dr. Ip effectively served as Managing Editor of this volume and spent numerous hours collating, re-editing and formatting these chapters, and has given his enthusiastic support to all of us throughout this venture. We are also particularly grateful to Mr. Arthur Hilgar and Mrs. Patricia Beers who were involved in the editing and re-typing of these chapters.

Daniel Medina, Ph.D.
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June, 1987

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CHANGES IN ANTIGEN PATTERNS DURING DEVELOPMENT OF THE MOUSE MAMMARY GLAND: IMPLICATIONS FOR TUMORIGENESIS

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I. INTRODUCTION

The development of the mouse mammary gland starts during fetal life around day 10 to 11 after fertilization (Fig. 1). By day 13, the epidermis shows focal outgrowths or invaginations into the underlying mesenchymal structures. In a few days, these foci of epithelial growth show a centerally located lumen and the earliest development of a duct. The basic design of the mammary gland is finished by day 17 of embryonal life (1,2). The second important phase in development begins around the 4th week of life in female mice. A system of branching ducts develops in the fat pads. The tip of the ducts show club-shaped structures, called endbuds.

A third phase of growth and subsequent maturation starts during early pregnancy when the so-called lateral ("alveolar") buds appear along the branched-out ducts. From these lateral buds, the alveoli emerge and organize into lobular structures in which milk is produced. Early (light microscopic) work on this sequence of events of the mouse mammary gland was presented by Cole (3).

At the cellular level, the differences between the two main cell types found in the fully differentiated mammary gland - the luminal cells and the myoepthelial cells - are very pronounced. The myoepithelial cells are located basal to the luminal cells, are basket-like with long cytoplasmic protrusions and function as contractile elements. In contrast, the luminal cells are cuboidal and secrete milk at their apical side.

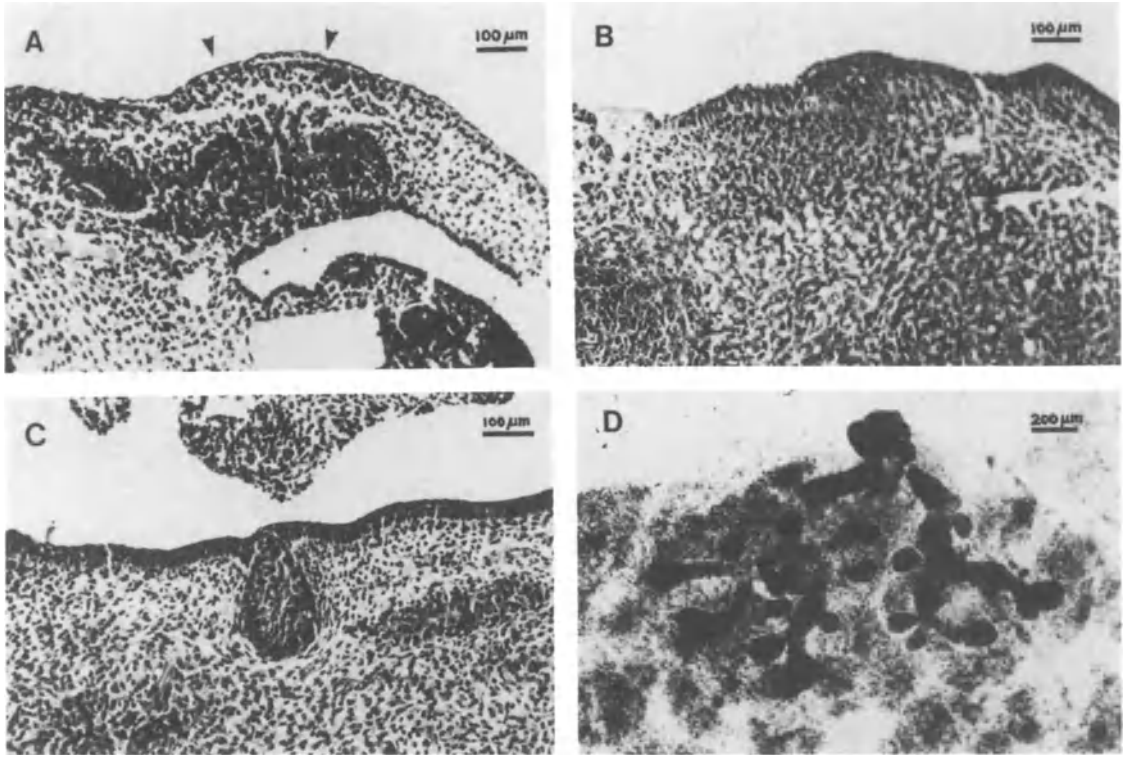


Figure 1. Development of the fetal mouse mammary gland. A,B and C: sections through the fetal skin, stained with hematoxylin-eosin. A: 11th day of pregnancy. Arrows indicate enlargement of the epidermis. B: 12th day. A focal outgrowth into the mesenchyme is visible. C: 14th day. Invagination of the anlage into the underlying structure. D: 19th day. Whole mount preparation of the anlage.

While the two functionally mature cell types of the mammary gland can easily be distinguished morphologically, this is not the case for immature types of cells in the developing gland. In the earliest phase, the 13-day old fetal anlage, no distinction can be made between cell types within the epithelial group of cells. At day 17, luminal markers appear at the apical side of the most centrally located cells in the anlage, while the cells closer to the basement membrane may express higher levels of basolateral membrane antigens or secretory products destined to become located in the basement membranes. Williams and Daniel (4) were able to distinguish with the electron microscope a cell type on the outer edge of terminal endbuds morphologically different from cells in the inner mass of the endbud. They called these cells "cap" cells and hypothesized that they may represent a bipotent stem cell for both the myoepithelium and epithelium.

It is our intention to describe some of the changes in morphology in mammary gland epithelium in relation to the expression of a series of antigens defined by a panel of monoclonal antibodies (5). We have defined at least 5 different cell types based on morphology and antigen expression in the neonatal and adult mammary gland. In this communication, we focus attention on the earliest phases of development during embryogenesis.

Immunohistological studies of antigen patterns of normal epithelial components of the mammary gland system are important because they can lead to a better classification of the numerous histological types of mammary tumors. The cell lineages which are often observed during tumor progression, can be traced using patterns of antigen expression. We illustrate this briefly using one of the better known types of progression in MTV-induced tumors, i.e. from hyperplastic alveolar nodules to adenocarcinomas of the A and B type and to carcinosarcomas (6,7,8).

II. MATERIALS AND METHODS

Table 1 shows the designations of the various monoclonal antibodies used in this study. The choice of this particular panel was based on the location of the markers in developing epithelial structures, particularly the terminal endbuds in puberal mice. Two markers, defined by JB6 and JsE3, are present in a single cell layer in the tip of such endbuds. Two markers, defined by 50B8 and YPC1/3.12, were chosen for the development at the basal side of the gland, and were present in the cells in contact with the basement membrane. Antibody 78B3 detects laminin. Two antibodies (50B8 and 33A10) detect different antigens expressed mainly at the luminal side of the epithelial cells. They react with different glycoproteins.

We included one polyvalent antiserum in this study against an intermediate filament system characteristic for epithelial cells, i.e. keratin. The distribution of the antigens detectable with this particular antibody has been described in detail for the rat and mouse mammary gland (11).

Immunoperoxidase Test on Frozen Sections

The antibodies were used on frozen sections, fixed for 3 minutes in acetone and washed twice in ethylether to remove a lipid film that otherwise inhibits the reaction. Incubation of sections with hybridoma culture fluids for 90 min. at room temperature was followed by three washes with PBS and incubation with peroxidase-conjugated secondary antibody. After a second series of washes, color was developed with aminoethylcarbazole in acetate plus hydrogen peroxide. The substrate was washed away with PBS and the sections were counterstained with hematoxylin and mounted in Aquamount. Inactivation of endogenous peroxidase was not necessary. For further details, see (5).

TABLE 1: ANTIBODIES AGAINST EPITHELIAL MARKERS OF THE MOUSE MAMMARY GLAND

Monoclonal antibodies[1]	Location in epithelium	Antigen definition
JB6	Tip of endbud	Glycoprotein
JsE3	Tip of endbud	Glycoprotein
78B3	Basement membrane	Laminin
50B8	Apical membranes	Glycoprotein (170 kD)[2]
33A10	Apical membranes	Glycoprotein
YPC1/3.12	Myoepithelial cells	unknown[3]
Polyvalent antiserum[1]		
/p-keratin	Various cell types	Keratin[4]

[1] All antibodies were also used by Sonnenbert et al. (5)
[2] See (9)
[3] See (10)
[4] See (11)

III. RESULTS

The anlage (Figs. 2 and 3A,B,C) of the 13-day fetus shows JB6 and JsE3 positive cells; these cells seem to originate from the basal cell layer of the epidermis. A basement membrane starts to develop as seen with 78B3 and keratins are expressed in every cell. No luminal markers are present during these earliest stages of mammary gland development. These appear around 18 days of fetal development with the formation of a lumen. At this stage, certain cells in the inner mass and along the lumen are negative for the JB6 and JsE3 antigens.

The second critical phase of development occurs at 4-6 weeks postnatally in female mice in the mammary endbuds. Here JB6/JsE3 positive cells are confined to one cell layer along the basement membrane, although an occasional cell in the inner mass of endbud may still be positive (Fig. 3D,E). Noteworthy is the difference in thickness of the basement membrane, which is absent or very thin at the tip of the endbud (laminin is seen in the cytoplasm of the cells at this location) and very thick and apparently folded in the neck of the endbud where elongation into ducts takes place. Luminal markers may be present in cells of the endbud, but not yet at the apical side of the cells along the lumen. Polarization of the antigens towards the lumen takes place during elongation of the duct. A new marker demonstrable with YPC1/3.12 appears in the maturing myoepithelial components with the elongation of the duct.

The third phase of development, which occurs during pregnancy, is characterized by lateral bud formation and subsequent maturation of the alveolus. JB6/JsE3 positive cells disappear and YPC1/3.12 positive cells appear. One luminal marker (50B8) disappears whereas another remains expressed (33A10). In the luminal cells, some keratin expression is lost, but keratin expression remains high in the mature myoepithelial cells. Luminal epithelial marker 33A10 is now present in secretions; this marker represents one of a series of milkfat globule membrane antigens (5).

The same markers can be used to define the antigenic phenotypes of various preneoplastic and neoplastic changes of the mammary gland, such as those induced by the mouse mammary tumor virus. The hyperplastic alveolar nodule is not homogeneous in its pattern of antigen expression. Hyperplastic alveolar nodules exhibit staining patterns resembling mature alveoli in one portion of the lesion and normal ducts in other portions with respect to markers as keratin and 50B8.

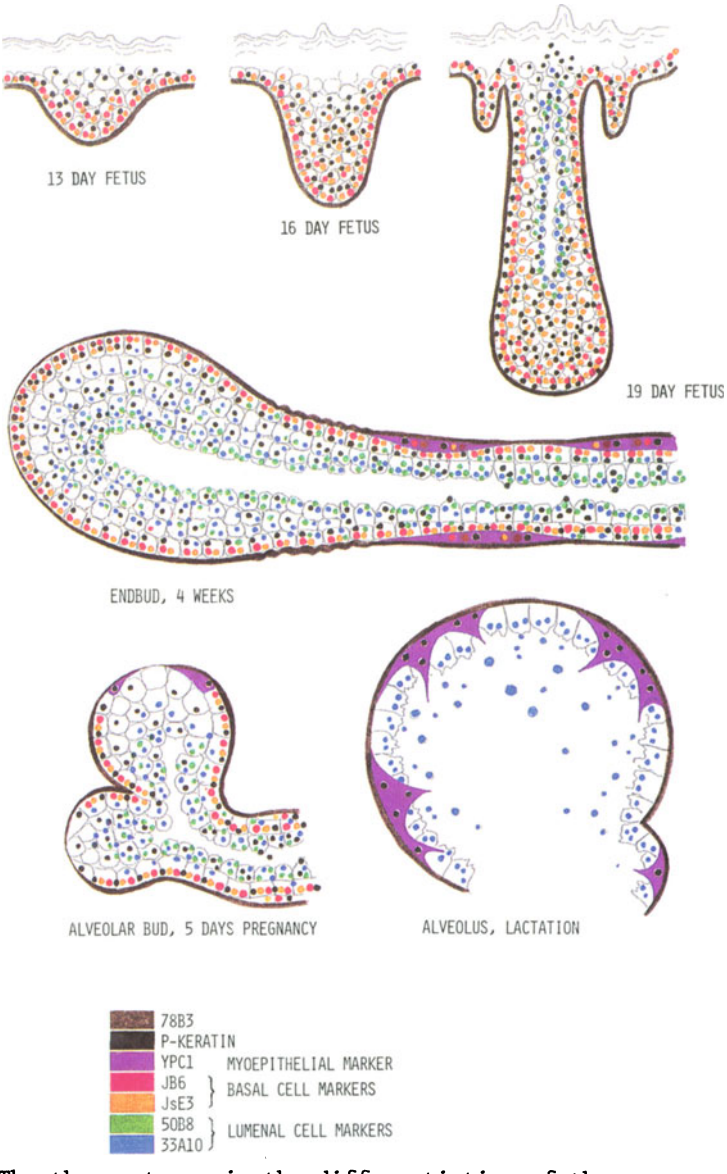
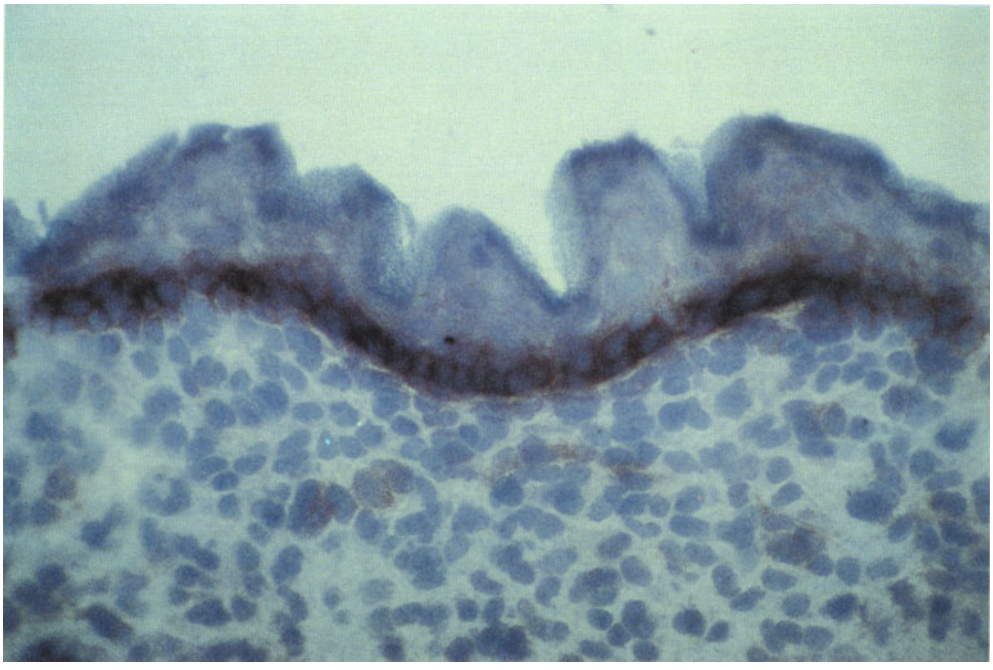
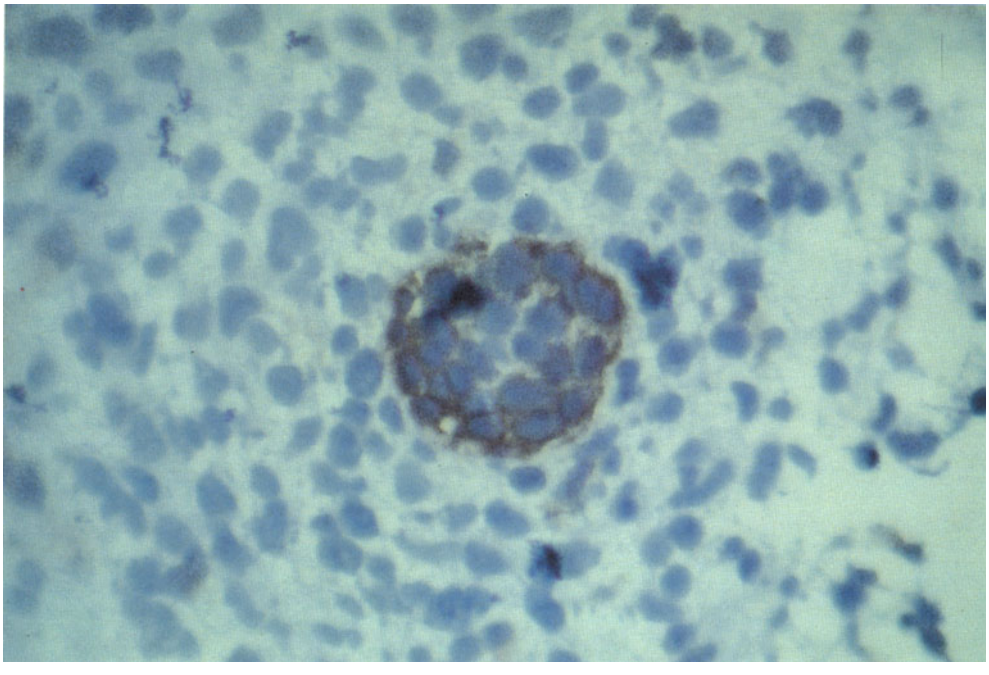


Figure 2. The three stages in the differentiation of the mouse mammary gland with schematic representation of the markers used in this study. Colored dots indicate the presence and roughly the location of the differentiation markers used in this study. In the more primitive cells of the fetal anlage and the puberal endbud, some markers are evenly distributed while later in the duct these antigens show polarization and are expressed in the basal or in the luminal cells. The antigen demonstrated by monoclonal JB6 is specific for the basal cell layer. In the fetal duct, the antigen defined by monoclonal 33A10 appears when the first lumen is formed, and it is the last antigen that remains in the luminal cell layer after differentiation into alveoli during pregnancy. Pre-keratin is always present in primitive cells, in basal cells and in myoepithelium, while in ducts it is absent in about half of the luminal luminal cells.

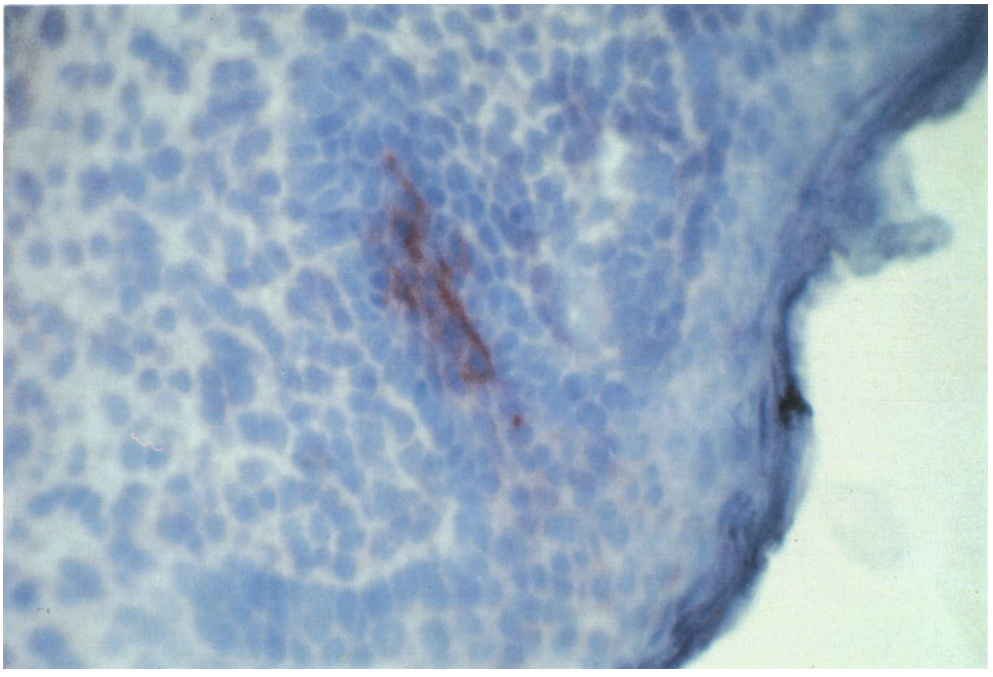


A. Fetal skin with basal marker JB6.

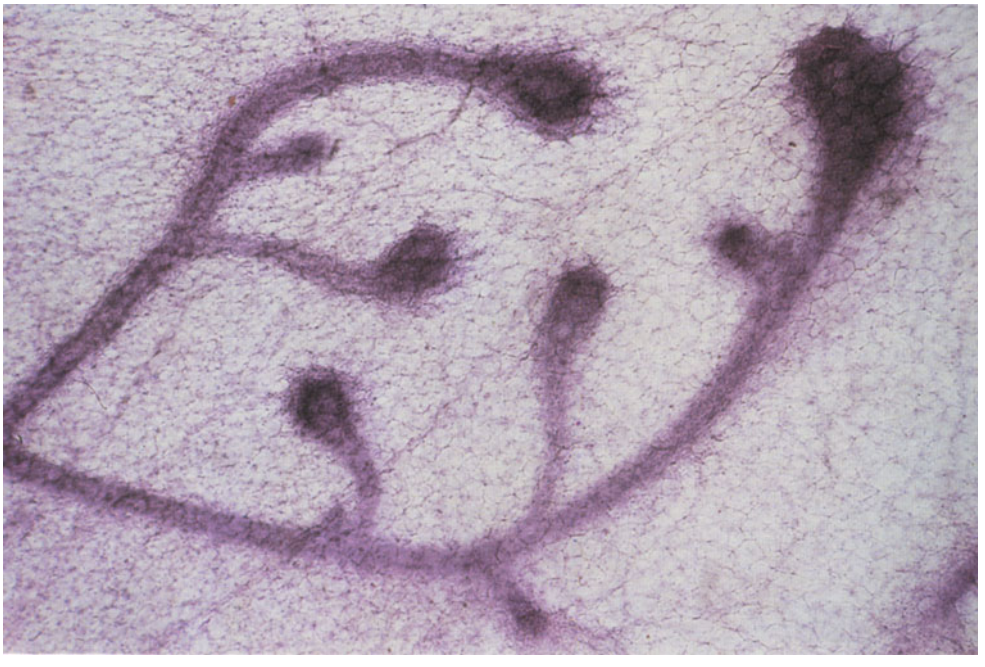


B. Anlage in 15 day fetus with JB6.

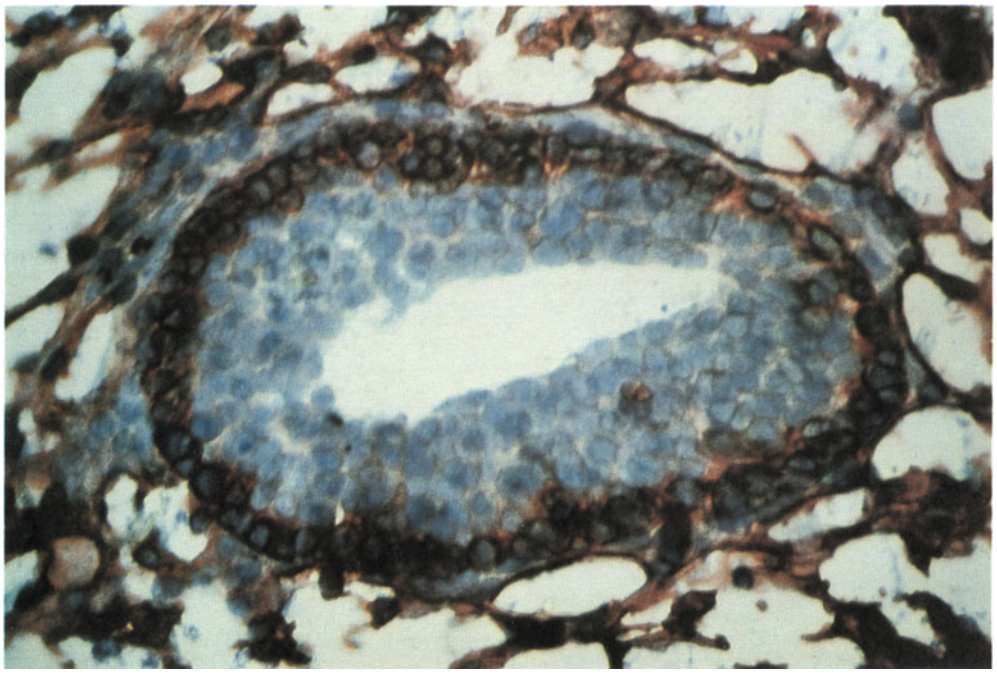
Figure 3. Frozen sections of mammary tissue stained by immunoperoxidase technique with amino-ethylcarbazole and counterstained with hematoxylin.



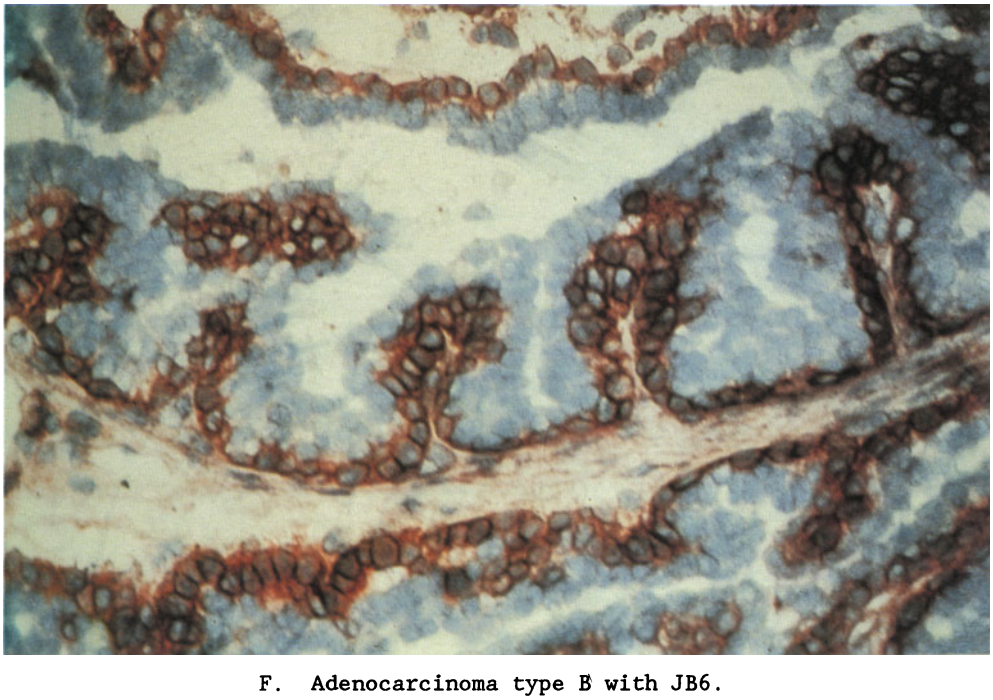
C. Anlage in 18 day fetus with 50B8. Only the newly formed lumen is stained.



D. Whole mount of terminal endbuds, stained with hematoxylin.



E. Tip of endbuds with JB6.



F. Adenocarcinoma type B with JB6.

Type A: Adenocarcinomas have marker patterns different from that seen in normal alveoli. The marker for mature myoepithelium, YPC1/3.12, is not present, but JB6 and JsE3 are weakly expressed in the cells along the basement membrane. Luminal markers are expressed even when a distinct lumen has disappeared. In hyperplastic alevolar nodules and the type A adenocarcinomas, a clear orientation with basal markers outside and luminal markers inside, as seen in the normal gland, is still maintained in contrast to other lesions.

Type B: Adenocarcinomas show numerous cells with the proper orientation. Basement membranes are very irregular. Lumina can still be seen and luminal markers may still be expressed. JB6/JsE3 antigens are very often present in one cell layer along basement membrane depositions, very similar to the situation in the terminal endbud (Fig. 3E,F).

Solid type carcinomas loose the JB6/JsE3 antigens; luminal markers can still be found and are indicative of some remaining polarization. Basement membranes are more often interrupted and appear as short regions.

Carcinosarcomas have lost all signs of glandular structure. There is only a weak expression of basal markers and no expression of myoepithelial markers. A distinct basement membrane is not visible although laminin may still be deposited outside the cells.

IV. DISCUSSION

The first visible evidence of the mouse mammary gland during embryogenesis is a slight enlargement of the epidermis of the embryo on the 10th to 11th day. It is a band extending from forelimb to hindlimb on both sides of the ventrolateral ectoderm. The epidermal cells migrate towards separate "centers of concentration" generating individual mammary buds on the 12th day. From days 12-16, the mammary rudiment grown slowly. At the 17th day, when a fatty substance first appears in the fat pad precursor tissue which develops separately posterior to the mammary epithelium (2), the mammary rudiment starts to elongate by rapid cellular proliferation forming the mammary sprout. The mammary epithelium penetrates the prospective fat pad making branching trees in the last few days of gestation (for reviews, see 12,13,14).

At the cellular level, patterns of antigen expression reveal certain critical moments of development. Originally the anlage exhibits antigens JB6 and JsE3 present in the basal cell layer of embryonal skin. The lumen develops later and parallels the expression of luminal markers. Thus, certain markers of the cells along the basement membrane are expressed earlier in prenatal life than the markers along the lumen. For example, laminin, but also intermediate filament antigens of the keratin class, are expressed very early, while antigens such as 50B8 and 33A10 come up later in embryonal life.

It should be noted that together with "polarization" and the occurrence of a lumen in the developing mammary epithelium, an "opening" has to be created to the outside. The destruction of the skin above the anlage may be the result of expression of proteolytic enzymes expressed only during luminal development at the apical side of the inner cells. It is well known that mammary gland cells produce a number of such enzymes (15), but it remains to be seen whether such enzymes are indeed expressed at this early stage of development.

Mammary ductal elongation was described in detail, using the electron microscope, by Williams and Daniel (4). Their study described a morphological different cell type in the terminal endbuds, called "cap" cells, and

they presented several convincing arguments to support their hypothesis that this cell type represents the stem cell for both the myoepithelium and the luminal epithelium. Cap cells are undifferentiated by morphological criteria; they are continuously proliferating; their layer is continuous with the myoepithelium and they appear to migrate to deeper regions of the endbud. The idea that cap cells may be stem cells can also be deduced from comparable work in the rat by Dulbecco and co-workers (17,18).

The distribution of the cap cell in the terminal endbud, based on morphological criteria, is identical with the distribution of the JB6/JsE3 positive cells; i.e. in a single layer of cells along the basement membrane and an occasional cell deeper in the bud. The "radial" structures of cell layers in the endbud suggests that from the tip cells, which originate at the top of the endbud, move into two directions, one alongside the basement membrane and one into the lumen. This movement and pathway of cells is schematically presented in Figure 4. We believe that occasional stem cells are still present in the ducts giving rise to luminal cells. It is also evident from transitional cell types between the primitive cap cell and the mature myoepithelial cells, that the latter derive from the former, as is shown very clearly by Williams and Daniel (4).

Later stages of normal development, except the process of involution of the lactating gland, have been described in detail (5). We were able to distinguish three types of luminal cells, called type I and II ductal luminal cells and alveolar luminal cells. In the ducts, expression of keratins could be related to morphological differences and may also be related to functional differences in cells of the ductal system.

The antigenic phenotype of the hyperplastic alveolar nodule is very similar to normal structures such as the alveolus and the mature duct. The main feature distinguishing this so-called preneoplastic lesions from the neoplastic ones described here (adenocarcinomas and carcinosarcomas) is the fact that the myoepithelium is fully developed, at least with respect to the emergence of the YPC1/3.12 marker and the disappearance of the cap cell markers and a continuous basement membrane.

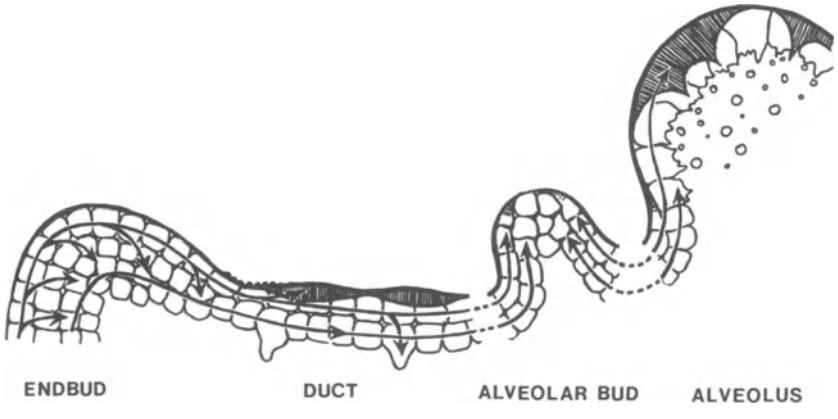


Figure 4. A proposed scheme of cell lineages in the growing endbud and the differentiating alveolus based on the experiments of Williams and Daniel (4) and Sonnenberg et al. (5). Basal (Cap) cells differentiate in longitudinal direction into myoepithelium of ducts and alveoli. Towards the lumen, they produce luminal cells, mainly in the tip of the endbud but probably also, with lower frequency in the duct. These luminal cells loose part of their luminal markers and prekeratin during differentiation into alveolar cells.

The type A adenocarcinoma contains myoepithelium and the individual lobes are close together with little stromal elements in between. Lumina and luminal markers are still present, basal markers are weakly expressed and there is keratin expression in the myoepithelial cells, The basement membrane is continuous, regular and intact.

The type B adenocarcinoma also does not contain mature myoepithelium as judged from YPC1/3.12 expression. One cell layer contains the cap cell markers JB6 and JsE3 and in this respect there is very close resemblance with the organization of the terminal endbud. If the cap cell is indeed the stem cell, these adenocarcinomas could be called stem cell tumors, with a defect in maturation of myoepithelium. Luminal epithelial characteristics are intact, but orientation may be disturbed. Also the basement membranes are irregular, sometimes deposited locally in enormous amounts and are very thick. Further progression from type B carcinomas to solid carcinomas without many lumina results in discontinuous basement membranes and loss of the cap cell markers. However, there are still groups of cell arranged around small lumina and some orientation into a basal versus luminal side is visible using some of these markers.

Carcinosarcomas do not resemble a normal mammary gland. All orientation is lost, although some laminin may still be produced and secreted by the tumor cells. There is a general loss of luminal markers. Keratins may still be seen to some extent.

Preneoplastic lesions, such as the hyperplastic alveolar nodule, have been regarded as precursory stages for adenocarcinomas in the mouse mammary gland (19,20); for review see (21). If this were the case, the defect in maturation of myoepithelium in adenocarcinomas of types A and B should occur in the progression from nodule to the carcinoma. However, genes involved in this step of progression have not been identified and it still remains to be seen whether this step might also occur in a normal mammary gland cell immediately leading to a carcinoma.

Further progression from adenocarcinoma towards carcinosarcoma is a rare event occurring infrequently in adenocarcinomas, often only after many transplant generations. This type of progression has been observed in a system of cloned mammary tumor cell lines, allowing us to study the genes involved in this step from non-invasive to invasive tumor cells (22).

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STEM CELLS IN MAMMARY DEVELOPMENT AND CANCER

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[2]The abbreviations used are: AB, alveolar bud; CALLA, common acute lymphocytic leukemia antigen; DMBA, dimethylbenz(a)anthracene; DMSO, dimethyl sulfoxide; EMA, epithelial membrane antigen; grade I tumor, lowest grading on the Bloom and Richardson Classification; GS-1, Griffonia Simplicifolia-1; HC, hydrocortisone; HMFG-1, human milk fat globule-antigen 1; Huma, human mammary cell line followed by the cell line number; I, insulin; LE61, monoclonal antibody to keratin filaments of simple epithelia; LICR-LON-M18, monoclonal antibody Ludwig Institute for Cancer Research - London Branch - milk fat globule membrane antigen 18; LICR-LON-23.10, monoclonal antibody to surface of basal cells of human skin; LP34, monoclonal antibody to keratin filaments of myoepithelial cells of rats; MFGM, milk fat globule membrane; MMTV, mouse mammary tumor virus; mRNA, messenger RNA; NMU, N,N'nitrosomethyl urea; p9ka, polypeptide of 9000 daltons; PGE_1, prostaglandin E_1; Prl, prolactin; PWM, Pokeweed Mitogen; Rama, rat mammary cell line followed by the cell line number; SV40, Simian Virus 40; SVE3, SV40-transformed human mammary epithelium; TDLU, terminal ductal-lobular unit; TEB, terminal end bud; Thy-1, thymocyte differentiation antigen-1.

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I. CELLULAR STRUCTURE AND DEVELOPMENT OF THE NORMAL RODENT MAMMARY GLAND

A major proportion of the development of the rodent mammary gland begins after birth and prior to puberty, and results in the progressive growth of the mammary parenchyma within the mammary fat pad (4). Extension of the mammary ductal tree and the generation of its branching pattern occurs in three main ways in varying proportions: first by the linear lengthening of existing ducts, secondly by dichotomous branching of the growing ductal tips, and thirdly by monopodial branching produced by the growth of collateral buds situated at the sides of existing ducts (22,120,142). During their period of rapid growth the ducts terminate in dilated TEBs[2], which are present until the ducts reach the limits of the mammary fat pad. The number of TEBs reaches a maximum in rats that are about 20 days old, and afterwards decreases. This decrease reflects not only their transition to terminal ducts but also their progressive differentiation to ABs with each estrous cycle (120). The ABs are direct precursors of the alveoli, which are then capable of synthesizing mammary specific products during pregnancy and lactation (120). The TEBs also contain cells with the highest mitotic activity in the gland, and this activity is decreased in alveolar buds and alveoli (28,121).

The mammary ducts are composed of one or more layers of cuboidal epithelial cells, some of which border a lumen that is continuous throughout the ductal systems, and a layer of elongated myoepithelial cells normally surrounds them (4). In the past these two cell types have been distinguished by their characteristic ultrastructural morphologies: epithelial cells possessing apical microvilli and specialized junctional complexes with associated desmosomes, whereas myoepithelial cells possess smooth muscle-like myofilaments with pinocytotic vesicles and basement membranes on their basal surfaces (99). More recently immunocytochemical stains have been used (20): in the rat antiserum to MFGM and keratin monoclonal LE61[3] (67) stain the epithelial cells, and antisera to vimentin, human keratin, actin, myosin, keratin monoclonal LP34, GS-1 and PWM lectins[4], and antisera to the basement membrane components laminin, Type IV collagen, Thy-1 (75) normally stain the myoepithelial cells/associated basement membranes more intensely (27,61, 119,138,149). A third functionally differentiated cell type, the secretory cell, is found in the mammary alveoli and this is characterized by its ultrastructure (100), and by staining with peanut lectin (80) and with antisera to casein (57,114).

Although the majority of the mammary gland consists of three discrete cell types, the TEBs, lateral buds and ABs of the developing rodent mammary gland are composed of a heterogeneous collection of cells (85,160). These include epithelial and myoepithelial cells, and irregular loosely-adherent cells. The irregular, loosely-packed cells or cap cells are situated mainly around the periphery of the TEBs and lateral buds, and are of rather undifferentiated appearance. They show gradations in ultrastructure to the myoepithelial cells of the subtending duct and also the the epithelial cells within the cortex of the TEB (85,160). The gradations to myoepithelial cells are accompanied by an increase in the staining with antisera to actin, myosin, human keratin, vimentin, laminin, Type IV collagen (30,85), keratin monoclonal LP34[3], and the lectins GS-1 and PWM[4]. The gradations to epithelial cells are accompanied by an increase in the staining with anti-MFGM (85), a monoclonal to rat epithelial cells (30), and with keratin monoclonal

LE61[3]. The peripheral cells of the ABs are more closely packed, contain a greater number of myofilaments and show increased staining with antisera to myosin (85), keratin monoclonal LP34[3], and the lectins GS-1 and PWM[4] than those of the TEBs. These results suggest that the undifferentiated cap cells do not represent a discrete cell type but show transitional forms to myoepithelial cells on the one hand and epithelial cells on the other, and that the tendency towards the myoepithelial phenotype is more predominant in the more differentiated budded structures, the ABs. Results from pulse-chase experiments with DNA precursors (28) and additional monoclonal antibodies to cytokeratins (3) are consistent with this interpretation. Similar types of transition have also been observed between epithelial cells in ABs, which only bind peanut lectin after treatment with neuraminidase and casein-secreting alveolar cells which can bind peanut lectin directly[5].

Whether any one of the three discrete types of mammary cell can directly regenerate the other cell types in vivo is unknown. Transplantation studies of mosaic tissue from two inbred strains of mice show that ductal and alveolar structures can breed true, and that neoplastic mammary tissue in similar transplant lines can be categorized into one of the three discrete types (126). However, in the former experiment the ductal and alveolar growths are always in the same proportion in a given pair of mouse strains, suggesting an alternative explanation based on hormonal differences between strains, and in the latter case the argument is complicated by other mammary cell types being found in some of the resultant tumors (14). Moreover, evidence is now mounting against completely immutable mammary cell types in vivo. Thus mammary ductal epithelial cells may eventually give rise to myoepithelial cells, since myoepithelial cells are absent from the ducts of embryonic and neonatal rats, and only appear about 7 days after birth (99,149). Moreover, different parts of the mammary gland have been dissected out and transplanted to other suitable sites in syngeneic animals. In all cases fully-developed mammary glands are generated which will secrete milk products in isologous, pregnant hosts (58,59), suggesting a more reversible state between ductal and alveolar cells. Finally bud-free ducts can regenerate the entire mammary tree, including TEBs (87) and casein-producing alveoli[5] in appropriate hosts, suggesting that the collection of intermediate cell types, including the cap cell, can also be generated from the epithelial and/or myopithelial cells of the severed ends of the bud-free ducts.

II. CELLULAR STRUCTURE AND DEVELOPMENT OF THE NEOPLASTIC RAT MAMMARY GLAND

The susceptibility of the rat mammary gland to rapid chemical carcinogenesis decreases with age after 50 days (23,60), and is correlated with the presence of TEBs and terminal ducts (122). Although the degree of glandular differentiation of DMBA- (60) and NMU- (46) induced tumors varies somewhat, the vast majority are delineated by a fibrous capsule showing little definite extracapsular infiltration or invasion, virtually no evidence of metastases, and, in most tumors the cells are cytologically benign with normal nuclear to cytoplasmic ratios, little cellular pleomorphism, and virtually no aberrant mitotic figures (158). By standard human histopathological criteria in our hands most of these tumors are benign, and are best classified as adenomas showing varying degrees of atypia. Serial transplantation of these immunogenic (65) tumors in syngeneic animals can yield weakly-metastasizing tumors (157), but chemical induction in partially immune-deficient rats that are then subjected to nonspecific immunostimulation produces nonimmunogenic tumors of much higher metastatic ability (64). The former tumors metastasize to lungs and lymph nodes only, primarily through the blood stream (159), while the patterns of metastatic spread of some of the latter tumors are similar in many respects to those of the human

disease, with involvement of lymph nodes, lung, bone, spleen and kidney, and dissemination by lymphatic and/or hematogenous routes (64).

Analysis of the cell types present using the immunocytochemical and ultrastructural criteria of the previous section shows that the benign tumors, both carcinogen-induced and the MT-W9 transplantable tumor, contain extensive areas of both epithelial and elongated, myoepithelial-like cells in duct-like arrangements (5,32,78). However, many of the elongated, myoepithelial-like cells possess a more variable and undifferentiated appearance than the myoepithelial cells of mature mammary ducts (32), and are therefore more similar to the basal cells of TEBs and ABs (85). All tumor lobules are surrounded by a basement membrane (32) which is often thicker than normal (89), and more comparable to that of the neck region of TEBs (160). In contrast, no cells with any myoepithelial characteristics are seen in the transplantable, metastasizing rat mammary tumors described above (SMT-2A, SMT-007, TMT-081, MT-450, TR2CL), and the majority completely lack any basement membrane (32,159). Similarly the carcinogen-induced benign tumors can synthesize small amounts of casein and produce alveolar-like cells in hormonally stimulated rats (57,114,137). However, the amount of casein mRNA (137) and the fraction of alveolar cells (57,114) is only 1-5% of that found in normal mammary glands of lactating rats. The ability to produce casein or alveolar-like cells is completely lost in the metastasizing rat mammary tumors described above[5].

Although there is usually a complete loss of the residual myoepithelial and alveolar features in most of the above metastasizing rat mammary tumors, the weakly-metastasizing tumor TR2CL contains undifferentiated elongated cells and patches of fragmented basement membrane (159). This suggests that there may also be a gradual loss of the remaining myoepithelial cell characteristics and basement membranes with increasing metastatic potential, at least within the rat systems employed. This loss of myoepithelial cells, basement membranes, and alveolar cells may not be true for other types of metastasizing mammary tumors. Thus transplantable tumors developed from the MMTV-induced mouse mammary tumors can metastasize and still retain a basement membrane (97), but they are noninvasive in the mammary fat pad and metastasize only to lungs (143). In these properties they may be more akin to the weakly metastatic TRC2L rat mammary tumor.

III. CULTURE OF NORMAL RAT MAMMARY GLANDS AND BENIGN TUMORS

Collagenase digestion of normal rat mammary glands and chemically-induced benign tumors severs the epithelial elements from most of the fatty stroma, yielding organoids of glandular elements and fragments of blood-vessels (55,113). If the digestion is terminated before destruction of the surrounding basement membrane, the organoids will adhere to a tissue culture vessel after 12-24 hr, and epithelial cells will grow out sometime later (55). The majority of the stromal cells will adhere to the substratum after 2 hr, and hence can be separated from the more slowly-adhering epithelial cell fraction (113). When re-introduced into cauterized fat pads of syngeneic rats the normal epithelial fraction gives rise to the entire mammary system, whereas the faster-sticking fraction causes regrowth of the fat pad (106). In primary cultures of normal glands and benign tumors, both epithelial (Fig. 1a) and myoepithelial-like cells can be distinguished at the ultrastructural level, although the latter tend to lose their myofilaments (113). Moreover confluent cultures yield hemispherical blisters or domes (Fig. 1b) (74) and small amounts of casein with the mammatrophic hormones Prl, E, HC, and I (113). The normal epithelial cells stain with antisera to MFGM and, after treatment with neuraminidase, some stain with peanut lectin, while the myoepithelial cells stain with antisera to vimentin (144), Thy-1[3], laminin, type IV collagen (63,144) and the lectins GS-1 and PWM[4], exactly as *in vivo*. However, staining with antisera to actin, myosin and human keratin

is different. The keratin antisera stain the cultured epithelial cells as well as some of the elongated, myoepithelial-like cells, although others are unstained; while action and myosin antisera stain only some of the elongated myoepithelial-like cells. In addition, larger transitional cells, intermediate in staining between epithelial and myoepithelial cells can be distinguished (144); they are similar to the intermediate cap cells seen in vivo. These cultures can also yield duct-like outgrowths when grown on a simulated mesenchyme of floating collagen gel (101,156).

Single-cell-cloned epithelial cell lines have been obtained from normal mammary glands of 7-day-old Wistar Furth rats, e.g., Rama 704 (86), and from DMBA-induced tumors of out-bred Sprague Dawley rats, e.g., Rama 25 (16) or inbred Furth Wistar rats, e.g., Rama 37 (34), and from NMU-induced rat tumors (29) (Table 1). All the primary epithelial cultures and the early stages of the epithelial cell lines require the presence of myoepithelial-like cells for satisfactory growth (106). Since all these epithelial cell lines produce similar results, those of Rama 25 are described in detail. This cuboidal epithelial cell line (Fig. 1a) although single-cell-cloned three times, still yields ridges of elongated cells, elongated cells floating freely in the medium, and 1-3% of elongated cell clones; one such clone is termed Rama 29 (Fig. 1c) (16,105). Similar morphological forms have also been generated from mouse mammary tumor epithelial cells (26,52). The elongated rat cells have been adjudged to be related to myoepithelial cells based on their ultrastructure and their immunocytochemical staining pattern (84,145,147). However, like the elongated, myoepithelial-like cells in primary cultures of rat mammary glands (144), although the elongated cells always express vimentin, laminin, type IV collagen, fibronectin, Thy-1 (29, 119,145,147,150) and the receptors for GS-1 and PWM lectins[4], the staining pattern with antisera to actin, myosin, and human keratin is variable; some clones stain well and others poorly, e.g., Rama 29 (118). On the whole the most myoepithelial-like cell lines have been produced from normal rat mammary epithelial cells, e.g., Rama 401 (150) and Rama 704E (86) (Table 1),

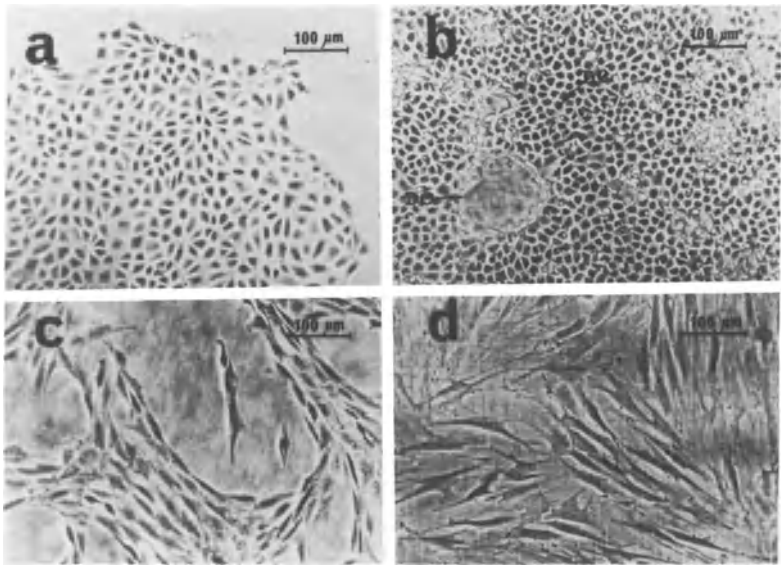

Figure 1. Morphology of rat mammary cell lines in culture: Living cells have been photographed with phase-contrast optics a. Colony of cuboidal epithelial cells, Rama 25; b. droplet cells (DC) in a dense culture of cuboidal epithelial cells with dome (DO); c. elongated, myoepithelial-like cells, Rama 29; d. mammary fibroblasts, Rama 27.

TABLE 1

Origins of Mammary Cell Lines Discussed

Mammary tissue	Cell Line	Identity	Reference
Normal rat	Rama 704	epithelial	(86)
	Rama 704E	myoepithelial-like	(86)
	Rama 401	myoepithelial-like	(150)
Benign DMBA rat tumor	Rama 25	epithelial	(16)
	Rama 259	epithelial, truncated alveolar-like pathway	(108)
	Rama 25-I	epithelial/myoepithelial intermediate cells	(118)
	Rama 29	myoepithelial-like	(16)
Benign DMBA syngeneic rat tumor	Rama 37	epithelial	(34)
Weakly-metastasizing rat tumor, TR2CL	Rama 600	epithelial	(159)
Moderately-metastasizing rat tumor, TMT-081	Rama 800	anaplastic epithelial	(33)
Strongly-metastasizing rat tumor, SMT-2A	Rama 900	anaplastic epithelial	(110)
Normal human transformed by SV40	SVE3	epithelial	(unpubl.)[9]
	Huma 7	epithelial	(unpubl.)[9]
Human ductal carcinoma	Ca2-83	epithelial	(112)

rather than from epithelial cells of benign mammary tumors, e.g., Rama 29 (16). This result is consistent with the better differentiated myoepithelial cells being found in normal rather than in neoplastic glands *in vivo*. However, it would also appear that the majority of the myoepithelial cell markers, including the myofilamental arrays (84) are found in the most recent converts from the cuboidal epithelial cells (86), and that subcloning *in vitro* can lead to selective loss of the microfilamental systems (61). For these reasons the elongated cells in primary cultures and cell lines are classified as myoepithelial-like cells (107) (Fig. 2).

When confluent cultures of the cuboidal epithelial cell lines become densely packed, they form small, dark, polygonal cells with small vacuoles or droplets at their peripheries (Fig. 1b). These have been termed droplet cells (16). Conversion of an initially homogeneous culture of cuboidal cells to droplet cells can be accelerated with agents that stimulate differentiation of Friend erythroleukemic cells (43), notably DMSO (16), PGE_1 (108), or retinoic acid (117) in the presence of Prl, E, HC, and I. These droplet-cell cultures contain domes (Fig. 1b) and synthesize increased amounts (20-40 fold) of immunoreactive casein, which has been authenticated as the p42ka component present in rat milk by peptide-mapping techniques (146). They also demonstrate an increase in staining with peanut lectin after treatment with neuraminidase (80). Based on the above criteria these cultures have been adjudged to be related to alveolar cells, although since the amount of casein synthesized is only 1-2% of that found in lactating mammary gland explants (146), they are classified as alveolar-like cells

(107) (Fig. 2). The cuboidal epithelial cell lines can therefore give rise to the major differentiated cell types of the mammary gland. They can also form branched, immature duct-like structures (15,84) with a correctly organized basement membrane (90), and budded, sac-like structures superficially similar to alveoli (88) on floating collagen gels. They or closely-related cells are therefore possible candidates for stem cells for the mammary gland and its mixed (epithelial and myoepithelial) tumors.

IV. IDENTIFICATION OF DISCRETE DIFFERENTIATION STAGES OF RAMA 25 IN VITRO AND THEIR EFFECTS IN VIVO

Differentiation of the benign epithelial cell line Rama 25 along both pathways occurs in discrete stages. Time-lapse cinematographic analysis along the droplet cell/doming, alveolar-like pathway indicates that a linear sequence of morphological stages exists, and that they are triggered, directly or indirectly, by the pumping action of the Na^+/K^+ ATPhase (93) in the order: cuboidal →grey →dark →dark-droplet →doming cells (96) (Fig. 3). The dark and dark-droplet cell stages are associated with increased peanut lectin-binding ability after treatment of the cells with neuraminidase, and the doming stage with the production of small amounts of casein (96). These results suggest that the dark±droplet cells correspond more to the epithelial cells in ABs which also require neuraminidase treatment before this lectin will bind, than to the extensive casein-producing and peanut lectin-binding alveolar cells in vivo. Each of these in vitro stages is associated with the increased synthesis of a novel polypeptide; a variant epithelial cell line derived from Rama 25, Rama 259 that has a truncated pathway produces only grey and dark cells and their associated polypeptides (94) (Fig. 3). DMSO or retinoic acid in the presence of Prl, E, HC, and I accelerates the overall pathway predominantly by increasing the rate of droplet cell formation, and this can be reversed by removing the inducers (96). Similar morphological stages have been observed in normal primary

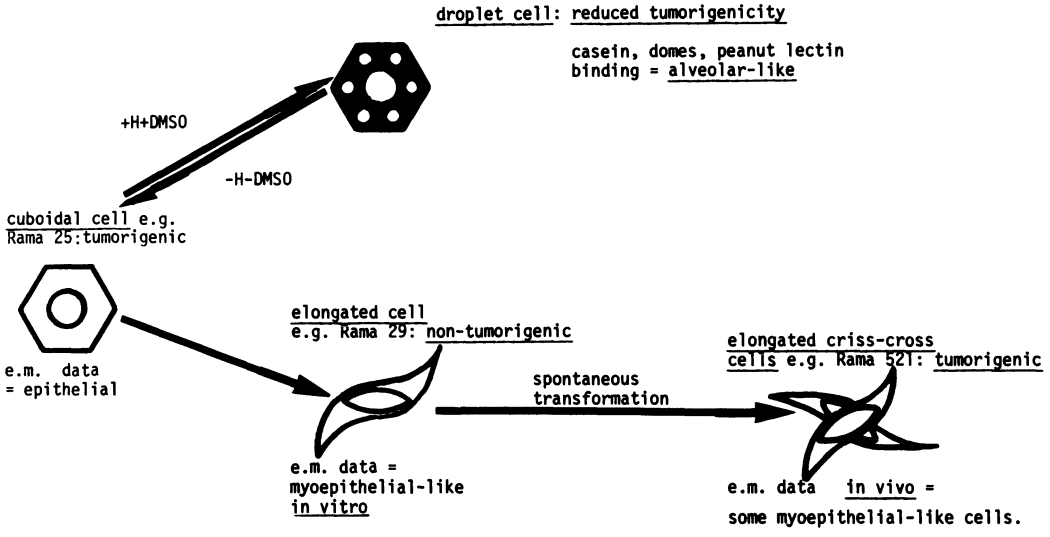

Figure 2. Diagram of the intercellular conversions of Rama 25 epithelial cells: Rama 25 cuboidal epithelial cells from a benign tumor can differentiate to droplet cell/doming-alveolar-like cells with mammatrophic hormones Prl, E, HC, I(H) and dimethylsulfoxide (DMSO) or to elongated myoepithelial-like cells (e.g., Rama 29). Spontaneous transformation of the elongated, myoepithelial-like cells can occur yielding morphologically transformed cells (e.g., Rama 521). The reversible (⇄) and irreversible (→) nature of the steps is also shown.

cultures[5] and cell lines (e.g., Rama 704) of rat mammary epithelial cells showing that they are not unique to epithelial cell lines from benign rat mammary tumors.

Clonal cell lines that are intermediate in morphology between Rama 25 epithelial cells and elongated, myoepithelial-like cells have been isolated, and they form a morphological series in the order: Rama 25 cuboidal cells, Rama 25-I2, Rama 25-I1, Rama 25-I4, and elongated cells e.g., Rama 29 (Fig. 4). This same order is maintained for increasing frequency of conversion to elongated cells, increased binding of antisera to laminin, vimentin, Thy-1, increased binding of GS-1 and PWM lectins[4], increasing abundance of 7 polypeptides characteristic of elongated, myoepithelial-like cells, and increasing myoepithelial ultrastructural features (Fig. 4). Similarly the same order is maintained for decreasing conversion to cuboidal cells, decreased binding of antisera to MFGM, decreasing abundance of 4 polypeptides characteristic of cuboidal epithelial cells, and decreasing epithelial ultrastructure (118). The most abundant protein characteristic of elongated, myopithelial-like cells, p9ka (8) increases mainly in Rama 25-I1 cells, laminin in Rama 25-I1 and Rama 25-I4 cells, and Thy-1 in Rama 25-I4 and elongated cells (118). That these morphological intermediates are also intermediates along the elongated, myoepithelial-like pathway is suggested by the results of pretreatment of Rama 25 epithelial cells with the microtubule-disrupting agent colchicine. This treatment progressively increases the percentage of elongated cell colonies after removal of the drug (95). When followed by time-lapse cinematography, this conversion process is seen to occur by sequential morphological stages, similar to those of the cell lines above. The last stage is the only irreversible one (Fig. 4). Increases in binding to Thy-1 antiserum and the changes in most of the 11 characteristic polypeptides after such treatment are also consistent with this model (95). However, there appears to be no consistent molecular alteration producing these effects: increases in p9ka (10) and Thy-1[6] are produced by increasing the level of cytoplasmic mRNA, while the increase in type IV collagen is produced mainly by a decrease in its degradation rate (148). These cellular intermediates have also been identified by immunocytochemical and morphological criteria in primary cultures of normal mammary glands (Section III), and several of the polypeptides, notably p9ka, have been found in different elongated myoepithelial-like cells and cell lines from normal rat mammary glands (9). These results suggest that both intermediate cells and p9ka

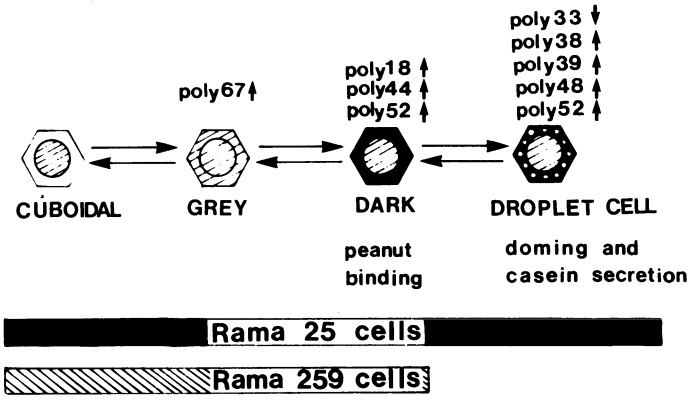

Figure 3. Diagram of the differentiation of Rama 25 epithelial cells along an alveolar-like pathway: The linear sequence of different morphological stages of Rama 25 epithelial cells is shown after treatment with DMSO and Prl, E, HC, I together with the corresponding changes in novel polypeptides. The truncated pathway of the variant Rama 259 cells is also shown. The polypeptide numbers are as described in Paterson and Rudland (94).

protein are therefore not unique to a single cell line from a benign rat mammary tumor.

When grown on floating collagen gels, the intermediate cell line Rama 25-I2 forms more mature, duct-like structures than the parent Rama 25, and these possess both epithelial and myoepithelial-like cells organized correctly within the tubules (118). The other two intermediate cell lines fail to form such structures, but yield instead giant, striated cells which have ultrastructural characteristics of skeletal muscle and which produce striated muscle-specific myoglobin (109) (Fig. 4). This result has been confirmed when the cells are grown in nude mice as tumors, and when similar cells from benign neoplastic Rama 37 (109) or normal Rama 704 (86) epithelial cell lines are grown on gels in vitro or, for Rama 37-derived cells in syngeneic rats in vivo. Ultrastructural and immunocytochemical analysis of cells on gels suggests that Rama 25-I1 and Rama 25-I4 resemble the intermediate/cap cells of TEBS and ABs respectively (118). Since the intermediate cell lines can give rise to epithelial- and myopithelial-like cells as well as to well-differentiated mesenchymal elements such as skeletal muscle (109), they are better candidates than the closely-related epithelial cell lines such as Rama 25 for both normal stem cells in mammary TEBs in vivo and for the neoplastic stem cells in the mixed tumors of glandular origin (56).

The epithelial cell lines from benign tumors, e.g., Rama 25 and Rama 37 yield progressively growing tumors within 20-40 days in nude mice (111) or syngeneic rats (34) respectively, while those from normal mammary glands, e.g., Rama 704 fail to yield tumors in syngeneic animals (86). The tumors consist of glandular areas and spindle-cell areas of varying myoepithelial phenotypes. The fact that most of the neoplastic mammary-growth-patterns can be identified within the above epithelial areas suggests that different

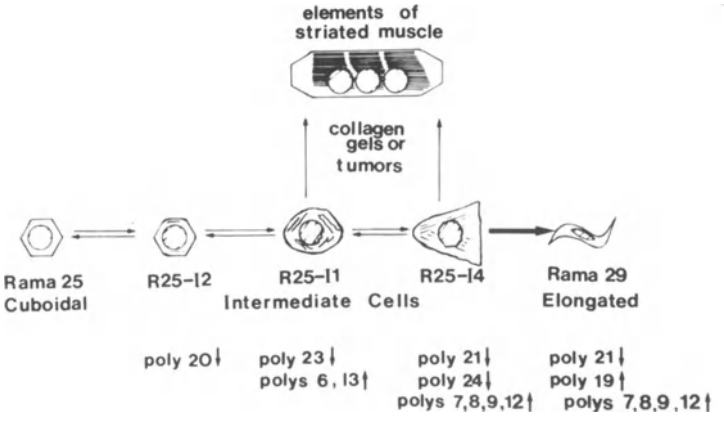

Figure 4. Diagram of the differentiation of Rama 25 epithelial cells along a myoepithelial-like pathway: The cell lines intermediate in morphology between Rama 25 cuboidal, epithelial and elongated, myoepithelial-like cells (e.g. Rama 29) are designated by R25-I (118). They are also thought to resemble similar cells in the direct conversion of Rama 25 cuboidal cells to elongated cells (95). Only the last stage is irreversible. The polypeptide changes associated with each stage are also shown; the numbers correspond to those reported previously (95,118).

histological forms of mammary tumor can be generated by a single, and/or closely-related type of mammary epithelial cell (111). Complete differentiation of the neoplastic epithelial cell line Rama 25 along either pathway often reduces its tumorigenic potential in nude mice. Thus most of the elongated, myoepithelial-like cell lines from Rama 25 (e.g., Rama 29) fail to induce tumors in nude mice, but spontaneous transformants from Rama 29 yield benign tumors composed of spindle cells of varying degrees of myoepithelial characteristics (111) (Fig. 2). This suggests that subsequent, and perhaps different transformation events are required to generate the spindle-cell tumor, although this may not necessarily be the case in all systems (34,52). Induction of differentiation of Rama 25 cells to droplet, alveolar-like cells by DMSO, PGE_1 (108), or retinoids (117) with Prl, E, HC, and I causes a large reduction in rates of DNA synthesis in vitro and in rates of tumor-formation in nude mice (Fig. 2). Moreover, the variant of Rama 25, Rama 259 which fails to complete the later stages of the droplet cell-alveolar-like pathway (94) also fails to reduce its DNA synthetic rate and tumor-forming-ability under the same conditions. Thus the effect of mammatrophic hormones, pregnancy (23,77), and agents such as vitamin A and the retinoids (132) in protecting the rat mammary gland from carcinogenic insult may be due to their ability to induce differentiation. This ability may be exerted either at an early stage (73) converting normal stem/cap cells in TEBs (122) to less susceptible, slower-growing differentiated cells (96,121), or at a later stage in the carcinogenic process (140) converting tumor stem cells (16,118) to differentiated cells having lower tumorigenic potential.

V. CHARACTERISTICS OF CARCINOMA CELLS FROM METASTASIZING RAT MAMMARY TUMORS IN CULTURE

Collagenase digestion of transplantable, metastasizing rat mammary tumors has not been very successful in obtaining cultured carcinoma cells, an its use has been limited largely to the weakly-metastatic tumors such as TR2CL (157,159). Thus other strategies have been employed. These include culturing cells from ascitic versions of the transplantable tumor, e.g., TMT-081 (44) and SMT-2A (110) or by selecting the most metastatic variants in vitro from cultured metastases (79), usually with other cell types present as feeders. The ascitic and solid forms of the tumor and the cell lines developed from them give the same histological appearance and patterns of spread in the syngeneic rat, eliminating the possibility of major changes being due to the cells growing as an ascites tumor or in culture (33,44,64,-110). For our cell lines and strains (e.g., Rama 600, Rama 800, and Rama 900) isolated from tumors of increasing metastatic potential (TR2CL, TMT-081, and SMT-2A respectively) (Table 1), there is a corresponding increase in the cells' ability to grow in suspension as loosely-adherent aggregates, a decrease in growth rate, and a greater dependency for support on feeder cells (33,110,159). In fact the most metastatic SMT-2A ascites tumor cells grow only in culture with a feeder layer of normal mesothelial cells from the same ascites fluid (110). There is also an increasingly anaplastic and heterogeneous cellular appearance, as manifested by a gradual loss of normal epithelial staining with antisera to MFGM and human keratin, a gradual loss of epithelial ultrastructure, and a gradual increase in membranous blebbing and shedding (33,110,159).

In contrast to cultured cells of normal and benign rat mammary glands, those from metastasizing rat mammary carcinomas yield no myoepithelial cells, and basement membranes are usually absent (33,110,159). The closest to the benign tumor cell lines in these respects is the weakly-metastasizing cell strain TR2CL which is composed not only of epithelial cells of the Rama 600 type (Table 1) but also of undifferentiated elongated cells. These elongated cells are similar in staining pattern and ultrastructure to the undifferentiated elongated cells generated by recloning normal myoepithelial-like cell lines Rama 401 and Rama 704E (61), in that they retain com-

ponents of the basement membrane but have lost most of the microfilamental systems. They do not metastasize on their own, however, and their presence in the parental strain may serve to enhance the growth rate of the Rama 600 cell type (159), similar to the effect of the myoepithelial-like cells on the growth of normal and benign epithelial cells in culture. In addition cloned epithelial cell lines from TR2CL, TMT-081 and SMT-2A metastasizing tumors fail to yield elongated, myoepithelial-like cells in culture (33,-110,159), even after treatment with colchicine (95). This lack of production of myoepithelial cells has also been observed in other metastasizing epitheloid cell lines developed from a chemically induced rat mammary tumor (79), although there may be exceptions for some MMTV-linked mouse systems (143). Even in the rat, the tumors of the weakly-metastasizing Rama 600 cell line also contain a more elongated-cell component, but if this represents differentiation towards a myoepithelial cell it is of a partial and incomplete nature, and may merely reflect a vestige of the complete pathway (159). This partial differentiation towards a myopithelial cell, however, may still be sufficient to generate the fragmented basement membrane which is sometimes seen in the Rama 600 tumors <u>in vivo</u>. Similarly no casein-producing or alveolar-like cells have been detected in any of our metastasizing cell lines under the same hormonal conditions that generate such cells in the benign cell lines (33,110,159). However, Rama 600 cells yield grey and dark cells under these conditions[5], possibly because of their retention of some vestige of the differentiation pathway to alveolar-like cells. Perhaps neoplastic transformation of the epithelial/intermediate cells results in a truncation of both differentiation pathways, and this truncation occurs earlier with increasing metastatic potential.

Most of the usual properties associated with viral transformation of cultured fibroblasts (115,116) are not applicable in our series of rat mammary cell lines of increasing metastatic potential (e.g., reduced, not elevated growth rates; reduced, not elevated ability to grow in semi-solid medium; reduced, not increased ability for autonomous growth (33,110,159)). Thus clonal growth of the moderately-metastatic cell line Rama 800, which metastasizes predominantly to lung and lymph nodes is stimulated substantially by co-cultivation with fragments of either of these tissues, but they are without effect on the non-metastasizing cell lines (31). Properties which do change radically when these cells become highly metastatic are probably those involved with increasing instability (81,83,98) or plasticity (103) of genetic expression. Thus, in addition to the loss of some or all of both differentiation pathways of the normal mammary epithelial "stem" cell, there is a large increase in chromosomal number up to near tetraploid (33,110), and a large increase in resistance to the cytotoxic effects of drugs such as ouabain and trifluorothymidine[7] (31). Similar but more heterogeneous results in another rat mammary metastasizing system have been reported for resistance to cytotoxic killing by adriamycin, 5-fluorodeoxyuridine (152), hyperthermia (141), and gamma irradiation (151). One final example of the ability of the highly-metastatic mammary carcinoma cells to adapt to their environment is the growth of the Rama 900 cells in nude mice. Although the non-metastasizing Rama 25 (111) and the weakly-metastasizing Rama 600 cells (159) grow readily in nude mice, cell strains of the moderately-metastasizing TMT-081 (44) and strongly-metastasizing Rama 900 (110) grow poorly from subcutaneous sites, and are eventually rejected, like their parental tumors. However, if Rama 900 cells are inoculated intraperitoneally, about half the nude mice develop ascites tumors, and these mainly contain mouse mesothelial feeder cells and Rama 900 tumor cells. The mouse ascites-derived tumor cells can now produce tumors and metastases in nude mice, but not in syngeneic rats (110). Results in this section, therefore, tend to reduce the importance of simple molecular explanations that are based solely on autostimulation of cell growth (116) for the metastatic properties of our rat mammary systems, and tend to emphasize the increasing

capability of the metastatic cell to adapt and take advantage of the (growth) environment of its host (104).

VI. CELLULAR STRUCTURE AND DEVELOPMENT OF THE HUMAN MAMMARY GLAND AND ITS TUMORS

Compared with the development of the rodent mammary gland, little is known about that of the human breast. Presumably the same principles apply, with the possible exception of the timing of those events that depend on hormonal changes which may themselves occur at different developmental stages in the two species. Most mammary ducts in humans are reported to terminate in TDLUs (92) which are probably closest in appearance to the ABs, and not the TEBs of rats (85). This finding probably arises because most of the analyses in humans have been conducted on tissues from mature, parous women, rather than from prepubertal girls, where the incidence of putative TEBs may be expected to be at a maximum. The TDLUs, however, still contain the highest mitotic activity in the gland (40,123), and there is some evidence that structures more akin to TEBs are seen in adolescent girls (123).

Ultrastructural and immunocytochemical analyses of the TDLUs tend to confirm their analogy with AB-like structures of rodents. Thus some of the more immature TDLU structures contain a heterogeneous collection of cells which include epithelial cells, myoepithelial cells, and peripheral cells of a more intermediate ultrastructure (129,135) and staining pattern with antisera to actin, myosin and keratin monoclonal LP34[3]. Although these peripheral cells show gradations in ultrastructure and staining patterns to the myeopithelial cells of the subtending terminal duct, in these respects they are still closer to the myoepithelial cell[3] (129,135), and as such they resemble the intermediate cells of ABs and not TEBs of rat mammary glands (85). The TDLUs, like the ABs of rodents eventually give rise to alveoli under the correct hormonal stimulation (13,124), and some monoclonal antibodies [LICR-LON-M18 (41), HMFG-1 (138)] to EMA (127), the equivalent of rat MFGM, as well as peanut lectin (80) preferentially bind to alveolar cells of the lactating gland without the necessity for treatment with neuraminidase. Thus some evidence exists in the TDLUs for cells that are morphologically and perhaps functionally (40) intermediate between epithelial and myoepithelial cells are for desialylating step(s) along a potential alveolar cell pathway. Definite proof, however, will have to await a morphological analysis of the terminal ductal structures of prepubertal and pregnant females.

Mammary dysplasia or fibrocystic disease can occur throughout the mammary system, but the specific phenotypic feature which best correlates with increased risk of neoplastic disease is the presence of atypical epithelial proliferations that arise in terminal ductal structures (153). These various lesions may be interpreted as either giant hyperplastic AB-like structures or as hyperplastic groups of end buds, and probably represent a spectrum from benign lesions to carcinoma <u>in situ</u> (154), the direct precursor to mammary carcinoma, although there are contrary views (6). Thus both benign and malignant human mammary neoplasias are thought to arise in terminal ductal structures, and in this respect are more similar to those of the rat than the MMTV-infected mouse (25).

Analysis of the cell types present in different neoplastic states using ultrastructural (1,6,45,71) and immunohistochemical staining techniques (2,11,18,72) for components of the microfilaments and basement membranes, in particular for myosin, human keratin, laminin and type IV collagen (49) has shown that some myoepithelial cells and basement membranes are always present in the major categories of benign breast disease (epitheliosis, adenosis and fibroadenoma). Moreover, mixtures of epithelial, myoepithelial and intermediate cell types (92) occur in epitheliosis, while myoepithelial cells form a major cellular component of sclerosing adenosis (6). However, in

infiltrating ductal carcinomas, the myoepithelial cells are almost entirely absent, and fragmented basement membranes are retained by only a small proportion, usually of the Grade I category (49). The premalignant carcinoma in situ retains the normal glandular myoepithelial cells and basement membrane surrounding the carcinoma cells, and often both are concurrently lost in the production of infiltrating carcinoma (49). These immunocytochemical results have been confirmed recently using monoclonal antibodies to novel antigens on the surface of myoepithelial cells, notably monoclonal LICR-LON-23.10 which recognizes basal cells of skin and blood vessels (47) and monoclonals to CALLA, the acute lymphoblastic leukemia antigen (48). Certain benign lesions also contain the well-differentiated mesenchymal elements of skeletal muscle, cartilage and/or bone (6,56) and others can be induced to yield alveolar-like cells by a suitable hormonal environment (7,134); both findings are extremely rare in infiltrating ductal carcinomas. Thus the broad pattern of the parenchymal cell types and occurrence of basement membranes in human breast neoplasms is therefore similar to that found in the corresponding rat mammary tumors of varying metastatic potential.

VII. CULTURE OF NORMAL HUMAN MAMMARY GLANDS AND MALIGNANT TUMORS

Human mammary glands and benign tumors have been digested with collagenase and cultured in a way virtually identical to that of the corresponding rat tissues (35,53,66,133). The organoids so produced are fully capable of yielding all the major cell types in the nude mouse after appropriate hormonal stimulation (50), and of producing very similar cellular morphologies and structures to those of the corresponding rat mammary tissues on plastic (35,133) and collagen gels (42,128,161). They therefore consist of epithelial cells and basal, elongated myoepithelial-like cells (42,128). The former stain with antisera (35) and monoclonal antibodies (36) to EMA, antisera to human keratin, keratin monoclonal LE61, and peanut lectin after neuraminidase treatment[8], and some of the latter stain with antisera to actin, human keratin, laminin, type IV collagen, vimentin, fibronectin, keratin monoclonal LP34, monoclonal LICR-LON-23.10 and monoclonals to CALLA[8], as in the human mammary gland (41,47,48,49,80,127,138). They also contain the larger, more open transitional cells (36,136) which are intermediate between epithelial and myoepithelial staining patterns[8]. Unlike the rat cultures, however, many of the cells lose their nucleus and desquamate into the medium (36,133). Since many of the different epithelial morphologies can rapidly and reversibly interconvert[8] (128), this system is probably unsuitable for the clonal separation of potential antigenic markers of different cell types (37). However, pulse-chase experiments with DNA precursors in organ cultures suggest at least that the myoepithelial cells originate from cells within the epithelial population (62).

Immortalization of primary cultures of human mammary epithelial cells by transformation with SV40 (21) yields ring clones (e.g., SVE3) and subsequent single-cell-clones (e.g., Huma 7) of cuboidal, epithelial cells (Table 1) which also produce elongated cells, but at less than a tenth of the frequency of the corresponding rat cells[9]. The epithelial cell lines produce all the cellular morphologies and structures on collagen gels and in tumors in nude mice that have been observed in the non-malignant rat epithelial cell lines, but in addition retain the desquamating properties of the primary cultures[9]. Ultrastructure, immunocytochemical staining and concanavalin A-reactive glycoproteins confirms the identity of the epithelial cell lines with that of the majority of the epithelial cells of cultured organoids[9]. In addition, some of the elongated cell lines on collagen gels and in tumors have similar staining patterns to the elongated, myoepithelial-like cells of primary cultures, and produce more basement membrane proteins and monoclonal 23.10-reactive glycoproteins than their parental epithelial cells[9]. The reduced production of different cellular morphologies and their differentiated characteristics by the original SV40-transformed

human mammary epithelial cell lines may be due to their different source of human milks (139), their selection for growth in soft agar (21), and/or their being hypotetraploid (102) compared with near diploid[9]. Results with our Huma cell lines, however, suggest that epithelial stem cell systems that can give rise to elongated, myoepithelial-like cells and droplet cell/doming, possibly alveolar-like cells may also exist in human mammary glands, in a similar fashion to those of the rat.

Like most of the metastasizing rat mammary tumors, the culture of human mammary carcinomas has been extremely difficult (17,53,66), although a few epithelial cell lines have been established (51,69,70,82). Routine digestion of over 100 primary ductal carcinomas with collagenase by slight modifications (54) of the methods used for the rat mammary benign tumors yields loosely-adherent (>72 hr), malignant-looking cell clusters and fast-adherent (<48 hr), less malignant-looking epithelium on collagen gels (54,-112). Metastases in axillary lymph nodes and pleural effusions yield only the loosely-adherent clusters, while normal mammary glands and fibroadenomas yield only fast-adherent colonies (54,112). These results suggest that the fast-growing, adherent sheets of epithelium from primary ductal carcinomas (130) do not usually represent the most-metastasizing cell populations, but, as in the rat the latter are probably best represented by the slow-growing, loosely-adherent aggregates (19,68,70,155) which normally die out on repeated transfer in vitro (39,82,91). Those rare instances where epithelial cells emerge as permanently-growing cell strains usually involve their passage through a period of crisis, which is often characterized by a switch from very slow-growing, loosely-adherent cell clusters to more rapidly-growing, adherent cell sheets (38,69,125). Continued passage of one preparation of loosely-adherent cell clusters has yielded a continuously growing cell strain, Ca2-83 which has not yet undergone a period of crisis and still grows with doubling times of 10-14 days (112) (Table 1). These cells are a good representative of malignant cells found in many secondary mammary tumors and can reproduce the fat-containing pleomorphic variants found in the original primary tumor and recurrent metastases of the patient (112). Moreover, the properties of Ca2-83 are also largely in keeping with those of the more metastatic rat mammary cell lines and with what is known of human mammary carcinoma cells (131), before their period of crisis in vitro, and their subsequent transformation into established cell lines (112).

Since the fast-adherent sheets of epithelium from cultures of different human mammary tissues nearly always contain elongated, myoepithelial-like cells, while the loosely-adherent clusters do not, myoepithelial-like cells are usually found in cultures of fibroadenomas and uninvolved peritumoral tissue adjacent to carcinoma (112). However, they are almost invariably missing from cultures of metastases, the malignant cell strains Ca2-83 (112) and PMC-42 (76,155), and the loosely-adherent aggregates of malignant cells of ductal carcinomas (112). Moreover, Ca2-83 cells fail to synthesize at least one basement membrane component, laminin (112) and fail to produce casein and alveolar-like cells in culture with DMSO and the mammatrophic hormones[5]. These results are consistent with both the pathology of benign and malignant human breast disease and the findings from culturing the equivalent rat mammary tumors. Whether loss of the basement membrane in most human mammary carcinomas is caused by a failure in its synthesis (49), as with Ca2-83, or by its enzymatic destruction (12) is unknown. The presence of abnormal organoidal structures of epithelial and myoepithelial-like cells in some of the primary ductal carcinomas and their absence in metastatic tumors (112) probably reflect progression of the primary tumor from a less malignant to a more malignant phase, and thus a larger proportion of organoidal structures in the primary may result in the fragmented basement membranes seen in some Grade I ductal carcinomas (49). These findings are also more likely to be consistent with a mutational event occurring in an epithelial stem cell with gradual truncation of its differentiation pathways

during the progressive phase of the disease, than with mutational events occurring simultaneously in the epithelial stem cell and an adjacent nondifferentiating epithelial cell which ultimately gives rise to the malignancy (138).

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STRUCTURAL COMPONENTS AS MARKERS OF DIFFERENTIATION AND NEOPLASTIC PROGRESSION IN MAMMARY EPITHELIAL CELLS

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I. INTRODUCTION

One of the primary aims in breast cancer research has been the development of molecular markers that can a) distinguish among the different cell types (epithelial, myoepithelial, stem cell, stromal, etc.) present in normal and abnormal mammary tissues and in cultures derived from these tissues, and b) identify a cell as normal, hyperplastic/preneoplastic, or malignant. Components of the cell surface and the cytoskeleton are especially promising candidates for such markers. A variety of surface antigens, usually defined by monoclonal antibodies, have now been described and were the subject of a number of recent reviews (1-5). The focus of the present chapter will therefore be on the cytoskeleton and in particular on intermediate filaments and their constituent proteins.

Several general reviews on intermediate filaments and on keratins have been published over the past few years and provide excellent introductions to these cytoskeletal elements (6-11). Of the five classes of intermediate filament proteins, only keratins and, in certain circumstances, vimentin have been found in mammary epithelial cells (6-11). The purpose of our discussion will be to summarize and evaluate the information currently available on the expression of these components in mammary epithelium and point out some of the advantages and problems associated with their use as markers.

Both epithelial and myoepithelial cells of the mammary gland contain filaments composed of keratins (12-16). As stromal cells do not have this

type of protein, the use of antibodies against keratins in immunocytochemistry has provided an easy, reliable means of characterizing a cell as epithelial or non-epithelial in most in vivo and in vitro situations (12-20). In certain circumstances, for example with some long-term cell lines, however, characterization of cells in this way becomes more difficult. This problem will be addressed later.

Like other epithelia, the mammary gland has a characteristic subset of the total of 19-20 keratins present in most mammals (8). The subset of keratins found in mammary epithelium of the three species, human, cow, and mouse, that have been analyzed to date is not the same either quantitatively or qualitatively (Table 1) (8,21,22). Eight keratins are present in the normal human gland, whereas cow and mouse mammary epithelium each have four keratins (8,21,22). Only a M_r 50,000 (50K) keratin with an isoelectric point of 5.2-5.3 appears to be common to all three species. Human and cow also have 58K and 58.5K keratins, respectively, with similar electrophoretic characteristics, and human and mouse have 52.5K and 55K keratins, respectively, and a 40K keratin that are similar. The differences, however, underscore the need for caution in making generalizations or extrapolations between species. Other complications arise because the epithelium can gain and/or lose particular keratins in benign and malignant disorders (8,23), growth in culture (8,21,22,23), growth on different types of substrata (24,25), and possibly in other conditions as well. Additional problems, associated mainly with monoclonal antibodies, involve "masking" of keratin epitopes (26). These factors can affect the reactivity of antikeratin antibodies with mammary epithelium and will be discussed where appropriate.

II. TECHNICAL CONSIDERATIONS REGARDING ANTIKERATIN ANTIBODIES

Several technical considerations should be mentioned regarding the use of antikeratin antibodies (monoclonal or polyclonal) in immunocytochemistry. Many antikeratin antibodies recognize formalin-fixed material, whether cultured cells or tissue sections, either poorly or not at all (13,15,19,27,

Table 1

Keratins Present in Normal Mammary Epithelium

	Keratins detected by 2D-PAGE	
Species	Basic	Acidic
Human[a]	58K(7.4)[b], [5][d]; 54K(6.0), [7]; 52.5K(6.1), [8]	50K(5.3), [14]; 50K(4.9)[c], [15]; 46K(5.1), [17]; 45K(5.7)[c], [18]; 40K(5.2), [19]
Cow[e]	59K(7-8), 58.5K(7-8)	53K(5.4), 50K(5.3)
Mouse[f]	57K(7.1), 55K(6.3)	50K(5.2), 40K(5.4)

[a]Data from (8).
[b]Numbers in parentheses indicate apparent isoelectric points.
[c]These keratins are either minor components or appear inconsistently in the tissue.
[d]Numbers in brackets refer to the keratin number designated in Moll's catalog of human keratins (8).
[e]Data from (21).
[f]Data from (22).

29). For this reason, the most commonly used fixatives for studies on keratins are alcohols, acetone, or a combination of the two. We routinely fix cultured cells in absolute methanol followed by absolute acetone with immediate rehydration in buffer (no air drying). Cultured cells can be kept in methanol at -20°C for as long as a month with good preservation of antigenicity. Frozen tissue sections are fixed in acetone. For paraffin embedding, pieces of tissue are fixed 24-48 hrs at 4°C in absolute ethanol, dehydrated, embedded in paraffin, and sectioned using the lowest possible temperature for each step (15). We strongly recommend testing any antikeratin antibody on specimens fixed by one of these methods along with formalin-fixed samples. Regardless of the fixation method, pretreatment of paraffin-embedded material with a protease such as Pronase (e.g., 0.1 mg/ml for 15 min at room temp) after deparaffinization is usually necessary to obtain optimal staining (19,27,29).

An important related point is that the validity of conclusions based on results with an antikeratin antibody/antiserum is dependent on the characteristics of the reagent. Keratins have a high degree of homology in primary structure and most antikeratin antibodies react within and between species with multiple keratins (8-11). However, no single antikeratin antibody or antiserum has detected all keratins in any species. Moreover, determining that an antibody reagent reacts with epidermal keratins in immunodiffusion or immunoblot assays has limited meaning when the tissue under investigation is the mammary epithelium, which may have only two or three keratins in common with epidermis. For example, human epidermis expresses keratins No. 1, 2, 5, 10, 11, 14, and 15, whereas human breast contains keratins No. 5, 7, 8, 14, 15, 17, 18, and 19 (the numbering scheme for human keratins is that of Moll et al., ref. no. 8). Thus, the two tissues share only keratins 5, 14, and 15, and keratin 14 is present in breast as a minor and/or variable component (8). Showing that an antibody recognizes keratins 1, 2, 10, or 11 in epidermis is not an indication of which keratin(s) it detects in the mammary gland. Even an antibody or antiserum against epidermal keratins that is cross-reactive with many keratins may not recognize mammary keratins. This problem provides one explanation for the lack of reactivity of some polyclonal and especially monoclonal antikeratin antibodies with rodent or human mammary epithelial cells (B. Asch and H. Asch, unpublished data). It is therefore important to know at least that an antibody detects one or more mammary keratins of the animal under study, and preferably, which mammary keratins are recognized.

By extension, if a mammary-derived cell does not react with a particular antikeratin antibody or antiserum, one cannot conclude solely on that basis that the cell lacks keratins. This point becomes especially important when dealing with tumors. In the only electrophoretic analysis of keratins in human breast cancers (8,30), all tumors had lost expression of keratins 5 and 15. Although only a few carcinomas were examined (8,30), the implications are of considerable consequence. For example, an antiserum against epidermal keratins that preferentially stained myoepithelial cells in normal human breast was found to have little or no reaction with human breast cancers (18). The authors' conclusion that the tumors have very few myoepithelial cells would be tenable if, for instance, the antiserum recognized only keratins 5 and 15 in mammary epithelium and if these keratins were restricted to myoepithelial cells. The antiserum used in the study was not characterized in this regard (18), however. Alternatively, myoepithelial cells may be present in the tumors but no longer express keratins 5 and 15. The cell type specificity of the latter keratins has not been established. In a related situation, we found that a commercial antikeratin antiserum strongly stained the luminal cells in normal human breast but gave no reaction with myoepithelium (B. Asch and H. Asch, unpublished results). When tested on several infiltrating carcinomas, the antiserum produced little or no staining. These data do not distinguish between the possibility that the

tumors are derived from the myoepithelium and the possibility that the keratin(s) in the epithelial cells recognized by the antiserum has been lost during the development of malignancy. Immunoblot experiments to clarify this point are in progress. The actual status of myoepithelial cells in human breast cancers will require further investigation.

III. EXPRESSION OF KERATINS IN NORMAL AND ABNORMAL MOUSE MAMMARY EPITHELIUM

In a series of studies to assess keratins as molecular markers of differentiation and malignant progression in mouse mammary epithelium (22,23,-26,31), we first examined the cytoskeletal composition of the adult female mammary gland during the four stages of the mammary developmental cycle represented in virgin, pregnant, lactating, and involuting animals (22). The polypeptides in cytoskeletal extracts from BALB/c mouse mammary tissues were analyzed by one- and two-dimensional polyacrylamide gel electrophoresis (1-D and 2-D PAGE) combined with immunoblots using polyclonal and monoclonal antikeratin antibodies. Two acidic polypeptides of 50K and 40K, along with two relatively basic components of 57K and 55K were identified as keratins. Very few differences were found in the set of polypeptides expressed by the gland in the various developmental stages, indicating that the cytoskeletal composition of the epithelium is not grossly affected *in vivo* by hormonal shifts, periods of minimal and maximal cell growth, or differentiated function. The similarity between extracts of mammary tissue from virgins, which have only ducts in the BALB/c strain, and pregnant and lactating mice, which have ducts and alveoli, implied that ductal and alveolar cells express the same set of keratins.

The keratins expressed during the entire spectrum of normal physiological states of the adult female gland were then compared with those expressed in mammary hyperplasias and tumors of hormonal, viral, and chemical etiologies (23). The most conspicuous feature on 2-D PAGE of the cytoskeletal polypeptide profiles from the different lesions was the similarity to each other and to that of normal tissue. However, important differences were also apparent. In particular, the 55K keratin was greatly increased and a 46K polypeptide that has now been identified as a keratin (R. Scott, B. Asch, and H. Asch, unpublished results) was prominent in one hyperplastic line and all mammary adenocarcinomas. As the 46K keratin has not been detected in any of the normal adult mammary tissues, it may provide a marker of neoplastic progression *in vivo*. We also discovered that abnormal differentiation in the hyperplasias and tumors, i.e. squamous metaplasia with keratinization, was associated with a marked increase in the 57K keratin (23).

The effects of growth *in vitro* on the set of keratins expressed by mouse mammary epithelium were also analyzed (22,23). Marked differences between the *in vivo* and *in vitro* patterns of keratins in normal cells were observed, including a substantial increase in the 55K keratin and the appearance of the 46K keratin (22). Other than the 55K keratin, most of the neutral-to-basic cytoskeletal polypeptides, including the 57K keratin, were reduced or absent compared to *in vivo*. Thus, normal cells in primary culture on plastic both gained and lost certain keratins, demonstrating that like several other epithelia, expression of keratins in mammary cells can be altered by environmental conditions. In contrast, the polypeptide patterns of cytoskeletal extracts from primary cultures of the preneoplastic and tumor cells were virtually the same as those of the corresponding tissues and that of normal cells in culture. These results show that although major differences exist in the set of keratins expressed by the normal and abnormal mammary cells *in vivo*, these cells have very few, and in some cases no differences in their keratin composition when they are cultured on plastic.

To determine which cell type(s), epithelial, myoepithelial, or both, expressed each keratin, we used antibody preparations specific for a limited set of keratins or only one keratin in immunocytochemistry on mammary tissues and cultured cells. An antiserum against the 50K keratin reacted strongly with myoepithelial cells of normal and abnormal mammary tissue in vivo and in vitro while producing a negligible or no reaction with epithelial cells (31,32), indicating that this component is characteristic of myoepithelial cells. An antiserum against the human 40K keratin (33) was found to be monospecific for the 40K keratin in immunoblots of cytoskeletal extracts of mouse mammary tissue, and in immunocytochemistry it stained all normal epithelial and myoepithelial cells in vivo and in vitro (S. Pocchiari, B. Asch, and H. Asch, manuscript in preparation). This antiserum produced a variable reaction with malignant tissue and cultured cells, consistent with electrophoretic data showing that the 40K keratin is present irregularly in these samples. These results contrast with the cell distribution in the human gland of the 40K keratin, which is limited to luminal epithelium of normal tissue but is found in almost all tumor cells (34). On the basis of immunocytochemistry with a monoclonal antibody, AE4 (26), and a monospecific antiserum (H. Asch and B. Asch, unpublished data), the 55K keratin is present in all normal and abnormal mouse mammary epithelial and myoepithelial cells in vivo and in culture. Initial experiments with an antiserum against the 46K keratin suggest that this keratin is present in all epithelial and myoepithelial cells in primary culture (R. Scott, B. Asch, and H. Asch, unpublished results). Immunocytochemical studies on mammary tissues with this antiserum are in progress. Monospecific antibodies against the 57K keratin are not yet available. A tentative model of the distribution of keratins in normal and malignant mouse mammary epithelium in vivo and in primary culture, based on our current data is presented in Fig. 1 (next page).

IV. MASKING OF KERATIN EPITOPES

Despite the almost identical keratin composition of normal and malignant mouse mammary cells growing in primary culture (23), use of the monoclonal antibodies AE1, AE3, and AE4 revealed another aspect of keratin expression in which differences were found between cultured normal and abnormal mammary cells (26). In immunocytochemistry, AE1 never stained normal cells but did stain a minority of preneoplastic and carcinoma cells. AE3 reacted with a subpopulation of epithelial cells in both the normal and abnormal cultures, except for a few cultures from one type of tumor wherein all of the epithelial cells were reactive. AE4 decorated an elaborate keratin filament network in all cultured mammary epithelial cells, regardless of neoplastic state. In double-label immunofluorescence, an antiserum which reacts preferentially with myoepithelial cells (13) stained the same cells as AE1 in the tumor cultures and AE3 in the normal and most tumor cultures, indicating that the cells in these populations recognized by the antibodies were myoepithelial. In contrast, immunoblot experiments with cytoskeletal extracts from the normal and tumor cells showed that the keratins recognized by each monoclonal antibody were the same in all cells except for the 40K component that was present in normal cells but absent or decreased in the cancer cells. Thus, while normal cells had keratins of 40K and 50K recognizable by AE1 in immunoblots, the epitope detected by this antibody was apparently concealed or "masked" in situ. Both AE3 and AE4 reacted with the 55K keratin in immunoblots. As immunofluorescence with AE4 showed that the 55K keratin was present in all mammary epithelial cells, the AE3-specific epitope must be masked in the majority of normal and tumor cells. Accordingly, epitopes on three keratins, the 40K, 50K, and 55K, were masked in normal cells, whereas masking in the tumor cells involved primarily the 55K keratin. Attempts to "unmask" the epitope recognized by AE1 in normal cells or to increase the number of cells reactive with AE3 in the normal and tumor cultures by perturbation with proteases, detergent, or other agents failed

(26). Thus, certain cultured preneoplastic and neoplastic mouse mammary cells with a myoepithelial phenotype have an altered organization of keratins that is manifested by a keratin antigenic determinant which is visible by immunocytochemistry in the abnormal cells but not in normal cells. This is the first demonstration that the immunoreactivity of keratins can be modified during neoplastic progression of epithelial cells. More recent results have shown that AE1 and AE3 strongly stain epithelial and myoepithelial cells in sections of normal mouse mammary tissues, but produce little or no reaction with cells in sections of carcinomas (S. Pocchiari, B. Asch, and H. Asch, manuscript in preparation). One explanation for the altered reactivity of the cells *in vitro* as compared to *in vivo* may be the drastic change that occurs in cell conformation which in turn may necessitate a rearrangement or reorganization of keratin filaments, consequently affecting the position and accessibility of keratin epitopes.

Several incidents of masking of keratin epitopes have now been confirmed or suspected (26,35-37) and more will undoubtedly be encountered. Masking is therefore not an uncommon situation. All of these cases have involved monoclonal antibodies that react with a keratin in immunoblots of a

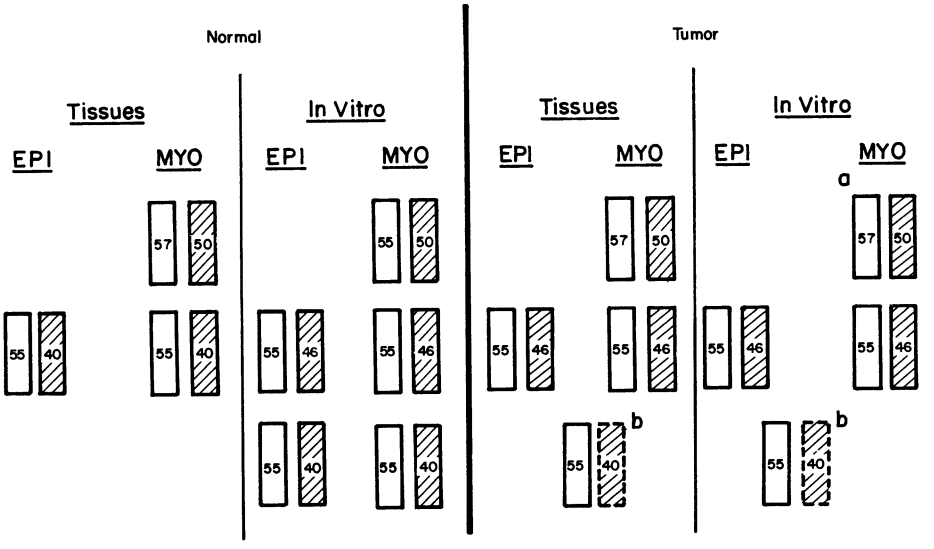

Fig. 1. Proposed distribution of keratins in normal and malignant mouse mammary epithelial cells *in vivo* and in primary culture on plastic. *Epi*, epithelial cells; *Myo*, myoepithelial cells. Rectangles represent keratins. Size of rectangle does not reflect the quantity of a polypeptide per cell but merely its presence. Keratins have been aligned in pairs, with one acidic of a relatively lower molecular weight (40K, 46K, 50K; hatched boxes) and one basic of a relatively higher molecular weight (55K, 57K; open boxes). This alignment is based on the concept of "keratin pairs" (reviewed in 9) and was derived from immunoblot and immunocytochemistry data. Direct biochemical evidence of these pairs is not yet available. Note that the 57K and 50K keratins are expressed only or primarily by myoepithelium. [a]*In vitro*, the 57K keratin has been found only in cultures derived from mammary tissues exhibiting squamous metaplasia with keratinization (23). [b]In tumor cells *in vivo* and *in vitro*, the 40K keratin is detected sporadically. The positioning of the 40K-55K keratin pair in tumor cells reflects the fact the cell type(s) expressing it is unknown.

cytoskeletal extract from a culture or tissue but fail to produce staining when used in immunocytochemistry. The lack of staining is not due to an inability of the antibody to recognize the native molecule or to an artifact of fixation (26,35-37). The finding that masking of keratin epitopes can occur in mammary cells, particularly in association with benign or malignant disorders, serves as a caveat for interpreting studies with monoclonal antibodies and emphasizes the need for biochemical data to support immunocytochemistry results. The implications are of special concern in experiments employing monoclonal antibodies to trace cell lineages in mammary gland development, differentiation and tumorigenesis. Nevertheless, such differences in immunocytochemistry may be exploited for differentiating cell types and as indicators of neoplastic progression if the keratins present in each cell type are known.

V. EXPRESSION OF KERATINS IN NORMAL AND MALIGNANT HUMAN BREAST EPITHELIUM

Moll et al. (8,30) demonstrated by 2-D PAGE that certain keratins are lost and others are gained in infiltrating ductal carcinomas of the human breast as compared to normal ductal epithelium. Moreover, the tumors could be subdivided into two groups based on their profile of keratins (8). Normal ductal epithelium contained keratins No. 5, 7, 8, 14, 15, 17, 18, and 19. Cancers in group I had keratins No. 7, 8, 18, and 19, and cancers in group II had keratins No. 6, 7, 8, 11, 14, 16, 17, 18, and 19. Keratins No. 7, 8, 18, and 19 are thus constant members in normal and malignant tissues. All of the cancers tested had lost expression of keratins 5 and 15. In addition, group I was missing keratins 14 and 17, while group II had gained keratins No. 6, 11, and 16. Benign lesions and other types of breast cancers were not examined. Current data indicate that keratins 18 (38) and 19 (34) are found in luminal epithelium but not myoepithelium. Interestingly, some luminal cells in ductules and in the terminal ductal lobular units appear to lack keratin 19 and may have the proliferative capacity appropriate for a stem cell population (34). The cell type distribution of the other six keratins in the gland is unknown, although at least one appears to be restricted to myoepithelial cells (39). The biological and/or clinical significance of the changes seen in the tumors has not been investigated, but the potential applications of these changes in keratin expression as molecular markers in human breast cancer warrant further investigation. An example of the information that can be derived from studies on keratin is found in Paget's disease of the nipple, wherein the profile of keratins along with the presence of milk fat globule membrane antigens has implicated ductal epithelium of the breast as the cell of origin of this disorder rather than epidermal cells (34,40,41).

The set of keratins expressed by normal and malignant human breast epithelium in primary culture has not yet been reported. However, Chang et al. (42) found that human milk epithelial cells transformed by SV40 in culture had an altered arrangement and distribution of keratin filaments as compared to their normal counterparts. Whether this alteration was related to a change in keratin content and/or was a peculiarity of virus-transformed mammary cells remains to be determined.

VI. DISTINGUISHING CELL TYPES IN MAMMARY CARCINOMAS

Two recent studies with monoclonal antikeratin antibodies have revealed new distinctions among different types of human mammary epithelial cells. Using two monospecific monoclonal antibodies, Bartek et al. (28,34) found that keratin 19, a 40K polypeptide, was present in most luminal epithelial but not in myoepithelial cells of the normal human gland. The antibodies produced heterogeneous staining (i.e., some positive and some negative) of cells comprising all benign tumors examined (28), while all tumor cells in

almost all malignancies were positive. Although most in situ carcinomas were homogeneously positive, a few were heterogeneous for keratin 19 (28). This raises some interesting questions. Are the homogeneously positive in situ carcinomas more likely to become infiltrating carcinomas than the heterogeneous ones? Are the former derived from the the latter? We have recently obtained the same staining results with human breast tissues (B. Asch and H. Asch, unpublished results) using a monospecific antiserum against keratin 19 (33). In this case, masking of epitopes is probably not a problem because an antiserum recognizes multiple antigenic determinants, and it is unlikely that all of them would be masked. Taken at face value, these data imply that myoepithelial cells, which apparently lack keratin 19 in normal tissue, are not present in breast cancers. However, it is possible that during the transition to malignancy, expression of keratin 19 is initiated in myoepithelial cells thereby making them indistinguishable from epithelial cells on this basis. Moreover, the recent report of Dairkee et al. (39) provides evidence that myoepithelial cells are present in many breast carcinomas. These investigators have developed a monoclonal antibody which is monospecific for a 51K keratin and recognizes myoepithelium but not luminal cells in normal gland. This antibody gave little or no reaction with 58% of the breast carcinomas examined, had a heterogeneous pattern of staining with 37% of the tumors, and produced a strong homogeneous reaction with 5% of the cancers. These results may be a direct reflection of the presence or absence of myopeithelium. However, neither masking of an epitope nor the selective switching off of expression of the 51K keratin can be excluded as explanations for at least some of the negative results obtained. As the 51K keratin was characterized by 1-D rather than 2-D PAGE (39), it is not yet possible to confirm its identity relative to Moll's catalog (8). Thus, the presence or absence of myoepithelial cells in human breast cancers is still unsettled. This point should be resolved as additional markers for myoepithelial cells become available. At the same time, the ability of the monoclonal antibody against the 51K keratin to subdivide ductal carcinomas into three categories may have other important biological or clinical significance, as discussed by the authors (39).

Recent studies in mice (13,32) with antikeratin antisera that selectively stain myoepithelial cells have confirmed and extended earlier morphological and ultrastructural studies (43-45) showing that myoepithelial cells are present in primary mammary carcinomas and their metastases. Myoepithelial cells were a substantial, albeit minority, population in the primary tumors, in the circulation of most tumor-bearers, and in all lung metastases (32). In the rat, myoepithelial cells are also present in primary neoplasms (46,47). However, studies on the cell types present in metastases of rat mammary tumors have been conducted on certain transplant lines that are capable of metastasizing (48,49). Based on immunocytochemistry with antibodies against keratin, myosin, laminin, and type IV collagen, which can delineate myoepithelium in the normal gland, results with such lines have indicated few if any myoepithelial cells are present in the metastatic lesions (48,49). However, a recent report (50) showing that such characteristics of myoepithelial cells can change or be lost due to dedifferentiation during passage in vivo or cloning in vitro leaves the status of myoepithelial cells in rat mammary metastases unresolved. This finding also brings into question the use of transplant lines or cloned mammary tumor cells in analyzing the role of myoepithelial cells in mammary tumors and their metastases.

We concur with other investigators (e.g. 20,46) that myosin and actin, which are also found in many mesenchymal cells, are at best ambiguous markers for myoepithelial cells, especially in tumors and cultures. Moreover, recent evidence (51,52) indicating that some fibroblastic cells can produce laminin and type IV collagen raises questions regarding the reliability of these proteins as markers for myoepithelium, unless other indicators are also used.

VII. EXPRESSION OF VIMENTIN BY MAMMARY EPITHELIAL CELLS

Vimentin is characteristic of mesenchymal cells and is found in epithelial cells *in vivo* in only a very few cases (e.g. 53,54). The bulk of evidence indicates that myoepithelial cells do not contain vimentin either in normal or abnormal mammary tissues (12, 13, 17, 20, 55, 56). However, two reports have claimed that vimentin is present in this cell type, particularly in the gland of virgin or non-pregnant animals (50,57). In both of these studies, the antivimentin antiserum was made in rabbits and was not characterized with a cytoskeletal extract of mammary tissue to verify that vimentin was the only component recognized. This point is important because rabbits have a high incidence (often exceeding 50% of the population) of naturally occurring antikeratin antibodies (58; B. Asch and H. Asch, unpublished observations). Moreover, double-label immunocytochemistry with a reagent known to react with myoepithelium, such as an antikeratin antibody, was not performed in either study, raising the additional possibility that the cells decorated by the antisera were the fibroblasts that are usually juxtaposed to the myoepithelium, although separated from it by the basal lamina. To examine this point in mammary tissue of a virgin mouse, we used a goat antivimentin antiserum prepared in our laboratory against vimentin extracted from a mouse mammary fibroblast cell line and purified by 2-D PAGE. In 2-D immunoblots of cytoskeletal polypeptides extracted from primary mouse mammary cell cultures, the antiserum was monospecific for vimentin (Fig. 2, a,b, next page). In double-label indirect immunofluorescence with the antivimentin antiserum and a rabbit antikeratin antiserum that stains all epithelial and myoepithelial cells in mouse mammary gland, no coincident staining of the two antisera was found (Fig. 2, c,d,). We obtained the same results with the antivimentin antiserum in immunoperoxidase and with human mammary tissue. Our results with human tissues agree with those recently published by Dairkee et al. (56). We have not yet tested rat mammary tissue and therefore cannot rule out a species difference between rats as compared to mice and humans.

Species differences do occur in expression of vimentin by mammary cells cultured *in vitro*. Using a combination of antikeratin and antivimentin antisera in double-label immunofluorescence, we previously showed that mouse mammary epithelial and myoepithelial cells in primary culture three to five days post-plating do not have detectable vimentin (13). Moreover, several mouse mammary cell lines have either no vimentin or a small percentage of cells expresses it (59,60). Schmid et al. (21,61) derived clonal cell lines of bovine mammary epithelium in the presence or absence of high concentrations of insulin, hydrocortisone, and prolactin. Lines propagated in the presence of the hormones did not contain vimentin while lines grown without the hormones did. The lines also differed morphologically and in the set of keratins they expressed (21,61). The data suggest that the hormones affected the expression of intermediate filament proteins and/or selected for growth of a particular cell type. Primary cultures of bovine mammary cells have not been examined for vimentin or keratin expression. Dairkee et al. (56) studied normal human breast cells in primary culture and found vimentin present in essentially all epithelial cells within four days of plating. In primary cultures from virgin rats, a heterogeneous expression of vimentin was reported by Warburton et al. (62). Small cuboidal cells were negative while large epithelioid and elongated cells were positive. The former cells appeared to be luminal epithelium whereas the two latter types had some characteristics of myoepithelium. Established mammary cell lines of human and rat origin, like most epithelial lines, usually express vimentin as well as keratin (8,48-50). It should be noted that the mouse and bovine cultures were derived from pregnant and lactating gland, respectively (13,21,61), while the human and rat cultures were prepared from the glands of non-pregnant and virgin individuals, respectively (56,62). With mice, whether the mammary tissue came from a pregnant or virgin animal

is probably not a factor in the expression of vimentin in mammary cells *in vitro* because cells in primary cultures derived from hyperplastic alveolar nodules and carcinomas, both of which were in virgin animals, also lacked vimentin (13,23). The expression of vimentin thus represents another cytoskeletal difference between mammary cells of different species.

VIII. EXPRESSION OF KERATINS BY MAMMARY EPITHELIAL CELL LINES

A most puzzling situation involves the expression of keratins by established mammary epithelial cell lines. Although some mouse mammary epithelial cell lines have retained a set of keratins almost identical to that of mammary cells in primary culture, their reactivity in immunocytochemistry with antikeratin antibodies can be quite different (Table 2).The COMMA-1D cell line, derived from mouse mammary gland, is inducible for casein synthesis in culture and produces normal ductal outgrowths when transplanted into the cleared mammary fat pad of a syngeneic host (63). By 2-D PAGE it has the same profile of keratins as mouse mammary cells in primary culture. However, immunocytochemistry with two antikeratin antisera and the monoclonal antibody AE4, which stain the entire population of epithelial cells in a primary culture, produced staining in only 36-75% of

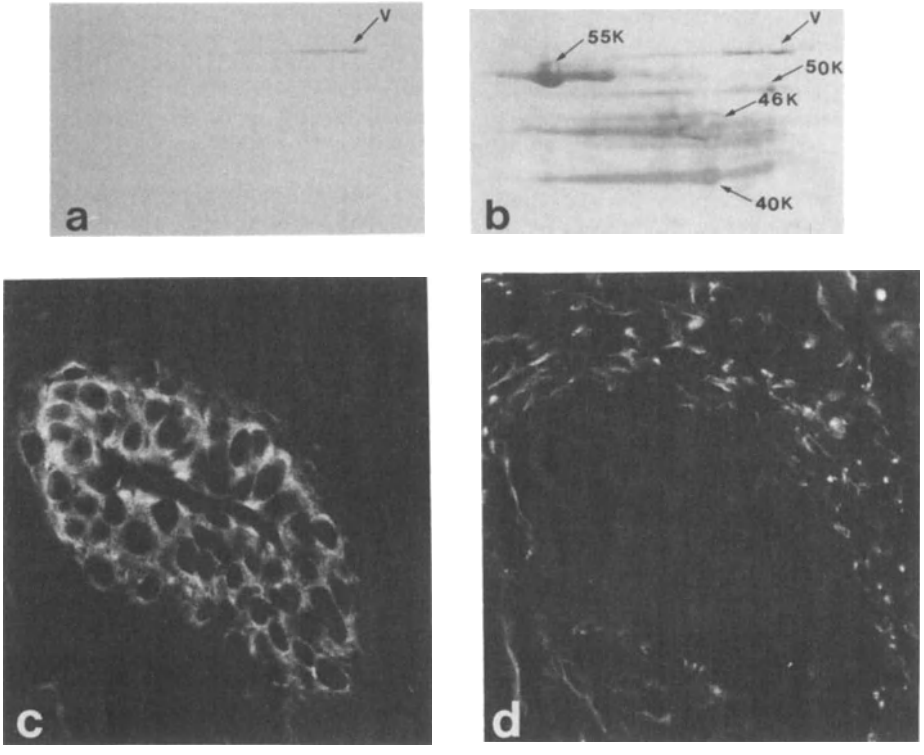

Fig. 2. Expression of vimentin in mouse mammary tissues. a, b, immunoblot characterization of the goat antivimentin antiserum. Cytoskeletal polypeptides extracted from normal mouse mammary cells were separated by 2-D PAGE, blotted onto nitrocellulose, and stained by a, indirect immunoperoxidase with the antiserum, and then by b, India ink to visualize all polypeptides. The numbers refer to the keratins present; v, vimentin. Only vimentin is reactive in a. c, d, double-label indirect immunofluorescence staining of virgin mouse mammary tissue. In the cross section of a large duct, the reactivity of a rabbit antikeratin antiserum in c, which stains both epithelial and myoepithelial cells, has no overlap with the goat antivimentin antiserum in d. X516.

Table 2

Expression of Keratins in Cultured Mouse Mammary Epithelial Cells

Cell type	Keratins present by 2D-PAGE[a]	Percent cells positive in indirect immunofluorescence staining			
		LE61[b,c]	Rab 3[b] (55K, 50K, 40K)[d]	AE4[b] (55K)[e]	DAKO (55K, 50K, 40K)[f]
Primary Culture	55K, 50K, 46K, 40K	ND[g]	100%	100%	100%[b]
COMMA-1D[h]	55K, 50K, 46K, 40K	90-100%	36%	50%	75%[b]
COMMA-T[i]	55K, 50K, 46K	ND	100%	100%	90%[a]
COMMA-F[i]	55K, 50K, 46K	ND	100%	100%	100%[a]

[a]Data from M. Zafari, B. Asch, and H. Asch
[b]Data from D. Medina (personal communication)
[c]The keratins in mouse mammary epithelium recognized by LE61 have not been determined.
[d]Mouse mammary keratins detected by Rab 3 in an immunoblot assay (22)
[e]Mouse mammary keratin detected by AE4 in an immunoblot assay (26)
[f]Mouse mammary keratins detected by an antiserum against bovine muzzle keratins (DAKO Corp., Santa Barbara, CA) in an immunoblot assay; data from M. Zafari, B. Asch and H. Asch
[g]Not done
[h]Cell line described in (63)
[i]Cell line described in (60)

the cells comprising the COMMA-1D line. Another monoclonal antibody, LE61 (64), stained 90-100% of COMMA-1D cells, indicating that most of the cells do have keratins and are bona fide epithelial cells. As compared to cells in primary culture, the altered reactivities of the cell line with the antibodies, excluding LE61, are difficult to explain. Although the keratins recognized by LE61 in mammary epithelium have not been determined, unless it is monospecific for the 46K keratin, its specificity must have some overlap with one or more of the other antibodies used in this study. The most obvious explanation is that some cells no longer express keratins detected by a given antibody. However, this would not explain the discrepancies such as the reactivities of AE4, which recognizes the 55K keratin, and the two antisera, Rab 3 and DAKO, which both detect the 55K, 50K and 40K keratins in immunoblots of cytoskeletal extracts from cultured mammary cells. Regardless of the molecular explanation, the results suggest that prolonged culture and passage in vitro can produce changes in keratin expression in mammary epithelial cells that are not only different from cells in vivo but also from cells in primary culture. Such changes could pose problems with the use of antibodies against keratins as indicators of cell type in these cultures. In contrast, two sublines, COMMA-T and -F, derived from the COMMA-1D cells, have immunocytochemical reactivities almost identical to those of cells in primary culture (Table 2). A similar situation may occur in some rat mammary cell lines that fail to react with antikeratin antibodies, possess vimentin filaments, but also have type IV collagen and/or laminin (e.g. 53,56). However, an analysis of keratins in the rat cells was not performed.

The behavior and differentiation of mammary epithelium is strongly influenced by the extracellular matrix (reviewed in 65). The cytoskeletal alterations may therefore be adaptations of the cells induced by contact with an alien (plastic) substratum. In fact, two studies have shown that the keratin phenotype of mammary epithelium is modulated by different substrata (24,25). Initiating and maintaining cultures of mammary cells on a suitable substratum might stabilize the cytoskeletal composition and preserve a cellular phenotype closer to that expressed in vivo. Other factors may also contribute to the cytoskeletal constitution of mammary cells. In parallel with the altered expression of keratins, Medina and coworkers (personal communication) have found that the percentage of COMMA-1D cells expressing vimentin varies with the culture passage level and the concentration of serum in the medium.

IX. EPITHELIAL CELL TYPES AND LINEAGES IN THE MAMMARY GLAND

At least five types of epithelial cells can be defined in mammary epithelium based on structure, function, and histological location: luminal and myoepithelial cells of ducts, luminal and myoepithelial cells of alveoli, and in the immature gland, cap cells of the end bud. The cap cells appear to be one type of stem cell in the gland (66). An elusive question is, what and where is the stem cell(s) in the adult gland that is responsible for expansion of the epithelium in response to the hormonal signals accompanying pregnancy? Dulbecco and colleagues (57,67,68) have been mapping cell lineages in the developing rat mammary gland using a polyclonal and various monoclonal antikeratin antibodies, antisera against myosin, vimentin, laminin, type IV collagen, and two other monoclonal antibodies. In their study, ten cell types were distinguished, each of which had a characteristic profile of reactivities with the panel of antibodies. Four cell types were identified in the end bud and two cell types each in ducts, ductules and alveoli. Developmental pathways have been proposed for luminal epithelial and myoepithelial cells based on the overlap of markers, with cells at the tip of the end buds representing a common stem cell for both. In addition, putative stem cells that may be precusors of luminal cells and alveoli were detected in the basal layer of the ducts. These cells were

distinguishable from myoepithelial cells by their staining pattern with the panel of antibodies. The specificity of the various antikeratin antibodies for mammary keratins was not reported. Sonnenberg et al. (69) have taken a similar approach in analyzing cell types in mouse mammary gland and have suggested that a basal cell, distinct from myoepithelial cells, is present in the ducts and can differentiate into both myoepithelial and luminal epithelial cells. It is interesting to note that a luminal, rather than basal, cell has been proposed as a possible stem cell in the human gland (28). In any case, direct evidence for the pluripotent ability of these putative stem cells is the critical requirement, especially in view of the recent results of Medina et al. (60), which suggest that more than one cell type may be necessary to obtain morphogenesis of the gland in vivo.

X. SUMMARY

Antibodies against components of the cytoskeleton, cell surface, and basement membrane are providing important probes for subdividing and classi fying normal and abnormal mammary tissues and individual epithelial cells according to new criteria based on structural composition and organization. However, the expression of keratins in particular is proving to be more complex in mammary cells than was originally appreciated. At the same time their complexity and heterogeneity expands the potential of their use as markers. The challenge is to understand the expression of these elements a the cellular and molecular levels.

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CHROMOSOMES IN BREAST CANCER

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I. INTRODUCTION

Observation of chromosome aberrations in association with tumors of particular morphology, developmental stage, or tissue of origin has generated new interest in cytogenetic analysis. Tumor-specific aberrations could be valuable in identifying tissue-specific developmental genes and in detecting sites within the genome that are critical for cell growth or control of cell division. Documentation of tumor-chromosome associations seems essential to understanding the roles that chromosomal events can play in the biology and evolution of malignancy.

For a few developmental tumors of childhood (retinoblastoma, Wilms' tumor), mutation or loss of certain genes linked to specific chromosomal deletion or recombination appears critical to tumor etiology and progression. These observations have provided strong impetus to the search for tumor-specific chromosome associations. The association can be sufficiently strong that it is used in diagnosing some malignancies. Therefore, it is also important to seek evidence for involvement of specific chromosomes or intrachromosomal sites in breast cancer. Moreover, aberrations, either numerical or structural, appear more extensive with increasing severity or extent of the malignant disease. There is evidence in breast cancer that altered DNA content (DNA aneuploidy) is correlated with histologic anaplasia, loss of hormone receptors, and clinical evidence of advanced tumor growth. If diploid DNA content confers a better prognosis, then quantita-

tion of chromosome changes, specific or otherwise, may have prognostic value and may correlate with tumor progression.

A new array of techniques permits molecular approaches to genomic disturbance. Specific chromosome aberrations can serve as signposts to point us in the proper direction for molecular studies. The identification of amplified sites within chromosomes (homogeneously stained regions or HSRs) or in the form of double minutes (DMs) indicates expansion of particular gene functions. In some tumors, these chromosome aberrations have been associated with drug resistance or with increases in oncogene copy number. Preferential recombinant sites for chromosomes have also been linked with mutation or altered activation of oncogenes in tumors. Thus, identification of preferential sites for aberration can lead to better understanding of mechanisms in tumor formation and progression and may provide us with the beginnings of a realistic explanation for the prevalence of chromosome changes in human cancer. The purpose of this review, therefore, is examination of data from breast tumors at different stages to weigh the evidence for extent and specificity of chromosomal alteration, to correlate quantitative increments of chromosome change with increasing severity of disease, and to question whether any studies thus far have led to understanding at the molecular level of this particular form of human cancer.

No clear picture has yet emerged of the prevalence or role of chromosome changes in human breast cancer. Remarkably few analyses of primary cancers have been published; much of the available data relates to cancer-derived material in culture and to direct and cultured analyses of effusion-derived malignancies. These facts are surprising since many breast cancer cases, primary and metastatic, were reported prior to the advent of chromosome banding techniques.

II. EARLY STUDIES

Several reviews of human breast cancers summarized results of roughly 100-150 samples from primary and secondary tumors (1,13,51). Most tumors were aneuploid, and despite the absence of banding, marker chromosomes were recognized in many. Only a few samples, a ductal carcinoma, two lobular carcinomas *in situ*, and one metastatic tumor, were described as diploid (51,62). Like the solid tumors, cells from malignant effusions were generally aneuploid. One review compared the mean modal numbers of grouped solid tumors and tumor effusions and it was not clear whether individual tumors contained diploid as well as aneuploid cells (13). In a second series (which reviewed some of the same original reports), modal numbers of individual tumors were separated into near-diploid and clearly aneuploid groups (1).

Some early work described chromosome analyses on proliferative lesions, benign or non-invasive, that were not fully developed malignant tumors. Studies of cystic disease, of fibroadenomas and of lobular carcinomas *in situ*, were reported. In most cases these non-malignant or premalignant lesions yielded normal diploid cells. Aneuploidy without structurally aberrant chromosomes was observed in five of six cases of fibroadenoma in culture (22). In fibrocystic disease, aneuploidy was found with structurally abnormal chromosomes in eight of ten cases. Abnormal findings were more frequent in cases classified as "proliferative" fibrocystic disease and the authors argued that their observations provided evidence for the precancerous nature of fibrocystic disease in some patients. Major structural aberrations and aneuploidy characterized every example of invasive carcinoma in their study.

In summary, early studies demonstrated that aneuploidy was widely prevalent and that chromosomal structural rearrangements marked primary carci-

nomas of the breast and even some benign lesions. In a few cases, tumors with no apparent chromosome aberrations were reported. Several reports emphasized similarities between findings in primary and metastatic cancers from the same patient. However, advanced stages of tumors such as effusions showed a greater degree of aneuploidy than did primary tumors (13).

III. PATHOLOGIC DIAGNOSIS AND STAGING

Precise identification of the lesion is crucial to interpretation of cytogenetic evidence in breast cancer. There is little evidence for a continuous histologic spectrum from normal to hyperplastic to neoplastic in the breast. (The reader is referred to Azzopardi (4) for an excellent discussion). Many lesions have been identified that are clearly proliferative but not malignant. Some, such as fibroadenoma, sclerosing adenosis, or blunt duct adenosis, have not been seriously challenged as other than benign growths. Fibrocystic disease which is widely prevalent and pleomorphic has, at times, been suspected as a tumor-predisposing or premalignant lesion. Non-banded chromosomes analyses (22) indeed suggested that cytogenetic evidence of aneuploidy and chromosome aberration could help to distinguish within fibrocystic disease those patients who were more at risk of progressing to frank malignancy. At present, fibrocystic disease is generally regarded as a benign condition, contributing only slightly to risk for the development of neoplasia. The disorder is so prevalent, approaching 50-60% in some series, that it often coexists with neoplasia; thus, an increased frequency in cancer patients has been difficult to demonstrate. It is important to remember that the association and the increased risk which has been inferred is not evidence of a precursor relationship, but could result from shared etiologic agents.

A few proliferative lesions in the breast are less easy to distinguish from true malignancy and it is crucial to separate them into either precancerous or truly benign lesions. Epitheliosis, or epithelial hyperplasia, is sometimes difficult to distinguish histopathologically from frank malignancy. The difficulty of accurate diagnosis makes correlation with the clinical course and prognosis uncertain. Similarly, benign papillomatous lesions must be differentiated from papillary carcinoma. There is little evidence that papillary carcinomas arise from preexisting papillomas nor that papillomas when recurrent are malignant lesions. For both epitheliosis and papilloma, better definition of their relationship to malignancy depends primarily on distinguishing them accurately from malignant lesions. Cytogenetic studies might aid in making the distinction.

In the breast, as in other glandular tissues, the epithelium is separated from underlying connective tissues by a myoepithelial cell layer and basement membrane. Thus, proliferation of the epithelium alone, even when cytologically malignant, may be classified as not fully malignant if it has not broached the basement membrane. Such a lesion is usually identified as carcinoma *in situ* and represents a pre-invasive stage of the disease. Carcinoma *in situ* may be papillary, trabecular, or solid, and is often found in the presence of invasive malignancy. When present without invasive disease, it represents an earlier stage of disease and therefore should be associated with a better prognosis and less deviant karyotype. However, the natural history of such lesions and their evolution into invasive disease is not well understood. Breast cancer is often multicentric and microscopic lesions are frequently found in the contralateral breast. Equivocal results from study of presumptive-control normal tissue may be obtained because of wide-spread microscopic precancerous or cancerous lesions.

Most cancers of the breast are classified as lobular or ductal. This classification is based upon differences in the geographic origin of the tumor cell mass. From the work of Wellings (64), there is good reason to

think that both lobular and ductal cancers are the same disease originating from cells in the terminal duct-lobular unit. There is therefore no reason to expect associations with different chromosome patterns or differing prognoses between the two major forms of breast cancer. The less frequent breast cancers, such as medullary carcinomas, infiltrating comedocarcinomas, and colloid carcinomas also seem to originate from duct cells. Other specialized forms of breast cancers should be considered separately as they may confer different prognoses and, if originating from different cell types, may also show differences in karyotypic picture.

Early metastases of breast are more often found in the skin, contralateral breast, and the ipsilateral lymph nodes. Distant metastasis to lung, bone, and other solid tissues, should be viewed as constituting a more advanced stage of malignancy. Prerequisites for metastatic growth include invasion into vessels, the ability of tumor cells to survive during transport in vascular channels, to extravasate, and to take up residence and grow in metabolically different environments. Metastasis therefore indicates acquisition of new biological properties by tumor cells. Growth of cells in malignant effusion fluids probably represents the most advanced stage of malignancy. Implants on pleural or peritoneal surfaces shed cells which are apparently capable of dividing in mesothelial-lined spaces, where transudation of fluid is stimulated by their presence. Even in poorly differentiated primary tumors, the cells usually adhere to one another in arrangements reminiscent of normal acini and ductules; and aggregates of tumor cells are supported by stromal elements and vascular channels. In contrast, in an effusion, single cells grow essentially autonomously with no attempt at gland formation and completely free of connective tissue framework. Thus, stages of malignant tumor growth reflect not only the severity of clinical disease but also acquisition of significant capacity for cellular autonomy. There is accumulating evidence that these stages in biological progression of a tumor may also be expressed as increases of karyotypic atypia.

IV. ANIMAL MODELS

Difficulties encountered in the study of human malignancies can sometimes be bypassed by studies of suitable animal model systems. One of the best-studied is the mouse mammary tumor model, originally described by Bittner in terms of milk factor transmission. The DBA and C3H strains of mice, regularly transmit a virus from mother to progeny by means of milk and the transmitted virus is responsible for high frequencies of breast cancer in these strains. Widely used lines of mouse ascites cells, derived from mouse mammary tumors (32), are highly aneuploid and contain rearranged chromosomes. However, early direct cytogenetic preparations revealed that the Bittner tumors were generally diploid (40 chromosomes). Tjio and Ostergren (61) studied 19 spontaneous tumors from high milk-virus strains of DBA and C3H mice. All but three of nineteen tumors were diploid with normal chromosome morphology. Of the remainder, one had a modal number of 39, one was bimodal with both diploid and tetraploid cells, and one tumor was entirely tetraploid. The authors observed that variation in chromosome number per cell was greater than for normal tissues, and also noted that no gross chromosome changes appeared after serial transplantations in culture.

Spontaneous mammary carcinomas from both high-virus strains and Swiss mice revealed the same patterns (33). Moreover, mammary tumors of virus-free strains induced by hormonal imbalance were similar, with eight diploid and one hyperdiploid tumor. Recently, a spontaneous mammary tumor from the C3H strain demonstrated extensive biological heterogeneity within a primary tumor (16). Chromosome numbers ranged from 40 to 130 per cell in highly heteromorphic cultures in first passage. Four cell lines established from the original cultures each differed in unique characteristics from the others. Two lines were diploid or pseudodiploid and two were highly aneu-

ploid. The above studies were limited in their ability to recognize structural rearrangements in the mouse karyotype with its entirely telocentric chromosomes.

The characterization of mouse mammary tumors as predominantly diploid was refuted when banding studies demonstrated a non-normal pattern in tumor cells that were diploid in chromosome number. Direct preparations of excised mammary tumors from GR, C3H, and some non-inbred strains of Swiss mice, using tryspin-Giemsa banding, demonstrated trisomy for chromosome 13 in most cells (17). In the GR strain, all tumors, although not all cells of each tumor, showed trisomy for #13. In the C3H and Swiss mouse tumors, trisomy for #13 predominated, and there was random chromosome loss. Some cells with normal karyotypes were found, and loss of an X chromosome was frequent. The specificity of trisomy for #13 in association with mouse mammary cancer was emphasized by its manifestation in mouse species that appeared to carry different mammary tumor viruses. In some tumors without the trisomy, translocation and partial replication of #13 was detected. Even more significant was evidence that mammary tumors induced by urethane displayed the same trisomy that was found in the spontaneous mammary tumors induced by milk virus (18). This model of site-specific chromosomal involvement in conjunction with mammary tumor induction by differing agents holds promise that similar chromosomal specificity might characterize human breast tumors and could lead to identification of factors critical in growth and development of mammary epithelium.

V. TECHNIQUES AND THEIR LIMITATIONS

The problems of quality of preparation, extent of analysis, and degree of representation of the tumor by cells in metaphase are similar for breast cancer to those for other primary tumors. Results from direct analysis of tumors (metaphase cells obtained by mincing of tissue and preparation for karyotypic examination within hours after excision) are limited to the small proportion of cells in mitosis in fresh tumor material. The poor technical quality of tumor-derived metaphase cells is well known to cytogeneticists. The limited numbers and incomplete analyses of breast tumors that have been examined attest to the problems of preparation. Since it is difficult to apply other markers to cytogenetic preparations, one cannot differentiate diploid tumor cells from cells of nonmalignant stromal or inflammatory origins. It has been suggested that many chromosomally aberrant cells observed in direct preparations may be cells that, by virtue of their aberrations, are arrested in metaphase and unable to complete the cell cycle. A fresh tumor may include a disproportionately large number of such "genetically dead" cells. If that is true, then the cytogenetically aberrant cells may not represent the main mass of tumor cells which continue to divide and propagate. In contrast, cells that grow in culture clearly derive from a subset of cells of the original tumor. They are able to adapt to survival and growth in culture, an environment which differs in many unknown ways from conditions for growth within the body. Moreover, it is known that cells and chromosome aberrations can evolve in culture, both spontaneously and as a result of culture conditions. Thus, new cytogenetic findings may appear which do not represent any cells of the original tumor. Primary tumors are heterogeneous with respect to their chromosome constitution as well as other parameters. Selective pressures or random events may result in removal of some chromosomal patterns and selection for a few or a single dominant pattern in cultured cells. The extent to which chromosome patterns in long-term culture represent the tumors from which the cells originated, thus becomes highly questionable. These and other considerations limit the value of karyotypic analysis of tumors, directly or in culture and must influence interpretation of results. For the reasons described, neither method is likely to yield an accurate picture of the characteristics of the dividing components within a tumor *in vivo*. Results from both sources must

be used to attempt to structure, as the blind men did with the elephant, a reasonable re-creation of reality.

Because of sampling problems of material for karyotypic analysis, another important source of information derives from studies on DNA content in breast cancers. Data are based on larger and presumably more representative samples of tumor cell populations. Samples for flow cytometry (FCM), however, usually include stromal or inflammatory elements as well as tumor cells and therefore will reveal diploid peaks, within which tumor cells cannot be identified. In contrast, microspectrophotometry, which combines morphologic recognition of tumor cells, with measure of the cellular DNA content, is free of that source of bias. Neither method is capable of detecting chromosome rearrangements that do not involve quantitative alterations in DNA nor, at present, of detecting small changes in DNA content that are amenable to cytogenetic definition. These methods, particularly FCM, are not entirely devoid of the danger of selective loss of subpopulations during sample preparation.

Data from cytophotometric studies usually based on Feulgen staining indicate that approximately one third of all breast cancers are composed largely or entirely of cells with diploid DNA content (56,58). Another third of the cases appear to contain diploid tumor cells as well as some that are aneuploid. The frequency of tumors that are recognized as diploid by FCM is more variable, ranging from a low of 8% to over 50% of the tumors in different series (56). Diploid and near-diploid tumors are more often estrogen-receptor positive and are less anaplastic (46). The hypothesis that all diploid cells are nontumor cells was tested by correlation of FCM data with cytologic analysis of the same samples (23). The proportion of aneuploid cells by FCM in several cases differed from the proportion of tumor cells identified by cytologic examination of the same suspension. Tumors that were diploid by FCM contained between 5-100% tumor cells (median = 50%). In the majority of tumors with small aneuploid peaks, cytology indicated a larger fraction of tumor cells and it was concluded that a second, diploid stem line of tumor existed. In correlative studies, bimodal tumors are usually classified on the basis of their aneuploid peaks. Recent data indicate that 66 to 85% of breast cancers have aneuploid cell lines but that heterogeneity within tumors is common (9). The frequent correlations of aneuploidy with undifferentiated morphology, greater proliferative fraction, and lack of estrogen receptors emphasize the importance of quantitative chromosome aberration in tumor progression.

VI. SPECIFICITY OF CHROMOSOMAL ABERRATIONS

A. Solid Tumors

The evidence for specific chromosomal findings in breast cancer will be divided into three sources: analysis of primary tumors by direct methods, studies of effusion fluids by direct methods, and a review of the relatively large literature on cultured cells, including short-term cultures derived from breast cancers of various stages, and cell lines.

The numbers of cases from which direct studies of primary breast cancers have yielded any results is extremely small in view of the many attempts that have been made. Only seventeen cases have been reported in which banding methods permitted complete or even partial analysis of chromosome number and marker identification (Table 1). Several investigators have obtained metaphase cells from fewer than half the cases attempted and karyotypable cells in much smaller proportions. A series of 110 primary breast cancers yielded analyzable metaphases in only 37 cases (49). Of these, 14 had counts in the diploid range; only 9 were karyotyped. While some cases were analyzed fairly extensively, 1 of the 9 was represented by a

Table 1: Karyotypes of Primary Breast Cancers

Modal # & Sex Chromosomes	Aneuploidy	Markers	References
46,XX	+5,+8,-20,-21	2p-,t(9q:17p), 16q-,t(9p:17q)	(42)
46,XX	-6,+7,-8,-15	t(3:11),t(5:11) 16q+, + 2 ring chr.	
46,XX/50,XXX	+8,+12,+21	---	(60)
48,XX	-1,+3,-20	+i(1q),+i(1q), +t(1:20)	(34)
68-70	not described	+i(1q)	(35)
39-41	variable	del(1),1p+,t(1:14), + many others	
52-55	variable	t(1:1),del(1),+ many others	
79,XXXX	multiple losses and gains	+i(1q),+t(1:3),+t(1:3), +t(1q:14q), +7 markers	(37)
55,X	+7,+22,(11 chr)	+22 markers*	(49)
45,X	(-15 chr)	+15 (14 markers + 1 abn)	
45,XX	-8,-16,-17	+del(1),+ins(17)	
41,X	(-19 chr)	+15 (12 markers + 3 abn)	
42,XX	(-12 chr)	+8 (6 markers + 2 abn)	
46,XX	-6,-8,-11	+del(1),+t(11:?),+?	
46,XX	(-6 chr)	+7 markers	
46,XX	-16	+del(1)	
50,XX	+5,+6,+9,+10,+20 (-7 chr)	+6 markers	

Karyotypes are presented completely where space permits and if cells were analyzed completely. The cases included were harvested directly. i.e. within a few hours after surgery. In reference (2), only cases 7 and 8 were identified as stemline; the referred to as "representative".

*A marker in this study was defined as a structural aberration appearing more than once, and an abnormal chromosome (abn) as an aberration found in a single cell.

single karyotyped cell. Other investigators were able to karyotype 10 of 78 tumors, and 4 of those analyzed were malignant effusions; 6 tumors were in the diploid range (12). In a recent report on preparations from 55 solid breast tumors, 23 cases were inadequate in either number or quality of metaphases and another 10 cases were excluded from consideration because only normal karyotypes could be found (24). They focused on evidence of DMs and did not present karyotypic analysis of the abnormal cases other than to indicate their non-diploid number and the presence, in some, of marker chromosomes.

Near-diploid cases are often more informative because they contain fewer aberrations and are more amenable to complete analysis. Of the 17 cases in Table 1, nine are diploid ±2 in chromosome number. Nevertheless, several are marked by large numbers of chromosome rearrangements. One observation which emerges from inspection of their karyotypes is the frequency of markers involving translocations of chromosome 1, resulting in excess copies of 1q. Only three cases (42,60) completely lack numerical or

structural aberrations involving the #1. In the largest series (49), marker analysis was sufficiently extensive to preclude complete reporting in Table 1, but each of the nine cases had at least one marker that included a part of chromosome 1. Formation of isochromosomes of 1q was another source of multiple copies for this chromosome arm. Chromosome 3 was noted in trisomy as well as in translocation in several cases (49) and in two other near-diploid cases (34,42). Substantial evidence of preferential loss or gain cannot be elicited from individual case reports, but in the series of Rodgers et al. (49), the most frequent losses noted are chromosome 8, 15, and 16. In contrast, two other near-diploid tumors (42,60) are trisomic for #8 (also commonly seen in myeloid leukemia). Another frequently lost chromosome, #16, shows structural rearrangements as well (42). The simplest case with aberrations is missing chromosome 16 and has gained a marker (a deletion of 1p).

A single case (50) provides direct cytogenetic analysis of cancer of the male breast. Seventy-six cells were examined, two of which showed a normal diploid male karyotype. The other karyotyped cells showed a mode of 44, loss of both copies of chromosomes 1, 8, and 12, and no aberrations of either X or Y. Several of the marker chromosomes involved the long arm of chromosome 1. These results on a cancer of the male breast do not differ significantly from patterns observed in a number of female breast cancers in the same laboratory (49).

Some authors exclude cases from consideration if only normal diploid karyotypes are found. One case in Table 1 is bimodal with a diploid normal mode and only numerical aberrations in the aneuploid mode. In another series, as yet only published in abstract form (12), three of ten cases are bimodal with a normal diploid mode; two additional cases contain 46,XX cells. A small proportion of cells from four of the series of nine cases (49) are diploid and in a bimodal case, one mode is entirely composed of normal cells. However, on the grounds that the diploid cells show better chromosomal morphology and that the single case with a diploid mode had infiltrates of mononuclear cells histologically, the authors interpreted these diploid metaphases to represent normal reactive cells rather than as part of the tumor cell population. Most near-diploid tumors showed no clonal changes in number or structure of the X chromosome pair, although an X was occasionally lost from individual cells (49). This is somewhat surprising considering the late replicating nature of the second X and the reported absence of Barr bodies from mammary tumor cells (51). Structural rearrangements of chromosome 11 are described in one case (42) and loss of a #11 was relatively common (49). However, given the relatively small number of cases, including several that were incompletely analyzed, the only consistent finding in these near-diploid primary tumors is that of 1q translocations and extra copy numbers. None of the other aberrations is present in sufficient frequency to be clearly indicative of specificity in breast cancer. Excess replication and translocation of the 1q region has been reported in several forms of cancer, including leukemias, ovarian cancers, testicular, and other solid tumors, (2,43,63). Three oncogenes, N-ras, B-lym and src, have been localized to the short arm of chromosome 1, and SK-1 to the long arm.

B. Effusions

Direct cytogenetic studies of effusion fluids are almost as uncommon as those of primary tumors. One reason for the paucity of data is that effusion fluids are often aneuploid and show such extensive chromosome rearrangement that karyotypic definition is difficult and often incomplete. Therefore, a case that showed a diploid chromosome number with loss of a #1, a #16 and a #22 and addition of three markers involving the same three chromosomes, is of particular interest (7). Another diploid case had lost

#16, gained #19 and had structural alteration of #17 (45). Further, in a hypodiploid effusion fluid where many chromosomes were missing, the only pairs for which normal copies were entirely absent were #3, 10, 16 and 22 (48). Two studies comprising 14 breast cancer effusions showed near-diploidy in half the cases (3,10). Two had no identifiable clonal aberrations, two were bimodal, and several cases showed rearrangement involving an X chromosome.

In one series, chromosomes 1, 3, 7, 9, and 14 participated in rearrangements considerably more often than expected on the basis of length alone (10). In the other, exchanges involving #1, 2, 5, 7, and 16 were emphasized (3). Attempts at culture led to growth from four effusions, which were harvested within five to 20 days of culture initiation (10). However, the cells which grew out were normal diploid, although in all but one case the original preparation had yielded aneuploid cells. The authors suggested that there was selection against nondiploid cells in vitro. Karyotypic variability is a more consistent feature of effusion fluid analysis than of primary tumors. Clonal evolution has been documented in two sequential effusions from the same patient (3). A few isolated cases have also been either hypodiploid or hyperdiploid with large numbers of structurally altered chromosomes and frequent isochromosome formation (28,29,48). Some near-diploid effusions, like some primary breast cancers, showed little deviation from the normal diploid karyotype.

The frequent superposition of large numbers of apparently random changes obscures identification of those which are truly nonrandom and significant in tumor evolution. The data available from direct examination of the small numbers of primary, metastatic, or effusion fluid breast cancers reveal a dominant but not uniform finding of multiple copies of 1q in both primary and metastatic breast cancers. A second observation that may also represent a nonrandom chromosome change based on the small numbers of near-diploid breast tumors, is monosomy or structural rearrangement for #16. Deletion or rearrangement of #16 has also been noted in leukemias, meningiomas, retinoblastomas, and tumors of lung, ovary and urinary tract (43).

C. Cell Lines

Cell lines, many derived from effusion fluids, are relatively more accessible for study than are fresh human tumors. Cell lines are more often heteroploid than are samples from direct tumor analysis. Relatively few are near-diploid; the majority show numbers ranging from 50 to 90 chromosomes. Usually the cells contain many marker chromosomes (sometimes exceeding the number of normal homologs). In contrast to direct analysis of breast cancers, markers in cell lines have often included translocations involving an X chromosome. Isochromosome formation involving the 1q is relatively frequent, and preferential rearrangement of #11 has been reported (31,39,-53,54). DMs or minute chromosomes have been observed (20,54); in the MCF-7 line either HSRs or DMs were observed in vivo and in vitro. A carcinosarcoma, a tumor most likely of different pathologic origin, gave rise to a cell line containing isochromosomes but was one of the few tumors that did not show 1q in marker formation (26).

Despite some striking examples of uniformity (14), cell lines often show evidence of instability and heterogeneity. Many lines of breast cancer origin maintain varied morphology during propagation (65). While some maintain considerable heterogeneity of karyotype, in others a predominant chromosome pattern emerges in culture. For example, in our studies, although different clonal karyotypes were observed in primary culture, by the third subculture, the pattern was uniform in almost all cells with markers involving chromosomes 1 and 11 (59). Moreover, chromosomal evolution in culture does not only result from early selection. One group reported dif-

ferences in modal chromosome number between the original effusions and long-term cultures and showed further changes in modal numbers long after culture initiation (11,27,53). MCF-7 sublines show extensive karyotypic variability with marked differences between cells grown in vivo and in vitro (54). A line that evolved from a bimodal primary culture with a minute chromosome, was unimodal and had lost the minute (20). In the BT474 line, multinuclear cells and multipolar mitoses have been observed (40). Thus, breast cancer lines appear heteroploid, show evidence of evolution during establishment in culture, and show considerable karyotype instability which can permit rapid evolution, either randomly or in response to selective influences.

The relative success of culturing cell lines from effusions rather than solid tumors suggests that effusion cells are better able to adapt to the environment in vitro than are cells from solid tumors. The conditions for growth of effusion fluids in vivo should provide a homogenous environment and opportunities for rapid selection. In contrast, a solid tumor may encompass a variety of local microenvironments that differ greatly within the same tissue mass (67). Given the inherent instability of tumor cell chromosomes, a more variable environment may promote greater instability and exert local selective influences on chromosome aberrations arising by chance. Thus, it would appear more reasonable to attribute an enhanced ability to adapt to culture and immortality to progressive alterations in tumor cell biology rather than to heterogeneity.

Short-Term Cultures. We have gained new perspective from a recently developed short-term culture system. The technique is based on propagation from organoid ductular aggregates after prolonged enzymic digestion in a medium designed to promote epithelial cell selection and growth. (For complete description, see chapter by Helene Smith). Although the cells grow slowly and are viable only for short periods (one to four months in culture), successful culture has been achieved for approximately 65% of the samples initiated. Cytogenetic analyses of these cultures from breast cancer have yielded mainly normal diploid karyotypes (66). Cultures of 15 primary breast cancers were analyzed and while individual cells with numerical or structural aberrations were found, no clonal aberrations were identified. Further, no consistent patterns of loss, gain, or rearrangement were detected. Cultures derived from solid metastases differed from those of primary tumors. All the cells that grew were near-diploid, but the frequency of aneuploid cells and of cells characterized by structural rearrangements was greater than that for primary cancers. Three of the 12 cases exhibited clonal karyotypic aberrations in culture.

A possible explanation for differences between these results and those of direct tumor analyses is that many primary tumors may be karyotypically heterogeneous. Our experience with short-term cultures may, in part, explain the paucity of data from primary breast cancers and difficulties in obtaining cultures from malignancies of the breast. Other investigators have observed normal diploid cells in fresh tumors but have assumed that they represented stromal or inflammatory rather than tumor cells (24,45,49). Evidence that the cultured diploid cells do, in fact, originate from the cancers, is based on their epithelial characteristics, immunologic data, and their ability to invade normal amniotic membrane (56,57). The method used for sample preparation involves prolonged enzymic digestion that may select for the most adhesive components within tumors. In breast cancer duct epithelium, one might expect cellular adherence to correlate with better differentiation. Cells derived from metastatic effusions were uniformly characterized by major structural rearrangements and significant aneuploidy, as noted by other investigators. Increase in chromosomal aberration with increasing tumor progression in this system are shown in Table 2.

As increasing biological deviation is acquired, aneuploid cells may

Table 2: Chromosome Aberrations in Cultures from Different Stages of Malignant Progression (Data summarized from reference 66)

Specimen (# cases)	No. of Cells Analyzed (with banding)	% Aneuploid Cells	% Cells with Structural Rearrangements
Nonmalignant (6)	98 (38)	0	0
Primary Carcinoma (15)	286 (133)	3.8	2.4
Metastatic Carcinoma - to other breast (3)	53 (37)	3.8	7.5
- to skin (7)	95 (75)	11.0	18.0
- effusion (3)	57 (57)	98.2	100.0

Chromosome harvests were prepared from primary cultures of benign and malignant mammary epithelium. Cultures from solid tissues were plated after prolonged incubation and enzymic disaggregation while cells from malignant effusion were plated directly. A striking increase in chromosomal aneuploidy and rearrangement accompanies advances in malignant progression. The 6 cases of nonmalignant epithelium were comprised of 4 reduction mammoplasties and 2 contralateral breasts.

then adapt better to growth in vitro. Nonetheless, fewer than 10% of such effusions are capable of long term propagation. We recently studied a patient in whom three effusion fluids were obtained over a period of two years. The third sample, obtained after the patient had received both radiation and chemotherapy, adapted repeatedly to growth as a cell line, whereas the first two samples grew in short-term culture but did not develop into lines. The chromosome patterns of the first two samples were not greatly dissimilar to those from the third effusion. All were near-diploid and contained several structural rearrangements. The karyotypic rearrangements observed are consistent with those reported for other breast cancers, both primary and secondary. Several markers involving extra copies of 1q were seen, the #16 was monosomic and translocations involving chromosomes 9, 11, and 17 were observed (Fig. 1). Like other cell lines, these far-advanced tumor cells may represent only a small fraction of the original tumor population.

Several conclusions can be drawn from our studies. First, the diploid cells observed so often in preparations from breast cancers may not be contaminating stromal or inflammatory cells but may represent a subset of truly diploid cancer cells. Second, our culture system, originally developed to propagate normal breast tissues, appears selective against aneuploid populations from primary and secondary tumors. And third, that only when aneuploid cells from tumors have acquired great biological autonomy, are they capable of growing under the culture conditions described. If these conclusions are correct, then the primary events in breast cancer may often not be associated with chromosomal aberrations. Many breast cancers are markedly heterogeneous, morphologically and cytogenetically, and the frequent chromosomal aberrations in primary and metastatic tumors may represent secondary events reflecting the acquisition of characteristics common to many tumor cell populations. Their presence within primary tumors may indicate the

extent of cytologic and cytogenetic evolution that has occurred within the developing primary lesions.

VII. GENE AMPLIFICATION

The morphologic anomalies described as homogeneously stained regions (HSR) or double minutes (DM) are generally accepted as chromosomal manifestations of gene amplification and have often been found in association with drug resistance. Both HSRs and DMs have been reported in breast cancers from various sources.

Three primary mammary cancers with HSR-bearing chromosomes were described, two of which had regions corresponding to the classic description of an HSR while in the third the abnormal region was partially stained by C-banding (36). An HSR was also reported in a direct preparation of cells from a breast cancer pleural effusion (47). HSR-positive chromosomes were noted in two cell lines, one derived from breast cancer and the second of suspected breast tumor origin. An unusual HSR, observed in a metastasis-derived cell line, was uniformly brightly fluorescent but with G-banding showed non-homogeneous repeated bands of the type referred to as ABR (abnormally banded regions) (5). In none of these cases was the chromosome bearing the HSR identified.

The frequency of chromosomal amplification was emphasized recently in a study of fresh solid tumors and malignant effusions (24). Many of the primary cancers in this study were from individuals who had received no

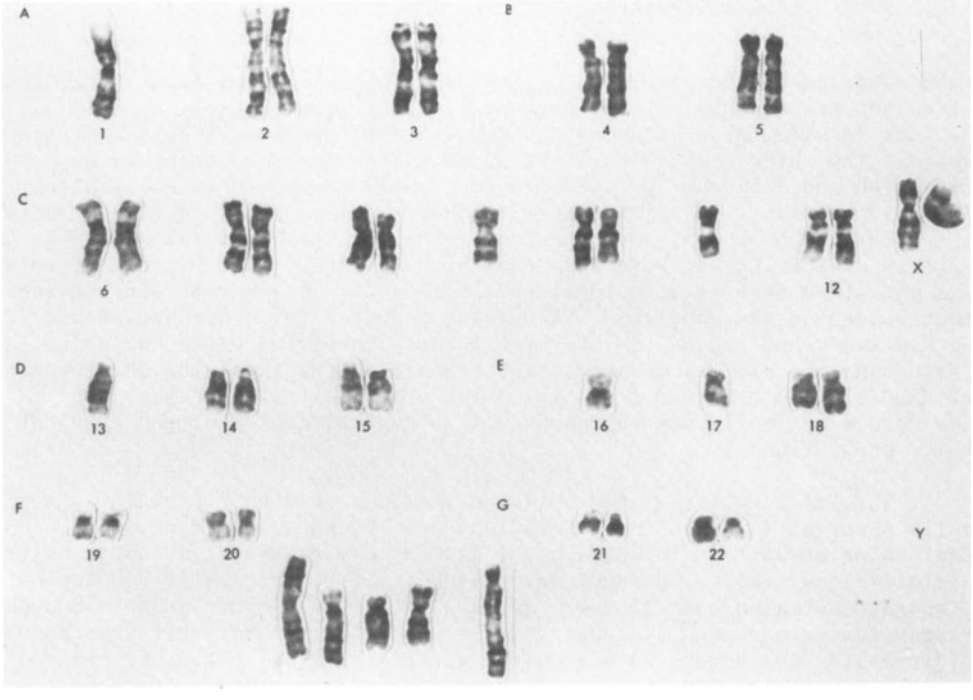

Figure 1: Karyotype from first passage of a breast cancer effusion fluid that developed into a cell line. It is interpreted as 45,XX,-1,-9,-11, -13,-16,-17,+5 markers. The markers in the bottom row reading from the left are identified as t(1q:13q), 1p-(p22), inv(11)(p15q13), t(9q:17q), and inv(1)(p36q21). The 1p-(p22) resembles a marker described in (30) as t(1:3)(p33:p25).

chemotherapy. DMs were observed in 15 of 22 solid breast tumor samples, including 2 of 4 metastases. In cases where more than 10 metaphases contained DMs, no more than 1 or 2 such pairs were found in most cells. Cell with more than 6 DM pairs were rare in any case. In contrast, effusion fluids from breast cancer patients tended to have large numbers of cells exhibiting the phenomenon and more frequent cells with larger numbers of DM (24). Since both DM frequency per cell and DM-bearing cells per tumor were low, these observations could have been overlooked easily in earlier studies. Small numbers of DMs are unlikely to be detected in flow studies. Clearly, chromosomal forms of gene amplification can be found in cases where no chemotherapy has yet been employed. It has been suggested that amplification is a manifestation of a far-advanced (aggressive, metastatic or effusion) form of cancer. Oncogene amplification has been associated with advanced stages of disease in neuroblastoma (8). If the chromosomal observations in breast cancer were correlated with evidence of oncogene amplification, this might also provide a measure of tumor cell progression. Retrospective correlation of the genetic findings with the clinical course of individual patients is therefore an essential next step.

VIII. ONCOGENES

The location of oncogenes within the karyotypes has provided clues to one role for chromosome modification in cancer biology. The proto-oncogenes, normal cellular components, appear to function in growth, regulation and differentiation. In malignant tissues, both structural (mutational) and quantitative alterations of these genes have been identified. Breast cancer, like other human tumors, has been the subject of intense search for alterations of oncogene expression, amplification, or structural modification. A screen of human malignancies for quantitative oncogene mRNA as a measure of gene expression included four cases of breast cancer (55); there was strong expression of c-fms in three cases and some expression of other oncogenes. In no case was there comparison between normal tissue and the breast tumor. More recent studies examined DNA rather than gene product, by Southern blot hybridization with nine cellular oncogene probes (68). Ten breast cancers were among the 71 epithelial tumors studied. The investigators reported increase in gene copy number for c-myc in two of the ten breast cancers. Both were relatively large tumors, both had positive axillary nodes, and were considered aggressive lesions. Decreased intensity of a c-Ha-ras band in one tumor was interpreted as loss of an allele. A similar altered allelic ratio at the c-myc locus, noted for a breast cancer metastasis, was not found in the primary tumor nor in normal breast tissue from the same patient. While the decreased band intensities were interpreted as allelic loss, the authors could not rule out chromosome loss or recombination as an explanation. Since the allelic bands were not completely lost in any case, these results might also suggest heterogeneity within the tumor cell population.

Other approaches to the study of oncogene alteration have employed breast cancer cell lines. Heterogeneity for expression of the oncogene, N-ras, is an aspect of variable phenotype in the MCF-7 line which also shows marked karyotypic instability (54). When seven sublines of MCF-7 were compared after digestion with Eco-Rl, variation over a ten-fold range in the amount of N-ras hybridization DNA was found (25). None of three other breast cancer cell lines or four primary breast cancers showed N-ras amplification. Amplification in MCF-7 was not associated with either DMs or an HSR (both present), but was localized to a marker chromosome of undetermined origins. Another breast cancer line, SKBR-3, demonstrated a ten-fold increase in c-myc copy number, together with increased expression of c-myc RNA (38). No genomic rearrangements of the c-myc was found. MCF-7 and three other breast cancer cell lines did not show amplification of c-myc. No cytologic localization of the amplified gene was presented. Yet another

breast cancer line, SW613-F, also revealed c-myc amplification, in this case correlated with increased number of DMs per tumor cell (44). However, oncogene amplification was maintained even when the DMs were lost with passage of time, and in those cells, the gene was integrated into a marker chromosome. Similar to other studies, no evidence of genomic rearrangement was found. The oncogene amplification was increased by passage through nude mice, suggesting an association with more aggressive components within the tumor. Another study which supported amplification of N-ras in the MCF-7 line described above, also recorded the presence of two other, heretofore unrecognized, transforming genes in DNA from the same cells (21). The results from cell lines must be viewed with caution, given doubts about the degree to which they represent primary tumors; however, together with emerging evidence of oncogene expression and gene amplification in fresh tumors they provide support for a role for chromosome aberrations in tumor progression.

IX. PREDISPOSING FACTORS

Inherited predisposition to cancer has been associated with chromosome instability that may be expressed spontaneously, after exposure to cancer-causing agents, or under certain culture conditions. From studies of patients with hereditary colon cancer syndromes, the hypothesis emerged that cells grown in culture that appear normal by other criteria could demonstrate a "mutant cancer-prone genotype" and that either increased tetraploidy or hyperdiploidy could be considered as expressions of cancer-prone genes (15). Similar studies of cells from breast cancer patients have compared results in individuals from hereditary breast cancer families with and without the disease, with individuals with breast cancer but without positive family history, and with selected controls (41). They report hyperdiploidy in vitro in six out of seven breast cancer patients from families with hereditary breast cancer and in one of four individuals with non-hereditary breast cancer. Similar positive findings were reported for several unaffected first degree relatives of the breast cancer patients. Increased tetraploidy was not observed and it was concluded that hyperdiploidy in culture was a marker in some individuals for heritable breast cancer (41).

Another chromosomal approach has been used to investigate propensity to increased cancer incidence. Constitutive heterochromatin (highly repetitive DNA sequences) may function in mitotic or meiotic pairing and segregation or may be involved in transpositions. Large blocks of heterochromatin are present on human chromosomes 1, 9, and 16 near the centromeres. Heteromorphisms of these regions, recognized as quantitative differences in C-band positive material between two homologs, have been sought in cancer patients. A group of 54 breast cancer patients, twelve with positive family histories, were compared with a control group of randomly selected blood donors (6). Statistically significant differences were found between the patients and the controls, with C-band increases in the cancer patients. Differences were also reported between sporadic and familial breast cancer patients and between pre- and post-menopausal cancer patients. The prevalence of inversions on chromosome 1 and 9 was also significantly higher in cancer patients. Thus a constitutional chromosomal pattern was identified which may confer greater risk for breast cancer development.

X. CONCLUDING REMARKS

Strikingly little cytogenetic data has been obtained directly from human breast cancers. Some are near-diploid with few karyotypic alterations while others show extensive chromosome rearrangement, whether the tumors are diploid or aneuploid. There is karyotypic variation among cells within the same tumor. Some tumors are bimodal and appear to contain normal diploid

cells, an impression that is reinforced by results from short-term cultures (10,66). Many breast tumor cells, from primary, metastatic, effusion or cell culture sources, show preferential involvement of 1q. Since most breast cancers originate from the same cell type, they should display a similar complex of associated chromosomal lesions. Evidence from the mouse mammary cancer model supports the same themes which emerge from study of human tumors -- that there is tumor-associated specificity of chromosome aberration and that there is cytogenetic heterogeneity within tumors. Limitations of each of the means available for study are so severe that we are only able to reconstruct an approximation of the genetic constitution of human tumors by using many different sources of data.

Sandberg has proposed that key events in tumor formation involve chromosome rearrangements (52). The critical alteration is rearrangement of a genetic locus unique for each cell and tissue type. Thus, a primary, specific, karyotypic change is proposed as relevant to tumor etiology. Other, secondary, karyotypic changes may vary among cells and tumors of the same site and type. The latter changes could be responsible for biological attributes such as invasiveness or metastasis and could be common to many tumors.

The common, apparently specific karyotypic alterations in breast cancers are more likely to be secondary changes for the following reasons: although prevalent, they are not present in all primary cancers. There is evidence that many primary cancers contain chromosomally unaltered cells. The most common observations, replications and rearrangement of 1q and, to a lesser extent, involvement of #16, have been found in other primary tumors including those of ovarian, endometrial, urinary tract and leukemic origins. Identification of these preferential sites is particularly intriguing in light of the postulated cancer-predisposing effects of increased heterochromatin. Last, the evidence for heterogeneity and evolution within primary tumors makes it likely that secondary karyotypic changes would be present within the primary lesion.

Molecular evidence, although scanty, also supports a role for chromosome changes in tumor progression. Neither chromosomal nor molecular data have yet contributed to our identification or understanding of initiating events in cancer. Dulbecco (19) has suggested sequencing the entire genome to resolve problems in the study of human cancer in terms that are particularly relevant to breast tumors -- "A major difficulty -- ("if important genes differ in cancers of different organs") -- is the heterogeneity of tumors and the lack of cultures representative of the various cell types present in cancer." At present, with ample evidence of heterogeneity in primary tumors and in culture systems and lacking evidence for specific primary karyotypic associations, we have little ammunition for a molecular attack on the primary genetic events in breast cancer.

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EPITHELIUM - MESENCHYME INTERACTION IN THE FETAL MAMMARY GLAND

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I. INTRODUCTION

Development of the vertebrate embryo depends heavily on cell and tissue interaction. In early stages, inductive interactions between blastemas and tissues are responsible for the diversification of cell types; later, interaction between already committed cell populations is required for the precise development of the complex structures of vertebrate organs (Kratochwil, 1983). These organs are typically formed by at least two tissues of different developmental history, e.g. epithelium and mesenchyme, which associate secondarily in development (Grobstein, 1967).

Developmental interdependence of these tissues has been demonstrated by means of tissue separation and experimental recombination. In the case of epithelial-mesenchymal organs, such studies (Grobstein, 1955) have shown that in almost all cases epithelial development depends on the presence of mesenchymal cells. The degree of organ specificity in mesenchymal requirement may vary (Grobstein, 1967, Kratochwil, 1972). Dependence on the mesenchyme is most obvious during the formation of an organ, in the case of glands or gland-like organs during the phase of most active epithelial growth and branching morphogenesis. Although experimentation is considerably more difficult in adult stages, it is assumed that epithelial-stromal interaction continue to play a role in the stabilization of organ structure

and cellular phenotype, or conversely, that disturbed tissue relationship may be involved in pathological processes (Tarin, 1972).

The mammary gland is a typical epithelial-mesenchymal organ, with some special features that make it particularly interesting: While the development of most comparable organs is completed before birth, growth, morphogenesis, cell diversification, and full phenotypic differentiation of the mammary gland occur in adult life. Extrapolating from other epithelial-mesenchymal organs it can thus be suspected that organogenetic interactions do indeed occur between adult tissues. Secondly, the cycles of development and involution of the adult gland are under obvious hormonal control. From other glands we know of the great importance of mesenchymal-epithelial interactions in particular for morphogenesis. It seems therefore reasonable to speculate that hormones may act (in part) by modulating tissue interaction processes.

In this brief chapter, I shall review information from classical tissue separation and recombination experiments done mostly in the fetal gland. The role of the stroma in the development of the adult gland is examined by Stockdale (see chapter in this volume), and the function of extracellular matrix molecules as possible mediators of the mesenchymal influence has already been discussed in the two preceeding chapters. Here, I shall concentrate on the role of the mesenchyme in the initiation and early stages of mammary development, and also on the interplay between hormone action and epithelial-mesenchymal tissue interaction.

II. EPITHELIUM-MESENCHYME INTERACTION IN THE FORMATION OF THE GLAND PRIMORDIUM

The epithelium of the mammary gland is derived from the embryonic epidermis. In most mammals, the earliest morphological indication of its development is the formation of a band or crest of thickened epithelium - the "mammary band (crest)" or "milk line". In species with numerous gland pairs, the milk line runs from the forelimb to the inguinal region, in others it may be restricted either to the thoracic or to the inguinal end. This mammary ridge then gives rise to the individual mammary buds. Even species with only one pair of glands, as the human, pass through the stage of the milk line, whereas the 5 gland pairs of the mouse apparently arise as individual thickenings of the epidermis (Balinsky, 1950; glands 4 and 5, though, are linked by thickened epidermis for a short time in 11-day embryos).

Tissue combination experiments aimed at elucidating the possible role of the underlying mesenchyme in this process have been done in the rabbit embryo (Propper, 1968). In this species, a prominent mammary band forms on day 13. If 12-day epidermis of the prospective mammary region was placed on mesenchyme from other areas, no mammary ridge could develop. On the other hand, 12-day "neutral" epidermis (from head or neck region) associated with 12-day "mammary" mesenchyme gave rise to mammary structures. The experiments suggest that it is the mesenchyme which initiates mammary development and that formation of the mammary ridge depends on mesenchymal induction. After that event, from day 13 onwards, mammary epithelium is capable of continuing its development even in association with "neutral" mesenchyme (but it should be noted that further differentiation has not been investigated beyond the formation of buds and sprouts).

This concept of mesenchymal induction of mammary development agrees with the experience derived from an analysis of skin development in general. It is (usually) the dermal component which determines position, patterning, and type of skin appendages formed in experimental dermis-epidermis combinations (for a review, see Sengel, 1976).

The response of the epithelium to the mesenchymal induction apparently does not include locally increased cell proliferation. To the contrary, Balinsky (1950) has found a reduced mitotic index in early mammary epithelium as compared to neighboring epidermis. Our own studies with tritiated thymidine (unpublished) showed even distribution of DNA-synthesizing cells throughout the epidermis during formation of the mammary buds in 11-day and 12-day mouse embryos, followed by conspicuous near-absence of TdR-incorporating mammary epithelial cells on day 13, while the bud still increases in size. Balinsky (1949) proposed that the thickening of the mammary placode and early growth of the bud is caused by migration of epidermal cells, a suggestion that might be supported by the appearance of the tissue in scanning electron micrographs (Propper, 1978).

While it seems plausible (from experience with other skin derivatives) that young epidermis from other body regions can also form mammary ridges and buds, the mammary-like ingrowths described when chick or duck epidermis was associated with rabbit mammary mesenchyme (Propper, 1969), would still seem to require more careful analysis. Unequivocal identification of the species origin of these epithelia has not been attempted, nor has the further development of these "buds" and "sprouts" been followed. Inductive interaction is known to occur between avian and mammalian tissues, with the restrictions imposed by the genetic repertoire of the species (for instance, chick epidermis responds to mouse dermis with an attempt to make feathers, not hair - see Sengel, 1976). If, however, the primary action of the mesenchyme of the mammary region is to cause cell streaming within the epidermal sheet, a response even of bird epidermis would not seem totally inconceivable.

At present, the processes underlying the first decisive event in mammary gland development are not understood at all, i.e. when the prospective mammary epithelial cells enter a developmental pathway different from their surrounding epidermal cells. The mechanism of mesenchymal induction is not known - as, unfortunately, is the case in all other inductive systems, as well. Neither do we know the primary differences in gene expression between the cells of the mammary bud and those of the epidermis. If the mesenchyme is indeed responsible for the position of the mammary ridge and/or buds, we have no indication as to what determines the "pattern" within the mesenchyme. At least at the morphological level, the mesenchyme under the mammary ridge, or under the individual mammary placodes of the mouse embryo, is not distinguishable from neighboring tissue. It is only during subsequent growth from of the bud that adjacent mesenchymal cells become oriented around the gland epithelium and thus can be recognized as "mammary" mesenchyme (below).

III. THE ROLE OF EPITHELIUM-MESENCHYME INTERACTIONS DURING THE "RESTING PHASE" OF THE MOUSE GLAND; THEIR IMPORTANCE FOR THE HORMONE RESPONSE OF THE MAMMARY RUDIMENT

Formation of the primordial buds of the mammary anlagen is not immediately followed by outgrowth and branching of the epithelium - as would be the case in other glands or gland-like organs. Instead, the mammary rudiment passes through a relatively extended period of slow growth and little or no morphogenetic activity. In the mouse gland, the epithelial buds form between day 11 and 12, but outgrowth of the primary sprout begins only at 16.5 days, just 2.5 days before birth. Such a period of quiescence has been observed in the development of the gland in a variety of species (Balinsky, 1950) and may thus be a general characteristic of mammary development.

A. Sexual Dimorphism in the Development of the Mouse Mammary Gland Rudiment

In the female mouse embryo, the epithelial bud changes very little between days 12 and 16. Originally attached directly to the epidermis, it acquires a "stalk" on day 13 which lengthens minimally during the next 2 days. Formation of this stalk on day 13 occurs almost without any cell division in the epithelium, suggesting cells flowing down from the epidermis. Epithelial DNA-synthesis is resumed on day 14 (unpublished). The mesenchyme under the 11-day placode and around the 12-day bud is morphologically entirely inconspicuous. During subsequent days, the nearest mesenchymal cells orient themselves tangentially around the epithelial bud and by day 14, a sort of mesenchymal "capsule" has formed around each bud. This "mammary mesenchyme" is distinct from the adjacent dermis by the orientation and shape of its cells, by the absence of all vascularization, and by an extracellular matrix that stains much more heavily with cationic dyes, such as Alcian blue (Kronberger, unpublished). The most characteristic differentiative property of this mesenchyme is its possession of sex steroid receptors (below), but it is never as clearly delineated from the rest of the dermal mesenchyme as is, for instance, the salivary gland's capsular mesenchyme in the lower jaw. The sequence of morphological changes suggest that this "mammary mesenchyme" forms under the influence of the epithelial bud.

More dramatic are the events taking place in the gland of male mouse fetuses during the "resting phase". The early development up to day 13 is the same in both sexes, but during the first hours of day 14, the "mammary mesenchyme" begins to condense in the male, eventually forming a very compact capsule around each bud. Later on the same day, the mesenchymal condensation slips upward to the stalk of the gland in the first 4 gland pairs, and moves under the bud in the fifth. In the anterior 4 glands the stalk stretches and eventually ruptures, the gland epithelium thus becoming permanently separated from the epidermis. The cells of the "stalk" remaining with the epidermis become necrotic and disappear, the lower portion of the gland epithelium may survive in part, depending on the position of the gland and the strain of mice (Raynaud and Raynaud, 1953 a; Raynaud et al. 1970; Kratochwil, 1971). If male mice possess remnants of a mammary gland, their ducts are never connected to the epidermis and they do not have nipples. The epithelial rudiment of the 5th gland pair (second inguinal) is pushed out through the epidermis and leaves no trace (Raynaud and Raynaud, 1953 b). This destruction of the male mammary anlagen is caused by testicular androgens, as shown by experiments in vivo (Raynaud and Raynaud, 1956) and in vitro (Kratochwil, 1971).

Although this sexual dimorphism in fetal mammary development is not typical for mammals, and not even present in all rodents (e.g. not in the guinea pig), its analysis has provided some valuable insight into the interplay between hormone action and epithelial-mesenchymal tissue interaction.

B. Hormone Effect Mediated by Tissue Interaction

Both tissues of the gland are visibly affected by the hormone: the mesenchyme condenses and the epithelium is destroyed. Organ culture experiments had shown that testosterone acts directly on the gland, (Kratochwil, 1971), but could not decide whether the hormone acts directly on both tissues. The mechanism of hormone action was elucidated in experimental tissue combination utilizing the androgen-insensitive Tfm ("testicular feminization") mutant of the mouse (Lyon and Hawkes, 1970). At the 12-day stage, the epithelial mammary bud can be cleanly separated from its endowing

mesenchyme and recombined with mesenchyme from another source. Recombination explants composed of androgen-insensitive epithelium and "wild-type" mesenchyme responded to testosterone (i.e. the mesenchyme condensed and the epithelium was destroyed), whereas the reciprocal combinations of normal epithelium with androgen-insensitive mesenchyme completely failed to react (Kratochwil and Schwartz, 1976; Drews and Drews, 1977). It was thus established that the hormone has only one target tissue in the gland and that its effect on the epithelium is mediated by the mesenchyme. The mechanism by which testosterone-stimulated mesenchymal cells destroy the epithelial anlage is still not known.

Tissue destruction may justly be considered an atypical result of mesenchyme-epithelium interaction. It is therefore important to note that the same situation prevails in the development of the urogenital sinus: During fetal sexual differentiation, testosterone causes the development of the prostate with its glandular epithelium, whereas the sinus epithelium remains smooth in the female. Experiments combining wild-type and androgen-insensitive (Tfm) tissues have established that here, too, testosterone acts on the mesenchyme (Cunha and Lung, 1978). Growth of the epithelium and its glandular development are the result of hormone-initiated mesenchyme-epithelium interaction.

The fetal development of the mammary gland is not only affected by androgenic steroids but also by estrogens. The latter effect is not physiological and can only be demonstrated experimentally. Injection of estrogens into pregnant mothers or directly into the fetus caused severe inhibition of gland development and nipple malformation (Raynaud and Raynaud, 1956). In organ culture, concentrations of estradiol as low as 0.1 nM can prevent outgrowth of the primary sprout (without causing effects related to the testosterone response). Although no receptor mutant is available for unequivocal experimentation, ^{3}H-estradiol autoradiographs provided quite convincing evidence that the mesenchyme is also the target for estrogenic steroids (Kratochwil and Wasner, in preparation). This result again implicates mesenchymal-epithelial interaction in the inhibition of epithelial outgrowth. As in the processes mediated by testosterone, the mechanism is not known. It cannot even be decided whether epithelial growth depends on mesenchymal factors, the production of which is blocked by the hormone, or whether estrogen-influenced mesenchyme actively inhibits further epithelial growth and development.

This negative effect of estrogens in the mammary gland again has a "positive" counterpart in the development of the female genital tract. In neonatal mice, estrogens stimulate growth and hypertrophy of uterine epithelium in the apparent absence of epithelial estrogen receptors. By autoradiographic demonstration, as well as by whole cell binding assays, high affinity and saturable estrogen-binding sites could be found in the mesenchyme only (Bigsby and Cunha, 1986). Mesenchymal sex steroid-binding was demonstrated for a number of embryonic organs in the chick, especially those subject to sexual differentiation (Gasc and Stumpf, 1981 a, b). This is an interesting finding in view of earlier tissue combination studies done in a variety of epithelial-mesenchymal organs, which had shown that organ morphogenesis, including the development of characteristic epithelial structures, is more dependent on the mesenchyme than on the epithelium itself (Alescio and Cassini, 1962; Kratochwil, 1969; Sengel and Dhouailly, 1977; Cunha et al., 1980). It would thus appear that a hormone controlling the formation of an organ (rather than its physiological function) could do so more efficiently by acting on the dominant partner in organogenetic tissue interaction, i.e., the mesenchyme (Cunha et al., 1983).

C. Tissue Interaction in the Development of Hormone Responsiveness

While the epithelium appear as the passive partner in hormone-induced tissue interaction, it was found to play the active role in the development of hormone responsiveness - at least in the mammary gland.

The testosterone-induced destruction of the mammary anlagen in male fetuses takes place on day 14. In vitro experiments have shown that there exists only a short androgen-responsive "window" during mammary development: The rudiment becomes sensitive to the hormone late on day 13, and is no longer affected on day 15 (Kratochwil, 1977). The development of androgen responsiveness from day 12 to day 14 correlates well with the development of specific androgen-binding sites (Wasner, Hennermann and Kratochwil, 1983). Most importantly, mammary rudiments explanted on day 12 or even day 11, become responsive in vitro in due time. Development (and also loss) of testosterone responsiveness must therefore be controlled by processes within the gland rudiment itself (Kratochwil, 1977). When, however, the mesenchyme (i.e. the target tissue of the hormone) was explanted without epithelium, it never showed any response to testosterone (e.g., condensation), and neither when mammary epithelium was replaced by epithelia from other organs (Durnberger and Kratochwil, 1980). In contrast, mammary epithelia from other species (rat and rabbit; the glands of the latter are not affected by testosterone) placed on mouse mammary mesenchyme caused a response to testosterone.

The function of mammary epithelium was eventually revealed by ^{3}H-testosterone autoradiographs of 14-day mammary rudiments: steroid-binding was restricted to the mesenchyme, as was to be expected from the Tfm - wild-type tissue combination experiments (above), but it was also seen that only a very few layers of mesenchymal cells around each gland bud, the "mammary" mesenchyme referred to above, retained the hormone (Heuberger et al., 1982). In view of earlier experiments that had excluded the migration of mesenchymal cells to the mammary bud (Durnberger and Kratochwil, 1980), this distribution (as seen in Fig. 1) strongly suggested that mammary epithelium had induced the formation of androgen receptors in the adjacent "mammary" mesenchyme. Experimental evidence for induction was finally obtained by combining 12-day epithelial buds with mesenchyme of the mammary region (but which had not been in contact with a gland before). During two or three days of subsequent culture, each of these epithelia became surrounded by receptor-positive mesenchymal cells (Heuberger et al., 1982).

Induction of testosterone receptors is organ-specific (i.e. epithelia from other organ rudiments are ineffective) but not species-specific, as seen by the positive results obtained in combinations of rat or rabbit mammary epithelia with mouse mesenchyme (Durnberger and Kratochwil, 1980). These are the characteristics of embryonic tissue interactions, as is also the short range of the inductive influence.

The same situation was found to prevail for the formation of estrogen receptors. Only the "mammary" mesenchyme proper binds ^{3}H-estradiol, and estrogen-binding sites can be induced in mesenchyme of the lateral body wall by its combination with epithelial mammary buds (Kratochwil and Wasner, in preparation). In autoradiographs of embryonic chick organs, ^{3}H-estradiol-binding mesenchymal cells were also found in the vicinity of epithelia (Stumpf et al., 1980), and it therefore appears possible that epithelial induction of mesenchymal steroid receptor synthesis is a more widespread phenomenon.

Hormone action and epithelium-mesenchyme tissue interaction are thus interdependent in at least two steps during mammary development: first, an inductive signal from the epithelium causes the synthesis of sex steroid

receptors in adjacent mesenchymal cells. (This dependence of one characteristic differentiative property of the mesenchyme on the presence of the epithelium provides the best evidence for the assumption - originally based on purely morphological observations - that "mammary" mesenchyme develops

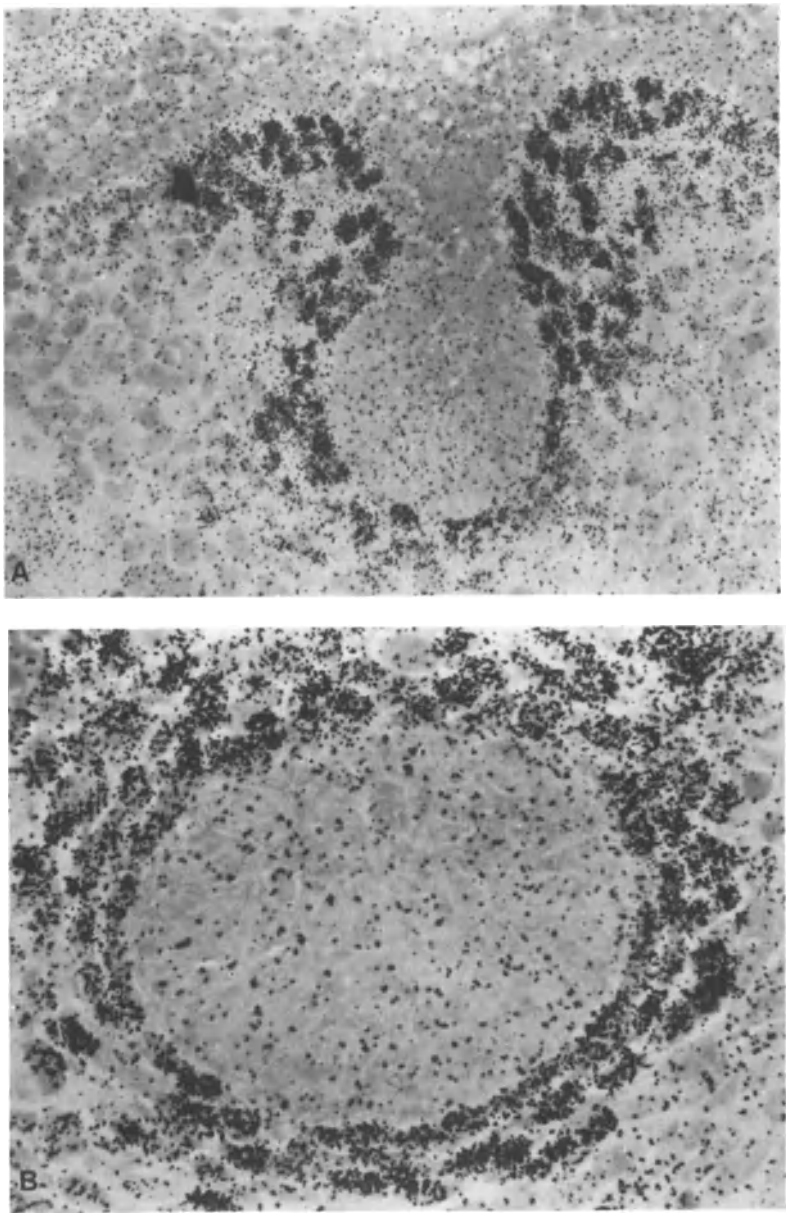

Figure 1: ^{3}H-Dihydrotestosterone autoradiographs of 14-day mouse mammary rudiments.

A. Median section through a mammary rudiment, epidermis on top. Note that steroid-binding mesenchymal cells are found only around the gland bud and its stalk. (From Heuberger et al., 1982).
B. Oblique section through an epithelial gland bud. Note the well defined envelope of receptor-positive mesenchymal cells around the epithelium, indicating the short range of the inductive influences.

secondarily under the influence of the epithelial bud.) In the second step, testosterone-stimulated mesenchymal cells destroy the epithelial anlage in male fetuses, thus mediating the effect of the hormone on the bud. Similarly, outgrowth of the epithelial sprout can be prevented by estrogens acting on the surrounding mesenchyme.

IV. OUTGROWTH AND EARLY BRANCHING

A. Outgrowth of the Primary Sprout

Eventually, after this extended period of conspicuous morphogenetic inactivity, one or several ducts grow out from the primordial mammary bud. In the (female) mouse fetus, outgrowth of a single primary sprout occurs at 16.5 days in the thoracic glands, at 17 days in the inguinal pairs. The time course of this early development, especially the transition from "resting phase" to outgrowth, does not appear to be regulated by changes in the hormonal milieu of the fetus: explanted gland rudiments adhere to the same strict temporal program in vitro (Kratochwil, 1969). It is not known whether outgrowth is triggered by mesenchyme-epithelium interaction, although its inhibition by estrogens (which act on the mesenchyme - above) indicates active participation of the "mammary mesenchyme" in this process. From heterochronic combinations of 16-day epithelium with 12-day mesenchyme, which had shown that the time of outgrowth is determined by the stage of the epithelium (Kratochwil, 1969), does not necessarily follow that the "clock" for development resides in the latter tissue; the older epithelium could have been exposed to the decisive mesenchymal signals before recombination. Although the sprout grows by active cell proliferation, especially at its tip, its outgrowth from the bud is not simply correlated with the resumption of epithelial DNA-synthesis, which occurs 2 days earlier.

B. Mammary Epithelium Interacts with Two Types of Mesenchyme

The outgrowing epithelial sprout breaks through the capsular "mammary" mesenchyme and enters the mesenchyme of the future fat pad. The fibroblastic "mammary" mesenchyme stays behind and - still identifiable by its steroid receptors - eventually becomes the mesenchyme of the nipple (Wasner, Hennermann and Kratochwil, 1983). All branching activity of the gland takes place in this "second" mesenchyme which undergoes adipose conversion 2 days after birth (Sakakura et al., 1982) and independently on its invasion by mammary ducts. During the earliest, organogenetic phase of its development, mammary epithelium is thus associated with two different types of mesenchyme in sequence, a unique feature of its development (Sakakura et al., 1982).

C. Mesenchymal Control of Early Epithelial Branching

In relatively short-term organ culture, the development of mammary epithelium does not appear to depend specifically on the fat pad precursor mesenchyme. It can grow and branch in dermal and subdermal mesenchyme from other body regions, in jaw mesenchyme, and also in mesenchyme of the lateral body wall of chick embryos (unpublished). The overall morphology with the characteristic monopodial branching pattern of the gland remains rather typical in these associations. An entirely different result is obtained, however, when mammary epithelium is forced to grow into mesenchyme of the submandibular salivary land. Aside from a markedly increased growth rate, the morphology of the gland epithelium and especially the pattern of its branching activity is modified in a way to resemble a salivary rather than a mammary gland (Kratochwil, 1969): instead of slim ducts with lateral secondary buds ("monopodial" branching), the epithelium forms adenomere-like end bulbs which are cleaved as in the salivary gland. A particularly high growth-promoting activity of salivary mesenchyme (which can also be deduced

from experiments with pancreas epithelium (Rutter et al., 1964) might be responsible for the enhanced epithelial growth but it cannot easily explain the effect on the branching pattern. Experiments on the lung (Alescio and Cassini, 1962; Wessells, 1970) and the urogenital sinus (Cunha et al., 1981) have shown that the mesenchyme governs epithelial morphogenesis. In these cases, however, the effect seen was an induction of budding per se, which to a large part could be accounted for by mere stimulation of growth. By comparison, mammary epithelium growing in salivary mesenchyme shows that the mesenchyme controls epithelial morphogenesis in a much more delicate way. Interestingly, the cytodifferentiation of mammary epithelium is not affected by salivary mesenchyme. Upon implantation into lactating hosts, such combination cultures produced milk (Sakakura et al., 1976).

Since mesenchymes from diverse sources can exert such characteristic effects on epithelial development (as shown not only in the mammary gland), the question arises to the significance of the sequential association of mammary epithelium with 2 types of mesenchyme. Essentially all of the gland's development takes place in the second mesenchyme, the fatty stroma. This tissue is the ideal grafting site for mammary epithelium and allows histotypic reorganization even of cells previously grown in monolayer cultures (Daniel and DeOme, 1966). The responsiveness of the adult gland epithelium to its mesenchymal environment was shown in the experiments by Sakakura and collaborators (1979 b) who implanted pieces of fetal mesenchyme (the fibroblastic "mammary" mesenchyme, or salivary mesenchyme) into the fat pad. The host gland colonizing these transplants underwent localized hyperplasia and formed irregular ductal and ampullar structures. The authors propose that it is the fatty mammary stroma which determines the characteristic morphology of the adult gland with its widely spaced ducts and branch points (referred to as "stretching out" of the duct system by Sakakura, 1983).

It is not known whether the transient association of mammary epithelium with the fibroblastic "mammary" mesenchyme during the resting phase is of importance for its further development. The only established function of this mesenchyme - destruction of the epithelial bud in male fetuses under the influence of testosterone - is of no obvious biological significance and, in addition, is a phenomenon found only in mouse-like rodents. After outgrowth of the primary sprout, this fibroblastic mesenchyme populates the nipple (then, perhaps, its estrogen receptors may become relevant). The question remains whether the "mammary" mesenchyme of the resting stage interacts with the newly formed epithelial bud to allow its developmental segregation from the epidermis and/or maturation before actual outgrowth. Sakakura (1983) has attempted to "skip" the association between mammary epithelium and the fibroblastic "mammary" mesenchyme by combining young epithelium directly with the fat pad (precursor). In an earlier study (Sakakura et al., 1979 a) they found that 13-day epithelia, separated from their mesenchyme and transplanted into the cleared fat pad of juvenile female mice, could develop to an apparently normal gland. More recently (Sakakura, 1983), however, it is reported that combinations of 12-day and 14-day mammary epithelia with fat pad precursor mesenchyme failed to develop, whereas the same experiment done with 17-day mammary epithelium (taken after the resting phase) was reasonably successful. Unfortunately, in this latter study, young mammary epithelia (12 and 14 day) separated from, and recombined with, their own fibroblastic "mammary" mesenchyme gave just as negative results (Sakakura, 1983), rendering the experiment somewhat inconclusive. Although the unusual high sensitivity of the 12 to 15-day mammary bud to separation from its mesenchyme (also in our experience) may suggest some specific dependence on tissue association, we have at present no idea about the developmental role played by the "mammary" mesenchyme during the resting phase.

V. SUMMARY

An attempt to summarize our current knowledge of epithelial-mesenchymal interactions during the fetal development of the mammary gland is found in the following Table and in Fig. 2.

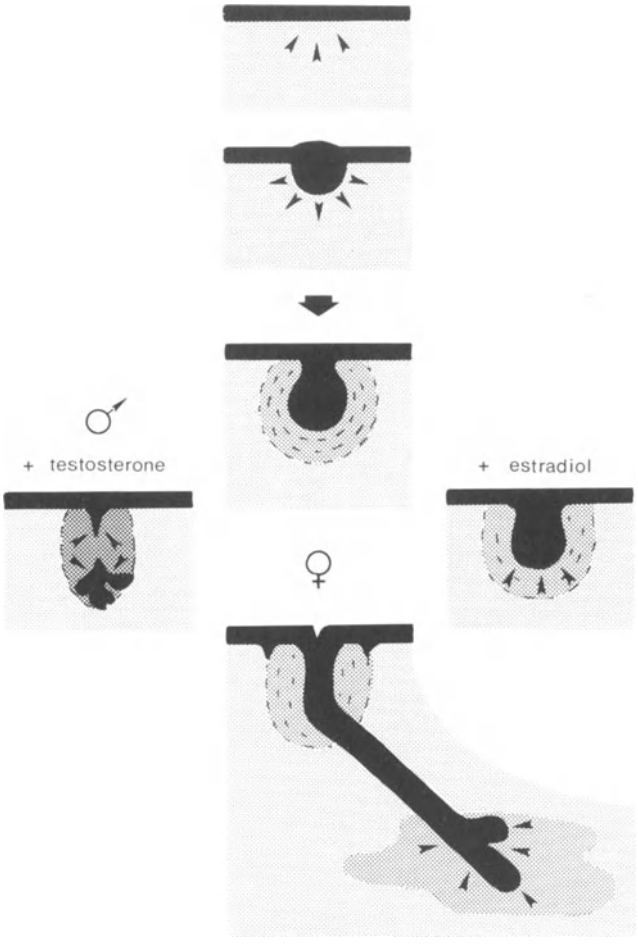

Figure 2: Schematic representation of the known interactive events during fetal development of the mammary gland. First, the mesenchyme induces formation of the epithelial mammary bands or buds. Then, the young bud induces the development of the fibroblastic "mammary" mesenchyme, one characteristic of which is the possession of androgen and of estrogen receptors. Next, on day 14 in male (mouse) fetuses, testosterone acts on these mesenchymal cells which then in turn destroy the epithelial anlage (left). After experimental administration of estrogens (right), estrogen-responsive mesenchymal cells prevent outgrowth of the primary epithelial sprout. Bottom: In 16 to 17-day fetuses, the primary sprout pushes through the fibroblastic "mammary" mesenchyme and invades the fat pad precursor. The properties of this mesenchyme determine the further growth and branching pattern of the gland epithelium.

Stage (age of embryo in the mouse)		Epithelium	Mesenchyme	Ep ⟷ Mes	Interaction
10 (?)		epidermal/ectodermal sheet	inconspicuous loose mesenchyme	E ← M	Induction of mammary buds (mammary band in the rabbit)
11		mammary placode	inconspicuous		
12		mammary bud	inconspicuous		
13		stalked bud; temporary arrest of DNA-synthesis	beginning orientation of mesenchymal cells around epithelial bud; synthesis of steroid receptors	E → M	Organization of "mammary" mesenchyme Induction of androgen and of estrogen receptors
14		stalked bud; resumption of DNA-synthesis	testosterone response in males: mesenchymal condensation and destruction of epitheium	E ← M	Testosterone-induced destruction of epithelial mammary buds by "mammary" mesenchyme
				E ← M	experimentally: Estrogen-influenced mesenchyme prevents outgrowth of the gland sprout on day 16 (precise time of interaction not known)
15	"resting" period	bud with longer stalk	fat pad precursor mesenchyme becomes identifiable; fibroblastic "mammary" mesenchyme loses androgen-sensitivity		
16	growth period	outgrowth of the primary sprout	fat pad precursor mesenchyme distinct		
17		outgrowing sprout leaves the fibroblastic "mammary" mesenchyme, enters fat pad precursor; beginning of epithelial branching	"mammary" mesenchyme is incorporated into the nipple;		
				E ← M	Mesenchymal control of epithelial growth and branching activity
21 (= day 2 p.p.)			adipose conversion of the fat pad precursor		

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EXTRACELLULAR MATRIX EFFECTS ON MAMMARY CELL BEHAVIOR

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I. INTRODUCTION

Developmental biologists have long recognized that the extracellular matrix (ECM) plays a vital role in various developmental processes such as tissue morphogenesis and cytodifferentiation (1,2,3). Expression of the proliferative, morphogenetic and functional potentialities of the mammary epithelium *in vivo* and in culture are clearly influenced by the ECM (4,5). The mechanistic details of how the ECM affects mammary epithelial cell behavior remain to be defined. As the ECM supports the mammary epithelium *in vivo* includes both acellular and cellular domains, ECM-mediated effects are likely to include interactions between different acellular ECM components, between cells and acellular ECM components and between different cell types. Additionally, such interactions must be coupled to the hormonal and growth factor requirements known to exist for expression of the mammary cell phenotype.

In an effort to dissect the complex process that ensures proper mammary gland function, mammary gland biologists have devoted a substantial amount of time to developing culture systems that permit growth and differentiation of mammary epithelial cells. As a result, normal mammary epithelial cells can be dissociated in a viable form from their *in vivo*

topological relationships, can be cultured for extended periods of time and can be hormonally modulated to express differentiation markers. Four different culture systems have been shown to support the expression of tissue-specific markers by dissociated mammary cells. These are: (a) cell culture on two-dimensional floating collagen (stromal) gels (6,7); (b) cell culture within three-dimensional collagen gels (8,9); (c) cell culture on complex biomatrices (10,11,12,13,14); and (d) cocultivation of mammary epithelial cells with adipocytes (15) or fibroblasts (16).

Different questions have been explored with each of these approaches and each system presents advantages and limitations. A unifying conclusion however, is that an important determinant in the expression of the mammary cell phenotype is the ECM used as culture substratum, be it part of the acellular or cellular compartment of the ECM. These approaches with cells in culture thus provide good model systems to investigate the role of the ECM in normal mammary cell growth and differentiation and in the neoplastic process and to explore how the expression of specific genes is regulated by hormones.

This review will focus on studies concerning the modulation of mammary cell behavior in culture in relation to the ECM used as substratum. The discussion presented will be dominated by data on the mouse model system as our most extensive knowledge on the subject derives from studies with this system. Special emphasis will be placed on contrasting results with the different culture systems listed above both with respect to cell growth and differentiation. The behavior of "normal" mammary cell lines as well as that of neoplastically altered mammary cell populations will be compared for conditions which provide a culture environment permissive for growth and/or differentiation of normal cells. It is beyond the scope of this review to discuss the vast number of studies that have advanced our knowledge of the hormonal and growth factor requirements of the mammary gland <u>in vivo</u> and in culture; the readers are referred to two recent and excellent reviews that address this topic thoroughly (17,18).

II. THE TWO-DIMENSIONAL COLLAGEN GEL CULTURE SYSTEM: EXPRESSION OF TISSUE-SPECIFIC MARKERS

The differentiated phenotype of mammary epithelial cells cannot be maintained when these cells are cultured on a conventional plastic substratum. A notable advance in the field of mammary epithelial cell culture was the development by Emerman and Pitelka (6) of culture conditions that support expression of mammary cell differentiation. Based on studies by Michalopoulos and Pitot (19) on adult rat liver epithelium, Emerman and Pitelka introduced the use of a floating stromal collagen gels for the cultivation of dissociated mouse mammary epithelial cells. Mammary epithelial cells from pregnant mice express features of the differentiated phenotype in culture in response to lactogenic hormones only when plated on floating collagen gels. Differentiation markers maintained or enhanced include cell surface polarization, microvilli, epithelial junctional complexes, a well-developed secretory apparatus and synthesis and secretion of the milk protein, gamma casein (6,7,20,21). Correlating with expression of cell differentiation is a change in cell shape from squamous, if cultured on an inflexible surface, to columnar on floating collagen gels (6,21). The ability to modulate expression of mammary cell-specific functions by the culture substratum is well illustrated by experiments where "dedifferentiated" cells, as a result of culture either on plastic or on inflexible collagen gels, reexpress differentiation markers when cycled to floating collagen gels (6,22).

The recent studies of Bissell and collaborators with the two-dimensional collagen system provide the most detailed data on the modulatory

effects of the ECM on the synthesis and secretion of milk components (13,23,24,25,26). Using mouse mammary cells from mid- to late-pregnant mice, these studies have begun to address the important issue of whether the ECM on which the cells are cultured can modulate the expression of the full repertoire of biochemical products characteristic of the in vivo phenotype and, if so, whether expression of different products is regulated coordinately.

In the course of pregnancy, mammary epithelial cells must activate a complex program of hormone-responsive differentiation in order to achieve their fully functional state at the onset of lactation namely, milk secretion (17,18). The constituents of milk are diverse and include proteins, sugars, lipids and salts. The precise timing of their synthesis must be synchronized with the onset of milk secretion; the details of how this is accomplished have not been elucidated. The milk protein genes are good markers for monitoring distinct synthetic activities that must be induced, maintained and modulated if the in vivo secretory mammary phenotype is to be studied under controlled culture conditions. Briefly, the major protein components of mouse milk (Table 1 and Fig. 1), the caseins, are a family of phosphoproteins comprising several molecular species referred to as alpha-1 (43 Kd), alpha 2 (39 Kd), beta (30 Kd), gamma (23 Kd), delta (21 Kd) and epsilon (14.5 Kd) (27). The mouse whey proteins include: (a) α-lactalbumin

Table 1

Synthesis and secretion of caseins by mouse mammary cells from pregnant mice in short term cultures

Culture conditions[a]		Relative amounts of mouse caseins[c]									
		Cell-associated					Secreted				
Substratum	Lactogenic hormones[b]	α_1	α_2	β	γ	δ	α_1	α_2	β	γ	δ
Freshly isolated uncultured mammary cells	h^+	3+	3+	4+	+	+	3+	3+	4+	2+	+
Floating collagen gel	h^-	2+	+	-[d]	-	-	-	-	-	-	-
	h^+	4+	4+	4+	+	2+	4+	4+	4+	+	+
Attached collagen gel	h^-	+	-/+	-	-	-	-	-	-	-	-
	h^+	+	+	2+	-	-	-/+	-	-/+	-	-
Plastic	h^-	+	-/+	-	-	-	-	-	-	-	-
	h^+	-/+	-/+	+	-	-	-	-	-	-	-

[a] 7-12 days in culture

[b] h^+ with insulin, prolactin and hydrocortisone, h^- without these hormones

[c] compiled from references (13,23-26)

[d] not detectable

+ to 4+ reflect relative intensity of casein bands detected either by the immunoblot procedure or by immunoprecipitation of labeled proteins with antiserum to mouse caseins (normalizations for equal amounts of samples were assumed)

(a moiety of lactose synthetase and thus, a cofactor in the synthesis of lactose; ref. 28) and the whey acidic protein (WAP) both with apparent molecular weights of 14,000 (27,29,30); (b) the iron-binding protein, transferrin (24,27) and (c) the serum protein, albumin which is not synthesized by mammary cells but sequestered from the blood (31).

The specific proteins identified in cultures of mouse mammary cells as a function of the substrata have been grouped into four classes (13,24,25): (a) milk proteins whose hormone-dependent synthesis and secretion are modulated by the culture substrata (e.g., the caseins); (b) milk proteins that are not responsive to hormones and exhibit only marginal quantitative modulation by the culture substrata (e.g., transferrin and butyrophilin, a major glycoprotein in milk fat globule membranes); (c) milk proteins whose hormone-dependent synthesis cannot be maintained or elicited by the otherwise differentiation permissive flexible collagenous substratum (e.g., α-lactalbumin and WAP); and (d) a set of "culture-specific" proteins that are hormone and substrata independent and which are not detected in freshly isolated mammary cells or in mouse milk. Data on the expression of some of these proteins relative to the culture substrata and the hormonal environment are summarized in Tables 1 and 2. Note that these data were compiled from the gel patterns and text descriptions provided by several studies (13,23,24,25,26). For the sake of simplicity, the relative levels of the protein products indicated may not always reflect the quantitative complexities of the original data, particularly with respect to mRNA levels. Instead, such information is noted in the table below.

Mouse mammary cells continue to synthesize and secrete all casein polypeptides for up to one month in culture to the same extent as freshly isolated uncultured mammary cells only when maintained on floating collagen gels (Table 2; 13,24,25,26). On attached (or glutaraldehyde-fixed) collagen gels or on plastic, low levels of some of the caseins can be detected intracellularly even after 2 weeks of culture; these proteins are not secreted (Table 2; 13,25).

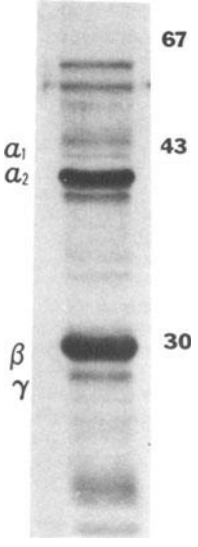

Figure 1. Mouse milk proteins separated in a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis stained with coomassie blue. Molecular weight markers: bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (21K) and lysozyme (14.3K).

Table 2

Expression of milk components other than caseins by mammary cells in culture

Source of tissue	Culture conditions[a]			Relative levels of expression[c,d]						
				Whey proteins		Transferrin (80 kd)		Butyrophilin (67 kd)		
	Type of culture	Culture substratum	Lactogenic hormones[b]	Alpha-lactalbumin	WAP	Cell associated	Secreted	Cell associated	Secreted	Lactose secretion
Mid-pregnant mice	Primary dissociated	Plastic	h-	-	-	++	++	NE[e]	NE	-
			h+	-	-	++	++	+	+	-
		On attached collagen gel	h-	-	-	++	++	NE	NE	-
			h+	-	-	++	++	++	++	-
		On floating collagen gel	h-	-	-	+++	+++	NE	NE	-
			h+	-/+	-/+	+++	+++	++	++	-
Mid-pregnant rats		On floating complex mammary-gland derived biomatrix	h+	4+	NE	NE	NE	NE	NE	3+
		On floating collagen gel	h+	-/+	NE	NE	NE	NE	NE	-
Mid-pregnant mice	Explant culture		h+	+	NE	NE	NE	NE	NE	+
			h+	4+	NE	NE	NE	NE	NE	3+

[a,b,c] as for Table 2 [d] compiled from references 9,12,17,22-24 [e] not examined

Caseins synthesized by cells on floating collagen gels are phosphorylated to a higher degree than those from cells cultured on inflexible substrata (13,25,26) and it has been suggested that alterations at this level may affect their secretory fate (25,26). Additionally, collagen gels that are freed to float contract to varying degrees (6,19) and this phenomenon is cell-mediated. Myoepithelial cells in vivo are known to facilitate milk ejection by their ability to contract around the secretory units and ducts in response to oxytocin (32). Although there is no evidence that myoepithelial cells can perform their contractile activities in culture, it is conceivable that the mere process of collagen gel contraction mimics myoepithelial cell behavior and thus potentiates the secretory response.

Metabolic labeling experiments detected low levels of α-caseins (in particular α-1) in the absence of lactogenic hormones and in serum-free medium on the three culture substrata examined (Table 2; ref. 13,25). Thus, in contrast to the other caseins, the α-caseins are less responsive to the modulatory effects of hormones and the ECM. The first caseins we have detected in the course of pregnancy is β-casein in mammary gland extracts from mice on the 10-12th day of pregnancy (ref. 33; and unpublished observations). Mammary epithelial cells from unprimed virgin mice can be induced to synthesize substantial amounts of β-caseins (but little or no α-caseins) in the three-dimensional collagen gel system (section III, B) corresponding to the midpregnant state (33). These observations, in agreement with those of Lee et al. (25), suggest that the expression of α-caseins need not be coordinate at least with that of β-casein.

Regulation of the synthesis and secretion of mouse milk proteins with respect to ECM influences has been studied in most detail for β-casein. β-casein expression is regulated at the transcriptional level by the hormonal environment, as is also observed for mammary cells in organ culture (17,18). In the absence of prolactin hydrocortisone, β-casein mRNA (and protein; Table 2) is not detected regardless of the substrata used for culture (25).

The ECM does modulate the magnitude of the response at the level of β-casein mRNA and by affecting the stability of the protein itself. This is illustrated by the observation that on floating collagen gels, mammary cells accumulate, in response to hormones, β-casein mRNA levels comparable to those of a lactating gland. These values are 3-10-fold lower when the same cells are maintained on inflexible substrata in the presence of hormones (24). And while the mRNA is translated under these latter culture conditions, as previously mentioned, β-casein is degraded intracellularly and is never secreted. Mammary cells from pregnant rabbits were reported to exhibit a transcriptional block with respect to the casein genes when grown on attached collagen gels (34,35). These apparent mechanistic differences are not readily accounted for by differences in the culture procedures, although they could be explained by lower sensitivities of the assays used in the rabbit system. Further studies should reveal whether species differences indeed exist with respect to the mechanism of casein gene regulation modulated by the ECM.

Culture conditions that elicit casein synthesis and secretion comparable to levels found in vivo do not support synthesis of mouse α-lactalbumin, WAP, and the milk sugar, lactose (Table 3, ref. 13,23,24,25,36). This is in contrast to rabbit mammary cells on floating collagen gels (35), to mouse mammary cells in explant cultures (17,18) and to rat mammary cells on a complex mammary gland-derived biomatrix (section III: 10,11,12), all of which synthesize and secrete α-lactalbumin and/or lactose in response to hormones. Also relevant are the observations of Cline et al. (37) who showed that mammary cells from lactating mice can synthesize and secrete

lactose on floating collagen gel cultures if the integrity of the alveolar units is maintained upon dissociation of the tissue.

Thus, factors additional to interactions with a flexible collagenous substrata appear to be necessary to elicit and maintain expression of a more "complete" functional mouse mammary phenotype in culture. Obvious parameters that could enhance the differentiative response in culture are a substratum closer in composition and structure to the mammary ECM in vivo (10,11,12), an additional or a different requirement for growth factors and hormones (17,18), and the maintenance or generation of tissue topological relationships (37,38). These parameters of course need not be exclusive of each other.

From the studies summarized in Table 3, mouse transferrin and butyrophilin have emerged as examples of milk protein genes which may be insensitive to hormones and are not modulated by the ECM. Similar observations have been reported for synthesis and secretion of transferrin by rabbit mammary cells (35). Little is known about the physiological regulation of these proteins in the course of pregnancy; thus, it will be important to expand these observations in the future and in particular to correlate them with in vivo mammary gland behavior.

Epithelial cells from the mammary glands of unprimed mice do not respond to the modulatory effects of the two-dimensional collagen substratum in the same manner as cells that have already begun the program of cell

Table 3

Proliferative responses of mammary epithelial cells

as a function of the culture substratum

Source of cell pool	Medium supplements	Culture substratum	Approximate increase in cell number 2 wks in culture
Mammary glands from midpregnant mice or spontaneous mouse mammary adenocarcinomas[a]	25% porcine serum + cholera toxin	Plastic	3 fold
		On attached collagen gels	6-10 fold
		In collagen gels	20 fold
Mammary glands from midpregnant rabbits[b]	Serum-free/ chemically defined and progesterone prolactin	On attached collagen gels	2 fold
		On floating collagen gels	No measurable increase
		In collagen gels	6 fold

[a] reference 42

[b] reference 35

differentiation in vivo. The differentiation potential of mammary cells from unprimed adult virgin mice on the two-dimensional collagen system has been examined using culture conditions analogous to those of Bissell and collaborators (namely, serum-containing medium for 24 hrs followed by serum-free medium supplemented with insulin, hydrocortisone and prolactin). Even after 3 weeks of culture in the presence of hormones, caseins are not detected either intracellularly or in the culture medium (see section III, B for a detailed discussion). It remains to be examined whether casein mRNAs are present in these cultures. The same cells, however, can be induced to synthesize levels of β-casein comparable to those present in mammary cells from a lactating gland if grown for 2-3 weeks in the three-dimensional collagen system in medium containing high serum and lactogenic hormones (33). These results illustrate the complexity of the interactions required to induce (as opposed to those required to maintain) the differentiated mammary phenotype for which the collagenous substratum is likely to be but one element.

An intriguing observation which needs further clarification is the finding by Bissell and collaborators that mammary cells activate in culture synthesis and secretion of a group of hormone and substrata-independent proteins not present in differentiated mammary cells in vivo or in mouse milk (13,24,25). Whether synthesis of these "culture-specific" proteins is due to limitations of the culture system or rather is a reflection of disruption of a physiologically important parameter such as the tissue architecture is presently unknown.

The studies discussed above have led to several important observations worthy of re-statement: (a) interaction of mammary cells with a two-dimensional flexible collagenous substratum elicits and maintains expression of markers of morphological functional differentiation; (b) while the culture conditions provided by a floating collagen gel are sufficient to permit hormonal modulation of the mouse casein genes, the maintenance and/or induction of other mammary cell-specific synthetic activities (e.g. α-lactalbumin, WAP, and lactose) require a more complex environment; and (c) given an optimal collagenous culture environment, the complexities of the hormonal requirements of the mammary gland emerge. Thus, β-casein expression is regulated primarily at the transcriptional level by hydrocortisone and prolactin, in agreement with organ culture studies. The α-casein genes are expressed in the absence of these hormones, albeit to a lesser degree, whereas transferrin expression is completely insensitive to the hormonal environment and culture substratum.

III. THE THREE-DIMENSIONAL COLLAGEN GEL SYSTEM

A. The Proliferative Response

A disadvantage of the two-dimensional stromal collagen culture system is its limited ability to support normal mammary cell proliferation (6,36). In contrast, mammary epithelial cells proliferate well in two-dimensional cultures if provided with a substratum of basement membrane collagen type IV (39) which, in fact, significantly reduces dependency on epidermal growth factor (EGF) and dexamethasone for attachment and growth (40). Collagen type IV is also required for the continued growth and survival of the mammary epithelium in vivo (41).

Yang et al. (8,9) utilized stromal collagen as a three-dimensional matrix by suspending mammary cells within it prior to its gelation. The resulting culture environment was shown to support two unique features: (a) sustained proliferation of normal and tumor mammary epithelial cells in primary culture and (b) generation of three-dimensional outgrowths through a

process reminiscent of the branching typical of morphogenesis. Mammary cells from mice (8,9,42), rats (43,44), rabbits (34,35), and humans (38,45, 46) exhibit a similar growth behavior when embedded within collagen gels. However, mammary cells derived from lactating glands exhibit a decrease growth potential in collagen (33), perhaps reflecting the initiation of the program of terminal differentiation which leads to gland involution in vivo (17,18). The initial studies on this system by Nandi and collaborators (8,9) utilized medium supplemented with relatively high serum concentrations; they later developed a serum-free medium that supports as well the proliferative features of mouse mammary cells mentioned above (47).

In an attempt to define the elements of the collagen system that are important in eliciting sustained proliferation, Richards et al. (42) examined the growth behavior of normal and neoplastic mouse mammary cells plated either on plastic, on attached or embedded with collagen gels. Comparable studies are available for rabbit mammary cells (34,35); these experimental contributions are summarized in Table 3.

The initial growth rates of normal and neoplastic mammary cells either on attached gels or within them is approximately the same; growth ceases, however, on the two-dimensional gels when the cells form monolayers (6-8 days after plating). Mammary cells embedded within collagen continue to grow at approximately the same rate for several weeks. Quiescent cells on attached collagen gels can be stimulated to proliferate again in response to a collagen overlay which they invade generating three-dimensional colonies (42). From these observations, it was concluded (42) that a three-dimensional matrix merely provides a greater surface area for cell growth. While Richards et al. (42) did not examine the proliferative behavior of the mouse cells on floating gels, it has been noted by others (48) that contraction of a collagenous gel, as happens upon floating, is in itself inhibitory to cell proliferation. The lack of a proliferative response under the latter conditions has been observed for normal mouse mammary cells (6,36), even in subconfluent cell monolayers (J. Rosen, personal communication), and is supported by the observations with the rabbit system (Table 3). Induction of a quiescent state by gel contraction may well be a consequence of a change in cell shape, a well-documented correlate of the transition between the "dedifferentiated" and the differentiated mammary cell phenotypes on collagen gels.

It has been proposed by Kidwell et al. (49) that a stromal collagen matrix potentiates mammary cell growth and differentiation since it provides a physiological environment upon which the cells can deposit ECM components and assemble a basement membrane. Several experimental observations lend support to this proposal. First, growth of mouse mammary cells on or in collagen gels correlates with deposition of collagen type IV, laminin and fibronectin at the cell-collagen interface (23,42) where a basement membrane-like structure has been shown to assemble (6,50). Rat (51) and human mammary cells (38; Bartek and Taylor-Papadimitriou, unpublished observations) cultured within stromal collagen also deposit collagen type IV, laminin and fibronectin at the cell-collagen interface. Second, inhibition of collagen type IV synthesis results in a marked decrease in cell growth in or on collagen (42) in agreement with previous studies (39,41). Lastly, a stromal collagen matrix modulates the quantitative and qualitative deposition of glycosaminoglycans by mammary epithelial cells (23,52,53). For instance, mammary cells on a differentiation-permissive flexible collagen gel deposit significant amounts of dermatan sulfate whereas on plastic or on attached gels hyaluronic acid production is favored (53). Glycosaminoglycan metabolism in the course of sustained mammary cell proliferation within three-dimensional collagen matrices has not been examined thus far, and this would be interesting to know.

Thus, a proliferative mammary epithelial cell response in culture correlates with deposition of ECM components; quantitative and qualitative parameters of this process are modulated by the culture substratum. The growth response can be either magnified by an increase in permissive surface area such as that provided by a three-dimensional stromal collagen matrix (8,42) or abolished by the signal(s) provided for example by collagen fiber contraction (48).

Mammary cell proliferation in vivo is a hormone-dependent process; the precise hormonal and growth factor requirements vary with the developmental stage of the gland (17,18 and for example 54). The growth promoting effects of EGF and glucocorticoids on mammary epithelial cells plated on stromal collagen appear to be mediated through their effects on collagen type IV synthesis, secretion and stabilization within the ECM (40,55), suggesting an indirect effect of these hormones on the proliferative response. It is emphasized, however, that to separate experimentally direct from permissive effects is not an easy task and it is likely that in vivo ECM components modulate cell growth by both types of effects. The ability to modulate the mammary proliferative response in the three-dimensional collagen system discussed above should facilitate experimental examination of these problems (56).

B. Morphogenesis and Differentiation Markers

Depending upon the source of tissue (e.g., normal or neoplastic) and the culture conditions (e.g., hormones, collagen gel strength), the three-dimensional mammary cell structures that result from growth within collagen exhibit various morphologies ranging from duct-like with extensive branching to cyst-like with few projections (8,9,45,57). In general, normal mammary cells, regardless of the animal species, express a ductal-type morphology within collagen gels (9,33,34,38,42-46,57) and the extent of branching can be enhanced by freeing the gels to float (38,46). The generation of a typical three-dimensional ductal pattern within a collagen matrix is illustrated in Fig. 2. Histological sections show that many of these outgrowths are composed of a single layer of low columnar epithelial cells arranged around a lumen (Fig. 2D) that increases in size in older cultures and often contains secretory material (33,38,50). The duct-like structures are very primitive when compared to the functional units of the mammary gland (46). Their generation in culture is a direct response to contact with stromal collagen, a phenomenon that can also be induced in vivo (58).

The cells of the three-dimensional outgrowths behave like polarized epithelia with numerous microvilli projecting towards the inner lumina (33,38,50); mammary cells on two-dimensional floating collagen gels also become polarized except that their apical surfaces face the culture medium (6,36). Intercellular junctions such as desmosomes and tight junctions are present between many of the cells (33,38,50). As previously mentioned (section III, B), a basement membrane-like structure is sometimes visible at the cell-collagen interface (50) where collagen type IV, fibronectin and laminin can be detected by immunocytochemical procedures (38,50).

Morphological features of secretory differentiation comparable to a late pregnant state occur in three-dimensional outgrowths upon addition of lactogenic hormones, in particular prolactin (59; unpublished observations). Collagen embedded mouse and rat mammary cells from virgin mice accumulate fat droplets which readily stain with oil Red O (59; Fig. 3). This behavior is best observed in cultures that have reached the stationary phase of growth (Durban, unpublished observations). Tubular branches generated by mouse mammary cells have been noted to thicken and develop end bud-like structures (60).

C. Expression of Mammary Cell-Specific Products, Serum Factors, Three-Dimensional Cellular Organization and a Flexible Three-Dimensional Collagenous Substratum

The potential of the three-dimensional collagen culture system to support expression of biochemical markers of mammary cell differentiation is largely unexplored to date. This is reflected by the handful of studies available on this topic (33,59,61,62) in contrast to the growing literature with the two-dimensional floating culture system. Existing evidence,

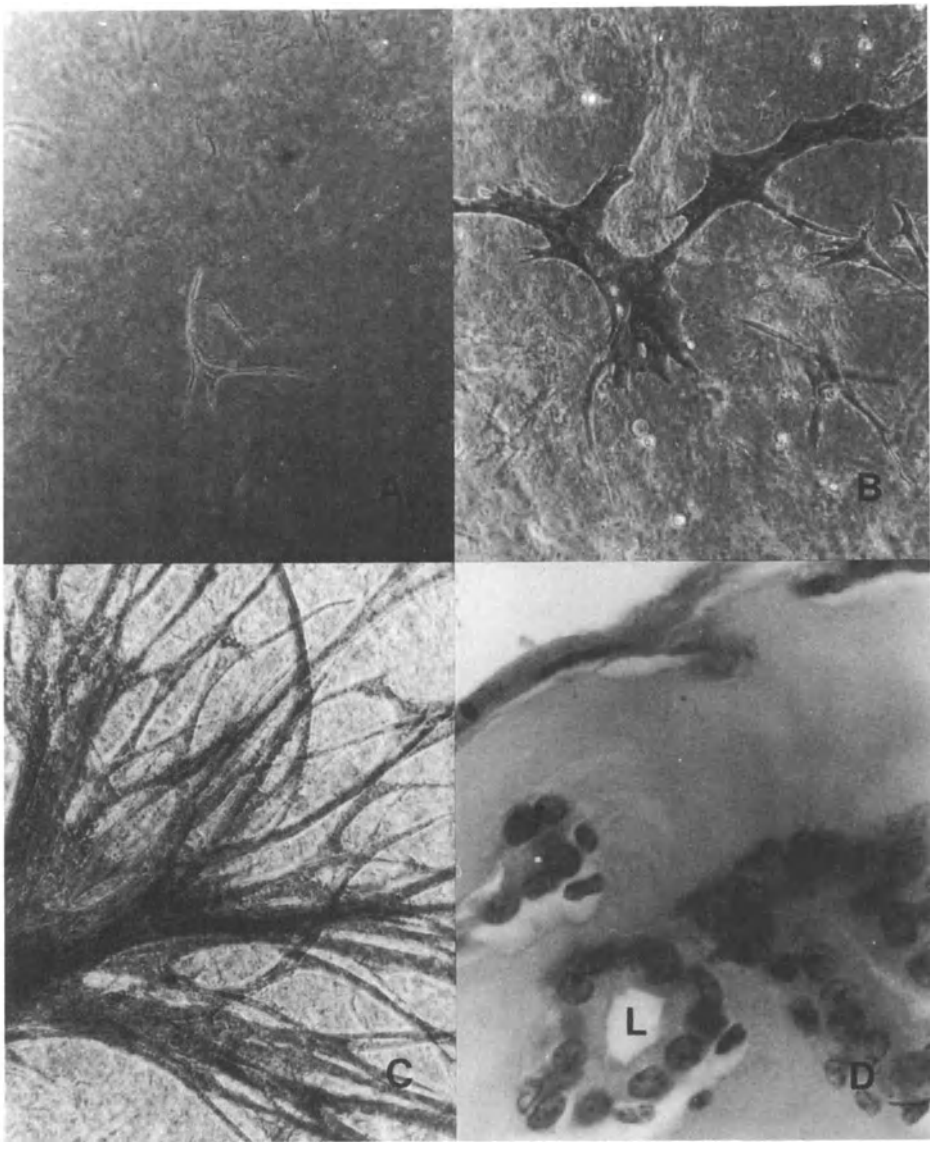

Figure 2. Generation of three-dimensional outgrowths within collagen gels. Phase micrographs (a-c) of mammary cells from virgin mice at (a) 3 days, (b) 1 week, and (c) 3 weeks of embedding. Collagen gel was floated for the last week of culture. Note that photographs (b) and (c) were fixed and stained with Giemsa. x75. (d) Cross section of three-dimensional outgrowths. Lumen (L). x250.

however, indicates that growth of dissociated cells within collagen with generation of three-dimensional structures is a powerful experimental approach both to define physiologically important parameters of the induction of mammary cell-specific products and to dissociate hormonal effects on mammary cell proliferation (54,56) from those on cell differentiation (33,59,61).

As first shown by Tonelli and Sorof (61), it is possible to hormonally induce dissociated mammary epithelial cells not primed for differentiation in vivo to synthesize caseins within three-dimensional collagen gels. Significant levels of β- and η-caseins and low levels of α-caseins accumulate during a 2 week period of hormonal stimulation (61,33). It has not been possible to examine the secretory process in these cultures as secretions are released into the lumens of the three-dimensional outgrowths and to a lesser extent into the surrounding collagen matrix (34,35,38,59). The expression of mouse milk proteins other than caseins has not been examined thus far in this system. Normal rat mammary cells within collagen gels were shown to express α-lactalbumin in response to hormones (44).

Using mammary cells from unprimed virgin mice with collagen, the kinetics of the "induction" process and quantitative and qualitative parameters of casein accumulation relative to mammary cells from lactating glands were examined (33). An unexpected finding was that a time period within collagen (2-3 weeks) appears to be necessary to induce and accumulate detectable levels of caseins in mammary cells from unprimed virgin mice. This time period is independent of three-dimensional growth and lactogenic hormones. Hence, mammary cells stimulated with lactogenic hormones at 1 week of growth in collagen require approximately three more weeks to accumulate detectable levels of caseins (i.e., 1-5 ng caseins/100 ug protein by an ELISA competition assay and 30-50 ng caseins/100 ug protein by immunoblotting gel separated proteins). A longer period of growth in collagen prior to hormonal stimulation, e.g. 2 or 4 weeks, shortens the time needed to

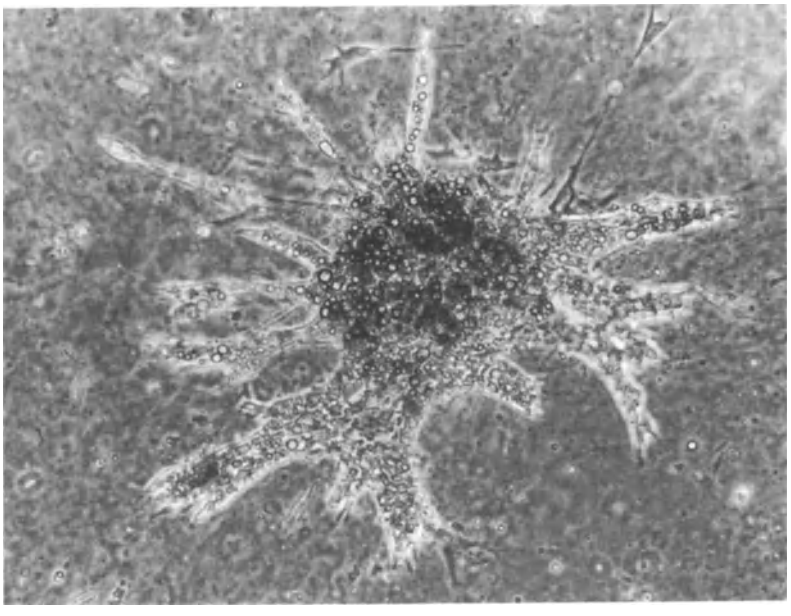

Figure 3. Phase micrograph of oil Red O stained mammary cells from virgin mice hormonally-stimulated with lactogenic hormones after 2 weeks of growth within collagen for 3 days. Note accumulation of lipid droplets, in particular, towards the center of the outgrowth. x100.

detect caseins to approximately 2 weeks and 3 days, respectively. Given sufficient time in collagen, these cells accumulate casein levels comparable to those of a lactating gland although the ratio of individual casein polypeptide favors β-casein, in analogy to the midpregnant state (33). As these experiments did not examine casein mRNA levels and/or turnover, it is presently unknown at which level protein accumulation is being regulated in this system.

In the first week of culture within collagen and in the presence of lactogenic hormones, mammary epithelial cells taken from midpregnant mice have intracellular casein levels lower than those of freshly dissociated cells. During this time, the cells are rapidly increasing in number (62). Thereafter, levels of caseins comparable to those at plating are detected. Thus, a stimulus to proliferate, such as that provided by the three-dimensional collagen matrix, affects the magnitude of the differentiative response. The delay in the detection of caseins upon induction of mammary cells from unprimed virgin mice cannot be explained totally on the basis of the proliferative stimulus provided by the three-dimensional collagen matrix. For example, growth plateaus at about 1 week in these cultures (initial seeding density: 10^5 cells/cm^2) and yet cells stimulated with lactogenic hormones at this time do not have measurable levels of casein until about 2 weeks later (33).

Vonderhaar and collaborators have shown (17) that estrogen/ progesterone priming of immature virgin mice both increases synthesis of EGF by the submandibular salivary gland and induces synthesis of an EGF-related growth factor by the mammary cells. This growth factor appears to be required for induction of hormone-dependent morphological and functional differentiation of mammary cells from immature mice in explant cultures. The identity of the mammary growth factor and of the mammary cell population responsible for its synthesis remains to be defined (17).

Of interest in this context is that the synthesis and accumulation of casein by mammary cells of unprimed virgin mice in collagen correlates with high concentrations of serum in the hormone-supplemented medium (61,33). A change to low serum medium at the time of stimulation with lactogenic hormones results in the accumulation of much lower levels of caseins (61; Durban, unpublished observations). And, in fact, we have not succeeded in inducing mammary cells from unprimed virgin mice to synthesize casein either on two-dimensional or three-dimensional floating collagen gels unless serum is added to the cultures (see below). Detection of α-casein by immunofluorescent staining in mammary gland end buds from immature, unprimed rats cultured within collagen in low serum medium required addition of estrogen and progesterone besides lactogenic hormones (59).

Thus, mammary cells not primed for differentiation *in vivo* and which may not be synthesizing the putative "EGF-like growth factor" could conceivably require an exogenous source of it for induction of differentiation markers. High serum concentrations in the culture medium could partially meet this requirement, albeit not as efficiently. This would explain, in part, the additional time period needed to see a detectable response with mammary cells from unprimed virgin mice. Given that mammary cells induced to differentiate *in vivo* prior to culture have a reduced dependency on exogenous serum components for the expression of various milk proteins (13, 23-25), these cells may well continue to synthesize in culture the putative mammary growth factor (17). Certainly this would be important to know.

As discussed in previous secretions, EGF and hydrocortisone have been shown to mediate their effects on mammary cell behavior by stimulating deposition of collagen type IV (40,55). An exciting possibility for which there is presently no evidence is that the putative mammary growth factor

could potentiate deposition of important ECM components such as collagen type IV. Were this to be true, then an endogenous or exogenous supply of such factor in culture could influence synthesis of ECM components, quantitatively and qualitatively, which in turn could affect deposition of basement membrane-like structure with overall consequences on mammary cell proliferative and differentiative responses.

Hormonal induction of casein synthesis in mouse mammary cells not primed for differentiation in vivo was examined as a function of serum factors, the flexibility of the collagenous matrix and the generation of three-dimensional ductal-type structures. Three questions relevant to ECM influences on the induction of the transition from the "undifferentiated" to the differentiated mammary cell phenotype were addressed: (a) does induction of casein synthesis on two-dimensional floating collagen gels, where the proliferative stimulus is insignificant and where a striking change in cell shape occurs, also require serum components?; (b) is generation of three-dimensional cellular organization a necessary element of the induction of casein synthesis?; and (c) can induction of the transition to a differentiated phenotype be enhanced if the three-dimensional collagen matrix is made flexible by floating? Dissociated cells from unprimed virgin mice were plated either on or within collagen gels, then hormonally-stimulated in either serum-free or in serum-supplemented medium. Collagen gels from each culture condition were then freed to float 48 hrs after plating as indicated (Table 4).

A few points are noteworthy from these experiments. (a) Growth of mammary cells, not primed for differentiation in vivo, on a two-dimensional flexible collagenous substratum is not sufficient to induce casein synthesis in the absence of serum (samples 1 and 2, Table 4). (b) The same cells can be induced to synthesis caseins on two-dimensional floating collagen in the presence of serum and with a minimal growth stimulus, although a better response is obtained when the cells are grown within collagen (compare sample 3 with samples 5 and 7, Table 5). In agreement with our previous observations (33) β-casein is the major casein polypeptide which accumulates regardless of whether the cells are plated on or in collagen (Fig. 4). (c) Generation of three-dimensional growth within collagen does not appear to be a requirement for the induction of casein synthesis by mammary cells not primed in vivo (compare sample 3 with samples 5 and 7, Table 4) although, as expected, it greatly enhances the proliferative response (compare samples 1 and 3 with samples 5 and 7, Table 4). These observations do not exclude the possibility that topological relationships generated by three-dimensional growth may be necessary to potentiate ECM effects on the differentiated phenotype. (d) The flexibility of the three-dimensional collagen matrix does not appear to influence either the proliferative or the differentiative responses of these cells (compare samples 5 and 7, Table 4), although it does enhance the extent of branching of the ductal-type outgrowths (38; and unpublished observations). A point that needs clarification is whether cells primed for differentiation in vivo are more dependent on a change in cell shape for maintenance of differentiation in this system.

It should be emphasized that while the three-dimensional gels also contract when floated, it is presently unclear whether changes in cell shape actually occur in the three-dimensional outgrowths. Electron microscopic examination of these samples has not been undertaken to date. The observations presented above emphasize the possibility that the effects of the ECM and/or its components on the mammary cell phenotype need not be direct. Depending upon whether phenotypic features are to be induced or maintained, the temporal sequence of regulatory events may either be different or may require somewhat different elements.

Table 4

Behavior of mouse mammary cells from unprimed virgin mice as a function of the culture substratum and serum factors

Mice	Sample number	Culture substratum	Medium	Hormones[a]	Increase in cell number[b]	Intracellular caseins[c] 1 wk	3 wks
Unprimed virgins	1	On attached collagen	Serum free[d]	h+	2 fold	NE[e]	-[e]
	2	On floating collagen	Serum free	h+	none measurable	-	-
	3	On floating collagen	20% HS	h+	1.8 fold	-	+/++
	4	In attached collagen	Serum free	h+	none measurable	NE	-
	5	In attached collagen	20% HS	h+	8 fold	NE	+++
	6	In floating collagen	Serum free	h+	none measurable	NE	-
	7	In floating collagen	20% HS	h+	7.5 fold	-	+++
Mid-pregnant		On floating collagen	Serum free	h+	NE	++++	NE

[a] insulin, prolactin, hydrocortisone and aldosterone
[b] at 1 week of plating; initial seeding density 3x 105 cells/cm^2
[c] cell extracts were analyzed by immunoblot procedure for gel separated proteins as described (33); samples were normalized for protein concentrations
[d] 24 hours in medium containing 20% horse serum (HS) then switched to serum free medium with hormones
[e] NE = not examined - = none detectable

IV. COMPLEX EXTRACELLULAR MATRICES AND CELL-CELL INTERACTIONS IN CULTURE

The studies relevant to these topics will be discussed here only briefly as treatises on these subjects are presented elsewhere in this volume (see chapters by Wicha and by Stockdale).

A. Complex Extracellular Matrices

Based on the rationale that the ECM that supports the mammary epithelium *in vivo* is a complex structure composed of type IV collagen, laminin, glycosaminoglycans and glycoproteins, Wicha et al. (10) used as culture substratum a complex ECM derived from pregnant rat mammary glands. Rat mammary cells plated on this acellular biomatrix exhibited enhanced growth and differentiation and remained viable for several months. Production of α-lactalbumin in the presence of hormones was 5-fold greater than the levels obtained for cells on floating collagen gels. Synthesis and secretion of lactose could be maintained for up to 1 month in contrast to the rapid loss of this marker on floating collagen gels. A floating biomatrix enhanced both cell proliferation and differentiation and correlated with a change in cell shape (10,12).

Recently, a complex ECM prepared from the basement membrane producing Engelbreth-Holm-Swarm (EHS) tumor was found to be as efficient as the mammary gland biomatrix for maintaining α-lactalbumin secretion by rat mammary cells (12). The EHS matrix is also a very effective culture substratum for mouse cells eliciting high levels of beta casein mRNA in response to hormones (13,14). The EHS matrix has not been used thus far as a three-dimensional substratum for mammary cell growth. Future studies should establish whether matrices closer in composition to the *in vivo* ECM can, in fact, promote the full range of synthetic activities specific to mammary cells.

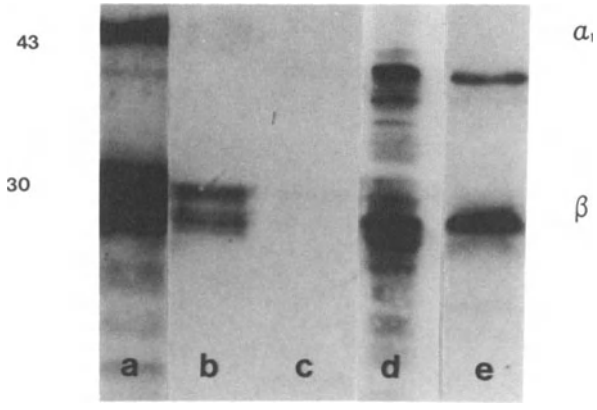

Figure 4. Immunoblot detection of casein polypeptides induced with lactogenic hormones in collagen gel under various culture conditions. Lanes a-c, mammary cells from unprimed virgin mice. Lane a, cells cultured for 4 weeks within an attached collagen gel in the presence of serum. Lane b, cells cultured for 4 weeks on a floating collagen gel in the presence of serum. Lane c, cells cultured for 4 weeks on a floating collagen gel in serum-free medium. Lane d, mammary cells from midpregnant mice cultured on a floating collagen gel for 4 weeks in serum-free medium. Lane e, purified mouse caseins. Molecular weights as in Figure 1.

B. Cell-Cell Interactions: Heterogeneous vs. Homogeneous Populations of Cells

The contribution of cell-cell interactions to the proliferative and differentiative responses of mammary epithelial cells is not a parameter that can be easily analyzed in culture. Yet, it is possible that a differentiation-permissive culture environment facilitates physiologically relevant interactions between different epithelial cells (e.g., secretory, ductal, myoepithelial) or between epithelial cells and cells which are part of the ECM in vivo (adipocytes and fibroblasts). All of the studies available up to date on the modulatory effects of the ECM used mammary cells in primary culture; these, while enriched for epithelial cells, are still heterogeneous in terms of epithelial cell phenotypes and are likely to contain as well small populations of fibroblasts and adipocytes.

Recently, Levine and Stockdale (15) showed that coculture of mammary epithelial cells from midpregnant mice with irradiated 3T3-L1 adipocytes promotes hormone-dependent synthesis and secretion of caseins. The effects appeared to be specific to adipocytes, as coculture with 3T3 cells, foreskin fibroblasts or a matrix derived from the adipocytes, did not support casein synthesis. Although these studies indicated a response only during a short period of culture (1 week), in contrast to studies with a complex biomatrix (10,11,12) and floating collagen gels (13,25), they nevertheless provide an important experimental approach to the study of cell-cell interactions in culture. It will be interesting to determine in the future whether coculti-vation with adipocytes promotes deposition of basement membrane components as well as a change in cell shape. Also, the response of cells not primed for differentiation in vivo will be important to examine in this system.

Relevant to the subject of cell-cell interactions, we have not succeeded in inducing homogeneous populations of mammary cells to express differentiation markers in culture. For example, human milk cells, a rather homogeneous population of secretory cells, favor the pathway of squamous differentiation within collagen even in the absence of cAMP elevating agents (38). Additionally, cloned populations of mammary cells such as that provided by established mammary cell lines (e.g., ClS1, MOMA-1) are not inducible for casein synthesis in collagen although they are capable of three dimensional growth (Durban and Butel, unpublished observations). Bissell et al. (13) have noted that the NMuMg line is also not inducible for casein synthesis on floating collagen gels.

In our experience to date, the only cell line that has exhibited inducibility for the synthesis of caseins in collagen is the mouse mammary epithelial cell line, COMMA-1D (63). This cell line is a heterogeneous population of cells which also exhibits normal morphogenesis in vivo. Recent studies have demonstrated that the heterogeneity of cell phenotypes must be maintained both for morphogenesis in vivo and differentiation in vitro as clonal isolates of the line obtained to date do not exhibit either of these properties (64; J. Rosen, personal communication; Durban, unpublished observations). The possibility has been suggested that a "stem" cell capable of generating heterogeneity of cell phenotypes is present in mass culture but has not yet been described (K. Danielson, personal communication).

With the caveat in mind that the cells may no longer be responsive to lactogenic hormones, we have speculated that the inability of homogeneous populations of mammary cells to differentiate is a reflection of their limited ability to synthesize and secrete the various molecules needed to deposit a basement membrane (38). For example, myoepithelial cells are believed to be the main producers of collagen type IV and this cell population is not present in milk cells or in any of the cloned mammary cell lines

studied. Studies with complex matrices such as that of the EHS tumor should establish in the future whether the importance of cell-cell interactions in the differentiative response is indeed through the deposition of basement membrane components.

V. NEOPLASTICALLY ALTERED CELL POPULATIONS

It is important to establish whether the regulation of phenotypic features is similar in altered mammary cell populations and in normal cells. Such information can enhance our understanding of how a normal cell becomes neoplastic and can have practical applications as in the determination of drug sensitivities and the design of drug treatments.

The culture systems discussed here provide an experimental approach to study both the behavior of altered mammary cell populations under controlled and defined conditions and to examine their interaction with ECM components. A few relevant studies will be discussed below to illustrate the usefulness of these approaches in studies of mammary tumorigenesis.

Hormone-responsive mouse and rat mammary tumor cells can maintain their in vivo phenotypic properties when plated on collagen gels. Studies by Emerman and Worth (65) indicate that androgen-responsive and androgen-independent derivatives of the Shionogi mouse mammary tumor are stable when cultured on collagen gels maintaining growth rates, morphologies and tumorigenic potentials identical to tumor cells transplanted in vivo. Hormonal regulation of milk protein genes in hormone-dependent DMBA-induced rat mammary adenocarcinomas has also been examined with this system (66,67). These tumors contain a small subpopulation of cells (less than 5%) that produces significant amounts of casein; the overall casein message in the tumor is less than 1% of the level in a lactating gland (68). Using a cell separation procedure to enrich for the casein producing cell population which was then cultured on collagen gels, the casein-producing tumor cell population was amplified for further studies (66). Casein synthesis by these cells could be maintained upon culture on attached collagen gels. Furthermore, as for normal cells, the accumulation of casein and WAP mRNA was shown to be regulated by prolactin, in the presence of insulin and hydrocortisone (67). As illustrated by these studies, the ability to examine the behavior of different tumor cell populations under culture conditions that promote in vivo behavior will permit the exploration of areas such as the development of tumor cell heterogeneity and the relationship between cell differentiation and transformation.

The unique ability of the three-dimensional collagen culture system to support sustained proliferation of mammary cells has made it possible to consistently propagate mouse mammary preneoplastic (Durban and Butel, unpublished observations) and neoplastic cell populations (8,47,56,69) in primary culture. As a result, the growth behavior of spontaneous primary mammary tumors has been examined in reference to hormonal requirements (47,69) and to agents known to promote tumorigenesis (70). The transition from a normal to an altered mammary cell phenotype is particularly amenable to study with this system as a rapidly proliferating cell population is a better target for agents such as tumor promoters and chemical carcinogens (71).

The potential of preneoplastic and neoplastic mammary cell populations to express markers of cell function in vivo is variable and this may be a reflection of their hormonal responsiveness. Additionally, alterations may be present at the level of matrix interactions which could affect the differentiative potential of the cell. For instance, DMBA-induced rat mammary tumor cells have lost the property of preferential attachment to collagen

type IV (49), and spontaneous mouse mammary tumors derived from the hyperplastic alveolar nodule D2 line have an altered capacity to interact with fibronectin in vivo and in culture (72). In this context, we have observed a range of differentiative responses, measured by the presence of casein, by mouse mammary preneoplastic cell populations cultured within collagen; such responses do not always reflect their responsiveness to lactogenic hormones in vivo (Durban, Medina, and Butel, MS in preparation). Although the metabolism of ECM components in these cells has not been analyzed, the ability or inability of these cells to modify a collagenous matrix in culture could well alter the expression of the hormone-dependent differentiative response. By comparing the potential of preneoplastic and neoplastic cell populations for differentiation in vivo to that on or in a collagen gel, it should be possible to identify alterations in matrix interactions as a result of the neoplastic process. Characterization of such alterations could then be approached by examining the response of the cells when individual ECM components (singly or in combination) are added to the stromal collagen matrix and also by studying their behavior on a more complex matrix. These approaches are being undertaken with normal mammary cells (13) and certainly also deserve consideration in the study of neoplastically altered mammary cell populations.

VI. CONCLUDING REMARKS

The observations and culture systems discussed here provide a framework to define further the modulatory effects of the ECM on the mammary cell phenotype. It is clear that the ECM brings about changes in gene expression (Fig. 5). One of the present challenges is to define the temporal sequence of events that mediate such effects. Overwhelming evidence exists to indicate that changes in cell shape brought about by the ECM are one of the important elements of this sequence. Whether a change in cell shape is the result of another event in the sequence such as deposition of a basement membrane and/or cell-cell interactions should be clarified in the future by ongoing studies in several laboratories.

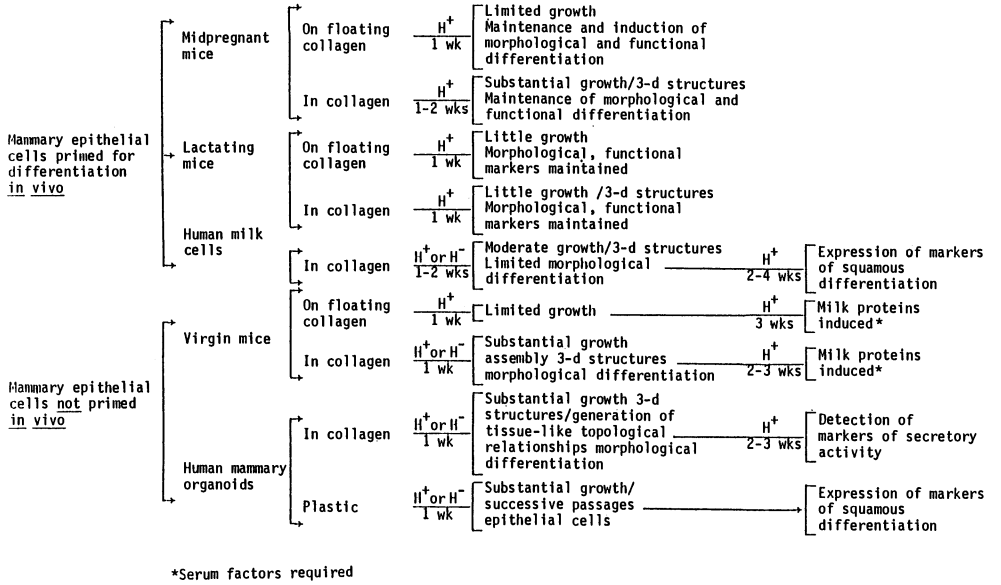

Figure 5. Schematic outline of mammary cell growth and differentiation in culture in response to a stromal collagen matrix.

One aspect of the effects of the ECM on mammary cell behavior emphasized in this review is that to elicit the transition from the undifferentiated to the differentiated mammary cell phenotype additional interactions may be required. It remains to be established whether ECM influences on mammary cells derived from different developmental stages are mediated via similar regulatory mechanisms. The possibility that a mammary gland-derived growth factor (17) is one of the required elements of the sequence of events that mediate ECM effects is an interesting one, worthy of study. Finally, as profound changes in the ECM correlate with the transition of a normal cell to a malignant one (73), the ability to study preneoplastic and neoplastic mammary cells in culture under conditions that support in vivo behavior offers an experimental approach to identify the alterations in cell-ECM interactions that can bring about changes in proliferative and differentiative responses.

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EXTRACELLULAR MATRIX: STRUCTURE, BIOSYNTHESIS, AND ROLE IN MAMMARY DIFFERENTIATION

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I. INTRODUCTION

Many studies have shown that the extracellular matrix (ECM) has a profound effect on morphogenesis, cellular shape, and the regulation of cellular growth and differentiation. Although the molecular mechanisms by which matrix molecules exert these effects are unknown, progress in understanding these complex events has been made by a number of approaches. One approach involves the isolation, purification, and characterization of individual ECM components and their receptors in order to investigate how these molecules interact with each other and with the cell surface. Another approach has been to investigate the effects of matrix components on the regulation of gene expression. Based on these kinds of studies, it is now possible to examine the mechanisms through which matrix components may mdiate their effect on cellular growth and differentiation. One such mechanism is the ability of ECM components, by interaction with specific cell surface receptors that are functionally associated with the cytoskeleton, to regulate gene expression through physical and/or chemical signal transduction.

In this chapter, we summarize recent findings on the structure, biosynthesis, and function of the extracellular matrix in the mammary gland.

In Section II, we provide an overview of the structure and composition of basement membranes. For more detailed summaries, a number of recent reviews are recommended (1-10). In Section III, the evidence for specific receptors for these basement membrane components is reviewed. In Section IV, we review data on the composition of the ECM of the mammary gland and on the development of an *in vitro* system to examine the role of ECM components in mammary growth and differentiation. Recent studies on the role of the cytoskeleton in mammary differentiation are described. Finally, we propose a model for the modulation of mammary gene expression by ECM and cytoskeletal components.

II. STRUCTURE AND COMPOSITION OF BASEMENT MEMBRANES

Basement membranes are thin, continuous sheets of extracellular matrix material separating epithelial, endothelial, adipocyte, muscle, and endocrine cells from the underlying collagenous stroma. Three layers, a 20-300 nm thick layer, the lamina densa, an electron lucent lamina lucida, 10-50 nm thick, between the lamina densa and the cell surface, and a reticular lamina connecting the lamina densa to the stromal ECM below have been described (11). Inoue et al. have shown that in some organs basement membranes lack this ultrastructural organization (12). The composition of basement membranes has been determined by the biochemical analysis and fractionation of tissues such as the lens capsule (13) and the glomerulus (14) and by the analysis of basement membrane producing tumors such as the Englebreth-Holm-Swarm (EHS) tumor (15-18), and an embryonal carcinoma cell line (19,20). Laminin, fibronectin, entactin, nidogen, collage types IV and V, and heparan sulfate proteoglycans have been isolated and purified from these sources. Antibodies raised against these components have allowed immunohistochemical localization of these basement membrane components at the light and electron microscopic level.

A. Laminin

Laminin, isolated from several sources (16-17,19-25), is a glycoprotein with asparagine-linked oligosaccharide chains of approximately 900 kd consisting of three types of disulfide linked subunits, a 360 kd subunit A chain, a 225 kd B_1 chain and a 205 kd B_2 chain (26). Rotary shadowing electron microscopy has demonstrated that laminin is a cross-shaped molecule with two globular domains at the ends of the short arms and one globular domain at the end of the long arm (27). Protease digestion has demonstrated that the cell binding domain is on a 260-300 kd fragment of the 3 short arms without the globular domains (P1 fragment) (28). Neurite outgrowth and hepatocyte attachment domains have also been identified (29-30). Laminin molecules can form polymers and aggregates by binding to the globular domains (31). Laminin also binds to other ECM components, including heparan sulfate proteoglycans (32), type IV collagen and to nidogen (33-34). Therefore, laminin is a large, complex molecule with multiple functional domains which enable the molecule to self-assemble and interact with other ECM components and with cells. These characteristics may be important in the assembly of the basement membrane and in cell-substrate interactions.

B. Fibronectin

The fibronectins are a group of disulfide linked dimeric glycoproteins which are components of the ECM of many different tissues and which are important in cellular adhesion, morphology, cytoskeletal organization, cell migration, hemostasis, differentiation, and malignant transformation (reviewed in 6-8). The subunits of the fibronectins are approximately 220-250 kd and contain multiple, well-defined functional domains capable of binding to cells, bacteria, collagens, fibrin, heparin, proteoglycans, and actin. The fibronectins have been characterized at both the protein and DNA levels.

A tetrapeptide Arg-Gly-Asp-Ser within the cell binding domain is required for cell binding activity although the serine can be replaced by other amino acids (35-36). The tripeptide sequence arg-gly-asp is shared by other cell binding proteins, including fibronectin, fibrinogen and von Willebrand factor. This sequence may provide a common signal for cell protein interactions (37).

C. Other Glycoproteins

Entactin is a 158 kd sulfated glycoprotein that was identified in a murine endodermal cell line and in the EHS tumor (38,39). Entactin has been localized in basement membranes of a variety of tissues including the mammary gland (40). Nidogen, a glycoprotein isolated from the EHS tumor, has been described in Reichert's membrane and is expressed during embryogenesis (41-42).

D. Collagens

The collagens, the major structural and supportive elements of the body, form a heterogenous family of at least 10 proteins (see reviews 2-4). The collagens are characterized by a triple helical domain with the repetitive sequence gly-X-Y, with the X position amino acid often being proline and the Y position amino acid often being hydroxyproline or hydroxylysine. Types I, II, III, and V are fibrillar interstitial collagens (43); type IV collagen is a non-fibrillar protein found in basement membranes (44). The more recently described collagens, types VI, VII, VIII, IX, and X are also non-fibrillar (45-49).

Type IV collagen, the major and ubiquitous collagenous component of basement membranes, is a heterotrimer composed of pro-α1(IV), 185 kd, and pro-α2(IV), 170 kd chains (50). It has a 400 nm triple helical portion with a globular domain at the carboxy terminus (NC1 domain) (44). Aggregation of type IV collagen monomers occurs *in vitro* and appears to be mediated by covalent interactions between the noncollagenous domains of the molecule. A model for assembly of type IV collagen has been proposed by Timpl et al. which involves interaction between the NC1 domain of two molecules and among the 7S domains of four molecules (44).

Interactions between type IV collagen and laminin have been proposed. Kleinman et al. have shown that there is an increase in turbidity when type IV collagen and laminin are incubated at 35° C (16) although Engvall and Ruoslahti failed to detect a specific interaction between type IV collagen and laminin (51). Charonis et al. reported that laminin and type IV collagen form complexes that can be observed by rotary shadowing electron microscopy (33). Laurie et al. have also shown by similar techniques that laminin and type IV collagen interact at specific sites, although at different locations on the type IV collagen molecule than these reported by Charonis et al. (52). Heparan sulfate proteoglycans have similarly been shown to associate with type IV collagen (52). Although functional interactions have not yet been proven *in vivo*, they may prove to be extremely important in the organization and synthesis of basement membranes.

Type V collagen is a hetero or homotrimer, variably composed of α1(V), α2(V), or α3(V) chains. The exact relationship of type V collagen to the basement membrane is not clear but it appears to act as an anchoring protein of the cell and its basement membrane to the interstitial collagen below (53,54).

E. Proteoglycans

Proteoglycans are a diverse group of ECM components composed of core proteins with glycosaminoglycan chains. There is considerable heterogeneity in the core proteins and in the size, composition, and amount of sulfation of the carbohydrate side chains. This heterogeneity appears to be tissue specific and may affect the properties and function of the proteoglycans (see 10 for review). Studies have shown that basement membranes contain heparan sulfate proteoglycans (15). Proteoglycans also bind to other ECM macromolecules such as laminin (55), fibronectin and type I collagen (56). Proteoglycans may play a role in tissue specific gene expression. Reid has shown in vitro that proteoglycans from a number of tissue sources augment levels of liver specific mRNAs and reduce levels of housekeeping gene mRNAs. These effects appear to be regulated at transcriptional and post-transcriptional levels (57).

III. EXTRACELLULAR MATRIX RECEPTORS

There is increasing evidence that cells interact with extracellular matrix components via specific cell surface receptors. Receptors have now been isolated for laminin, fibronectin and collagen.

A. Laminin Receptors

Work from our laboratory, as well as others, indicates that laminin binds to a variety of normal and malignant cells via a specific 68-70 kd cell surface receptor. This receptor, purified by laminin affinity chromatography, binds laminin with a K_d of approximately 2×10^{-9}M. It has been isolated from human breast cancer cells (58), mouse fibrosarcoma cells (59), myoblasts (60), murine melanoma cells (61), human neutrophils (62), and mouse macrophages (63). The receptor may be involved in the adhesion of both normal and malignant cells to extracellular matrix substrata. A 50 kd laminin receptor with similar affinity has recently been isolated from Staphylococcus aureus (64). In addition to this high affinity laminin receptor, laminin has been found to bind to some cell types with lower affinity via cell surface glycolipids (65). The CSAT (cell substrate attachment antigen) proteins have also been shown to bind both laminin and fibronectin with a K_d of approximately 10^{-6}M (66).

B. Fibronectin Receptors

A 140 K_d cell surface glycoprotein isolated from a variety of cells by affinity chromatography has been identified as a fibronectin receptor. This receptor binds to the arg-gly-asp-ser cell binding region of fibronectin. It has been iodinated at the cell surface and can be inserted into liposomes suggesting that it is an integral membrane cell surface protein (67-68). In addition to the fibronectin receptor, cells can attach to fibronectin via the CSAT, a complex of 3 polypeptides of approximately 140 daltons which has been identified through the development of a monoclonal antibody which blocks cell attachment (69) as noted above.

C. Collagen Receptor

Several molecules which specifically bind collagen have been isolated from a variety of cells. A 47 kd cell surface receptor for type IV collagen has been isolated by affinity chromatography (70). An integral membrane proteoglycan which binds type I and III stromal collagen has been isolated from mouse mammary epithelial cells (71). This cell surface proteoglycan may act as a receptor for interstitial fibrillar collagens.

D. Interaction of Matrix Receptors with the Cytoskeleton

A number of studies have indicated that ECM may affect cell shape and the cytoskeleton. Sugrue and Hay demonstrated that when corneal epithelial cells are removed from their basement membrane, the basal cell surface forms numerous blebs which are associated with a disorganized cytoskeleton. Addition of soluble matrix molecules such as laminin or fibronectin causes actin to form filaments at the basal cell surface (72). On the basis of these studies, they postulated that cells have transmembrane matrix receptors capable of mediating the interaction of extracellular matrix and the cytoskeleton. Work in our laboratory has provided evidence that cell surface laminin receptors interact with cytoskeletal actin. Purified laminin receptor protein is capable of bundling f-actin filaments in vitro (73). Furthermore, we have demonstrated that laminin can be clustered on the cell surface and that these clusters are resistant to extraction with non-ionic detergents (74). On the basis of these experiments, we have proposed a model in which laminin at the cell surface can cause rearrangement of the cytoskeleton by clustering cell surface laminin receptors which in turn interact with actin in the cytoskeleton (see below).

Studies from other laboratories have indicated that cell surface receptors for other ECM components may also be capable of interacting with the cytoskeleton. The 140 kd fibronectin receptor has been shown to co-localize with actin filaments at points of cell contact with the substratum (75). Fibronectin and actin appear to form a close transmembrane association termed the fibronexus (76). Rapraeger and Bernfield have provided evidence that the integral membrane cell surface proteoglycan which is a receptor for interstitial collagens is also capable of interacting with the cytoskeleton (77). Taken together these studies demonstrate that cells have multiple ECM receptors which are transmembrane proteins and which may be capable of interacting with the cytoskeleton in vivo.

IV. EXTRACELLULAR MATRIX AND MAMMARY GLAND FUNCTION

There is considerable evidence that both extracellular matrix and hormonal components are involved in mammary gland morphogenesis, growth and differentiation. We here review evidence dealing with the extracellular matrix composition of the mammary gland and the role of extracellular matrix components in mammary growth and differentiation. We also summarize our recent findings relating to the regulation of mammary gene expression by extracellular matrix components.

Immunohistochemical studies demonstrated that basement membrane composition and architecture change during mammary gland morphogenesis (40,78,79). In the virgin gland, myoepithelial cells form a continuous layer around the epithelial cells. These cells rest on a continuous basement membrane containing laminin, type IV collagen and heparan sulfate proteoglycan. Fibronectin, types I and V collagen, and entactin are associated with the interstitial connective tissue and are not in the basement membrane of the virgin gland. With the alveolar growth of pregnancy and lactation, the epithelial cells directly abut the basement membrane which remains continuous. In the involuting gland dissolution of the basement membrane occurs (80). As confirmed by experimental model, inhibition of type IV collagen synthesis leads to an involuting morphology (81). Thus an intact basement membrane is required for the maintenance of the architecture of the gland. In addition, growth and attachment of mammary cells in vitro also require type IV collagen. Cis-hydroxyproline reduces the attachment and growth of mammary cells on type I but not type IV collagen (82). These findings suggest that basement membrane components play an important role in the morphogenesis of the mammary gland.

A number of studies have indicated that the synthesis and deposition of ECM components by mammary epithelium is influenced by both soluble factors and other extracellular matrix components. Type IV collagen deposition by virgin rat mammary cells is hormone dependent (83). Glucocorticoids have been found to suppress type IV collagenolytic activity leading to increased type IV collagen deposition (84). In addition, a variety of growth factors including EGF (84) and mammary-derived growth factor-1 (85) modulate type IV collagen synthesis. Thus, basement membrane deposition may be regulated by soluble factors. In addition, the synthesis and deposition of ECM components by mammary epithelium is also influenced by extracellular matrix components themselves. It has been shown that incorporation of ECM components into a basement membrane is altered when mammary cells are cultured on floating collagen gels in vitro. On these gels there is enhanced incorporation of SO_4^{-2} and glucosamine into proteoglycans in the ECM. As determined by pulse chase studies, this is partially due to reduced degradation (86,-87). Parry et al. have demonstrated that mammary cells on floating collagen gels incorporate glycosaminoglycans into an ECM whereas those on tissue culture plastic or attached gels secrete most of the synthesized glycosaminoglycans into the medium (88)

Role of the ECM in Mammary Differentiation

A number of studies have shown that ECM components can influence mammary differentiation in vitro. Most of these studies have utilized floating gels of stromal collagen to maintain the expression of milk proteins as evidenced by increase in the synthesis of casein mRNAs and proteins (89-92). The ability of collagen gels to induce and maintain mammary differentiation is associated with a contraction of the gels. If gel contraction is prevented either by glutaraldehyde fixation or by leaving gels attached to the culture dish, the expression of differentiated function is greatly reduced (91,93). These observations have led to the suggestion that cell shape changes are necessary for the expression of differentiated function. However, it is unclear whether the effects of floating collagen gels on mammary differentiation are direct or are indirectly mediated by other ECM components deposited on collagen gels by mammary epithelium. The latter hypothesis is supported by the observation that the expression of milk proteins by mammary cells on floating collagen gels is accompanied by the deposition of other basement components into the ECM (94,95). Basement membrane deposition and milk protein synthesis are significantly reduced on tissue culture plastic or on attached collagen gels. Furthermore, the addition of the proline analog L-azitidine-2-carboxylic acid to inhibit collagen synthesis inhibits casein synthesis in primary mammary organ cultures (96). These experiments suggest that ECM components synthesized by mammary epithelium in vitro may be important in the maintenance and induction of mammary differentiation.

In an attempt to more closely simulate the in vivo environment, we have cultured rat mammary epithelium on complex mixtures of ECM components and defined basement membrane components. We have previously shown that virgin rat mammary cells can be induced to synthesize the milk protein α-lactalbumin (α-LA) when plated on a complex ECM extracted from pregnant rat mammary glands. Furthermore, this complex ECM, which contains both stromal and basement membrane components, has organ specificity since ECM extracted from other organs was not as effective in supporting mammary differentiation (97).

In vivo, mammary epithelium rests on a basement membrane composed of components described in Section II. We therefore investigated the effects of basement membrane components on the differentiation of rat mammary epithelium. As an initial step in determining the level of regulation by ECM

components, we measured the effect of these components on the steady state accumulation of mRNAs and proteins for α-, β-, and γ-casein and α-LA.

The substrata utilized included a mixture of ECM components that we extracted from the EHS tumor and which contains laminin, type IV collagen, entactin, nidogen, and proteoglycans (39), a purified basement membrane component laminin, stromal collagen gels, and tissue culture plastic. We hypothesized that the self-polymerizing EHS gel, which has been shown to induce the differentiation of Sertoli cells (98) and melanoma calls (99), might provide a useful substratum for the induction of differentiation of virgin mammary cells. Laminin was utilized since it is a major component of the ECM of the in vivo mammary gland basement membrane. Stromal collagen gels were utilized so that we could compare the effect of basement membrane components to stromal components and because all previous studies of differentiation in primary cultures have utilized the collagen gel as a model system. Differentiation on these substrata was compared to that on tissue culture plastic. Previous in vivo and organ culture studies in the rat (100-103), mouse (104,105), and rabbit (106,107) have shown that casein gene expression is modulated by insulin, prolactin, and hydrocortisone. Our studies have been performed in a serum-free defined medium containing these lactogenic hormones.

We examined the effect of basement membrane and stromal collagen substrata on the morphology of primary rat mammary cultures. Cells on tissue culture plastic or laminin demonstrated a flattened morphology. However, refractile lipid vacuoles were detected in the cells on laminin-coated dishes (Fig. 1a and 1b), an appearance consistent with differentiated function (93,108-110). Cells on "plain" collagen gels or collagen gels to which laminin had been added to the medium also developed these vacuoles (Fig. 1c and 1d) and at the ultrastructural level, demonstrated the characteristic rounded morphology (Fig. 2c). Cells on the EHS gel developed a globular appearance in the absence of detectable gel contracture (Fig. 1e and 2d). The morphology of cells on the EHS gel was similar to that seen on the mammary gland ECM which we had previously shown induced the secretion of α-LA (97), (compare Fig. 2a and 2b).

These experiments demonstrated that both basement membrane and stromal components induce morphologic differentiation of virgin rat mammary cells. In order to better understand these events at a molecular level, we examined the effect of basement membrane and stromal components on the steady state accumulation of milk proteins and their mRNAs.

We utilized RNA dot blot analysis with cDNA probes of rat α-, β-, and γ-casein provided by Jeffrey Rosen (111-112) and a mouse cDNA probe of α-LA provided by M.R. Banerjee. Cytoplasmic RNA was isolated from mammary cells grown on various substrata essentially as described by Maniatis and Falvaro (113,114) and equal amounts were dotted onto nitrocellulose filters (115) and hybridized to ^{32}P-labelled nick translated cDNA clones. Following autoradiography, the steady state levels of cytoplasmic RNA for the four milk protein genes were measured by densitometry. Fold increase in the RNA steady state levels was obtained by normalizing all data to results on tissue culture plastic. Representative dot blots shown in Fig. 3 are quantified in Table 1. We found that cytoplasmic RNAs for the various milk proteins are regulated differentially by matrix components. The accumulation of α-casein mRNA was up to 8 fold more on ECM components than on tissue culture plastic; β-casein mRNA was stimulated up to 3 fold; α-LA mRNA showed less than 3 fold modulation and γ-casein was unaffected by these substrata. Basement membrane components, the EHS gel or laminin, as well as stromal collagen gels induced the accumulation of α-casein mRNA. This modulation by matrix components was not due to changes in total RNA synthesis since no significant change in the amount of 28S ribosomal RNA was detected (Fig.

3e). We conclude from these experiments that ECM components have differential effects on the steady state accumulation of milk protein mRNAs. Whether changes in mRNA accumulation are regulated at the transcriptional or post-transcriptional levels remains to be determined. In order to show whether these findings were reflected at the protein level, we measured the effect of ECM components on casein and α-LA protein synthesis.

We utilized a polyclonal antibody against an acid precipitable fraction of rat milk as described by Lee, et al. (91) to immunoprecipitate milk proteins synthesized by primary culture. Fig. 4 demonstrates that this antibody detects α-casein (40 kd), β-casein (28 kd), and γ-casein (18-22 kd). Transferrin (80 kd), xanthine oxidase (150 kd), butyrophilin (67 kd), κ-casein (32 kd) and a 16,000 unidentified protein are also detected by this antibody.

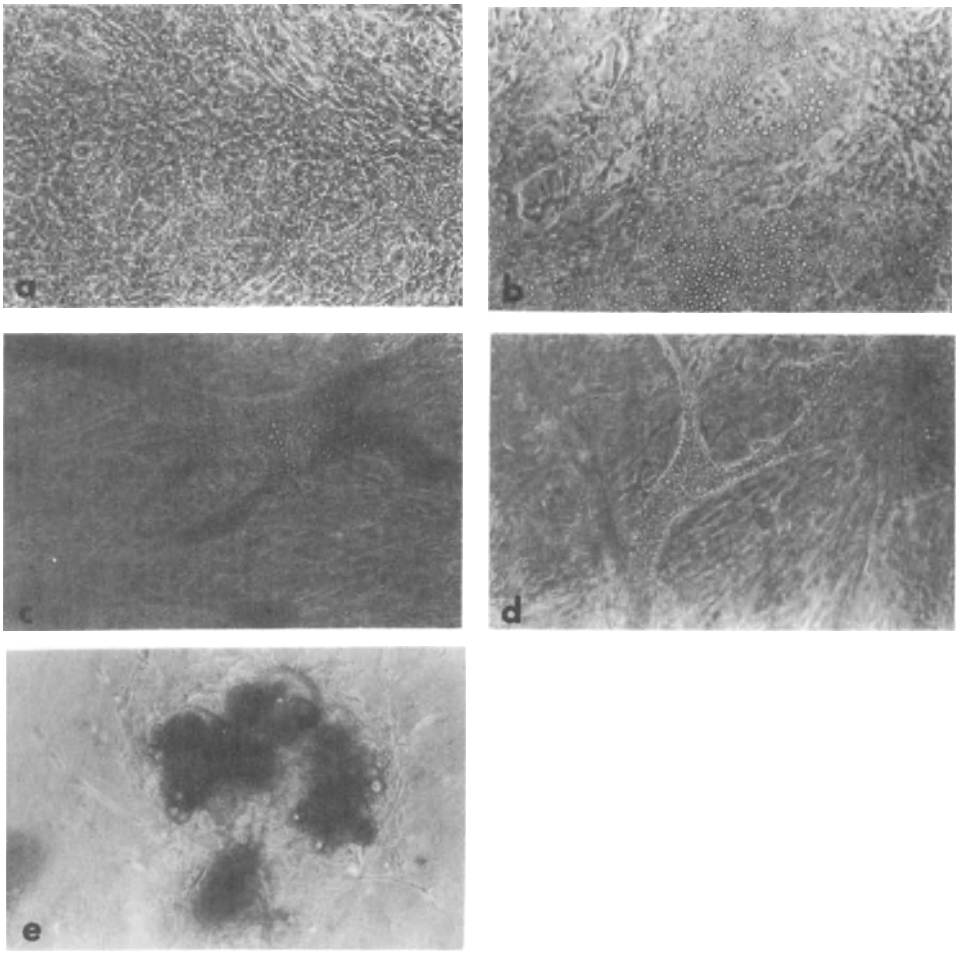

Figure 1: Morphology

Mammary cells were maintained in culture for two weeks in a serum-free medium composed of ovine prolactin 0.3 μg/ml, insulin 5 μg/ml, hydrocortisone 0.2 μM, transferrin 5 μg/ml, epidermal growth factor 10 ng/ml, fetuin 1 mg/ml, and gentamicin 50 μg/ml. They were plated on a) tissue culture plastic, b) laminin coated plastic, 5 $\mu g/cm^2$, c) floating collagen gel, d) floating collagen gel with soluble laminin added to the medium, 200 μg/ml, and e) EHG gel. Phase contrast micrographs were taken at 100 x magnification.

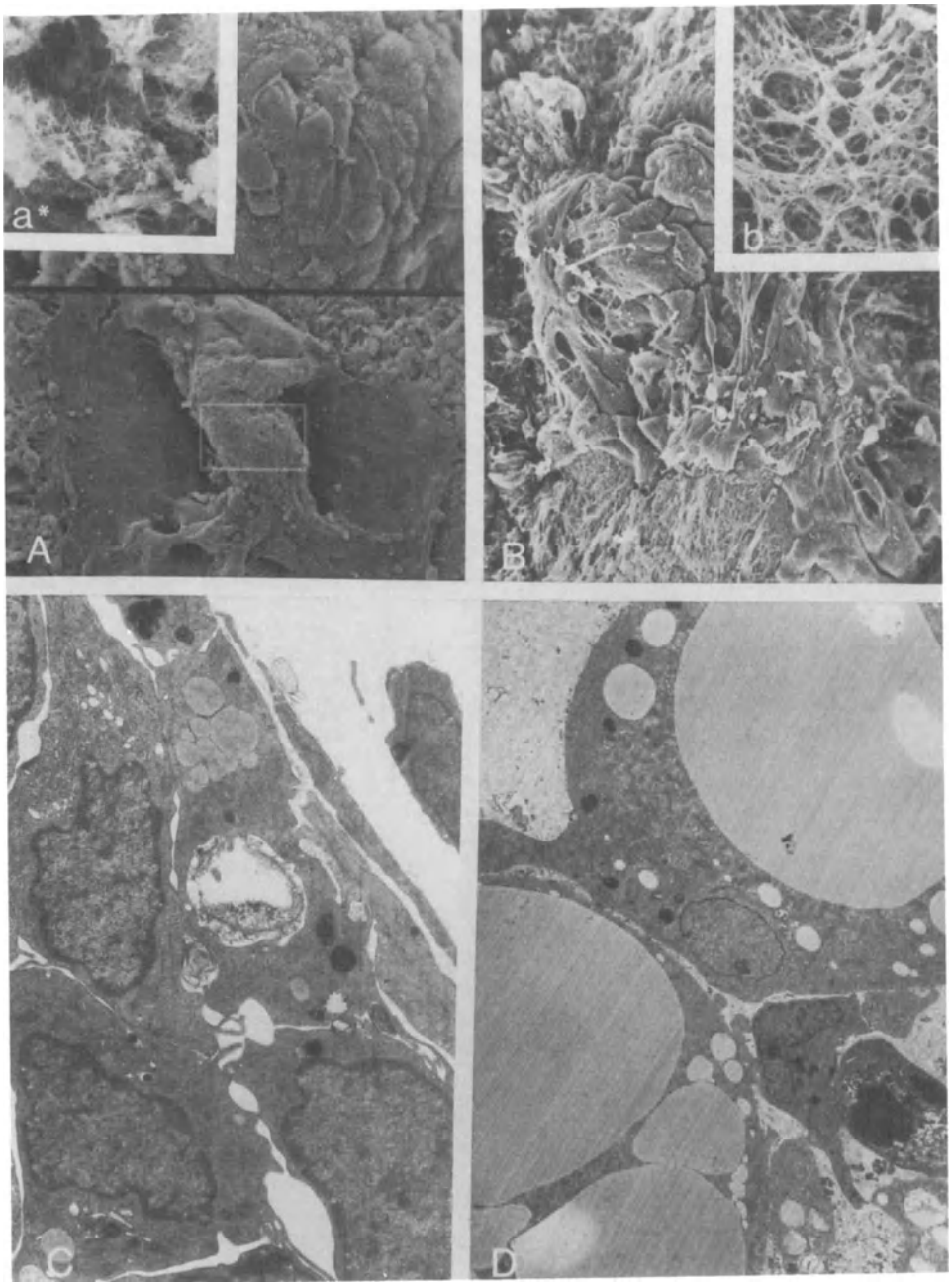

Figure 2: Morphology

Mammary cells were maintained in culture for two to four weeks on a) a matrix derived from pregnant rat mammary glands, x 150 below, x 790 above, b) EHS gel, x 400, c) floating collagen gel, x 3150, d) EHS gel, x 1600. The inset a* demonstrates the SEM appearance of the mammary matrix, 2090; b* demonstrates the SEM appearance of the EHS gel, x 2870.

Cultures were maintained in a serum-free defined medium and labelled with 100 μCi/ml [^{35}S]-methionine for 24 hours on days 2, 5 and 13. Medium and cell layer fractions were obtained, total protein was determined in each fraction by TCA precipitation, and a comparable number of TCA precipitable counts was preabsorbed with pre-immune rabbit serum and Staph protein A and then immunoprecipitated by the polyclonal anti rat milk antibody and Staph protein A. The immunoprecipitates were reduced and fractionated on 10% SDS polyacrylamide gels, dried, and fluorograms obtained. The steady state level of α-casein synthesized in these cultures was measured by densitometry and the fold increase in α-casein on ECM coated dishes was normalized to that on tissue culture plastic. Data from multiple experiments are summarized in Table 2. These data show that both basement membrane and stromal components enhance α-casein synthesis compared with culture on tissue culture plastic. These differences were detected as early as day 3 in culture. In contrast, the induction of the steady state accumulation of α-casein mRNA by ECM components was detected only later in culture, by day 6. α-Casein synthesis was enhanced up to 12 fold on a laminin substratum and 14 fold on the EHS gel. On collagen gels, α-casein synthesis was enhanced up to 30 fold. We postulated that the addition of soluble laminin might further

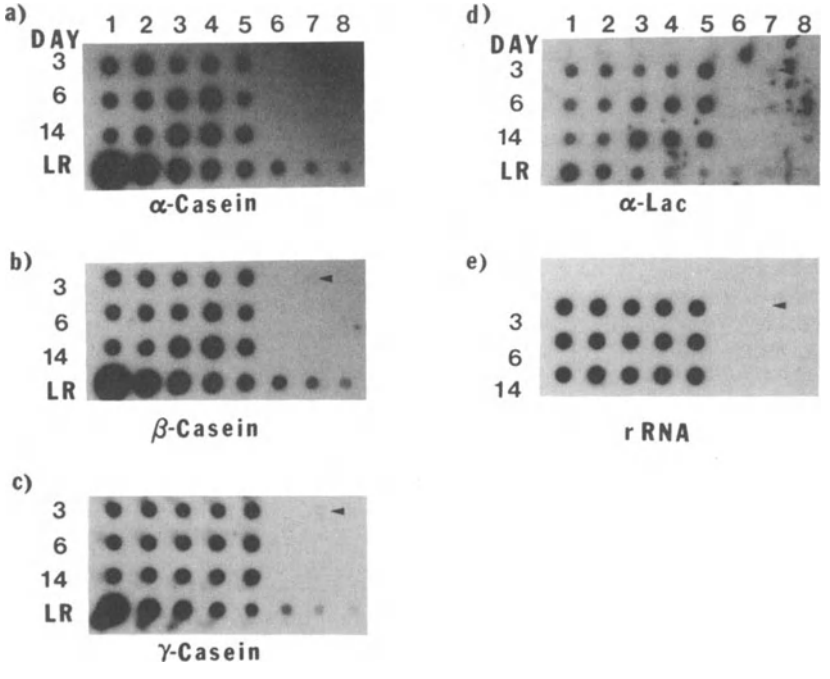

Figure 3: Dot Blots of Cytoplasmic RNAs

Cytoplasmic RNAs isolated from primary rat mammary cells cultured on various substrata were dotted onto nitrocellulose filters in 2 μg aliquots and hybridized to ^{32}P-labeled cNA probes as follows: a) α-casein, b) β-casein, c) γ-casein, d) α-lactalbumin, and e) rRNA. Primary cell cultures were harvested at days 3, 6, and 14 following initial plating on matrix substrata. Lanes (1) tissue culture plastic, (2) laminin coated plastic, (3) collagen gel, (4) collagen gel and soluble laminin, and (5) EHS gel. LR designates lactating RNA isolated from 3 to 4 day lactating rat mammary gland and serially diluted as follows, Lanes (1) 2.0 μg, (2) 1.0 μg, (3) 0.5 μg, (4) 0.25 μg, (5) 0.125 μg, (6) 0.062 μg, (7) 0.31 μg, (8) 0.015 μg. Yeast t-RNA was dotted on each filter in a 2 μg aliquot as indicated by the arrowhead.

Table I. RNA Accumulation

	Substrata	Day 3	Day 6	Day 14
α-casein	Plastic	1.0	1.0	1.0
	Laminin	1.6 (0.2)	2.3 (0.6)	4.2 (0.7)
	Collagen Gel	0.7 (0.1)	5.6 (2.7)	8.0 (1.8)
	Collagen Gel & Laminin	0.9 (0.2)	8.0 (3.0)	6.8 (0.8)
	EHS Gel	0.3 (0.1)	0.7 (0.1)	2.2 (0.2)
β-Casein	Plastic	1.0	1.0	1.0
	Laminin	1.0 (0.1)	1.5 (0.2)	1.4 (0.2)
	Collagen Gel	0.6 (0.0)	1.9 (0.7)	2.7 (0.5)
	Collagen Gel & Laminin	0.7 (0.1)	2.2 (0.8)	2.9 (0.9)
	EHS Gel	0.7 (0.0)	0.8 (0.0)	1.4*
γ-Casein	Plastic	1.0	1.0	1.0
	Laminin	1.0 (0.1)	1.2 (0.1)	1.0 (0.1)
	Collagen Gel	0.6 (0.0)	0.9 (0.2)	1.0 (0.0)
	Collagen Gel & Laminin	0.6 (0.1)	1.0 (0.2)	1.0 (0.0)
	EHS	0.9 (0.3)	1.1*	1.0 (0.1)
α-Lac-talbumin	Plastic	1.0	1.0	1.0
	Laminin	0.9 (0.1)	1.3 (0.1)	1.2 (0.1)
	Collagen Gel	0.6 (0.1)	1.8 (0.8)	2.1 (0.5)
	Collagen Gel & Laminin	0.6 (0.2)	2.4 (1.0)	2.2 (0.6)
	EHS Gel	1.3 (0.9)	1.4 (1.1)	1.8 (0.5)

The data represents the mean value of 3 to 4 experiments obtained by densitometry of RNA dot blots. The standard error of the mean is given in parenthesis. Data are expressed as fold increase relative to tissue culture plastic. The * denotes a single determination on a given substrata.

increase the expression of specific milk proteins since we and others have shown that laminin binds to the cell surface (58-63,116) and may cause rearrangement of the cytoskeleton (72-74). We found that the addition of soluble laminin appears to enhance α-casein synthesis although this was not apparent at all time points tested. In contrast the addition of soluble laminin had no additional effect on the accumulation of α-casein mRNA.

α-LA was detected by indirect immunofluorescence and quantified by a sensitive radioimmunoassay. As seen in Fig. 5, cells on a laminin substratum contain considerably more α-LA than those on tissue culture plastic. The effect of various substrata on α-LA accumulation was determined by radioimmunoassay. The fold induction of α-LA synthesis on matrix substrata compared with that on plastic is shown in Table 3. As was the case for α-casein, α-LA synthesis was enhanced by basement membrane components and collagen gels. α-LA was stimulated more than 30 fold on the EHS gel compared with that on tissue culture plastic, 16 fold on collagen gels; the addition of soluble laminin enhanced α-LA synthesis more than 40 fold. Thus, collagen and laminin appear to have an additional effect on α-LA accumulation.

Unlike α-casein, the synthesis of butyrophilin and transferrin was not modulated by ECM components. In addition, we detected small amounts of β- and γ-casein in some experiments but this was not a consistent finding. Other investigators have shown that β-casein is the most prominent milk protein detected in mouse mammary cultures of floating collagen gels (89,-92). Using comparable concentration of lactogenic hormones, we found no

additional effect on the accumulation of mRNAs or proteins. We conclude that difference in culturing conditions, the developmental state of the gland, and species differences may account for different profiles of casein synthesis.

These studies demonstrate that both basement membrane and stromal components induce the synthesis of the milk proteins α-casein and α-LA. However, the expression of milk proteins, like the expression of the milk protein mRNAs, is not coordinated. In addition, the fold induction of α-casein and α-LA at the protein level exceeds the fold induction of the mRNAs for these proteins for most time points and on most substrata. These data suggest that the accumulation of mRNA is not the rate limiting step in the synthesis of α-casein and α-LA and may indicate levels of regulation at the translational and/or post-translational levels. This effect of matrix is not due to selection for a particular cell type by ECM. Epithelial cells remain present on tissue culture plastic as determined by immunofluorescent staining for the milk fat globular membrane component PAS-1, a marker for mammary lumenal epithelial cells (117), (data not shown). Therefore, the lack of milk protein gene expression at the mRNA of protein levels on tissue culture plastic is not due to the absence of lumenal epithelial cells.

V. THE ROLE OF THE CYTOSKELETON IN GENE EXPRESSION

Studies outlined above demonstrated that ECM components may regulate mammary gene expression at multiple levels and that these ECM molecules interact with the cell surface via specific receptors. Furthermore, these receptors are capable of interacting with the cellular cytoskeleton. These

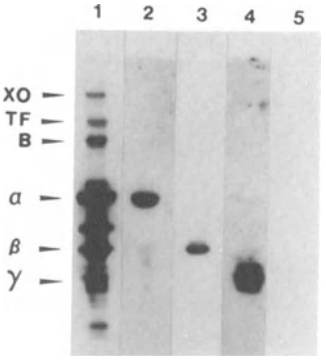

Figure 4: Identification of Proteins Detectedy by the Anti-Rat Milk Antibody by Western Blot Analysis

An acid precipitable fraction of skimmed rat milk was electrophoresed on a 10% SDS:PAGE, transferred electrophoretically to nitrocellulose, and probed with 1) polyclonal rabbit anti-rat milk antibody, 2) rat anti-mouse monoclonal antibody against α-casein provided by F. Stockdale, 3) mouse anti-rat monoclonal antibody against β-casein provided by C. Kaetzel, 4) rat anti-mouse monoclonal antibody against γ-casein provided by F., Stockdale, and 5) pre-immune rabbit serum. The monoclonal antibody lanes were incubated with a Staph A binding second antibody and all were incubated with [^{125}I] Staph A, washed, and an autoradiogram obtained. xo - xanthine oxidase, TF - transferrin, B - butyrophilin, α - α -casein, β - β -casein, γ - γ -casein.

observations suggest that ECM might affect mammary gene expression through cytoskeletal changes mediated by these receptors. The cytoskeletal changes might be reflected in cell shape changes. A number of studies in different systems have shown that cell shape is related to specific gene expression.

Table 2 α-Casein Synthesis

Medium	Day 3	Day 6	Day 14
Plastic	1.0	1.0	1.0
Laminin	3.5 (0.5)	11.5 (3.4)	2.9 (0.6)
Collagen Gel	16.8 (3.2)	23.9 (2.9)	3.1 (1.5)
Collagen Gel & Laminin	21.4 (9.0)	12.0 (3.2)	0.6 (3.0)
EHS Gel	4.3 (3.2)	5.8 (4.2)	8.3 (2.8)
Cell Layer	**Day 3**	**Day 6**	**Day 14**
Plastic	1.0	1.0	1.0
Laminin	2.3 (0.4)	1.0 (0.2)	1.3 (0.3)
Collagen Gel	2.5 (1.2)	8.4 (4.0)	5.0 (2.4)
Collagen Gel & Laminin	8.4 (2.4)	9.6 (7.2)	9.7 (5.3)
EHS Gel	1.0 (0.2)	1.7 (1.3)	6.2*

α-Casein secreted into the medium or in a detergent extract of the cell layer is reported as a mean fold increase relative to that on tissue culture plastic. These data were obtained by densitometry within the linear range of exposure of fluorograms of ^{35}S-methionine labeled immunoprecipitates fractionated on 10% SDS polyacrylamide gels. All values represent the mean of 2-3 experiments. The standard error of the mean is shown in parenthesis. The * denotes a single determination.

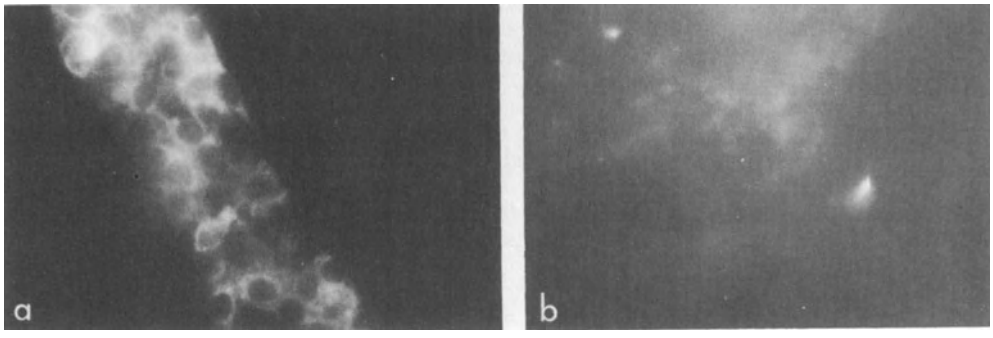

Figure 5: Detection of α-Lactalbumin

Mammary cells were maintained in culture for two weeks. Indirect immunofluorescence was performed using a rabbit antibody against rat α-LA: a) cells on a laminin coated slide, b) cells on uncoated tissue culture plastic slide, 630 x.

Table 3 α-Lactalbumin Synthesis

Medium	Day 3	Day 6	Day 14
Plastic	1.0	1.0	1.0
Laminin	1.2 (0.3)	3.0 (0.8)	4.9 (1.7)
Collagen Gel	3.0 (1.9)	11.5 (4.7)	7.9 (2.5)
Collagen Gel & Laminin	3.1 (1.5)	18.8 (7.8)	9.4 (3.9)
EHS Gel	2.4 (1.3)	7.6 (2.3)	6.7 (2.0)
Cell Layer	**Day 3**	**Day 6**	**Day 14**
Plastic	1.0	1.0	1.0
Laminin	1.5 (0.4)	1.4 (0.3)	1.0 (0)
Collagen Gel	1.8 (0.6)	5.2 (1.7)	8.0 (2.7)
Collagen Gel & Laminin	2.0 (1.0)	13.5 (6.6)	32.1 (4.1)
EHS Gel	2.6 (2.1)	13.1 (6.9)	23.6 (4.1)

α-LA secreted into the medium or in the cell layer is reported as a fold increase relative to that on tissue culture plastic based on a radioimmunoassay for α-lactalbumin. Each experiment was performed on quadruplicate dishes for each time point. The data are normalized for cell number, per 10^5 cells determined by cell counts on replicate samples at each time point. The values for the medium represent the mean of 4 to 6 separate experiments. The values for the cell layer represent the mean of 2 to 4 separate experiments. The standard error of the mean is indicated in parenthesis.

In the mammary gland, this was first suggested by the collagen gel studies of Pitelka et al. (93,94,108,109). The role of the cytoskeleton in mammary gene expression was further suggested by cytoskeleton inhibitor studies which showed that casein RNA and protein synthesis was inhibited in mammary organ cultures by cytochalasin B and colchicine (118,119).

Since we have developed an *in vitro* culture system in which a purified basement membrane component, namely laminin, modulates milk protein gene expression and since laminin has been shown to affect the cellular cytoskeleton, we have tested the hypothesis that laminin's effect on milk protein gene expression is mediated by the cytoskeleton. We determined the effect of cytochalasin D on α-LA production by rat mammary epithelium cultured on laminin coated substrata. As demonstrated by rhodamine phalloidin staining (Fig. 6), mammary cells plated on a laminin substratum display an organized actin cytoskeleton. The actin organization is disrupted by addition of cytochalasin D to the culture medium. This effect is reversible by removal of the cytochalasin D (data not shown). The induction of α-LA synthesis by rat mammary cells on a laminin substratum is blocked by cytochalasin D treatment (Fig. 7a). This inhibition is not the result of a general inhibition of protein synthesis since total protein synthesis, as determined by TCA precipitable counts, is unaffected by this concentration of cytochalasin D (Fig. 7b). Although studies are necessary to determine if other subpopulations of proteins are increased or decreased by these inhibitors, these

experiments suggest that laminin induction of mammary differentiation requires an intact cytoskeleton.

Several studies have indicated potential mechanisms through which the cytoskeleton affects gene expression. Egly et al. have isolated a nuclear fraction from HeLa cells which appears to be a transcription initiator factor for RNA polymerase II and is identical with actin (120). Furthermore, microinjection of actin binding proteins or anti-actin antibodies into the nuclei of amphibian oocytes has been found to inhibit the transcription of lampbrush chromatin without affecting rRNA gene transcription (121). Other have shown that actin (122-124) and intermediate filaments (125) are part of the nuclear matrix. Several investigators have demonstrated that tissue specific gene expression may be partially mediated through interaction of chromatin with the nuclear matrix (126,127). It is possible that cytoskeletal components may also be involved in control of gene expression at the transcriptional level. Actin may also affect transport rates across the nuclear pores (128), affecting post-transcriptional events. Additionally, Penman and colleagues have proposed that the mRNAs for the cytoskeletal proteins are bound to the cytoskeleton and that assembly of the cytoskeleton occurs at the site of synthesis (129). Therefore, the cytoskeleton might serve to stabilize specific cytoskeletal mRNAs and thus act at a translational or post-transcriptional level. Taken altogether these studies suggest that the cytoskeleton may regulate gene expression at multiple levels.

The previous literature and experiments described in this chapter are consistent with the model presented in Fig. 8. This model for the regulation of gene expression by extracellular matrix is similar to the theoretical model proposed by Bissell et al. (130). According to this scheme,

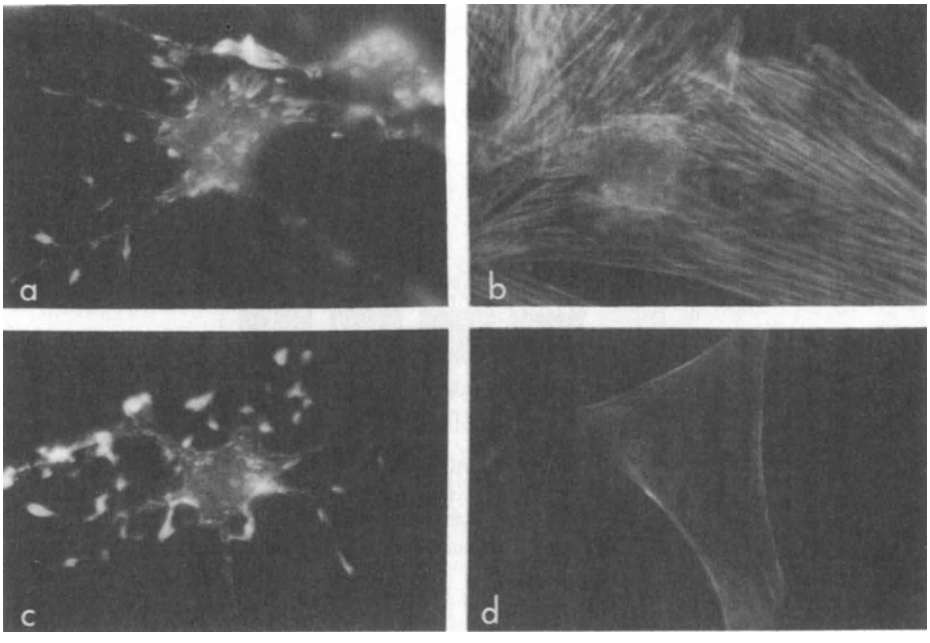

Figure 6: Effect of Cytochalasin D on the Actin Cytoskeleton
Mammary cells were maintained in culture for two weeks on laminin coated plastic (a and b) or on uncoated tissue culture plastic (c and d). Cytochalasin D (2 μM) was added for 48 hours to a and c. The cells were permeabilized with Triton X-100 and incubated with rhodamine conjugated phalloidin and visualized in a Leitz epifluorescene microscope, 650 x.

extracellular matrix components interact with specific receptors on the cell surface. These receptors are transmembrane proteins capable of interacting with the cellular cytoskeleton. Thus, extracellular matrix molecules affect the cellular cytoskeleton which in turn is capable of affecting gene expression at multiple levels. Further experiments are necessary to elucidate elements within this model. The mammary gland offers an ideal system to study the molecular mechanisms by which extracellular matrix and soluble factors regulate gene expression.

Acknowledgments

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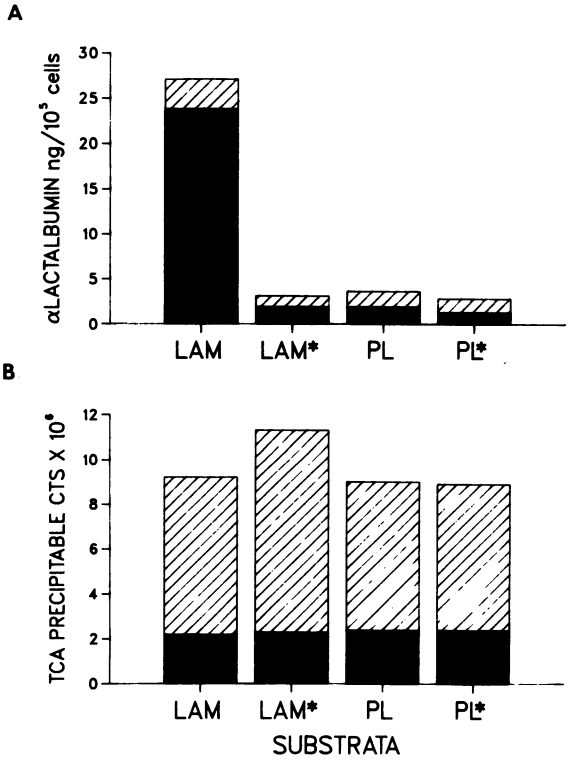

Figure 7: Effect of Cytochalasin D on α-LA Synthesis

Mammary cells were maintained in culture for two weeks on laminin coated plastic or uncoated tissue culture plastic. Cytochalasin D (2 μM) was added for 48 hours. A) A radioimmunoassay aliquots of the culture on medium and a detergent extract of the cell layer was performed and normalized for cell number. All points represent the mean of 4 replicate wells with variation less than 5%. B) An aliquot of [^{35}S]-methionine labeled medium or cell layer was incubated with 10% TCA for 30 minutes and the precipitate collected on Whatman glass filters and counted. Lam - laminin coated plastic; Lam* - laminin coated plastic with cytochalasin D added; PL - tissue culture plastic; PL* - tissue culture plastic with cytochalasin D added.

Medium, Cell layer.

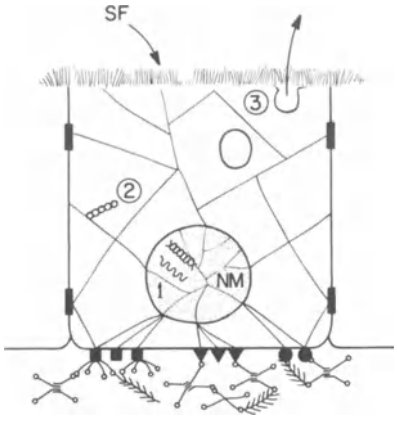

Figure 8: Model of the Effect of ECM and the Cytoskeleton on Mammary Milk Protein Gene Expression

Mammary cells exposed to soluble hormones or growth factors (SF) are anchored to a basement membrane. These cells have specific ECM receptors on their basal surface. The clustering of these transmembrane receptors induces cytoskeletal reorganization. The cytoskeleton in turn affects milk protein gene expression at multiple levels. 1. The cytoskeleton may interact with the nuclear matrix (NM) affecting milk protein gene expression at transcriptional and/or post-transcriptional levels. 2. The cytoskeleton may affect gene expression at the translational level by stabilizing mRNA or increasing the efficiency of translation. 3. The cytoskeleton may have effects at the post-translational level by altering protein stability or secretion.

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ADIPOCYTE, PREADIPOCYTE AND MAMMARY EPITHELIAL CELL INTERACTION

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I. INTRODUCTION

The development of mammary epithelium and its supporting tissues to form a mammary gland can be used as a paradigm for the analysis of several important questions in cell and developmental biology. Are cell interactions that result in cytodifferentiation and morphogenesis instructive or are they only permissive? Are cytodifferentiation and morphogenesis mutually interdependent processes? What controls cell positioning in a tissue so that morphogenesis can occur? How is hormone sensitivity acquired? And how are cell proliferation and differentiation integrated? We have developed a tissue culture model system of mammary gland differentiation to investigate these and other related questions. Analyses of mammary epithelium interacting with adipocytes in cell culture have led us to propose that cell interactions are required for cell proliferation and acquisition of prolactin sensitivity and that differentiation of mammary epithelium in vitro consists of both cytodifferentiation and morphogenesis, processes that are not necessarily interconnected.

Mammary epithelium proliferates within a fat pad to form a highly organized ductal and alveolar structure which synthesizes and secretes a variety of specific proteins, lipids, and carbohydrates (Anderson, 1974; Banerjee, 1976; Topper and Freeman, 1980). Development of the gland in the embryo and in the pre- and post-pubertal period suggests that interaction between the mesenchymal and the epithelial elements of the gland may be important for cytodifferentiation and morphogenesis. Mammary duct formation in the embryo does not occur if the epithelium fails to come into contact with mesenchymal cells (Kratochwil, 1969), and mammary epithelium grows best when transplanted to sites containing adipose tissue (Hoshino, 1972). Post-pubertal gland development is characterized by the epithelial bud below

Table 1

MILK PROTEIN EXPRESSION IN CELL CULTURES OF MOUSE MAMMARY EPITHELIUM

Substrate	Intracellular	Medium
Plastic	7	17
Foreskin Fibroblasts	9	18
Preadipocytes	14	30
Adipocytes	100	100
Adipocytes (insulin only)	21	15

All values are presented as percent effect and were normalized to the amount to β-casein detected in the culture with the adipocyte substrate (100%) This culture contained 101 ng β-casein intracellularly and 117 ng in the medium. The same number of mammary epithelial cells from a mid-pregnant mouse was plated on the various substrates. All cultures contained insulin, hydrocortisone and prolactin in the medium except (insulin only). β-casein was quantitated in these cultures using an immunoassay dot blot method (Wiens et al., 1986).

However, if subsequently, hydrocortisone and prolactin were added, casein synthesis and lipid accumulation commenced within a day. It is not known if there is a particular window of time during which hydrocortisone and prolactin must be added if cytodifferentiation is to occur. But the data suggest that the interaction of epithelium with adipocytes establishes a lactogenic hormone responsive state which can be maintained for days.

Several lines of evidence suggest, but do not prove, that the initiation of milk protein synthesis in mammary epithelial cells may not be dependent upon adipocytes *per se*. There is evidence that a substrate of living cells may not be required and that matrix components from tissues other than adipocytes or mammary gland can foster differentiation. As shown above, when a suspension of mammary epithelium is placed on tissue culture plastic the epithelial cells do not differentiate. But if mammary epithelial cells are cultured on other non-living substrates such as extracellular matrix derived from mammary gland (Wicha et al., 1982a, b), within collagen gels (Yang et al., 1979; Tonnelli and Sorof, 1982; Durban, et al., 1985; Suard et al., 1983); or on floating collagen gels they will differentiate in response to lactogenic hormones (Emerman et al., 1977; Burwen and Pitelka, 1980; Shannon and Pitelka, 1981; Lee et al., 1984; Suard et al., 1983; Lee et al., 1985; Rocha et al., 1985) These experiments differ from one another in the medium required for differentiation, the milk proteins synthesized, the time required to see a hormonal effect, and the percentage of epithelial cells that respond. The interaction of mammary epithelial cells with adipocytes may optimize the hormone-dependent differentiation of mammary epithelial cells, but these experiments using just matrix components suggest that the adipocyte *per se* may not mediate cytodifferentiation. It is likely that murine mammary epithelial cells from animals in all physiological states are committed cells that express milk proteins in permissive environments. Overt differentiation of these committed cells is based upon interactions which are permissive. Most likely they are mediated by components in the

the nipple growing under the influence of hormones to populate the fat pad anlagen of the gland. The work of Daniel and his colleagues (1984) shows that the internal organization of the mammary fat pad influences the pattern by which advancing mammary ducts populate the fat pad. Thus understanding mammary gland development requires investigation into the interaction of mammary epithelium with its surrounding stromal tissue, the adipose tissue. A tissue culture system was developed to analyze the interaction of the two major cellular constituents of the mammary gland - the epithelium and adipose tissue (Levine and Stockdale, 1984, 1985).

II. CYTODIFFERENTIATION OF MAMMARY EPITHELIUM IN CO-CULTURE WITH ADIPOCYTES

A monolayer system of murine adipocytes and mammary epithelium was developed to study adipocyte-mammary epithelial interactions (Levine and Stockdale, 1984, 1985). We took advantage of the fact that a variant of Swiss 3T3-Ll preadipocyte cells, growing in monolayer, form adipocytes when exposed to insulin and to agents that elevate cyclic nucleotides (Green and Kehinde, 1973). When 3T3-Ll preadipocytes reach confluence they can be converted with high frequency to adipocytes. These adipocyte monolayers can be used as an adipocyte-cellular substrate on which to culture mammary epithelial cells and study the interaction of adipocytes with various types of mammary epithelium. This system has been used to analyze questions which concern cell interactions and proliferation, differentiation and morphogenesis (Levine and Stockdale, 1984; Levine and Stockdale, 1985; Wiens, Park and Stockdale, 1986; Stockdale et al., 1986).

The interaction of mammary epithelial cells with adipocytes in vitro resulted in the cytodifferentiation of the epithelium. When mammary epithelial cells from pregnant mice were cultured on adipocytes in the presence of lactogenic hormones (insulin, hydrocortisone, and prolactin), there was a marked increase in the synthesis of casein (Table 1). The same is true using epithelium from non-pregnant, lactating and involuting mice (Wiens et al., 1986). In each case epithelium attached to the adipocytes as single or small clusters of cells and nearly all the epithelial cells in the culture were immunoreactive with antibody to β-casein by the first day of exposure to lactogenic hormones (Levine and Stockdale, 1985; Wiens et al., 1986). Caseins and α-lactalbumin accumulated within the epithelial cells themselves and were secreted into the surrounding medium. A negligible amount of casein was synthesized in the absence of lactogenic hormones. The magnitude of the effect on casein secreted into the surrounding medium varied with the physiological state of the donor mouse, ranging from 13 ng of casein per 16 mm culture of epithelium from non-pregnant mice to 75 ng of casein per 16 mm culture per day of epithelium from lactating mice. When the same epithelial cell suspension was cultured with lactogenic hormones on tissue culture dishes lacking adipocytes, no immunoreactive cells were seen and only small amounts of casein were found in the tissue culture medium. This response was dependent upon the cell type that formed the cellular substrate, since human foreskin fibroblasts (HNFF) and Swiss 3T3 cells (Levine and Stockdale, 1985) did not support the casein response to lactogenic hormones, nor did the matrix remaining on culture dishes (substrate attached material, SAM) from which adipocytes had been removed (Levine and Stockdale, 1985). Preadipocytes support cytodifferentiation to some degree as do other cell lines derived from the parental line of 3T3-Ll cells (Levine and Stockdale, 1985).

The response of mammary epithelium to lactogenic hormones was prompt. When mammary epithelium was cultured on adipocytes and allowed to attach and grow in the presence of only insulin, the epithelium did not react with monoclonal antibodies to α-, β-, γ-casein or antiserum to α-lactalbumin.

extracellular matrix produced by adjacent cells, particularly adipocytes, rather than substances of a humoral nature.

However, cytodifferentiation of mammary epithelium may not be solely dependent on matrix constituents or as proposed, changes in cell shape without a requirement for interactions with the cellular stroma of the gland. In none of the experiments using normal murine, rabbit or rat, mammary cells, are cultures composed solely of mammary epithelial cells. In most of the culture models mentioned above other cells that constitute the cellular environment in which the epithelial cell differentiates (myoepithelial cells, preadipocytes, or cells lacking a fat vacuole that are designated fibroblasts, adipocytes, and perhaps others) were present in mammary cell suspensions. Thus, both mammary epithelium and stromal cells were present in the experiments performed in collagen gels and on the various non-cellular substrates, even though in some experiments efforts were made to reduce the number of the non-epithelial cells (Tonnelli and Sorof, 1982; Lee et al., 1984; Lee et al., 1985). It may be that differentiation occurs on severely contracted, detached, floating collagen gels, for example, because with contraction, non-epithelial cells are brought into closer proximity to the mammary epithelial cells permitting the interactions seen with adipocyte cellular substrates. Likewise anywhere from four to six weeks of incubation are required for mammary epithelial cell suspensions cultured within collagen gels to respond to hormones. The apparent requirement for a long incubation of mammary cell suspensions in collagen gels may indicate that the non-epithelial cells must increase in number in proximity to the epithelial cells before an increase in milk protein synthesis can be detected. This hypothesis may explain why not every epithelial cell within a collagen gel was immunoreactive when co-cultured with adipocytes. Also the presence of stromal cells may explain the small amount of cytodifferentiation that we are able to detect in mammary epithelial cells grown on non-permissive cellular substrates such as human foreskin fibroblasts after long incubation times (Levine and Stockdale, 1985; Wiens et al., 1986). Likewise there is little evidence in this adipocyte-mammary epithelial cell model system that changes in cell shape per se are responsible for cytodifferentiation of mammary epithelium. Cells attached to adipocytes may very well have a different shape than those attached to plastic, but cells attached to human foreskin fibroblasts appear to be the same shape as those attached to adipocytes, yet mammary epithelium does not differentiate on human foreskin fibroblasts or plastic. The hypothesis that cytodifferentiation requires the interaction of mammary epithelial cells with living stromal cells is testable with cloned primary mammary epithelial cells cultured in collagen gels with and without accompanying adipocytes.

Findings from all the systems used for the study of mammary epithelial differentiation in vitro raise the question of what controls sensitivity of the epithelium to prolactin. Mammary epithelium expresses prolactin sensitivity only in certain environments. Such sensitivity must be a prerequisite for the cyto-differentiation seen in the model cell culture systems. When mammary epithelial cells were cultured on tissue culture plastic, the addition of prolactin did not result in milk protein production. The cells lacked sensitivity to prolactin. When mammary epithelium was cultured on adipocytes, but not on Swiss 3T3 cells, or on human foreskin fibroblasts, daughter cells were formed in response to the serum mitogens that did not make milk proteins unless prolactin was added to the medium (Levine and Stockdale, 1985). Co-cultivation of mammary epithelial cells with adipocytes established a state of prolactin sensitivity that was sustained for weeks in the absence of prolactin and expression of casein synthesis (Wiens et al., 1986). It should be possible to determine if this effect was a manifestation of increase rates of prolactin receptor synthesis, increased rates of insertion of the prolactin receptor into the epithelial cell membrane, or increased stability of the prolactin receptor. The surrounding

extracellular matrix components may be crucial in one or more of these processes.

III. CELL PROLIFERATION IN CO-CULTURES OF MAMMARY EPITHELIUM AND ADIPOCYTES

It was possible to dissociate proliferation from cytodifferentiation in the adipocyte-epithelial cell model. When suspensions of mammary epithelial cells were cultured on adipocytes, there was a 5-fold increase in the rate of DNA synthesis compared to the same cell suspension cultured on tissue culture plastic (Levine and Stockdale, 1984). The concentration of the mitogens present in serum was not the sole factor in effecting cell proliferation on these two culture substrates. The nature of the substrate as well as the concentration of mitogens determined the extent of epithelial cell proliferation (Levine and Stockdale, 1984). As the concentration of serum in the medium increased, the number of mammary epithelial cells on adipocyte substrates also increased. This response to mitogens was not dependent upon adipocytes themselves. If the adipocytes were removed from the tissue culture plate with EDTA leaving only substrate attached material (SAM), such dishes supported mammary epithelial cell proliferation to the same extent as adipocytes. When mammary epithelial cells were cultured on tissue culture plastic without either adipocyte SAM or adipocytes themselves, there was increased mammary epithelial cell growth at every increase in serum concentration, but the increase was less than it was when the substrate was adipocyte SAM or adipocytes themselves.

Analyses of proliferation of mammary epithelial cells on an adipocyte cellular substrate suggested that factors other than extracellular matrix alone may regulate the growth of mammary epithelium. Mammary gland growth *in situ* is generally attributed to ovarian steroid hormone stimulation. But there also may be local environmental factors produced by stromal elements that regulate growth. When mammary epithelium was cultured in medium in which adipocytes have previously grown there was a marked increase in epithelial cell proliferation. The adipocytes conditioned the medium in a fashion that enhanced mammary epithelial cell proliferation (Fig. 1). The epithelium responded to the growth stimulus of conditioned medium whether it was cultured on SAM or on tissue culture plastic. This conditioned medium effect on mammary epithelial cell growth suggests that growth regulation within the mammary gland may have a paracrine as well as an endocrine basis. Though there is abundant evidence that steroid hormones are responsible for the growth of the gland *in vivo* (Topper and Freeman, 1980), these observations on the paracrine effects of adipocyte conditioned medium, raise the possibility that the adipocyte may produce substances that regulate growth *in vivo* as well.

IV. MORPHOGENESIS OF MAMMARY EPITHELIUM IN CO-CULTURES WITH ADIPOCYTES

Duct branching, alveolus formation and mammary epithelial cell proliferation must be highly regulated if morphogenesis of the ductal-alveolar system is to occur. Studies in the adipocyte-mammary epithelium co-culture system indicate the regulatory controls for morphogenesis of the gland are separable from those of cytodifferentiation (Wiens et al., 1986). Cytodifferentiation and morphogenesis in a ductal-alveolar system are not mutually interdependent processes. When individual mammary epithelium cells were cultured on adipocytes and the three lactogenic hormones were added to the medium, individual cells synthesized caseins, α-lactalbumin and lipids (Wiens et al., 1986). This was true even though they were not in contact with other epithelial cells or part of a ductal structure. When these individual cells were cultured in the presence or absence of prolactin, they continued to grow on the adipocyte cell substrate and to form plaques or

colonies of epithelial cells which only later formed ducts. Thus cytodifferentiation was independent of morphogenetic events since it occurred in mammary cells in isolation.

Morphogenesis readily occurred in co-cultures of mammary epithelium and adipocytes and occurred concurrently or independently of cytodifferentiation. Near the end of the first six days of co-culture of the epithelium with adipocytes in the presence of insulin, hydrocortisone and prolactin, or insulin alone, it was apparent that morphological changes were occurring within the plaques of epithelial cells that formed on the adipocyte substrate (Fig. 2). First short oblong epithelial duct-like structures were noted, then over the next week these grew in length, branched, and developed central lumina (Fig. 3). Thus, within the first two weeks of co-culture two-dimensional branching ducts with rounded ends appeared. These ducts when formed in the presence of insulin, hydrocortisone and prolactin were composed of cells with lipid vacuoles as well as casein (Fig. 2, 3a). Branching mammary ducts developed in these cultures whether or not prolactin and hydrocortisone were added to the medium (Fig. 3b). In the presence of insulin alone, non-functional ducts (ducts that did not synthesize casein or contained lipid vacuoles) formed and remained non-functional. With the subsequent addition of hydrocortisone and prolactin, both casein synthesis and lipid accumulation appeared.

Ultrastructural analysis of the ducts that formed in mammary epithelial cell-adipocyte co-cultures revealed a well developed basal lamina, and epithelial cell polarization toward a central lumen that was lined by microvilli (Fig. 4). A complete basement membrane surrounded an entire

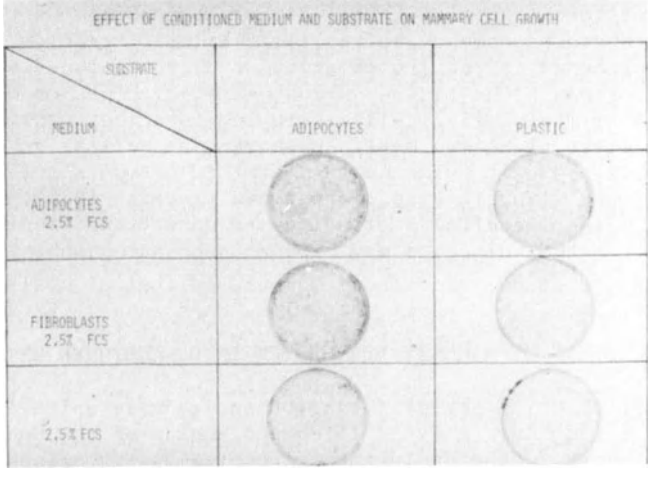

Figure 1. The same number of mammary epithelial cells from mid-pregnant BALB/c mice was plated on two types of substrates - adipocytes or tissue culture plastic. Medium was added to these dishes in which adipocytes alone, preadipocytes (fibroblasts) alone, or no cells had previously grown (2.5% FCS) and the dishes were incubated for 3 days and then fixed and stained with hematoxylin. Serum in the medium was intentionally used at low concentration (2.5%) to make apparent the growth enhancement of conditioned medium. Thus growth of mammary epithelium on adipocytes or tissue culture plastic was enhanced by factor(s) in medium conditioned by adipocytes and preadipocytes (fibroblasts).

duct. The basement membrane was about 54 nm thick and consisted of a lamina lucida (20 nm thick) and lamina densa (34 nm thick) (Wiens et al., 1986). The cytoplasm contained large lipid vacuoles and large amounts of rough endoplasmic reticulum. These structures did not appear if the epithelium was cultured on HNFF fibroblasts or plastic. In addition to the basement membrane, a stroma rich in interstitial collagen fibrils also formed adjacent to the basement membrane on which the epithelial cells rested (Fig. 5). The luminal surface of the ducts was lined by epithelial cells covered with microvilli and the lumen frequently contained secretory material. As in normal development the formation of a basement membrane appeared to be

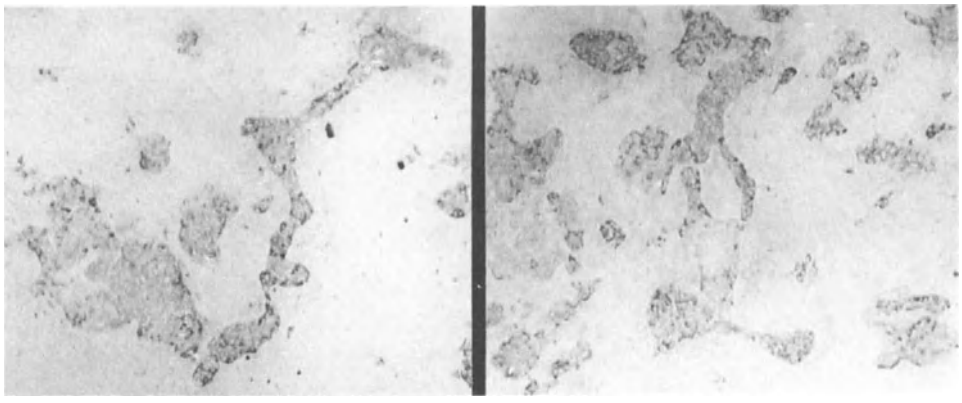

Figure 2: Examples of the early ducts formed by two weeks of incubation of mammary epithelium from mid-pregnant BALB/c mice on an adipocyte substrate in the presence of insulin, hydrocortisone, and ovine prolactin. The cultures have been stained with a monoclonal antibody to murine β-casein. Virtually every cell in these structures stained. Final magnification, 340 X.

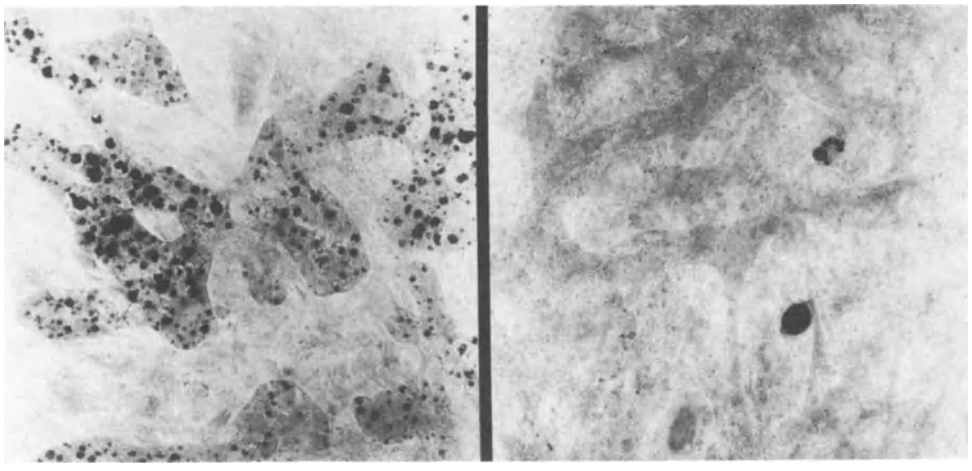

Figure 3: Mammary epithelium from a mid-pregnant BALB/c mouse was plated on adipocytes and incubated for 14 days in medium containing: (Left panel) insulin, hydrocortisone, and prolactin (all at 5 μg/ml); or (Right panel) only insulin (5 μg/ml). The cultures were then fixed and stained in 1% osmium tetroxide. Note the network of ducts and the presence of large fat vacuoles in the epithelial cells of the ducts incubated in the three lactogenic hormones. In the ducts formed in the presence of insulin alone no fat vacuoles are seen. Final magnification, 640 X.

important for the polarization of epithelial cells and their organization into ducts. When insulin, hydrocortisone and prolactin were present in the medium, these ducts manifested all the features of a secretory mammary epithelium, i.e., abundant RER and secretory vesicles, dense casein micelles, and a well developed Golgi apparatus. But prolactin was not necessary for basement membrane formation. If the same epithelial cells were cultured on plastic substrates or HNFF cells, ducts did not appear and the epithelium developed only rudimentary evidence of cell polarization and no basement membrane.

Morphogenesis also occurs in culture systems that do not employ living cell substrates (Bennett, 1980; Omerod and Rudland, 1982; Foster et

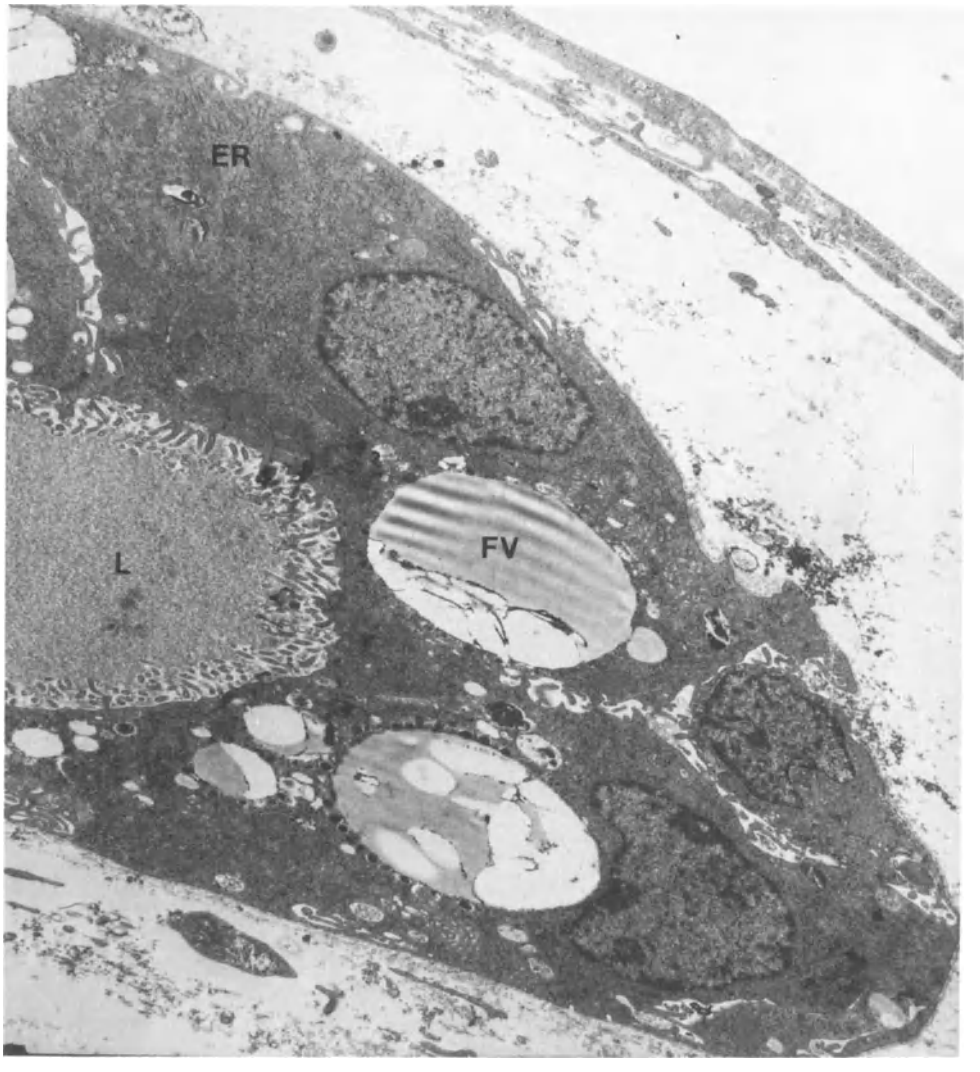

Figure 4: Transmission electron micrograph of a duct formed in co-culture of mammary epithelium from a mid-pregnant BALB/c mouse with adipocytes in the presence of insulin, hydrocortisone and prolactin. The lumen (L) is lined by microvilli and the surface of the duct is completely encircled by a basement membrane. The cytoplasm contains large fat vacuoles (FV) and many profiles of rough endoplasmic reticulum (RER). Final magnification, 13,900 X.

al., 1983; Danielson et al., 1984). When suspensions of primary mouse and rat mammary epithelial cells or even mammary tumor cells are embedded on collagen gels or are cultured on floating collagen gels, they will form duct-like structures. Epithelial cells in these culture systems form stellate 3-dimensional branching ductal structures usually with pointed (spike-like) ends that emerge from a central cellular mass. The collagen gel experiments suggest that stromal cell-epithelial cell interactions may not be required for morphological events to occur. However, as indicated in the discussion on cytodifferentiation, it is unknown if the stromal cells in such cultures are important for the morphogenesis that does occur.

The adipocyte co-culture system mimicked the sequence of events in normal murine mammary gland development. At puberty the main mammary ducts

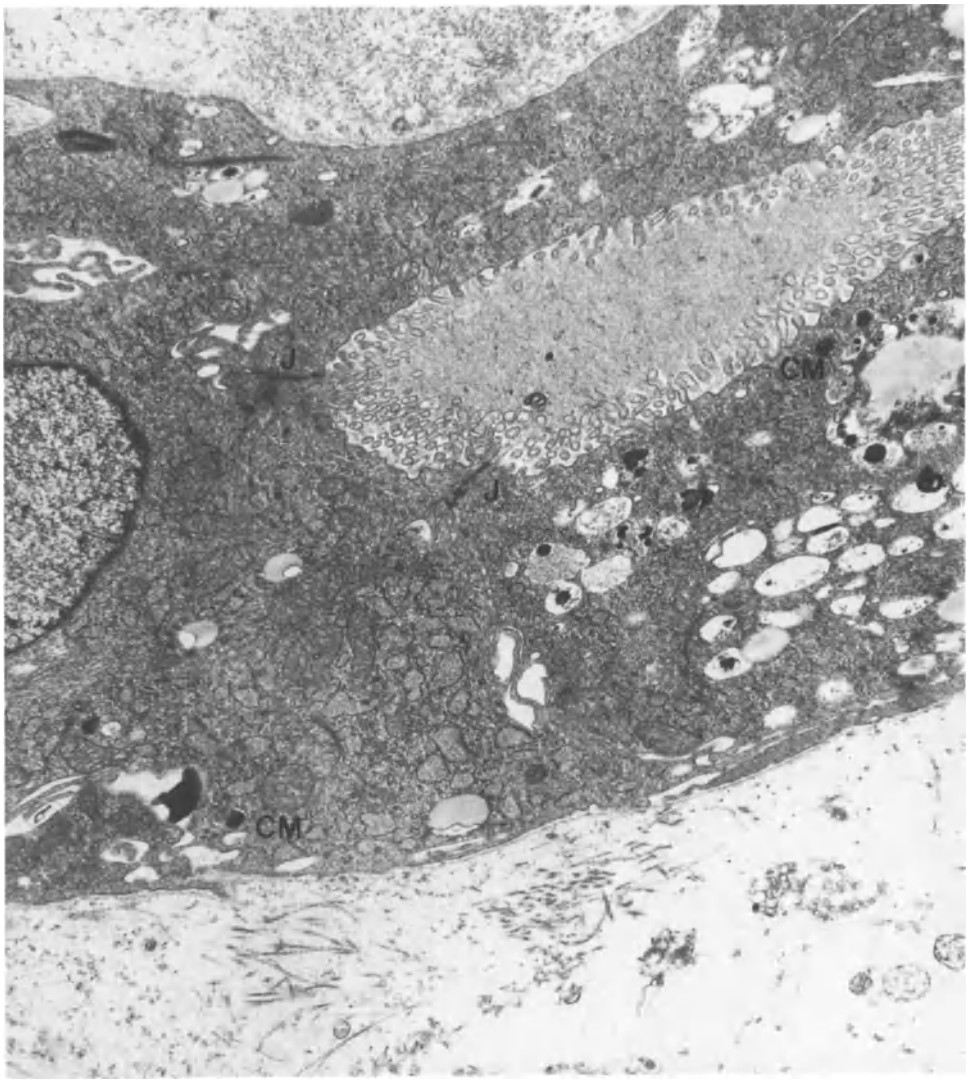

Figure 5: Transmission electron micrograph of another duct as described in Fig. 4. This micrograph illustrates the interstitial collagen fibrils formed adjacent to the basement membrane on which the epithelium rests; casein micelles (CM); and junctional complexes (JC) between epithelial cells. Final magnification, 19,500 X.

grow in length and form a branching ductal system that populates the mammary fat pad of the mouse. The ducts formed during the pubertal period do not synthesize milk proteins. This ductal system remains relatively dormant until pregnancy when there is a marked increase in epithelial cell proliferation. It is after this period of rapid ductal and alveolar growth that the epithelium begins milk protein synthesis. In the adipocyte-mammary epithelial cell co-culture system ducts formed in the absence of prolactin also remained dormant until exposed to prolactin. These ducts can remain non-secretory for at least 14 days but can begin the synthesis of the complete complement of milk proteins when hydrocortisone and prolactin are added to the culture medium.

V. SUMMARY

A co-culture system of adipocytes and mammary epithelium was developed to study the role of cell interactions in mammary gland cytodifferentiation and morphogenesis. Analysis of the interaction of adipocytes with murine mammary epithelium in cell culture reveals that the processes of proliferation, cytodifferentiation, and morphogenesis are not interdependent processes. However, these processes have in common that components of the extracellular matrix and/or cellular stromal elements influence them. Mammary epithelial cells are quite capable of cytodifferentiation before morphogenesis occurs, since it can be elicited in single isolated epithelial cells. Cytodifferentiation is a prolactin-dependent process. Morphogenesis on the other hand, can occur in the absence of cytodifferentiation (milk protein synthesis) and the complete complement of lactogenic hormones. An important element in distinguishing those substrates that support cytodifferentiation and morphogenesis was the ability of the substrate to foster the deposition of a basement membrane. It is proposed that the presence of stromal cells or certain matrix components permits the deposition of the basement membrane by the mammary epithelium. The possibility also was raised that paracrine interactions between the adipocyte and epithelium of the gland may complement the role of the matrix components in modulating epithelial cell proliferation. This model system therefore, supports the general contention that interaction between elements within the microenvironment of the mammary epithelium are required for proliferation, cytodifferentiation, and morphogenesis.

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INTERACTION OF MAMMARY TUMOR SUBPOPULATIONS

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Preceding chapters have been concerned with the ways in which normal and neoplastic cells react to and influence their microenvironment in the development and functioning of normal, as well as malignant, mammary tissues. This environment consists of other cells, of extracellular matrices, and of a wide variety of growth modulating factors. Looked upon from this perspective, tumor subpopulation interactions are but a special case of a general biological principle, namely, that cells, as well as whole organisms, do not exist and function in isolation but rather as mixed populations in which characteristics of individual components are modified by characteristics of the other members of their "ecosystem" (1). The purpose of this chapter is to review the evidence that tumor cell subpopulations within a single neoplasm can affect each other's phenotype and to speculate on the variety of mechanisms that can be involved. A prerequisite to this discussion is consideration of the concept of tumor heterogeneity, especially as it applies to breast cancers.

I. Mammary Tumor Heterogeneity

Recent work in the heterogeneity of experimental tumors has been focused on the identification and characterization of different "clonal" subpopulations within single cancers. This work has generally required that tumors be subjected to a variety of dissociation and cloning procedures,

usually carried out in vitro, and that the subpopulations obtained be further subjected to prolonged periods of independent cultivation in order to show that, at least for the period of observation, they "breed true." The earliest experimental report, of which we are aware, that mammary tumors can contain heterogeneous, neoplastic components was that of Henderson and Rous (2) who sprayed fragments of individual mouse mammary tumor virus (MMTV)-associated tumors over the surfaces of subcutaneous connective tissue that had been split by forcibly injecting Locke's solution and air into the skin of young mice. This procedure revealed a variety of benign and neoplastic structures which could then be separately propagated and studied. More recent studies have utilized less imaginative methods to isolate different subpopulations, for example, directly picking out patches of "interesting" cells from monolayer cultures (3), plating on different substrata (3), gradient separation (3,4), cloning in agarose (5,6), agar (7), or on plastic cell culture dishes (8), and selecting cells from individual metastases or pieces of "primary" implants (9,10). The distinctive characteristics of subpopulations obtained in these ways suggest that the extensive heterogeneity in morphology, histology (11), and antigen (12) and hormone receptor expression (13) that has been noted in human breast cancers has its basis in a coexistence of multiple tumor subpopulations. Indeed, two subpopulations have been isolated from a single biopsy fragment of a human breast cancer (14), and human breast cancer cell lines, including MCF-7, have been shown to be heterogeneous in respect to a variety of characteristics (15,16,17,18). A clonal basis for heterogeneity in growth properties (16), cytogenetic characteristics (18), and ER status (16,18) has been established in MCF-7 cells. Altogether the evidence suggests that the observations on heterogeneity of experimental tumors can be extended to human breast cancer. However, there are at least three notes of caution to accepting this statement at face value. Firstly, as discussed previously (19), there are sources other than differing clonal subpopulations for the heterogeneity of tumors: differences in three-dimensional architecture, in relationships to vasculature and connective tissue stroma, in distribution of inflammatory cell infiltrates, and in cell cycle, to name a few. Secondly, observations on tissue sections, etc. cannot distinguish clonal heterogeneity from heterogeneity imposed by these other sources. Thirdly, in order to prove clonal heterogeneity, investigators must subject tumors to procedures which may themselves promote further genotypic diversification, leading to the emergence of new clones. Thus, one is never absolutely certain that the characteristics of a given isolate are truly reflective of its characteristics prior to isolation. This is true even without the confounding potential for tumor subpopulation interactions which, as will be discussed below, can modify these characteristics in unexpected ways.

Keeping these reservations in mind, heterogeneity among tumor subpopulations has been shown in a variety of experimental breast neoplasms, including those of the mouse (2,3,5,6,20) and the rat (4,8,10) and those which have a viral (2,3,20), chemical (4,10) and "spontaneous" (5,7) etiology. Phenotypic properties that can be distributed differentially in breast tumors include karyotype (3,21), histology and cellular morphology (3,7,22), growth rate in vitro (3,6), capacity for anchorage-independent growth (5), expression and inducibility of tumor-associated antigens (23), immunogenicity (24), hormone receptor expression (20,25) and, in the case of MMTV-associated tumors, patterns of acquired viral DNA copies (20). Mammary tumor subpopulations have been shown to vary in capacity for differentiated function (4,26), as well as in expression of more generalized abilities such as fibronectin production (8). Important behavioral characteristics are also distributed differentially among subpopulations from single breast tumors, for example, ability to engage in contact-mediated intercellular communication, i.e., "metabolic cooperation" (27) and, of premier importance, ability to metastasize (6,7,10,28,29). An additional important parameter by which tumor subpopulations can differ is sensitivity to

therapy, including chemotherapy (9,30,31), radiation (29,31,32,33) and hyperthermia (31). In addition to the many questions concerning the biological significance of such variablility, this extensive heterogeneity poses numerous logistical problems in the design and execution of investigations utilizing rodent breast cancers.

II. Origins of Tumor Heterogeneity and Tumor Progression

The necessity of demonstrating that isolated subpopulations breed true has been mentioned as an important criterion in establishing clonal heterogeneity. Yet it seems obvious that this criterion cannot be applied too stringently. Many cancers are considered to be clonal in origin; how then can clonal diversity arise? Furthermore, "genetic instablility" is thought to be a hallmark of neoplastic cells (24). Thus, clonal diversification is a characteristic feature of cancer development and growth and one cannot expect that an isolated subpopulation would never undergo change. The rate at which new variants emerge within clonal subpopulations can be unexpectedly quick (on the order of 10^{-2} variants/cell/generation), or, alternatively, many orders of magnitude less frequent (35). The "stability" of isolated subpopulations varies greatly, even within one tumor (28,35) and, as will be discussed below, can be modulated by the coexistence of other subpopulations. Different phenotypic characteristics can change, independently and at different rates, within single clones (29,30,31,35). This "phenotypic drift," which is most easily quantitated in vitro, bears great similarity to the phenomenon of neoplastic progression as defined by Foulds (36) and experimentally demonstrated by him and others in mammary tumor systems (36,37,38). Although the generation of variants is often considered to be a random process, it can be remarkably reproducible: repeated tests on multiple samples of the same clone can reveal phenotypic drift in the same characteristics at essentially the same passage level (35). Again this reproducibility has been noted in studies of progression in which mammary tumors, serially passaged through syngeneic rodent hosts, have been observed to change phenotypically in a repeatable (39), even cyclical (40), manner. As has been discussed by Welch, et al. (35), there are several possible explanations for this apparent regulation of clonal diversification: 1) it may be analogous to events in embryonic development, with some sort of preprogrammed schedule, 2) it may actually be random but, in the face of common selective pressures, the mixture of cell populations which survive would, as a whole, express similar phenotypes, and 3) subpopulation interactions may modulate the expression of divergent, clonal phenotypes so, as in 2), the characteristics of the mixed population, whether in a culture dish or growing as a tumor, would represent a "consensus" phenotype.

Irregardless of the validity of these speculations, one is still faced with attempting to explain the molecular mechanisms responsible for clonal diversification. Current opinion holds that no one class of mechanism is the sole source of tumor heterogeneity. Other chapters in this book are devoted to mechanisms which certainly could be involved: chromosomal abnormalities (Wolman); subchromosomal events, such as gene amplification, genomic rearrangements, "conventional" mutations, as well as alterations in gene expression, as by DNA methylation; and neoplastic stem cell differentiation, which as in normal cell differentiation, gives rise to phenotypically distinct cellular subpopulations (Rudland). In this latter regard our laboratory has described a mouse mammary tumor "stem" cell line, 68H, that is capable of producing a wide range of clonal variants distinct in morphology, antigenicity, and growth potential (41).

Other postulated mechanisms of clonal diversification invoke events occurring within the tumor microenvironment, rather than the genomic instability or developmental program of the neoplastic cells per se. Cell

fusion between tumor cells or between tumor and normal cells, followed by chromosomal redistribution, is one such possibility (42). We have been studying another mechanism that requires the participation of other cells, in this case tumor-infiltrating macrophages. We have reported that macrophages isolated from tumors produced by a series of cell lines originally derived from a single mouse mammary tumor are themselves a heterogeneous population (43,44,45) In this system tumor-associated macrophages from cell lines that are able to metastasize are enriched for the more mature, activated subtypes, as compared with macrophage populations from nonmetastatic tumors. A characteristic of activated macrophages is the production of active oxygen metabolites which can be involved in mutation, promotion, and neoplastic transformation (46,47,48). We have shown that tumor-associated macrophages are mutagenic to bacteria in the Ames' assay (49) and able to induce 6-thioguanine resistant variants in a mouse mammary tumor cell line (50). We propose that inflammatory cells, such as macrophages, are a source of "endogenous mutagen" that fuels the process of clonal variation and tumor progression.

Tumor-associated macrophages may also effect tumor progression by a mechanism suggested by Schimke (51). Schimke has proposed that any toxic agent or circumstance (for example, hypoxia), when it does not directly kill tumor cells, but only interferes with their proliferation, could cause areas of gene amplification and destabilization of the genome. This line of thinking can readily be applied to mammary cancers in which areas of necrosis, low growth fractions, etc. are commonly observed.

The purpose of this somewhat lengthy introduction to the main subject of this chapter is to familiarize the reader with current ideas about tumor heterogeneity in mammary cancers in a context that emphasizes the entire tumor ecosystem. Tumor subpopulation interactions both make use of, and alter, the circumstances of that ecosystem. As will be detailed below, subpopulation interactions have consequences on expression of a wide variety of phenotypic traits.

III. Consequences of Tumor Subpopulation Interactions

A. Alteration of Tumorigenicity or Growth Rate

The possibility that tumor subpopulations might influence each other's growth properties was recognized thirty years ago by Hauschka (52) when he found that subpopulations of the Krebs 2 tumor were individually more tumorigenic than was the parental tumor line. This suggested to him that the subpopulations had been "held in check by other elements in the parental" tumor. On the other hand, Woodruff et al. (53) has postulated that "some sublines require the cooperation of others to survive" because clones are often less tumorigenic than their parental, methylcholanthrene (MCA)-induced tumors. Both consequences of subpopulation interaction have been observed on several other occasions.

A common, perhaps related, phenomenon is that of a primary tumor influencing the growth of metastatic nodules (54-57). Fisher et al. (54) found that the removal of a primary mouse mammary tumor resulted in increased mitotic indices in established metastases. Similarly, the effect of a primary tumor growing s.c. on the ability of tumor cells to form lung colonies after intravenous (i.v.) injection depended upon the size of the primary for mouse sarcoma L1 (56) and 3LL Lewis lung carcinoma (55) lines. Such interactions have been attributed to both immunologic and nonimmunologic mechanisms. A second challenge with Walker 256 carcinoma cells stimulated the growth of an earlier transplant despite the fact that the second transplant did not grow (57). By using an unrelated fibrosarcoma, it

was demonstrated that the resistance to the second challenge was not tumor specific but the augmentation of the first was (57).

We have examined interactions between subpopulations of a BALB/cfC_3H mouse mammary tumor by injecting the isolated subpopulations on contralateral flanks (58). Growth of a subpopulation in this "bilateral protocol" was compared to single implant growth and to growth when the same subpopulation was transplanted on both sides. Two subpopulations, line 66 and line 67, mutually inhibited each other's growth as determined by changes in incidence or latency. A highly immunogenic subpopulation, line 410, inhibited the growth of a poorly immunogenic subpopulation, line 168. The presence of line 168 had no effect on the growth of a second line 168 tumor or of a line 410 tumor. The basis of the 410-168 interaction appears to be immunologic because it did not occur in irradiated mice and because resistance to line 168 could be transferred adoptively with lymph node cells from line 410-immune mice (58).

Caignard et al. (59) used a similar bilateral protocol to detect interactions between two subpopulations, TR and TS, of a 2,2-dimethylhydrazine induced rat colonic carcinoma. Line TR was tumorigenic and TS was nontumorigenic in normal rats. However, prior inoculation of TS cells inhibited tumor formation by TR cells and TS cells formed tumors if injected into rats bearing TR tumors. Both effects could be adoptively transferred with splenocytes (59). These studies illustrate the presence of interacting immunologic properties which both stimulate and suppress host responses.

A bilateral protocol was also used to demonstrate the ability of one slow growing clone of a C_3H mouse mammary tumor to stimulate the growth of a second slow growing clone (6). This interaction was not reciprocal; clone MT-2D stimulated the growth of MT-2ES, but clone MT-2ES did not effect the growth of MT-2D. Preirradiation of the host mice had no effect on the interaction. Single tumors formed from mixtures of MT-2D and MT-2ES (at a 1:1 ratio) grew faster than tumors formed from either individual clone (6).

Newcomb et al. (60) described interactions between B16 melanoma clones which could be demonstrated only when the clones were mixed together, not when a bilateral protocol was used. One clone, which was not tumorigenic, prevented the growth of two tumorigenic clones in normal mice. The nontumorigenic clone differed from the two tumorigenic clones in its inability to produce plasminogen activator (PA). In cocultures, the nontumorigenic clone inhibited the production of PA by either of the two tumorigenic clones. The nontumorigenic clone did grow in mice immunosuppressed with antithymocyte serum treatment and the cells established in tissue culture from the resulting tumors were transiently 1) able to produce PA, 2) able to produce tumors in normal mice, 3) unable to inhibit growth of the tumorigenic clones, and 4) less able to depress PA production by the tumorigenic clones. Newcomb and associates suggested that PA production suppressed local, but not systemic, expression of host resistance (60).

Butler et al. (61) described the ability of two nontumorigenic MCF-7 clones to produce tumors when co-injected into nude mice. Four nontumorigenic clones, D6, E6, K2, and 4R, were tested, two at a time. Only the combination of D6 plus E6 was tumorigenic.

Nowotny and Grohsman found that two variants of the TA3 mouse mammary carcinoma interacted so that both or neither would grow in allogeneic mice depending upon the ratio of the two variants injected (62).

There is some evidence that tumor subpopulation interactions may result in the establishment of an end state ratio. Tumor line ELD, selected from the Ehrlich ascites mammary tumor, maintains a stable ratio of 96%

diploid cells and 4% tetraploid cells in vitro (63). The ratio can be altered by selecting for tetraploid cells but, once the selective pressure is removed, the 96:4 ratio is again achieved. A similar event has been described in vivo in which a 9:1 ratio between two clones of a human colon carcinoma was established and maintained in nude mice, irrespective of the starting ratio (64).

Tofilon et al. (65) found that mixed spheroids, made up of a fast growing and a slow growing subpopulation of a rat brain tumor, grew at the same rate as spheroids made entirely of the faster growing subpopulation. In this case the starting ratios of fast to slow subpopulations were maintained. Although growth in spheroids allows maximum cell-cell contact, the necessity for contact was not established.

The ability of conditioned media from one tumor subpopulation to stimulate the growth, in vitro, of a second tumor subpopulation has been demonstrated for 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors (66,67). Isacke and Deller (68) reported an interaction between malignant embryonal carcinoma cells and a benign subpopulation in which each reciprocally stimulated the growth of the other. Both soluble factors and contact-mediated mechanisms were implicated in the stimulation of the malignant cells by the benign variant cells.

In our own studies of growth interactions in vitro between subpopulations of a mouse mammary tumor, both contact-mediated and soluble factor-mediated interactions occur (69). We compared the ability of tumor subpopulations that were plated on separate cover slips (preventing cell-cell contact) to alter each others growth rate when bathed in a common media. Coculture with lines 168 or 66 inhibited the growth of other subpopulations, but lines 68H, 67, or 410 had no effect. However, line 68H cells could alter the growth of line 168 if cell contact was allowed between the subpopulations.

B. Alteration of Metastatic Phenotype

That tumor subpopulation interactions might affect metastasis is suggested by the suppression of metastasis which can result from the induction of a host immune response to a primary tumor (55,56,70,71). Subpopulation interactions might also result in an increased incidence of metastasis. Slemmer (72) reported that the presence of normal myoepithelial cells was essential for metastasis of neoplastic mammary cells. Other studies have shown interactions in which a metastatic subpopulation increases the metastatic proficiency of an otherwise nonmetastatic subpopulation. Poste and Nicolson (73) increased the lung colony forming efficiency of the poorly metastatic B16 melanoma F1 subline by fusion with membrane vesicles from the highly metastatic subline F10. Malinoff et al. (74) found that highly metastatic variants of two MCA-induced mouse sarcomas produced high levels of laminin whereas poorly metastatic variants did not. Exogenous laminin increased the lung colony forming efficiency of the latter sublines.

We have reported that metastases which develop in the simultaneous presence of metastatic and nonmetastatic subpopulations of a mouse mammary tumor can contain clonogenic cells of the nonmetastatic subpopulation (75,76). In one protocol, mixtures of the highly metastatic line 410.4 and poorly metastatic line 67 were injected i.v. Resulting tumor lung colonies were dispersed and placed into primary tissue culture. Both line 67 and line 410.4 colonies were found in these "experimental" metastases. In a second protocol, the nonmetastatic line 168 was transplanted into the subcutis. Metastatic line 410.4 cells were then injected i.v. to produce lung colonies which were dispersed and placed into primary tissue culture. Both line 168 and line 410.4 colonies were identified in these colonies. Injec-

tion of the nonmetastatic 168 cells i.v. into mice bearing line 168 tumors s.c. did not result in the formation of lung colonies (75). In both these protocols the existence of the "nonmetastatic" cells in the lung colonies was determined by morphology. Recently, a drug resistant variant of the metastatic line 66 has been used to verify these results (76). This variant, 66cl4, is resistant both to 3 mM ouabain and to 60 μM thioguanine, the latter because of a deficiency in the enzyme hypoxanthine guanosine phosphoribosyl transferase ($HGPRT^-$) which makes it unable to grow in HAT medium (hypoxanthine, aminopterin, thymidine). HAT medium can be used to selectively grow wild type cells from mixtures with line 66cl4 and the identity of the cells so selected can be confirmed by their sensitivity to both thioguanine and ouabain. Lung colonies formed after i.v. injections of mixtures of line 67 and line 66cl4 cells did indeed contain tumorigenic cells which grew in HAT medium but not in ouabain or thioguanine and, therefore, presumably were line 67 cells (76).

It is not clear if these synergistic interactions between tumor subpopulations are related to the observations made by Slemmer (72) with genetically mosaic tissues.

C. Alteration in Drug Resistance

Cancers often regress upon initial administration of chemotherapeutic agents but then resume growing and are refractory to further treatment. This suggests that resistant tumor subpopulations were present in the tumor or were generated subsequent to the initiation of therapy. We have questioned whether tumor cells resistant to a drug could transfer that resistance to other subpopulations, a well known phenomenon in bacterial cells where resistance to antibiotics is transferred by episomes from cell to cell under the selective pressure of antibiotic treatment (77).

Our first experiments utilized mammary tumor subpopulation 168, which is sensitive to cyclophosphamide (CY), and subpopulation 410, which is not. With the bilateral protocol described for studying growth interactions, line 410 tumors were measurably sensitive to CY in animals that also carried line 168 tumors (78). Thus, unlike the bacteria system, sensitivity rather than resistance was transferred. Preirradiation of the hosts did not negate the ability of 168 tumors to enhance the sensitivity of 410 tumors to CY, suggesting that an immune reaction was not involved. We have also analyzed the effect of subpopulation interactions on sensitivity to methotrexate (MTX). These studies were carried out <u>in vitro</u> by plating different subpopulations on separate coverslips and growing them in a common, drug-containing medium. Once again, the dose response to drug was unchanged for the relatively sensitive subpopulation 410.4, regardless of the subpopulation with which it was cocultured, but three relatively resistant subpopulations, lines 67, 168, and 68H, uniformly became more sensitive to MTX when cocultured with 410.4 (78). Both the CY and MTX experiments were done under circumstances in which the interacting subpopulations were separated physically. The transfer of sensitivity to thioguanine from $HGPRT^+$ to $HGPRT^-$ cells via a mechanism requiring cell to cell contact, that is, the transfer of thioguanine nucleotides through gap junctions ("metabolic cooperation"), is a widely recognized way by which drug sensitivity can be transferred between cells. We have demonstrated the transfer of thioguanine sensitivity between our mammary tumor subpopulations, but we have also found quantitative differences between the abilities of different subpopulations to participate in this interaction (27).

In contrast to these results, Tofilon et al. (65) were able to demonstrate a transfer of resistance between tumor subpopulations. In this work 1,3-bis(2-chloroethyl)-1-nitrosourea resistant (R3) and sensitive (9L) rat brain tumor subpopulations were grown as spheroids. At a single drug dose,

survival of sensitive 9L cells in mixed spheroids increased as the percentage of resistant R3 cells was increased.

Recently we, too, have been able to demonstrate the transfer of drug resistance between our mammary tumor subpopulations. In this case a reciprocal transfer of resistance occurred so that two tumor subpopulations survived in the presence of a drug combination which killed either subpopulation when growing individually (79). Line 66 cells grow in HAT medium, but not in ouabain, which inhibits the active transport of Na^+ out of the cell. Line 66cl4 cells are resistant to ouabain but are unable to grow in HAT medium. Mixtures of the two lines, at ratios of 10:1 to 1:10, grew in medium containing both HAT and ouabain. For this to occur, cells must be able to undergo metabolic cooperation: purine nucleotides made by 66 cells presumably serve as a source of purine nucleotides for 66cl4 cells and 66cl4 cells remove excess Na^+ for both subpopulations.

Our previous experience with the transfer of MTX sensitivity between mammary tumor subpopulations in vitro (78) led us to develop a system to determine whether similar interactions could be observed in vivo. We have derived variants of our original mammary tumor lines that are resistant to thioguanine, 2,6-diaminopurine, and/or ouabain. With medium containing the appropriate selective drug, these variant subpopulations can be isolated from mixtures with other subpopulations. Mixtures of MTX sensitive 410.4, or its thioguanine and ouabain resistant variant 44FTO, with MTX resistant lines (in a ratio of 1:1) were injected into mice one day before noncurative MTX treatment. The observed growth delay subsequent to MTX treatment was as great for the mixtures as for 44FTO or 410.4 alone. Thus, tumors initiated with 50% of cells from the MTX resistant lines were not detectably more resistant to MTX than tumors initiated with 100% of cells from the sensitive line. Tumors were removed, enzymatically dispersed, and cloned in selective medium to determine the proportion of each tumor cell line in the resulting tumors. The expected shift to the MTX resistant subpopulation in the treated tumors was observed in only one of three experiments. Thus the "therapeutic response" did not necessarily correlate with enrichment for the MTX resistant line (80).

D. Alteration in Radioresistance

Although several reports have shown that tumor subpopulations are heterogeneous in radiosensitivity, only the reports of Dertinger et al. (81,82) indicate that tumor cell interactions can effect radiosensitivity. In these studies, tumor cell lines able to form gap junctions were more resistant to gamma irradiation than were tumor lines which were less able to do so. Furthermore, resistance required a three-dimensional structure because cells in monolayer were more sensitive than cells in a spheroid; cells in monolayer were no more resistant than were dispersed cells.

E. Interactions in Hormone Response

Demonstrations that one cell type of a hormone responsive tissue may be directly acted upon by hormones and then secondarily control the response of a second cell type (83,84) suggest that similar interactions might occur between tumor subpopulations. However, experimental evidence is not compelling. Line MCF-7 does grow preferentially in estrophilic sites (85) but cell interactions are not necessarily involved. Sluyser et al. (86) found that hormone independent (HI) strain GR mouse mammary tumors were not made hormone dependent (HD) even when HI cells constituted only 10% of the transplanted tumor cells. However, the constitution of the resulting tumors could not be determined, so the possibility that HD tumor cell replication was altered by the presence of HI cells was not excluded. Danielpour and Sirbasku (87) found that MTW9/PL, an estrogen responsive rat mammary tumor,

was composed of estrogen dependent, estrogen responsive, and estrogen independent clones. An estrogen independent line, MTW9B, was selected by serially passing MTW9/PL tumors in castrated male rats. Of seven clones derived from an apparently autonomous tumor, four were estrogen independent, two were estrogen responsive, and one clone was estrogen dependent. Thus, in the presence of HI cells, HD cells had been able, apparently, to survive and thrive in vivo. Estrogen independent clones produced a factor which stimulated the incorporation of thymidine by cultures of both estrogen dependent and estrogen independent clones. Production of this autocrine (and paracrine) growth factor was inducible by estrogen in a HD clone. Danielpour et al. (88) also reported the production of a growth factor by an estrogen responsive clone of rat GH3/C14 pituitary tumor cells and postulated that progression from estrogen responsive growth to autonomous, hormone independent growth is due to cells acquiring the ability to produce the autostimulatory factor without estrogen stimulation.

F. Progression and Clonal Stability

As mentioned above, Woodruff et al. (53) have suggested that cooperation with more aggressive sublines allow less tumorigenic cells to grow. Clones derived from mouse hyperplastic alveolar nodules (HANs) are generally not transplantable. For example, C1-S1, a clone of the D1 HAN line of BALB/c origin (89), does not produce outgrowths when transplanted into cleared mammary fatpads. Perhaps subpopulations of HANs are interdependent also, requiring cooperation for growth and progression. Enzymatic dispersion of apparently normal mammary tissue before transplantation results in the appearance of HANs (90), and enzymatic dispersion of HANs before transplantation accelerates their progression to tumors (91). These studies emphasize the potential importance of cell interactions in mammary tumor progression.

Interactions between cells have been implicated in drug-induced differentiation of P19 embryonal carcinoma cells. Variants of P19 were selected which were indifferent to treatment with dimethylsulfoxide (DMSO) or retinoic acid. Induction of differentiation of the parental cells when mixed with the variant cells still occurred after treatment with retinoic acid but not with DMSO. Thus, induction of differentiation with DMSO could be altered "by interactions between neighboring cells" (92).

Ling et al. (93) demonstrated that clones of the KHT mouse fibrosarcoma line are highly unstable with respect to the ability to metastasize. New, nonmetastatic variants develop at a high rate. Poste et al. (94) and Miner et al. (95) reported similar metastatic instability in B16 melanoma clones but found that it could be stabilized by growing the clones as mixtures, either in vitro or in vivo. The ability to stabilize was independent of the metastatic phenotype of the interacting cells. When clones were reisolated, metastatic heterogeneity was reestablished, whether the clones were maintained in vivo or in vitro.

Thus, subpopulation interactions may modify either the generation or expression of those mechanisms which are responsible for the production of the subpopulations to begin with.

IV. MECHANISMS OF SUBPOPULATION INTERACTIONS

Tumor heterogeneity is no longer a novel concept. The subject is now reviewed about as frequently as it was mentioned a decade ago. Although the concept of subpopulation interactions often appears in these discussions, the underlying mechanisms remain almost totally undetermined. Thus, the present discussion must primarily be conjecture and must rely on various models, not only those of tumor subpopulations. As will be seen, depending

on the specific interaction, either host or tumor factors may be responsible, and, if the latter, either external factors or cell-contact mechanisms may be involved.

A. Host-Mediated Tumor Subpopulation Interactions

Many tumor subpopulations are immunologically similar and immunization with any one of them seemingly induces (or fails to induce) the same response. Such reactions will not be considered here but, rather, we will concentrate on interactions that achieve a net result different from the responses to the individual subpopulations. One such interaction is the so-called "innocent bystander effect" (96,97) which results in the suppression of growth of a subpopulation which is not itself immunogenic but which coexists with an effectively immunogenic subpopulation. As an illustration, in syngeneic strain 2 guinea pigs, line 1 hepatoma is immunogenic and does not grow progressively in intradermal (i.d.) sites, whereas the nonimmunogenic line 10 hepatoma does (note: line 1 and line 10 are not subpopulations of an individual tumor). If lines 1 and 10 were injected i.d. at separate sites, no interaction was observed but if mixtures of line 1 and line 10 were injected i.d. into normal guinea pigs, tumors did not grow progressively, even when the number of line 10 cells was 100 times the tumorigenic dose. This bystander effect was attributed to injury to the shared microvasculature of the mixed tumor which, although initiated by a specific immune effect, had a nonspecific outcome (96,97).

We described the inhibition of nonimmunogenic subpopulation 168 tumors in mice bearing the immunogenic subpopulation 410 in a bilateral protocol (58). Thus, the mechanism of this interaction is clearly not the "innocent bystander effect." The interaction did not occur in preirradiated mice and could be adoptively transferred with lymph node cells. We suspected that the presence of line 410 altered the host-immune infiltrate into the contralateral line 168 tumor, leading to growth inhibition. The presence of a tumor can have systemic effects on the immune system reflected by changes in bone marrow composition (98) and the effects can be different for subpopulations of single tumors (99,100). Tumor-associated lymphocytes were obtained from enzymatically dissociated tumors by isokinetic separation and characterized according to reactivity with antilymphocyte serum (ALS), anti-Thy 1.2, anti-Lyt 1.2, and anti-Lyt 2.2 antibodies (101). The lymphocyte infiltrate from line 410 tumors differed from the infiltrate from line 168 tumors. In contrast to our expectations, these differences were still present in line 410 and 168 tumors growing bilaterally in individual mice (101). Thus, at least with these techniques and lymphocyte markers, we could not demonstrate that an alteration in the infiltrate was involved in this subpopulation interaction.

This and other descriptions of "one-way cross reactivity" between subpopulations (24,102) suggest that the failure of a subpopulation to induce immunity may be irrelevant in a heterogeneous tumor if that subpopulation is sensitive to the immune response induced by a sister subpopulation. However, it is also possible that subpopulation coexistence could result in the suppression of an otherwise effective immunity. Naor (103) suggested that the presence of "suppressogenic" epitopes on a tumor cell could result in the failure of the host to respond to immunogenic epitopes which might also be present. He recognized that the two epitopes could be present on distinct subpopulations. Whether or not an immune resistance to the tumor was induced would depend upon the balance between the two. The results of Nowotny and Grohsman (62) suggest that the balance between resistance and suppression can be tipped by altering the relative content of two tumor subpopulations. Perhaps the TR subpopulation of the rat colon carcinoma discussed earlier primarily expressed suppressogenic epitopes whereas the TS subpopulation primarily expressed immunogenic epitopes (59).

Legrue and Hearn (70) extracted B16 melanoma F1 and F10 variants with butanol. F1 extracts, but not F10 extracts, immunized mice against s.c. challenge with either F1 or F10 melanoma variants. Immunization with F1 extracts inhibited lung colonization by both F1 and F10 cells injected i.v. but pretreatment with F10 extracts enhanced lung colonization. Thus, a common determinant was apparently expressed on both variants but functioned as an immunogen on F1 cells and as a suppressogen on F10 cells (70).

In addition to the possible role of suppressogenic epitopes in host-mediated subpopulation interactions, an epitope expressed on one subpopulation may not induce a response when in the presence of an "immunodominant" antigen on another subpopulation (104). The ultraviolet light-induced 1591-RE mouse fibrosarcoma regresses in normal mice but occasionally grows progressively (1591-PRO). Immunization with either 1591-RE or 1591-PRO induced an immune response against 1591-RE but only 1591-PRO induced immune resistance to 1591-PRO. Urban et al. (104) concluded that 1591-RE had two tumor associated antigens but only one was immunogenic whereas the 1591-PRO did not have the antigen which was immunogenic on 1591-RE but the second epitope, which was antigenic but not immunogenic as expressed on 1591-RE, was immunogenic when expressed on 1591-PRO.

Finally, as if all these possibilities aren't enough, mixtures of subpopulations have been reported to act synergistically to induce host resistance not inducible by any of the individual subpopulations (105).

Although all the above examples of host-mediated subpopulation interactions seem to have an immune basis, this is not necessarily so. Besedovsky et al. (106) demonstrated that two fibrosarcomas altered several serum hormone levels in rats and that the changes were different for individual tumors. Theoretically, then, it may be possible for HI subpopulations to induce the host to produce hormones which enhance HD subpopulation growth.

Tumor subpopulations may also alter drug metabolism by the host (78,107-109). Cytoxan (CY) requires metabolic activation by microsomal cytochrome P450 mixed function oxidases, primarily in the liver. In our subpopulation experiments in which line 168 rendered line 410 sensitive to CY (78), we found that the toxicity of CY was greater for mice bearing line 168 than either for normal mice or mice bearing line 410 tumors, suggesting that the basis for the apparent transfer of drug sensitivity from 168 to 410 tumors was a more effective activation of CY by the 168 bearing host. Alternatively, deactivation of CY may be inhibited in 168 bearing mice.

B. Diffusable Mediators of Subpopulation Interactions

A currently held notion is that self-stimulating growth factors produced by tumor cells (i.e., autocrine factors), perhaps products of oncogenes (110), allow escape from dependence on host growth regulators and lead to the uncontrolled growth of tumor cell populations. From this concept it is a short step to postulate that such growth factors can be produced by one subpopulation, either spontaneously (67) or in response to hormones (111), and then stimulate growth of a non-producing variant. As assessed by the ability of conditioned media to stimulate the formation of colonies in soft agar by NRK (normal rat kidney) cells, different clones of MCF-7 produced different amounts of transforming growth factors (TGF)(112). Although TGF production did not correlate with the ability of a clone to form colonies in soft agar, one clone was shown to be sensitive to stimulation by a "crude mammary derived TGF" (112). Different cells respond to different growth factors (112-114) and, thus, subpopulations may make one factor but respond to a different one. Smith et al. (114) reported that a factor derived from pituitary gland stimulated division of cuboidal rat

mammary epithelial cell lines, whereas fibroblast growth factor stimulated stromal and myoepithelial lines. Epidermal growth factor (EGF) stimulation was variable but unrelated to lineage. In a heterogeneous tumor, subpopulations may be making and responding to different growth factors such that complex interdependencies are established.

In our laboratory the production of prostaglandin E (PGE) by mouse mammary tumor subpopulations was found to vary by more than 20 fold between the highest and lowest producers; the level of PGE produced correlated with metastatic phenotype (115). Subpopulations may also differ in their response to prostaglandins; a loss of PGE receptors on rat mammary tumor cells occurs during progression to hormone independence (116). The role of prostaglandins in mammary tumor biology is discussed in detail by Fulton elsewhere in this volume but it is possible that high PGE levels produced by one subpopulation in a mixture could alter the behavior of other subpopulations present.

Clonal variation in the production of a platelet derived growth factor (PDGF)-like substance has been described in a human malignant glioma (117) and breast cancer cell lines may also produce factors similar to PDGF (118). PDGF can down regulate EGF receptors (119) and the down regulation of EGF receptors has been reported to correlate with an elevation of plasminogen activator activity (120). PDGF is also able to stimulate collagenase expression by fibroblasts (121). Thus, the production of PDGF-like substances by one subpopulation, itself possibly unresponsive, could conceivably induce other subpopulations, nonproductive but responsive, to produce enzymes believed to be important in invasion and metastasis.

Finally, the production of tumor growth inhibitory factors has been described for the Ehrlich ascites tumor (122), for a human rhabdomyosarcoma (123), and for our mouse mammary tumor subpopulations (69). In the latter studies, the inhibitory factor was not produced by all of the subpopulations but both producers and nonproducers were inhibited. These tumor factors may be related to normal tissue chalones such as that prepared from bovine mammary gland which inhibited Ehrlich ascites mammary carcinoma cells (124).

C. Contact Dependent Mechanisms

Intercellular communication via the passage of small molecules through gap junctions between neighboring cells may be essential in the maintenance of normal tissues (reviewed in 125). The loss of competence in intercellular communication or an alteration in the specificity of communication may allow cancer cells to evade normal growth control signals. Because communication occurs very efficiently between some subpopulations, and poorly between others (27), growth signals transmitted via gap junctions could be nonrandomly distributed through a tumor. Interactions altering drug sensitivity (27,79) or radiation resistance (81,82) would also be expected to be asymmetrically distributed throughout a tumor.

Another type of contact-dependent mechanism may be involved in the appearance of "nonmetastatic" tumor cells in metastases. The formation of tight junctions, intermediate junctions, or desmosomes between neighboring cells could result in heterotypic emboli forming distant metastases containing cells with inheritantly different metastatic behavior.

Another contact-requiring cellular interaction, the ability of one cell type to influence the transcription of mRNA by a second cell type, has been reported recently by Fraslin et al. (126). This interesting finding

suggests the possibility that cooperation between oncogenes in the multistep development of cancer (127,128) may not require that the cooperating oncogenes be expressed in the same cells.

Other contact dependent mechanisms that may be involved in tumor subpopulation interaction are fusion of tumor cells, both with normal cells and other tumor cells (reviewed in 42 and 129) and, perhaps fancifully, "cannibalization" of some tumor cells by other tumor cells, as has been reported in small cell carcinoma of the lung (130).

D. Alteration of Extracellular Matrix

The role of the extracellular matrix in mammary tumor biology is the topic of another chapter (Durban) in this text. We will only briefly mention certain subpopulation interactions that may involve the extracellular matrix. Thus, subpopulations of mammary tumors may produce type IV collagen which, as a component of basement membrane, is required for the growth of other, nonproducing subpopulations (66). The elaboration of laminin (74) or hyaluronic acid (131) by highly metastatic subpopulations could possibly promote the metastasis of nonproducing, poorly metastatic subpopulations.

Interactions in the degradation of extracellular matrix have been observed frequently. Plasminogen activator (PA) can activate procollagenase (132) and induce platelet aggregation (133) as well as convert plasminogen to plasmin. Carlsen et al. (134) compared the PA production in vitro of 10 clones of the 13762 rat mammary tumor. Clones which released high levels of PA were better able to form lung colonies after i.v. injection than were poor producers. However, in mixed tumors, metastasis of neigh-boring cells might be effected as well as that of the producers themselves. Furthermore, because some growth factors can induce PA production (135), it may be that a factor produced by one population could induce PA in another.

Cell interactions can both enhance (136-139) or suppress (140,141) production of proteases. Inhibitors of proteinases secreted by one tumor subpopulation (142) might diminish the invasive capacity of a second subpopulation in a mixture. The products of the digested extracellular matrix may stimulate additional subpopulations. Both fibronectin (143) and laminin (74), for example, are matrix components which are also chemotactic for some tumor cells.

V. Conclusion

We have discussed the concept of clonal heterogeneity and reviewed the literature as it concerns mammary cancer. We have presented the evidence that tumor subpopulations within a single neoplasm can interact to alter each other's phenotype and have speculated upon a few of the possible mechanisms of interaction. It is obvious that no single mechanism is responsible for all of these interactions. Rather the capacity for interaction and the events responsible depend upon the specific characteristics of the subpopulations and of their environment. Our major conclusion is that, regardless of the characteristics of the component cells, one must view cancers as populations of cells, dynamic and interacting, in order to more fully understand the behavior and progression of malignant disease.

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RODENT MODELS TO EXAMINE IN VIVO HORMONAL REGULATION OF MAMMARY GLAND TUMORIGENESIS

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I. INTRODUCTION

The experimental demonstration of the influence of hormones on the genesis of mammary tumors in rodents was begun in 1913 by Lathrop and Loeb, who demonstrated that mammary tumors were more frequent in outbred multiparous mice than in nulliparous mice of the same strain (1). Three years later, Lathrop and Leob reported that ovariectomy of mice at an early age either completely inhibited or greatly delayed the appearance of mammary tumors (2). Since these reports, countless numbers of studies have been reported documenting the exquisite control by the endocrine system of the genesis, development and growth of the rodent mammary tumor. The purpose of this chapter is to provide an assemblage of the current in vivo mouse and rat mammary tumor models most commonly utilized by the tumor biologist intent on understanding mammary tumor - hormonal regulatory processes; a succinct review of the endocrine responsiveness of these mammary tumor models is provided.

II. SPONTANEOUS MOUSE MAMMARY TUMORS

At the turn of the century, the experimental study of mammary tumorigenesis in laboratory animals had its inception (3). The laboratory animal model which initiated the study of experimental mammary gland tumorigenesis was the spontaneous mouse mammary tumor model. This laboratory animal model has provided substantial insight into the process of mammary gland tumorigenesis. For example, the spontaneous mouse mammary tumor was the first experimental animal mammary tumor that was successfully and continuously transplanted. This mammary tumor was the first to be selected out and concentrated in high tumor inbred strains. This was the first experimental

animal mammary tumor model in which the influence of hormones, viruses and stress were found to play an important role. The concept of tumor progression and preneoplastic lesions were primarily developed during the study of this laboratory animal model.

The role of the endocrine system in spontaneous mouse mammary gland tumorigenesis has been examined by many laboratories. Enhancement of mouse mammary gland tumorigenesis by chronic administration of 17 β-estradiol (4), estrone (5), estriol (6), diethylstilbestrol (7), certain steroid contraceptives (4) and progesterone (8) has been reported. Induction of a chronic hyperprolactinemia by pituitary grafting (9), pituitary tumor grafting (10), administration of certain tranquilizers (11), induced hypothalamic lesions (12) or prolactin injections (13) also enhances the development of mammary tumors in mice. In accord, ovariectomy (2), ovariectomy-adrenalectomy (14), hypophysectomy (15) or a drug-induced hypoprolactinemia (16) sharply suppresses or totally abolishes the development of mouse mammary tumors. Drug-induced hypothyroidism also has been reported to suppress the spontaneous development of mammary tumors in mice (17). Removal of the submandibular gland or chronic administration of epidermal growth factor (EFG) suppresses or enhances, respectively, the developmental growth of spontaneous mouse mammary tumors (18); this very recent report is the first evidence of endocrine salivary gland activity in the in vivo stimulation of murine mammary gland tumorigenesis.

In many strains of mice, e.g., C3H, A, DBA, 020, CBA and certain substrains of BALB/c, multiple pregnancies or pituitary isografting enhances the development of spontaneous mammary tumors (1,9). In other strains of mice, e.g., RIII, BR6, DD, and GR, the mammary glands are particularly sensitive to the hormonal milieu of pregnancy, i.e., mammary tumors often develop during the first pregnancy; such mammary tumors usually regress during lactation (19). In other strains of mice, e.g., certain substrains of BALB/c and C57BL, the mammary glands are relatively resistant to a tumorigenic hormonal milieu, i.e., the administration of an array of hormones, pituitary isografting or multiple pregnancies induces only a relatively low incidence of mammary tumors, often accompanied by prolonged latency periods of mammary tumor appearance (20). Perhaps the mouse strains most resistant to hormonal induction of mammary tumors are the TSI and STS strains; mammary tumor incidence in multiparous TSI mice is 0% and in multiparous STS mice, only 13% (mean latency period of 670 days). The STS strain appears, further, to be totally refractory to the mammary tumorigenic stimulating activities of pituitary isografting (21).

A microscopic mammary gland dysplastic lesion, with high neoplastic potential, termed preneoplastic lesion, is seen in mice which develop spontaneous mammary tumors (22). The most thoroughly examined spontaneously arising preneoplastic lesions are hyperplastic alveolar nodules (23) and plaques (24). Hyperplastic alveolar nodules grossly appear as small nodules associated with ducts. Microscopically, they consist of proliferating alveolar structures that cannot be differentiated from normal tissue. Some of these nodules regress, others are static in growth and some develop into palpable tumors. Certain hyperplastic alveolar nodules are hormone responsive (prolactin and/or ovarian steroids), others appear to be hormone-independent (22,23,25). Grossly, plaques are ovoid or disc shaped. Histologically, they consist of branching tubules lined by hyperplastic epithelium and a central core of loose connective tissue. Plaques are hormone-responsive, for they grow during pregnancy and regress after parturition (24).

Spontaneous mammary tumors that arise in mice, depending on the mouse strain, may be carcinomas (adenocarcinomas of alveolar or ductal origin), carcinomas with squamous cell differentiation (e.g., adenoacanthomas) or

carcinosarcomas. With the exception of the pregnancy-induced mammary tumors in the RIII, BR6, DD and GR mouse strains, palpable mammary tumors that arise spontaneously in mice are almost always hormone-independent, i.e., these tumors will continue to grow even in ovariectomized and/or hypophysectomized hosts (15). The pregnancy-dependent mammary tumors require either the hormonal milieu of pregnancy or a hormonal combination of estrogen, progesterone and prolactin for optimal growth (26,27).

Two types of mouse mammary tumor viruses (MTV) are present in all inbred strains of mice, i.e., endogenous and exogenous mouse MTVs (28). The exogenous MTV is usually transmitted via the milk at nursing and is instrumental in the early development of mammary tumors in susceptible mice. The endogenous MTV is in a proviral form transmitted as a gene and is most often associated with late onset of mammary tumors. In contrast, certain feral mice possess no organized MTV; these mice have normal mammary development with concomitant hyperplasia (29). Spontaneous mammary tumors rarely occur in feral mice; the mammary tumorigenic sensitivities of these wild mice to a hormonal stimulation is at present unknown.

III. CARCINOGEN-INDUCED MOUSE MAMMARY TUMORS

During the past 50 years an array of carcinogens, e.g., 3,4-benzopyrene, 3-methylcholanthrene (MCA), 1,2,5,6-dibenzanthracene, 7,12-dimethylbenzanthracene (DMBA) and urethan, has been utilized to induce mammary carcinomas in a number of mouse strains that have low spontaneous mammary cancer incidence in virgin animals (3). Among these mouse strains which are susceptible to chemical carcinogenesis of the mammary gland are A, BALB/c, $BD2F_1$, C3Hf, C57BL, x IF, CBA, CE, DBA, ICR/Ha, IF, LAF, NH and NZY. Carcinogen-treated mice develop an array of mammary gland dysplasias (30), e.g., hyperplastic alveolar nodules, ductal hyperplasia, squamous metaplasia, area nodules, alveolar proliferation along ducts, noduloids, inflammatory nodules and keratinizing nodules; of these dysplasias, the ductal hyperplasias are usually the most frequent (dependent on type of carcinogen and mouse strain) and five give rise to mammary adenocarcinomas upon transplantation into syngeneic recipient mice. The type of mammary tumors that arise in carcinogen-treated mice are most often mammary adenoacanthomas and type-B mammary adenocarcinomas; the adenoacanthomas appear, as a rule, earlier than the type-B mammary adenocarcinomas. The major disadvantage of the carcinogen-induced mouse mammary carcinoma model is that the mice require multiple carcinogen injections for optimal tumor yields. Multiple carcinogen injections not only impair the health of the animals but hinder the ability to study initiation and promotion tumorigenic processes. In addition, the mean latency period of mammary tumor appearance in carcinogen-treated mice is relatively long, albeit considerably shorter than that observed in the spontaneous mouse mammary tumor models.

Prolonged hormone stimulation enhances the induction of mammary tumors in mice by chemical carcinogens. For example, MCA-treated BALB/c mice produce a high incidence of mammary tumors only in the presence of concomitant hormone stimulation (pituitary isografts); prolonged hormone stimulation before and during carcinogen treatment resulted in far more mammary tumors than did the identical hormone treatment initiated after carcinogen treatment (31). Concomitant hormone and carcinogen treatment in mice results in larger numbers of mammary hyperplastic alveolar nodules and keratinizing nodules and fewer ductal dysplasias (32), the latter lesion being the most commonly observed dysplasia seen in mice treated only with chemical carcinogens (33).

The hormonal responsiveness of mammary tumors derived from carcinogen-treated mice has not been examined extensively. Pituitary or pituitary tumor grafting of mice previously treated with a chemical carcinogen,

enhances mammary tumor development (31,34). Ovariectomy of mice following carcinogen treatment suppresses mammary tumor development (35). The growth of 90% and 44% of urethan-induced and DMBA-induced mammary tumors, respectively, has been reported to be suppressed by ovariectomy (36). Thus, carcinogen-induced mouse mammary tumors are often hormone-responsive; the relative importance of pituitary and ovarian hormones for development and growth of these tumors is unknown.

IV. TRANSPLANTABLE MOUSE MAMMARY TUMORS

A. GR/A Mouse Mammary Tumors

The most extensively examined hormone-responsive, transplantable mouse mammary tumor is the GR/A. Mammary tumors are readily induced in GR/A mice by the administration of estrogen and progesterone or by repeated pregnancies (19) These tumors, upon transplantation, retain their hormone responsiveness. The administration of estrogen and progesterone to mice bearing grafts of these tumors results in increased tumor growth (37). Chronic administration of a nonsteroidal antiestrogen inhibits the growth of these tumors (38). The administration of prolactin to these animals has been reported to enhance the growth of the tumors, whereas drugs that suppress prolactin secretion caused growth inhibition (39). The transplanted GR/A mouse mammary tumors are ductal carcinomas; occasionally, these tumors progress to hormone independence; such tumors are designated GR/A-HI.

B. TPDMT-4 Mouse Mammary Tumor

The TPDMT-4 transplantable mouse mammary tumor was derived from a pregnant DDD mouse (40). The growth of these tumors is enhanced by estrogen and progesterone; these hormones administered singly, however, do not appear to enhance tumor growth. Increase of prolactin secretion enhances tumor growth; hypophysectomy causes tumor regression. The tumors are ductal carcinomas.

C. Shionogi Carcinoma 115 Mouse Mammary Tumor

The Shionogi carcinoma 115 is a transplantable mouse mammary tumor that arose spontaneously in a DD/S mouse (41). The growth of this tumor is stimulated by androgens; growth of these transplants do not appear to be affected by estradiol, progesterone, cortisol or prolactin. The histopathology of these tumors are medullary carcinomas; loss of androgen dependence results in a tumor of spindle cell morphology.

D. UHDMT-26 Mouse Mammary Tumor

The UHDMT-26 transplantable mouse mammary tumor arose in a urethan-treated BALB/c mouse (41,42). Growth of this transplantable mammary tumor is inhibited by ovariectomy; such tumors are ductal carcinomas.

E. MXT Mouse Mammary Tumor

The MXT transplantable mouse mammary tumor was derived from a carcinogen-treated $BD2F_1$ mouse (43,44). The MXT is ovarian-dependent, i.e., the growth of this tumor is enhanced by estrogen, progesterone or an estrogen-progesterone combination. Ovariectomy significantly reduces tumor growth. The MXT is a ductal carcinoma.

F. C3H/HeJ Mouse Mammary Tumor

Mammary tumors developing spontaneously in C3H/HeJ mice can be readily transplanted into syngeneic hosts. Although the primary tumors or the

transplants are not recognized as being hormone-responsive (pituitary and ovarian hormones), the growth of the transplanted (and primary) tumors can be inhibited by chronic treatment with a number of glucocorticoids, e.g., dexamethasone and methylprednisolone (45). The transplanted mammary tumors are adenocarcinomas.

G. Preneoplastic Mouse Mammary Gland Dysplasias

The mammary tumor-producing potential of an array of preneoplastic mouse mammary gland dysplasias, upon transplantation to the gland-free mammary fat pad, can be influenced by endocrine secretion. Keratinizing nodules, found in carcinogen-treated BALB/c mice, give rise to greater numbers of mammary tumors when transplanted into mice bearing pituitary isografts (46). The progression of transplantable, carcinogen-induced hyperplastic alveolar nodules (HAN lines C3-C6) to mammary tumors in BALB/c mice is often inhibited by ovariectomy of host mice (46). The progression to neoplasia of certain transplantable, nodule-outgrowth lines (D2, C4, C4) in BALB/c mice is inhibited by a nonsteroidal antiestrogen or testosterone; the progression to tumors of such lines, however, does not appear to be influenced by prolactin secretion (47). Many carcinogen-induced, ductal hyperplasias (CDl-CD5, HD-4, HD-7), upon transplantation into pregnant BALB/c or $BD2F_1$ mice, give rise to increased numbers of mammary tumors; ovariectomy often impedes the progression of these preneoplastic lesions to tumors (48). Although it is evident that many carcinogen-induced, preneoplastic mammary gland dysplasias in mice are hormone-responsive, it should be pointed out that a number of these dysplasias are hormone-nonresponsive.

Hormone-induced preneoplastic mammary dysplasias in GR mice (plaques) can be readily transplanted into the gland-free mammary fat pad. Transplantation of these lesions to virgin hosts gives rise to relatively normal ducts; upon transplantation to pregnant hosts or estrogen and progesterone-treated hosts, mammary tumors develop in the transplant sites (49,50). Adding prolactin and growth hormone to the ovarian steroid hormonal milieu does not appear to enhance mammary tumor development (50). Spontaneously arising preneoplastic mammary gland dysplasias in C3H mice (hyperplastic alveolar nodules) can also be readily transplanted to the gland-free mammary fat pad. The transformation of these lesions to mammary tumors can be enhanced by concurrent transplantation of pituitary isografts (51).

V. SPONTANEOUS RAT MAMMARY TUMORS

Mammary tumors are the most common type of neoplasm to occur spontaneously in female rats (3,52). Spontaneously developing mammary tumors have been observed in a variety of rat strains, e.g., August, Albany-Hooded, Copenhagen, Fischer, Lewis, Osborne-Mendel, Sprague-Dawley, Wistar and Wistar/Furth. Carcinomatous spontaneous mammary tumors in the female rat have been classified as adenocarcinoma, solid carcinoma, cystadenocarcinoma, papillary carcinoma and (rarely) squamous cell carcinoma. Mammary fibrosarcomas and mammary tumors with mixed histopathological features are also occasionally encountered. Benign mammary tumors (fibroadenoma, fibroma and occasionally adenomas or adenolipomas) arise spontaneously in female rats far more frequently than do carcinomatous or sarcomatous mammary tumors. Benign mammary tumors usually constitute greater than 85% of the mammary tumors that develop spontaneously in female rats that are allowed to live a full life time. The percent of female rats that develop spontaneous mammary tumors (benign and adenocarcinomas) will vary as a function of strain and time period of observation; percentages of 50-70% mammary tumor incidence in two-year-old female rats are not uncommon.

The development of spontaneous mammary tumors in the rat is influenced by endocrine secretion (3,52). Female rats are far more susceptible to

spontaneous mammary tumorigenesis than are male rats (52). Spontaneous mammary tumors are observed more frequently in multiparous rats than in nulliparous rats (53). Early ovariectomy nearly completely prevents spontaneous mammary tumorigenesis in rats (54); early ovariectomy-adrenalectomy (54) or hypophysectomy (55) completely blocks this tumorigenic process.

Chronic administration of moderate dose levels of estrogens to female rats markedly increases the incidence of mammary tumors. Significantly, the histopathology of these mammary tumors is influenced by chronic estrogen treatment, i.e., between 75-90% of the estrogen-induced mammary tumors are adenocarcinomas (52). Strain susceptibility to estrogen-induced mammary tumorigenesis, in descending order of susceptibility, is Albany-Hooded, Sprague-Dawley, Lewis, Wistar and Fischer (56). Prolonged and continuous exposure to estrogen appears to be more important that the actual dose (57). Ovariectomy or adrenalectomy of estrogen-treated rats increases the latency period of estrogen-induced mammary tumor appearance; hypophysectomy completely prevents the induction of these tumors by estrogen (58). Administration of progesterone or testosterone to the estrogen-treated rats increases the latency period of mammary tumor appearance (58). Chronic administration of relaxin has been reported to enhance the development of mammary tumors induced in rats by estrogen (57).

Chronic administration of prolactin to female rats, via multiple pituitary homografts (53) or induced hypothalamic lesions (59), significantly increases mammary tumor incidence. In contrast to estrogen-treated rats, chronic administration of prolactin induces an increase only in benign mammary tumors, not in carcinomatous lesions (53,59). Similar results have been reported with a growth hormone preparation (55). Thyroidectomy does not appear to reduce spontaneous mammary tumor incidence in rats but does appear to increase the latency period of mammary tumor appearance (54). Chronic administration of progesterone or testosterone to female rats, intact or ovariectomized, does not appear to enhance mammary tumor development (60).

The growth of established, spontaneously developing benign mammary tumors has been reported to be suppressed by a chronic, drug-induced inhibition of prolactin secretion (61). The growth of estrogen-induced mammary tumors in the rat is dependent upon continuous estrogen stimulating; hypophysectomy causes rapid mammary tumor regression even in the presence of continuous estrogen stimulation (58).

In general, the use of the spontaneous rat mammary tumor model, particularly during the past 20 years, has not been extensive. The need to maintain large numbers of animals for nearly their entire life span obviously limits the utility of this model for studies on experimental mammary gland tumorigenesis.

VI. CARCINOGEN-INDUCED RAT MAMMARY TUMORS

The carcinogen-induced rat mammary carcinoma model, during the past two decades, has been the most extensively examined animal model of human breast cancer (62). Although an array of carcinogens have been used to induce mammary tumors in the rat, e.g., MCA, 2-acetylaminofluorene, 3,4-benzopyrene, ethylnitrosourea, butylnitrosourea, etc., the most frequently used carcinogens during this period of time have been DMBA and methylnitrosourea (MNU). The DMBA and MNU-induced mammary carcinoma models have a number of features that make these models attractive to the experimental oncologists, e.g., tumor induction ease and reliability, organ site specificity, tumors of ductal origin, tumors of predominantly carcinomatous histopathological characteristics (benign fibroadenomas develop at later time periods after carcinogen treatment), tumors of varying growth factor

and/or hormone responsiveness, and the potential to examine tumor initiation and promotion processes (a single dose of the carcinogen results in large numbers of tumors). The strain of rat that is most susceptible to DMBA or MNU-induced mammary tumorigenesis (and most frequently examined) is Sprague-Dawley. Using an appropriate dose of carcinogen and optimal timing (30-65 days of age), 100% of female rats of this strain will develop multiple mammary carcinomas within 3-4 months after carcinogen treatment. Strains of rats which are also susceptible to these carcinogens (albeit, of substantially lesser susceptibility) are AxC, Buffalo, Fischer, Lewis, Long-Evans, Wistar and Wistar/Furth; Long-Evans rats are the least susceptible.

Perhaps the most attractive feature of the DMBA or MNU-induced rat mammary carcinoma model is the hormone responsiveness and/or dependence of the induced tumors; a normally functioning endocrine systems is prerequisite for optimal tumor development, both in the initiation and promotion stages of this tumorigenic process. Hypophysectomy of rats after carcinogen treatment blocks mammary tumor development or causes prompt mammary tumor regression (62,63). Clearly, prolactin is important for the developmental growth of both DMBA and MNU-induced rat mammary carcinomas (63-65); MNU-induced carcinomas, however, may be less dependent on this pituitary hormone for growth than the DMBA-induced tumors (65-67). Growth hormone does not appear to be crucial for growth of the DMBA-induced rat mammary carcinomas (68), but may be important for growth of MNU-induced cancers (65).

Both DMBA and MNU-induced mammary carcinomas are responsive to ovarian hormone secretion. Ovariectomy of rats after carcinogen treatment inhibits mammary tumor development or causes tumor growth suppression (62,63). Estrogens, in moderate dose levels, stimulate the growth of these tumors (66,69). Growth enhancement of DMBA-induced mammary carcinomas by estrogens is pituitary dependent (70); estrogens appear to be able to induce some growth of MNU-induced mammary carcinomas in the absence of pituitary gland secretion (66). Moderate dose levels of progesterone administered to rats treated with DMBA almost invariably enhances tumor development and growth (71); when combined with moderate dose levels of estrogen, growth stimulation is further enhanced (72). The effect of progesterone on growth of MNU-induced mammary carcinomas, in contrast, may be one of inhibition (73). Although it is clear that prolactin and ovarian steroids enhance the growth of carcinogen-induced rat mammary carcinomas, these hormones, when administered prior to or on the day of carcinogen treatment, inhibit this tumorigenic process (62,63). Hypophysectomy or ovariectomy prior to carcinogen treatment virtually completely blocks chemical carcinogenesis of the rat mammary gland (62,63).

Moderate dose levels of adrenal steroids appear to inhibit (74), whereas moderate dose levels of insulin (75) and thyroid hormones (70) appear to stimulate the growth of DMBA-induced rat mammary carcinomas. The influence of these hormones on growth of MNU-induced rat mammary carcinomas is unknown (adrenal steroids and insulin) or uncertain (thyroid hormones) (62).

TPA (12-0-tetradecanoylphorbol-13-acetate) is a well known tumor promoter (growth factor) in a variety of tumor systems. Although TPA <u>per se</u> has not been administered to rats bearing carcinogen-induced mammary tumors, the unesterified parent compound of TPA, i.e., phorbol, has been examined in this animal model. In Wistar rats treated with DMBA, phorbol acts as a "classical" tumor promoter, i.e., chronic administration of phorbol commencing after carcinogen treatment significantly enhanced mammary tumorigenesis (77). In contrast, utilizing an identical experimental protocol, but substituting Sprague-Dawley rats for Wistar rats, no evidence of mammary tumorigenesis promotion by phorbol was observed (78).

VII. RADIATION-INDUCED RAT MAMMARY TUMORS

Whole-body radiation (x-rays, γ-rays, neutrons) is an effective means of inducing mammary tumors in laboratory rats (79). Strains of rats which are susceptible to radiation-induced mammary gland tumorigenesis are AxC, Fischer, Lewis, Long-Evans, Wistar/Furth and Sprague-Dawley. Of these rat strains, Sprague-Dawley and Lewis rats are the most susceptible to the mammary tumor-inducing activities of radiation; Long-Evans rats are the most resistant. A single administration of radiation can induce a high yield of mammary tumors, facilitating the study of initiation and promotion tumorigenic processes. Mammary tumors that develop in the irradiated rat are adenocarcinomas and/or benign fibroadenomas; the mammary tumors occur at later time periods after radiation treatment. Histologically, the radiation-induced rat mammary tumors resemble the mammary tumors induced in rats by chemical carcinogens.

The hormone responsiveness of the radiation-induced rat mammary carcinomas has been examined, but to a substantially lesser degree than have the chemical carcinogen-induced rat mammary tumors. Ovariectomy (80), chronic treatment with a nonsteroidal antiestrogen (81), or a drug suppressing secretion (82), administered post radiation, inhibits the development of mammary tumors. In accord, treatment of rats with estrogen and/or prolactin immediately after or several months after radiation enhances mammary tumor development (83,84). Adrenal steroids inhibit the development of radiation-induced rat mammary tumors (85); progesterone appears to inhibit the development of these tumors in estrogen-treated animals (86). Phorbol, the unesterified parent compound of TPA, does not appear to be a tumor promoter in the radiation-induced rat mammary tumor model (78). Thus, the endocrine responsiveness of the radiation-induced rat mammary tumor in the developmental growth stages is very similar to that of mammary tumors induced in rats with chemical carcinogens.

Although there are a notable number of physiological similarities between radiation-induced and chemical carcinogen-induced rat mammary carcinomas, there are also striking differences between these models (79). First, the anatomical distribution of radiation-induced mammary tumors in rats is random in the four quadrants of the rat mammae; mammary tumors in chemically carcinogen-treated rats tend to occur more frequently in the anterior quadrants. Second, the induction of mammary tumors in rats by radiation is not age-dependent, i.e., mammary tumors occur as frequently in older rats (≈225 days of age) as in younger rats (42 and 84 days of age); the induction of mammary tumors in young rats (30-65 days of age) by chemical carcinogens is considerably more efficient than that observed in older rats (≈80 days of age). A third difference is the carcinogenic sensitivity of the mammary gland during different physiological states. The pregnant or lactating rat is relatively refractory (compared to virgins) to a chemical carcinogenic stimulus; in contrast, virgin, pregnant and lactating rats appear to be equally sensitive to the carcinogenic activities of radiation.

VIII. TRANSPLANTABLE RAT MAMMARY TUMORS

A. MTW9 Rat Mammary Tumor

The MTW9 transplantable rat mammary tumor was procured in the early 1960s from a carcinogen-treated Wistar/Furth rat. To date, a number of variants of MTW9 are available for study. Among these variants are the ovarian and pituitary hormone-responsive MTW9A, the hormone-autonomous MTW9B and the androgen-responsive MTW9C (87). The progression of this tumor from hormone responsiveness to androgen responsiveness and to hormone autonomy has been examined (87). The MTW9A tumor is particularly sensitive to pituitary hormone secretion, i.e., growth of these tumors is stimulated in

intact, ovariectomized and hypophysectomized rats bearing grafts of prolactin and growth hormone-secreting pituitary tumors (88). The growth of the MTW9A tumor is also stimulated by ovarian hormones (89); progesterone may be more important than estrogen in stimulating the growth of these tumors (90). MTW9/PL is another variant of MTW9. The growth of this tumor is stimulated by prolactin, estrogen, progesterone and testosterone; hydrocortisone inhibits tumor growth (91). Histopathologically, the MTW9 rat mammary tumors are adenocarcinomas.

B. 13762 Rat Mammary Tumor

The 13762 transplantable rat mammary tumor was derived from a mammary tumor excised from a carcinogen-treated Fischer rat (92,93). Small doses of estrogen or large doses of androgens stimulate in vivo growth of this tumor; progesterone appears to have no effect on tumor growth, whereas glucocorticoids suppress tumor growth. Enhancement of prolactin secretion increases the growth of these tumors. The 13762 transplantable rat mammary tumors are adenocarcinomas.

C. MRMT-1 Rat Mammary Tumor

The MRMT-1 transplantable rat mammary tumor was derived from a carcinogen-treated Sprague-Dawley rat (94). The tumor cells readily metastasize to regional lymph nodes and lungs after subcutaneous transplantation. Hypophysectomy causes tumor regression and disappearance of lung metastasis. Histopathologically the tumors are adenocarcinomas.

D. DMBA-4 Rat Mammary Tumor

The DMBA-4 is a transplantable mammary tumor that was obtained from a carcinogen-treated Wistar/Furth rat (95). The tumor cells readily metastasize through the lymph system, migrating to the liver, lung and spleen. Growth and metastasis of these tumors are inhibited by chronic treatment of host animals with nonsteroidal antiestrogens. The tumors are adenocarcinomas.

E. SNMU And ANMU Rat Mammary Tumors

The SNMU and ANMU tumors are transplantable mammary carcinomas derived from carcinogen-treated Fischer and Buffalo rats (96). The growth of SNMU tumors is stimulated by estrogen; the ANMU tumors are hormone-autonomous.

F. R3230AC Rat Mammary Tumor

The R3230AC tumor is a transplantable mammary carcinoma that was obtained from a spontaneously developing mammary tumor which arose in a Fischer rat (97,98). The growth of these tumors is inhibited by prolactin, estrogen, androgens and insulin.

G. Nb-HD And Nb-HA Rat Mammary Tumors

The Nb-HD transplantable mammary tumor was derived from female Nb rats chronically treated with estrone (99). The growth of these tumors is initially dependent upon estrogen. Transplanted Nb-HD mammary tumors that progress to hormone independence are referred to as Nb-HA tumors (hormone-autonomous). Both tumors are carcinomas.

H. MCCLX Rat Mammary Tumor

The MCCLX transplantable rat mammary carcinoma was derived from an estrogen-treated AxC rat (100). The growth of this tumor is dependent upon

estrogen and prolactin. Hypophysectomy causes tumor regression even in the presence of estrogen administration.

IX. MAMMARY XENOGRAFTS IN ATHYMIC NUDE MICE

Medical researchers, intent on understanding human disease processes, have long sought to carry or maintain grafts of normal or pathological human tissue in experimental animals. Attempts to transplant human tissue into immunologically deficient hosts (e.g., thymectomized-irradiated mice) or to immunologically privileged sites (e.g., hamster cheek pouch) have generally been unsatisfactory; eventual rejection of the xenografts appears inevitable. The most recent development in this field has been the introduction of the athymic nude mouse, a mutant born without a functional thymus and capable of maintaining grafts of foreign tissue for indefinite periods of time (101,102). Lacking a thymus gland, therefore T-cell (lymphocyte) deficient, the athymic nude mouse has been shown to be capable of accepting and maintaining grafts of an array of foreign tissue, e.g., tissues derived from amphibians, reptiles, birds, rats, rabbits, cats, dogs and humans.

To date, grafting of xenogeneic normal mammary tissue to the athymic nude mouse has been accomplished by a number of laboratories. Normal mammae derived from rats (103,104), cow (105,106) and humans (107,109) have been successfully transplanted into this immunologically deficient rodent. Grafts can be maintained in these mice for their entire life span, i.e., > two years (110). Importantly, the grafted mammary tissue is capable of responding to a hormonal stimulus. Administration of human placental lactogen (107), estrogen (108,109) or thyroxine (109), or co-grafting of a rat pituitary tumor (108) to athymic nude mice bearing grafts of normal human breast tissue, causes increased DNA synthesis of the ductal epithelium within the grafts. Administration of bovine mammogenic hormones to athymic nude mice bearing grafts of normal bovine mammary tissue causes increased DNA synthesis and lactogenesis of the epithelium contained within the grafts (105,106). Also, transplantation of a prolactin-secreting pituitary tumor to athymic nude mice bearing grafts of normal rat mammae results in extensive developmental growth of the rat mammary epithelial cells (104). In effect, the normal rat mammae when grafted to athymic nude mice (as subcutaneous tissue slices or as dispersed epithelial cells innoculated into the cleared mammary fat pad) respond to a mammogenic hormonal stimulus with extensive growth resembling that of late pregnancy. In contrast, grafts of normal human or bovine mammae (s.c. slices or fat pad innoculates), although responsive to a hormonal stimulus, do not show extensive hormone-induced growth; expansive proliferation of these cells, comparable to that seen in late pregnancy, has not yet been demonstrated.

In contrast to normal human breast tissue, transplants of primary carcinomatous human breast tissues are not readily accepted by the athymic nude mouse (≃90% rejection rate) (111,112). Transplants of primary carcinogen-induced rat mammary carcinomas are likewise not readily accepted by these immunologically deficient animals (104). In contrast, cell lines of human breast carcinomas are readily transplanted into these mice; over two dozen such cell lines to date have been successfully maintained in these animals (113). The hormone responsiveness of a number of human breast carcinoma cell lines can be observed in athymic nude mice. For example, ovariectomy of these mice suppressed the growth of transplants of the MCF-7 human breast carcinoma cell line; estrogen administration caused enhanced growth of this cell line (114-116). The secretions of a transplantable rat pituitary tumor have been reported to further enhance estrogen-induced growth of transplants of MCF-7 in athymic nude mice (115). In accord, the administration of a nonsteroidal antiestrogen to athymic nude mice bearing MCF-7, or the hormone-responsive human breast carcinoma cell line ZR75-1, has been reported to cause tumor growth inhibition (116). The human breast

carcinoma cell line T-47D can be readily transplanted to the athymic nude mouse. Growth of this cell line in athymic nude mice is stimulated by estrogen; secretions of a transplantable rat pituitary tumor further enhance estrogen-induced growth of this cell line (117). The growth in athymic nude mice of the human breast carcinoma cell line Br-10 appears to be enhanced by estrogen administration and reduced by ovariectomy or testosterone treatment (18). Human breast carcinoma cell lines which are not hormone responsive, upon transplantation into athymic nude mice, do not respond to host endocrine manipulations. Cell lines of rat mammary carcinomas (e.g., Rama 25, R3230AC and 13762) have also been successfully transplanted into the athymic nude mouse (119, 120); the hormonal responsiveness of these cell lines in this immunologically deficient animal has not been examined.

The athymic nude mouse is clearly an important and unique addition to our consortium of animal models for the study of human breast cancerigenesis. It is the first laboratory animal which provides the experimental potential for an ex vivo-in vivo analysis of the factors prerequisite for growth, differentiation and ultimately tumorigenesis of the human breast. The degree to which this laboratory animal can realize this important potential will hopefully soon be defined.

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PRIMARY CULTURE SYSTEMS FOR MAMMARY BIOLOGY STUDIES

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I. INTRODUCTION

The importance of hormones in the growth, morphogenesis, differentiation and neoplastic transformation of the mammary gland is well recognized due to a large number of experiments in vivo (for review see 1). However, the precise nature of hormonal involvement is not clearly understood because of the difficulties in analyzing complex in vivo interactions. The role of growth factors in any of these processes is, perhaps, even less well understood, due in part to the complexity of in vivo interactions and in part to the general lack of knowledge of the role of growth factors in any in vivo process. The recent advances relating some viral oncogene products to growth factor receptor variants or growth factor sub-units makes it important to understand more fully the role of these molecules in the processes listed above. Culture systems of various types have been used to try and address these questions and are, theoretically, of great advantage since they allow observations and manipulations of cells and tissues in isolation, where experimental variables can be controlled or minimized. The

goal of these investigations is to be able to reproduce in culture a system that mimics the in vivo physiological and pathological phenomena of interest. No individual system completely meets this goal, but recent advances allow a better simulation of some of the processes that occur in vivo. Unfortunately, even those systems which closely mimic the in vivo situation in terms of growth and differentiation cannot yet mimic the process of neoplastic transformation in culture.

A variety of culture systems have been employed to analyze the dynamic interactions among mammary epithelial and stromal elements and the regulatory effects that hormones and growth factors have on them. In this chapter we briefly discuss each of the major types of culture systems which have contributed significantly to our understanding of growth and differentiation in the mammary gland, and evaluate the advantages and disadvantages of each of these systems. We then review the more limited use of some of these systems to analyze the processes of morphogenesis and transformation in culture. As the literature on the use of these systems for growth and differentiation is extensive and our space here limited, we will only cover studies of mammary biology in primary culture, i.e., the cultures started from cells, tissues or organs obtained directly from the subject organism. To illustrate better the strengths and shortcomings of each system for studies of growth and differentiation, we will compare each culture to a hypothetical, idealized system for growth and differentiation.

II. CULTURE SYSTEMS

Culture systems are broadly categorized into three groups: tissue culture, organ culture and cell culture. The first, which is little used today, consists of cultivation of explants of small fragments of tissues in a plasma clot and will not be further considered in this review. Organ cultures use conditions in which the normal interrelationships between cell types are maintained as they exist in the organism. Cell culture involves dispersal of the cells of the gland by enzymatic or other means and culture of individual cell types or mixtures of cell types. Organ culture has been the system of choice in mammary biology for many years, but with advances in the understanding of the interactions between epithelial cells and the extracellular matrix, the influence of cell shape and other factors, cell culture has gone through a rapid evolution and now can be used for a variety of experiments. Below we briefly summarize the techniques currently used for mammary culture.

Organ Culture

Two types of organ culture are used for mammary biology studies. The first is called fragment organ culture and uses small pieces of tissue, approximately 1 mm^2, supported on a siliconized substrate and suspended in a watch glass containing appropriate medium in moist CO_2/O_2 environment. Modification of fragment organ culture has produced the second system, which uses whole mammary glands from young rats or mice rather than the fragment explants.

Cell Culture

After appropriate dispersal, cells may be cultured in either two-dimensional (2D) or three-dimensional (3D) systems. The 2D systems are categorized into two groups: monolayer plastic with or without a feeder layer, and monolayer non-plastic. In the former, cells are cultured on a plastic substratum with or without feeder cells. The latter involves culturing the cells on top of extracellular matrix components or a layer of collagen film or gel. The 3D culture systems include embedding the cells in a type I collagen gel or a "biomatrix" gel composed of basal lamina

components. In addition, 3D soft agar culture systems have also been used occasionally for studies of mammary tumor cell growth.

III. CRITERIA FOR AN IDEALIZED CULTURE SYSTEM

In this section we will outline a set of purely hypothetical parameters which we consider to be important in an ideal culture system. Since the final nature of an ideal system will depend on the questions being addressed, these criteria will not be the same for each investigator. No culture system meets all of these criteria, and it is very likely that none will in the near future. We will use these criteria as an exercise for discussion of the advantages and disadvantages of each culture system for growth and differentiation. The list of criteria is as follows: 1) the system should be easily managed, should use chemically defined medium and substratum and should be experimentally reproducible; 2) the system should be easily manipulable for biochemical, histological, immunocytochemical and cell biological procedures; 3) if desired, the system should be able to select for and maintain pure, identifiable populations of cells; 4) the system should allow multifold, easily quantifiable growth when stimulated by mammogenic hormones and other agents; 5) the system should allow good morphological definition with apical-basal polarization, maintenance of a partition between the basal and apical compartments, and in 3D cultures should allow tubule formation if that is a characteristic seen in vivo; 6) the system should allow analysis of the role of cell shape and basement membrane components in growth and functioning of the mammary epithelial cells; 7) the system should allow all stages of mammary development to be mimicked, and additionally, allow transformation and discrimination of transformed cells from normal counterparts under physiologically relevant concentrations of hormones, growth factors and other conditions; 8) finally, the system should allow the use of tissue or cells from any physiological state of the animal, as well as tissues from immature or endocrine-ablated or -manipulated donors.

IV. SYSTEMS USED FOR STUDIES OF GROWTH AND DIFFERENTIATION

Since understanding of the regulation of growth and differentiation in normal and neoplastic cells is likely to be a major factor in the analysis of neoplastic transformation, much emphasis is given to this area of mammary biology studies using a variety of culture systems. We have chosen to cover growth and differentiation together since in many ways they are vitally linked processes. Many of the criteria for a good system for studying growth also apply to a system for studying differentiation. Indeed, one important aspect of any study of growth is that the cells responding to a stimulus are truly mammary cells and are phenotypically normal after growth. An easily assessed marker for the normality of mammary cells after culture is synthesis of differentiated products. Although a few growth studies have utilized mammary tissues from bovine and rabbit sources, most of these studies have been conducted with mouse, rat and human mammary cells. Studies on differentiation are less restricted in species selection; however, we will primarily consider work using these three species. First, we will briefly review those components of the idealized culture system important for studies of growth and differentiation, and then discuss the advantages and disadvantages of the culture systems that have been used in these studies with mammary tissue from mice, rats and humans.

While most of the criteria laid out in Section III are useful in a culture system for studying growth, the most important components are that multifold growth of identifiable populations of cells should be inducible using several independent compounds (criteria 3 and 4), that the system should be easily managed and reproducible (criteria 1 and 2), and that it should be usable for all stages of mammary development under physiologically

relevant conditions (criterion 7). The only addition to these requirements, for a system in which to study differentiation in culture, is that the cells should maintain their polarity and morphological definition (criterion 5). In the rodent systems discussed below, retention of normal phenotype by the cultured explants or cells has been examined by subsequent implantation of the explants or by eventual injection of the cultured cells into the cleared mammary fat pad of syngeneic female animals or nude mice (2-4).

With use of any of the systems below for differentiation, one given is that they should actually allow differentiation to occur in response to hormonal stimulation and to be measured in some fashion. Although the biochemical and molecular biological methods used for identification of the products made by differentiated cells are widely varied, they will not be discussed here, and each culture method may have had one or more of these methods applied to it. The relative hormonal responsiveness of differentiated product synthesis in each system will also not be discussed in this section, except to say that all systems respond to the hormones generally considered lactogenic; these hormones are insulin, prolactin and cortisol or other glucocorticoid. Another given in this chapter is that some non-morphological method for identification of the differentiated state is required, since only one system below (whole organ culture) has a morphological marker for differentiation. Some markers for differentiation, such as ultrastructural morphology or immunohistochemical methods, are usually equally applicable in all the systems.

A. Whole Organ Culture

This culture system has been used successfully for mouse and rat tissues (5-12), but for obvious reasons has not been reported for human tissues. This system grew out of the fragment organ culture system (see below) as an answer to some of its shortcomings. One major advantage is that the tissue architecture is preserved, allowing apparently normal morphogenesis through early alveolar development to take place concurrently with growth and differentiation. This is important, since growth, development and differentiation of the mammary gland are dependent on a close, two-way relationship between the stroma and parenchyma, and early hormonal actions are in part mediated through the stroma (13). Growth, morphogenesis and differentiation in this system can be coupled to transformation (see Section VI), forming an integrated system which may be used for comparatively short term investigations of less than three weeks. A more recently discovered feature of this system is that up to two rounds of growth, morphogenesis and differentiation can be induced if epidermal growth factor (EGF) is included in the medium (14,15). Finally, up to a 10-fold increase in epithelial cell number can be achieved with this system, more than with fragment organ culture (10).

There are significant problems with whole gland organ culture, the most serious being carryover of undefined endogenous hormones and growth factors in both the mouse and rat systems. There has been little study of this problem (16), and it is, thus, difficult to approach other than empirically. This problem can never be completely eliminated, but only minimized through the use of cell culture techniques or endocrine-ablated animals. The priming required in the mouse suggests that, in addition to carryover of hormones, undefined changes in the gland *in vivo* are necessary before a response will occur in culture. This is the exact problem primary culture systems were created to avoid. These problems complicate the analysis of the strict hormonal/growth factor requirements for growth and differentiation. Since one use of this culture system has been to determine the absolute requirements for casein gene expression in the mouse (10), the undefined nature of this priming step is of great concern. Although maintenance of the epithelial/stromal interactions is normally considered a

strength of this system, it poses theoretical problems when attempts are made to assess the direct effects of hormones solely on the epithelial component of the gland. The only way to assess these direct effects unequivocally is to remove the stromal component altogether and culture the epithelium in the absence of stromal interactions or in the presence of a defined stromal element. If direct effects are not a concern, this is of little importance. Growth is fairly limited in any one round of development in organ culture, and is difficult to quantify with certainty, since separation of the epithelial and stromal elements by collagenase digestion is required (10). This difficulty makes normalization of product synthesis to epithelial cell number problematic at best.

Another shortcoming is that the bulkier glands from animals larger than mice or immature rats are not usable due to diffusion problems (5,17). Finally, one of this system's major strengths is also a weakness. Although the duct network of the gland is maintained, the primary duct seals off at the nipple, preventing secreted products from reaching the medium. This system thus requires termination of culture and homogenization of the explant(s) to examine the products synthesized.

B. Fragment Organ Culture

Fragment organ culture has been used for decades for mouse, rat and human glands, and was the progenitor of the whole organ culture technique. Tissue from any source is reduced to a number of 1 mm^2-explants, which are placed on siliconized lens paper or other support and cultured in much the same way as whole organ culture (18,19, see 20 for review). For human tissues, this is the system generally referred to when the term "organ culture" is used. This system is simple and generally reproducible and utilizes a simple defined medium (18,19). The basic tissue level architecture is also maintained, but the overall organ architecture is destroyed. Thus stromal interactions are maintained, and morphogenesis on a micro level may be followed histologically. Tissue from different physiological states (i.e., virgin, mid-pregnant, etc.) and from many different species have been used successfully (21-26). Diffusion is much less of a problem than for whole organ culture, since the size of the explants is much reduced. There is a very large reference base for all three organ sources, and it has been used for many different types of investigation in growth and differentiation. In contrast to the situation in whole organ culture, α-lactalbumin, if not casein, will escape the explants and can be found and measured in the medium (27).

This system shares some problems with whole organ culture, and also has some that are unique. Carryover of hormones and growth factors is less of a problem since priming is not required in virgin tissue, but still exists and is dependent on the source of the tissue (16). Direct effects of medium supplements on the epithelial cells are, as in whole organ culture, difficult to assess. The tissue in this system is generally viable only for a short time, usually less than 10 days (28). These time constraints, combined with the small size of the explants, limit the amount of growth that can occur. Thus coupled growth and differentiation are more difficult to study, although it has been done (28,29). The extent of growth is difficult to assess, and is usually measured by [^{3}H]-thymidine incorporation, labeling index, or mitotic index (20,24-26,28). None of these methods can follow the actual increase in cell numbers quantitatively, although the responding cell type can be identified. In human explants, differing cellularity in fragments in different experimental series is a major problem in total [^{3}H]-thymidine uptake studies, and labeling and mitotic indices obtained from different parts of the breast epithelium (lobules, terminal ducts, and bigger ducts) may give different pictures (30). The tedious and time consuming nature of labeling index or mitotic index measurements will limit the number

of cultures that can be analyzed, and this makes numerous time points, hormone combinations, and dose-response experiments difficult to perform. As was the case for whole organ culture, it is possible to dissociate the epithelial component from the stromal cells by collagenase digestion for DNA quantification (29,31,32), but this remains quite tedious and recoveries are problematic.

In differentiation studies, this system is useful mainly for tissue from midpregnant or primed mice and rats (19,20), as well as tissue from humans (33,34). Tissues from virgin mice are less responsive (20,28,35) and tissues from ovariectomized mice or ovariectomized and adrenalectomized rats have not been responsive (31,36,37). Since cell number is difficult to quantify absolutely, the amount of differentiated product synthesis is usually normalized to the wet weight of the explants (20). Although α-lactalbumin is released into the medium, product synthesis is usually measured by homogenization of the explants, followed by quantification by various methods (19,20,33,34).

The use of human breast cancer (23) in fragment cultures has presented more difficulty than has non-cancerous human breast tissues (22), especially in cases of carcinoma with dense hyaline stroma. This is believed to be due to limited access of the breast cancer cells to the culture environment, because of the extensive surrounding stromal tissue. It has been reported that survival in these cases can be prolonged if collagen is dissolved around the epithelial cells, either by the addition of collagenase or by estrogen, which is believed to enhance collagenolytic activity (23). Nonetheless, despite the well established phenomenon that about a third of the breast cancer patients in an unselected population will respond to various hormone therapies (38), human breast cancer explants in fragment organ culture have not responded to various hormone additives. As mentioned earlier, problems with differing cellularity in the fragments and with growth assessment may make it difficult to unequivocally demonstrate the hormonal regulation of growth of tumor tissue in this system.

C. Monolayer Cell Culture on Plastic Substrata

Culturing cells as monolayers has been the most popular in vitro method employed in studying the effects of various agents on the growth of mammary cells (2,39-50), but has been little used for differentiation studies. Although new problems are introduced by this methodology, significant advantages can also be realized. The cells are most often obtained by enzymatic digestion with collagenase or trypsin (51,52), but dissociated human mammary epithelial cells for monolayer cultures may also be obtained from the spilling technique (53), breast milk colostrums and milk (54), as well as from the pleural effusion from breast cancer patients (55). These cells are cultured using serum, organ extracts, and/or conditioned medium on plastic culture dishes developed for fibroblast culture. Since diffusion to a monolayer is not a problem, the cells are exposed to the desired dose of any given agent added to the medium. Quantitative analysis of growth should be much easier than in either type of organ culture, although when feeder cells are used this is not always the case (56,57). The cells are easily observable microscopically during culture, and immunohistochemical analyses of cell type are thus, much easier after culture (58). Monolayer culture can allow greatly increased expansion of a population of interest, facilitating its further analysis. Finally, monolayer culture and other types of cell culture allow the culture of cells in the absence of a stromal influence or in the presence of a more defined stromal influence than is possible in organ culture.

This culture method has not been widely used for studies of differentiation because mammary epithelial cells from most species will not differ-

entiate on normal tissue culture plastic (59). Rat cells have recently been induced to secrete casein and α-lactalbumin in this culture system, but serum-containing media and long culture periods were required. Little growth was found in this case, and growth was measured by increase in protein content of the cell layers (60,61).

Though theoretically appealing, monolayer culture of mammary epithelial cells on plastic substrata developed for culture of fibroblast cell types has often fallen far short of the ideals. Most importantly, growth of mouse and rat mammary cells is usually limited to a doubling over the first few days of culture and is often measured by [^{3}H]-thymidine labeling in autoradiographs or by changes in DNA synthesis (39,42-46). The rodent cells also often become polyploid (39), especially at low cell density, and do not respond to various additives in terms of increased cell number (44,62). Early studies using human mammary epithelial cells also failed to show an unequivocal increase in cell number and relied on labeling index, colony formation, in situ area measurement, or [^{3}H]-thymidine uptake as a growth parameter (54,63-65). However, more recent studies have reported substantial progress by the use of various factors including the conditioned medium (66,67), serum-free medium (66), and low calcium (68). Growth of human mammary cancer cells in primary culture has also been reported (41,69-72), but the major difficulty lies in the lack of markers for cancer cells in culture (73). This makes interpretation of growth regulation in response to additives difficult to interpret. Though not always considered a shortcoming, the collagenase digestion most often used for separating epithelial cells from the stromal cell types is a process that is often difficult for the inexperienced. The culture media used almost always contain serum, conditioned medium, or organ extracts, all of which add to the complexity of the system. Overgrowth of the cultures by contaminating fibroblasts can also be a problem, albeit one that can be solved by the addition of D-valine (74) or cholera toxin (CT) (75,76) to medium, or by selective trypsinization.

Recent attempts to use this culture system have involved feeder cells. For mouse cells, at least two separate systems have been developed. In the first, mouse mammary epithelial cells are seeded onto irradiated, insulin-induced, 3T3-L1 preadipocytes (56,58), and in the second, irradiated LA-7 cells are added to a culture after the epithelial cells are attached to the culture dish (57). Human mammary epithelial cells have been seeded onto feeder layers comprised of human skin fibroblasts (69), foam cells, mouse fibroblast lines, human mammary fibroblasts and calf lens epithelial cells (64). Under these conditions, multifold growth of the epithelial cells is reported, dependent not only on the medium-conditioning effect of the feeder cells, but at least partially on the cell-to-cell contact with the feeder cells themselves (56,57). The 3T3 preadipocyte system has also been shown to increase greatly the amount of casein synthesized and secreted by the cells (58). While exciting, the use of feeder cells introduces a new set of undefined interactions with the epithelial cells and medium conditioning, all of which must be carefully considered. In a sense, they reintroduce the stromal element and unknown cellular interactions into the cultures, albeit a somewhat more defined stromal element.

D. Monolayer Cell Culture on Non-Plastic Substrata

Much recent work has focused on the interaction of cells with various basement membrane components, collagen gels or dried collagen films supported by a tissue culture plastic dish. Many of these investigations have been done with mouse cells, but tissues from rats and humans have also been used in this system with some success. The non-plastic substrata most often used are gels and films made from stromal type I collagen (46,48,59,64,78,79), extracellular matrix (ECM, 80), or biomatrix (81). Mammary cells show more

proliferative potential on these non-plastic substrata and will grow for a longer period of time in culture compared to growth on plastic. Growth can actually be measured by increase in cell number (82). It is possible to demonstrate a response to mammogenic hormones, although in most cases in which they have been used for growth studies, serum or other undefined medium components have been included (46,81). Rat cells grown on type I collagen gels have been repeatedly passaged (48). Work done with ECM and biomatrix indicates that collagen, laminin and other components of this matrix have important roles to play in the regulation of growth (79,81). The cells are easily observed microscopically in culture except on biomatrix, but immunohistochemical methods are somewhat more difficult to perform than in monolayer culture on plastic. Cells cultured on either of these substrata have an improved ultrastructural morphology compared to those cultured on plastic surfaces, although this ultrastructure may not resemble the in vivo situation as closely as culture in collagen gels (see Section V) unless the collagen gels are released to float in the medium and contract. All of these culture systems are usable for short or long term studies (59,81,83). The use of these non-plastic substrata seems to partially substitute for the missing stromal cells in these cell cultures. This potentially allows use of this system to follow direct effects of hormones and growth factors in the presence of defined substrata.

Cells cultured on these substrata also show an increased ability to differentiate when compared to cells cultured on plastic. Both collagen gel, when released to float in the medium, and biomatrix show good potential for the differentiation and allow secretion of products into the medium. The use of the collagen gel system allows the use of many different species (59,83-86), and allows for authentic vectorial ion transport by the cells (87). The use of biomatrix has only been reported for rat (81) and rabbit (88) cells; the report on rat cells indicates that differentiated product synthesis is greater on biomatrix than on collagen gel (81).

As always, there are also shortcomings in this system. As was the case for culture on plastic the cells must be isolated by some means, usually enzymatic digestion. The non-plastic substrata are expensive and biomatrix is also technically difficult to prepare and handle. Biomatrix has, thus far, only been successfully used for rat and rabbit cultures. ECM and biomatrix are also comparatively undefined, containing many non-collagenous proteins in addition to the major basement membrane components, and even type I collagen gels as commonly used have non-collagenous impurities (our unpublished data). For differentiation studies the collagen gel system requires a high initial seeding density (89). Collagen gel and biomatrix also seem to require cells from hormonally-primed animals (59,81), but this has not been well investigated.

E. Three-Dimensional Cell Culture

With the exception of soft agar culture (see below), this system represents an extension of the on-collagen-gel work presented above. Instead of plating mammary cells on top of a gel of collagen, the cells are embedded within the fibrous collagen matrix (90-92). Tissues from many species have been used (75,86,93,94). Cells in this system show good growth potential and will grow for more than one month free of contamination by fibroblasts or other cell types (95). Cells inside the collagen gels will grow more than cells seeded on top of collagen, because growth can be three-dimensional with a greater percentage of the cell population participating in proliferation (82). A unique feature among the cell culture systems is that the cells in collagen gel will organize themselves into hollow colonies with correct apical-basal polarity, small lumina in the center of the colonies, and a partial basal lamina adjacent to the collagen gel (see Section V). A result of this colony topology is that cells that would otherwise appear

similar in monolayer culture will form colonies with different morphologies in collagen gel, facilitating the isolation of populations of potentially differing cell types. The cells are easily observed microscopically in culture. Serum-free media have been developed for mouse, rat and human cells, and the cells will respond to various supplements including hormones, growth factors, lipids, prostaglandins, tumor promoters and other agents that have been found to be growth stimulatory either in vivo or in vitro (93,94,96-101). As above, growth is easily measured by increase in cell number or by DNA content of the cultures (75). Although this system has not been much used for studies of differentiation, some reports exist of its use in this type of study (102, 103). In this system, cells from ovariectomized and adrenalectomized mice will respond to lactogenic hormone combinations with casein synthesis (our unpublished observations), which distinguishes it from any of the other systems mentioned above.

Although this system may fulfill the largest number of criteria for the ideal culture system outlined above, it also has its own shortcomings. The collagen gel used is expensive, and placing the cells in the gel often is a problem for those inexperienced with its use. Passage is difficult in this system, although not impossible. Diffusion through the collagen gel is inhibited, prohibiting pulse chase experiments or rapid switching from one medium type or set of components to another. Rapid isolation of the cells for biochemical work is also difficult, and the use of immunohistochemical techniques requires that the gels be fixed and sectioned in much the same way as a tissue specimen (100). A shortcoming this system shares with whole organ culture is that the topology of the colonies in the collagen gels prevents secreted products from entering the medium (96,102,103). Thus, a culture must be terminated to obtain a measurement of the extent of differentiation.

Three-dimensional growth of rat and human mammary cancer cells in response to various additives have also been reported in soft agar (104-108). Although it is commonly believed that only cancer cells will form colonies in soft agar, some normal cells will also grow, and this may complicate interpretation (108). In addition, the plating efficiency is very low and the extent of growth is difficult to quantify, and for this reason the quantification is usually performed on the basis of colony formation. Recovery of these colonies for subsequent studies is a major problem.

F. Summary

Clearly, the culture systems that have been the most useful for studying the growth of mammary cells in culture have been the whole organ system, and monolayer culture on non-plastic substrata, and cell culture in collagen gel. While none of these systems fully meets the criteria set out in Section III for the ideal culture system, one can consider that these three systems come the closest. Of these systems, the in-collagen-gel system has probably been the most useful in demonstrating that mammary epithelial cells from rodents can be grown in response to many different growth promoting agents in a serum-free medium. The monolayer non-plastic systems have been less used for growth in the past, but the purification and use of some of the individual components of the basal lamina found in ECM hold great promise for those systems also. In the human system the development of complex undefined media that allow culture on plastic represents a great step forward.

Although some of the culture systems have not provided much convincing data when used for growth studies, this is not the case for studies of differentiation. Each of the culture systems discussed has been used successfully for studies of differentiation, and each also has its own parti-

cular shortcomings. The monolayer non-plastic culture system is probably currently the most useful, since it allows study of the widest range of phenomena, including product synthesis and secretion, interaction with basal lamina components both as ECM and as individual purified components, ion transport, and ultrastructural morphogenesis; potentially it also allows for combined studies of growth and differentiation. However, more work needs to be done using this system for growth studies. The organ culture systems are also useful for more specific purposes, especially when an easily managed system is desired, but these do not permit controlled study of the cellular interactions, and carryover of endogenous hormone cannot be eliminated. Three-dimensional culture in collagen gels has permitted preliminary investigation of the hormone and growth factor responsiveness of endocrine-ablated cells, so this is also, a very potentially useful system.

V. SYSTEMS USED TO STUDY MORPHOGENESIS IN CULTURE

In vivo, the form and function in a tissue or organ are inseparable. As we have discussed above, culture systems used for the mammary gland have allowed investigation of the function of mammary cells in culture, both in systems in which all cellular components are present, as well as in systems in which individual cellular components are examined. Although we have touched on morphogenesis in examining whole organ culture and fragment organ culture, we have not discussed morphogenesis in any of the other systems, nor how they might be used for study of morphogenesis. Epithelial morphogenesis is a complex phenomenon, encompassing both intercellular recognition and adhesion as well as binding to and invasion of the local substratum. Although the use of cell culture systems to analyze morphogenesis in culture has been limited, and focused mostly on the rodent systems, we will examine what has been done and compare morphogenesis in these systems to that occurring during the cyclic growth and development of the adult mammary gland.

The mammary gland is composed of multiple epithelial cell types including myoepithelial, ductal and alveolar cells, as well as several types of stromal cell (109). During pregnancy the predominately ductal tree characteristic of the non-pregnant state is transformed into a lobuloalveolar structure (1). The cells of the ducts and alveoli are cuboidal to columnar in shape and are polarized around a central lumen. Surrounding the ducts and alveoli is a basement membrane, which modulates the interaction of the epithelial cells with the surrounding stroma and helps maintain the structural integrity of the epithelial parenchyma. This complex organization is determined both by intrinsic factors, carried in the genetic program of the cells, and by extrinsic factors, such as humoral factors and the stromal matrix within which the cells are growing.

In an ideal culture system, the growth and differentiation of mammary epithelial cells would occur in a manner similar to that seen in vivo. That is, one would see the formation of branched tubular structures, which, under the influence of lactogenic hormones, would become biochemically and morphologically secretory and release secretory products into an open lumen. The complete recapitulation of a mammary tree in vitro with the proper multicellular organization and morphogenesis may be a distant goal, yet certain aspects of this developmental scheme can be observed in vitro. These include: a) growth and differentiation mediated by hormones and growth factors, b) basement membrane synthesis, c) intercellular communication and adhesion, d) modulation of cell shape, and e) the formation of branched tubular structures. It would seem that the easiest way to observe morphogenesis in culture would be to use the whole-gland organ culture system, since it represents the actual gland and not a mimicry of the morphogenesis seen in the gland in vivo (8). However, as with experiments on growth and differentiation conducted in vivo, the interactions may prove to be too

complex to sort out without the help of simplified model systems. It is obvious that in order to observe morphogenesis in culture similar to that seen in vivo, mammary cells must be cultivated in a three-dimensional culture system. Culture of cells on the surface of culture dishes only permits an examination of mammary function in a two-dimensional space. In this arrangement, morphogenesis is guided by the flat culture surface resulting in mostly monolayer formation. Three-dimensional culture can be achieved by the use of a collagen gel culture system (75). Because the two-dimensional and three-dimensional culture systems are so different in their morphogenetic properties, we will discuss them separately.

A. Two-Dimensional Culture Systems

Mammary epithelial cells have been cultured on plastic, collagen-coated plastic, on top of collagen gels, and on ECM or biomatrix. The influence of these substrata on growth and differentiation is discussed above. There are two major influences that these substrata have upon mammary epithelial cell morphogenesis. The first is adhesion of cells to a substratum, in that mammary epithelial cells must grow attached to a surface. One determinant of attachment is the synthesis and formation by the cells of a basal lamina containing molecules modulating attachment of cells to the surface (110). Cells rarely attach and grow on a surface unless attachment factors are present, perhaps provided by serum (40), or unless conditions are provided that stabilize basal lamina formation (111). The type and amount of glycosaminoglycans produced by cells cultured on plastic substrata differ from those produced by cells cultured on top of collagen gels (112). Basal lamina components are released into the extracellular medium when mouse mammary epithelial cells are plated on plastic, but not when they are cultured on top of collagen gels (111). If mouse cells are cultured for a prolonged time on the top of collagen gels, an underlying basement membrane can be observed (113).

The second feature of the cells which can be modulated in two-dimensional culture is the cell shape. If cells are plated on plastic or collagen gels, they form flattened colonies that are squamous in appearance. If the attached collagen gels are then released to float in the culture medium, the cells will contract the gel and become cuboidal/columnar in shape (113). Numerous microvilli appear on the "lumenal" surface and, interestingly, the cells become competent for hormone-stimulated synthesis of milk products such as casein. The cells are refractory to hormonal stimulation of milk product synthesis if the gels remain attached (89).

B. Three-Dimensional Culture Systems

Human (114,90,91), rat (93,100), and mouse (75,96,115) mammary epithelial cells have all been cultured inside collagen gel matrices. In this system, small epithelial cell clumps are embedded within a type I collagen gel. The cells attach to the collagen, then grow by sending projections into the gel matrix. When using mouse mammary epithelial cells these projections appear "duct-like" in that they are hollow tubes. Growth apparently occurs at the periphery of the colonies and at the ends of the growing branches (82). These three-dimensional structures, in which basement membrane formation can sometimes be seen (82), are produced from isolated epithelial cell populations and are observed in serum-free medium. Long tubules can be formed, around which the cells become polarized and, when stimulated by lactogenic hormones, appear secretory, often ultrastructurally indistinguishable from mammary tissue in vivo (96). Bilateral branching of the tubules can be observed, particularly when growth is stimulated by progesterone and prolactin, as well as bulbous growing ends (see Figure 1). This system thus mimics morphogenesis, using an isolated epithelial population.

This three-dimensional culture system with collagen gel mimics in vivo morphogenesis, using mouse mammary epithelial cells, only under certain hormonal and growth factor conditions. Rat or human mammary epithelial cells cultured in collagen gel have a more varied morphology, including tubules like those seen in the mouse, spherical cysts, larger masses of cells with many small lumina, or masses of loosely adherent cells with the appearance of fibroblasts but with marker proteins that show them to be epithelial cells. These differences in morphology may reflect different cell types giving rise to different colony shapes when allowed to express themselves in the collagen gel.

C. Summary

Cell culture on two-dimensional surfaces can produce epithelium similar in morphology and function to the in vivo state, if the cells are cultured on floating collagen gels or substrata made up of extracellular matrix components, either ECM or biomatrix. Morphogenesis approaching that seen in vivo or in organ culture is possible using three-dimensional culture, such as the collagen gel culture system with mouse mammary epithelial cells. Morphogenesis in three-dimensional culture is more varied, using cells from other sources and under differing hormonal and growth factor conditions.

VI. TRANSFORMATION OF MAMMARY CELLS IN VITRO

One of the ultimate goals in mammary biology has been the development of a system or systems to study the process of neoplastic transformation of mammary epithelial cells in culture. The establishment of an efficient system in which to transform mammary epithelial cells neoplastically in culture would greatly facilitate the study of the interactions between mammary cells and the hormones and carcinogens leading to the phenomenon known as breast cancer. A truly successful system has yet to be worked out, as will be evident from the ensuing discussion, and transformation in culture remains the least tractable problem for mammary culture biologists.

An idealized system for transformation of mammary epithelial cells in culture should meet the same requirements as those needed for growth and differentiation, as described in Section III. In addition, a high frequency of detectable transformants is needed, with neoplastic significance for cancers observed in vivo. These transformants should be induced quantitatively by either direct or indirect acting chemical carcinogens. Finally, the ability to maintain untreated normal cells in culture is required for the purposes of comparison. Tissues from both humans and rodents have been used in the past, and in summarizing this work, we will concentrate not only on the relative advantages and disadvantages of the systems used, but also on the criteria that were used to determine the extent of the transformation, the nature of the transformants derived, and the efficiency of the transformation process. We will discuss this information by species studied, and on a case-by-case basis, rather than by the culture system, since relatively little work has been done in this area.

A. Studies on Mouse Tissue

Treatment of mouse mammary glands with 7,12-dimethylbenz(a)anthracene (DMBA) in whole-gland organ culture in a chemically defined medium, results in the transformation of lobuloalveolar structures to hormone-independent foci called nodule-like alveolar lesions (NLAL); these persist after regression in culture is allowed to occur (3,116). The NLAL are morphologi

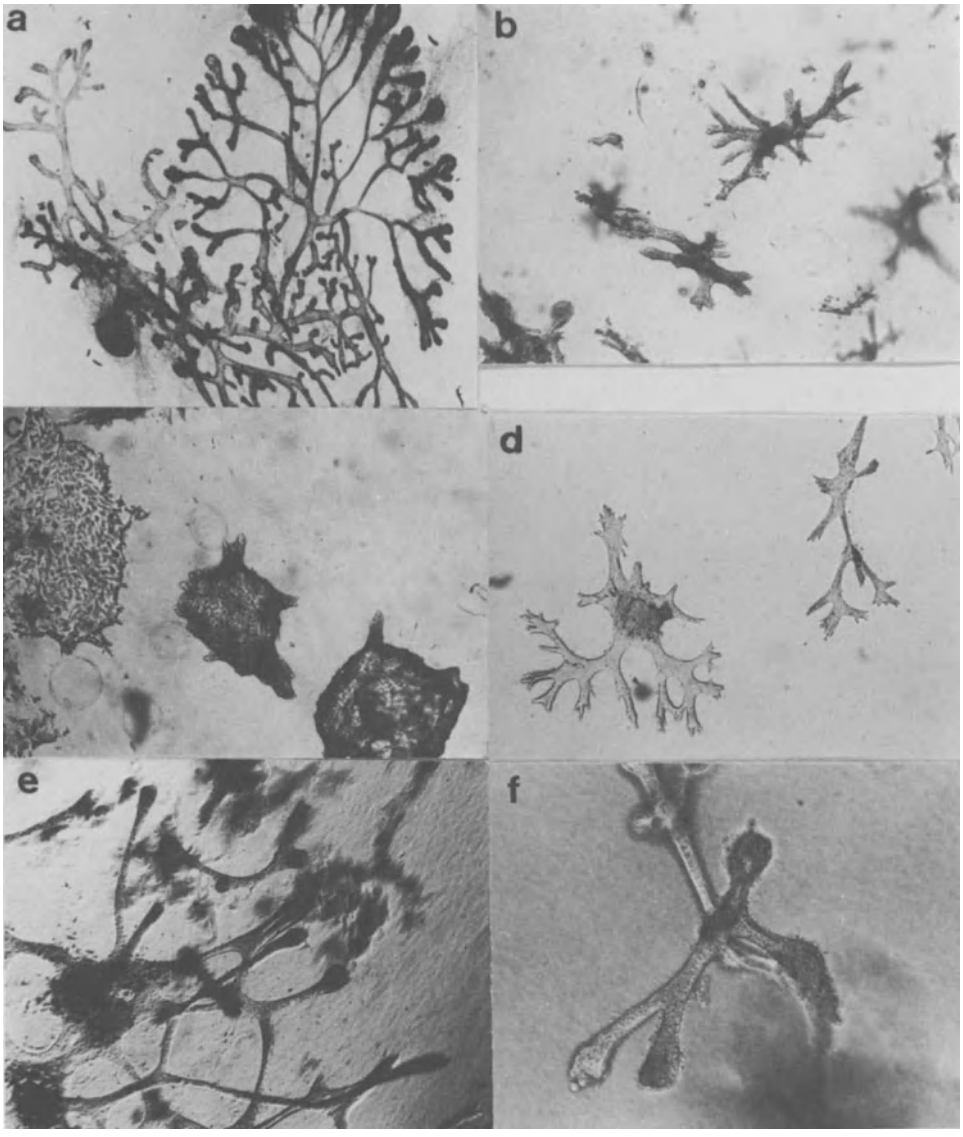

Figure 1. Morphology of mouse and human mammary epithelial cells grown within collagen gels. (a) Whole mount of a mammary gland from a virgin mouse showing a ductal tree with end buds. (b) Human breast epithelial cell colonies exhibiting duct-like morphogenesis (14x). (c) Human breast epithelial cell colonies exhibiting spherical morphogenesis (14x). Colony on the left seems flat since it is growing in two dimensions between the bottom and top layers of the collagen gel. (d) Mouse epithelial cell colony exhibiting a "stellate" morphology with numerous arms or projections with pointed ends (33x). (e) Mouse epithelial cell colony exhibiting long duct-like tubules with blunt ends (20x). (f) Mouse epithelial cell colony exhibiting branching morphogenesis (83x).

cally detectable in culture, occur at a high frequency, and produce adenocarcinomas when transplanted to the gland-free mammary fat pads of syngeneic female hosts. Only DMBA at highly cytotoxic concentrations has been demonstrated to produce NLAL that produce hyperplastic outgrowths and carcinomas when transplanted. Other carcinogens or lower concentrations of DMBA have not been shown to produce either hyperplasias or carcinomas after transplantation (117), although the NLAL do appear in culture. An important problem is that the fate of the carcinogen used is difficult to analyze in organ culture, since there is a mixture of stromal cells and epithelial cells and there is a tendency for the lipophilic polycyclic hydrocarbons to be sequestered in the adipocytes surrounding the epithelium.

Mouse mammary epithelial cells have been grown inside collagen gels, in serum-free media containing either EGF or progesterone and prolactin (see above, 95). Treatment of the culture with multiple exposure to N-nitroso-N-methylurea (NMU)/or DMBA produces a high incidence of ductal and alveolar hyperplasias after transplantation to the gland-free mammary fat pad of syngeneic mice. No obvious carcinomas were detected in the primary transplants. There was a higher frequency of lobuloalveolar hyperplasias produced from treated cells grown in media containing hormones than in those grown in media with EGF. The lobuloalveolar hyperplasias produced carcinomas after transplantation. No in-culture parameters for transformation were observed.

B. Studies on Rat Tissue

Using fragment organ culture, explants of rat mammary glands were cultured for 9-12 days in serum-containing media supplemented with hormones, and treated one to three times with DMBA (118). The explants were then transplanted to syngeneic hosts, and adenocarcinomas developed by 90 days. This report did not determine whether induction of carcinogenesis actually occurred in culture or whether there were any changes related to transformation.

Several groups have used monolayer culture on plastic to investigate transformation. Rat cells grown for a brief period in monolayer culture on plastic with serum, and exposed to DMBA or NMU, produced lobuloalveolar and ductal hyperplasias and adenocarcinomas at a low frequency when transplanted to the gland-free mammary fat pads of syngeneic hosts (62). No in-culture parameters for transformation were used. An increase in concanavalin-A binding to rat mammary cells exposed to DMBA in serum-containing monolayer culture on plastic has been described as an indicator of transformation, but the neoplastic significance of these observations was not established (119). Rat cells treated in monolayer culture on plastic with DMBA had an enhanced life span when grown in serum-containing media supplemented with hormones. After 199 days in culture a small percentage of the treated cells acquired anchorage independence and formed carcinomas in athymic nude mice (120).

Only one report using monolayer non-plastic culture exists. In this case rat mammary cells were grown and passaged on top of type I collagen coated dishes, in serum supplemented with growth factors and hormones. Exposure of cultures to N-methyl-N-nitro-N-nitrosoguanidine enhanced the proliferative potential of the cells, and cell lines were obtained from treated cultures (48). No evidence for neoplastic potential of the cells was reported.

C. Studies on Human Tissue

These studies have all involved a single culture system, i.e., monolayer culture on plastic. Human breast epithelial cells obtained from milk, and grown as monolayers in serum supplemented with hormones and cholera

toxin, have been transformed with SV-40 (121). The SV-40 infected cells grow in soft agar and have large-T antigen. One cell strain gave rise to cell lines. However no neoplastic significance of the transformed cells was described. Primary cultures of normal human mammary epithelial cells that metabolize benzo(a)pyrene (BP) produce cultures with extended life spans when treated multiple times with BP (122). Two immortal cell lines were recovered from the enhanced life span cultures. The lines do not appear to be malignantly transformed, as they do not form tumors in nude mice and show little anchorage independence.

D. Summary

Attempts to transform mammary epithelial cells neoplastically have been hampered by the problem of correlating the transformed phenotypes observed in culture with hyperplasias or neoplasias observed in vivo, and, in most systems described, by the low frequency of any type of transformation observed; these handicaps make further analysis of the conditions necessary for transformation difficult. A high incidence of transformed populations has been obtained, using either fragment or whole organ culture, but restricted growth in culture and the short length of culture period limits the potential for studying the complete neoplastic process in culture. Monolayer culture has been used to a great extent, but all of these studies suffer from serious shortcomings of one sort or another. Well-defined conditions for the growth and differentiation of mammary epithelial cells are being developed, but have not yet been achieved, and reliable, in-culture markers for the different phenotypes of mammary cells or hyperplasias and neoplasias have not been developed. The establishment of a culture system in which the mammary epithelial cells can be grown, their phenotype modulated, and efficient neoplastic transformation achieved and detected would greatly facilitate any study of the complex interaction of growth factors, hormones and carcinogens with mammary epithelial cells that results in breast cancer. Although certain portions of this set of goals have been achieved, it should be apparent that much remains to be done.

VII. CONCLUSION

The goal in the development of a mammary gland culture system is to be able to reproduce in culture the physiological and pathological phenomena that occur in vivo. It is apparent from the information presented here that no single complete system exists, and indeed, it may not be possible to produce a single system to investigate all in vivo phenomena in culture. Even the direct roles of hormones and, especially, of growth factors on differentiation, the most extensively investigated phenomenon in mammary biology, is still subject to debate. The debate becomes more intense as one moves to less investigated phenomena: growth, morphogenesis, and, most strikingly, transformation. This debate reflects both the complexity of the mammary gland and the shortcomings of the systems which have been used to date. Although we have outlined an idealized culture system in Section III, it is likely that this hypothetical system is also flawed in some respects due to the partial state of knowledge and our own views and prejudices.

The simplest culture systems, fragment organ culture and whole organ culture, have been much used for investigations in growth and have been especially useful for investigations in differentiation. Their retention of the stromal influence and ability to mimic stages of differentiation in a serum-free medium are major strengths. However, assessment of the role of stromal elements in differentiation, morphogenesis and transformation is exceedingly difficult in these systems. The limited duration of such cultures also impairs their usefulness in the study of transformation in culture.

Two-dimensional cultures on plastic substrata eliminate the undefined stromal influences and replace them with other interactions, both defined and undefined. Although this system has been much used, its actual utility as a simple system for rodent tissue is arguable; however, development of a new serum-free media have allowed work with human tissue to progress rapidly. The recent supplementation of this system by the use of feeder cells and/or complex media has increased its utility at some cost to its defined nature. These last improvements have come too recently for much more than preliminary studies to have been accomplished as yet.

The recent development of culture systems which utilize stromal collagen or basal lamina components as a substratum or matrix provide promising techniques for studying mammary epithelial biology. These systems allow both growth and differentiation over a long term in culture, in the absence or presence of stromal elements as desired. They can utilize serum-free media, and allow study of extensive growth and differentiation in one system. This system probably fulfills the greatest number of criteria for the idealized culture system, albeit not all of these criteria.

If there is any conclusion to this chapter, it is that the most exciting work in mammary gland culture lies ahead. The work that has been done with the culture systems available has led to a greater understanding of the complex nature of hormonal and growth factor interactions in mammary growth and development. The role of individual hormones is still only partially understood, however, and the role of growth factors is still a subject largely of speculation. The transformation of mammary cells in culture is a goal that remains elusive, with rare exception, but one that further use and development of existing culture systems should soon begin to address.

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PROLACTIN EFFECTS AND REGULATION OF ITS RECEPTORS IN HUMAN MAMMARY TUMOR CELLS

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I. INTRODUCTION

It is a well known and accepted fact that estrogens are the predominant hormone involved in human as well as rodent mammary cancer development and growth regulation. The possible physiological role of other hormones, particularly prolactin, in the etiology and progression of this disease is not as well established. However, the use of several rodent model systems has established that this lactogenic hormone plays an important role in the induction and progression of mammary tumors in experimental animals.

II. RODENT MODEL SYSTEMS

A. Mice

Much of the work with rodents has made use of the drug CB-154 (2-bromo-α-ergocryptine), a potent suppressor of prolactin secretion by intact and/or grafted pituitaries (1-4). This drug has the advantage of not markedly interfering with the secretion of other anterior pituitary hormones (3-6). Initial studies by Muhlbock and Boot (7) showed that multiple pituitary isografts, which secrete large amounts of prolactin (8), significantly increased the incidence of spontaneous mammary tumors in mice. Liebelt and Liebelt (9) obtained similar results with a single pituitary isograft. Subsequently, Yanai and Nagasawa (1) showed that CB-154 suppression of prolactin secretion by the isografts resulted in a significant reduction in the mammary tumor incidence. In addition, Welsch and Gribler (10) showed that suppressing the normal levels of prolactin secreted by the intact

pituitary, by chronic treatment of young mice with CB-154, virtually prevented the development of spontaneous mammary tumors. More direct evidence that prolactin is a key hormone in mammary tumor development in mice was obtained by Boot et al. (11), who obtained significantly more tumors in animals given daily injections of the lactogen.

B. Rats

A similar body of evidence supporting the concept of a key role of prolactin in mammary tumor development exists for rats. Unlike mice, which can develop mammary tumors spontaneously, primarily due to the presence of the endogenous mammary tumor virus (MMTV) (12) or to induced hormonal imbalances as mentioned above, most mammary tumors in rats arise in response to physical or chemical carcinogen treatment (13-14). However, some studies have also been performed to examine the effects of induced pituitary hormonal imbalances on mammary tumor incidence in rats. Welsch et al. (15) reported that there was a much higher mammary tumor incidence in rats receiving multiple pituitary isografts than in controls, regardless of parity status. These data were consistent with previous work by Kwa et al. (16) showing a positive correlation in aged rats between elevated serum prolactin levels and spontaneous breast tumor incidence. Similarly, lesions of the median eminence, which resulted in an imbalance in the levels of circulating pituitary hormones in favor of prolactin, resulted in an increase in the incidence of spontaneous breast tumors in rats (17-19). In addition, growth of the spontaneous mammary tumors in rats has been shown to be prolactin-dependent. Treatment of such tumor-bearing rats with ergot drugs caused reversible regression of the tumors (20).

Chemical carcinogen-induced breast cancer in rats is also dependent on prolactin for sustained growth. Not only is there a direct correlation between serum prolactin levels and susceptibility of various rat strains to induction of tumors by these agents (21), but there are also correlations between drug-induced hypoprolactinemia and retarded tumor growth (22-26) and between induced hyperprolactinemia and increased tumor growth (27-32). Since administration of prolactin to intact animals may stimulate tumor growth by an indirect mechanism, several types of experiments were performed to clarify this problem. Increased tumor growth was obtained by administration of prolactin to rats bearing carcinogen-induced mammary tumors if the rats were subsequently ovariectomized (33), ovariectomized and adrenalectomized (34), or ovariectomized, adrenalectomized, and hypophysectomized (29); thus rendering an absolute requirement for other defined hormones less likely. In addition, Welsch and Rivera (35) and Welsh et al. (36) reported that prolactin can stimulate [^{3}H]-thymidine incorporation into DNA, in explants from 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary tumors cultured in the presence of insulin with or without corticosterone as well. Labeling index and cell number were also increased when prolactin was added to DMBA-induced tumors in either organ or primary cell culture (36).

The N-nitrosomethylurea (NMU)-induced mammary tumor in rats is also dependent on prolactin for growth *in vivo* (37,38) or *in vitro* (39,40). Arafah et al. (48) reported that growth of regressing NMU-induced tumors in hypophysectomized rats was stabilized by administration of either prolactin or estradiol to the animals. Active growth was restored when both prolactin and estradiol were given simultaneously. Further studies by Manni and coworkers (39,40) showed that cells from the NMU-induced tumors, grown *in vitro* in soft agar, were stimulated to grow by the addition of prolactin to the culture medium or by exposure to conditioned medium collected from cells previously treated with prolactin. Thus, the NMU-induced breast cancer in rats, like the DMBA-induced tumor, appears to be responsive to prolactin in terms of sustained growth.

III. HUMAN BREAST CANCER

A. Studies In Vivo

In contrast to the studies in the rodent model systems mentioned above, attempts to establish a clear function for the lactogenic hormones in the etiology and progression of human breast cancer have not been as successful. Much of the problem has centered on the lack of a good, reproducible model of prolactin responsiveness for the human disease. Several studies have shown that basal, serum levels of bioactive lactogenic hormones are significantly elevated in a subset of women at risk for familial breast cancer (41-44), as well as in U.S. white, Japanese, and South African black breast cancer patients (45). Other studies, however, have failed to find any differences in the serum prolactin levels in Indian (46) or English white (47) breast cancer patients, compared to matched controls free of the disease. In addition, studies attempting to correlate elevated prolactin levels with increased risk of breast cancer or with poor prognosis have shown conflicting results. Initial examination of several small groups of women given reserpine, which depletes dopamine and enhances prolactin secretion, suggested that there was a two- to six-fold greater risk of breast cancer with chronic use (48-50). However, subsequent large-scale investigations involving 12,000 patients have failed to confirm this observation (51). Recently, Holtkamp et al. (52) reported a long-term followup study on 149 women with advanced metastatic breast cancer. They found that hyperprolactinemia occurred in 44% of patients with metastatic breast disease during the course of the disease. In patients experiencing several episodes of disease remission and relapse, the incidence of hyperprolactinemia increased with each relapse. Prolactin blood levels returned to normal if hyperprolactinemic patients experienced remission after chemotherapy. On the other hand, there has been only limited success with attempts to alter the course of the disease by lowering serum prolactin levels with either hypophysectomy (53), L-dopa (54), CB-154 (55-58), or agonists of luteinizing hormone-releasing factor (59,60). Thus, while there is some evidence suggesting a role for prolactin in human breast cancer, the clinical date are, as yet inconclusive.

B. Studies with Breast Biopsy Material

Since the conventional wisdom is that prolactin must first bind to specific receptors on the surface of the cells before it can affect a biological response, a great deal of research effort has concentrated on defining the receptor status of human breast tumors and cell lines. Several laboratories have established the presence of specific prolactin receptors in human breast cancer biopsies (58,61-71). Initial studies, which measured only free binding sites, found that 35-60% of biopsy samples were positive for prolactin receptors, and there was no correlation of this with estrogen and/or progesterone receptor status nor with menopausal status (61,62,64,-65,68,71). However, when a desaturation assay that allowed for detection of total binding sites was used, as many as 75% of the samples were positive for prolactin receptors, (63,65,67,71). In general, no correlation was found between prolactin receptors and age, weight, menopause status, or pathological features such as differentiation, histoprognostic grading, and cellular density (65,67), although a lack of prolactin receptors was reported in better differentiated (grade I) carcinomas (65). Using the desaturation assay, conflicting studies have reported no correlation (63) or, in contrast, a highly significant one (66) between the presence of prolactin receptors and that of estrogen and progesterone receptors. The level of prolactin receptors was not altered by pretreating patients with CB-154 prior to mastectomy (58). Using immunocytochemical techniques, prolactin has been localized in up to 56% of breast carcinomas, and the

presence of the hormone was related to good histological differentiation (72).

Biopsy samples are not always suitable for the multiple, long-term tissue culture experiments which are necessary to establish a relationship between receptor status and biological response. Hence, such responses have been sought using athymic nude mice, short-term primary cultures, or breast cancer cell lines in long-term tissue culture. Primary breast biopsy samples grown in nude mice respond to lactogenic hormones with increased thymidine-labeling index when the animals are also given estradiol (73). These data in vivo would suggest that prolactin plays a role, either direct or indirect, in the growth of human breast cancer cells. This is further supported by recent results from several laboratories, using primary cultures of mammary epithelial cells isolated from breast biopsy material or reduction mammoplasty samples. Kleejer-Anderson and Buehring (74) reported that pharmacological concentrations of ovine or human prolactin can affect the growth rate of malignant and nonmalignant human epithelial cells. Malarkey et al. (75) found that physiological levels of human prolactin and human growth hormone (hGH), but not ovine prolactin, increased the population doubling of primary breast tumor cultures. Similar results were also obtained by Welsch and coworkers (76,77), using benign human breast tissue. Hammond et al. (78) reported that multiple serial passages of human mammary epithelial cells under serum-free conditions require the presence of bovine pituitary extract. Replacement of this extract with prostaglandin E_1 plus ovine prolactin yields a defined medium that allows for three to four serial passages. Likewise, Manni et al. (79) found that, under the serum-free conditions of a soft agar clonogenic assay, ovine prolactin exerted a dose-dependent, growth-promoting effect on human breast cancer cells in primary culture. The effect of prolactin was more pronounced with cells from estrogen receptor-positive tumors.

C. Studies with Established Breast Cancer Cell Lines

Despite the evidence for a physiological role of prolactin from experiments in vivo or using primary breast biopsy material in culture or in nude mice, it has been difficult to obtain a clear and consistent growth response in established breast cancer cell lines. Both T47D and MCF-7 cells responded to lactogen stimulation when grown in solid tumors in nude mice (80-82). As with primary breast biopsy material (73), this effect was only apparent when the animals either had intact functioning ovaries or were supplemented with estrogens. The presence of prolactin receptors has been established in several cell lines, but the number of binding sites observed varied from cell line to cell line and from laboratory to laboratory (83-85). In addition, the level of binding could be modulated by addition of exogenous hormones such as estradiol (86), antiestrogens (87), prolactin (86), progesterone (86,88,89), dihydrotestosterone (86), and thyroid hormones (unpublished observation). The presence of abundant receptors for prolactin on these cells, however, has not always been associated with consistent responses to lactogenic hormones in terms of growth stimulation. While T47D cells have been reported to alter their shape, adhesion and lipid accumulation in response to hGH prolactin (1 μg/ml) was without effect on the CAMA-1 cells (91). However, the newly established line EFM-19 was stimulated by human prolactin to grow in serum-free conditions, with other lactogenic hormones giving little or not response (86). In studies using the MCF-7 cell line, until recently no positive growth responses to prolactin in vitro have been reported. Shafie and Brooks (92) reported that prolactin increased the number of estrogen receptors in these cells but that no increase in growth rate resulted. Shiu (84) also was unable to demonstrate an effect of either human prolactin or hGH (1 μg/ml) on growth of these cells, either in the presence of 1% charcoal-stripped serum (CSS) or under serum-free conditions. Likewise, Jozan et al. (93) reported negative results with

ovine prolactin when cells were grown on extracellular matrix under serum-free conditions.

In light of the results with growing cells in nude mice and the similar results using primary breast biopsy samples, we recently re-examined the role of prolactin in the growth of human breast cancer cells in long-term tissue culture, using the MCF-7 cells as the model. We found that these cells were responsive to physiological concentrations of human lactogens under proper growth conditions, even in serum-containing medium. This is illustrated in Figure 1. When MCF-7 cells were grown for three or six days in the presence of 10% fetal calf serum (FCS) containing exogenously added human prolactin, little or no enhancement of cell growth was observed (94). However, if the serum was first stripped of endogenous hormones by pretreatment with dextran-coated charcoal, prolactin-stimulated growth was observed. The prolactin-stimulated growth occurred more rapidly than estrogen-stimulated growth. After three days in the presence of 10% CSS, estradiol (10^{-8}M) produced a 30% increase in cell number, compared to 10% CSS alone (a two-fold increase over plating density). Human prolactin (100 ng/ml), on the other hand, gave a 2.4-fold increase in cell number over CSS alone, and a 4.4-fold increase over plating density. After six days in culture, in the presence of either of the hormones, the same number of cells were present. This represented a five-fold increase in cells over the number plated, but only 80% more cells than in the absence of added hormones (i.e., with 10% CSS alone). The prolactin-stimulated growth of these cells was more evident in the presence of 1% CSS, compared to 10% CSS. More than a three-fold increase in cell number was seen after three days in the presence of 1% CSS

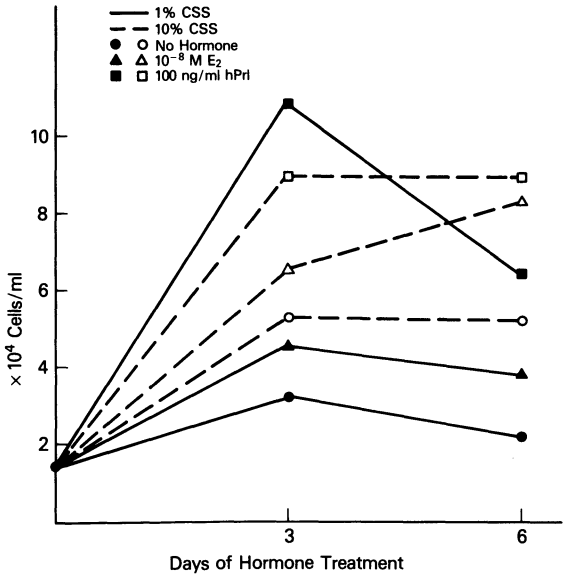

Figure 1. Time course of growth of MCF-7 cells in 1% (——) or 10% (---) CSS in the absence (● ○) or presence of 100 ng human prolactin/ml (■ □) or 10^{-8} M E_2 (▲ △). Cells were grown for 1 day in the CSS before the addition of the hormones on day 0. Cells were collected by brief trypsinization and counted with a hemacytometer using trypan blue exclusion to determine viability. In all cases shown, viability was >95%.

and prolactin, compared to 1% CSS alone. This represented a 7.5-fold increase in cell number compared to the plating density. Under these same conditions, estradiol produced only a 40% increase in cell number (three-fold over plating density). After six days, total cell survival in the presence of 1% CSS, even with hormonal supplements, was significantly decreased. Five percent CSS gave results similar to those in 10% CSS (data not shown). The estradiol concentration used (10^{-8}M) was optimal for the MCF-7 cells grown in our laboratory. Altering the estradiol concentration only resulted in fewer cells present, under all growth conditions tested.

The ability of various lactogens to stimulate growth of the MCF-7 cells in the presence of 10% CSS is shown in Figure 2. Human prolactin was the most effective and consistent in the stimulation of growth over a three-day period. The minimal effective concentration of this hormone in the presence of 10% CSS was 10-50 ng/ml (94), which is in the physiological range. The maximal response was obtained at 100-250 ng/ml, after which there was a dramatic decrease in the hormone's effect (see Figure 4 later). The other lactogens which stimulated the cells to grow in CSS, such as hGH, human placental lactogen, and ovine prolactin (not shown), were generally less effective or less consistent in their response, compared to human prolactin. While the others occasionally gave responses at 50 ng/ml, at least 100-250 ng/ml were required for more consistent responses. All lactogens tested showed a decrease in stimulation at concentrations of 500 ng/ml or higher (94). The degree of the decrease varied with different hormones and experiments. The reason for this is unknown but may be related to down-regulation of the receptors by the hormone (95). The decreased response at very high concentrations of even the human lactogens may explain the lack of prolactin-induced growth reported by several investigators who did use CSS, but used human prolactin at 1-10 μg/ml (84,92).

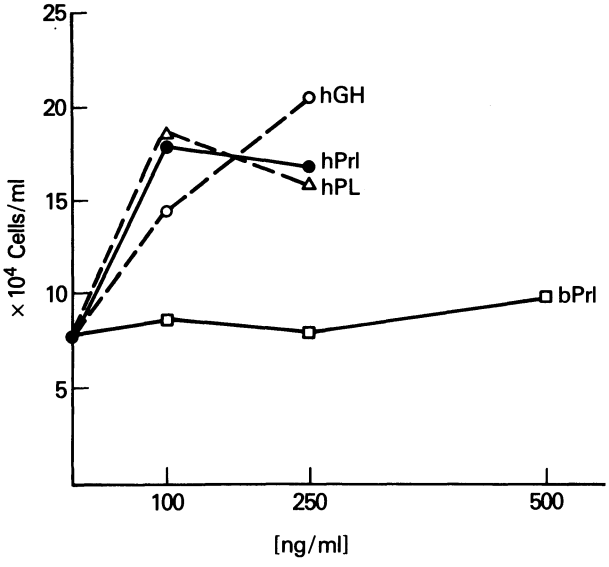

Figure 2. Effect of different concentrations (0 to 500 ng/ml) of various lactogens on the growth of MCF-7 cells for 3 days. Cells were grown in the presence of 10% CSS for 1 day prior to the addition (day 0) of human prolactin (●—●), human placental lactogen (Δ---Δ), human growth hormone (O---O), or bovine prolactin (□—□). Three days after hormone addition, cells were collected by brief trypsinization and viable cells counted by trypan blue exclusion using a hemacytometer.

Table 1

Specific Binding of Various Lactogens to

MCF-7 Cells Grown in CSS

Competing hormone	Specific binding (cpm/10^6 cells)
human prolactin	2495 ± 120
human growth hormone	1977 ± 204
human placental lactogen	2456 ± 55
bovine prolactin	2309 ± 193
ovine prolactin	2353 ± 164

MCF-7 cells were grown for 4 days in the presence of 10% CSS before specific lactogen binding was determined. Specific binding of ^{125}I-labeled human growth hormone was determined after 4 hours at room temperature as described (94). Each competing unlabeled hormone was present at 1 μg.

The MCF-7 cells in the presence of CSS appeared to respond maximally to lactogens of human origin. While ovine prolactin stimulated the cells to grow, once the endogenous bovine lactogen was removed from the serum, the response was neither as consistent, nor as great, as that with human prolactin (94). This observation may explain the negative results reported by some authors who used MCF-7 cells and ovine prolactin (84,91-93). The maximal effectiveness of homologous hormonal systems is a well established endocrinological phenomenon. As shown in Figure 2, bovine prolactin did not stimulate the cells to grow better than in CSS alone, even at concentrations ranging from 10-500 ng/ml.

The ability of these various lactogens to compete for the prolactin receptor on the surface of the MCF-7 cells is shown in Table 1. All of the lactogens-human prolactin, human placental lactogen, hGH, and ovine and bovine prolactin-effectively competed with labeled hormone for binding to the receptor. In fact, more detailed analysis of binding competition data on the MCF-7 cells suggested that the bovine prolactin used for these studies was a more effective competitor for the lactogenic binding sites than was human or ovine prolactin (94).

Bovine prolactin bound to the receptors but did not transmit a growth signal to the cells (Figure 2). As a result, if bovine lactogens were present in the medium, either as the endogenous hormones of FCS, or by simultaneous addition to CSS as shown in Figure 3, no stimulation of the cells was achieved when human prolactin was added at concentrations ranging from 50 to 250 ng/ml. This effect was achieved, at least in part, at bovine prolactin concentrations as low as 50 ng/ml (94). FCS, which is routinely used in most laboratories to support the growth of the MCF-7 cells, has a lactogen content as high as 250 ng/ml by radioreceptor competition assay (94). The level of the lactogen remaining in the serum after stripping with dextran-coated charcoal was an important factor in the subsequent response of the cells to exogenous prolactin. After a single cycle of stripping, an average of 75% (range 56-85%) of the endogenous lactogens were removed (94). Thus, the lack of response of the cells to human prolactin, in the presence of 10% FCS, was probably due to the receptor-blocking action of endogenous

lactogens in the serum. The majority of this was most likely related to bovine placental lactogen. Such a mechanism (i.e., one hormone blocking the action of a related hormone) is not without precedent. Recently, Gertler et al. (96) reported a similar situation using a recombinant form of hGH that is missing 13 amino acids at the amino terminus (Met 14-hGH). This synthetic hormone was unable to elicit a growth response in the rat lymphoma cell line Nb2 and blocked the growth response to other active lactogens when added to culture medium simultaneously with them. It also inhibited bovine prolactin-stimulated fat synthesis and α-lactalbumin secretion in explants from bovine mammary glands. Met 14-hGH bound to the prolactin receptors on the cell surface and competed with prolactin for these binding sites.

CSS has been extensively used in studies on cell growth and responses under the influence of steroid hormones. However, it is now clear that, in addition to removing steroids, dextran-coated charcoal treatment also removes the majority of the lactogenic hormone receptor-binding activity. This stripping procedure is essential if a response to exogenous lactogens is being sought. Since the cells are initially grown in the presence of CSS for one to five days before the addition of the hormones, it is essential to remove as much of the endogenous lactogen as possible, even if it means performing multiple cycles of charcoal stripping. The endogenous lactogens in FCS will diminish the subsequent growth responses to human prolactin, even if CSS is added simultaneously with the hormone. Hence, it is essential that cells be grown in the CSS for one to five days prior to hormone addition to desaturate the receptors and allow for maximum response.

While it is now clear that the MCF-7 cell line does respond to prolactin with increased growth in culture, this is not true for all breast cancer cell lines examined. As shown in Figure 4, the ZR 75.1 cell, which has prolactin receptors at a level equal to that in the MCF-7 cell (85), also responded to this hormone under appropriate culture conditions. However, not all of the clones of MCF-7 that we have examined were able to

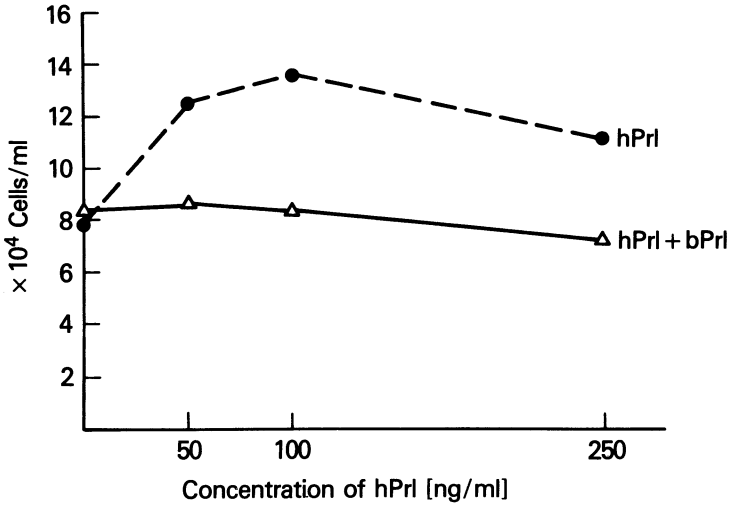

Figure 3. Effect of bovine prolactin on MCF-7 cells grown in the absence or presence of various concentrations of human prolactin. MCF-7 cells were grown for 1 day in the presence of 10% CSS before the simultaneous addition of the human and bovine prolactins on day 0. Three days later the cells were harvested by brief trypsinization and viable cells were counted by trypan blue exclusion using a hemacytometer. Bovine prolactin was used at 500 ng/ml.

respond to prolactin in terms of growth in the presence of lactogens, even though they have abundant receptors for the hormone (unpublished). In addition, in agreement with Shiu (84), we found that the T47D breast cancer cells, which are reported to have as many as three times more lactogen receptors than MCF-7 or ZR 75.1 (83-85), did not respond to human prolactin in terms of growth, even in the presence of CSS. This was true whether the cells were examined after three, four, or seven days in culture and in the presence and absence of insulin (not shown). The lack of a growth response by these cells, however, does not necessarily mean a lack of hormone response. Shiu (90) has reported a change in cell shape, adhesion, and lipid accumulation by T47D cells in response to human lactogens. These same cells also responded to prolactin with specific induction of a protein (PIP) which existed as three distinct glycosylated forms (97).

IV. CONCLUSION

The role of prolactin in the etiology of human breast disease may not be a simple one: rather, it may function in a complex, multiresponse system that we can only investigate under culture conditions properly staged by removal of impeding endogenous hormones from the culture medium. Under the proper conditions of culture, the human breast cancer cell lines MCF-7 and ZR 75.1 are highly responsive to growth stimulation by homologous lactogenic hormones. These afford us excellent models for further studies on the possible role of prolactin in growth and maintenance of human breast cancer.

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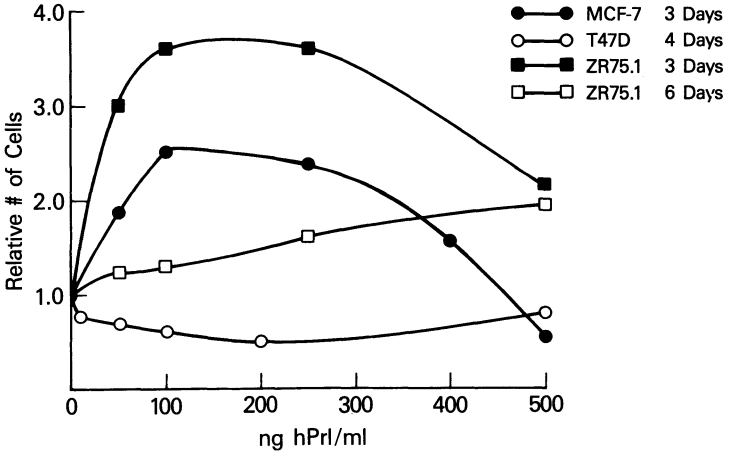

Figure 4. Effect of human prolactin on growth of various human breast cancer cell lines. Cells were grown for 1 day in CSS prior to addition of human prolactin on day 0. All cell were harvested by brief trypsinization and viable cells counted by trypan blue exclusion using a hemacytometer. MCF-7 cells were grown in 10% CSS and harvested after 3 days with the hormone (no prolactin = 7.7×10^4 cells; ●—●) T47D cells were grown in 5% CSS and harvested after 4 days with the hormone (no prolactin = 6.6×10^4 cells; O—O). ZR 75.1 cells were grown in 5% CSS and harvested after 3 (no prolactin = 5.8×10^4 cells; [] [] or 6 days (no prolactin = 15.8×10^4 cells; □—□) in the presence of the hormone.

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REGULATION OF GROWTH AND SECRETION OF GROWTH FACTORS BY 17 B-ESTRADIOL AND V-RAS^{H} ONCOGENE IN HUMAN MAMMARY CARCINOMA CELL LINES

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Abbreviations used: E_2, estradiol; IMEM, improved modified Eagle's medium; CM, conditional medium; CME_2, medium conditioned by cells pretreated with estradiol; HPLC, high performance liquid chromotography; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor; FGF, fibroblast growth factor.

I. INTRODUCTION

Breast cancer is unique among cancers in that its growth is strongly regulated in about one third of clinical cases by estrogenic hormones or antiestrogenic antagonists (1). Breast cancer occurs in women who never had functional ovaries with only 1% of the frequency of that in women with intact ovaries. Thus, estrogens are stimulatory, at least initially, in nearly all breast cancers. This hormonal component of growth control appears to be a remnant of normal, differentiated epithelial proliferation. During puberty or pregnancy-lactation, estrogen exerts mitogenic, anabolic and secretory effects on mammary epithelium. While estrogen is a proximate mitogen for either normal or malignant breast epithelium, the hypothalamus-pituitary axis is indirectly in control of ovarian estrogen secretion by virtue of gonadotropin releasing hormone and gonadotropin stimulation (2).

In addition, the pituitary gland (or other organs) may secrete other direct or indirect acting mitogens as yet undefined. Such hypothetical, estrogen-induced endocrine mitogens have been termed estromedins (3,4).

This review will concentrate primarily on the biochemical events, induced by estrogen, which are associated with stimulation of proliferation of human breast cancer cell lines in vitro and in vivo. Using these clonal lines of cells, usually derived from plural or ascites fluid of patients, we and subsequently others have succeeded in demonstrating receptors for, and direct proliferative responses to, physiologic doses of 17 β-estradiol (E_2) (5-8). Several such estrogen-responsive lines exist, including MCF-7, ZR-75-1, and CAMA-1 (9). MCF-7 is among the best characterized of these. In the last part of this article we will review some recent experiments whereby the tumorigenic properties of MCF-7 cells are enchanced by v-ras^H oncogene transfection, bypassing estrogen controls. Throughout this review we will attempt to summarize the literature supporting the hypothesis that polypeptide growth factors might be common mediators of growth control for both estrogen-regulated and autonomous breast cancer.

II. ESTROGENIC EFFECTS ON PROLIFERATION-ASSOCIATED ENZYMES, MAJOR SECRETED PROTEINS, AND TUMOR INVASIVENESS MARKERS

We and others began the study of estrogen-stimulated breast cancer growth in model cell systems (usually MCF-7 cells) by analyzing principal growth-related enzymes and major secreted proteins. A systematic search led to observations that estradiol (E_2) induces a large number of enzymes involved in nucleic acid synthesis, including DNA polymerase, thymidine and uridine kinases, thymidylate synthetase, carbamyl phosphate synthetase, aspartate transcarbamylase dihydro-orotase, and dihydrofolate reductase (10, 11). Physiologic concentrations of E_2 stimulate DNA synthesis by both salvage and de-novo biosynthetic pathways. In two instances recently reported, estrogen regulates thymidine kinase and dihydrofolate reductase at the mRNA level (12,13). Regulation of thymidine kinase mRNA is at least in part at the transcriptional level (13). To summarize the results of many studies, estrogen does not appear to modulate any particular key enzyme involved in growth. Rather, growth is induced by pleiotropic or cascade mechanisms. The existence of "second message" regulatory systems in this process is possible, but has not yet been proven. We have recently observed that E_2 stimulates, with an exceptionally long lag time, the turnover of phosphatidyl inositol in MCF-7 cells (14). In a variety of other model systems, this metabolic effect is quite rapid and tightly coupled to growth control by proteases and hormones, particularly the polypeptide growth factors (15,16). Thus, phosphatidyl inositol turnover, with its associated stimulation of protein kinase C and Ca^{2+} fluxes, could be a fundamental metabolic mediator of the mitogenic effects of E_2. Estrogen-induced growth factors could explain the delayed time course of the phospholipid effects.

Alternate avenues of investigation have identified the progesterone receptor (17) as an additional protein induced by estrogen. Although, progesterone is not directly growth modulatory for human breast cancer, the presence of progesterone receptor does appear to be tightly coupled to functional growth regulation by estrogen. Thus, progesterone receptor content of human breast tumors is used (along with estrogen receptor) as a marker for estrogen and antiestrogen responsiveness of tumors in clinical therapy (1).

Aside from regulation of these essential growth-controlling enzymes and of the progesterone receptor, estrogens (and antiestrogenic compounds) alter the cellular or secreted activity of several other proteins whose function in growth control remains less well characterized. These include plasminogen activator and other collagenolytic enzymes (18), several rela-

tively abundant secreted proteins, including a 24 kd protein described by McGuire and colleagues (19), 52 and 160 kd glycoproteins described by Rochefort and colleagues (20), a 39 kd glycoprotein complex (21), a 7 kd protein initially identified by Chambon and colleagues by detection of an estrogen-induced mRNA species (termed pS2) (22), and the cytoplasmic enzyme lactate dehydrogenase (23). Plasminogen activator (along with other proteases) is thought to contribute to tumor progression and growth by allowing the tumor to digest and traverse encapsulating basement membrane (24). While this is likely, it is conceivable that proteases may serve additional roles such as facilitating release of mitogenic growth factors like IGF-I (somatomedin C) from carrier proteins, or processing inactive precursor growth factors and proteases to active species (25). Interestingly, one of the major secreted proteins, the 52 kd glycoprotein, is also reported to have biologic activity -- it is mitogenic for breast cancer cells when tested in vitro (26). The activities of the 160, 39, 24, and 7 kd proteins are unknown at present. It is of note that the 160, 52, and 7 kd secreted proteins may be dissassociated from estrogen and antiestrogen modulation of MCF-7 cell growth using two MCF-7 clonal variants aberrant in their growth control by these agents (27-29). These three proteins species are decreased by antiestrogen to the same extent in MCF-7 and in LY2, a stable, antiestrogen-resistant variant of MCF-7. This suggests that a significant reduction in secretion of these proteins has no impact on growth in the case of LY2. In I-13, an MCF-7 clonal variant in which growth is arrested by physiologic concentrations of E_2, the same three proteins are induced to the same extent as in MCF-7.

Finally, we have recently been able to demonstrate that estrogen induces another function in MCF-7 cells, the cell surface "receptor" or binding protein for laminin. The laminin receptor is thought to mediate attachment of cells to basement membrane laminin (24) and contribute to tumor invasion from the primary tumor confines into new colonization areas of the host (see Chapter by L. Liotta, this volume). E_2 treatment of MCF-7 cells increases I^{125}-laminin binding, cell attachment to artificial laminin-coated membranes, and migration of the same cells across an artificial membrane toward a diffusable source of laminin (30).

In summary, while estrogens may have a considerable number of influences in vivo that may indirectly alter breast cancer progression, direct effects of estrogens on isolated breast cancer cells in vitro are well established. These effects include growth regulation itself, as well as modulation of enzymes and other activities thought to mediate mitogenic and metastatic events. Later in this review we will consider estrogenic influences on a class of secreted proteins which is minor in abundance but actively biologically -- the polypeptide growth factors. These factors, along with some of the above-mentioned major secreted proteins, are likely candidates as "second messengers" in the actions of estrogen on breast cancer. Milk, the normal secretory product of untransformed mammary epithelium, is an abundantly rich source of growth factor activities (see Chapter by W.R. Kidwell and D. Salomon, this volume). Presumably, these factors in milk are important in neonatal development and nutrition. Since breast cancer cells still produce and respond to some of these growth factors, it seems possible that growth factor secretion may take on a new, deleterious role in the context of malignant transformation.

III. ANTIESTROGENS

The triphenylethylene antiestrogen known as tamoxifen has become a mainstay in treatment of early breast cancer as well as advanced disease is used in combination with cytotoxic chemotherapy. In contrast to clinical chemotherapeutic agents, antiestrogens appear to be cytostatic rather than cytocidal and have a remarkably low incidence of significant side effects.

Many investigators have noted the close correlation of the clinical response to antiestrogens with the presence of the estrogen receptor (and its induced product -- the progesterone receptor) and the high affinity of antiestrogens and their active metabolites for the same receptor. Based on these considerations, the most likely explanation of antiestrogen action appears to be simple antagonism of the growth-promoting effects of estrogen (31,32). However, alternate views invoking other microsomal binding sites for antiestrogen have been presented (33).

Antiestrogen treatment leads to cell cycle block (in early G_1 phase) of most of the cells in vitro and to arrest of tumor growth in vivo (32,34-36). It had been initially observed that MCF-7 cells responded in vitro to both estrogens and antiestrogens under normal cell culture conditions (6). While these experiments could be interpreted as suggesting that antiestrogens could act (to arrest growth) independently of an occupied estrogen-receptor complex, recent work by Katzenellenbogen and co-workers has clearly shown that a high concentration of phenol red present in the culture medium of the cells in these studies was a highly significant estrogenic stimulus (37). Removal of phenol red, for which the structure resembles that of certain nonsteroidal estrogens, abrogated antiestrogen action on MCF-7 cells and dramatically enhanced the responsiveness of the cells to estrogen induction of cell growth and progesterone receptor (8). There remains little compelling evidence at present for antiestrogens acting in any fashion other than by direct antagonism of the initiation of signals generated by an agonist-occupied receptor.

The principal limitation to the clinical utility of antiestrogens is the gradual resistance which develops in tumors treated with these agents. Although some antiestrogen-resistant tumors lack the estrogen receptor, it is unlikely that loss of the estrogen receptors explains most of the instances of in vivo loss of antiestrogen sensitivity during treatment (1,2). It is of interest that a model system for acquired resistance, a stable clone of MCF-7 cells stepwise selected in vitro for antiestrogen resistance, still contains high levels of the estrogen receptor (28). It is, therefore, of interest to determine if alternate positive and negative hormonal growth effectors for breast cancer exist. The chief growth-modulatory, polypeptide hormones described in the literature for other cancer model systems, such as transformed fibroblasts, are the polypeptide growth factors.

IV. GROWTH FACTORS AND THE TRANSFORMED PHENOTYPE IN FIBROBLAST MODEL SYSTEMS

A standard model system for the study of polypeptide growth factor action has been the growth of rodent fibroblasts in vitro. Studies were initially carried out in cell monolayers on plastic surfaces. Smith, Scher and Todaro, among others, identified "restriction points" in the cell cycle of "normal" (but immortalized) fibroblasts. Various growth factors abrogated these restriction points, allowing the cell cycle to progress (38). Platelet-derived growth factor (PDGF), a "competence" growth factor, allowed cells to pass a restriction point in early G_1, epidermal growth factor (EGF or the related transforming growth factor α, TGFα) acted later, while insulin-like growth factor - I (IGF-I or somatomedin C) acted still later in G_1 (39). EGF and IGF-I are "progression" growth factors. Malignant transformation was proposed as resulting from ectopic production of growth factors, abolishing both competence and progression restriction points in a cell's own cycle. One consequence of this was proposed to be the serum-independent growth of some cancer cells (40-42).

In addition, an "anchorage-independent" growth assay was developed using agar or agarose suspensions of cells. It had been observed that the ability of cells to grow in colonies under anchorage-independent conditions

was correlated with their tumorigenicity or state of malignant "transformation" (43). Research from a number of laboratories over the past few years has identified at least four growth factor activities which, together, can reversibly induce the transformed phenotype in murine fibroblasts. These studies have identified PDGF, EGF (TGFα), IGF-I (or IGF-II, a different somatomedin activity) and an additional growth factor, transforming growth factor β (TGFβ) (38,44,45). Based on these data, these growth factors are considered likely to be involved in cancer growth control. However, it should be emphasized that the murine fibroblast model system may not apply to cancers of other tissues or in other species.

It is not yet known what are the principal restriction points for epithelial-cell growth. A major departure from the fibroblast model, however, is the fact that TGFβ is a growth inhibitor for many types of primary and malignant epithelial cells (46,47). Therefore, it is likely that while some of the same growth factors may facilitate passage through the cell cycle in fibroblasts and epithelial cells, control of anchorage-independent growth may involve another less well defined growth factor(s). A candidate for such a growth factor is provided by the work of Halper and Moses (48). Basic pituitary fibroblast growth factor (FGF) can subserve such a function in cloning of an adrenal carcinoma cell line (SW13), and epithelial cancers produce a related activity which remains relatively uncharacterized at present. Not withstanding the unknown features of cell cycle control in breast cancer, we have begun the analysis of its secreted growth factors with the study of all five of the activity classes mentioned above: PDGF, TGFα, IGF-I, TGFβ, and the epithelial transformation factor. We have placed special emphasis on the regulatory effects of a proximate mitogen for breast cancer, i.e. estradiol.

V. REGULATION OF BREAST CANCER GROWTH BY HORMONES OTHER THAN ESTROGEN

We and others have shown growth regulation of MCF-7 cells in monolayer culture by a variety of non-proteinaceous, trophic hormones other than estradiol. These include glucocorticoids, iodothyronines, androgens and retinoids. MCF-7 cells have receptors for, but very little growth response to, progesterone and vitamin D. Additional studies have demonstrated receptors for and responses to the polypeptides, insulin, EGF, and IGF-I. Receptors, but little cellular response, have been demonstrated for other hormones, such as prolactin and calcitonin (49,50). The multiplicity of growth-stimulating hormones for breast cancer cell culture systems *in vitro* suggests the possibility that serum-borne, indirect, hormonally mediated effects of E_2 (by estromedins) may play important contributory roles *in vivo* (51). Alternatively, growth factors with a similar spectrum of activities could be elaborated by the breast cancer cells themselves. In the nude (athymic) mouse model system, E_2 is an absolute tumor-growth requirement for two human cell lines, MCF-7 and T47D, and a growth stimulator for a third cell line, ZR-75-1 (18,35,52). McGrath and his colleagues have further defined this system by showing that E_2 need not enter the systemic circulation in nude mice to promote sufficient MCF-7 tumorigenesis; elevation of local E_2 concentration near the tumor was adequate (53). This suggests that although estrogens might be needed to induce a host of modulatory factors (such as estromedins) required by the tumor or host for tumorigenesis, the production and action of such factors is probably restricted to the local area of the tumor. *In vivo*, mammary stroma is likely to provide factor(s), as yet unidentified, that contribute to full mitogenicity of estrogen (54). Other human breast cancer cell lines, such as Hs578T and MDA-MB-231, lack estrogen receptors and form rapidly growing, estrogen-independent tumors in the nude mouse (9,52,55). These five human cell lines (MCF-7, ZR-75-1, T47D, Hs578T, MDA-MB-231) are being studied in detail by our laboratory in an attempt to understand better growth regulation of breast cancer *in vitro* and *in vivo*.

IV. ESTROGEN-MODULATED GROWTH FACTOR ACTIVITIES - AUTOSTIMULATING FUNCTIONS

Our attention was drawn to the possible involvement of secreted growth factors in growth control of breast cancer by an observation made with MCF-7 cells plated at various densities. We found that initial growth rate was proportional to number of cells plated (56). While multiple interpretations of these data are possible, they are consistent with the production of autostimulatory growth factors by the MCF-7 cells. In preliminary experiments we found that conditioned medium harvested from MCF-7 cells treated with E_2 (CM-E_2) was capable of stimulating thymidine incorporation and proliferation of other MCF-7 cells (57). In contrast, conditioned medium prepared without E_2 (CM) was much less effective. Residual E_2 was removed from CM-E_2 prior to analyses. This kind of result had also been obtained by Vignon and Rochefort and their colleagues (58) who had noticed that MCF-7 cells grew faster when medium was changed less frequently, as compared to cells for which medium was changed every other day. They had also noted that CM-E_2 was directly capable of stimulating other MCF-7 cells.

We wished to determine if proteins in the conditioned medium were capable of acting humorally *in vivo* in the nude mouse to stimulate MCF-7 tumorigenesis (59). For this purpose a serum-free culture system was developed which supports cell growth for all five of the above mentioned cell lines for up to one week. The medium consists of Richter's IMEM + 2 mg/l transferrin + 2 mg/l fibronection. MCF-7 cells, $\pm$ E_2 pretreatment (10^{-9} M, 4 days), were used to condition the serum-free medium, which was collected over a subsequent 2-day period (CM and CM-E_2). Media were dialyzed extensively against 1 M acetic acid, lyophilized, reconstituted in phosphate-buffered saline and precipitated protein was removed. This extraction also removed 99.98% of the residual E_2. Reconstituted CM or CM-E_2 was infused into athymic female oophorectomized mice via Alzet minipumps. The equivalent of 10 ml/per day of CM or CME_2 (concentrated to 20 ul) was infused for 4 weeks from a mid-dorsal, subcutaneous location. MCF-7 cells were injected (2 to 5 x 10^6 cells/injection) at 4 different mammary fat pad locations in each mouse. Small tumors (up to 0.5 cm diameter) appeared at MCF-7 sites within 2 weeks. Tumors in CM-E_2-infused animals appeared with 2- to 3-fold greater frequency than in CM-infused animals; animals inoculated with only MCF-7 cells and sham pump infusions did not have tumors. CM and CM-E_2-supported tumors reached maximum size in 2 to 3 weeks of treatment, usually declining in size thereafter. Positive controls, i.e., E_2-pellet-implanted animals, had continuously growing tumors for up to 4 weeks. CM- and CM-E_2-induced tumors were verified as adenocarcinomas by histologic analysis. While the CM-E_2 supported tumor growth, uterine weight was unaffected. In addition, CM-E_2 activity was decreased by treatment with trypsin or a reducing reagent, or by heating to 56°C for 1 hour. Therefore the tumor-growth-promoting substance(s) in CM-E_2 was unlike E_2, and likely to be similar to a polypeptide growth factor(s). These data suggest that cultured human breast cancer cells under estrogenic stimulation release a tumor-promoting factor(s) which can act *in vivo* after release into the general circulation of the athymic mouse.

A. Identification of Transforming Growth Factors, IGF-I and PDGF

Fractionation of conditional medium from MCF-7 and other breast cancer cell lines was then undertaken to identify the growth factors present. These cell lines secrete stimulatory activity for MCF-7 and 3T3 fibroblas monolayer cultures as well as "transforming growth activity" (TGF) for anchorage-independent growth of NRK and AKR-2B fibroblasts in soft agar culture (52,57,60,61). In initial studies using acid Biogel P60 and P150 chromatography, we identified a 30 kd (apparent molecular weight) peak of transforming activity for NRK fibroblasts. This peak also coincided with a

peak of growth-stimulating activity for MCF-7 cells, as well as the predominant species of EGF-receptor competing activity (52,57). Thus, this activity may be related to TGFα, but it appears to be larger than the cloned and sequenced 6 kd species from transformed fibroblasts (62). The 30 kd, TGFα-like species is induced in conditioned medium by E_2 pretreatment of MCF-7, T47D, and ZR-75-1 cells; the concentration increases 2- to 8-fold, depending on cell type and culture conditions. Current experiments are focused on regulation, purification and characterization of this activity. The expected 4.8 kilobase (kb) TGFα-mRNA species has been detected by Derynck and coworkers in MCF-7 and some other human breast cancer cell lines (62). It is of interest that some, but not all, estrogen-independent breast cancer cells secrete high levels of the TGFα-like activity (52).

Using radioimmunoassay, we and others have also noted that a second potential autostimulatory mitogen, IGF-I is also secreted by all human breast cancer cells examined to date (63,64). This species, partially purified from MCF-7 cells, comigrates with authentic serum-derived IGF-I after acid-ethanol extraction. A 600 base-pair mRNA species was also detected with Northern blot analysis using a DNA probe to authentic IGF-I (65). In the same study, mRNA corresponded to the smallest of three RNA transcripts observed in poly A-selected RNA from human liver. Initial studies indicated no E_2 induction of secreted IGF-I in standard culture conditions employing phenol red-containing medium. Subsequent studies, utilizing the more substantially estrogen-depleted (phenol red-free) medium, have shown a 2- to 3-fold induction of IGF-I with E_2 treatment (66). IGF-I secretion is inhibited by the growth-inhibitory antiestrogens (in phenol red-containing medium) and by glucocorticoids. Current work is focussed on the mechanism of IGF-I induction and its possible biological role(s). Interestingly, two highly malignant estrogen-receptor-negative breast cancer cell lines (MDA-MB-231 and H5-578T) secrete high levels of IGF-I and have low responsiveness to exogenous IGF-I (63).

All breast cancer cells examined to date also secrete a TGFβ-related activity (61,67). In acid Biogel chromatography a major peak of radioreceptor-competing, and AKR-2B fibroblast transforming, activity comigrates with authentic platelet-derived TGF-β. In contrast to its transforming effects on some fibroblasts, authentic TGFβ is growth-inhibitory for many breast cancer (and other epithelial-derived) cell lines (46,47,61). All breast cancer cells examined expressed the expected 2.5 kb mRNA species. Interestingly, TGFβ secretion is inhibited by treatment of MCF-7 cells with growth-stimulatory E_2 or insulin. Growth-inhibitory antiestrogens and glucocorticoids strongly stimulate its secretion. Intracellular TGFβ does not appear to be modulated. TGFβ from antiestrogen-induced MCF-7 cells strongly inhibits the growth of another estrogen receptor negative cell line, MDA-MB-231. This growth inhibitor was reversed in the presence of a polyclonal antibody directed against native TGFβ. Interestingly, in the antiestrogen-resistant but not TGFβ resistant, MCF-7 variant LY2, antiestrogens do not significantly induce TGFβ secretion. Current work is further addressing the mechanism of TGFβ regulation (68).

Finally, all breast cancer cell lines examined to date secrete a PDGF-related activity, detected by anchorage-dependent growth stimulation of 3T3 fibroblasts in the presence of platelet-poor plasma (69,70). Immunoprecipitation of metabolically labeled MDA-MB-231 breast cancer cell extracts and medium detected the expected 28 kd and 14 kd species. Northern blot analysis of poly A-selected RNA from breast cancer cells yielded the expected 4.2 kb mRNA transcript using a c-sis probe (69). Current research is focused on determining if the PDGF-like species is regulated and what its function might be. Human breast cancer cells are not known to be growth regulated by PDGF. Rather, its role might be paracrine in nature. Interestingly, the

highly tumorigenic MDA-MB-231 cell line produces the most PDGF of the cell lines examined so far (69).

B. Identification of a Novel Anchorage-Independent Epithelial Growth Factor

In contrast to fibroblasts, the hormonal controls over the cell cycle in epithelial cells are only poorly understood. While it is known that EGF and IGF-I are commonly mitogenic and TGFβ commonly growth inhibitory for epithelial cells, the restriction points in the cell cycle where these growth factors might act are largely unknown. In addition, the controls governing anchorage-independent growth are also largely unexplored. Halper and Moses (48) have established a model system with human SW-13 adrenal carcinoma cells in soft agar culture. These cells clone poorly unless basic FGF or conditioned medium from certain epithelial cancers is applied. No other growth factors are known to be active. This activity has been only partially characterized from kidney but appears to be 40-42 kd in size.

We have begun the purification of a related activity from human breast cancer cells (71). The most tumorigenic lines, MDA-MB-231 and Hs578T, produce high levels of the activity, while estrogen-receptor containing lines produce much lower levels. The activity from MDA-MB-231 cells has a very acidic isoelectric point, and is approximately 60 kd in size by gel filtration and gel electrophoresis. It has been purified to near homogeneity by an acid-ethanol extraction, isolectric focussing, and HPLC sizing. Current work is directed toward complete purification and characterization of this activity and examination of its regulation (71).

In summary, our observations are that estrogen regulation of MCF-7 cells is coupled to inductions of TGFα and IGF-I and repression of TGFβ. Two other growth factors are also secreted, PDGF and a partially characterized epithelial transforming factor. These two activities (sometimes in association with high levels TGFα, TGFβ, and /or IGF-I) are produced in very large amounts by estrogen-receptor-negative, highly tumorigenic lines. It is possible that estrogen - antiestrogen regulation of MCF-7 cells is at least partly mediated by coordinate effects on growth-stimulatory and growth-inhibitory factors, "second messengers". Future studies with blocking antibodies against growth factors and against these receptors should help to evaluate this hypothesis. Estrogen-independent cancers are associated with increased output of a large number of growth factor activities.

VII. IN VIVO TESTS OF GROWTH FACTOR ACTIONS

As an initial step in evaluation of possible autoregulatory growth factors, MCF-7 cells have been grown as xenografts in the nude mouse. As previously mentioned, conditioned medium from E_2-treated MCF-7 cells stimulate limited growth of MCF-7 tumor in the absence of E_2 itself. As a test of the hypothesis that E_2-induced growth factors may mediate this effect, we have directly infused human EGF (1 μg/day) and/or human IGF-I (0.6 μg/day) into female, oophorectomized nude mice injected at four mammary fat pad locations with MCF-7 cells (2 to 5 x 10^6 cells/injection site. These concentrations corresponded to those observed in the conditioned medium utilized in the previous studies. As before, growth factors were infused with Alzet minipumps, and the experiment was carried out for 2 weeks. Both growth factors induced tumors, but EGF induced more than twice as many tumors as IGF-I. EGF supported development of tumors up to 0.5 cm in diameter. As expected, E_2-pellet-implanted control animals had a high incidence of continously growing tumors up to 0.8 cm in diameter, over the time period of the experiment (59). Thus, based on these experiments with authentic growth factors, it is likely that growth factors closely related to IGF-I and TGFα are produced by cells and may have some autostimulatory actions on

tumor growth in vivo. In addition, the EGF-related induction of TGFα by E_2 may be relevant in E_2 may be relevant in E_2-stimulated tumor growth. Greater availability of TGFα, TGFβ, PDGF, and epithelial transforming activity in the future should facilitate the testing of these activities in this in vivo reconstitution system.

In independent studies (72) with mouse mammary carcinogenesis, Oka and coworkers have recently demonstrated a likely role of EGF in both mammary tumor onset and subsequent growth support. Using a mouse strain highly susceptible to spontaneous mammary tumors, removal of the submandibular glands (sialoadenectomy) dramatically reduced the incidence of tumor formation and/or the rate of growth of the breast tumors to form. The submandibular gland is a major source of EGF in mammals and reinfusion of EGF into such sialoadenectomized mice restored incidence and growth rate of tumors to their normally high level. TGFα- and EGF-like activities may have endocrine functions in tumor support. As the data with MCF-7 cells shows, one mechanism of tumor progression might involve local production of TGFα by the tumor (but still under estrogen control). Clearly, TGFα- or EGF-like growth factors may well be important regulators of mammary tumor progression, by a variety of possible mechanisms.

VIII. POSSIBLE PARACRINE AND OTHER FUNCTIONS OF GROWTH FACTORS

At least some of the growth factor products of breast cancer cells appear related to growth factors in milk (73), e.g., TGFα. This and other factors are treated in the chapter by Salomon and Kidwell in this book. The function of such factors may be related to offspring growth rather than to parental mammary growth; e.g., TGFα (and EGF) can promote eyelid opening in mice (74). Though growth factors such as IGF-I and TGFα may be capable of autocrine stimulation of tumors, they and other growth factors may have paracrine effects on extracarcinoma tissue. PDGF promotes fibroblast growth and chemotaxis, and its secretion may contribute to the marked stromal proliferation characteristically surrounding breast carcinoma (75). In addition, TGFα and TGFβ stimulate bone resorption and hypercalcemia, also characteristic of breast cancer (76). Other paracrine effects of growth factors might be immunomodulatory in nature. Finally, potentially the most important paracrine function of factors secreted by cancer cells is angiogenesis. Though many activities may contribute to this phenomenon, both growth factors and proteolytic degredation products of basement membranes are likely candidates (77-79). The factors secreted by breast cancer cells leading to vascular infiltration of the tumor have not yet been identified. However, Vallee and coworkers have recently isolated, sequenced, and cloned an angiogenic protein secreted by human colon carcinoma cells (80).

IX. MALIGNANT PROGRESSION OF BREAST CANCER FROM ESTROGEN DEPENDENCE TO INDEPENDENCE - ROLE OF ONCOGENES?

Recent studies in rodent systems have implicated specific genetic alterations leading to malignant transformation and tumor progression. In the mouse, mouse mammary tumor virus (MMTV) inserts itself into the genome at specific sites and generally induces expression of at least two cellular genes (81). In the rat carcinogenesis model system, activation of the oncogene known as Harvey ras ($c\text{-}ras^H$) occurs by point mutation (82). At the present time, no such unifying statements can be made about human breast cancer. Rather, diverse observations of oncogene activation suggest a plethora of mechanisms at work in malignant progression. In one human breast cancer cell line, Hs578T, an activated $c\text{-}ras^H$ oncogene has been observed, as predicted based on the rat model system. However this potential mechanism appears far from universal (83). Second, a whole series of cellular proto-oncogenes are observed to be expressed in diverse studies employing cell lines and tumor specimens (84,85). These oncogenes (all mem-

bers of the ras family, myc, myb, fms, fos, fes) include ones localized in plasma membrane, nucleus, and cytoplasm. Two other oncogenes cErb B and neu (or cErb B_2) are both closely related to the EGF receptor and have also been detected in breast cancer cell lines and tumor biopsies (85,86). Interestingly, cErb B (the EGF receptor) is expressed to the greatest extent in estrogen-receptor-negative cell lines and in tumor biopsies (87,88). It may represent a new marker for dedifferentiation or malignancy in breast cancer. Recent studies have suggested that a mechanism for its high level of expression is at the transcriptional level. It is not yet known whether overexpression of the cErb B in cancer directly contributes to the transformed phenotype or indirectly mediates the effects of EGF (or TGFα) produced in an autocrine-type loop. Finally, as previously mentioned, PDGF, the product of the c-sis proto-oncogene, is expressed by a variety of breast cancer cell lines (69,70). Though PDGF itself is not generally growth stimulatory for epithelial cells, it may contribute in other ways to the transformed phenotype (such as through paracrine actions). It is possible that additional oncogene activities will be observed in breast cancer using different techniques in the future. One such possibility is that an epithelial cell test system will detect transforming genes which go unrecognized by the well established NIH 3T3 fibroblast test system.

One interpretation of the diversity in observations of activated oncogenes and expressed cellular proto-oncogenes is that many mechanisms or steps exit in the malignant progression of breast cancer. Alternatively, observations of expression of some of these cellular proto-oncogenes could reflect rather than induce, malignant status. Clearly, to test hypotheses concerning oncogene activity in breast cancer it is necessary to directly insert the oncogene of interest into a relevant cell test system. This objective has been recently achieved using normal diploid human mammary epithelion first immortalized with brief benzo(a)pyrene treatment and then transferred with oncogenes (89,90). Stampfer has observed that treatment of normal mammary epithelial cells in culture with benzo(a)pyrene achieved immortalized but non-tumorigenic lines (89). These lines appear nearly normal by several criteria. Subsequently, Clark has inserted, using retroviral vectors, various oncogenes into one of these lines to determine the phenotype effects (90). Insertion of v-ras^H, v-mos, and SV40 T antigen rendered the cells capable of growth in high levels of serum, but did not confer tumorigenicity. However, transfectants containing SV40 T plus either v-ras or v-mos were strongly tumorigenic in nude mice.

The next section addresses the activity of v-ras^H oncogene in MCF-7 cells. In this model system, effects on malignant status have been systematically observed and assessment has been made of which growth factor changes are associated with the changed phenotype. This type of test has not yet been carried out for other oncogenes of possible importance in human breast cancer.

X. RAS ONCOGENE AND ESTROGEN INDEPENDENCE: A MODEL SYSTEM

As previously mentioned, the differences between E_2-responsive and E_2-autonomous breast cancer have been of major interest to our group. In many cases, breast cancer patients present initially as responsive to hormonal (tamoxifen) therapy. Following extended treatment, the breast cancer may become hormone-unresponsive. A model system was needed to study the conversion from hormone-responsiveness to hormone-independence. For this purpose, we chose to transfer DNA from the tumor-causing, Harvey sarcoma retrovirus to MCF-7 cells. The tumor-inducing portion of this viral DNA (the oncogene) is called v-ras^H, and is closely related to the most commonly detected activated oncogene in some highly malignant human cancers. MCF-7 cells did not initially contain this oncogene, but one estrogen-independent cell line did:

Hs578T (83). The v-ras^H oncogene was transferred cells by the calcium phosphate method (91).

Stably transfected MCF-7 cells (MCF-7$_{ras}$) integrated into their DNA several copies of the v-ras^H gene, had five to eight times the level of ras mRNA as did control cells, and had detectable phosphorylated p21 (the protein which is the ras gene product). The cellular p21 is not a substrate for phosphorylation. MCF-7$_{ras}$ cells displayed unaltered growth rate under control conditions in vitro but had resistance to growth inhibition by antiestrogens. The transfected cells were tumorigenic, in the absence of estrogen, in 85% of inoculated female, oophorectomized nude mice (91). Interestingly, the MCF-7$_{ras}$ cells also exhibited increased rates of turnover of phosphatidyl inositol, analogous to the effect of E_2 treatment of MCF-7 cells (14). In addition, these cells also expressed increased levels of the laminin receptor on their surfaces (30).

We next examined growth factors secreted by these MCF-7 cells. CM prepared from MCF-7$_{ras}$ cultures, as compared with that from control cultures, contained 3- to 4-fold elevated levels of radioreceptor-assayable TGFα and bioactive TGFα as assayed by anchorage-independent growth of NRK fibroblasts. A single peak of TGFα-like activity with an apparent molecular weight of 30 kd was eluted from acid gel chromatography of MCF-7$_{ras}$ CM. Also, secretion of immunoreactive IGF-I and TGFβ were augmented 3- to 4-fold in MCF$_{ras}$ cells, but PDGF was not elevated. These growth factors appear to be biologically active in vivo. Tumors grown from MCF-7$_{ras}$ cells in the nude mouse were associated with the development of small tumors derived from MCF-7 cells separately implanted at a distant site in the nude mouse (92). Ras oncogene activation could bring about phenotypic and tumorigenic changes in human breast cancer cells, some which may also be induced by estrogens. However, the cells retained the capacity to bind estrogen and respond to estrogens, as shown by E_2 induction of the progesterone receptor. Thus ras oncogene transfection bypasses estrogen activation of the transformed phenotype, but induces that phenotype via a pathway that appears to be similar, but not identical, to the E_2 induction pathway. Future studies will more clearly delineate the similarities and differences between E_2- and v-ras^H-induced malignant progression of MCF-7 cells.

XI. CONCLUSION

In conclusion, evidence has been presented in this review for involvement of TGFα (EGF-like), IGF-I, and TGFβ-related growth factors in E_2 induction of E_2-receptor-containing breast cancer growth. Elevated levels of these and other growth factors have also been observed in E_2-receptor-negative, highly tumorigenic cell lines. Purified EGF has been found to be capable of partially replacing E_2 as tumor growth stimulator in vivo in the nude mouse, and is therefore likely to play a role in vivo as an autostimulatory tumor growth factor. As an independent test of association with of growth factor secretion with tumorigenesis, a cell line has been rendered E_2-autonomous, and more tumorigenic, by DNA-mediated transfection of the v-ras^H oncogene. Increased TGFα and IGF-I secretion were observed in this line confirming their close association with malignant status. It is anticipated that future studies will define the mechanisms of growth factor regulation; attempts can then be made to attenuate malignant status by interfering with their action.

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GROWTH FACTOR PRODUCTION BY MAMMARY TUMOR CELLS

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I. INTRODUCTION

Much attention has recently been focused on the possibility that the behavior of tumorigenic cells (anchorage independent growth, loss of growth factor dependency, loss of contact inhibition, etc.) are caused by changes in the control of expression of growth factors and/or their receptors (1,2). Indeed, the transforming genes of a number of tumor cells and viruses have been identified as genes for growth factors or receptors (1,2). Our aim here is not to present a complete review of this area, but rather to compile the published data from our lab and others on the types of growth factors that have been detected in primary and established mammary cell lines and in extracts of normal and neoplastic mammary epithelium from rodent and human tissues. The list is quite long, even for human mammary tumors and tumor cell lines. It includes mammary-derived growth factor I (MDGFI), mammary-derived growth factor II (MDGFII), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), insulin-like growth factor I (IGFI),

platelet-derived growth factor (PDGF), gastrin-releasing peptide (GRP), 52 K protein, epidermal growth factor (EGF), and human tumor growth factor (h.TGF). Rodent mammary tumors contain these factors and some additional ones as well. For example, 7,12-dimethylbenz(a)anthracene-induced rat mammary cell lines produce an activity that greatly stimulates myoepithelial, but not epithelial, cell growth. Rat mammary tumors also contain a growth inhibitory activity similar to one that Grosse's laboratory has purified from bovine mammary tissue (3).

The presence of so many types of growth factors in mammary tumors or cells could mean one of several things. The factors might be produced ectopically. This may or may not play a role in tumor growth or progression. The factors might represent secretory products of the normal lactating mammary gland; most of the growth factor activities found in tumor tissues or in cell lines established from it, are present in substantial amounts in human milk (4). Regarding the potential physiological and/or pathophysiological role of tumor-associated growth factors, it will be important to determine whether their production is amplified by mammotrophic hormones and whether antibodies against the factors affect normal or neoplastic mammary epithelial cell growth in culture.

II. ANCHORAGE-INDEPENDENT GROWTH-CONFERRING ACTIVITIES DERIVED FROM MAMMARY TUMORS OR TUMOR CELLS

There are at least five anchorage-independent growth promoting activities that have been detected in human and rodent mammary tumors or tumor cell lines to date. The first was an Mr 68,000 acidic protein (pI 5.2) isolated by Zwiebel et al. (5) from primary, well differentiated rat mammary tumors. A factor which is possibly the human counterpart was recently described by Swain et al. (6). This group of factors also includes TGFα, TGFβ, IGFI, and MDGFII, and EGF, all of which have recently been found in human mammary tumors and human milk. Current information on each factor will be considered.

A. Mammary Tumor Factor (MTF) and Swain Factor

Todaro's lab first detected anchorage-independent growth inducing factors (SGFs) in the culture medium of sarcoma virus transformed cells (7). Following that report Zwiebel et al. (5) assessed the possibility that SGF-like factors were also produced by carcinogen-induced rat mammary tumors. Two factors that, like SGF (or the TGFα component of SGF) compete for epidermal growth factor receptor binding, were found in primary, nitrosomethylurea (NMU) and DMBA-induced tumors (5). The factors were extracted into acidified ethanol and precipitated with ether. Molecular sieve chromatography indicated the presence of two major species, a Mr 68,000 acidic protein and a Mr 6000 protein that was active in stimulating the growth of normal rat kidney (NRK), 3T3 and chick embryo fibroblast cells in soft agar. The low molecular weight species appears to be authentic TGFα since it competes with chemically synthesized TGFα for binding to antibodies against TGFα. The Mr 68,000 species was shown to be protease and heat sensitive, properties not dissimilar to another anchorage-independent growth-conferring factor isolated by Swain et al. (6) from HS578T and MDA MB 231 human mammary carcinoma cell conditioned medium. The Swain factor is a heat and protease sensitive, acidic protein (pI = 4.2-4.5) with an Mr of 62,000. Swain et al. (6) have purified their factor to apparent homogeneity as judged by electrophoresis on SDS-acrylamide gels. At present little is know about the abundance of Swain Factor in tumors. Interestingly, MTF appears to be present in much higher amounts in well differentiated rat mammary tumors than in poorly differentiated ones (5,8).

B. TGFα, TGFβ and EGF

TGFα and TGFβ are separate growth factors that were resolved from crude preparations of sarcoma growth factor, or SGF (9). TGFα is a slightly acidic (pI 6.5) peptide having a Mr of approximately 6000. It competes with EGF for EGF receptor binding (7) and, along with TGFβ, greatly stimulates the anchorage-independent growth of a variety of cell types, especially normal rat kidney (NRK) cells (7). In most assays, TGFα and EGF are interchangeable, exceptions being that TGFα is more potent than EGF in promoting re-epithelization of burn wounds (10), as well as in facilitating bone resorption (11) and angiogenesis (12).

Two groups have shown that TGFα-like factors are synthesized by human mammary tumors and tumor cell lines. We have found that about 50% of human mammary tumors possess readily detectable TGFα mRNA (13). In general, the mRNA positive tumors also contain estrogen and progesterone receptors, whereas the mRNA negative tumors usually lack steroid receptors (13). Not only is the mRNA detectable, it is also transcribed. Using a bioassay (anchorage independent growth assay), or a radioimmunoassay with anti- TGFα antiserum, TGFα was found in mammary tumor cell-conditioned growth medium and in acid-ethanol extracts of freshly isolated human breast tumor samples (14). Lippman's group (15) demonstrated, using bioassays, that estrogen enhanced the release by MCF-7 cells of TGFα into the growth medium. We confirmed this finding and additionally, showed that normal mammary ductal and alveolar cells from reduction mammoplasty samples also elaborate TGFα in culture (14). These facts, plus the observation that ovariectomy results in a decrease in TGFα mRNA levels in rodent mammary tumors (as demonstrated in Northern hybridization experiments (16) makes it probable that TGFα production is normally regulated by ovarian steroids. It has been suggested that this is the mechanism by which estrogens effect growth stimulation of mammary cells (15).

TGFβ is a basic protein composed of two identical peptide chains that are linked by disulfide bridges. TGFα and TGFβ have both been cloned and their sequences determined (7,18). We have assessed the production of TGFβ by normal and malignant human mammary epithelial cells grown in serum-free medium. Production rates were about 20 ng/ml/10^5 cells/day for either cell type (4). Recent experiments by Sporn's and Moses' groups (19,20,21) have raised the possibility that TGFβ is a negative regulator of cell growth since the factor has been shown to slow the growth of a variety of cell types, including human mammary cell lines. These actions of TGFβ are variably counteracted by TGFα or EGF, depending upon the growth conditions and cell type. Thus TGFβ is a growth promoter for NRK cells in soft agar cultures if TGFα (or EGF) is also present. At any rate, since, according to our findings, TGFβ production by normal and neoplastic cells is about equal, a major growth regulatory role of the factor might be dependent on whether TGFα or EGF are also produced. It might also be dependent on whether binding proteins for TGFα or TGFβ are expressed by the cells, since binding proteins usually attenuate the potency of growth factors. Preliminary evidence that TGFβ binding proteins might act in this manner has been presented (22).

Epidermal growth factor, or EGF, is a small, acidic peptide that is derived from a precursor protein having a Mr of about 128,000 (23,24). The corresponding mRNA for the preproEGF is 4.8 Kb long. EGF and TGFα share the property of being derived from very large precursors (17). Recently we demonstrated that mouse mammary epithelium derived from normal or tumor tissues contains a 5 Kb mRNA species that hybridizes with a cDNA containing the coding sequence for mouse EGF (25). Additionally, we found that antibodies against mouse EGF suppress the growth of both normal and neoplastic mammary epithelium in primary cultures (25). More recently, EGF production by human mammary cell lines has been reported (26). Also, the fluid from human

breast cysts has been shown to contain very large amounts of EGF,(27) levels that approach those found in human colostrum (28). The regulation of EGF production by mammary cells remains to be elucidated. Unlike our observations with TGFα, we did not find any effect of ovariectomy on the levels of EGF mRNA expression in the normal mouse mammary gland, nor could we induce EGF mRNA expression by the administration of estradiol (unpublished observations). Nevertheless, it is tempting to postulate that the production of EGF varies depending on the physiological state of the gland. Cells that are producing significant amounts of an autocrine (self-stimulating) growth factor are generally unresponsive to the same factor added to culture medium. Is it possible that the changing sensitivity to EGF of cultured normal mouse mammary cells in various physiological states, as reported by Nandi's lab (29), is due to differences in EGF (or TGFα) production?

C. Mammary-Derived Growth Factor II (MDGFII)

MDGFII has been included in this group of group factors, not only because it stimulates the anchorage-independent growth of various cells (3T3, chick embryo fibroblasts, mouse mammary tumor cells, MCF-7 cells) but also because it brings about its biological effects via interaction with the EGF receptor system (30). MDGFII has been purified about 10,000 fold from human milk following fractionation by isoelectric focusing, reverse phase and gel permeation chromatography (30). The factor is inactivated by protease digestion or treatment with disulfide reducing agents. Chromatography on sizing gels under high ionic strength conditions reveals a Mr of 17,000. At low ionic strength, the protein co-elutes with authentic human EGF, indicating a size of approximately 6000 (30). However, human EGF and MDGFII are completely resolved by chromatography by reverse phase HPLC on C_{18} columns, using trifluoroacetic acid in a gradient of acetonitrile (30). Furthermore, MDGFII activity in a soft agar growth assay is not neutralized by adding an excess of anti-human EGF monoclonal antibody (30). The possibility exists that MDGFII is derived from preproEGF since that protein contains about 7 sequences that are EGF-like. These peptide sequences can be folded via disulfide bonding to give structures with internal loops similar to those in mature EGF and TGFα (23,24). Human mammary tumors apparently contain MDGFII. Acid-ethanol extracts of human mammary tumor biopsy material have been fractionated on isoelectric focusing columns and the resultant fractions analyzed in radioreceptor and in soft agar growth bioassays. Material positive in both assays were found to focus at a pH of 4-4.2, identically with the MDGFII of human milk. These activities, like those in milk, were heat insensitive but were inactivated by dithiothreitol or pepsin treatments (30). Compared to the activity in milk, that of the tumors was considerably lower.

D. Insulin-Like Growth Factor I (IGFI)

Insulin-like Growth Factor I, or IGFI, is a single peptide growth factor that is folded by disulfide bridges. The factor has a Mr of about 7000 and a pI of 8.4 (31). A slightly shorter, very acidic, form has also been detected (32). IGFI acts on cells primarily via its own receptor, but also, to some extent, via the insulin receptor. Using specific radioimmunoassays, two groups have identified IGFI in the medium in which human mammary tumor cell lines have been cultured (33,34). Using similar methods and reagents, we failed to detect the factor in conditioned medium in which normal human mammary cells had been grown (19). Presumably, expression of this factor is related to the neoplastic state of the cells. Because the concentration of IGFI in milk is very low compared to that in serum (35), it has been proposed that IGFI is not a product of the normal lactating gland, but rather, that it is derived from the circulation (19). IGFI has been considered in the present section because it is apparently essential for stimulating the

anchorage independent growth of NRK cells in soft agar (36), although IGFI is normally not considered to be a transforming growth factor.

III. MDGFI AND CSSF: FACTORS THAT STIMULATE EXTRACELLULAR MATRIX BIOSYNTHESIS

MDGFI and CSSF were purified from human and rat mammary tumors, respectively (37,38). Because of their similar biological properties they are considered together here. In the ng/ml concentration range MDGFI and CSSF differentially stimulate the biosynthesis of collagen and other extracellular matrix proteins by as much as ten fold. As indicated below, there is reason to believe that this action is physiologically important for the growth of the normal mammary gland and of some tumors.

A. Mammary-Derived Growth Factor I (MDGFI)

MDGFI action on mammary cells has been extensively investigated. It interacts with high affinity membrane receptors on mammary and other cell types (Kd = 10^{-10}). Within 15 minutes, it causes an increase in collagen biosynthesis, an effect that is accompanied by a comparable increase in collagen mRNA expression (38). Preliminary date indicate that MDGFI is synthesized by mammary epithelial cells. Conditioned media from primary cultures of normal and malignant human mammary epithelium have been shown to inhibit the binding of ^{125}I-MDGFI to its receptors (4). MDGFI is large in size for growth factors, having an Mr of 62,000. Like MDGFII and the factor purified by Swain et al. (6) MDGFI is a highly acidic protein (pI 4.8). Its activity is relatively heat stable and it is not inactivated by disulfide bond reaction. A limited digestion with pronase completely destroys its biological activity. A unique characteristic of MDGFI is that its ability to stimulate the proliferation and basement membrane protein synthesis in primary cultures of normal mammary cells is dependent on the substratum on which the cells are plated. On plastic or type I collagen surfaces, a very great stimulation is seen, whereas on type IV collagen there is almost no stimulation. These results have led to the postulate that type IV collagen, the collagen type found in basement membranes, is a negative feed back regulator of its own biosynthesis. Data consistent with this thesis are presented below in discussions of CSSF, the rat equivalent of human MDGFI.

Thus far, four primary human mammary tumor samples have been analyzed for the presence of MDGFI. All have been positive. Additionally, using radioreceptor assays with ^{125}I-MDGFI and mammary epithelial cell membrane preparations, we have demonstrated that both normal and malignant human mammary epithelium in primary culture release substance(s) into the medium which competes with labeled MDGFI for receptor binding (4). On a per cell basis, the tumor cells produce about three times as much of this receptor binding competing activity as do the normal cells.

Two other facts are worthy of mention. First, fibroblasts contain MDGFI receptors on their cell membranes and they respond to MDGFI by increasing their collagen biosynthetic machinery (i.e., their collagen I mRNA levels). However, unlike normal mammary epithelial cells in primary culture, the division of fibroblastic cells is not stimulated by MDGFI. Second, even normal mammary epithelial cells do not respond to MDFGI in the absence of estrogens. Thus, when female mice were ovariectomized and several days later mammary cells were isolated and tested for MDGFI responsiveness, no growth stimulation was seen. Responsiveness to MDGFI was restored by implanting an estrogen pellet at the time of ovariectomy (37).

B. Collagen Synthesis Stimulating Factor (CSSF)

Collagen Synthesis Stimulating Factor, or CSSF, appears to be the rat counterpart of MDGFI, as mentioned. This factor was detected in acid-ethanol extracts of DMBA and NMU-induced, primary mammary tumors (38). It is a Mr 68,000 protein whose activity is relatively heat stable, but destroyed by pepsin. It has been highly purified in small amounts by isoelectric focusing (pI 5.9), gel permeation HPLC chromatography and finally, by gel electrophoresis. Like MDGFI, CSSF was found to be very efficient in differentially stimulating labeled proline incorporation into protease-free, collagenase-sensitive protein (i.e., collagen) in cultures of normal mouse mammary, NRK and 3T3 cells. As was true for MDGFI, CSSF does not produce this effect if normal mammary cells are grown on basement membrane collagen-coated dishes.

The distribution of CSSF in a variety of rat mammary tumors has been investigated. The results suggest that CSSF may be an autocrine factor that is made by the mammary epithelium (normal mammary cells and the well differentiated tumors derived from it) and that it regulates basement membrane production. The tumor types expressing the activity, as well as the fact that CSSF action is suppressed in mammary cells growing on basement membrane collagen, supports this hypothesis. CSSF abundance was assessed in two types of primary rat mammary tumors (DMBA and NMU-induced) as well as in four transplantable rat mammary tumors, T-DMBA, T-NMU, MTW9 and MTW9a. The abundance of CSSF (as judged by the amount of Mr 68,000 protein recovered after purification) was compared to the amount of basement membrane present in the tumors. The T-DMBA, T-NMU and MTW9a tumors lacked both the growth factor and basement membranes. The primary tumors were strongly positive for both. The MTW9 tumor was weakly positive for CSSF protein and for basement membrane presence (38).

These observations provide the strongest available proof that CSSF and, by analogy, MDGFI act to regulate basement membrane synthesis in vivo. Because synthesis of basement membranes appears to be essential for the growth and survival of normal mammary epithelium and of well differentiated mammary tumors as well, the factors would appear to be important physiological regulators of proliferation in these types of tissues (37,38,39,40,41, 42). It is also interesting that the transplantable tumors that don't make CSSF or basement membranes are metastatic, unlike the primary tumors or the MTW9 tumor. Other groups have suggested that the basement membrane serves as a limiting barrier against invasion and metastasis (43). Several changes thus characterize the T-NMU and T-DMBA tumors. They are not sensitive to proline analogs that block basement membrane deposition. They are metastatic and they do not produce growth factors that autostimulate the tumor cells to make basement membranes (44). The key question is whether changes in growth factor production are involved mechanistically in the other changes that occur as the tumors undergo progression to their most virulent state, metastasis.

As mentioned earlier, the evidence from ovariectomy studies and from comparisons of transplantable vs primary tumors suggests that TGFα levels are hormonally regulated, being highest in those tumors (human or rat) that possess estrogen and progesterone receptors. TGFα is like CSSF and MDGFI in its distribution and ability to stimulate collagen synthesis.

IV. OTHER WELL CHARACTERIZED GROWTH FACTORS FROM MAMMARY TUMORS OR MAMMARY TUMOR CELL LINES

This group of factors includes 52 K protein, gastrin releasing peptide (GRP), platelet-derived growth factor (PDGF), mammary cell growth inhibitor (MCGI) and h.MTGF. These factors have either been purified to homogeneity,

and their presence confirmed in mammary cells by specific radioimmunoassay, or Northern blot hybridizations have shown that cells or tumor tissues contain growth factor mRNA.

A. 52 K Protein

In attempting to identify the mechanism whereby estrogens stimulate MCF-7 cell growth, Rochefort's group found that the steroids cause the cells to release glycoproteins into the culture medium (45). One of these proteins, the 52 K protein, was isolated using immunoaffinity columns of bound monoclonal antibody against 52 K protein. The purified protein has been extensively characterized. It is a N-glycoside linked protein with a high content of mannose oligosaccharide chains and phosphorylated mannose (46). Current evidence indicates that the protein is normally shunted to lysosomes in the cell via mannose-6-phosphate receptor mechanisms. However, as a result of estrogen stimulation of the cells, the 52 K protein is secreted. The secreted form is then internalized via receptors and, after cleavage, becomes localized in lysosomes. The mature form of this protein is 34,000 daltons. Rochefort's group now believes the 52 K protein is a cathepsin D-like protein (47). However, since it can stimulate the growth of estrogen-deprived MCF-7 cells at nanomolar concentrations (48), it is an interesting candidate for the regulation of normal and/or neoplastic human mammary cell growth. Rochefort and associates have analyzed the distribution of the factor in human mammary tissue samples by immunoperoxidase staining. The protein was in high amounts in benign mastopathia (ductal hyperplasia and cysts), as well as in the epithelial compartment of some breast cancers (49). However, it was absent in non-proliferating normal human breast, lobular diseases or other non-proliferating atypical lesions (49). These histological observations are more consistent with 52 K protein's role in pathological diseases of the breast, rather than in normal growth regulation.

B. Gastrin Releasing Peptide (GRP)

Bombesin is a tetradecapeptide isolated from the skin of frogs. Janke and Lazarus discovered that cow milk contains a component that is immunologically related to the gly-asn-gln-trp sequence of bombesin (50). A second group later showed that a similar substance is present in human milk and identified it as GRP (51). These findings led to analyses for the presence of bombesin-like activities in tumors of the breast of both rodents and humans. Chemically induced rat mammary tumors were found to contain substantial amounts of GRP (52). Also, cell lines established from an androgen dependent mouse mammary tumor (the 64/24 cell) and from a 7,12-DMBA-induced rat mammary tumor (the WRK 1 cell line) were highly positive (52). A number of human breast tumor samples were analyzed but only a few of these were positive for GRP (53). Unlike their effects on small cell carcinoma of the lung (54), GRP and bombesin fail to stimulate the growth of either normal or neoplastic rat mammary cells grown in primary cultures (Kidwell, unpublished observations). Either the mammary cells already produce sufficient amounts of GRP for optimal cell division in culture or GRP is not able to stimulate these cells.

C. Platelet-Derived Growth Factor (PDGF)

PDGF is a very basic protein (pI = 9.8) that is present in high amounts in platelets, as the name implies. The factor is composed of A and B hetero- and or homodimers. The chains are held together by disulfide bonds that are essential for biological activity. PDGF ranges in size from 28,000 to 35,000 due to differences in glycosylation (55). PDGF has been isolated from the colostrum of goats, cows and sheep (56). The oncogene equivalent of PDGF from simian sarcoma virus appears to be a homodimer of

the B chain of PDGF; this is apparently also true for an oncogene present in a human glioma cell line. In contrast, a human osteosarcoma derived oncogene appears to be composed of two A chains (57).

An activity that is immunologically related to PDGF has been isolated from several mammary tumor cell lines (58). Radiolabeling studies and Northern blot analyses have demonstrated that a functional mRNA for this protein is expressed (58). It remains to be seen, however, whether the PDGF-like protein is composed of A or B chains. Studies by Heldin's group strongly suggest that neither PDGF nor its oncogene counterparts are active directly on epithelial cells because all epithelial cells tested lacked PDGF receptors (59). Most mesenchymally-derived cells were, however, receptor positive (59). For this reason, Rosengurtz et al. (58), have proposed that the PDGF-like activities made by the mammary epithelial cell lines are targeted for the stromal cells of the gland. They further postulate that production of this type of activity might enhance tumor encapsulation by activating the stromal cells to deposit collagen and other extracellular matrix proteins (58). Thus far, however, the degree of encapsulation of mammary tumors has not been assessed relative to their PDGF production levels.

D. Mammary Cell Growth Inhibitor (MCGI)

MCGI is a Mr 13,000 acidic protein (pI 5) that was originally described by Grosse's laboratory (60). The protein, which is apparently not glycosylated, was isolated from lactating bovine mammary glands using molecular sieve and ion exchange column chromatography, followed by gel electrophoresis. MCGI, at ng/ml concentrations, inhibits the proliferation of rat mammary ascites tumor cells in culture (60). Growth inhibition in vitro appears to be mediated by a block in de novo purine biosynthesis at the ribonucleotide reductase step since the inhibitory effects of MCGI are overcome by adding purine nucleotides to the culture medium (61). Polyclonal antibodies against bovine MCGI have been utilized in enzyme-linked immunoassays to determine the amount of MCGI present during various physiological states of the bovine mammary gland. The results suggest that MCGI may be an important negative growth regulator since low levels of the inhibitor are found in proliferating glandular tissue, whereas high levels are present in non-proliferating glands (3). Material which is immunologically related to MCGI has been detected in human milk (3). Low, but detectable levels of MCGI cross-reacting material have also been found in acid-ethanol extracts of human and rodent mammary tumors (Kidwell, unpublished observations). Whether MCGI interacts through membrane receptors on mammary cells is still to be determined. The factor is very unstable to a variety of storage conditions and does not retain biological activity following radioiodonation using Bolton-Hunter or lactoperoxidase methods.

E. Human Mammary Tumor Growth Factor (h.MTGF)

The purification of h.MTGF from pooled human breast tumors has recently been described (62). The factor has a molecular size of 16,000. It is a basic protein (pI 8.0) that is inactivated by heat, acid, trypsin and disulfide reducing agents. Purification included ion exchange on carboxymethyl cellulose, heparin-Sepharose, and copper-Sepharose affinity columns. The protein gave a half-maximal stimulation of the growth of rabbit fetal chondrocytes at a concentration of about 50 ng/ml; dose response curves for other responsive cell types (T47D, human foreskin fibroblasts, bovine endothelial cells) were not presented. Two mammary cell lines, BT 20 and BT474, were not stimulated by h.MTGF.

On SDS-polyacrylamide gels, apparently run under non-reducing conditions, there appeared to be a single protein species in the most highly

purified preparations. It remains to be seen whether h.MTGF is a unique and new growth factor. Many of its properties are the same as those of basic fibroblast growth factor, which is widely distributed in human tissues, both normal and neoplastic.

V. PARTIALLY PURIFIED GROWTH FACTORS FROM MAMMARY TUMORS OR TUMOR CELLS

Included in this category are a myoepithelial cell growth factor and Mammary Tumor-derived Growth Factor. To date only scant information on the purification of either factor has been reported. It is possible that they may be the same as other, more well characterized factors.

A. Myoepithelial Cell Growth Factor (MyCGF)

This factor, like MDGFI, is more active on some cells than others. Hiragun et al. (63) isolated two cell types, E4 and M2, from 7,12-DMBA-induced rat mammary tumors. By means of electron microscopy and immunohistochemistry, E4 was shown to be epitheliod, whereas M2 appeared to be of myoepithelial origin. The conditioned medium from E4 cells was found to stimulate dramatically the proliferation of M2 cells in culture. Although conditioned medium from the M2 myoepithelial cell line did not stimulate the E4 cells, we found that myoepithelial cells from primary 7,12-DMBA-induced rat mammary tumors release some factor(s) that greatly enhance the attachment and growth of epithelial cells from these same tumors (64). Hiragun's factor from the E4 cells is not specific for M2 cells. Dramatic growth responses were also seen in cultures of BALB/3T3, mammary stromal fibroblasts, NRK and Rat-1 cells (63). The activities present in E4 cell-conditioned medium were heat and protease sensitive and were larger than 10,000 daltons, based on ultrafiltration studies. The fact that fibroblasts are so responsive to MyCGF leads one to believe that the factor(s) might be related to PDGF or fibroblast growth factor, a factor which is also active on myoepithelial cell lines.

B. Mammary Tumor Derived Growth Factor (MTDGF)

Sirbasku's laboratory has developed the concept of estrogen effected growth control via a group of growth factors called estromedins, factors that are produced in various organs in response to estrogen. These growth factors are postulated to act on the mammary gland via the circulation (65). His group also suggested that such factors might be induced and act directly in the mammary gland. Thus, a transplantable rat mammary tumor, MTW9/PL, was shown to contain an extractable growth factor(s) as does the human mammary cell line, MCF-7 (66). The MCF-7 activity is extractable from the cells with acetic acid. Increased amounts are detected if MCF-7 cells are treated with estrogens (67). Purification included acid extraction and heating, followed by ion exchange and gel permeation chromatography. The resultant preparation was judged to be only 1% pure. Further purification will be required to determine whether this activity is the same of different from that of the known growth factors. It should be mentioned that TGF is acid-extractable and heat stable and that its production by MTW9/PL tumors and MCF-7 cells is influenced by estrogens (14-16).

VI. CONCLUSIONS

A number of growth factors can be detected in normal and/or neoplastic mammary tumors of rodent and human species. Both indirect and direct evidence suggest that many of these factors are glandular products. In assessing the possible role of the factors, it is important to keep in mind that many are also found in milk. Consequently, they may play some role in the neonate as well as acting as autostimulatory factors for the mammary gland itself. A most important question regarding targets of the factors may be

answered by determining whether their biosynthesis is associated specifically with proliferative or with differentiative states of the normal gland. High production in the latter state would favor a role in the nursing infant. Production in proliferative stages would more likely involve autocrine actions on the mammary gland itself. Also, most studies to date have focused on neoplastic, rather than normal tissues, exceptions being TGFα, TGFβ, MDGFI and IGFI, the production of which we have assessed in primary cultures of human mammary epithelium. Since ectopic production of hormones, such as HCG, is a well known pathological event, a more detailed analysis of production of the other growth factors in normal mammary cells is warranted to ascertain whether production is physiologic or pathologic.

It is also relevant to point out that a physiological role (whether in the mother or the offspring) of a growth factor made by the mammary gland is more likely if mammotrop(h)ic agents stimulate its production. This seems to be the case for TGFα, MTDGF, and 52 K protein, at least. A physiological role is also made more likely when a substance accumulates in the mammary gland or in a mammary tumor and when biosynthesis of that substance is known to be enhanced by a mammary-derived growth factor. An example of this type is CSSF, a factor that greatly stimulates basement membrane biosynthesis and is present in tumors making a basement membrane but absent from those that are not. A physiological role is also favored if one can show that an antiserum against a mammary-produced growth factor alters the behavior of mammary cells in vivo or in vitro. Thus, antibodies against either EGF or TGFα can reduce the growth of normal or neoplastic cells in monolayer and in soft agar cultures, respectively (5,25). Further studies along these lines will help to clarify the physiological vs pathological roles of IGFI, MDGFII, PDGF, MCGI, Swain Factor and MTF. Although the large number of growth factors described to date are intimidating, from a teleological standpoint, they may be understood from the complex nature of cell-cell interaction and from the fact that multiple, synergistic growth factor stimulations may be required to produce either a physiological or a pathological response.

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PROSTAGLANDINS IN BREAST CANCER

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I. CHARACTERISTICS OF PROSTAGLANDINS

The prostaglandins (PGs) are a group of lipids synthesized from arachidonic acid via the cyclooxygenase pathway; other cyclooxygenase products, including thromboxane and prostacyclin, as well as lipoxygenase-dependent products of arachidonate, which include the leukotrienes and hydroxyeicosatetraenoic acids, are collectively referred to as eicosanoids (reviewed in 1). While interest in all eicosanoids has increased vis a vis cancer (reviewed in 2), this review will focus on the primary prostaglandins, i.e., 20-carbon, unsaturated lipids, designated by letters, e.g., PGE_2, $PGF_{2\alpha}$, PGD_2, etc. PGE_2 has been studied most extensively.

The prostaglandins are synthesized by nearly every cell type and have potent regulatory roles in both physiologic and pathologic states. The prostaglandins are not true circulating hormones, due to their rapid degradation in the circulation; however, they are believed to act in a manner analogous to hormones, at or near the site of their synthesis. While their functions are many, including induction of labor, regulation of gastric secretions, and regulation of renal perfusion, this discussion will focus on their role in tumor growth regulation and immune defense.

II. Prostaglandins and Cancer

Many human and experimental tumors are active synthesizers of one or more prostaglandins, usually at much higher levels than are equivalent

normal tissues (reviewed in 2). A great deal of evidence has been provided from nutritional studies (3,4,5) that prostaglandins may act as promoters, or growth enhancers, in mammary carcinogenesis.

III. EXPERIMENTAL TUMORS (NON-BREAST)

In one of the earliest studies of experimental tumors, Levine (6) reported that $HSDM_1$ mouse fibrosarcoma cells synthesize high levels of PGE_2, *in vitro*, whereas normal mouse fibroblasts synthesize little PGE_2. Strausser and Humes (7) reported that fibrosarcomas induced by Moloney sarcoma virus are rich in PGE_2, as compared to normal muscle tissue. As these tumors regress spontaneously, PGE_2 levels decline. Similarly, high PG synthetic activity has been reported in the VX_2 carcinoma of rabbits (8), Lewis lung carcinoma (9), hepatomas (10) and the B16 mouse melanoma (11).

IV. EXPERIMENTAL MAMMARY TUMORS

Tan et al. (12) showed that PGE_2 concentrations are higher in Sprague-Dawley rat DMBA-induced mammary tumors than in normal mammary gland. An extensive study by Karmali et al. (13) showed that four eicosanoids - PGE_2, $PGF_{2\alpha}$, PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) and TXA_2 (measured as the degradation product TXB_2) - are all elevated in NMU-induced rat mammary adenocarcinomas. The NC mouse mammary carcinoma also exhibits high PGE_2-like activity (14).

We have studied prostaglandin levels in a variety of mouse mammary lesions, including a series of tumor subpopulations derived from a single, spontaneously arising BALB/cfC_3H mammary adenocarcinoma (15). These lines differ from each other by a number of immunologic, biologic and biochemical criteria. We have examined PG levels in lesions varying from preneoplastic, hyperplastic alveolar nodules (HAN), and slowly growing, nonmetastatic tumors to highly metastatic tumors (Table 1, ref. 15). We have confirmed the reports that homogenates of malignant tissues have higher levels of PGE_2 than do pre-neoplastic tissues and, in addition, that increased aggressiveness is associated with higher PG levels. These lesions have high levels of $PGF_{2\alpha}$ as well. High levels of both prostaglandins are also correlated with metastatic potential - a topic to be discussed later.

We, like many others, conducted these experiments using tumor homogenates containing host inflammatory cells in addition to tumor parenchymal cells. In addition, we studied cultured tumor cells, freed of host cells, and have confirmed that two metastatic tumor lines, 4526 and 410.4, release more PGE_2 into the medium (490 ng/10^6 cells and 350 ng/10^6 cells, respectively), than does the nonmetastatic tumor line 410 (130 ng/10^6 cells) (Table 1, ref. 16). This information is important because we have shown that macrophages infiltrating these tumors also synthesize PGE_2 (17).

V. ROLE OF PROSTAGLANDINS IN TUMOR GROWTH (STUDIES *IN VIVO*)

While it now seems clear that many tumors have active cyclooxygenase systems to synthesize one or more prostaglandin, the role of these products in tumor growth and metastasis is complex and would appear to depend on the nature of the tumor studied and the major eicosanoids that are synthesized. The subcutaneous growth of many experimental tumors can be inhibited by oral or intraperitoneal administration of PG synthesis inhibitors. Indomethacin (Indo) is most commonly employed, but other inhibitors such as aspirin and flurbiprofen have shown similar effects. These studies have employed carcinomas (9,10,14,18,19) and sarcomas (20,21) as well as ascitic leukemias (22,23).

Table 1. Characteristics of Mammary Lesions

Cell	Phenotype	PGE[a] (ng/g)	$PGF_{2\alpha}$ (ng/g)	PGE[b] (ng/10^6 cells)	NK[c] target	MO[d] target
C4 HAN	Preneoplastic hyperplastic	9	62	n.d.[e]	n.d.	n.d.
C4 Tumor	Nonmetastatic immunogenic	35	161	n.d.	n.d.	n.d.
68H	Slow growing, non-metastatic, immunogenic	29	385	525	n.d.	n.d.
67	Nonmetastatic	n.d.	n.d.	n.d.	n.d.	S
410	Nonmetastatic immunogenic	75	58	130	S	n.d.
168	Nonmetastatic[f] low immunogenic	98	n.d.	n.d.	S	S
410.4	Metastatic immunogenic	321	563	350	R	R
66	Metastatic nonimmunogenic	337	n.d.	n.d.	R	R
4501	Metastatic nonimmunogenic	580	489	n.d.	n.d.	n.d.
4526	Metastatic nonimmunogenic	689	434	490	n.d.	n.d.

[a]Tumor homogenates are solvent extracted, chromatographed and PGE and PGF determined by radioimmunoassay.
[b]PGE levels in medium of tissue-cultured tumor cell lines.
[c]S, R indicates relative sensitivity or resistance to killing by poly i.c. activated normal spleen cells.
[d]S, R indicates relative sensitivity or resistance to growth inhibition mediated by MVE-activated macrophages.
[e]n.d. = not determined.
[f]Tumor 168 does not form metastases spontaneously, but will form lung colonies following intravenous injection.

Additionally, Powles et al. and others have reported that prostaglandin synthesis inhibitors can prevent the osteolytic bone deposits and hypercalcemia associated with growth of the Walker tumor in rats, the VX_2 rabbit carcinoma and the HSDM, murine fibrosarcoma (8,18,24,25). A later study, however, suggests that this hypercalcemia is responsive to dexamethasone and not Indo and may, therefore, be mediated through the lipoxygenase, rather than the cyclooxygenase, pathway of arachidonate metabolism (26).

While the majority of studies show an inhibition of tumor growth by PG inhibitors, in a few systems the opposite effect is seen. Growth of the B16 melanoma in C57B1/6 mice is inhibited by the subcutaneous injection of 16,16-dimethyl PGE_2 (a stable analogue of PGE_2) (27), and tumor latency is shortened by Indo. Administration of PGA_1 has a similar tumor inhibitory effect (28). Inhibition of the ascitic hepatoma-130 with 6-keto-PGE_1 (a degradation product of 6-keto-$PGF_{1\alpha}$) and acceleration of growth with TXB_2 has been reported (29). Growth of a neuroblastoma is also inhibited by PGD_2 (30).

Arguments can also be made for both tumor-accelerating and inhibiting effects of PGs on mammary tumors. Hillyard and Abraham reported that Indo could block the growth-promoting effect of corn oil on a murine mammary

tumor (31). Daily oral administration of flurbiprofen to mice bearing the transplantable NC mammary carcinoma reduces tumor volume, increases survival time and reduces the extractable PGE-like material obtainable from tumor homogenates (19). In a more recent study these workers have shown that the increase in survival time induced by Indo treatment is reversed when Indo is combined with administration of the PGE_2 analogue 16,16-dimethyl PGE_2 (14).

We have found that continuous administration of oral Indo in the drinking water (7 μg/ml) from the day of tumor transplantation leads to complete regression of a nonmetastatic, low-PGE-producing mammary tumor, designated tumor line 410 (Table 2, ref. 32). Two highly metastatic cloned lines, obtained from a metastatic subpopulation isolated from the same tumor as 410, are less inhibited by Indo treatment. The incidence of progressive tumors in tumor 4501-implanted mice is not altered by Indo, but the mean survival time for drug-treated animals is increased by 68% (Table 2). Tumor 4526, another highly metastatic, high-PGE tumor, is markedly inhibited by Indo, with 26% of mice developing progressive tumors compared to 80% of the vehicle-treated controls.

We have maintained mice on similar or slightly higher doses of oral Indo (10 μg/ml) for as long as nine months without observing overt toxicity and with normal weight gain. We have shown with both the mammary tumor and a murine fibrosarcoma that these low doses effectively reduce the levels of PGE_2 in tumor homogenates by 50-70% (32,33).

The role of PGE in rat mammary tumors appears to be different from that in mouse mammary tumors. This may be related to possible interactions of prostaglandins with the hormones upon which rat mammary tumors are characteristically dependent or responsive. Jubiz reported that growth of the hormone-dependent rat mammary tumor MTW-9 is inhibited by $PGF_{2\alpha}$ (34). This was felt to be mediated indirectly through the $PGF_{2\alpha}$-mediated reduction of serum progesterone concentrations upon which these tumors are dependent. Jacobson reported similar findings (35). Foecking et al. (36) found that, in Sprague Dawley rats, DMBA-induced tumors with the longest latency have the lowest levels of PGE_2 in tumor homogenates. The induction of tumor regression by ovariectomy results in tumors with higher PGE_2 content (120 ng/g) compared to progressively growing tumors in intact rats (65 ng/g tissue). Hormone reconstitution experiments indicated that the change in PGE_2 levels is associated with the decrease in prolactin levels, although no causal relationship was established. Thus, in this system, tumor regression is associated with high PGE_2 *in situ*. A later study links the elevation of PGE_2 to elevated cyclic AMP (cAMP) (37). The induction of cAMP by prostaglandin stimulation has been studied extensively and will be discussed later in this article. A similar elevation of PGE_2 and $PGF_{2\alpha}$ in regressing nitrosomethylurea-induced mammary tumors in Buffalo rats has been reported (38).

The estrogen-responsive rat mammary adenocarcinoma R3230AC is inhibited (as measured by tumor weight and volume) by flurbiprofen or by reduced glutathione (GSH) (39), an antioxidant which has a number of activities including the inhibition of PG synthesis. GSH treatment reduces PGE_2 synthesis in these tumors suggesting that, in this system, reduced PGE_2 is associated with inhibition of tumor growth. This conclusion is confused, however, by the finding that GSH treatment increased the amounts of four other eicosanoids: 6-k-$PGF_{1\alpha}$, TXB_2, PGE_1 and $PGF_{2\alpha}$.

VI. ROLE OF PROSTAGLANDINS IN EXPERIMENTAL TUMORS (STUDIES *IN VITRO*)

While the majority of studies *in vivo* suggest a tumor-growth-promoting role for PGs, the *in vitro* data are much more divided. Indo or ibuprofen at relatively high concentrations has been shown to inhibit the replication of Raji lymphoid cells (40), rat mammary tumor cells (13), Walker carcinoma

Table 2. Effect of Oral Indomethacin on Subcutaneous Tumor Growth

Tumor	Indo[a]	Progressive[b] Tumor (%)	Mean Survival[c] Time (Days)
410	+	1/12 (8)	353[d]
	-	10/12 (83)	143
4501	+	18/27 (67)	89
	-	17/23 (74)	53
4526	+	6/23 (26)	111
	-	16/20 (80)	91

[a]On the day of tumor transplantation, mice were switched to drinking water containing ethyl alcohol vehicle (1%) or Indo (7 lg/ml).
[b]Incidence of progressively growing tumors.
[c]Mean survival time for mice bearing tumors.
[d]One animal with progressive tumor died day 353

(41) and VX2 rabbit carcinoma (42). Similar or lower concentrations of Indo, sufficient to inhibit PG synthesis in vitro, have led to stimulation of cell replication in HEp-2, L and HeLa cells (43); equivalent results have been obtained with flurbiprofen in human breast tumor cells (44). Our murine mammary tumor cells are stimulated by Indo (16). Undoubtedly, some of the effects seen at high Indo are related to nonspecific effects, such as inhibition of phosphodiesterase and accumulation of cAMP, or to toxic effects of the drug.

When individual prostaglandins are examined for their effects on cell replication, diverse effects again are seen. In the majority of studies, the addition of PGE_1, PGE_2 or the stable analogues of PGE_2 inhibit cell proliferation (2,27,41,43,45). Raji lymphoid cells show a biphasic response, with PGE_1 stimulating cells at low concentrations (100 pg/ml) and inhibiting at higher doses (40). The exception is a clone of the VX2 carcinoma which is stimulated to proliferate with PGE_1 or PGE_2 at concentrations of 10^{-6} M to 10^{-8} M (42).

It has been assumed that the inhibition of cell proliferation by the E series PGs is due to the well documented rise in intracellular cAMP that ensues (46). However, recent studies by Honn et al. (47,48) and Bregman et al. (45) showing inhibition of cell proliferation with PGA analogues, suggest that the inhibition of growth of B16 and Cloudman melanoma cells is independent of the cAMP effects. Indeed, Honn and Marnett postulate that the growth inhibition is due to "chemical reactivity rather than hormonal activity" (48). Again, exceptions exist, with the Raji cell unaffected by PGA_1 (40) and proliferation of the VX2 carcinoma stimulated by PGA_2 (42). Thomas (43) reported an inverse relationship between rates of cell proliferation and PGE production; however, in our system, the higher PGE levels are seen in lines with the shortest or longest doubling times (16).

Unlike these PGs, $PGF_{2\alpha}$ more consistently shows either growth stimulation or has no effect. Jimenez de Asua showed, a number of years ago, that $PGF_{2\alpha}$ initiates DNA synthesis in mouse 3T3 cells (49). This PG also stimulates Raji lymphoid cells (40) and the VX2 cell line (42). PGD_2 also, rather consistently, inhibits the proliferation in vitro of murine melanomas

(45,47), mastocytoma (50) and the L1210 leukemia (51), and PGJ_2, a decomposition product of PGD_2, inhibits normal mouse fibroblasts and smooth muscle cells (52). In a study showing that TXB_2 stimulates B16a proliferation while lowering intracellular cAMP, whereas PGI_2 inhibits proliferation, Honn suggests that TXB_2 and PGI_2 (which have opposing effects on platelet aggregation) may also function as bidirectional regulators of tumor growth (2).

Although most prostaglandins appear to inhibit cell proliferation in vitro, these findings are often based on pharmacologic concentrations. While these may prove useful in designing possible therapeutic strategies, they do not necessarily clarify the physiologic role of these lipids in tumor growth. The fact that Indo and other prostaglandin inhibitors so often inhibit tumor growth in vivo suggests that the net effect of increased cyclooxygenase activity in tumors is to promote tumor growth. Clearly, the action of some prostaglandins in vivo may be to down-regulate tumor growth, while others, such as $PGF_{2\alpha}$, may stimulate tumor cells directly.

In the normal mammary gland, linoleic acid (the precursor of arachidonate) is necessary for normal lobular-alveolar development to occur (53). Rudland et al. (54) have shown that low concentrations of PGE_2 stimulate DNA synthesis in normal rat mammary epithelium and have proposed that PGE_2 is a growth factor for rat mammary epithelium. Other studies showing interactions of PGs with various mammatrophic hormones suggest that these lipids are important in normal mammary gland development and function, as well as in the cancerous state.

VII. HUMAN BREAST CANCER

Two early studies by Bennett et al. (55,56) reported high levels of bioassayable, PGE-like material present in breast tumors that had metastasized to the bone, and found that post-surgical survival time is shorter in women with high-PGE tumors. In related studies Powles et al. (57) showed that osteolytic activity expressed by tumors taken from women with hypercalcemia or bone metastases is, in some cases, inhibitable by aspirin, suggesting a prostaglandin-mediated mechanism. A follow-up study of these patients has, however, shown no relationship between this osteolytic activity in vitro and recurrence rates or survival times (58). Data showing that breast tumors with high PGE_2 activity tend to have histological evidence of lymphatic, nodal and vascular invasion led Rolland to propose that high PGE_2 is a marker for high metastatic potential (59).

Malachi and colleagues (60) found that breast tumor PGE levels fall into a high and a low group, but these two groups do not segregate in regard to prognosis based on histological grade, clinical stage, degree of lymph node involvement or, ultimately, survival time. A 12-36 month follow-up study of 17 patients showed no correlation between PG levels and incidence of metastasis or death. Karmali measured tissue content and production of five prostanoids: PGE_1, PGE_2, $PGF_{2\alpha}$, 6-k-$PGF_{1\alpha}$ and TXB_2 (61). Although the content of all five compounds is higher in the tumor than in uninvolved breast tissue, only TXB_2 levels are related to other clinical, prognostic parameters. Elevated TXB_2 is associated with larger tumors and number of involved nodes. There is no relationship between any eicosanoid and the presence of distant metastases. A study using established, metastatic breast tumor lines found no relationship between PGE_2 levels in vitro and donor age, race, survival time, metastatic site, presence of estrogen receptor or presence of chromosomal abnormalities in cultured cells (62).

Others have reported elevated prostaglandins in breast tumors (63-65) but these were not examined in relation to clinical parameters other than

tumor grade. One study reported a possible inverse correlation of PG levels and tumor grade in a small number of samples (63), whereas another reported higher PGE_2 levels in higher grade tumors (64).

A prospective study by Watson et al. analyzed 100 breast tumors for PGE_2 and $PGF_{2\alpha}$ (66). Higher levels are seen in estrogen-receptor-positive tumors but there is no apparent relationship to nodal involvement. These patients are being followed for clinical outcome. Vergote reports that $PGF_{2\alpha}$ is significantly elevated in malignant breast tissue from patients with no evidence of distant metastases, when compared to normal or benign breast disease specimens. Tumors with the best prognosis (small, node-negative, differentiated) have higher $PGF_{2\alpha}$ levels (67).

Plasma levels of 6-k-$PGF_{1\alpha}$ and TXB_2 are significantly higher in patients with malignant breast tumors, in comparison to patients with either benign breast tumors or to healthy controls (68); however, no relationship is seen between levels of 6-k-$PGF_{1\alpha}$ and any histological or clinical predictor of prognosis. A recent prospective study by Aitokallio-Talberg et al. (69) of the production of 6-k-$PGF_{1\alpha}$ and TXB_2 showed higher levels in tumors than in mastopathic specimens. In this study 15 of 23 patients developed metastases during the 3.7-year follow-up, but prostanoid production in tumors from these women did not differ from nonrecurrent specimens.

We have been participants in a long-term, prognostic study of breast cancer in women with no clinical evidence of metastatic disease at diagnosis beyond axillary lymph nodes; in this study many parameters have been studied for their relationship to recurrence. We have analyzed the levels of PGE_2 and $PGF_{2\alpha}$ in tumor homogenates of approximately one hundred specimens (70). Median levels (and range) of PGE_2 and $PGF_{2\alpha}$ are 10 ng/g (1-125 ng/g) and 29 ng/g (2-627 ng/g), respectively. There is a highly positive correlation between levels of the two PGs. Watson has recently reported a similar correlation (66). We have found no apparent relationship between estrogen or progesterone receptor content and levels of either PG. In addition, no relationship is seen between degree of lymph node involvement and PG levels. A tendency for tumors with worse prognostic grades to have more PG is seen but is not statistically significant. At the present time, these women have been followed for an average of 30 months, during which time 20% of the tumors have recurred (71). Analysis of these data by lifetable analysis or Cox regression revealed no effect of PG levels on time to recurrence or death. When cases with bone metastases were analyzed separately, we found lower than average PG levels in patients with skeletal involvement.

Thus, based on our studies and those of others (58,60-71), determination of PG levels in tumor homogenates does not appear to be valuable as a predictor of early recurrence in women with limited disease at the time of surgery. The early, promising studies by Bennett (55,56) were carried out retrospectively, on samples from women with known skeletal involvement and known survival times. The association of PGs with specific factors e.g., bone involvement, may not correlate well with the survival rate for the entire patient population. The complex and diverse effects of various PGs *in vivo* and *in vitro* and the extreme heterogeneity of breast cancer would predict that the role of any one PG in tumor growth (and thus its predictive value) would differ in different tumors. It is obvious, but sometimes ignored, that any one experimental mammary tumor system is a model of only one woman with breast cancer. It is hoped that as our understanding of the roles of individual PGs increases, we will enhance our ability to use these quantitatively abnormal products in diagnosis, prognosis and perhaps even in therapeutic intervention.

VIII. PROSTAGLANDINS AND METASTASIS

I have presented a body of evidence indicating that prostaglandins play a role in the growth of primary or transplanted tumors and that, in many systems, the inhibition of the cyclooxygenase pathway results in inhibited tumor growth. Because of the absolute clinical importance of preventing metastatic spread, particularly in the case of breast cancer, much interest has also focused on the role of PGs in metastasis.

Many steps appear to be required for tumor cells to complete the cascade leading to successful metastasis (reviewed in 72). Among factors believed to contribute to the completion of these steps, is the ability of tumor cells to aggregate with each other (homotypic) or with platelets (heterotypic aggregation) to form emboli, which, due to enhanced lodgement, endothelial adherence, resistance to immune attack or other factors, are more successful in metastasis than are individual tumor cells. Thus, a great deal of interest has been directed towards platelet aggregation inhibitors. Normally, platelet aggregation is promoted by thromboxane A_2 (released by platelets) and inhibited by prostacyclin synthesized by vascular endothelial cells. Perturbations in this homeostatic mechanism lead to hyper- or hypo-aggregatory activity.

Early studies by Gasic et al. showing that tumor cells can induce platelets to aggregate in vitro and that aspirin can reduce both experimental and spontaneous metastasis (73), suggest that PGs do play a role in metastasis. There is additional evidence that platelets affect metastatic spread, based on studies with anti-coagulants (74-77); however, this discussion will be restricted to PG-mediated platelet effects.

To test more directly the hypothesis that platelet aggregation contributes to tumor spread, Honn and co-workers (78) tested the effect of various PGs on the incidence of experimental metastasis in the lungs following the intravenous injection of B16 amelanotic melanoma (B16a) cells. PGI_2, an anti-aggregatory compound, reduces the number of detectable lung colonies, while PGE_2 and $PGF_{2\alpha}$ are inactive. PGD_2 reduces the number of lung colonies but to a lesser extent than does PGI_2. PGI_2, in combination with theophylline to maintain high levels of intracellular cAMP, is the most effective in reducing lung colonies. PGI_2 does not alter the degree of initial tumor cell arrest in the lungs; rather, it appears to alter later steps, since treatment is equally effective if given one hour after tumor cell injection. Thus, these workers propose that tumor cells can induce platelet aggregation and that endogenous PGE_2 is a natural deterrent to dissemination. Interestingly, Mehta et al. (79) report that exogenous PGI_2 loses bioactivity more rapidly in the plasma of patients with malignant bone tumors than in normal controls.

This study corroborated earlier studies by Fitzpatrick and Stringfellow (80,81) showing an inverse correlation between metastatic potential and levels of PGD_2 (another anti-aggregatory PG) in the B16 melanoma. In that system, the highly metastatic B16 F10 population synthesizes lower levels of PGD_2 than does the weakly metastatic B16 F1. Unlike many experimental tumors, these melanomas synthesize little PGE_2, nor do they form other platelet-active eicosanoids such as PGI_2 or TXA_2. Studies in vivo show that preincubation of either line with Indo increases the number of lung colonies following intravenous injection, whereas preincubation with PGD_2 reverses the Indo-mediated enhancement. Fitzpatrick and Stringfellow proposed that the anti-metastatic effect of PGD_2 is attributable to its inhibitory effect on platelet aggregation.

The antithrombotic drug, Nafazatrom, has been tested in these systems, based on its ability to stimulate PGI_2 synthesis by vascular endothelium and

to prevent the enzymatic degradation of this compound (82). Nafazatrom treatment of mice reduces both the experimental and spontaneous metastasis of B16a cells. Maniglia et al. (83) have reported similar results; however, they conclude that the antimetastatic effects of Nafazatrom are due to the inhibition of endothelial extracellular matrix degradation.

Using the B16a tumor, Lewis lung carcinoma or the CT26 colon adenocarcinoma, Karpatkin et al. (84) and Haas et al. (85) were not able to reduce experimental metastases with prostacyclin or Nafazatrom. While diverse tumors can be expected to respond differently, the reasons for lack of response of the B16a tumor in this study, in contrast to the great reduction in lung metastases shown by Honn et al. (78) and Maniglia et al. (83), are not clear.

Certainly, the role of tumor PGs in metastasis is as complex and varied as it is in control of primary tumor growth. Much attention has focused on the role of PGI_2 and TXA_2 in metastasis, due to their potent platelet effects; evidence that PGE_2 plays a role in metastasis also exists. The metastasis of Lewis lung carcinoma, which synthesizes PGE_2, is inhibited somewhat by oral Indo (9); however, clones derived from metastatic nodules produce less PGE_2 in culture than does the parent tumor, suggesting that the inhibition of metastasis is not mediated through PGE_2 inhibition. This finding may be analogous to that of Fitzpatrick and Stringfellow (80,81), in which another anti-aggregatory prostaglandin (PGD_2) was elevated in non-metastatic tumors (see above). These same authors demonstrated that oral Indo inhibits the appearance of culturable EL4 leukemia cells from the lungs following intraperitoneal introduction of tumor cells (86). An in vitro system showed that PGE_2 enhances the migration of EL4 cells out of glass capillary tubes.

As reported above, we have found a direct correlation between levels of PGE_2, $PGF_{2\alpha}$ and metastatic potential (Table 1, ref. 15). Thus, the mouse mammary tumor system differs from both the B16 system in which PGD_2, the major metabolite, is inversely correlated with metastatic potential (80,81), and the Lewis lung carcinoma in which dissemination is seen in tumor cells with low PGE_2 activity (87). That PGE_2, or another cyclooxygenase metabolite, contributes to the ability of mammary tumor cells to disseminate is shown by our studies, in which the spontaneous metastasis of mammary tumor lines 410.4 and 66 is greatly reduced in mice treated with oral Indo from the time of tumor transplantation until surgical removal of the primary tumor. (Figure 1). While we have not ruled out a platelet effect, it appears that other actions of tumor PGs are also important. PGE, because of its anti-aggregatory activity, would be expected to decrease metastatic dissemination, but the correlation is just the opposite. In addition, in this system, Indo treatment of the host is not effective in inhibiting experimental lung metastases resulting from intravenously injected tumor cells, again suggesting that platelet effects are not critical (Figure 1). Interestingly, pretreatment of the tumor cells with Indo (1 μM), prior to their intravenous injection, markedly reduces lung colony forming ability (Table 3), which is the opposite of the results seen by Fitzpatrick using the B16 melanoma (80,81).

We think it likely that these effects, in our system, are mediated by interactions of immune effector cells, particularly natural killer cells and macrophages, with tumor cells.

IX. PROSTAGLANDINS AND THE IMMUNE RESPONSE

The idea that prostaglandins play a role in immunoregulation was initially described in a tumor system by Plescia et al. (22). In that in vitro system, cultured tumor cells suppress the antibody response of spleen cells

to sheep erythrocytes. Aspirin or Indo blocks this suppression and exogenous PGE_2 mimics it. Thus, Plescia proposed that tumors can mediate the well documented immunosuppression that occurs in cancer-bearing subjects. Shortly thereafter, Webb and Osheroff (88) demonstrated that heightened PGE_2 release occurs shortly after antigenic challenge in the normal host and thus serves to act as a negative feedback regulator of the immune response. Goodwin and co-workers first showed a similar function for the cell-mediated arm of the immune response, namely, in the regulation of the T cell proliferative response to mitogenic stimulation (89). Since that time, PGE_2 has been shown to inhibit many T cell functions (reviewed in 90). In addition, and particularly important in relation to metastasis, it has been demonstrated that both macrophages and natural killer (NK) cells are inhibited, under certain conditions, by PGE_2 (91-93). The role for other cyclooxygenase products in immunoregulation is less certain.

While exogenous PGE_2 can directly suppress immune effector cell functions, this molecule is also believed to function, *in vivo*, by the induction of suppressor cell activity (90). A number of such suppressor cells have been described, including ones with properties of both T lymphocytes and macrophages. In addition, PGE_2 can have positive stimulator actions in the development of immune responses, particularly in the case of immature immune effectors, and can have both positive and negative regulatory effects on macrophage and NK function (94-96) depending on the basal activation state, the timing of the PG, and the nature of the inducing (antigen) signal.

A role for PGE_2-mediated immune suppression has been described in a number of disease states (90) and can be demonstrated in the tumor-bearing state as well. As described above, Plescia and co-workers first showed

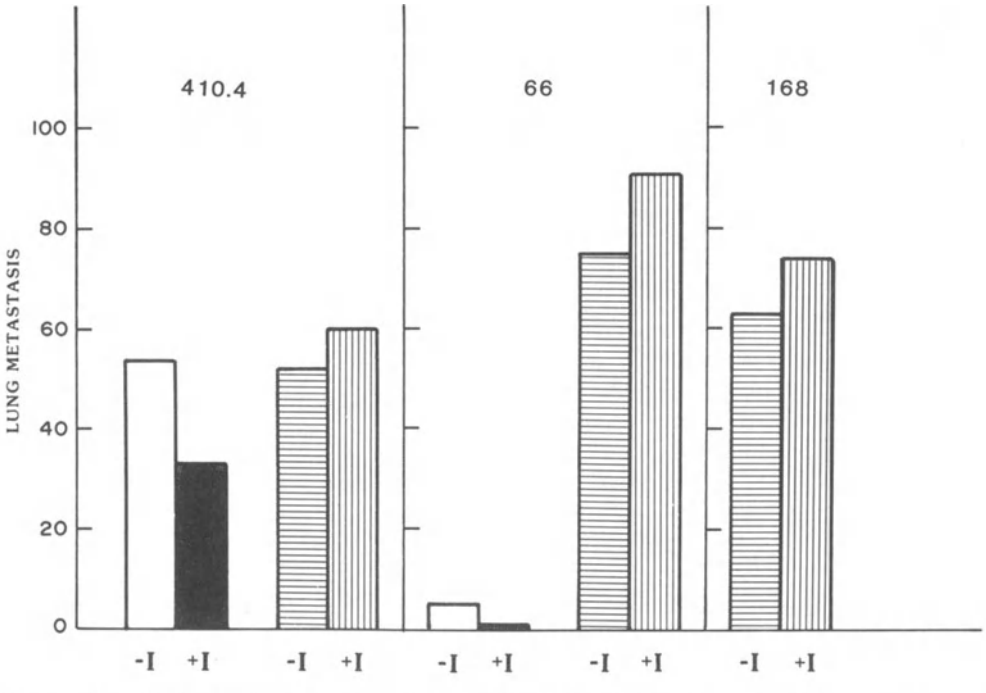

Figure 1. Number of lung metastases determined for 3 tumor lines. Spontaneous metastasis from subcutaneous tumor is indicated as open bar ☐ for controls, closed bar ■ for INDO treatment; ▤, experimental metastasis after intravenous injection for control mice; ▥, experimental metastasis for INDO treated mice.

Table 3. Effect of Tumor Cell Pretreatment (Indo) on Experimental Metastasis of Tumor 410.4

Treatment[a]	No. Lung[b] colonies	Incidence	X ± S.E.	Median
- Indo	5,9,24,27,38,39, 46,55,138,140	10/10	52.1 ± 15.3	38.5
+ Indo	0,0,0,0,1,2,2	3/7	0.7 ± 0.4	0

[a]410.4 tumor cells were cultured for 48h in the presence of ethyl alcohol (vehicle) or 1 μM Indo prior to intravenous injection.
[b]On day 21, mice were sacrificed and lungs examined under a dissecting microscope for the presence of surface colonies.

immune suppression mediated by PGE released by tumor cells. In the B16 melanoma system described earlier, in which 16,16-dimethyl PGE_2, or PGA_1 inhibits tumor growth (97), immune suppression is also prevented. That the effect is due to a direct reversal of immune suppression, rather than related to the lowered tumor burden in these animals, is suggested by similar findings in non-tumor bearing mice immunosuppressed with adriamycin.

Poor mitogenic responses seen in mice bearing several tumors, including a mammary adenocarcinoma, are reversed with oral Indo (98). Lynch et al. (99), while reporting that Indo inhibits the growth of a murine fibrosarcoma, conclude that the effect is not mediated by the reversal of immune suppression, since these mice are not immune to rechallenge with the same tumor. However, immunosuppression of NK and macrophage function could still have played an undetected role, as these cells do not display immunologic memory.

In addition to T and B cell-mediated immunity, natural cytotoxicity and antibody-dependent cellular cytotoxicity towards tumor targets are also suppressed by PGE_2 (100). This PGE_2 is synthesized by the human bladder tumor target cells and its synthesis is stimulated by contact with lymphocytes. The enhancement of cytotoxicity with PG inhibitors is dependent on the presence of macrophages. Complex interactions between macrophages, lymphocytes and tumor targets are likely to be involved.

Suppressor macrophages induced by, and/or acting via release of, PGE_2 have also been described in both experimental and clinical cancer. The poor phytohemagglutinin mitogenic response of peripheral blood lymphocytes from Hodgkin's disease or colon cancer patients is reversible by either Indo or by removal of macrophages from the test system (101,102). Macrophages from tumor-bearing mice have been shown to be high producers of PGE_2 (103). Peripheral blood monocytes of breast cancer patients release high PGE_2 and exhibit poor cytotoxicity towards a prostatic tumor cell line unless Indo is present (104). Although reversal of suppression by Indo has been demonstrated in many systems, Tilden and Balch have pointed out that this effect in melanoma patients is not related either to abnormal production of PGE_2 by patient monocytes or to increased sensitivity of their lymphocytes to PGE_2, nor is it affected by another PG inhibitor, RO-205720 (105). Thus, the reversal of immunosuppression by Indo in this system does not appear to be due to inhibition of PGE_2 synthesis by effector cells. This study does not rule out, however, the induction of suppression in vivo by tumor synthesized PGE_2.

As described above, growth of our immunogenic mammary tumor line 410 is almost completely inhibited by Indo (ref. 32, Table 2). Three metastatic lines, all of which produce high levels of PGE_2, and are relatively less immunogenic, are less completely inhibited by Indo. Subcutaneous growth of another metastatic, high PGE_2 line that is not immunogenic, line 66, is not inhibited by Indo, suggesting that the Indo effects are, in part, mediated through immune effector function (15). Growth of these tumors is inhibited slightly by the interferon inducer, polyinosinic-polycytidylic acid (poly I.C.); however, greater inhibition is seen in combined treatment with poly I.C. and oral Indo, suggesting that activation of nonspecific effector cells (NK and macrophages), coupled with abrogation of immune suppression, contributes to the growth inhibition.

We have shown that high PG levels are positively associated with metastatic potential. We have hypothesized that this PGE_2 functions to prevent the successful control of metastasis by macrophages and NK cells. Hanna and Fidler (106) and Gorelik et al. (107) have shown that NK cells are important in controlling the metastatic dissemination of B16 melanoma and Lewis lung carcinomas. Cell lines that are more successful in colonizing the lung are also more resistant to the cytotoxic effects of NK cells. We have shown that the ability of our mammary tumor lines to form experimental metastases is also increased when NK function is impaired by treatment with anti-asialo Gm_1 (an anti-NK) serum *in vivo* is decreased by treatment with poly I.C. In addition, the spontaneous metastasis, but not experimental metastasis, of tumor 410.4 and 66, is inhibited by treatment of the host with Indo (Figure 1). This somewhat surprising result led us to propose the following: (1) since spontaneous metastasis of both an immunogenic (410.4) and nonimmunogenic (66) tumor is inhibited, specific (T cell) immune effector mechanisms are probably not involved in control of metastasis; and (2) because prior treatment of the host with Indo does not affect the experimental metastatic rate, the Indo effects are more likely to be directed towards the tumor than towards the immune effector cells. Thus, we proposed that the effect of Indo treatment in the spontaneous metastasis model is to increase the sensitivity of the tumor target to NK killing. Indeed, we showed that the metastatic tumors are more resistant to killing *in vitro* by poly I.C.-activated normal spleen cells than are nonmetastatic tumor lines and, in addition, the sensitivity of all targets is increased by pre-culturing the target cells in Indo (15). Further, while pretreatment of the host has no effect on the pulmonary growth of iv-injected tumor cells, pretreatment of these same target cells with Indo, before injecting them into untreated hosts, completely inhibits the ability of these cells to form pulmonary colonies (Table 3). Thus, tumor PGs may serve to render the target cells more resistant to cytotoxic mechanisms due to inherent changes in the tumor cells or due to an ability to repel the attack by NK cells.

Studies by Wei and Heppner (personal communication) show that NK activity is very low in lymphocytes isolated from tumors 410.4, 66 and 168. Interestingly, high NK activity is detectable only in the poorly tumorigenic line 68H, the preneoplastic HAN and the C4 tumor arising from it. It should be noted that these are the three lesions with the lowest PGE levels (Table 1). Studies are currently in progress to determine if Indo treatment alters the nature or function of these NK cells.

Macrophages have been examined in many systems for their role in metastatic control. Early studies claimed that the degree of macrophage infiltration was less in metastatic tumors (108); however, our studies and those of others have shown that this is not a general phenomenon (109). We have shown, in fact, that while the number of macrophages *in situ* is relatively constant among tumors of our different mammary lines, the macrophages isolated from metastatic tumors are more likely to be activated (as determined by ectoenzyme characterization) and cytotoxic towards mammary

tumor cells (17,109). Tumor-associated macrophages are heterogenous in phenotype; metastatic tumors contain macrophages with high levels of the ectoenzyme and leucine aminopeptidase, and low PGE_2 synthetic activity (17).

We found that both metastatic and nonmetastatic tumors are equally sensitive to macrophage-mediated cytotoxicity; however, the nonmetastatic tumors are more sensitive to macrophage-mediated cytostasis (growth inhibition) than are the metastatic tumors (Table 1, ref. 110). As with NK sensitivity, the cytostatic sensitivity of metastatic targets is increased by pre-culture with Indo. In this case, increased activation of the effector cells (with lipopolysaccharide) also diminishes the differences in sensitivity between metastatic and nonmetastatic cells.

Thus, in parallel with the NK studies, inhibition of PG synthesis by tumor cells renders them more susceptible to macrophage attack. Studies to test the role of macrophages and the interaction of these cells with PGs in vivo are now in progress.

X. PROSTAGLANDIN RECEPTORS

While there is ample evidence that prostaglandins can function to help tumor cells escape immune attack, these lipids, in all probability, have other activities in terms of growth regulation. As reviewed above, the literature on the function of individual PGs in cell proliferation studies in vitro and tumor growth inhibition studies in vivo is contradictory. These results undoubtedly reflect the diversity of cancer cell types, hormonal responsiveness, the PGs that these cells make and, equally important, the PGs to which these diverse cell types are capable of responding. It is this latter point that we have begun to address in an attempt to explain some of the diverse cell reactions. We have undertaken studies to identify and characterize the binding sites for PGs on mammary tumor cells.

Using a ^3H-PGE_2 ligand-binding assay, we have shown that ^3H-PGE_2 binds with high affinity to viable line 410.4 tumor cells (ref. 111, Figure 2). This binding is almost completely blocked if endogenous PGE_2 synthesis is not inhibited with Indo. The binding is enhanced at 37° C versus 5° C, is saturable and reversible with PGE_2. PGD_2 and $PGF_{2\alpha}$ partially inhibit binding of PGE_2 whereas PGA_2 is a poor competitor. PGE_1 and 16,16-dimethyl PGE_2 block binding of ^3H-PGE_2. Scatchard analysis of equilibrium binding data reveals a dissociation constant (K_d) of 1.1 nM, and an average of 14,000 binding sites per cell.

There is evidence for PG receptors in many normal cells, including platelets (112), lymphocytes (113), macrophages (114) and adrenal medulla (115). To our knowledge, few tumors have been examined (116). High affinity binding sites for PGE_2 have been described in the hormonally responsive rat mammary tumor MT-W9 (117). This high affinity binding is not seen in a hormone-independent tumor. In contrast, no high affinity binding of PGE_2 is detected in the NMU-induced rat mammary tumor (38) but a high affinity receptor for $PGF_{2\alpha}$ (K_d=2.9 nM) was described. In both these rat mammary tumors, PG binding and synthesis is increased during ovariectomy-induced tumor regression.

We initiated these studies, in part, to resolve the question of what role tumor PGs play in tumor growth. Many in vitro studies showing inhibition of cell replication with PGE would suggest that the endogenous product should serve to limit tumor growth; and yet, the net effect in vivo of inhibiting PG synthesis is tumor growth inhibition. Interestingly, PGE_2 appears to be a growth stimulator for normal rat mammary epithelium and may participate in the induction of differentiation as well (54). Clearly, other PGs, notably $PGF_{2\alpha}$, could serve to stimulate DNA synthesis, overriding

the inhibitory effects of PGE. Our studies in vitro, showing that murine mammary tumor cell proliferation is not inhibited by PGE_2, suggested that these cells are unresponsive to the high endogenous levels of PGE_2 in situ due to a paucity of PGE receptors. The present data, however, shows that high affinity binding does occur. Studies to determine if this receptor binding is coupled to adenyl cyclase activity (as measured by increased intracellular cAMP) are in progress.

Thus, the rat and murine mammary tumor systems differ in hormone responsiveness, PG responsiveness and PG receptor binding. These differences raise numerous questions regarding the interactions of hormones, growth factors and PGs. While there is some evidence supporting interactions between these compounds, as well as interactions between PGs and other tumor growth factors, the possibilities are only now being explored to any great degree.

XI. CONCLUSIONS

Since the relatively recent discovery of the prostaglandin compounds, much effort has been directed towards determining their physiologic functions. It is well established that some of the PGs are elevated in both experimental and human cancers but the particular classes found varies from tumor to tumor. In fact, no tumors have been characterized in regard to all known cyclooxygenase products, and new metabolites are continually being described. This review has focused on the primary PGs, particularly PGE, about which the most is known; however, other PGs or their metabolites may in fact have more important roles in tumor growth. Other cancer-related phenomena, such as hypercalcemia, appear to be related to high PG levels as well (118). While the studies in vivo with PG-synthesis inhibitors show that the net effect of PGs is the stimulation of tumor growth, this is not true in all systems. The role of PGs in mammary tumor systems is clouded by

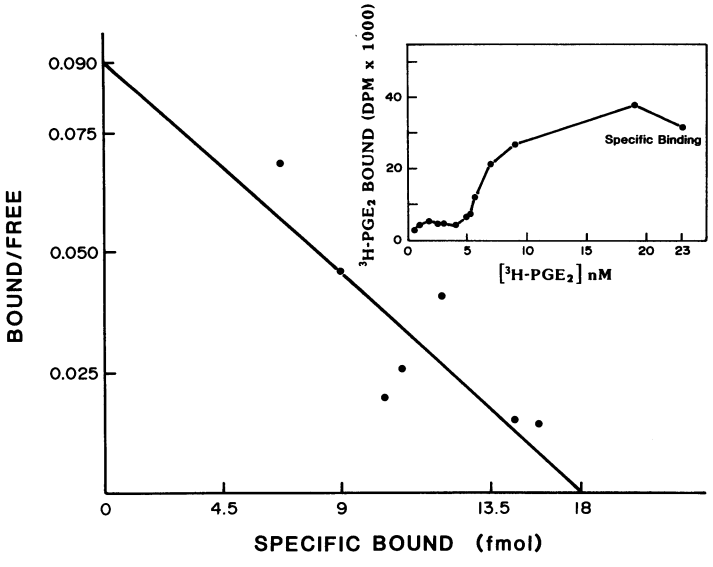

Figure 2. Scatchard analysis of equilibrium binding data for binding of ^{3}H-PGE_2 to viable 410.4 tumor cells. The insert shows the specific binding data. The lower part of the curve was used for the Scatchard analyses. The correlation coefficient for the line is 0.79. X-axis intercept indicates 18 fmoles per $1x10^6$ cells, or an average of 14,000 binding sites per cell. The dissociation constant, Kd = 1.1nM.

the observation that PGE stimulates replication of normal rat mammary epithelium (54).

Conclusions drawn about the effects of Indo must take into account the fact that inhibition of the cyclooxygenase pathway may make intermediate products more available to the lipoxygenase pathway. Only with the availability of inhibitors of specific PGs or PG analogues will it be possible to ascribe roles to individual PGs. Nevertheless, even given the limitations of our present knowledge, it is possible to conclude that prostaglandins do play a role in the growth and metastasis of tumors, that some may promote and others inhibit tumor growth, and that indirect effects such as those on immune effector cells, platelets and other host factors are likely to be involved.

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EXOGENOUS AND ENDOGENOUS MOUSE MAMMARY TUMOR VIRUSES: REPLICATION AND CELL TRANSFORMATION

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I. INTRODUCTION

The concept that mouse mammary tumorigenesis involves a transmissible genetic factor first arose from the observation that mammary carcinomas tend to occur in familial patterns (1,2). The development of inbred strains of mice with high and low mammary tumor incidences provided additional evidence for the involvement of some transmissible agent (3). Crosses performed

between high and low mammary-tumor-incidence strains yielded progeny whose tumor incidence was most strongly influenced by that of the mother, suggesting that a nonchromosomal factor was involved (4). This nonchromosomal factor was transmitted via the milk from mother to offspring (5), and the agent, mouse mammary tumor virus (MMTV), was isolated (6,7). Decades later, MMTV and the mouse mammary tumor system remain the only animal model for the study of a possible viral etiology of human breast cancer.

MMTV, a member of the family Retroviridae (8-10), possesses several features that distinguish it from other retroviruses. The most noticeable distinction of MMTV is its tropism for the mammary gland. Although MMTV antigens can be detected in a variety of non-mammary tissues (11-14), newly introduced viral DNA can be detected in lymphoid tissues of infected mice (15), and several heterologous cell types can be infected with MMTV (16-19), only alveolar epithelial cells of the mammary gland are believed to undergo transformation. Secondly, viral gene expression, as well as MMTV-induced carcinogenesis, is markedly influenced by glucocorticoid hormones (19). Finally, the MMTV genome contains the coding potential for an additional gene not found in the slowly transforming type C retroviruses (20-23). Although this additional gene may not behave as a classic viral transforming gene, the conservation of its coding sequences among different MMTV isolates suggests that it provides some essential function during MMTV infection or transformation.

MMTVs are divided into two main groups, based on mode of transmission. Most of the known molecular biology of MMTV was generated from studies of exogenous, or milk-transmitted, MMTVs. The exogenous MMTVs are responsible for the high mammary tumor incidence of inbred mouse strains of American (e.g., C3H, DBA, A) and European (e.g., RIII, DD) descent (24). MMTV particles are found in the milk and mammary tumors of such high-tumor-incidence mice (24).

Less is known about the second group of MMTVs, those that are transmitted endogenously in all inbred strains of mice via germ line DNA (25). In general, those inbred strains that harbor only endogenous MMTV, in the absence of exogenous MMTV (e.g., BALB/c, C57BL, 020), demonstrate a very low incidence of spontaneous mammary tumors (26). One exception to this rule is the GR strain of mouse in which an endogenous provirus is actively expressed and associated with a high incidence of spontaneous mammary tumors (26, 27). The role of endogenous MMTVs, if any, in mammary tumorigenesis is unclear. However, as more is learned of the mechanism(s) of cell transformation by exogenous MMTV, potential contributions of the endogenous MMTVs to the tumorigenesis process will be more readily assessed.

II. EXOGENOUS MMTV

The retrovirus MMTV is designated genus Type B Oncovirus Group (10) because of its distinctive, eccentrically located viral core (9,28). MMTV is an enveloped, RNA-containing virus, measuring 110-130 nm in diameter (29), and is 70% protein and 1.9% RNA by weight (30). Several variants of exogenous MMTV have been identified and are designated by the strain of mice from which they were isolated. The MMTV harbored by C3H inbred mice, designated (C3H)MMTV, is considered the standard MMTV to which other isolates are compared (31).

The genomes of the various exogenous MMTVs are similar, sharing approximately 90-95% sequence homology (32-34). The polypeptide contents of virions of the different isolates are also similar (35). The viral polypeptides are immunologically cross-reactive with the analogous protein of other MMTV isolates due to the presence of group-specific antigenic determinants shared by all MMTVs (34,36). In addition, competition radioimmunoassays (RIAs) have demonstrated the presence of class-specific (shared by certain

MMTVs) and type-specific (unique to each MMTV) antigenic determinants on viral proteins (34,35).

The biologic activities *in vivo* of various MMTVs have been compared. Viral infectivity was determined by injecting different MMTVs into recipient low-mammary-tumor-incidence mouse strains, followed by assaying milk samples for the presence of viral proteins. None of the four recipient strains tested (Af, C57BL, BALB/c, RIIIf) was uniformly resistant to infection by all exogenous MMTVs (37). Interestingly, C57BL mice were resistant to infection by the otherwise highly infectious (C3H)MMTV and were susceptible only to those MMTV isolates that are associated with mammary tumors of nonalveolar morphology (e.g., GR, RIII, DD) (38). BALB/c mice were susceptible to all of the exogenous MMTVs. Foster-nursing experiments were used to compare the tumor-inducing capabilities of the various isolates. The tumor potential of a given isolate was influenced by the host strain of mouse, suggesting the importance of virus-host interactions in mammary tumorigenesis of exogenous MMTV etiology (38).

A. The Virus Particle

Cell lines that produce MMTV have been established from virally induced mammary tumors (39-42), providing adequate amounts of virus for structural analyses. When purified MMTV is analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, seven distinct viral proteins are detected whose apparent molecular weights depend on both the gel system used and the source of the virus (35). The nomenclature of Teramoto et al. (43) and Cardiff et al. (44) will be used to denote these viral proteins.

The Viral Genome

The genetic material of MMTV consists of two apparently identical single-stranded RNA molecules that are held together by an associated cellular transfer RNA (45,46). Each subunit of RNA has a sedimentation coefficient of 35S (47) and consists of approximately 9000 bases of genetic information. In agreement with other retrovirus systems, the MMTV genome codes for three classes of viral structural proteins, including the nonglycosylated proteins of the viral core (*gag* gene), the viral reverse transcriptase (*pol* gene), and the glycoproteins of the viral envelope (*env* gene) (see Fig. 1) (35).

In addition to the *gag*, *pol*, and *env* genes, MMTV has the coding potential for a fourth gene. The existence of such a gene was first suggested by the *in vitro* translation of 3′-specific MMTV RNAs. The resulting protein products (36K, 24K, 21K, and 18K in size) (48-51) were related to each other, but could be distinguished from the *gag*, *pol*, and *env* proteins by tryptic peptide mapping (48). Subsequent cloning and sequencing of portions of the MMTV proviral genome revealed the existence of an approximately 1000 base pair (bp) open reading frame (orf) within the U3 region of the proviral long terminal repeat (LTR) (Fig. 1) (20-23). The function of this gene remains unknown, but is considered below and in the chapter by G. Smith in this volume.

Structural Proteins of the Virus Particle

The proteins comprising the envelope of the MMTV particle are glycosylated, a feature in common with other enveloped viruses (52). The proteins of the viral core are nonglycosylated. The nomenclature for the MMTV structural proteins, in keeping with that of other retroviruses (53), includes the prefix gp (for the glycoproteins), p (for the nonglycoproteins), or pp (for the major phosphoprotein of the virus), followed by a number designating the mass of the protein in daltons ($\times 10^{-3}$).

gp52. The most abundant protein of the MMTV particle, gp52, comprises approximately 25% of the protein of the virus (35). The spikes of the viral envelope (Fig. 2) (29) are believed to consist almost entirely of gp52, since trypsin (44) or low pH (54) treatment results in "bald" particles that lack this protein. gp52 is the only protein of the particle accessible for lactoperoxidase-catalyzed iodination (55, 56), confirming its surface localization on virions. Bifunctional protein cross-linking reagents have shown that gp52 is anchored to the lipid bilayer of the envelope via disulfide bonds with the transmembrane MMTV glycoprotein, gp36 (57-59).

gp52 is modified by glycosylation. When analyzed by isoelectric focusing procedures, gp52 migrates with a variable pI (5.8-7.0) (60),

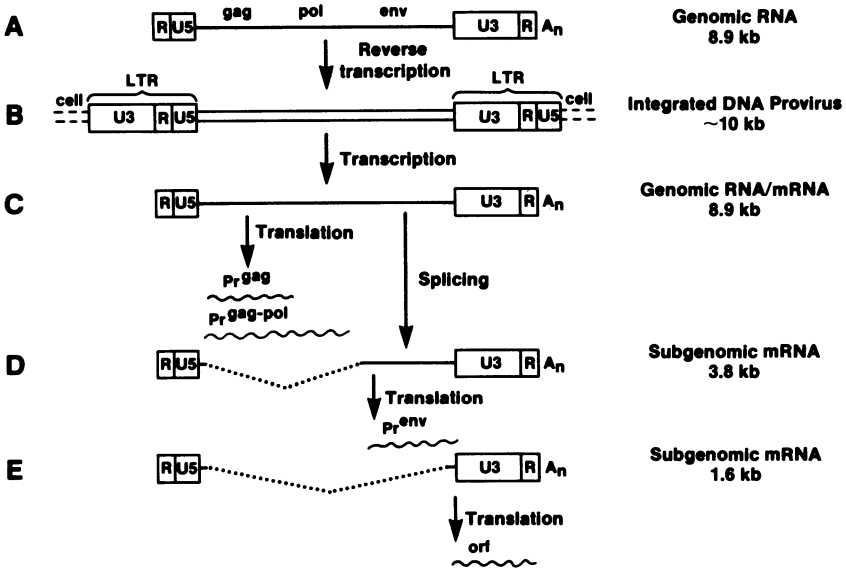

Figure 1. MMTV replication cycle. Genomic RNA (A) is reverse transcribed into double-stranded DNA which gets inserted into host cell DNA (dashed lines) as a DNA provirus (B). The provirus serves as a template for MMTV mRNA synthesis (C), some transcripts of which undergo splicing (D, E). The mRNAs are translated into gag (C), gag-pol (C), and env (D)-specific polyprotein precursors [and perhaps an orf protein (E)]. The structural protein precursors are processed and assembled with genomic RNAs to produce progeny virions. See text for details. [Modified from Dickson and Peters (35).]

consistent with the presence of several carbohydrate groups. Additionally, gp52 binds the lectin concanavalin A (58, 61) and can be metabolically labeled with radioactive sugars (62-64), with mannose and glucosamine being the predominant sugars of the protein. As gp52 is sensitive to digestion with the glycosidic enzyme endoglycosidase H, it is classified as a high-mannose-type glycoprotein (64, 65).

gp36. The second viral glycoprotein present in the lipid envelope of the MMTV particle is designated gp36. gp36 is not accessible to labeling by lactoperoxidase-catalyzed iodination of intact virus particles, but can be

labeled within the particle by the NaB(^{3}H)$_4$ oxidation-reduction procedure that labels exposed galactose residues (64,66). It exhibits a variable pI on isoelectric focusing (60) and can be metabolically labeled with radioactive sugars (35). In contrast to the high-mannose carbohydrates found on gp52, gp36 contains less mannose and relatively more glucosamine, galactose, and fucose residues and is considered to be a "complex-type" glycoprotein. The gp36 protein moiety contains two major regions of hydrophobicity (23,67,68), supporting its proposed function as a transmembrane protein which anchors gp52 in the lipid bilayer of the envelope.

p28. One of the most abundant proteins of purified virus, p28 is the

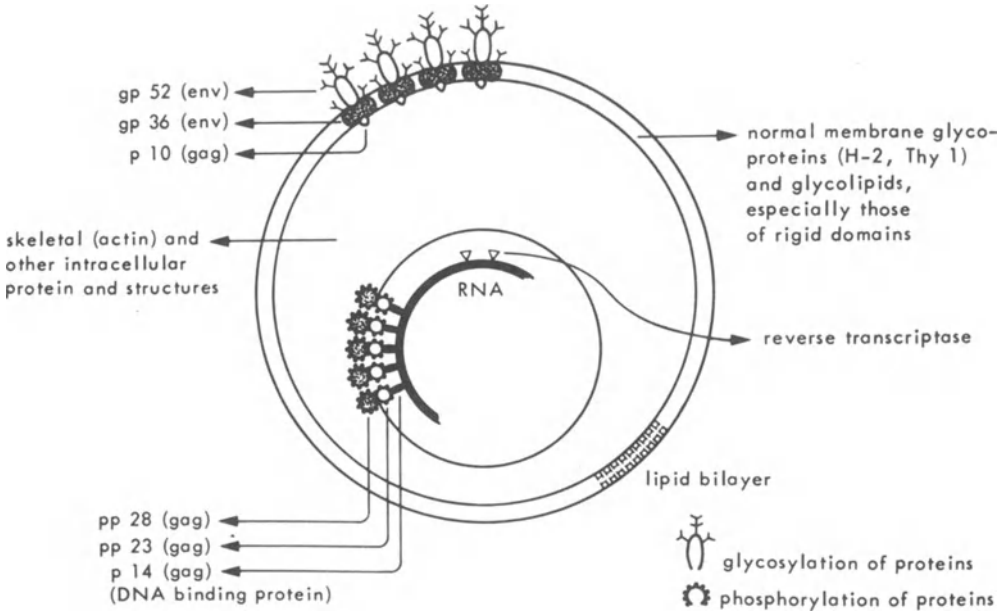

Figure 2. The MMTV particle. The type B retrovirus particle consists of an RNA-containing, electron-dense core surrounded by a lipid envelope. The two viral glycoproteins, gp52 and gp36, are localized within the lipid bilayer. The gag- specific proteins p28, pp23 and p14 constitute the viral core. The smallest gag protein, p10, becomes associated with the lipid envelope during a late stage of virus maturation. [Taken with permission from Bentvelzen and Hilgers (38).]

major protein of purified viral cores (Fig. 2) (35,43,69-72). The majority of the p28 molecules of the virus are phosphorylated (73,74), but the degree of phosphorylation is not uniform, as reflected by variability in the isoelectric point of this protein (pI 6.5-6.8) (60).

pp23 Although a very minor component of the virus particle, pp23 is the major phosphoprotein of MMTV (57,73,74). Several species of pp23 exist and can be separated by isoelectric focusing (pI 4.5-5.0) (60). Similarly sized phosphoproteins of an avian retrovirus (pp19) (75) and a murine retrovirus (pp12) (76) are associated with their viral RNAs. These phosphoproteins may be involved in the regulation of viral RNA expression, although

their precise function remains to be determined. A similar role for pp23 of MMTV may eventually be defined.

p14. As observed with several avian and murine C-type retroviruses, MMTV cores contain a highly basic (pI 8.8) (60), low-molecular-weight protein, p14, which has the capacity to bind single-stranded nucleic acids (77-80). As p14 can bind single-stranded RNA, it is possible that the protein is involved in packaging viral RNA into the core.

p10. The most hydrophobic protein of the MMTV particle is p10, a major constituent of the virion (Fig. 2) (67). p10 is considered to be gag-related because the high-molecular-weight gag polyprotein precursor reacts with antisera monospecific for p10 (44,81). However, the absence of p10 from purified preparations of MMTV cores suggests that in mature virus particles p10 may be associated with the viral envelope, perhaps being responsible for the eccentricity of the viral core by binding it to a site on the interior of the envelope (44).

p30. A protein sometimes found within the MMTV particle is p30. Although this protein shares some antigenic determinants with p14 (82-84), it contains several unique tryptic peptides not found in any gag-related protein (82, 85). Its presence in viral particles is variable and little is known about p30. It may represent a read-through product that may accidentally get packaged into virions due to the interaction of its p14 determinants with viral RNA.

Non-structural Proteins

Polymerase As with all replication-competent retroviruses, MMTV codes for and carries within the virus particle an RNA-dependent DNA polymerase. The polymerase, which reverse transcribes the viral RNA into DNA during initial stages of infection by MMTV, is present in approximately 70 molecules per virus particle (86). The MMTV reverse transcriptase displays a strong preference for magnesium over manganese as the divalent cation in *in vitro* assays (35), a feature in common with the polymerases of the human T-cell lymphotropic viruses (87,88).

B. Virus Replication

Infection of Cells

Initiation of infection by MMTV involves a specific interaction between the surface membrane of the viral envelope and a receptor on the exterior cell surface. This specific interaction presumably involves the MMTV glycoprotein, gp52. Electron microscopic examination of MMTV-infected cells shows that the interaction of the virus with the cell involves the spikes of the viral envelope (89). Further, the infectivity of virus pseudotypes that contain an MMTV envelope around the genome of vesicular stomatitis virus (90,91) or Kirsten sarcoma virus (92) can be neutralized by antisera specific for MMTV gp52.

The mechanism of viral penetration into the cell is unknown. The virus may enter via a phagocytic mechanism (89) or, by analogy with C-type murine leukemia virus, the host cell membrane may dissolve at the site of virus attachment due to enzymes present in either the cell membrane or the viral envelope (93).

DNA Synthesis and Integration of MMTV DNA into Host DNA

Once inside the cell, MMTV is uncoated in the cytoplasm (89) and the virus-encoded reverse transcriptase (94,95) transcribes a double-stranded (ds) DNA molecule from the template viral RNA genome (96). The dsDNA is

then transported to the nucleus where it becomes circularized into a covalently closed circle (96,97) prior to integration into the chromosomal DNA of the cell (98-100).

The process of MMTV DNA synthesis and circularization of the dsDNA molecule results in the duplication of 3'-specific viral sequences at the 5' end and vice versa (Fig. 1). This repeated area at the ends of the integrated provirus is known as the viral LTR. Whereas the LTRs of other retroviruses are approximately 300-600 bp in length (101), the LTR of MMTV is considerably larger and approaches 1300 bp in size (20-23, 97).

The precise mechanism by which the MMTV circular dsDNA integrates into host DNA is unknown. The integration event is site-specific with respect to the virus (98,100,102) and shares at least three features in common with insertion events of movable genetic elements (103). First, sequencing data show that the ends of the MMTV LTR contain a 6 bp complementary palindrome (21,104,105). Second, 6 bp of host sequences are duplicated at the site of integration, immediately adjacent to both ends of the provirus (21,104,105). Similar-sized host sequence duplications are observed for murine sarcoma virus (4 bp) (106) and avian spleen necrosis virus (5 bp) (107). Finally, the terminal 2 bp (TG....CA) of the LTRs are the same as those at the end of all eukaryotic movable genetic elements (108). The integration of MMTV into cellular DNA requires no apparent specificity in the nucleotide sequence of the host DNA and appears to occur at random sites (98, 102, 109).

Synthesis of Viral RNA

Although MMTV circularized dsDNA can serve as the template for viral transcription in rare cells that carry MMTV in episomal form (27), it is believed that the integrated MMTV provirus usually serves as the template for transcription of viral mRNA.

MMTV transcription has been studied by the isolation of poly-A-containing RNA from MMTV-producing cells, size fractionation of the RNA via velocity sedimentation centrifugation or agarose gel electrophoresis, followed by detection of the separated MMTV-specific RNAs by molecular hybridization to a radiolabeled MMTV cDNA probe. Two major classes of MMTV-specific mRNA have been identified: 8.9 kb (or 35S) and 3.8 kb (24S) in size (110-114). A less abundant, virus-specific mRNA, 1.6 kb in size, has also been detected (51,115,116). The 3'-specific termini of the 8.9-, 3.8-, and 1.6-kb RNAs are polyadenylated (51,115,116). All three mRNAs share sequences at their 5' termini (111,115), suggesting that the 3.8- and 1.6-kb species are generated from the 8.9-kb species by a splicing event.

The coding capacity of each of the three size classes of MMTV mRNA has been determined by *in vitro* translation followed by immunoprecipitation of the resultant proteins using monospecific antisera. The 8.9-kb mRNA serves as a template for the translation of the proteins of the MMTV core, as well as the RNA-dependent reverse transcriptase (48,73,114,117,118). The 3.8-kb mRNA codes for the envelope glycoproteins of MMTV (48,113,114,119). The 1.6-kb mRNA contains the information for the orf protein encoded by the MMTV LTR (51,115,116) (Fig. 1).

Synthesis of Viral Proteins

The primary translation products of the MMTV 8.9- and 3.8-kb mRNAs consist of high-molecular-weight polyprotein precursors, designated $Pr77^{gag}$, $Pr110^{gag}$, $Pr160^{gag\text{-}pol}$, and $Pr73^{env}$ (35). The precursors undergo a series of co- and post-translational modifications and are processed into the mature structural proteins of the virus. *In vitro* translation of either genomic RNA or the 8.9-kb mRNA (48,50,73,113,114,117), as well as metabolic

labeling of MMTV-producing cells (62,82,120-122), has yielded three high-molecular-weight proteins (77, 110 and 160K daltons in mass) that react with antisera monospecific for gag-related p28. The 77K protein represents the gag-specific polyprotein precursor (82), and the 110K and 160K proteins likely arise as a result of read-through into the adjacent polymerase gene of the virus (Fig. 1) (123-126). The order of viral proteins within the gag gene has been established as NH_2-p10-pp23-p28-p14-COOH (82-84).

Similar analyses identified the 3.8-kb mRNA as being env-specific (48,50,65,111,113,114,119,121,127). The 66-68K size of the precursor protein translated *in vitro* is considerably larger than the 60K apoprotein defined *in vivo*, indicating the existence of a cleavable signal sequence that is co-translationally removed *in vivo* (23,65,68,119,128,129). The env 60K apoprotein is co-translationally modified by the addition of 5 asparagine-linked, mannose-rich oligosaccharides (23,64,68). The resulting mature env precursor, designated $Pr70^{env}$ (130) or $Pr73^{env}$ (64,119), then undergoes cleavage into gp52 and gp36 en route to the plasma membrane (34,127). The order of the individual MMTV glycoproteins within the env precursor has been established as NH_2-gp52-gp36-COOH (23,64,68,84,131).

Assembly of virus particles in MMTV-producing cells involves an interaction of the $Pr77^{gag}$-containing viral core particles (28,44, 81,132,136) with the viral glycoproteins at the cell surface. Cleavage of $Pr77^{gag}$ into the individual gag structural proteins occurs during, or possibly after, budding of the newly formed virus particle into the extracellular medium. The budding process occurs without apparent harm to the cell, and the released, mature virus can proceed to infect another cell via gp52-cell surface interactions.

III. ENDOGENOUS MMTV

Nucleic acid hybridization studies have demonstrated that many normal animals from several different species contain DNA homologous to the genomes of certain weakly transforming retroviruses (137-139). For MMTV, these virus-related sequences are estimated to be present in about 3-6 genome equivalents per diploid genome (140,141). The endogenous MMTV-related sequences have been found in germ line DNA of all inbred strains of mice and in most, but not all, feral mice (25,109,142). The absence of detectable endogenous MMTV-related sequences in occasional feral mice indicates that these virus-related sequences are not required for normal mammary gland development (143,144). A similar conclusion was reached regarding the lack of involvement of endogenous retrovirus sequences in normal development of chickens (145).

A. Organization of Endogenous MMTV within Normal Cell DNA

Endogenously transmitted MMTVs with transforming activity have been isolated from certain inbred strains of mice (11,24,146). This observation suggested that at least some of the endogenous MMTV-related DNA must code for an entire viral genome. Indeed, the endogenous MMTVs are organized into discrete units termed proviruses (38), which are flanked on each end by viral LTRs (Fig. 1) (147). Because the organization of the endogenous MMTVs so closely resembles that of proviruses which arise during infection of cells by milk-transmitted MMTV, the endogenous MMTVs are believed to be the result of rare infections of germ line DNA (137,141,147).

At least 15 different endogenous MMTV loci have been identified in germ line DNA of inbred mice (141), with each inbred strain harboring a distinct subset of these 15 loci (80,102,147-151). The endogenous MMTVs are designated numerically (Mtv-1, Mtv-2, etc.) and are distinguished from one

another by the size of MMTV-specific fragments generated by an EcoRI digestion of liver DNA (141).

B. Regulation of Expression of Endogenous MMTVs

The expression of 2 of the 15 endogenously transmitted MMTVs is directly related to tumorigenesis. Both the Mtv-1 locus in DBAf and C3Hf mice (152-155) and the Mtv-2 locus of GR mice (154,156,157) are expressed and directly responsible for mammary tumorigenesis via an insertional mutagenesis pathway. These two examples, however, are the exception to the general rule that the expression of endogenous MMTVs is under some type of regulation that results in proviruses that are silent or expressed only at very low levels (27). Several different mechanisms have been proposed to explain this regulation of endogenous MMTV expression, although a certain degree of overlap exists among the different proposed categories of regulation.

Defective Nature of Certain Endogenous MMTVs

The simplest explanation for a silent MMTV provirus is that the provirus itself is defective and, therefore, incapable of serving as a template for complete virus replication. Such a defect might be either in the regulatory region (LTR) or in a coding region (gag, pol, env) of the provirus. Two examples of such defective proviruses exist. The Mtv-6 endogenous MMTV of BALB/c mice lacks much of its coding sequences, but contains sequences that react with an LTR probe (115,147). Although this provirus is not capable of supporting virus replication, it may be able to serve as a template for the expression of LTR-specific mRNA (158).

A second defective endogenous provirus is the Mtv-8 locus (found in BALB/c, CBA, C3H, C57BL, DBA, and GR mice). Cloned Mtv-8 DNA, when transfected into heterologous cells, directs the synthesis of MMTV RNA and proteins, yet no virus particles are made (27). DNA sequencing of the Mtv-8 provirus demonstrated the existence of a stop codon in the env gene that would result in a truncated env precursor protein (159). This defect is believed to be responsible for the lack of virus production. The remaining endogenous MMTVs have not been analyzed in detail, so it is unknown if any of the other proviruses contain similar defects.

Regulation of Endogenous MMTV Expression by Neighboring Control Elements

Some element adjacent to an MMTV provirus may regulate the expression of that provirus. This concept gained support with the observation that crosses between two different mouse strains that lacked MMTV expression produced progeny in which the expression of endogenous MMTV was readily detected (24). It was speculated that the crosses had removed a neighboring controlling element, thus releasing the MMTV from some type of regulatory constraint. Because the 15 endogenous MMTV proviruses have been mapped to many different chromosomes (141,151), such a hypothesis for gene regulation would require the existence of a neighboring controlling element for each of the proviruses. Recent analysis of MMTV expression in inbred hybrid mice has revealed the existence of nonviral gene products that can influence MMTV expression (160).

Regulation of Endogenous MMTV Expression by Modification(s) of Proviral DNA

Endogenous MMTV transcription might be reduced or prohibited due to constraints exerted on the proviral DNA. Methylation of DNA and ADP-ribosylation of chromosomal proteins represent two such types of mechanisms.

Methylation of specific cytosine residues in the DNA of eukaryotic genes has been proposed as a means by which the expression of such genes can be regulated (161,162). The possibility that methylation of endogenous MMTV is responsible for the regulation of its expression has been examined using restriction enzymes that are sensitive to the presence of methyl groups. DNA is digested with enzymes that are unaffected by the presence of methyl groups or with enzymes that are sensitive to the presence of methyl groups and the MMTV-containing DNA fragments compared by Southern blot analysis.

The endogenously transmitted MMTVs are usually heavily methylated and resistant to digestion with methylation-sensitive enzymes (163-168), while newly acquired proviruses introduced by foster-nursing experiments are hypomethylated (163,165). However, notable exceptions exist. Hypomethylation of endogenous MMTVs has been observed in certain normal and tumor tissues (155,164,165,167-174). Specific transcription of MMTV sequences usually correlates with hypomethylation, but exceptions do occur (171). Therefore, it is difficult to conclude whether hypomethylation of an endogenous MMTV provirus is biologically important. A causative link between hypomethylation and endogenous MMTV expression in the etiology of mammary tumorigenesis has certainly not been proven.

A possible role for ADP-ribosylation in regulating MMTV expression has been considered. Certain ADP-ribosylated chromosomal proteins lose ADP-ribose in the presence of either dexamethasone or inhibitors of ADP-ribosylation (175). As MMTV expression is enhanced by either of these two treatments, a correlation exists between the level of ADP-ribosylation of specific chromosomal proteins and MMTV expression. However, the cell line used for these experiments contained both exogenous and endogenous MMTVs and whether a similar mechanism might affect endogenously transmitted MMTVs has not been addressed. Further, the precise mechanism by which an ADP-ribose-mediated regulatory pathway might function remains to be determined.

Post-Transcriptional Blocks in Endogenous MMTV Expression

Once an endogenous provirus has been transcribed into RNA, the next level at which control of virus expression might be achieved is that of translation of the mRNA. One example of such regulation exists in the low-tumor-incidence C57BL strain of mice. The lactating mammary glands of C57BL females contain large amounts of MMTV-specific mRNA (176-178), but no viral proteins are detected. These viral mRNAs are processed and transported to the cytoplasm where they associate with polyribosomes. However, no translation occurs *in vivo*, although the mRNAs can be translated *in vitro* (179).

It is interesting to note that C57BL mice are resistant to infection by the prototype (C3H)MMTV, suggesting that a similar block in virus replication might extend to other exogenous MMTVs introduced into these mice. However, C57BL mice are susceptible to infection by (RIII)MMTV and (GR)MMTV (37). These two latter MMTV isolates are associated with mammary tumors of ductal, rather than alveolar, morphology, suggesting that those particular isolates replicate in ductal cells. Therefore, the block in virus expression observed in C57BL animals appears to be limited to the alveolar cells of the mammary gland.

Several examples exist in which endogenous MMTV sequences are transcribed and translated but mature virus particles are not formed due to a post-translational block. Thymic lymphoma cells of GR mice express the MMTV *env* precursor that is rapidly metabolized without being processed into gp52 and gp36 (180). Similarly, leukemic cells of DBA/2 mice have been shown to contain a hyperglycosylated *env* precursor that is not processed into gp52 and gp36 (181). In both instances, the block in *env* maturation is assumed

to be responsible for the lack of virus production. The processing block could reflect either a defective provirus template or an inability on the part of the leukemic cells to support step(s) necessary for the complete replication of MMTV.

Influence of the Differentiated State of the Cell on Endogenous MMTV Expression

Cell differentiation involves the expression of new sets of genes. Depending on the site at which an endogenous MMTV resides in the cellular DNA, the expression of that provirus might be influenced by the physiologic state of the cell. This idea has support from studies of MMTV proviruses of exogenous origin. McGrath and coworkers (182) found that mammary tumor cells that retained the ability to organize into alveolar structures produced MMTV particles. However, after serial transplantation, the tumor cells lost the ability to form alveoli concomitant with a loss in virus expression. Thus, differences in cell phenotype could be correlated with virus expression. Recent studies in BALB/c mice have shown that endogenous MMTV expression varies during mammary gland development associated with pregnancy and lactation (173). Different provirus templates appeared to be regulated independently. The precise mechanisms underlying the influence of the differentiated state of the cell on endogenous MMTV expression remain to be determined.

IV. TRANSFORMATION OF CELLS BY EXOGENOUS MMTV

Although MMTV was demonstrated to play a causative role in mouse mammary tumorigenesis nearly 50 years ago, the complex biology of the mammary tumor system has thwarted understanding of the precise mechanism by which the virus transforms cells (26,30,183). Whereas transformed fibroblasts display alterations in growth properties and cell interactions, no such changes are reproducibly observed in comparisons of normal mammary epithelial cells and their transformed counterparts (184-186). The lack of an *in vitro* transformation assay in the MMTV system has severely restricted the ability to define the molecular events required for the initiation and maintenance of the tumor phenotype.

The formation of a tumor is a multi-step phenomenon well demonstrated in a variety of model systems (187-189). This multi-stage concept has recently received support from the field of oncogene research in which oncogenes have been categorized as providing either a nuclear (immortalizing) or cell surface (transforming) function; both types of activity are usually required to mediate the complete transformation of a primary cell (190,191). Although that classification of oncogenes is overly simplistic, it serves to emphasize that multiple cooperating changes must occur before a normal cell assumes a completely transformed phenotype. The precise nature of the growth changes that take place in mammary epithelial cells during mammary tumorigenesis, however, are not yet understood.

Exogenous MMTV is transmitted to suckling mice through the milk from a virus-positive mother. Such a route of inoculation is not particularly efficient for virus transmission; newborns must nurse for approximately 3 days on a virus-positive mother in order to ultimately develop mammary tumors at an incidence predicted by the maternal tumor incidence (192). Once introduced into newborn mice, MMTV is taken up by the gut, becomes blood-borne (193), and spreads to various organs of the mouse (24).

The amount of MMTV present in the milk is directly related to the percentage of alveolar cells that are MMTV antigen-positive (183) and correlates with the mammary tumor risk of both the mother and the offspring (183,194,195). Therefore, effective transmission of the milk-transmitted

virus and subsequent tumor formation is dependent on the expression of high levels of MMTV in the mammary gland.

A. Preneoplasia in the Mouse Mammary Tumor System

Consistent with tumor formation being a multi-step phenomenon, an intermediate stage composed of preneoplastic cells, designated the hyperplastic alveolar nodule (HAN), is recognized in the mouse mammary tumor system. A HAN is a focal area of hyperplastic alveolar growth in the mammary gland which appears spontaneously with high frequency in certain inbred mouse strains (146,152,196), as well as in rats, dogs and humans (197). This hyperplastic nodule can be visualized *in situ* within the mammary gland of anesthetized mice. A HAN is considered to be preneoplastic because, when transplanted into cleared mammary fat pads, it progresses to form tumors at a rate significantly higher than does similarly transplanted normal mammary tissue (198).

In a low-HAN-incidence strain of mouse, such as BALB/c, HAN can be induced by the introduction of exogenous MMTV by foster-nursing (199) or by hormonal or chemical carcinogen treatment of the mice (200). The HAN produced by these different types of treatments appear morphologically similar (200).

Individual excised HAN can be maintained *in vivo* by transplantation into cleared mammary fat pads of syngeneic mice (201). The tissue will grow to fill the mammary fat pad and will organize into either alveolar or ductal morphology. The resultant outgrowth tissue can be serially transplanted indefinitely, and thus is distinguishable from normal mammary tissue which has a finite life span *in vivo* (5-6 transplant generations) (200). The preneoplastic outgrowth tissue can be distinguished from neoplastic mammary tissue in that the growth of preneoplastic cells is mammary fat-pad-dependent and subject to local growth regulation controls. In contrast, mammary tumor growth will not remain confined to the fat pad.

The morphological and growth characteristics of a preneoplastic outgrowth line remain stable during serial transplantation (201). Additionally, preneoplastic outgrowth lines progress to form tumors at a frequency characteristic for each line. Although the tumor potential of a preneoplastic line is generally stable over prolonged serial transplantation, the addition of various agents can influence that tumor potential. The same types of agents that induce nodule formation (e.g., viral, hormonal, carcinogenic) also can act upon preneoplastic cells to increase the tumor potential of a given line (201).

The preneoplastic HAN populations provide a powerful tool for studying molecular events involved in mammary tumorigenesis. It is difficult to draw meaningful conclusions regarding mechanisms of transformation by studying gene expression only after a tumor has developed. As tumors continue to undergo changes, a process termed "tumor progression" (202,203), it is not possible to distinguish between an event that led to tumor formation and one that resulted from tumor progression. By evaluating gene expression in preneoplastic cells, it may be possible to dissect cause and effect relationships in tumor development. The role provided by MMTV in mammary tumorigenesis may be in the initiation of the preneoplastic nodule, or in the preneoplastic-to-neoplastic conversion, or both.

B. Mechanism of Cell Transformation by Exogenous MMTV

The approaches utilized for studying MMTV-induced tumorigenesis have drawn heavily from other RNA tumor virus systems. The mammary-specific nature of MMTV-induced carcinomas suggests that MMTV does not share a

transformation mechanism with any other known RNA tumor virus. However, as most tumors probably arise following multiple cellular alterations, it may be that certain events in the MMTV transformation pathway will be found to have common features with those of other retroviruses.

The RNA tumor viruses can be divided into two basic groups with respect to transforming capabilities. Transformation by "acutely transforming" retroviruses (e.g., Rous sarcoma virus, Abelson leukemia virus) is mediated by a single unique gene carried within the virus, termed the viral oncogene, or v-onc (190, 204-207). In contrast, the "weakly transforming" retroviruses (e.g., avian leukosis virus (ALV) and murine leukemia viruses) lack a characteristic viral oncogene and utilize a separate type of pathway to accomplish transformation of cells. ALV-induced tumors arise from an infected cell in which a provirus has fortuitously inserted in a chromosome next to a silent cellular oncogene (c-onc), with subsequent activation of that gene (208-211).

MMTV is considered to be a replication-competent, weakly transforming virus because the tumors it induces do not arise until 6 to 12 months after infection (26). Because MMTV does not contain a viral oncogene analogous to those of the acutely transforming retroviruses, efforts have been directed at elucidating a mechanism of viral transformation more similar to that described for ALV. Two general processes can be envisioned for the involvement of MMTV in tumorigenesis. First, the site at which the MMTV provirus integrates into cellular DNA may be crucial. Alternatively, a viral gene product might mediate some step of the transformation process. As MMTV possesses no transforming gene per se, this hypothetically important viral gene would be the orf region of the LTR.

MMTV Proviral Integration and Mammary Tumorigenesis

The single-cut restriction enzyme EcoRI has been used to analyze the proviral content of MMTV-induced mammary tumors. EcoRI digestion of MMTV-containing cellular DNA generates two MMTV-containing fragments per provirus, the size of which depends upon the location of the next EcoRI site in the flanking cellular DNA sequences. Whereas proviruses insert randomly (with respect to the cell) in a large number of sites in the infected mammary gland (98), most subsequent mammary tumors consist of a clonal expansion of a single infected cell as evidenced by a uniform provirus pattern. Amplification and reinsertion of new proviruses is noted in nearly all MMTV-induced tumors (98,149,154,155,212-214). The exception to this general rule is found in the European strain GR mouse in which mammary tumors are quasi-clonal with respect to new proviral inserts (148,154).

When the lengths of virus-host cell DNA fragments from different EcoRI-digested mammary tumor DNAs are compared, a very complex pattern emerges. Although proviral integration always utilizes the same sites on viral DNA (and, therefore, preserves both LTRs), the cellular site of integration appears to be different in each tumor.

An alternative approach revealed that MMTV proviral insertion into a given region of cell DNA (as opposed to a specific site) may be important in tumorigenesis. An EcoRI fragment from an MMTV-induced mammary tumor that contained a single new provirus was isolated, and the cellular DNA flanking the new insert was cloned. This cloned cellular DNA was used to retrieve a 25-kb region of normal (uninfected) cell DNA from a mouse DNA library. This region of cellular DNA, termed int-1, was then shown to be occupied by a new proviral insert in 19 of 26 virus-induced mammary tumors of C3H mice (214, 215). Although MMTV proviruses can insert randomly in cell DNA, those infected cells that contain a provirus inserted into the int-1 region appear to be selected and expand clonally to form tumors. A second such preferred

integration region, termed int-2, which shares no sequence homology with int-1, has been described for the BR6 strain of mouse (216,217).

Insertion of a provirus into int-1 or int-2 apparently activates the expression of int-specific mRNA that is not expressed in normal mammary tissue (215,217). Analysis of the orientation of proviruses inserted within the int regions suggests that activation of int gene expression is by means of enhancer activity by the MMTV LTR (215,217). The int genes are conserved across species (215), and the 2.6-kb (int-1) and 3.2-kb (int-2) mRNAs share no homology with known oncogenes (215). As only 75% of mammary tumors in the systems examined contained proviral inserts in either int-1 or int-2 (217), the possibility exists that additional int regions may remain to be identified in the MMTV system.

The positioning of an MMTV provirus in a crucial region of cellular DNA appears to be an early event in the multi-step process of mammary transformation. Preneoplastic HAN tissue is often clonal with respect to proviral inserts (158,174,218-220), although such homogeneity may be altered during passage of the HAN tissues in vivo (220). Tumors that arise from such clonal preneoplastic populations may contain proviral contents identical to the parental HANs (218-220), although some tumors may differ (174). It is evident that repositioning of MMTV proviruses is not an absolute prerequisite for the transition of mammary cells from the preneoplastic to the tumor phenotype.

The Involvement of Cellular Genes in MMTV-Induced Cell Transformation

Other contributing events in addition to proviral insertion must occur within a cell to prompt its eventual autonomous growth as a tumor cell. These events most probably involve the perturbation of normal cellular functions. Although our understanding of normal eukaryotic cell physiology is severely limited, studies of the v-onc genes and their cellular counterparts (c-onc) have led to certain unifying hypotheses concerning possible mechanisms of cell transformation. It is reasonable to consider that MMTV-induced mammary tumorigenesis may involve the cooperation of certain c-onc genes.

Cellular oncogenes have been discovered in either of two ways. Some c-oncs have been transduced into viral genomes by a recombination event between a retrovirus and the cellular gene, resulting in the acutely transforming retroviruses described above. Alternatively, c-oncs have been identified in transfection assays using tumor cell DNA. As many as 40 different oncogenes have been identified thus far (190,191,221,222).

The mechanism of involvement of activated c-oncs in transformation is unclear, although two general modes of action have been proposed. Transformation could be due either to the inappropriate expression of a normally silent or strictly regulated gene, or to a mutated gene product no longer able to perform its normal cell function. Numerous examples exist that support each hypothesis (190,221,222). It also appears that a single onc gene may act by either of these basic mechanisms.

An important concept in onc-gene-mediated transformation was elucidated using the 3T3 transfection assay. Whereas certain onc genes are capable of inducing foci when transfected onto 3T3 monolayers, others are not (191,223,224). This observation prompted the hypothesis that known onc genes can be divided into two categories based on function. The first group of onc genes provides an "immortalization" function, while the second provides the "transforming" function that converts an immortalized cell to the tumor phenotype (190,191). The immortalizing genes, whose products

localize to the nucleus (e.g., myc, fos, myb), are unable to induce foci when transfected onto established (immortalized) cells, such as 3T3. The second group, whose gene products are found in the cytoplasm or in the plasma membrane, are able to induce foci in immortalized cells but not in nonimmortalized primary cells. Although exceptions exist, both types of functions are usually required to attain complete transformation of primary cells.

The conservation of c-onc gene sequences across species suggests that these genes provide some function basic to normal cell metabolism. This concept has been verified by the identification of three of the onc genes. The sis transforming gene, isolated from the simian sarcoma virus, shares amino acid sequence homology with platelet-derived growth factor, whose normal cell function is to stimulate cell growth (225,226). The erb-B transforming gene of avian erythroblastosis virus (227) shows approximately 90% homology with the trans-membrane and internal portions of the epidermal growth factor receptor (227), and the v-fms gene from feline sarcoma virus exhibits extensive homology with the receptor for colony-stimulating factor (228). Although the precise pathways of action of these oncogenes are not understood, it is conceivable that the elevated levels of the normal cell proteins present in virus-infected cells upset the delicate balance of one or more growth factor pathways within the cell and lead to the uncontrolled growth of the transformed phenotype.

Role of the LTR in MMTV Transformation

The second possibility for viral involvement in MMTV-induced tumorigenesis centers on the viral LTR. Potentially, the LTR could contribute to tumorigenesis by activating the expression of cellular genes due to enhancer-like activity (discussed above) or, alternatively, the protein encoded by the LTR orf could provide a function partially responsible for phenotypic alterations of the mammary cell.

The coding region of the LTR is sufficiently large to code for a 36K protein (20-23), but the functional role of the LTR orf protein remains speculative. The fact that this orf region is highly conserved in all MMTV LTRs analyzed, whether from endogenous or exogenous viruses, suggests that the gene product has some important role in the life cycle of MMTV. However, any role of the orf protein in the hormone induction of MMTV expression has been discounted because deletion mutants lacking the entire orf region remain responsive to hormones (19,229). Whether the orf protein is involved in the development of either MMTV- or carcinogen-induced mammary tumors is also unknown. A putative role as a transforming gene analogous to those carried by the acutely transforming retroviruses is unlikely in view of the long latency of MMTV-induced tumors.

Although the mRNA from the LTR orf has been identified (51,115,116) and this mRNA is translatable in vitro (51), no protein product has yet been identified in vivo. As more than one event is required for the transformation of epithelial cells by MMTV (and those specific events remain unidentified), the identity and function of the LTR gene product may provide insight into the mechanism(s) of mammary tumorigenesis.

V. ENDOGENOUS MMTV AND MAMMARY TUMORIGENESIS

As all inbred strains of mice harbor endogenously transmitted MMTVs, a role for these viral sequences in mammary tumorigenesis has been sought. Similarities between the genome organization of endogenous MMTVs and the new proviruses that arise during infection by exogenous MMTVs lend credence to a possible role for the endogenous MMTVs in the genesis of mammary tumors of nonviral (i.e., carcinogen, irradiation) etiology.

The contribution of endogenous MMTVs to tumorigenesis has been studied in strains of mice, such as BALB/c, C57BL, and 020, which lack a milk-transmitted MMTV and consequently experience a low incidence of late-occurring, spontaneous mammary tumors (26,27). A high incidence of early-onset mammary tumors can be induced in these inbred mice by the introduction of exogenous MMTVs by foster-nursing, as well as by treatment of the animals with carcinogens, irradiation, or hormonal stimulation (230-233). When the expression of endogenous MMTV was examined in non-virally induced mammary tumors of BALB/c mice, MMTV-specific RNAs were detected in some, but not all, such mammary tumors. Therefore, it appears that the expression of the entire endogenous MMTV genome is not required for the maintenance of the tumor phenotype of all mammary tumors (230, 232-234). Similar results were obtained in studies of C3Hf, C57BL, and 020 mice (177,235).

Although the expression of full-length, endogenous MMTV mRNA does not correlate with the transformed phenotype, the possibility exists that low levels of subgenomic mRNA may be important in tumorigenesis. Because cDNA probes representative of the entire genome were used for the analysis of MMTV RNA expression in the described studies, the expression of a crucial subgenomic viral mRNA may have escaped detection. It is noteworthy that preferential transcription of 3′-specific MMTV sequences was observed in BALB/c lactating mammary glands and mammary tumor cells by Dudley and coworkers (236). Those subgenomic transcripts map to the 3′ LTR of MMTV and are detected in preneoplastic tissues and mammary tumors of BALB/c mice as a 1.6-kb mRNA (115,116). Since the gene product of the 1.6-kb LTR mRNA has not been identified in vivo, the significance of its expression, if any, to mammary tumor development remains unknown.

As mammary tumorigenesis by exogenous MMTVs appears to include insertion of new proviruses into critical cellular loci as an important predisposing factor to tumor formation, one might expect the appearance of one or more new proviruses in mammary tumor DNA if endogenous MMTVs are involved in tumorigenesis by an analogous mechanism. However, when mammary tumors of mice that lack exogenous MMTV (e.g., BALB/c, C57BL, 020) were examined for MMTV proviral content, only some (not all) tumors contained newly acquired proviruses (150,164,165,237).

Together, these data suggest that an insertional mutagenesis-type model, as described for exogenous MMTV-positive mice, is not consistent with mammary gland transformation in low-mammary-tumor-incidence strains of mice. However, the contributions of endogenous MMTV to tumorigenesis by an unknown mechanism cannot be excluded.

The potential involvement of endogenous MMTV in the etiology of other types of tumors in mice has recently been suggested. Certain mouse leukemia cells of T-cell origin reportedly contain endogenous MMTV proviral sequences that are amplified or rearranged (238-240). The oncogenic effect(s) of such proviral rearrangements remain(s) to be proven.

VI. RELEVANCE OF MMTV TO HUMAN BREAST CANCER

The familial pattern of breast cancer in mice is due primarily to the presence of a milk-transmitted virus. The discovery of the Mason-Pfizer virus in a mammary tumor of a rhesus monkey (241) was the first demonstration of an MMTV-like virus in primates and provided impetus for searching for an analogous agent in human breast cancer.

The familial nature of breast cancer in humans has been known for many decades. Women who have family members with breast cancer are at a much

higher risk of developing the disease than are women who lack such a family history (242,243). Efforts to demonstrate a viral etiology for human breast cancer have focused primarily on finding a particle that possesses properties similar to those of MMTV, including immunological cross-reactivity. The inherent weakness in such an approach is that a virus only distantly related, or completely unrelated, to MMTV might not be detected.

Nevertheless, several lines of evidence are compatible with the association of an MMTV-related virus with human breast cancer. MMTV-related antigenic reactivity is detectable in human milk (244,245), in tissue sections of human breast cancers (246-248), and in cyst fluid recovered from benign fibrocystic breast disease (249,250). Antibodies reactive to MMTV are present in the sera of breast cancer patients (251-253) and in cyst fluid (250). [The virus specificity of these antigen and antibody reactivities was established by preadsorption of antisera with intact virus or purified structural proteins of MMTV (247,248,250-253).] Further, MMTV-related sequences have been found in human DNA (254,255) and MMTV-related RNAs detected in human breast cancer cells (256).

It is difficult to interpret the described antigenic and immunologic MMTV-related activities. The percentage of breast cancer patients positive for either antigen or antibody varies greatly from study to study (0 to 70%), suggesting that MMTV-related markers may be associated with only a subset of human mammary cancers. If an MMTV-related virus is a contributing factor in some, but not all, human tumors, care must be exercised in choosing a population of individuals at risk for the virus-associated type of breast cancer. Since women with family histories of breast cancer are much more likely to demonstrate type B virus particles in their milk than are control women (60% positive vs. 7% positive) (257), this group of high-risk women represents a logical choice for further studies in establishing a viral etiology for human breast cancer.

Several recent advances have provided new directions in efforts to identify a human mammary cancer virus. The MMTV-related sequences present in human DNA are only detected under low stringency conditions, suggesting that the MMTV-reactive, 3.7-kb EcoRI fragment is not highly similar to MMTV. The 3.7-kb fragment, which hybridizes with probes specific for the LTR-gag, gag-pol, or env regions of MMTV, has been cloned (254). Detailed analysis of the cloned DNA should establish the degree of relatedness to MMTV.

A newly established human mammary cancer cell line, T47D, offers promise in the search for a human mammary cancer virus. T47D cells produce virus particles with characteristics of a retrovirus, including a buoyant density of 1.18 g/ml, 70S RNA, reverse transcriptase activity, and gp52 (but not gp36 or p28) reactivity (258). The 70S RNA does not hybridize to MMTV probes under conditions of high stringency, but it is not known if the RNA hybridizes to the MMTV-related, 3.7-kb EcoRI DNA fragment cloned from human DNA (254). It is also unclear whether the T47D cells produce enough virus to allow the preparation of human mammary virus-specific immunological reagents.

It should be emphasized that the applicability of the MMTV system to human breast cancer does not depend on finding a related virus particle in humans. Any insights into the molecular events associated with MMTV-induced transformation should broaden our understanding of human breast cancer. For example, the region of normal human DNA homologous to int-2 has recently been cloned (259), so it will now be possible to determine whether this gene is activated in cases of human breast cancer or benign breast disease.

VII. FUTURE DIRECTIONS

The ultimate emergence of a tumor cell from a population of MMTV-infected cells occurs after a long latent period. This latent period possibly reflects the time needed for multiple rare events to accumulate within a cell before it becomes fully transformed. One recognized early event in mammary cell transformation is the insertion of an MMTV provirus into a discrete region of cellular DNA, with subsequent activation of a nearby normal cell gene. This observation emphasizes the importance of virus-induced host cell changes in MMTV tumorigenesis. To better understand the mechanism of MMTV involvement in the transformation process, it will be necessary to identify the int genes and determine their function in normal mammary epithelial cells. The availability of preneoplastic HAN lines will aid in assigning the expression of specific host cell genes to early or late events in the tumorigenesis process.

The etiologic involvement of exogenous MMTVs in mammary gland transformation in the mouse is firmly established. However, the inbred mouse strains also carry related endogenous MMTV sequences, and the potential contribution of these endogenous sequences to the development of mammary gland tumors of nonviral etiology remains unknown.

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ON THE MECHANISM OF CARCINOGENESIS BY MOUSE MAMMARY TUMOR VIRUS

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I. INTRODUCTION

Mouse mammary tumor virus (MMTV) is a classic example of a latently oncogenic retrovirus, its life cycle and tumorigenicity showing many parallels with the leukemia viruses of domestic cats, chickens and laboratory mice (1). Although indisputably linked to the development of particular cancers, such viruses do not themselves encode the oncogenes responsible for cell transformation. Epidemiologically, they are therefore very different from the various isolates of acutely oncogenic retroviruses, in which cellular proto-oncogene sequences have become transduced within the viral genomes (2). While the latter transform cells rapidly and efficiently, both *in vitro* and *in vivo*, the latently oncogenic viruses have little or no influence on cultured cells, and in the animal there is characteristically a long delay between exposure of the virus and overt manifestations of neoplasia. With MMTV, mice are naturally infected by milk-borne virus at birth, yet tumors rarely arise before about 4 months of age, the norm being closer to 6 to 9 months (3,4; and chapter by Slagle and Butel). Most female mice are detectably viremic at pregnancy, and shed high levels of virus in the milk, but although a high proportion of the mammary epithelial cells can become productively infected, they remain apparently normal. Indeed, only very rare infected cells undergo transformation and expand clonally to dominate the resultant tumors, suggesting some stochastic process as opposed to a direct influence of a viral gene product.

II. INSERTIONAL MUTAGENESIS

The clonal nature of MMTV-induced tumors can be effectively assessed by analyzing viral DNA sequences on Southern blots (5-10). As with all retroviruses, replication of MMTV requires the establishment of a DNA copy

of the viral genome, integrated as a provirus within the chromosomal DNA of the host cell (11; and Fig. 1). Insertion of such a large segment of DNA, approximately 10 kilobase pairs (10 kb), inevitably introduces new restriction enzyme sites and creates novel restriction fragments. The most inform

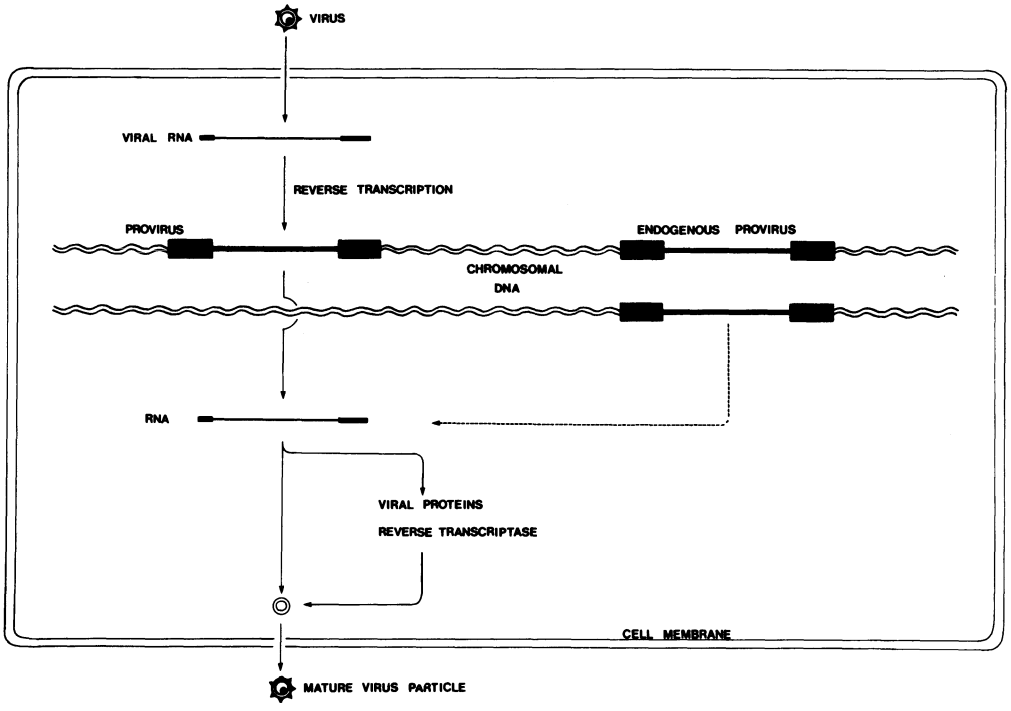


Figure 1. Life cycle of mouse mammary tumor virus.

The diagram depicts some essential features in the replicative cycle of MMTV that may be pertinent to carcinogenesis. Upon entering the cell, the viral particle is disrupted, to expose two copies of single-stranded RNA that comprise the viral genome. This RNA acts as a template for synthesis, by the viral reverse transcriptase, of a double-stranded DNA intermediate which becomes integrated as a provirus within the chromosomal DNA of the host cell. The weight of current evidence suggests that integration can occur at essentially random sites in cellular DNA and that once established, the provirus behaves as a stable genetic element. In infection by horizontally transmitted MMTV, each acquired provirus will be haploid, residing on only one of any chromosomal pair. This contrast with the endogenous proviral copies, which were presumably established by infections of the germline and have subsequently been rendered diploid in common laboratory mouse strains by inbreeding. Such endogenous proviruses are therefore vertically transmitted and common to all tissues, but apart from a few well-documented exceptions, they are only rarely expressed into viral RNA.

Each provirus is flanked by two long terminal repeats, or LTRs, (indicated as boxes) formed by duplication of sequences at the 5′ and 3′ ends of the viral RNA. It is these LTR elements that control expression of the viral genes, but the actual processes of transcription and translation are carried out by host functions. The primary RNA transcript performs the dual role of messenger RNA for viral proteins, either directly or after splicing, and of genomic RNA to be packaged into progeny virions. In the case of MMTV, virus assembly begins intracytoplasmically, resulting in the formation of what are termed A-type particles. These then bud through the plasma membrane, without concomitant cell lysis, and mature into the B-type particles typical of MMTV.

ative are fragments which span the junctions between viral and cellular sequences, since they are recognized by both viral and cellular DNA probes and effectively define each site of proviral integration. In a clonal population of MMTV-infected cells, any new junction fragment will be present in every cell and will therefore be readily detectable as a discrete band on Southern blots (Fig. 2). In contrast, a mixed population of infected cells will contain proviruses at many different sites, yielding junction fragments of random size which would appear as a smear on such blots (5-10).

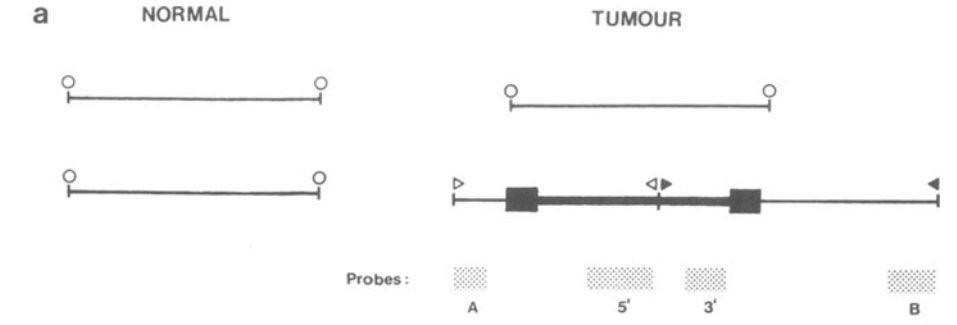
Additional indications of clonality arise from the fact that proviruses acquired by infection will generally reside on only one of any chromosomal pair. This has two major consequences. The first is that using virus-specific DNA probes, acquired proviruses will appear on Southern blots at only half the intensity of the endogenous MMTV sequences present in all laboratory strains of mice, since the latter will have been rendered diploid (homozygous) by inbreeding (Figs. 1 and 2b). The second consequence is that in a clonal tumor, a probe specific for the cellular sequences adjacent to any newly inserted provirus will recognize two restriction fragments at similar intensities, one corresponding to the novel virus-cell junction on one chromosome, the other to the normal allele on the uninterrupted chromosome (9,10; Fig.2).

In the analysis of MMTV proviruses in mammary carcinomas, it quickly became apparent that these virally induced tumors are indeed clonal or approximately so (the exact stoichiometry of the fragments can be distorted by inclusion of normal tissue in the tumor biopsy), and that they all contain at least one and more commonly several proviruses acquired by viral infection (5-10). The remainder of this discussion therefore rests on the premise that the presence of one (or more) of these integrated proviruses is responsible for some facets of the neoplastic phenotype.

Clearly, insertion of such a large segment of DNA within a functional gene could have serious consequences for gene expression. However, since this is more likely to be a negative effect and would occur on only one of the two chromosomal copies of the gene, it is also likely to be phenotypically silent without the concomitant loss of the second allele. While such recessive, mutagenic events could conceivably unleash some transforming factor, it is known that retroviral proviruses carry strong transcriptional promoters and enhancer elements within their long terminal repeats (LTRs) so that they may also exert positive effects on the expression of adjacent genes, provided they are in appropriate juxtaposition (12). Thus, the chance proviral activation of a cellular proto-oncogene, for example, could conceivably trigger the events leading to neoplastic transformation, by providing that particular cell clone with a selective growth advantage. Such notions now dominate current thinking on carcinogenesis by MMTV, and indeed of other latently oncogenic retroviruses, and some supporting evidence is summarized below.

III. PROVIRAL ACTIVATION OF CELLULAR GENES IN MMTV-INDUCED TUMORS

In invoking oncogene activation to account for MMTV's role in tumorigenesis, one clear expectation would be that proviruses in different tumors should reside close to the same cellular gene or at least within the same region or regions of the host genome. Over the last few years, therefore, several laboratories have undertaken to test these predictions by isolating recombinant DNA clones of virus-host junction fragments from tumor tissue and preparing unique sequence cellular DNA probes specific for the sites of proviral integration (9,10,13-15; illustrated in Fig. 2). Such studies have established the existence of at least four distinct cellular domains which are commonly occupied by an MMTV provirus in virally induced tumors. Two of these, termed int-1 and int-2, are now well characterized, and together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Probes :
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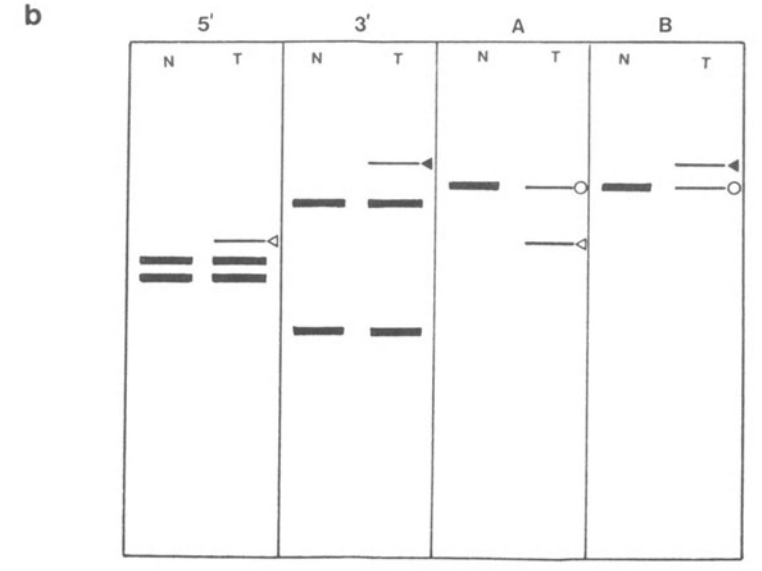
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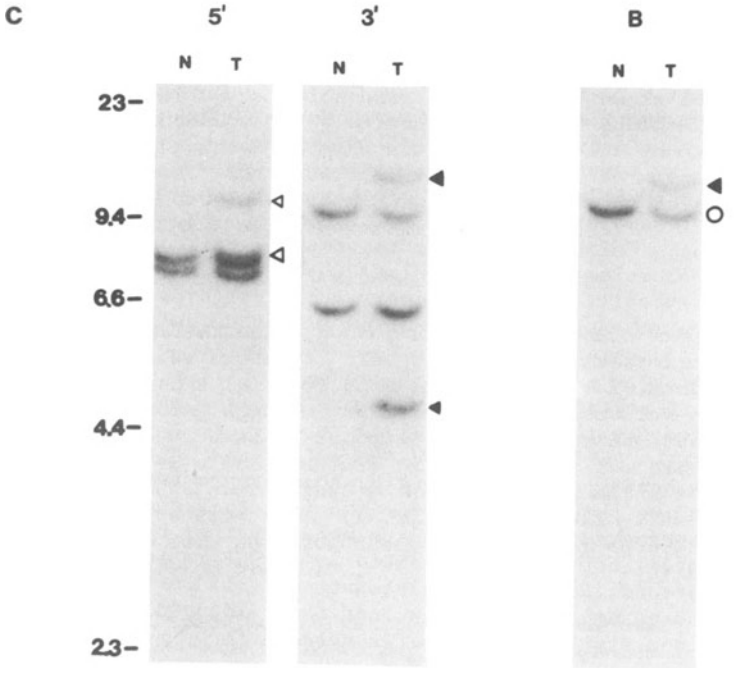
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account for a high proportion (80-90%) of the spontaneous mammary tumors encountered in the commonly studied susceptible strains of laboratory mice (9,10,13,14,16). A third region, int-3, was identified in a particular feral mouse (Czech II) colony, but its wider significance in other strains remains to be established (D. Gallahan and R. Callahan, personal communication). Similarly the so-called int-41 locus is so far implicated in only one mammary carcinoma and potentially in kidney carcinomas related to MMTV infection in a particular subline of BALB/c mice (15). All of these regions are discrete loci, mapping to different mouse chromosomes: int-1 is on chromosome 15 (13,17), int-2 on 7 (17), and int-3 on 17 (R. Callahan, personal communication). There is therefore no a priori reason why they should be preferred targets for MMTV integration, strengthening the conviction that they may include functional genes which contribute to neoplasia. Indeed it is now clear that at least the better understood loci, int-1 and int-2, do represent cellular genes, and that they are transcriptionally activated by the presence of an MMTV provirus in nearby DNA (13,14).

Figure 2. (shown on previous page) Southern blotting analysis of DNA from MMTV-induced tumors.

The figure illustrates both schematically (a and b) and with an actual example (c) the strategy for examining MMTV proviruses and their sites of integration.

a. A specific restriction fragment is depicted, generated by digestion of cellular DNA at the unique sites (o). Two copies of the fragment will be present in all diploid cells. However, if the MMTV provirus also carries a cleavage site for the restriction enzyme (EcoR1 for example) insertion of a provirus into one of the alleles of this region will create two novel fragments (◁ and ◀) each spanning the junction between viral and cellular sequences. By isolating recombinant DNA clones of these junction fragments, it is possible to generate hybridization probes (stippled boxes) specific for the 5′ and 3′ portions of the viral genome and for the cellular DNA sequences adjacent to the 5′ and 3′ ends of the provirus, designated A and B respectively. The use of these probes in Southern blot analysis of normal and tumor DNAs would yield results exemplified in b and c.

b. The schematic diagram illustrates data expected from the analysis of normal (N) or mammary tumor (T) DNA, digested with a restriction enzyme as in (a) and hybridized with the indicated probes. Both the 5′ and 3′ viral probes detect endogenous MMTV sequences in all tissues, revealed as unique 5′ and 3′ junction fragments in diploid amounts (see Fig. 1). In tumor tissue, however, novel junction fragments (◁ and ◀), created by insertion of an acquired provirus, are superimposed on the endogenous pattern. In a monoclonal tumor cell population, these acquired fragments would be expected to appear at half the intensity of endogenous sequences. Hybridization of the same blot with either of the cellular probes A and B identify the unaltered cellular domain (o) on both chromosomes of normal tissue, whereas in the tumor, one of these copies has been disrupted. The tumor therefore displays two fragments, in equal amounts, corresponding to the unoccupied integration site on one chromosome (o) and the novel junction fragment (◁ or ◀) on the other. From a knowledge of restriction enzyme cleavage sites in proviral DNA and the estimated sizes of fragments detected on the blots, data of this type also provide information on the position and orientation of each provirus relative to the integration site.

c. A typical example of a southern blot is shown in which a tumor containing two acquired MMTV proviruses is compared to normal, in this case spleen tissue. Two new 5′ junction (◁) and corresponding 3′ junction fragments (◀) are indicated. For simplicity, only one example is included of hybridization with a flanking cellular DNA probe (B).

IV. THE int-1 GENE

The identification of the int-1 locus hinged on the recombinant DNA cloning of a virus-host junction fragment from a C3H mouse mammary tumor containing only a single acquired MMTV provirus (9). By preparing unique sequence probes specific for this region, Nusse and Varmus demonstrated that approximately 80% of the spontaneous C3H tumors they analyzed had acquired MMTV provirus within this limited chromosomal domain. Although the boundaries of the region remain nebulous, proviral insertion sites in different tumors are distributed across at least 20 kb of cellular DNA (9,13,16,18), for which representative recombinant DNA clones have now been isolated and characterized (see Fig. 3). More significantly, sequences from within int-1 were found to be expressed in polyadenylated RNA specifically in tumors in which the locus is perturbed (13,16).

These latter observations pointed to the existence of an int-1 gene, a conclusion now verified both by sequencing of the genomic DNA and by S1-mapping and cDNA cloning of the 2.6 kb int-1 mRNA (18-20). Despite some residual uncertainties as to the precise 5′ end of the RNA and the presence of alternative poly A-addition signals, the essential architecture of the gene is now clear, comprising four coding exons and spanning roughly 5 kb of the genome (Fig. 3). The presumed open reading frame, encoding 370 amino acids, is followed by a long 3′ untranslated trailer. Confirmation of these

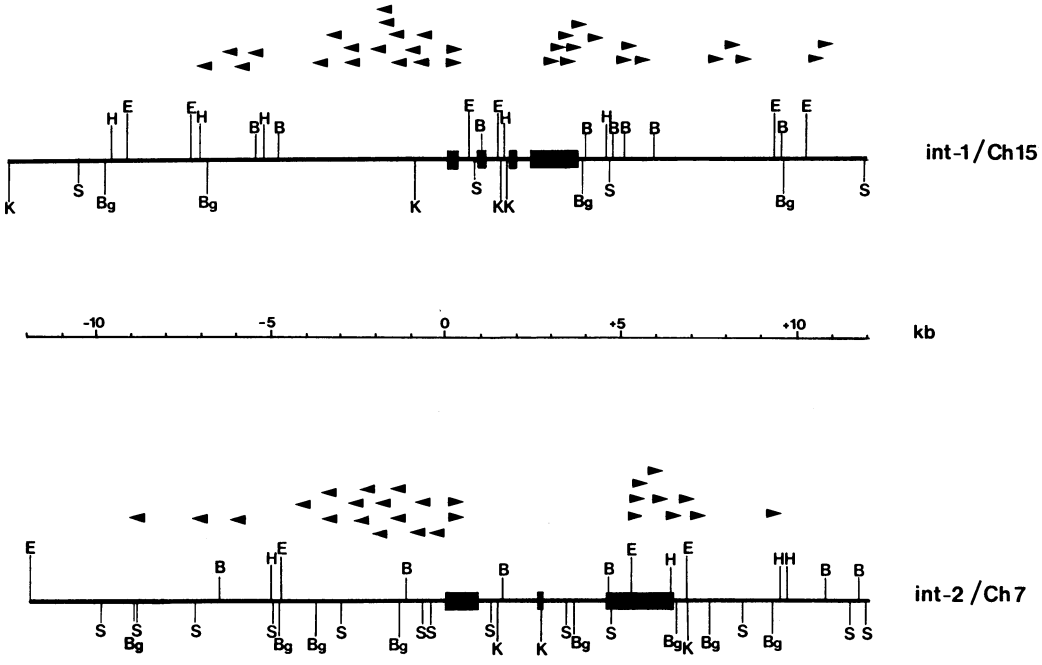

Figure 3. Topography and sites of proviral insertion in the int-1 and int-2 loci.

Restriction maps are presented for DNA spanning over 20 kb of the respective int regions in which the sites of cleavage for the enzymes Bam HI (B), Bgl II (Bg), Eco RI (E), Hind III (H), Kpn I (K) and Sac I (S) are indicated. The boxed segments on the linear maps depict the presumed exons of the corresponding cellular genes. Arrowheads above each map signify the location and transcriptional orientation of proviruses mapped within int-1 or int-2 in individual mammary tumors. The figure represents a compilation of data reported in references 13, 14, 16, 18, 20 and 21.

features has relied principally on *in vitro* expression systems in which the predicted 41 kilodalton primary translation product is readily observed (19,20), but preliminary experiments using antisera raised against synthetic *int-1* peptides also indicate that *int-1*-specific products are detectable in cells transfected with and expressing the *int-1* gene (A.M.C. Brown and H.E. Varmus, personal communication). The latter situation is complicated by the likelihood that the *int-1* product is glycosylated, the sequence predicting four potential asparagine linked glycosylation sites and a candidate signal peptide in the hydrophobic amino-terminal domain (18,20). Apart from these features, the *int-1* protein and gene sequences show no obvious similarities of homologies to know cellular oncogenes or to other entries in the DNA and protein sequence data bases.

V. THE *int-2* GENE

Although identified by an almost identical experimental approach and showing many analogies to *int-1*, the *int-2* locus is a completely separate chromosomal domain and encodes a quite distinct cellular gene (10,14,17). The original characterization of *int-2* relied on the analysis of two BALB/c mouse mammary tumors, induced by neonatal inoculation of the C3H strain of MMTV, each of which contained two acquired proviruses. By preparing probes for the different proviral integration sites, two of them (one from each tumor) were shown to be closely linked, again establishing the existence of a 20-25 kb domain of cellular DNA which was a frequent target for MMTV insertion (10,14; Fig.3). The majority of subsequent analyses were performed in the BR6 strain of mice in which around 60 to 70% of the spontaneous mammary tumors were found to contain an MMTV provirus in the *int-2* region. Moreover, independent confirmation of the existence of *int-2* came from parallel investigations by D. Morris and R.D. Cardiff (personal communication) on tumors in GR mice.

Further indications of the significance of *int-2* transpired when it too was shown to contain a cellular gene whose transcriptional activation in mammary tumors could be attributed to a proximate MMTV provirus (14). The apparent transcription unit of *int-2* has been sequenced and cDNA clones analyzed, revealing a gene comprising three coding exons and spanning about 8 kb of genomic DNA (21). However, recent investigations on mouse teratocarcinoma cell lines and on cells transfected with the *int-2* genomic DNA suggest that the pattern of *int-2* gene expression may be complex, with up to four discrete RNA transcripts discernible (G. Martin and H.E. Varmus, personal communication; C. Dickson, R. Smith and G. Peters, unpublished observations). These appear as two doublets of approximately 3.2 and 3.0 kb and 1.9 and 1.6 kb respectively in length, presumably derived by differential splicing or use of alternative initiation or polyadenylation sites. Resolution of these issues will clearly require further analyses of cDNA clones since current evidence suggests that the cDNAs isolated to date account for only the largest, 3.2 kb transcript (21).

Notwithstanding these uncertainties, it is possible to draw several conclusions regarding the predicted *int-2* gene product. A single open reading frame can be distinguished in the cDNA sequences, capable of encoding 245 amino acids, followed by a long untranslated 3′ trailer (21). The 27 kilodalton product would have a hydrophobic amino terminus, though not as extensive as that of *int-1*, a single potential N-linked glycosylation site, very little cysteine, and multiple clusters of arginine and lysine residues. However, this predicted sequence, as well as that of the genomic DNA, bears no resemblance to that of know oncogenes or other recognizable cellular genes, including *int-1*. Present efforts are therefore concentrated on exploitation of antisera raised against synthetic *int-2* peptides or products expressed in bacteria, to shed more light on the nature and function of the *int-2* protein.

VI. THE MECHANISM OF GENE ACTIVATION BY MMTV

Although direct proof of biological activity remains elusive, the int-1 and int-2 genes are now considered to be cellular proto-oncogenes and are cited as classical illustrations of how such genes may be identified by the principles of "transposon tagging" (12). Nevertheless, there are several precedents in which a known oncogene, previously defined by its appearance within the genome of an acutely transforming retrovirus, has also been the target for insertional mutagenesis (12). The earliest observations along these lines were made in chicken lymphomas induced by avian leukosis virus in which proviral integration commonly occurs adjacent to the proto-oncogene c-myc (22-24). The conclusion from these studies was that transcriptional activation of c-myc generally results from the intrusion of the promoter carried in the viral LTR in such a position that it supplants the normal transcriptional control sequences of the cellular myc gene (see Fig. 4). However, while promoter insertion accounts for around 90% of these neoplasms, the remaining 10% show a different pattern, with a provirus located either upstream or downstream of the intact c-myc gene (25). Curiously, the exact converse appears to hold for activation of int-1 and int-2 by MMTV, in that the majority of proviruses map outside the gene, often quite distant from the 5′ end, and only a few cases of promoter insertion have been described (13,14,16,18, 21). Moreover, virtually all proviruses located upstream of either int-1 or int-2 are in the opposite transcriptional orientation to that of the gene. Those proviruses integrated 3′ to int-1 or int-2 are transcribed in the same direction as the gene (see Fig. 3).

The favored rationalization for these observations is that activation of gene expression is mediated by cis-acting enhancer elements within retroviral LTRs (12-14; Fig. 4). Not only is there direct evidence for the existence of such enhancers (see refs. 26 and 27 for example), but there are also indications that they may display inherent tissue specificity, potentially reflected in the tissue tropism of the virus (28,29). Since the activity of enhancers is reportedly maximal on the proximal promoter (30,31), the orientation of proviral LTRs to point away from the target gene would avoid interposition of the viral promoter between the enhancer and the 5′ end of the target gene (Fig. 4). However, it should be stressed that these notions represent merely a rationalization of the data and no rigorous proof of their validity for MMTV is currently available.

Irrespective of the molecular details of activation, the mapped positions of integrated proviruses in different MMTV-induced tumors are additionally revealing. In both int-1 and int-2, no provirus has yet been found to disrupt the presumed protein coding sequence of the gene (16,18, 21). This is despite frequent disruption of the 3′ untranslated regions, in one instance as close as five base pairs distal to the translational stop codon (18). These latter tumors express novel int-1 or int-2 RNAs as a result of transcriptional read-through into the MMTV LTR and termination at the viral poly-A addition site (13,16,18; G. Peters and C. Dickson, unpublished observations). At the 5′ end, proviruses, and in one case a solo MMTV LTR in a promoter insertion mode also disrupt untranslated exon sequences, sometimes within a hundred base pairs or so of the proposed initiation codon (16,18,21). An obvious conclusion, therefore, which is equally valid for int-1 and int-2, is that the integrity of the putative gene product is crucial to its oncogenic properties. This contrasts strikingly with the situation for c-erb-B or c-myb, for example, where activation frequently results from proviral insertion, by the respective avian or murine leukemia viruses within the body of the gene, implying that some altered or truncated form of the normal gene product is required for tumorigenesis (32,33).

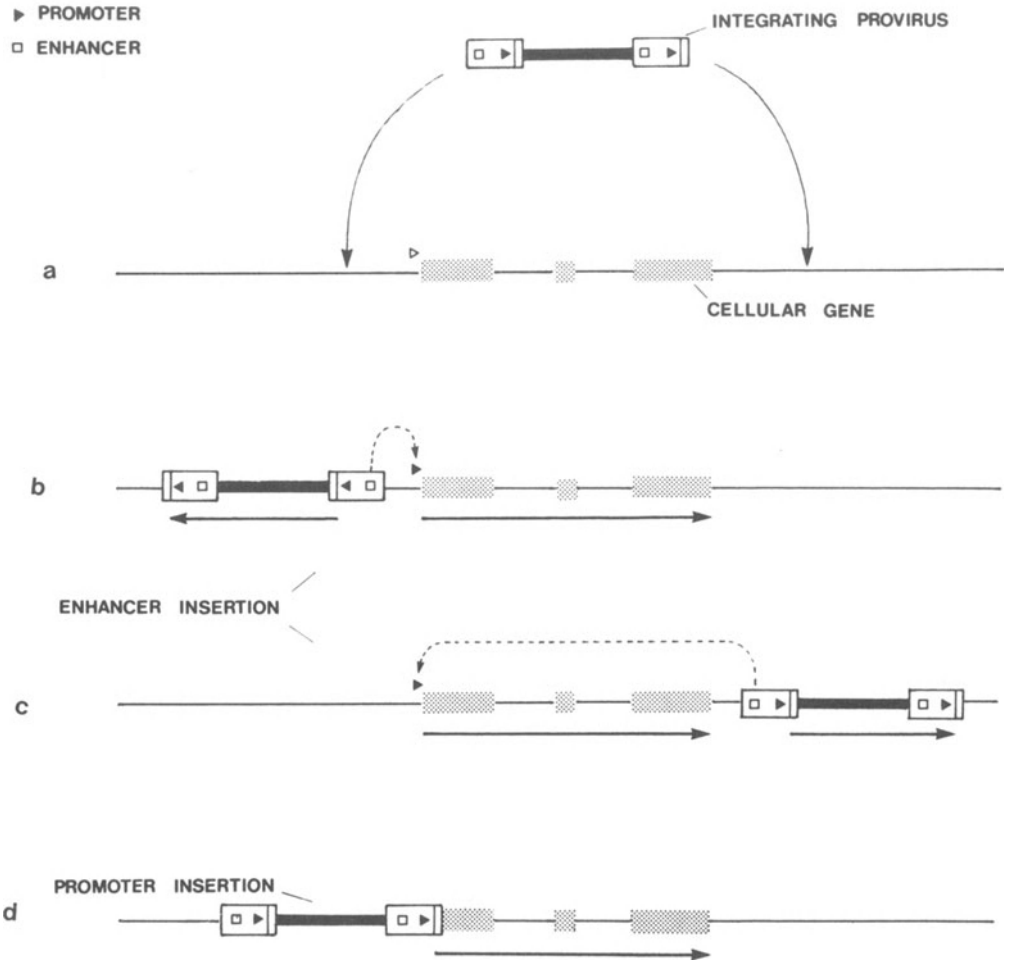

Figure 4. Proviral activation of a cellular gene.

The figure depicts a provirus, carrying active transcriptional promoter (▶) and enhancer (□) elements in each LTR, integrating on one or other side of a normal cellular gene (a). For illustrative purposes, the gene is shown with three exons (stippled boxes) transcribed from a cellular promoter (▷) which is quiescent in uninfected cells. Three scenarios are considered, all of which are exemplified by MMTV proviruses in virally induced mammary tumors (see Fig. 3). In (b) and (c), the viral enhancer is presumed to function in *cis* to activate the cellular promoter. This provides a rationalization for the orientation of the proviruses relative to the gene in that it avoids interposition of a viral promoter between the enhancer and the target promoter. In (d), the transcriptional orientation of the virus and the cellular gene are the same but the viral promoter has replaced that of the gene so that expression is directed by viral elements. that some altered or truncated form of the normal gene product is required for tumorigenesis (32,33).

VII. THE ROLE OF int-1 AND int-2 IN BREAST CANCER

MMTV is undoubtedly one of the principle causative agents of breast cancer, as it is manifest in viremic laboratory mice, and we have argued here that the major impact of MMTV in these tumors is to switch on the expression of the int-1 and int-2 genes. Current indications are that this may occur in up to 90% of the spontaneous tumors arising in susceptible strains of mice (16). However, this fact alone highlights the issue of the remaining 10% of such tumors, as well as the late arising or chemically induced tumors in non-viremic mice, in which the int-1 and int-2 genes are not implicated. Are there other int-like genes yet to be discovered or can a different category of oncogene play a role? Certainly no rational explanation can yet be proposed as to why MMTV should home in on int-1 and int-2, particularly as it has been shown that artificially constructed MMTV-myc chimeras can induce mammary tumors in transgenic mice (28), and that a transforming ras^H allele is detectable in N-nitroso methyl urea-induced mammary carcinomas in the rat (34).

A further consideration must be how to reconcile the switch on of a single, apparently normal cellular gene such as int-1 or int-2 with the prevailing prejudice that malignant carcinomas arise as a consequence of multiple events. Several points are germane here. The first concerns the nature of the int-1 and int-2 genes and their respective products, although so little is known at this stage that discussion must remain speculative. Nevertheless, there are now tentative indications that both genes are expressed transiently in early to mid embryogenesis (G. Martin and H.E. Varmus, personal communication) so that it would not be unreasonable to propose that they may have powerful influences on the growth and differentiation of cells in which they become expressed. Even in tumors, levels of int-1 and int-2 RNA rarely exceed ten copies per cell (13,14, 16), suggesting highly potent products. Moreover, the predicted int-1 protein bears several hallmarks of a secreted or cell surface glycoprotein (18,20), raising spectres of growth factors and their receptors. The second point is that there is no a priori reason to require int-1 or int-2 to be single step carcinogens. Exactly the opposite, in fact, since it is well known that mouse mammary tumorigenesis progresses through stages of pre-malignant hyperplasia, and that in some strains the tumors display pregnancy- or hormone-dependence (3,4). Examination of int-1 and int-2 status in these situations has suggested that though int gene expression may be a contributory factor, it may not be sufficient for frank malignancy (35). Indeed a recent survey of BR6 mouse mammary tumors indicated that a surprising number (approximately 50%) expressed both int-1 and int-2 RNAs in ostensibly monoclonal cell populations (16). Since the probability of insertional mutagenesis of both genes in the same cell is so low, these observations have been interpreted as evidence for cooperativity between the int-1 and int-2 gene products in tumor progression.

Finally, the status of all oncogenes, whether uncovered in acutely or latently oncogenic retroviral models, must be put in perspective by their relevance to human disease. With int-1 and int-2, both human homologs have been cloned and characterized and shown to be located on human chromosome 12 and chromosome 11q1.3 respectively (36-38). However, apart from citing their remarkable degree of conservation as further evidence for their potential importance in cellular physiology, there are too few data to justify a comprehensive discussion at this time. Attempts to detect int-1 or int-2 expression in human tissues, to detect rearrangement or amplification of the respective DNAs in breast carcinomas, or to establish linkages to human genetic disorders or predispositions, have unfortunately yet to bear fruit. Nevertheless, our prejudice is that int-1 and int-2 will inevitably prove to be functional and significant genes in mammalian development and that

irrespective of their role in human neoplasia, their identification will more than justify investigation of MMTV and its mechanisms of tumorigenesis.

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HORMONAL CONTROL OF MOUSE MAMMARY TUMOR VIRUS TRANSCRIPTION

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I. INTRODUCTION

Mouse mammary tumor virus (MMTV) is a RNA-containing tumor virus that causes mammary cancer in mice. MMTV has a life cycle similar to that of other characterized retroviruses, yet MMTV is not completely analogous to the other viruses (1). Some of its unique features have attracted considerable attention in the past few years. First, MMTV transcription is regulated by glucocorticoid hormones. The isolation of MMTV proviruses by gene cloning followed by their transfer into cultured cells has allowed major insights into the mechanism of steroid hormone action (2,3). Second, MMTV belongs to the group of retroviruses that do not carry an oncogene. Recent results suggest that the transformation of mammary gland cells is related to the integration site of the MMTV viral DNA in the host genome. Thus, MMTV most likely transforms cells via insertional mutagenesis (4,5). In this article we will limit our discussion to experiments concerning the hormonal control of MMTV transcription. We will show that glucocorticoid as well as gonadal steroid hormones can enhance MMTV transcription. The role which MMTV plays in mammary tumor formation will be described in other chapters in this book.

II. MOUSE MAMMARY TUMOR VIRUS AS A MODEL SYSTEM FOR STUDYING HORMONE ACTION

The life cycle of MMTV is similar to that of other retroviruses (1). Following virus infection the RNA genome is reverse transcribed into a double stranded viral DNA which integrates into the host genome. The integrated proviral genome has a characteristic structure. The viral

structure genes are flanked by long terminal repeats (LTR) and viral RNA synthesis begins in the U5 region of the 5′ LTR and ends in the U3 region of the 3′ LTR (Fig. 1 line A). The function of the LTRs is two-fold. They contain signals important for the integration of viral DNA into the host genome and, as we shall discuss in this review, signals which are important for the control of viral transcription.

Early studies using MMTV-infected cell cultures showed that the virus production was highest when cells were grown in medium containing glucocorticoids (6,7). This enhancement of virus expression by glucocorticoids has since been shown to be a primary response to the hormone. This makes the MMTV proviral gene an attractive model system to describe how steroid hormones control gene expression.

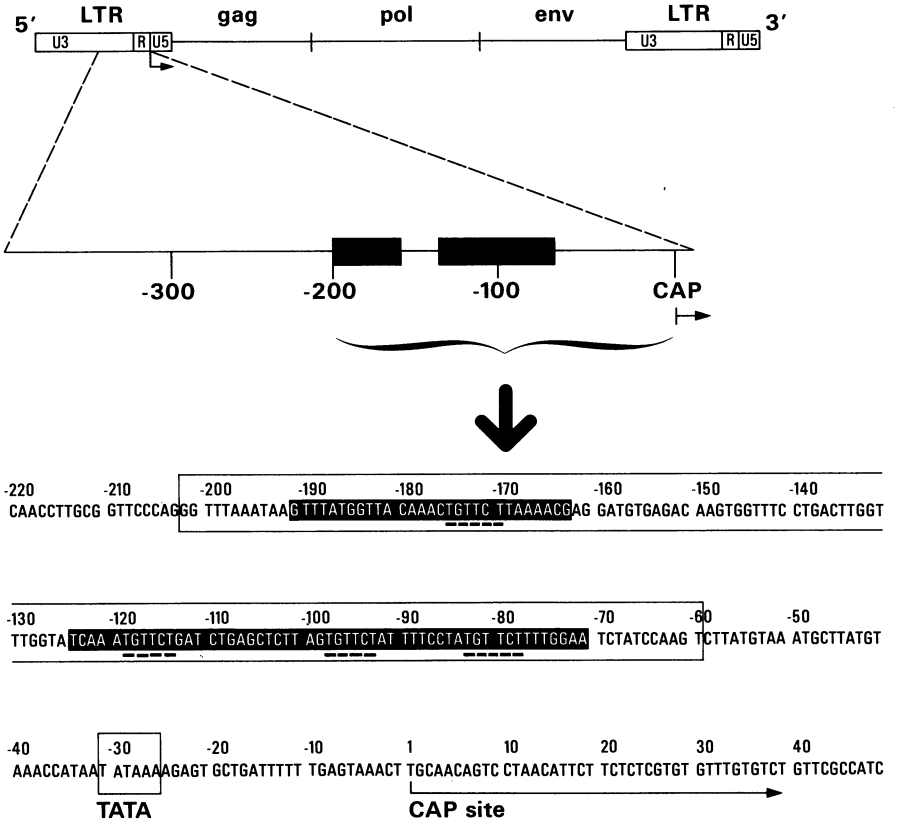

Figure 1: Line A shows the structure of MMTV proviral DNA. Viral RNA initiates at the R region of the 5′ LTR, proceeds through the U5, gag, pol, env genes and the U3 and terminates in the R region at the 3′ LTR.

Line B shows schematically the two regions upstream of the RNA initiation site which have been shown to bind the glucocorticoid hormone receptor.

Line C gives the nucleotide sequence of the LTR starting 220 nucleotides (-220) upstream of the RNA initiation (CAP) site and proceeding to nucleotide +50. The regions boxed in between -59 and -202 represents the hormone responsive element (HRE) which has been defined by deletion analyses described in the text. The shaded areas show the nucleotides which bind to the hormone receptor. The hexanucleotide "TGTTCT" appears four time within the HRE. These sequences are underlined by dashes.

Mouse mammary tumor cell lines as well as heterologous in vitro infected epithelial cells produce MMTV (7,8,9,10,11). In all cases virus production is stimulated 10- to 20-fold when the cells are grown in dexamethasone, a synthetic glucocorticoid (12). The stimulation of viral RNA synthesis is rapid, independent of simultaneous protein synthesis and appears to be caused by an increase in the rate of transcription of the MMTV provirus (13,14,15). These results suggest that glucocorticoids exert their effect directly at the level of viral RNA transcription.

The availability of molecular clones of the MMTV proviral DNA has allowed the analysis of the control of MMTV transcription to proceed. Different MMTV proviruses have been introduced into cultured cell lines by a DNA-mediated gene transfer technique. The transfected cells were analyzed for MMTV RNA transcription and the effect of glucocorticoids on MMTV RNA expression. In each case cells grown in dexamethasone contained 5- to 10-fold more MMTV specific RNA (16,17,18,19). These experiments suggested that the DNA sequence which confers hormone sensitivity on MMTV transcription is contained within the proviral genome. The recipient cells provide the components which allow the transcription of the transfected MMTV viral DNA to be influenced by glucocorticoids.

Viral RNA transcription initiates in the R region of the MMTV LTR (Fig. 1). The LTR contains signals important for the transcription of RNA polymerase II dependent genes (20-24) and it seemed reasonable to assume that signals important for the hormonal response would also be harbored in the LTR. This assumption has been experimentally verified. MMTV LTR sequences have been recombined in vitro with several indicator genes (25-29). These chimeric genes have been transfected into cultured cells and each has been shown to respond positively to glucocorticoids. Therefore, the DNA of the LTR was shown to be sufficient to trigger the hormonal response.

To define a minimal DNA sequence capable of conferring hormonal regulation onto adjacent promoters the MMTV-LTR chimeric gene constructs were subjected to in vitro mutagenesis. Progressive deletions starting from the 5′ end of the LTR (U3) placed the limit of the hormone response sequence to within 202 nucleotides upstream of the LTR RNA initiation site (29-31). Most chimeric constructions have used the LTR RNA initiation to determine hormonal inducibility. One chimeric molecule containing an LTR-tk gene contains two RNA initiation sites, one in the LTR and a second one representing the authentic tk-specific RNA start site. Both start sites are used in transfected cells, and the transcription of both resulting RNA molecules in under hormonal control (30). The presence of a hormonally controlled initiation site outside the LTR allowed the determination of the sequence requirements from the 3′ side - i.e., the question could be asked if the LTR promoter structure with the TATA box and cap site is part of the regulation region. Deleting from the RNA initiation site in the 5′ direction towards the transcriptional regulatory sequences showed that when more than 59 nucleotides were removed, tk transcription was no longer increased by glucocorticoids (32). Therefore, the proviral initiation site and TATA element in the LTR promoter are not part of the hormone regulatory sequence. This is shown schematically in Fig. 1, line C. In addition, the integrity of the entire sequence delimited from the 5′ side (-202) or the 3′ side (-59) does not seem to be essential. Internal deletions and substitutions have been introduced around position -107 (31,33) and, to a limited extent, can be accommodated without functional consequence.

In summary, the results of the in vitro mutagenesis of the MMTV LTR have defined a minimal sequence of 143 nucleotides, encompassing from -59 to -202 nucleotides upstream of the LTR RNA initiation site, as the hormonal response element (HRE).

The preceding section has described the experiments which show that the HRE of the MMTV LTR has the ability to act on heterologous promoters independent of the LTR RNA initiation site. Other experiments have shown that the HRE has additional enhancer-like properties. Enhancers are a class of regulatory elements which were initially discovered in viruses and appear to increase the transcriptional efficiency of a promoter in a manner relatively independent of their position and orientation (34).

An HRE containing DNA fragment has been isolated from the MMTV-LTR and recombined with a plasmid containing the α-globin gene. The HRE was attached to either the 5′ or the 3′ and of the globin gene in both possible orientations (Fig. 2). The distance between the HRE and the α-globin promoter was 1.1 kb. The plasmids were transfected and the transcriptional activity of the α-globin RNA initiation site was quantitated in the presence and absence of glucocorticoid hormone by the S1 nuclease mapping technique. The results are shown in Fig. 2. RNA initiated at the α-globin cap site protects three fragments from 90-96 nucleotides in length from nuclease digestion. All four of the constructs containing the HRE show hormonal inducibility of the α-globin transcripts. No increase in α-globin mRNA in the presence of hormones is observed in cells transfected with construct 5 which is devoid of HRE sequences. Therefore the HRE of MMTV fits the characteristics of known enhancers, i.e. it acts independently of distance and orientation, and can be described as a hormone-dependent enhancer element.

In the previous sections we have described the experiments which have led to the definition of the region of DNA (HRE) which is responsible for hormone sensitive MMTV transcription. The role of the HRE in glucocorticoid-induced transcription has been further investigated. The binding of the glucocorticoid receptor protein complex to specific fragments of the MMTV genome was tested. Nitrocellulose filter binding assays and DNA cellulose competition assays were used to show that the glucocorticoid receptor protein binds specifically to MMTV DNA. In the filter binding assay, partially purified receptor is mixed with various purified DNA fragments then passed over a nitrocellulose filter. Only those fragments bound to protein are specifically retained on the filter. Using this technique it has been shown that the receptor recognizes and binds preferentially to DNA fragments containing the MMTV LTR, but it does not bind to, for example, pBR322 DNA (35,36,37). The second assay which has been used to study receptor-DNA interactions is based upon the fact that activated steroid-receptor complex binds strongly to DNA coupled to cellulose. It is possible to compete the bound hormone-receptor complex from the DNA cellulose by the addition of excess DNA. This technique has been used to show that fragments of DNA containing the MMTV LTR are better competitors for the hormone-receptor complex than an unspecific DNA such as calf thymus (38,39).

The precise nucleotide sequence of the MMTV LTR which interacts with the hormone receptor-complex has been defined using the technique of "DNA-foot-printing" (40). When fragments of the MMTV LTR are incubated with the activated glucocorticoid receptor those nucleotides which are bound to the receptor are less sensitive to nuclease digestion. The specifically protected nucleotides can be visualized on a DNA sequencing gel. Two binding regions for the glucocorticoid receptor have been defined in the MMTV LTR. These sequences lie between position -71 to -124 and -163 to -191 upstream from the LTR RNA initiation site (39,40) (see Fig. 1 lines B and C). As discussed previously this same region has been biologically defined as the hormonal responsive element. In these receptor binding regions the hexanucleotide sequence 5′-TGTTCT-3′ is found four times. The guanosine residues of these hexanucleotides are protected by the receptor protein from methylation by dimethylsulfate (41). This suggests that there is a direct contact between the receptor protein and the DNA double helix at these sites. Another gene which is regulated by glucocorticoid hormones, the human metal-

lothionine IIA gene, also contains this hexanucleotide sequence within its glucocorticoid regulatory element. This consensus sequence appears to directly contact the receptor protein as similar to the case for the MMTV promoter (42).

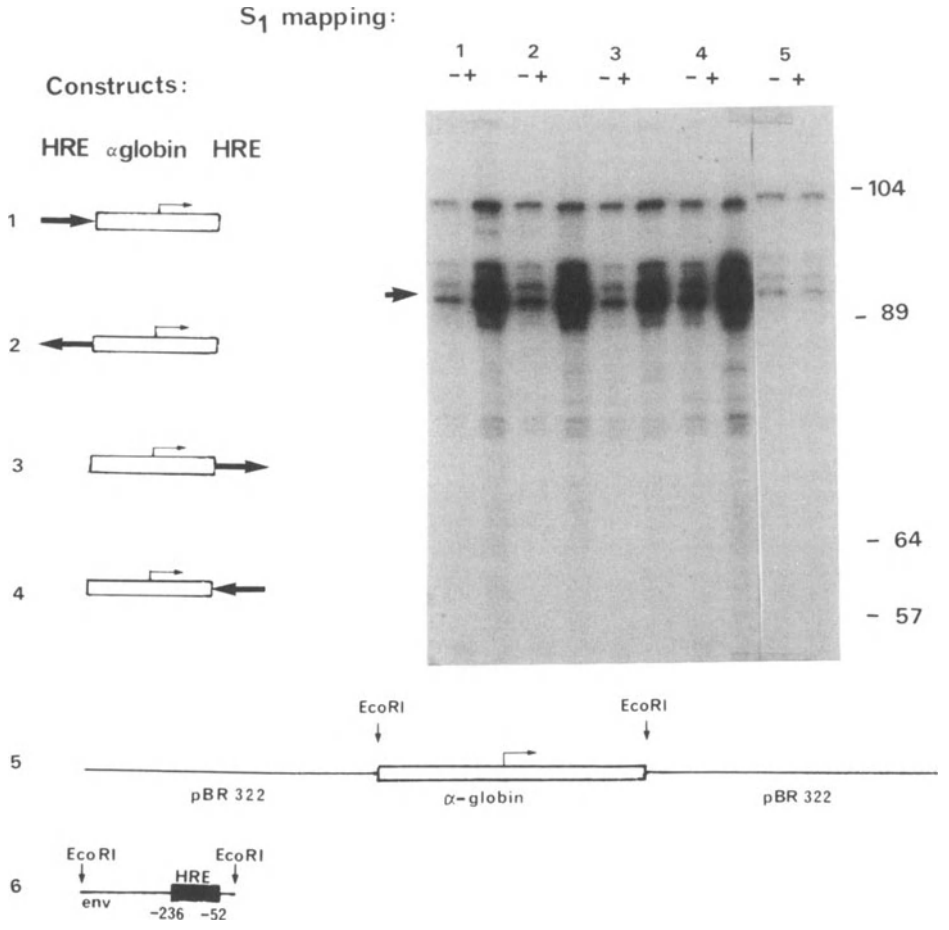

Figure 2: The hormone responsive element of the MMTV LTR acts as a hormone dependent enhancer.

The HRE of the MMTV LTR, shown as the EcoR1 fragment in line 6, was inserted into a plasmid containing the α-globin gene (line 5). The HRE was inserted in the EcoR1 site either 5′ to the globin gene in the syn (line 1) or anti (line 2) orientation, or 3′ to the gene in the syn (line 3) or anti (line 4) orientation. The plasmids were transfected into L cells and the effect of glucocorticoids upon α-globin transcription was analyzed in a S1 nuclease analysis. Total RNA was isolated from the transfected cells and hybridized with a 109 nucleotide ^{32}P-end labeled DNA fragment. RNA which initiates at the α-globin cap site protects a fragment of 90 nucleotides from S1 nuclease digestion (shown by the arrow). The numbers at the top of the film refer to the results obtained with the corresponding construct on the left either in the absence (-) or presence (+) of dexamethasone.

III. INDUCTION OF MMTV GENE EXPRESSION BY OVARIAN STEROID HORMONES

In the previous sections we described the mode of regulation of MMTV expression by glucocorticoids. The involvement of ovarian hormones in mouse mammary tumorigenesis makes it tempting to speculate that steroid hormones other than glucocorticoids could be involved in MMTV gene expression.

The high incidence of mammary tumors in the GR mouse strain is dependent upon the expression of the mouse mammary tumor provirus associated with the MTV-2 locus (43-45). Breeding females of this strain of mice develop mammary tumors under the hormonal influence of pregnancy but these tumors regress after parturition (46). These tumors are described as pregnancy dependent. Although ovariectomy reduces the subsequent occurrence of these tumors, a 100% reoccurrence has been observed when the ovariectomized mice are treated with a combination of progesterone and estrone or with 17 α-ethynyl-19-nortestosterone, a compound with high progestational activity coupled with a relatively low estrogenicity (47). Thus both the presence of the Mtv-2 locus and the presence of ovarian steroid hormones appear to be important for mammary tumor formation.

To test for the regulation of MMTV expression by gonadal steroid hormones the MMTV long terminal repeat (LTR) was linked to both sides of the bacterial neomycin (neo) resistance gene (48) to yield a provirus-like construct in which the neo gene was flanked on either side by LTR sequences (Fig. 3a). This construct was transfected into the human mammary tumor cell lines MCF7 and T47D both of which possess functional gonadal steroid receptors (49,50). The transfected cells were then treated with different hormones. Transcripts, starting at the authentic MMTV LTR initiation site, were quantitated by the S1 nuclease mapping procedure. The amount of RNA initiating at the correct start site was increased by all the gonadal steroid hormones used (Table 1). In T47D cells the progestins R5020 and medroxyprogesterone acetate (MPA) yielded the highest induction, which was about 15-fold above the expression in untreated cells. Dihydrotestosterone (DHT) caused a 7.5-fold increase and 17 β-estradiol had the weakest effect and caused only a 2.5-fold induction

Compared with the T47D transfectants, MCF7 cells transfected with the LTR-neo construct exhibited lower induction ratios. The presence of the two progestins and estrogen in the culture medium lead to a 2- to 4-fold increase in LTR-neo transcription. The differences observed in the induction ratios might reflect differences in the active receptors present in both cell lines. In the MCF7 cells, dihydrotestosterone appeared to have no effect upon the transcription of the LTR-neo gene. MCF7 cells have androgen receptors but it is known that functional receptors can be lost easily in cells growing in tissue culture (51).

The hormone responsive element (HRE) of the MMTV LTR is capable of binding the glucocorticoid hormone receptor complex and can confer hormone inducibility on heterologous promoters. We have also shown that the HRE functions as an enhancer, i.e. it is capable of inducing transcription when it is present in either orientation at various distances from a promoter. In the preceding section the gonadal steroid hormones were shown to have an effect upon transcription in a chimeric molecule containing two intact MMTV LTRs. In this section we will show that the glucocorticoid HRE from the MMTV LTR can also respond to gonadal hormones. These experiments were done in a transient transfection assay (52) with the plasmids shown in Fig. 3B. The HRE was combined with a chimeric gene containing the TK promoter linked to the chloramphenicol acetyl transferase gene (CAT). The HRE was linked 5' to the TK promoter in both possible orientations. The constructs were transfected into T47D cells and analyzed in transient assays. Transfected cells were incubated in medium without hormones for 24 hrs. Hormone was

then added to the medium for an additional 24 hrs and the CAT enzyme activity in the cellular protein extract was determined (52). The results, expressed as the ratio of the amount of converted chloramphenicol in the hormone-treated cultures to that in the untreated culture are shown in Table 2. The TK promoter is not responsive to treatment with steroid hormones. When the HRE was linked either in the normal 5′ position and syn orientation (pHRE TK CATa) or in the anti-orientation (pHRE TK CATb) to the TK CAT gene, CAT enzyme activity was increased following incubation with the progestin R5020 and the androgen DHT. Estrogen treatment of the transfected cells had no effect on the CAT activity.

From these experiments we conclude that the hormone responsive element contains signals to which the progesterone and androgen receptors can respond. Using the DNA "foot-printing" technique it has been seen that the progesterone-receptor complex does bind to the HRE of the MMTV LTR (53).

Binding studies using the androgen receptor and the MMTV LTR have not yet been carried out. But the functional analysis suggests that a regulatory region for this receptor might also be present in the LTR. The fact that there was no induction in CAT enzyme activity in the transfected cells treated with estrogen suggests that the DNA elements necessary for the estrogen response lie in part, or totally, outside the HRE of the MMTV LTR. As is the case for the androgen receptor, no binding experiments have been carried out using the estrogen receptor and the MMTV LTR.

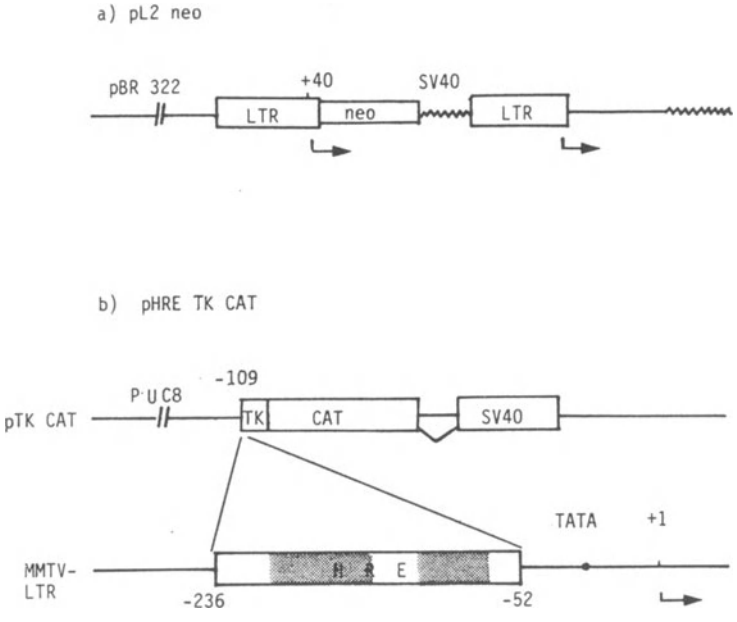

Figure 3: MMTV LTR chimeric constructs used for transfection of human mammary carcinoma cells.

a) A schematic representation of the plasmid pL2neo is shown. The gene coding for resistance to G418 (neo) is flanked by 2 MMTV LTRs. Transcription initiates in the left MMTV LTR at the usual site and proceeds through the neo gene.

b) The construction of pHRE TK CAT is shown. Plasmid pTK CAT was constructed by fusing the 160 nucleotide fragment (-109 to +51) containing the herpes simplex virus TK gene promoter with the CAT coding region. The HRE of MMTV (-52 to -236) was cloned into the single Bam HI site of pTK CAT in both orientations. The HRE does not contain an RNA initiation site in plasmid pHRE. TK CAT RNA initiates at the authentic TK RNA initiation site. The shaded areas represent the two glucocorticoid receptor binding domains.

Table 1: Induction of transcription from a MMTV LTR-neo construct by gonadal steroid hormones.

	Hormone			
Cell-line	R 5020	MPA	E_2	DHT
T47D	10.2	15.0	2.0	7.5
MCF7	3.3	2.2	3.9	1.1

T47D and MCF7 cells were transfected with pL2neo (Fig. 3a) and G418 resistant cell clones were selected. Pools of pL2neo transfected cells were grown for one week in charcoal stipped fetal calf serum then various hormones were added to the medium for 24 hrs. Total cellular RNA was isolated and the amount of RNA correctly initiating in the MMTV LTR in the presence or absence of steroid hormones was quantitated by the S1 nuclease mapping technique. The S1 nuclease resistant fragments were separated on a gel and the amount of radioactivity in the fragment indicative of correctly initiated RNA was quantitated by densitometric scanning of the autoradiographs. The numbers in the table reflect the induction factor i.e. the ratio of the intensity of the band following hormone treatment to its intensity in untreated cells.

The concentration of hormones was as follows: R5020, 30nM; medroxy-progesterone acetate (MPA), 30nM; Estrogen (E_2), 0.1 M for T47D cells and 50nM for MCF7 cells; dihydrotestosterone (DHT), 0.1 M.

Table 2: The HRE of the MMTV LTR confers progesterone and androgen inducibility on a heterologous promoter.

	Hormones		
Plasmid	none	R5020	DHT
p TK CAT	0.3	0.3	0.3
p HRE TK CATa	0.5	97.3	2.4
p HRE TK CATb	0.4	97.9	2.0

The plasmids shown in Fig. 3b were transfected into T47D cells. The cells were grown 24 hrs. in medium containing no exogenous hormones then for an additional 24 hrs. in medium containing 30nM of the progestin R5020, or 0.1μM dihydrotestosterone (DHT). The transfected cells were then harvested and the CAT enzyme activity (S2) was measured in protein extracts. The numbers represent the ratio of the amount of converted chloramphenicol in the cells grown in medium containing exogenous hormone to the amount converted in non-treated cells.

The role of two of the gonadal hormones estrogen and progesterone in the etiology of murine mammary tumor formation is well documented. Our experiments suggest that one of the consequences of the involvement of these hormones in tumorigenesis is a direct activation of MMTV proviral gene expression. The role which androgens play in mouse mammary tumorigenesis is not clear. In transplantation experiments testosterone only slightly enhanced the growth of hormone-dependent mouse mammary tumor grafts in gonadectomized hosts as contrasted to a clear increase in tumor growth by a

combined treatment with estrogen and progesterone (47). However, an androgen-dependent Shionogi carcinoma (SC-115) has been obtained from a spontaneous mammary carcinoma in a female mouse from the DS strain (54,55). A cell line derived from this carcinoma has recently been shown to synthesize MMTV specific RNA only if the cells are cultured in the presence of androgens (56). Thus, androgens may also have the potential to regulate MMTV expression in vivo.

IV. DISCUSSION

The mechanism of steroid hormone action and the effects of steroids on the transcription of individual genes has been studied in selected model systems. Estrogen and progesterone action has been described in great detail in, e.g., the chicken oviduct where the synthesis of the egg white proteins is controlled by these hormones (57). Androgen action has been described in the testis and prostate (58), and glucocorticoid hormone action has been studied in the liver (59). In all cases specific hormone responsive genes have been molecularly cloned and efforts have been made to define hormone regulation signals on the DNA preceding or contained in the regulated genes. The most advanced description of a steroid hormone regulated gene has been obtained by studying the proviral DNA of mouse mammary tumor virus and its induction by glucocorticoid hormones. The reasons for the advantageous use of this model system was the early availability of a molecular clone of the proviral DNA and the ability of many different transfected cell lines to properly use and regulate the expression of the exogenously acquired MMTV DNA. Many cultured cell lines maintain the potential to express glucocorticoid receptors and to utilize them in the regulation of endogenous and transfected genes. This property is not found for many other steroid receptors. The expression of the estrogen and progesterone receptors is in most cases rapidly lost after in vitro culture of primary cells thus making it difficult to study the expression of genes which respond to these hormones. The human breast carcinoma cell lines MCF-7 and T47D maintain the expression of estrogen, progesterone, androgen and glucocorticoid receptors (49,50). Therefore, it has been possible to transfect MMTV LTR containing plasmids into these cells and study the effects of gonadal steroids upon transcription. The results suggests that the MMTV LTR is responsive to the three gonadal steroids in addition to glucocorticoids. Thus, the mechanism of action of these hormones mediated by the binding of the receptor-steroid complex to DNA sequences in the LTR might be very similar. It is possible that receptors acquire their specificity from differences in the steroid binding domain. The receptor-steroid complexes, however, might recognize similar DNA sequences leading to similar consequences on transcription.

Do these observations shed light on the MMTV induced tumorigenesis in mice? A long standing question concerns the tissue specificity of MMTV induced tumors, which is the transformation of breast epithelial cells, and the specificity of steroid hormone control, which is the induction of MMTV transcription by glucocorticoids. Glucocorticoids regulate many processes in different tissues and glucocorticoid receptors are present ubiquitously. Therefore it is difficult to imagine that the glucocorticoid regulation of MMTV expression would have its primary effect on breast epithelial cells?

The integration of MMTV proviral DNA into specific cellular loci (int-1 and int-2) has been recognized in a high percentage of MMTV induced tumors (4,5, see chapter by Peters and Dickson). The activation of these cellular loci might be the role which MMTV has in mammary tumorigenesis. The prerequisite for int-1 and/or int-2 integration of MMTV is the spread of virus. The integration of proviral DNA in a large number of cells is dependent upon the transcriptional activity and the virus production in MMTV expressing cells. This production of virus particles could be enhanced in cells

containing receptors for, and responding to, estrogen and progesterone. Estrogen and progesterone receptors are present in mammary epithelial cells and these hormones play an important role in the growth and differentiation of breast epithelial cells (60). Our observation that the control of MMTV expression is not restricted to the glucocorticoids but is also responsive to other steroid hormones could in part explain the tissue specificity of MMTV induced mammary cancer.

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OVERT AND CRYPTIC FUNCTIONS OF THE MOUSE MAMMARY TUMOR VIRUS LONG TERMINAL REPEAT DNA SEQUENCE

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The abbreviations used are: MMTV, mouse mammary tumor virus; LTR, long terminal repeat, orf, open reading frame; DNA, deoxyribonucleic acid; RNA, ribonucleic acid, mRNA, messenger RNA; Dex, dexamethasone; ADP, adenosine diphosphate; HRE, hormone responsive element.

I. INTRODUCTION

As for other retroviruses, the synthesis of mouse mammary tumor virus (MMTV) proviral DNA by reverse transcriptase is a complex and indispensable process for establishment of a vegetative viral life cycle. MMTV contains a single-stranded genomic RNA with a sedimentation coefficient of between 60-70S and a molecular mass of 6.45×10^6 daltons, (1,2). This virion-contained RNA consists of two subunits (35S), each with molecular masses of 2.93×10^6 daltons. The 3′ terminus of MMTV genomic RNA is polyadenylated, and the 5′ terminus most likely carries the CAP structure 5′m7GpppG which is present in other retroviral genomic RNAs (3). Like other retroviruses, MMTV genomic RNA is copied into proviral dsDNA by a virally encoded reverse transcriptase. The proviral DNA is eventually integrated into the host cell chromosomal DNA in an apparently random fashion (4,5).

The MMTV proviral DNA copy integrated into the host cell genome is genetically co-linear with the viral RNA. However, the proviral DNA is larger because it is bounded at each end by two long terminal repeats (LTRs) which are generated by duplication of sequences from the 5′ (U5) and the 3′ (U3) ends of the genomic viral RNA. In addition to the LTRs at both sides of the integrated provirus, there is a direct cellular DNA sequence repeat of six nucleotides; this duplication is unique to each integration site and is probably generated during the integration process, most likely by

endonuclease activity which produces staggered ends in the cellular DNA (6). Similar direct repeats have also been found next to transposable elements in other organisms, suggesting a similarity in the mechanism of insertion of transposable elements and proviral genomic DNAs of retroviruses.

For discussion of MMTV and gene expression, two other chapters in this book are devoted to the biological and biochemical functions attributable to the MMTV LTR. Discussed will be MMTV LTRs function as a complex hormone responsive genetic regulatory element and its role as an enhancer of the transcriptional activity of nonviral host cellular genes. This latter function is currently proposed to represent the cardinal activity by which MMTV DNA can effect malignant transformation in mammary alveolar cells following its insertion, during vegetative viral reproduction into regions of the host cellular genome proximal to putative tissue-specific cellular proto-oncogenes, such as int-1 and int-2, and other putative mammary proto-oncogenes more recently discovered (7,8).

In this chapter, I will present a biologist's viewpoint of the significance of these properties of the MMTV LTR and also a consideration of those as yet undiscovered functions in the viral life cycle that may be specifically attributed to the polypeptide sequences encoded within the ~960 base pairs (bp)-long open reading frame (orf) contained within the U3 region of the MMTV LTR.

II. GENETIC STRUCTURE OF MMTV LTR

The MMTV LTRs are approximately 1350 bp in length and contain a number of features which should be described before proceeding to a discussion of LTR function in MMTV-induced tumorigenesis (Figure 1). Each LTR contains the following elements: an inverted repeat (IR) of six bases (AATGCCGC... ...GCGGCAGC); a unique U3 region derived from the 3' region of the positive strand MMTV genomic RNA (the U3 region is approximately 1190 nucleotides in length and contains several putative regulatory sequences and an orf of about 960 bp [9-11]); a short repeat (R) region of about 10 nucleotides

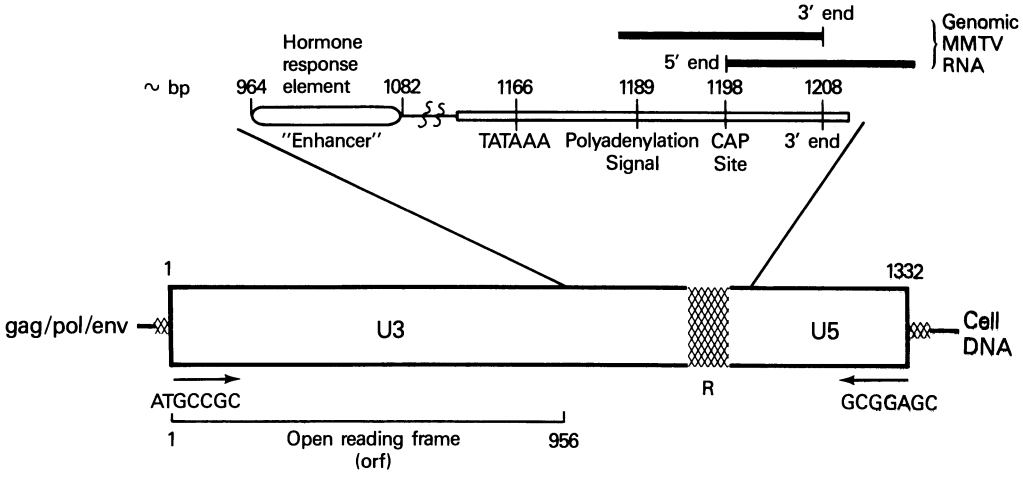

Figure 1. Points of interest of the MMTV-LTR are illustrated above in a 3' LTR from a chromosomally-integrated MMTV provirus. The features shown were taken from references 9, 10, 11, 26 and 67.

(12); a unique U5 region derived from sequences at the 5′ end of the viral genomic RNA; a Goldberg-Hogness TATAAA (13) sequence which is thought to be a signal for initiation by RNA polymerase II (~position 1166); a polyadenylation sequence AGTAAA (~position 1189); the 5′ CAP site of MMTV RNA (~position 1198) which is about 17 bases from the known 3′ end of the viral RNA. The 202 nucleotides preceding the LTR CAP site are involved in the hormone responsiveness of the MMTV LTR (14-19).

III. TRANSCRIPTIONAL EXPRESSION OF MMTV PROVIRUS

MMTV gene transcription is initiated from the 5′ proviral LTR and proceeds through the entire MMTV genome before termination within the 3′ LTR. This gives rise to a genomic-sized MMTV RNA (7.8 kb) which also serves as messenger for the gag-pol viral proteins and presumably for those proteins (integrase, endonuclease, polymerase) necessary for encapsidation of the viral RNA and for the production and integration of the reverse transcribed ds proviral DNA. A spliced message approximately 3.8 kb long is also initiated from the 5′ LTR and contains information for the translation of the envelope glycoproteins of the virion. A third message has been described in tissue expressing MMTV which comprises the coding region of the 3′ LTR or LTR orf. This message is also initiated at the 5′ LTR and has an approximate length of 1.8 kb (20-22). The LTR orf has the coding potential for a 36-37 kd protein (23-25). Graham et al. (22) reported the presence of yet a fourth RNA species abundant in mouse mammary tumors of the C3H/Sm mouse strain. This 4.8-kb RNA hybridized only with gag-pol sequences and could not be detected with env- or LTR-specific DNA probes. Further studies indicate that this RNA, although present in normal lactating mammary glands as well as tumors, does not fractionate preferentially with the polyadenylated species of total cellular RNA (G.H. Smith, unpublished observations); therefore, the significance of this RNA to viral replication or oncogenesis awaits further clarification. For review of the proteins encoded by the MMTV genome, see (26) and chapter by Slagle and Butel.

IV. INTRACELLULAR REGULATION OF MMTV EXPRESSION

To put this section into perspective relative to the infectious life cycle of MMTV, a short review of MMTV infection and gene expression in an intact susceptible mouse is important. The expression of MMTV in various tissues was originally determined by infectivity (27-30), later by immunological technique and/or electron microscopy (31-36), still later by examination of tissue RNA for MMTV homologous sequences (37-41), and most recently by tissue-specific MMTV provirus hypomethylation (42-46). Surprisingly, the list of tissues which support MMTV expression or replication by these criteria is fairly extensive. In the infected male mouse, MMTV can be detected in the bone marrow, peripheral blood, spleen, salivary gland, lung, kidney, and the male reproductive accessory organs, e.g., seminal vesicle, preputial gland, and epididymis. Infectious exogenous MMTV is present as a lifelong activity in the blood of mice of susceptible strains. Passage of MMTV blood-borne infectivity is restricted to other histocompatible strains and is mediated through the establishment of viable lymphocytes from the infected host in the recipient mouse. No infectious virions are found in peripheral blood, and bloodborne infectivity is not neutralized by antibodies. In infected female mice, all the organs mentioned previously are positive for MMTV expression with the exception of the female urogenital system. Most importantly in the female, the mammary glands show MMTV expression. Significantly, this is the only organ in the mouse where malignant transformation occurs as a consequence of MMTV expression. It should be mentioned that male mice do not develop glandular epithelium (see Kratochwil, this publication) in their mammary fat pads. However, following estrogenic stimulation of male mice, mammary epithelium proliferates, and mammary carcinomas which express MMTV are often induced in males of inbred

mouse strains which harbor infectious MMTV or posses oncogenic MMTV germline proviral genes such as Mtv-1 or Mrv-2 (47-49).

MMTV infection and expression are mediated through the transcriptional activity of the integrated MMTV proviral LTR. An integral part of this activity, but by no means the only factor, is the induction (i.e., positive regulation) of MMTV transcriptional activity by glucocorticosteroid hormones. A large body of literature attesting to the direct positive effect of glucocorticoids on MMTV transcriptional activity and virion production is available. These data have been adequately reviewed recently by Ringold (50,51) and others (19,52) and in this volume by N. Hynes. The location of the hormone responsive element (HRE) within the LTR has been firmly established to lie within the LTR DNA sequence -202 to -59 bp upstream from the RNA start site (16,53,54). As described in the chapter by Hynes et al., this LTR-derived DNA sequence can be transposed into recombinant DNA plasmids and shown to act on various heterologous gene promoters in syn or anti orientation over a distance of at least 1100 nucleotides. These properties prompt these authors to describe the LTR HRE as a hormone-dependent enhancer. It is this property of the MMTV LTR that is thought to be important in the development of mammary tumors following insertion of the proviral MMTV DNA into regions of the host cell genome containing as yet ill-defined mammary-specific proto-oncogenes (see chapter by Peters and Dickson).

Other investigators, specifically G. Hager and his collaborators, have recently demonstrated a potential negative regulatory aspect to the LTR HRE (55-57). In this viewpoint, the MMTV LTR HRE is seen as a negative cis regulator of gene expression whose negative influence can be relieved by the interaction with glucocorticoid-hormone receptor complex. Two types of experiments support this interpretation of LTR HRE function. Insertion of the MMTV LTR into recombinant DNA plasmids between a strong transcriptional enhancer (Harvey murine sarcoma virus long terminal repeat [HMSV-LTR]) and downstream coding sequences (Ha-v-ras or CAT) results in the hormone dependence of transcription enhancement by HMSV-LTR. Deletion of the HRE from the LTR sequences in these constructs has two effects: it abolishes the hormone-dependent enhancement of transcription and results in much higher constitutive levels of transcription, presumably due to the presence of the HMSV-LTR transcriptional enhancer. Further study demonstrated that prevention of protein syntheses in cells transformed with these recombinant plasmids resulted in a significant induction (depression) of transcription from the MMTV LTR in the absence of hormone even in those plasmid constructs where the exogenous enhancer (HMSV-LTR) was absent (57). Addition of hormone under these conditions led to a superinduction of initiation of transcription at the MMTV LTR start site. The authors have suggested from these studies and others that a labile protein mediates repression of transcription from the MMTV LTR. In support of this they observed that MMTV LTR DNA deleted of the HRE did not show superinduction following protein synthesis inhibition. These studies have been carried out in cell lines permanently or transiently transformed by recombinant plasmids carrying MMTV LTR, and similar results have not been reported in mammary cell lines expressing complete MMTV proviral DNA copies. Nevertheless, several recent observations have been made in 34I, a mouse mammary tumor cell culture. These cells were derived from a C3H mouse mammary carcinoma and possess stably integrated, glucocorticoid-responsive MMTV proviruses which express infectious virions. These experiments may be important to consider in light of the results from transfected cells relative to MMTV LTR HRE control. Johnson and his coworkers observed that treatment of 34I cells with inhibitors of $(ADPribose)^n$ synthetase resulted in an increase in MMTV RNA accumulation (58). In addition, the presence of added glucocorticoid resulted in a rapid decrease in ADP-ribosylation of selected chromosome proteins within the time frame of MMTV gene induction. Further studies indicate that glucocorticoid hormones are more effective agonists of MMTV gene transcription in cells

treated with ADP-ribosylation inhibitors. Moreover, other steroids, such as progesterone, 11-deoxycortisol, and RU-486, which bind competitively to steroid-specific receptor but which do not normally stimulate MMTV gene expression, become effective agonists of this activity in inhibitor-treated 34I cells (59). If, as Hager's experiments suggest, the MMTV LTR HRE is negatively regulated by a labile repressor protein, then these results could reflect the importance of ADP-ribosylation in the stability or effectiveness of the putative MMTV LTR HRE trans-acting repressor proteins(s).

Recent studies of the segregation of 11 endogenous MMTV proviral genes and their transcriptional expression in lactating mammary tissue of females of recombinant inbred strains provide additional support for the conclusion that cellular genes unlinked to MMTV gene loci can play significant roles in the regulation of viral expression (60,61). Comparison of the levels of MMTV RNA in lactating mammary glands showed no direct relationship between the presence of a particular MMTV proviral locus or to increasing numbers of proviral loci in individual strains. However, a direct relationship was demonstrated between the levels of MMTV transcripts in the mammary gland and the presence of the mouse LPS^n allele gene which segregates independently of MMTV proviruses (60,62). Therefore, the influence of LPS^n allele on MMTV expression must be exerted in trans, underlining the importance of cellular factors distinct from hormone receptors or the relative position of MMTV proviruses within chromosomal DNA in the regulation of MMTV gene transcription in mammary gland tissues.

Possibly the least understood and yet the most important characteristic of the MMTV LTR is its tissue-limited promotion of MMTV gene expression. As we have seen earlier in this discussion, transcription initiated from the LTR is under a variety of controls, both exogenous (e.g., hormones) and endogenous (e.g., heterologous enhancer sequences and/or repressor proteins). Nevertheless, it is significant that, in those mice susceptible to MMTV infection, expression, and tumorigenesis, it is the mammary epithelium that is exclusively subject to malignant transformation as a result of the genetic activity of MMTV. This selectivity of the oncogenic response to LTR-mediated gene expression has been dramatically underscored in recent studies of transgenic mice who received the mouse myc oncogene under control of the MMTV LTR (63). In these experiments, female mice which inherited the constitutively deregulated expression of LTR myc in their mammary tissue developed mammary adenocarcinomas by their fourth pregnancy (63,64). Other tissues in the transgenic mice, both male and female, showed elevated levels of MMTV LTR-initiated myc transcription, notably the salivary gland, testis, lung, preputial gland, small intestine, and spleen. However, none of these tissues had developed malignancies at last report. In one animal all the tissues assayed showed detectable levels of LTR myc RNA (63). Analyses of the mammary tumor DNA from these transgenic mice demonstrated the absence of MMTV proviral gene rearrangement, i.e., no newly acquired MMTV proviral genes were observed, and the LTR myc DNA construct had itself not been incorporated into regions of the mouse genome near int-1 or int-2, domains of mouse cellular DNA related to the expression of putative mammary-specific proto-oncogenes (Peters and Dickson, this volume; 7,8). Ignoring for the present the obvious implications of an overexpression of myc in the tumorigenic response of the mammary tissue, the major impact of this result is the confinement of the oncogenic response to the mammary gland. It is tempting to speculate that a promotionally active MMTV LTR conjugates those disparate events constitutive to mammary epithelial cell immortalization and subsequent progression to malignancy. Unfortunately, these transgenic mouse studies have not as yet focused upon the role of the MMTV promoter-enhancer (LTR) in the development of these mammary carcinomas. Before leaving the discussion of MMTV LTR myc "construct-positive" transgenic female mice and the probable role of the MMTV LTR in the development of their mammary tumors, two recent observations are worth mentioning. First, an interesting

study was recently published which demonstrated that the pathogenicity of two distinct "acutely transforming" murine sarcoma viruses, each containing the viral oncogene mos, is determined by sequences in the U3 region of the viral LTR. It was found that any mos gene, cellular or viral, in conjugation with the proper viral LTR DNA, was sufficient to produce the predicted disease syndrome when introduced into susceptible animals (65). Therefore, the U3 region of the viral LTR has a decisive function in determining target cell specificity. Second, transgenic mice bearing the cellular myc gene, coupled to immunoglobulin enhancer sequences μ or κ, frequently developed fatal B-cell lymphoma within a few months of birth (66). These results further support the conclusion that tissue-specific viral or cellular regulators can target the action of oncogenes to particular cell types. Nevertheless, the presence of tissue-specific regulators proximal to coding sequences is not sufficient to ensure their expression, as is clearly evident from Traina-Dorge's studies with recombinant inbred strains of mice bearing MMTV proviral genes whose expression, even in preferred tissues (mammary glands), is subject to some as yet undefined mechanism of regulation by unlinked host genes.

V. THE PROTEIN-CODING REGION (orf) OF MMTV LTR

As mentioned earlier, the U3-derived region of the MMTV LTR contains a long orf comprised of approximately 960 bp and possessing sufficient coding information to specify a polypeptide of approximately 36,700 daltons. Dickson and his coworkers originally discovered the LTR-associated orf when evaluating the protein-coding capacity of MMTV genomic RNA in a cell-free translation system. By partially degrading the MMTV virion-associated RNA which is in the positive sense and therefore serves as a translatable messenger RNA (mRNA) (as mentioned earlier), Dickson discovered a series of four methionine-rich polypeptides encoded within the 3′ portion of MMTV RNA (23-25). These four proteins were found to be completely unrelated to known viral structural proteins. i.e., gag, pol, or env by peptide mapping. The largest of these proteins was 36 kd; three smaller peptides 24, 21, and 18 kd which were predominant in vitro translation products were found to overlap with each other by peptide mapping and with LTR p36. Subsequent in vitro transcription and translation of LTR DNA, cloned into a bacterial plasmid, confirmed the presence of an LTR orf encoding an approximate 36 kd protein. The smaller species were derived from translation initiated at several different internal methionine codons (AUG) (24). Sequence analysis of cloned MMTV LTRs by Donehower et al. (9) and subsequently by Kennedy et al. (10) and Fasel et al. (11) independently confirmed the presence of a continuous orf beginning at the 5′ boundary of the LTR and extending approximately 960 nucleotides. Internal methionine codons were also discovered within the orf at which the translation of the smaller LTR-encoded polypeptides were presumably initiated during in vitro protein synthesis. Subsequent analysis of the LTR orf sequence suggested that it was not derived from host cellular sequences as, e.g., src or ras and other retrovirally associated oncogenes. Instead, these LTR orf sequences have apparently been conserved in at least three different strains of exogenous MMTV (9-11) and for at least one germline MMTV provirus Mtv-1 (L. Johnson, unpublished results). These observations argue strongly for a role of LTR orf gene product(s) in the MMTV viral life cycle and perhaps in MMTV-induced tumorigenesis. More details on MMTV RNA coding capacity and LTR orf is available in two excellent review articles by Dickson and Peters (26,67).

VI. TRANSCRIPTIONAL EXPRESSION OF THE LTR orf

Until quite recently, an RNA transcript of the LTR orf had not been recognized among the MMTV-specific mRNAs in infected mammary tissues and mammary tumors. Then, in rapid succession several reports were published describing subgenomic (1.6, 1.7, and 2.2 kb) MMTV RNA species comprised

predominantly of LTR homologous sequences. These RNAs contained leader sequences from the untranslated 5′ portion of MMTV DNA which suggested strongly that the transcripts had been initiated in the 5′ LTR, as are all other MMTV RNAs, but all the middle proviral DNA sequences (gag, pol, and env) have been spliced out of the mature polyadenylated orf mRNA (20-22). The LTR orf RNA was the only MMTV-specific RNA detected in BALB/c mice, which are not infected with MMTV and do not overtly express their MMTV germline proviral genes. Also, the expression of the LTR mRNA was limited in these mice to mammary tissues (20,21). In C3H and GR mice, where expression of MMTV germline proviral genes is dominant, 7.8-kb (genomic) and 3.8-kb (env) MMTV RNAs were detectable in mammary tissue as well as the smaller LTR transcript (21,22). While the first two reports (20,21) presented the biochemical characteristics of orf RNA structure which strongly support the conclusion that this transcript most probably represents the mRNA for the LTR orf protein, the latter study demonstrated a positive relationship between increasing steady-state levels of the LTR orf RNA and the progression of mammary epithelium from normal to preneoplastic to neoplastic tissue (22). Similar evidence was used to initially link increased cellular myc expression with lymphoma development (68-70). Nevertheless, the mechanism(s) by which myc RNA levels are maintained in neoplastic versus normal tissue is a complex and clearly unresolved question (71). This same appraisal can be made for the regulation of MMTV LTR orf RNA levels in normal, virus-infected, and neoplastic mammary tissue. This area clearly needs to be explored with relevance to MMTV's capacity to transform mammary epithelia.

Some clear statements can be made about MMTV LTR orf RNA regulation during mammary gland differentiation. However, caution is in order when attaching significance to these changes, because the cellularity of the gland is undergoing massive rearrangement with regard to the relative representation of epithelial versus stromal components. With that proviso, several studies indicate that LTR transcripts increase in abundance at midpregnancy in normal BALB/c mouse mammary tissue, where it is the only MMTV-specific RNA, and remain high throughout late pregnancy, lactation, and in 30-day post-weaning gland (72; M.S. Schwartz, personal communication). A similar pattern for LTR orf RNA regulation was observed in C3H/Sm mice, where all three MMTVmRNAs (genomic, env, and LTR orf) are constitutively expressed (G.H. Smith, unpublished data). Experiments to determine the effects of corticosteroid hormones on the regulation of LTR orf RNA have been attempted. Interestingly, both in vivo and in vitro treatment with dexamethasone results in a dose-dependent reduction of LTR orf RNA steady-state levels in transformed C3H/Sm mouse mammary tissues (Smith and Medina, unpublished results). This result is analogous to the negative regulation reported by Cordingley et al. (57) and discussed earlier, in mouse cells permanently transformed by DNA tumor virus episomes carrying genes under the transcriptional control of the MMTV LTR. Except for these preliminary indications, nothing is known of the regulation of this uniquely spliced viral LTR orf message. One difficulty encountered in studying this message in virally infected cells is related to the difficulty in detecting the absolute amounts of this transcript among the large excess of partially denatured virion-associated genomic RNA which contain LTR orf sequences proximal to the polyadenylated 3′ terminus. Thus, studies of LTR orf RNA and its regulation have been largely confined to the expression of MMTV proviral genes in animal models where the MMTV replicative cycle is blocked or incomplete (20-22). A second problem is suggested from the observation that LTR orf RNA levels are undetectable in preneoplastically transformed BALB/c mammary tissue outgrowth lines which harbor a single newly acquired exogenous MMTV provirus, whereas in BALB/c mammary outgrowth lines immortalized without acquisition of new MMTV DNA, the LTR orf transcript is relatively abundant and the only MMTV-specific RNA detectable (73,74). Unlike lactating mammary tissues, these lines are clonal. The absence of detectable LTR orf RNA

suggests that expression of the endogenous (i.e., germline) MMTV provirus responsible for the presence of LTR orf RNA (1.6 kb) in normal BALB/c mammary tissue is suppressed by the presence of the newly acquired active complete MMTV proviral gene in the tumor cell genome (73). This situation is analogous to the suppression of endogenous c-myc expression in the clonal B-cell lymphomas induced in transgenic mice carrying immunoglobulin-enhancer-linked c-myc in their germline DNA (66). The down regulation of the normal c-myc allele in this situation is thought to be due to autoregulation which is mediated via the gene products from the first coding frame of the newly acquired and transcriptionally activated c-myc gene. Therefore, the difficulty in detecting LTR orf RNA transcripts in MMTV-induced mammary tumors and their tissue culture derivatives may be due to down regulation of endogenous LTR orf RNA production within cells bearing newly acquired, fully expressed MMTV provirus(es). Conceivably, this might be explained by assuming that the LTR orf RNA translation products are stabilized in the presence of certain other MMTV viral gene products, e.g., gag or gag-pol, rendering the presence of LTR orf RNA unnecessary. In support of this hypothesis, induction of MMTV virion production by corticosteroid stimulation in MMTV-infected mammary tissues of C3H/Sm mice, which constitutively express MMTV LTR orf RNA transcripts, results in a dramatic reduction of LTR orf RNA and a concomitant increase in genome (8.1 kb) and env (4.4 kb) RNA (G.H. Smith, unpublished results). In vivo and in vitro replication systems for the study of infectious MMTV proviral DNA and its mutational derivatives will probably be required in order to fully appreciate the role of the LTR orf RNA transcript and its translational product(s) in the MMTV life cycle.

VII. MMTV LTR orf PROTEINS

Dickson's initial demonstration by cell-free translation of four methionine-rich polypeptides encoded by the LTR orf (23,24) stands as the seminal finding respective of definition of the orf "gene product(s)." Recent publication of similar sized proteins synthesized by cell-free translation of MMTV genomic RNA and immunoprecipitated by antibody raised against synthetic peptides derived from the coding sequences within the LTR orf provide further support of these initial studies (25; G.H. Smith, unpublished data). Nevertheless, to my knowledge, there is no published or submitted report identifying the natural LTR orf gene product(s) in fraction from MMTV-infected cells, tissues, or organs. The closest published account supporting the existence of a potential LTR orf-encoded protein is the demonstration of Rascevkis and Prasad (25) of a hybridization selectable RNA from BALB/c lactating mammary glands which produces upon cell-free translation a 36-kd protein immunoprecipitable by antibody raised against an LTR orf-derived synthetic peptide. As mentioned earlier, the 1.7-kb LTR orf transcript is the only MMTV-specific RNA present in BALB/c lactating mammary tissue. Attempts to use the anti-LTR peptide serum to identify the native protein in extracts from lactating glands were not successful. In my laboratory, two antisera raised in rabbits against two different LTR orf sequence-derived synthetic peptides produced immunoprecipitation lines when tested in Ouchterlony double gel diffusion plates against disrupted, gradient-purified, intracytoplasmic A particles. When tested together in the same agarose gel, these two antisera formed an immunoprecipitation line with identity when reacted with intracytoplasmic A particle proteins (76; A.M. McGrath, personal communication). In Western immunoblots of intracytoplasmic A particle proteins, several common polypeptides reacted with each of these sera. Absorption of the anti-LTR peptide sera with free peptide abolishes this reactivity. Preliminary experiments suggest that these LTR orf-related proteins possess binding affinity for nucleic acids. However, because of the presence of multiple sized gag and gag-pol precursor polypep-

tides in A particle SDS-PAGE gels, which possess determinants for the MMTV nucleocapsid ssDNA-binding protein, p14 (77,78), further experimentation is necessary to resolve the possible role of LTR orf peptide sequences in the nucleic acid binding activity.

What is the significance of finding LTR orf peptide determinants in association with intracytoplasmic A particles. These morphological entities are invariably present in the cytoplasm of cells replicating MMTV, and considerable evidence has accumulated demonstrating that these structures represent preprocapsids of MMTV (79-84). They possess a Mg^{++}-dependent reverse transcriptase with all the functional attributes of that found in the mature virion (85) and have been shown to contain newly synthesized DNA *in situ* by autoradiography (86). They contain MMTV-specific RNA with a complexity sufficient to protect full length radiolabeled MMTV cDNA from S_1 nuclease digestion following solution hybridization and cofractionate with DNA enriched for MMTV genomic sequences (87,88). In addition, A particle preparations have significant DNA binding and destabilization (unwinding) activities (78).

In general, authors of earlier reviews dealing with MMTV have underplayed the significance of A particles in the virus life cycle. At least in part, this is related to the absence of experimental data illustrative of a dynamic functional relationship between intracytoplasmic A particles and MMTV replication. Recently, however, studies of mutable transposable elements in *Drosophila* and yeast have clearly demonstrated the importance of subviral structural entities similar to MMTV-intracytoplasmic A particles in the production of efficiently transposed DNA copies of these genes following reverse transcription of their RNA. In a system developed for studying Ty (a transposable element of yeast), genetically tagged Ty was fused to the GalI promoter of yeast (89). In yeast cells containing this construction, Ty DNA transposition was inducible by galactose. Garfinkel *et al.* (90) reported that reverse transcription of Ty RNA and transposition of Ty DNA was invariably dependent upon the appearance of virus-like particles resembling A particles in the cytoplasm of induced cells. Reverse transcriptase, the ability to synthesize a genomic length Ty DNA and Ty-specified "*gag-pol*" antigen, all cofractionated with these subviral particles. These bodies are noninfectious, as are intracytoplasmic A particles (81) and accumulate in large clusters in the cytoplasm of galactose-induced cells (88), as do intracytoplasmic A particles in corticosteroid-treated MMTV-infected mammary tumor cells (91,92). Ty elements, like chromosomal MMTV proviral genes, are bounded by LTRs which bracket an internal DNA sequence of ~5.9 kb, the functional organization of which is similar to MMTV *gag-pol* (93). This central region is thought to encode a gene product which is produced in the form of a fusion polypeptide and gives rise to the functional "subviral" A-type particle. Two overlapping orfs in Ty specify proteins which have homology to retroviral DNA-binding proteins and to protease, integrase, and reverse transcriptase retroviral *pol*-encoded proteins. The DNA sequence of Ty predicts a primary translation product of approximately 200 kd; evidence that this prediction is correct and that the primary translation product is proteolytically cleaved to form various products. A similar activity has been detected in intracytoplasmic A particles (82-84) with the result that *gag*-related proteins with sizes similar to those found in the mature version are produced. Sequestration of one or more of the LTR orf gene products in intracytoplasmic A particles may suggest a role for LTR orf in one of the following viral functions: encapsidation, integration, reverse transcription, or the regulation of post-translational proteolytic processing of *gag* and *gag-pol* gene products. Transfection of LTR orf sequences under the control of a heterologous promoter into cells vegetatively replicating MMTV may serve to provide clues to the resolution of this hypothesis.

VIII. CONCLUDING REMARKS

Several interesting and functionally important attributes have been unequivocally assigned to the LTR of MMTV. These include: signals for promotion and initiation of viral-specific RNA transcription and its termination; a glucocorticosteroid responsive element for the positive regulation of transcription; sequences important for integration of MMTV proviral DNA into host genomic DNA; and finally, sequences which have the ability to enhance heterologous promoter-initiated gene transcription in *syn* or *anti* orientation. The latter characteristic has been designated the most important operative role in the promulgation of mammary oncogenesis by MMTV, i.e., through "insertional activation".

Features whose functions are less well documented, but whose existence may be equally compelling in MMTV-induced tumorigenesis, include the presence of a long orf in the U3-derived LTR component; evidence for negative regulation of viral transcription and the effect of heterologous enhancers of transcription through interaction with an unstable *trans*-acting "repressor" protein; tissue-specific direction of genetic activities leading to immortalization and malignant transformation of mammary epithelium in transgenic mice.

Two operationally distinct processes are paramount in the specific development of mouse mammary epithelial cell neoplasia, and these are generally representative of analogous phenomena in the mammary tissue of other mammals. Conveniently, these processes may be considered as two phenotypically distinct epithelial cell characteristics. They are: the acquisition of immortality (i.e., the incontrovertible ability to reproduce daughter cells with an indefinite reproductive capacity) and neoplastic transformation (i.e., the additional capacity to preemptively manipulate tissue-specific regulatory signals that are usually predictive of growth stasis, resulting in the expansion of the "immortalized" epithelial cell population). These two processes have been experimentally defined for mouse mammary epithelial cells by earlier experiments which have been adequately reviewed by Medina (94,95). Briefly, focal hyperplasias have been identified in mouse mammary tissue which possess, unlike normal mouse mammary tissue (96), unlimited division potential upon serial transplantation into the mammary fat pads of syngeneic hosts which have been cleared of mammary epithelium. These mammary epithelial populations are representative of the process of "immortalization" as described above. Compatible with this classification, these hyperplastic (preneoplastic) mammary populations are unable to proliferate except within mammary fat pads, cease to proliferate once the fat pad has been filled, and remain quiescent if placed in a fat pad already filled with normal mammary epithelium. Preneoplastic focal mammary lesions are found frequently in MMTV-infected mice, but they have also been found in MMTV-free mammary tissue of untreated mice. The rate of conversion from the preneoplastic (i.e., immortalized phenotype) to the transformed (neoplastic phenotype) state in these cellular populations is influenced by diet, hormonal stimulation, infection with MMTV, and exposure to chemical carcinogens (94,95). MMTV has been shown to have a strong stimulatory effect on both immortalization and neoplastic transformation of mammary epithelium (97,98,99). It is therefore reasonable to conclude that the presence of MMTV and its gene products accelerate the rate of these tumorigenic events. Since these two processes can be studied in mice that are not infected with exogenous MMTV (100,101) and in the absence of detectable MMTV *gag* or *env* gene products (102), it should be possible to pinpoint which viral gene products may be involved in the enhancement of these events. Two clues have emerged from such studies--a complete replicative viral life cycle is not necessary and concomitantly rearrangement or acquisition of MMTV proviral DNA is absent (44,102); however, in these "MMTV-free" systems, conversion of the preneoplastic (immortalized) cells to

transformed tumor cells is infrequent (101). These observations suggest that partial expression of MMTV proviral genes may be sufficient for the development and maintenance of the preneoplastic population. Evidence from two different experimental mouse mammary hyperplasia models show that the MMTV LTR orf transcript is increased in abundance in the preneoplastic (immortalized) tissue (22,72,74). Since preneoplastic mammary outgrowth lines have been shown to be clonal, or at a minimum quasiclonal (98,99), the apparent increase in the relative abundance of LTR orf RNA may reflect the differential expansion of cells partially expressing one or more of their germline MMTV proviruses. Acquisition of the transformed phenotype by cells within these outgrowth lines also does not result either in the appearance of newly acquired MMTV proviral gene copies or in the expression of a complete replicative viral life cycle (44,100,102). As mentioned earlier, the transformation event is rare in these preneoplastic outgrowth lines (101,-102). In a very well-studied BALB/c mouse preneoplastic mammary clonal outgrowth (D_2), an increase in tumorigenic potential was found to be associated with acquisition of a new MMTV provirus (74,75). The origin of this new MMTV provirus is as yet unclear. Nevertheless, one striking observation is that the chromosomal site of the new proviral integration is nearly identical in three independently isolated BALB/c hyperplastic mammary outgrowth lines which show frequent conversion of the malignant transformed phenotype (75). These studies tell us that MMTV tumorigenesis can be divided into two categories that are reflected phenotypically in the mouse mammary gland, i.e., preneoplastic immortalization and neoplastic transformation. They suggest further that the MMTV LTR plays an important role in each phase. In the initial phase, i.e., immortalization, no rearrangement of MMTV LTR within the context of the cellular genome is necessary; in the second phase, i.e., transformation, rearrangement and/or amplification of the MMTV LTR within the cellular DNA is associated with accelerated development of the neoplastic phenotype. Our current scientific technology in combination with the experimental biological systems mentioned within the text of this review are sufficient to the resolution of these LTR-associated elements of mouse mammary tumor virus-induced neoplastic transformation; only intellectual enthusiasm for the challenge is required.

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CELL BIOLOGY OF MOUSE MAMMARY CARCINOGENESIS IN ORGAN CULTURE

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I. INTRODUCTION

Mammary carcinogenesis including human breast cancer is a major cause of premature death of women in different parts of the world. Breast cancer also is the second highest cause of mortality of women in the United States.

Thus understanding of the pathogenesis of the mammary cancer has been of principal concern of the experimental oncologists.

Availability of the inbred strains of mice and the discovery of the mouse mammary tumor virus (MTV) in mouse milk (1-3) presented the opportunity for the experimental assessment of the tumorigenic characteristics of MTV in mouse mammary gland. The results showed that the virus is highly tumorigenic -- in certain strains of mice the incidence is near 100% and the process is modulated by the hormonal status of the animal (4,5). Also the tumor cells actively produce the virus. Advances in nucleic acid biochemistry have generated interest in the studies on the molecular basis of MTV-induced mammary carcinogenesis. Extensive studies during the past decade revealed that MTV has a single stranded RNA genome, it carries the enzyme, RNA-dependent DNA polymerase, which permits synthesis of a DNA copy of the RNA genome and its integration into the cellular genome as a provirus (6-8). Thus with regards to its genomic composition MTV resembles other RNA tumor viruses (retroviruses) including the Rous sarcoma virus (9). However, intensive efforts for over a decade have failed to find a link between the retroviruses, including MTV, and the human malignant disease. Although a viral etiology of human cancer is yet unclear, the knowledge and technology derived from these extensive studies have contributed significantly in the elucidation of the processes of normal differentiation in various organisms including human (10).

II. CHEMICALS AND MAMMARY CANCER

Meanwhile, contemporary studies revealed that a variety of environmental chemicals are also tumorigenic in experimental animals (11) and human exposure to these chemicals in industrialized societies is unavoidable. This striking realization then led to a focus on the elucidation of the processes of chemical carcinogenesis.

Huggins and his associates were the first to demonstrate that a single feeding of the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene (DMBA), to female rats would induce a high incidence of mammary cancer (12). Subsequently, Medina presented evidence that DMBA was also capable of inducing mammary tumors in female mice pre-stimulated with mammogenic hormones (13). Thus experimental models for studies on chemical transformation of the mammary cells *in vivo* became available in two different animal species.

Ichinose and Nandi in 1966 reported that full lobuloalveolar development of the mouse mammary parenchyma could be stimulated in organ culture of the whole mammary gland in medium containing mammogenic hormones (14). This accomplishment led to the question of whether DMBA-induced transformation of the mammary cells could be achieved in the isolated whole mammary organ in a mammogenic (mitogenic) hormone-supplemented culture medium. Studies in our laboratory then demonstrated DMBA-induced transformation of mouse mammary cells in isolated whole mammary gland in organ culture (16). This chapter includes discussion of the results of the recent studies obtained in our laboratory, using the *in vitro* transformation model: the mouse mammary cell in organ culture.

III. MOUSE MAMMARY GLAND DEVELOPMENT *IN VITRO*

The complex hormone-regulated developmental process of the mouse mammary gland, including the appearance of the mammary tumor *in vivo*, has been reviewed in recent years (17-21). A brief account is included in this section to provide background information.

In prepuberal female mice the mammary parenchyma is composed of ducts terminating as end buds and occasional alveoli. At pregnancy, the parenchyma undergoes hormone-stimulated morphogenesis and the development of lobuloalveolar structures. Subsequent functional differentiation of the alveolar structures then is marked by the appearance of the milk-proteins and their mRNAs (22). Weaning results in regression of the lobuloalveolar structures and the parenchyma returns to a predominantly ductal structure, with concurrent reduction of circulating hormone levels. The same sequential developmental events are repeated with each pregnancy. In the animal a combination of estrogen, progesterone, and prolactin constitutes the mammogenic hormone mixture which stimulates macromolecular biosynthesis and lobuloalveolar morphogenesis. A combination of prolactin and cortisol induces lactogenesis in the alveolar structures.

In post-partum animals lactogenesis is associated with increased levels of the milk proteins and their mRNAs, rough endoplasmic reticulum, and polyribosomes active in milk protein synthesis (22).

IV. MOUSE MAMMARY TUMORIGENESIS IN VIVO

The complexities of the multistep genesis of mammary tumors in mice have been discussed (23-26). Mammary tumors in mice often arise from alveolar dysplasia, referred to as hyperplastic alveolar nodule (HAN), regardless of their viral or chemical etiology. HAN is designated as preneoplastic tissue. Thus HAN can be considered as a morphological marker of preneoplasia. The multistep carcinogenesis of liver and skin are also believed to be marked by the appearance of the preneoplastic, hyperplastic nodules. In man a multistep process of neoplastic disease is also suggested by appearance of the dysplasias known as "carcinoma in situ" or "precancerous cystic hyperplasia". These "high risk" lesions may also represent a morphological marker of preneoplasia. HANs are microscopic alveolar nodules detectable in the involuted mammary glands that have been exposed to MTV and/or chemical carcinogens. After implantation into the gland-free mammary fat pad of syngeneic virgin host, HANs grow to form serially transplantable hyperplastic lobuloalveolar outgrowths that fill the mammary fat pad. HANs do not grow at ectopic sites and the alveolar tissue from HANs generally exhibit loss of hormonal responsiveness.

Hyperplastic lesions with possible preneoplastic characteristics have been observed also in the female breast tissue of several other species, including human. For additional details about the characteristics of the HAN tissue, the reader is referred to recent reviews (27-29).

Induction of HAN in mammary glands of virgin mice is dependent upon mammogenic hormone stimulation, a treatment that also enhances DNA synthesis and mitogenic activity in the mammary epithelium (21). Increased level of DNA synthesis in the tissue at the time of carcinogen treatment is thought to favor the tumorigenic process of the mammary epithelial cells (29).

V. ORGAN CULTURE OF THE WHOLE MAMMARY GLAND

Procedures for the culture of the whole mammary organ of the mouse have been described extensively (30-31). Priming of the immature female mouse by estrogen and progesterone is a prerequisite of a successful culture. Briefly, immature (3-4 week old) female BALB/c mice are primed by daily subcutaneous injections of a mixture of 1 μg estradiol-17β and 1 mg progesterone for 9 days. The primed animals are killed by cervical dislocation. The whole 2nd thoracic mammary gland is excised on a dacron raft. The gland, resting on the raft, is then transferred into a plastic culture dish containing warm, serum-free Waymouth's medium (MB752/1) supplemented with glutamine (350 μg/ml), penicillin (350 μg/ml) and different

combinations of steroid and polypeptide hormones. The glands floating on the medium are incubated in a humidified chamber in presence of 95% O_2 and 5% CO_2 atmosphere as described. The glands are first incubated for 6 days in a medium containing the different combinations of the mammogenic hormones, insulin, prolactin, estradiol, aldosterone and cortisol, to induce the lobuloalveolar development. This is followed by an additional 3-6 days of incubation in a medium containing the hormone mixture, insulin, prolactin, and cortisol, to stimulate functional differentiation (lactogenesis) of the alveolar structure. A subsequent incubation for 12-14 days in a medium containing insulin, or insulin and aldosterone, results in complete regression of the alveolar structures, leaving a ductal parenchyma. (Figure 1)

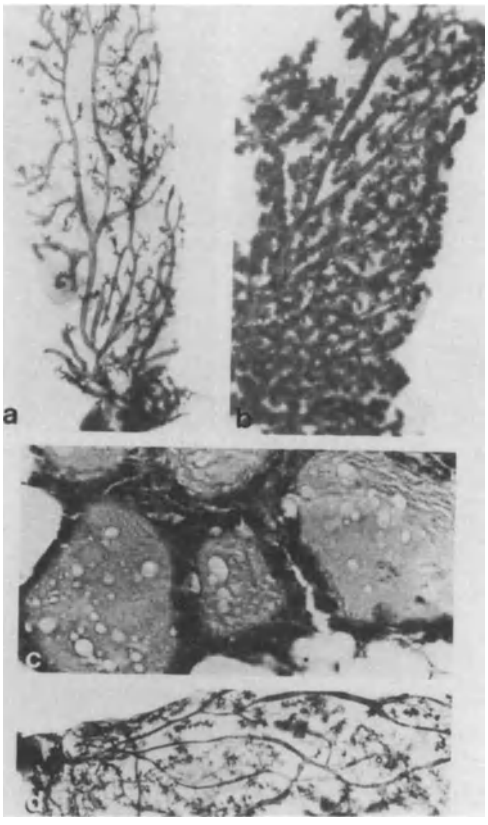

Figure 1. Growth and differentiation of the mouse mammary organ <u>in vitro</u>. (a) Second thoracic mammary gland from a 3- to 4-week-old female BALB/c mouse after nine daily injections of estrogen and progesterone. Only a ductal parenchyma is discernible. (b) Gland after 6 days of culture in medium containing the mammogenic hormone mixture of insulin, prolactin, growth hormone, estrogen, and progesterone. Note the extensive development of alveolar epithelial structures. (c) Histological section of a gland that was initially cultured as in panel b, and then incubated for a further 6 days in the presence of insulin and prolactin, and cortisol. The lumens of the alveoli are full of a milk-like secretion, containing proteins and fat droplets. (d) Gland cultured as in b, followed by 12 days in medium containing insulin and aldosterone. The alveolar structures can be seen to have regressed, leaving only a ductal parenchyma. (from M. R. Banerjee, 1976, Intern'l Rev. Cytol. 4: 1-97)

Like in the cultures of mammary explants from pregnant mice (32,33), insulin is needed for the viability of the parenchyma in cultures of whole mammary organs in serum-free medium. Also, a combination of polypeptide and steroid hormones are essential for lobuloalveolar development (34). In medium with aldosterone and/or cortisol plus prolactin, morphogenesis occurs, accompanied by an accumulation of casein mRNAs in the glands in vitro (35). Thus, like in the pregnant animal, lobuloalveolar morphogenesis is also accompanied by casein production in the glands in vitro. Thus the endocrinologically regulated morphogenesis that occurs in vivo can be mimicked in the glands in vitro by selectively controlling the hormones in the medium.

Consistent with the findings in vivo, morphogenesis of the glands in vitro is also accompanied by two waves of enhanced DNA synthesis during the 6-day culture period. The second wave of DNA synthesis corresponds with formation of the alveolar epithelium in the glands in vitro. The ductal epithelium in the glands involuted in vitro retains its potential to enter into a second round of lobuloalveolar development after continued incubation in medium containing epidermal growth factor (EGF) and mammogenic hormones (36).

VI. MAMMARY CELL TRANSFORMATION IN ORGAN CULTURE

The procedure for chemical carcinogen-induced transformation of the mammary cells in organ culture has been extensively described (31,32,37) and a brief account is included in this section. For additional details the reader is referred to the earlier reports.

Briefly, as a prerequisite of the procedure for culture of the whole mammary organ, the immature BALB/c female mice are primed for 9 days by daily injections of a mixture of 1 μg estradiol-17β and 1 mg progesterone. The entire 2nd thoracic gland is excised directly on a sterile dacron raft. The isolated whole mammary organ, resting on the raft, is then incubated in culture medium (Waymouth's medium, MB/752) containing glutamine, penicillin and the hormones insulin, prolactin, cortisol (5 μg/ml each) and aldosterone (1 μg/ml). The glands then are treated with 7.8 μM DMBA for 24 hr between 3rd and 4th day of culture. The 24-hr DMBA treatment period is timed to correspond with the 2nd wave of DNA synthesis in the gland stimulated by the mammogenic hormones. After the carcinogen treatment, incubation is continued for another 5-6 days in a carcinogen-free fresh medium containing the mammogenic hormone combination. This incubation with the mammogenic hormones apparently provides the hormone-mediated promotional action on the transformed mammary cells in the glands treated with DMBA. Mammogenic hormones are believed to act as promoting agents (38). Subsequently, an additional period of incubation is done in a prolactin-free medium containing either insulin alone or a combination of insulin and aldosterone for 12-14 days to allow regression of the alveolar structures. Figure 2 is a diagramatic presentation of the in vitro mammary cell transformation model in organ culture. Microscopic examination of the stained whole mount preparation of the glands shows a complete regression (involution) of the lobuloalveolar structures in the control glands treated with DMSO (solvent for DMBA). In contrast, isolated areas of nodule-like alveolar lesions (NLAL) are retained in the glands treated with DMBA or other carcinogenic chemicals. Since prolactin is essential for maintenance of the lobuloalveolar structures in the mammary glands in vitro (21), the presence of non-regressed alveolar structures (NLAL) in the prolactin-free medium indicates that the epithelium within the NLAL apparently has escaped from the normal hormonal requirement, indicating a state of transformation. Hence NLAL appears analogous to HAN. Studies by us and others have further shown that, in addition to DMBA, transformation effected by a variety of other carcinogenic chemicals (both direct acting carcinogens and those requiring metabolic activation) is also accompanied by the presence of NLAL in the mammary

glands in vitro (37,39,40). Thus NLAL constitutes an in vitro morphological marker of transformation of the mammary cells in organ culture.

VII. BIOLOGICAL CHARACTERISTICS OF THE TRANSFORMED CELLS

A. Tumor Incidence

While NLAL provides a morphological marker of epithelial cell transformation, escape of the epithelium from normal hormonal control per se may not indicate that the transformed cells in the mammary organ in vitro are potentially neoplastic. Confirmation of neoplastic potential of the mammary cells treated with DMBA in organ culture was obtained by transplantation into the mammary fat pad as described by DeOme et al. (41). Briefly mammary cells treated with DMBA in organ culture were transplanted into the parenchyma-free mammary fat pad of syngeneic virgin female mice. These experiments showed that the DMBA-treated cells produced hyperplastic outgrowths of mammary cells (MH-outgrowth) which filled the parenchyma-free fat pad. Mammary carcinoma then appeared in the MH outgrowth tissue (Figure 3). Both the MH outgrowth and the mammary carcinomas can be serially transplanted, indicating the irreversible nature of the transformation. Transplants of control tissue consistently produced typical ductal outgrowth in the virgin host (42). Thus the mammary cells exposed to DMBA in organ culture go through a multistage mammary carcinogenic progression similar to that occurring in the animal.

Recently we have observed that the environmental carcinogen, N-nitrosodiethylamine (DENA), also induces NLAL in the same mammary cell transformation model. The treated cells, and after mammary fat pad transplantation, produce serially transplantable MH-outgrowths (Figure 4). The mammary tumor incidence in the DENA-induced MH-outgrowth is however, extremely low (Table 1). This characteristic is consistent with the fact that DENA is not efficient in inducing mammary tumors in animals.

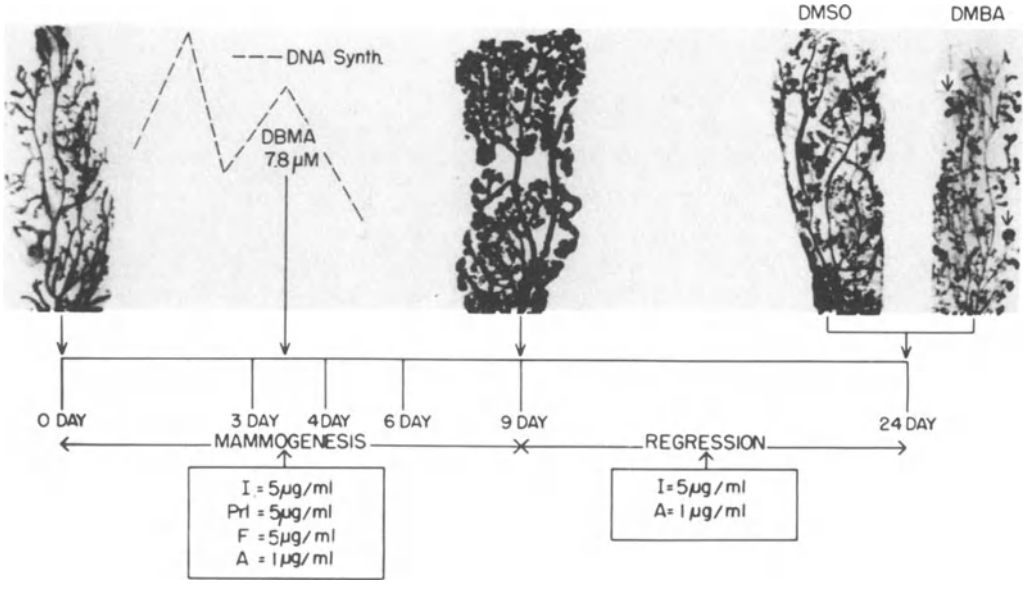

Figure 2. Experimental model for chemical carcinogen transformation of the mammary epithelial cells in organ culture. (from M. R. Banerjee, 1980, In: Cell Biology of Breast Cancer, Academic Press, NY, pp. 485-516)

B. Long Term Assessment of Tumorigenicity

The ability of DMBA-induced MH outgrowths to produce tumors was further analyzed in 12 outgrowth lines over 8-20 serial transplant generations (43). The results of this long term experiment showed that the characteristics of the individual MH outgrowth line were maintained through many generations of serial transplantation. The stable nature of the MH outgrowths was particularly evident in the MH-5 tissue. This was derived from a mixed alveolar and ductal tissue. The characteristic morphogenetic alteration of this outgrowth was maintained through serial transplantations of many generations. The twelve lines of MH tissues also remained tumorigenic during the 8-20 transplant generations. Evidence thus prompts the conclusion that, like the HAN, MH outgrowth lines derived from DMBA-transformed mammary epithelial cells in vitro are also preneoplastic.

VIII. HORMONE SENSITIVITY OF THE MH OUTGROWTHS

A. Growth Potential

The preneoplastic HAN present in mouse mammary glands generally have lost their normal hormonal requirements, regardless of their viral or chemical etiology (24). Accordingly, the influence of the mitogenic steroid and polypeptide hormones on the growth potential of four different MH outgrowth lines was analyzed. Samples of MH outgrowths were transplanted into the gland-free inguinal (4th) mammary fat pads of 3 wk old female mice (44,45). Cell proliferation in the implant was allowed to occur in the animal for 3 weeks filling 5% of the fat pad. The whole gland-free mammary fat pad carrying the implant was then excised on to a dacron raft and transferred

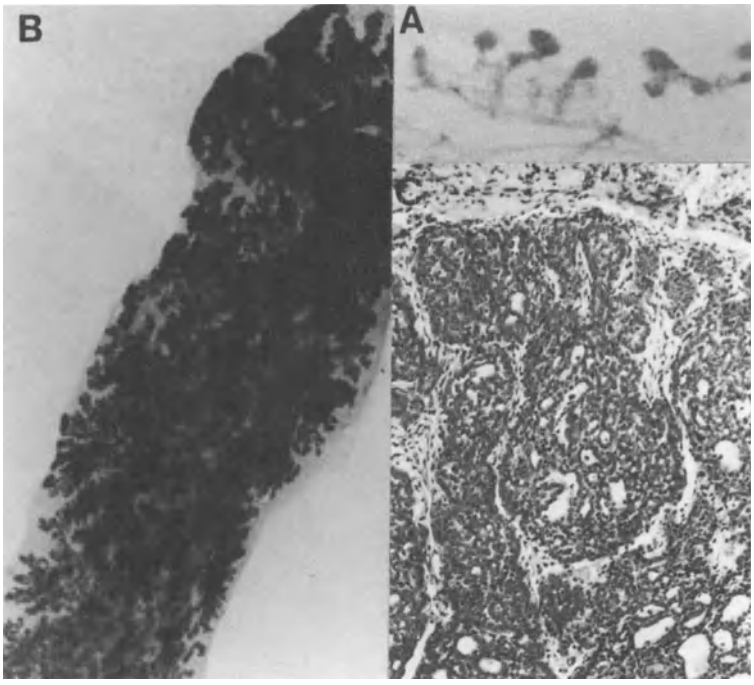

Figure 3. Hyperplastic mammary outgrowth (MH-outgrowth) and tumor obtained after transplantation of DMBA treated cells in organ culture. A, NLAL; B, MH-outgrowth; C, mammary carcinoma. Bar = 1 mm. x 10. Ln = lymph node. (from A. P. Iyer and M. R. Banerjee, 1981, J. Natl. Cancer Inst. 66: 893-905).

Table 1

N-Diethylnitrosoamine (DENA)-Induced Preneoplastic and Neoplastic Transformation of BALB/c Mouse Mammary Cells In Vitro

No. of Implants	No. of Ductal Outgrowth	No. of Hyperplastic Outgrowths	No. of Mammary Tumors
20	14	6	1

The mammary glands were digested with collagenase, 5 x 10^6 dissociated mammary epithelial cells from DENA treated glands were transplanted into one gland-free mammary fat pad of syngeneic virgin female mice. Contralateral fat pads received the non-treated control cells. Host animals were stimulated by a pituitary isograft into the renal capsule for 8-10 weeks. Stained whole mount preparation of the fat pads were examined 8 to 10 months after removal of the grafted pituitary. All fat pads carrying the control cells produced ductal outgrowths.

into the culture dish containing Waymouth's medium (MB 752/1) and the mammogenic hormones. The parenchyma-free mammary fat pad was found to support proliferation of the HAN tissue with over 60% of the fat pad filled with epithelium after 6 days of culture (Figure 5).

After 6 days of incubation in the medium containing the hormones, insulin, prolactin, aldosterone, and cortisol, the MH outgrowth tissue filled 60% of the fat pad. DNA synthesis in MH-1, MH-3, MH-5 and MH-9 increased to

Figure 4. Hyperplastic alveolar outgrowth (MH-outgrowth) obtained from the mammary cells transformed in organ culture after 24 hr treatment with N-nitrosodiethylamine (DENA). For additional detail, see table 1.

a peak on the 4th day and then declined to their respective basal level on the 6th day of culture. A similar single wave of DNA synthesis was also observed in a HAN line assayed under identical conditions. In contrast, DNA synthesis in the normal mammary glands in culture rises in two waves during 6 days of incubation in medium with the same hormone supplements. Thus the kinetics of DNA synthesis in the MH outgrowths and in the HAN tissue appears to be similar during morphogenesis in vitro, but the preneoplastic tissues exhibit a different pattern than that of the normal mammary glands in vitro. The increase in DNA synthesis was accompanied also by a rise in epithelial cell number. In the animal MH outgrowth grew and filled the fat pad by 8-10 weeks after transplantation. The same studies also showed that the MH-outgrowths are capable of extensive growth in fat pads in the growth medium containing insulin and prolactin, indicating that the MH-outgrowths, like HAN, also escape from the steroid hormone requirement for cell proliferation (4).

Development of the mammary fat pad culture method was prompted by the consideration of whether this tissue could serve as a site for heterotrans plantation in vitro. However, our preliminary studies showed that proliferation of the mammary tissue in the gland-free fat pad in vitro is dependent on priming of implant in animal for 3 weeks. Consequently the gland-free mouse mammary fat pad as an in vitro site for heterotransplantation is not possible.

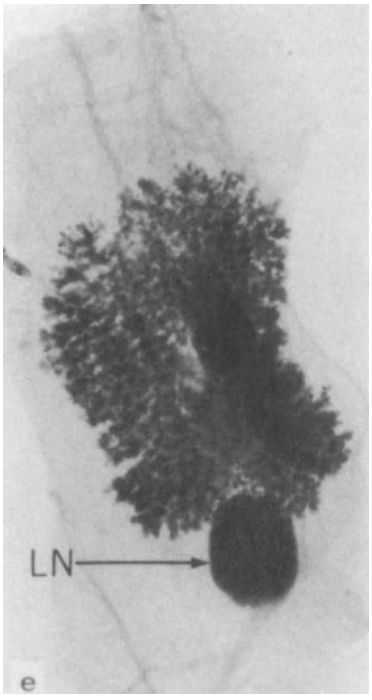

Figure 5. Spatial growth of the (hyperplastic) lobuloalveolar MH-9 outgrowth tissue filling the gland-free mammary fat pad. Arrows point to lymph nodes. Bar=1 mm x 10 (from N. Ganguly, R. Ganguly, N. M. Mehta and M. R. Banerjee, 1982, J. Natl. Cancer Inst. 69: 453-463)

Table 2

Levels of mRNAcsn in Total RNA from Preneoplastic MH Outgrowths Compared to mRNAcsn Levels in Normal Mammary Glands

Tissue	Host Mice	mRNAcsn,* %	Normal Gland mRNAcsn levels, % of Lactating mice	Normal Gland mRNAcsn levels, % of In Vitro
MH-1	Virgin	NM	-	-
	Lactating	0.0044	0.18	23.1
MH-5	Virgin	NM	-	-
	Lactating	0.0014	0.056	7.3
MH-9	Virgin	0.022	0.88	115.7
	Lactating	0.017	0.68	89.5
HAN-D8	Virgin	NM	-	-
	Lactating	0.006	0.24	31.5

*NM=not measurable.

For additional details, see reference 45. (from N. Ganguly, R. Ganguly, N. M. Mehta and M. R. Banerjee, 1982, J. Natl. Cancer Inst. 69: 453-463)

B. Milk-Protein Gene Expression

Functional differentiation of the MH outgrowths was determined in the animal by quantitating casein mRNA accumulation by molecular hybridization of cellular RNA to a casein cDNA probe (45). Table 2 shows that in the virgin host, mRNAcsn levels in MH-1, MH-5 and HAN-D8 remained suppressed, as expected. Although in lactating hosts, mRNAcsn was measurable in three transplanted preneoplastic lines, the concentrations of the mRNA in each of these tissues were only 0.056-0.88% of the levels present in the host's own lactating glandular tissue. Expression of the casein genes of MH-9 outgrowths, in virgin or lactating hosts, was also only at the levels of 0.68-0.88% of the normal lactation tissue. The extremely low levels of expression of the milk protein genes, even in the presence of the lactogenic hormone environment of the nursing mothers, indicate a virtual loss of sensitivity of the MH-cells to mammotrophic hormones. These results, obtained from the MH cells, are similar to those obtained with DMBA-induced HAN. Like in the mammary tumors derived from DMBA-induced HAN, casein mRNA was also generally not detectable in mammary tumors derived from MH outgrowths. Thus it appears that during the multistep transformation process the MH-outgrowth and the mammary tumor cells become insensitive to the normal lactogenic hormonal controls, regardless of whether transformation is induced *in vivo* or *in vitro*. Virtually identical results were obtained in another study measuring casein mRNA accumulation in DMBA-induced HAN and in mammary tumors *in vivo* (45a).

IX. PREVENTION OF MAMMARY CELL TRANSFORMATION IN ORGAN CULTURE

A. Inhibitory Action of Retinoids

Development of the mammary cell transformation model as described permitted analysis of the mode of action of several chemopreventive agents under defined conditions. The first of several studies in this series dealt with the mode of action of a retinoid on DMBA-induced transformation of the mammary cells *in vitro*. Incidence of NLAL in treated glands was used as marker for transformation *in vitro*. Retinoids include vitamin A (retinol) and its natural and synthetic analogs and these chemicals inhibit DMBA-induced mammary tumors *in vivo*. Certain retinoids can also modulate epithelial tissue differentiation, both *in vivo* and *in vitro*; progression of neoplastic growth is also known to be inhibited by retinoids. Moreover, retinoids also inhibit hyperplastic or metaplastic changes in the epithelial tissues caused by oncogenic chemicals. Retinoids also inhibit *in vitro* transformation of fibroblasts (46-49).

Dickens et al. (40) reported that retinylidene dimedone, a synthetic retinoid, can prevent DMBA-induced transformation of the mammary glands in culture at concentration as low as 1 nM, without any apparent cytotoxic effect. Exposure of the glands to the retinoid prior to (0-3 day) and during (3-4 day) DMBA treatment did not significantly inhibit transformation. However, when included in the culture medium after DMBA treatment (4-10 day) the retinoid caused a 90% inhibition of transformation. A similar level of inhibition was also observed when retinoid treatment was delayed until 3 days after DMBA exposure. Interestingly, a 72% reduction of transformation was also observed when the retinoid was present in the medium during the 10-24 day period of alveolar regression. We have postulated that the retinoid prevents transformation *in vitro* by acting mostly at the promotional stage. The loss of NLAL in the glands after incubation with the retinoid in the "regression" medium further indicates that the vitamin A analog can reverse the transformed state. These findings are consistent with earlier reports on anti-promotional action of retinoids *in vivo* on neoplastic transformation in a variety of organs, including the mammary gland. They are also consistent with reports showing that retinoids can cause regression of chemical carcinogen-induced benign lesions, hyperplasia, and metaplasia in different organ in culture (46-49).

Retinylidene dimedone also prevents mammary gland transformation caused by low concentrations of the procarcinogens, benzo(a)pyrene (BP) and FAA; but in presence of excess levels of the same procarcinogens the retinamide failed to block transformation. The same studies also showed that the retinoid was ineffective in blocking transformation by low levels of activated carcinogens such as BP-diol, BP-diol epoxide, N-OH-FAA, and 1-methyl-1-nitrosourea (NMU). Also the retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) failed to prevent the transforming action of NMU. Curiously, however, retinylidene dimedone was effective in preventing transformation caused by the direct-acting carcinogens, nitrosoamide, and MNNG. It is pertinent to mention that the studies summarized above were done using a non-oncogenic dosage of DMBA. As in animals, neoplastic transformation of mammary cells in organ culture with DMBA is dose dependent. Also, NLAL are induced at DMBA concentrations that are much lower than those required for tumorigenesis (39). (For additional details on this point, see reference 32).

Studies in our laboratory have shown that the potent environmental oncogenic compound, N-nitrosodiethylamine (DENA), can induce transformation of the mammary glands *in vitro* in a dose-dependent fashion. At the optimal concentration (1.5 μg/ml of medium) NLALs were present in 85% of the glands (9.5 NLAL per gland). Moreover, the transforming action of DENA was also

associated with a 3-fold increase of hydroxyurea resistant ^{3}H-thymidine incorporation into the DENA-treated glands, indicating an enhancement of DNA damage and repair activity. 4-HPR also caused a 61% inhibition of DENA-induced transformation of the glands with a concomitant decrease in the number of NLAL per gland.

B. Hormonal Influence on Retinoid Action

Retinoids are important modulators of differentiation. In the mammary glands, hormones play a critical role in normal and neoplastic development of the mammary cells. Thus, a possible influence of hormones on the chemopreventive role of retinoids during DMBA-induced transformation of the mammary glands in vitro was examined (50). Results showed that DMBA (7.8 μM) treatment of the gland, according to the standard protocol, induced transformation of 79% of the glands in medium containing insulin, prolactin, aldosterone and cortisol. After DMBA treatment (4-10 days of culture), the glands were exposed to 10^{-6} M 4-HPR and transformation of the mammary glands was reduced to 68%. The hormone mixtures insulin, prolactin, and aldosterone, or insulin, prolactin, growth hormone, estrogen and progesterone were equally conducive in DMBA transformation of the mammary glands in vitro. However, the preventive action of 4-HPR on DMBA transformation of the mammary gland was variable depending on the different combinations of steroid and polypeptide hormones in the medium. As indicated above, in medium containing the hormones, insulin, prolactin, aldosterone and cortisol, 4-HPR caused a 68% inhibition of transformation. However, the preventive action of the retinoid was reduced to only 15% when both estrogen and progesterone were also present. Moreover, in the presence of estrogen and progesterone plus insulin, prolactin and growth hormone, the retinoid only reduced transformation by 21%. These findings strongly suggest that the ovarian steroid hormones suppress the chemopreventive action of the retinoid. Further analysis revealed that when only progesterone was present in the medium along with insulin, prolactin, aldosterone and cortisol, the inhibitory action of 4-HPR was 52%; with estrogen but no progesterone in the medium containing insulin, prolactin, aldosteron and cortisol, the preventive action of 4-HPR was reduced to only 37%. The results thus indicate that among the two ovarian steroid hormones, estrogen is a potent antagonist to the action of the retinoid. These findings with mammary gland cultures are consistent with the report that ovariectomy enhances the chemopreventive action of 4-HPR as shown by the reduced incidence of mammary tumors in rats treated with NMU or DMBA (51). Results of the studies of the mammary gland in organ culture thus suggest that the enhanced preventive action of 4-HPR in ovariectomized rats is due to a depletion of endogenous estrogen. Although elucidation of the mechanism of estrogen antagonism on retinoid action will depend on future studies, evidence indicates that reduced prolactin levels in animals caused by 2-bromo-α-ergocryptin (a prolactin inhibitor) enhances the chemopreventive action of retinylacetate on NMU-induced rat mammary carcinogenesis (52). Since estrogen can modulate pituitary prolactin synthesis and release in the animal, ovariectomy is likely to lower the serum prolactin level and thus enhance the chemopreventive action of the retinoid. It is of interest to note that with a hormone combination of insulin, prolactin, aldosterone and cortisol, both mammogenesis and also DMBA-induced transformation is stimulated in vitro. In the in vitro system adrenal steroid hormone did not appear to affect the chemopreventive action of the retinoid.

C. Preventive Action of Selenium on Mammary Gland Transformation

Selenium (Se), a dietary element, has been reported to be effective in preventing neoplastic transformation by various oncogenic chemicals in a variety of organs. Epidemiological data also indicate that Se may be a protective factor against human breast cancer development (53,54). An

inhibitory action of Se on HAN induction by MTV or DMBA, also has been observed (55-58). The organ culture transformation model was used to assess the possible preventive action of Se on DMBA-induced transformation of the mammary epithelial cells (59). In these studies the mammary glands were treated with various concentrations of Se (Na_2SeO_3) at different times, before and after the 24-hr DMBA treatment of the glands during the total 9 day culture period in the medium containing the mammogenic hormone mixture, insulin, prolactin, and aldosterone.

As previously observed, DMBA (7.8 μM) treatment induced a 77% transformation of the mammary glands with an average number of 5 ± 0.6 NLAL per gland. Our results showed that 10^{-5}M of selenium (Se) in the medium prior to and during (0-4 days) DMBA treatment caused only 18% inhibition of NLAL development. However, if the same concentration of Se was added in the medium after DMBA treatment (4-10 day) there was an 84% inhibition of transformation. This indicated that while Se at 10^{-5}M concentration can prevent DMBA-induced transformation in vitro, its protective action is more pronounced when the treatment is given after carcinogen exposure (promotional stage). In the mammary system, then, selenium acts during the promotional stage. Hormones in the medium apparently act as promotional agents. The studies in vitro also revealed that the preventive action of Se is strictly dose-dependent. At lower concentrations (10^{-8} and 10^{-7}M) Se enhanced the transformation process during the initiation and promotional stages. These results, obtained from the mammary gland in vitro, appear to corroborate the epidemiological data which show that increased tumor incidence occurs in human populations in Se-deficient geographical areas. The mechanism of this Se mediated enhancement of transformation at low concentrations is unclear, although a dose-dependent differential action of Se has been observed on proliferative potential of mouse mammary cells in culture (60). Since increased cell proliferation is believed to be conducive to the neoplastic process, both at the initiation and promotional stages, it is conceivable that Se, at trace concentrations, may enhance the transformation process by maintaining an elevated level of cell proliferative activity in the glands in vitro. Se mediated reduced or enhanced frequency of transformed glands was also accompanied by decreases or increases, respectively in the number of NLAL per gland. This suggests that both the preventive and the enhancing actions of Se is mediated at the level of expression of the transformed cells in the glands in vitro. An inhibitory action of Se on expression of the transformed mammary cells as HAN also has been observed in mice exposed to the transforming actions of MTV and DMBA (55-58). Results of these studies also suggest that expression of the transformed cells as discrete lesions involves a unique process, apparently separate from the initiation events.

The mechanism of action of Se inhibiting the neoplastic process remains to be understood. A number of possible mode of action both at the initiation and promotional stages have been suggested. As an antioxidant Se may interfere with the metabolism of the procarcinogens, reducing the "initiation" process. Reduced DNA synthesis and cell proliferation caused by Se also has been suggested as possible mode of action during the post-initiation events. The observation that Se inhibits virally-induced mammary cell transformation, however, suggest that its action may not be limited to changes in the level of metabolic activation of chemical carcinogens.

D. β-Carotene Action on Mammary Cell Transformation In Vitro

Epidemiological studies suggest that β-carotene, the dietary vitamin A precursor, may exert some modifying influence on tumor incidence in humans living in different geographical areas (61). Several studies have indicated that carrot-rich diet or injections of β-carotene results in reduced incidence of tumors in rats and mice treated with carcinogenic chemicals or

ultraviolet radiation (62-66). However, these studies done in vivo do not permit a quantitative estimate about how much of the preventive action is due to β-carotene itself or by its metabolic product, vitamin A.

The chemopreventive properties of β-carotene was also tested during DMBA-induced transformation of the glands in organ culture (67). The mammary glands were exposed to β-carotene (carrot extracts, free of α and γ carotene; Sigma Chemical Corp., St. Louis, MO) before and after the 24-hr treatment of the glands with DMBA (7.8 μM), that is, between the 3rd and 4th day of the 10-day culture period in medium containing insulin, prolactin, aldosterone and cortisol according to the standard procedure. The glands were exposed to 10^{-6}M β-carotene (in n-hexane) between 0-3, 3-4, 0-4, 4-10 and 0-10 days of culture. β-Carotene inhibits DMBA-induced transformation of the mammary glands in vitro, as indicated by a reduced NLAL incidence. Incubation of the glands with β-carotene between 0-3 days (before DMBA treatment) produced a modest 37% inhibition of transformation. This relatively low level of NLAL incidence, however, may not be because β-carotene confers a resistance to transforming action of DMBA. Rather the results likely reflect a modifying action of the residual β-carotene retained in the glands exposed to the carotene before DMBA treatment (initiation stage) of the glands. The inhibition of transformation caused by β-carotene was 68% when the glands were exposed to β-carotene simultaneously with DMBA treatment (between 3rd and 4th day of culture). Exposure of the gland to β-carotene during the entire 0-10 day culture period, which includes the period of DMBA treatment, also showed a high level of inhibition of transformation (63%). The results further showed that incubation of the glands with β-carotene after DMBA treatment (4-10 days) also caused a 49% inhibition of DMBA transformation of the mammary glands in vitro. The findings summarized above thus indicate that the vitamin A precursor exerts a significant level of inhibition of DMBA-induced transformation of the mammary cells in organ culture, acting both at the initiation and the promotional stages. Nothing is known about the mechanism of β-carotene-mediated prevention of the neoplastic process, although several potential pathways have been discussed (61).

Our studies also showed that the chemopreventive action of β-carotene in the culture medium is not accompanied by any retinol accumulation (Figure 6), indicating that the results reflect action of β-carotene itself and thus mammary cells in vitro apparently do not metabolically convert the vitamin A precursor to retinol (vitamin A).

β-Carotene, as a free radical trapping agent, may reduce cellular accumulation of the electrophilic reactants, derived from the hydrocarbon. β-Carotene, acting as a scavenger of singlet oxygen, may also limit metabolism of DMBA by interfering with the cellular P-450 monooxygenase system. The action of β-carotene during the promotional stage may relate to its scavenging property for singlet oxygen, an entity which may cause cell membrane alteration by affecting lipid peroxidation (68). Alteration of cell membrane has been suggested to play a role during the promotional stage of transformation (69). Future studies in the isolated whole mammary organ in vitro should permit elucidation of the mechanism of action of the vitamin A precursor in preventing the processes of neoplastic transformation.

X. CARCINOGEN-INDUCED SISTER CHROMATID EXCHANGE (SCE)

Chromosomal aberrations and SCE are cytological manifestation of DNA damage and/or chromosomal rearrangement. SCE is characterized by double stranded DNA recombination at homologous sites of sister chromatids (70). Thus SCE provides an important morphological marker for the detection of the effects of physical and chemical agents on the cellular genome. Also, compared to chromosome aberrations, SCE is a markedly more sensitive indicator

of DNA alterations, because a notably higher incidence of SCE are caused by carcinogenic or mutagenic agents at concentrations much lower than the clastogenic levels (72). However, studies on the induction of SCE have usually been studied with cells in monolayer culture systems, principally because of the difficulty in obtaining good cytological preparations in cells from solid tissues. This problem has been overcome by modification of an existing method for the preparation of chromosomes from solid tissues (72). Also, by permitting the mammary epithelial cells to go through several cell cycles during hormone-stimulated morphogenesis, the differential labeling of the chromatids in organ culture was achieved. Thus the whole mammary organ culture was usable for measuring SCE (Figure 7) with the fluorescence plus giemsa staining procedure (73). Since the mammogenic hormones stimulate proliferation of the epithelial cells preferentially (21), SCE observed by the differential staining represents the chromosomal alterations produced primarily in the epithelial cells, the cell type of interest to mammary carcinogenesis. Data in Table 3 clearly show that the three carcinogens tested caused a significant enhancement of SCE in the mammary cells in organ culture over that seen in the control glands treated with DMSO, the solvent for DMBA. The high incidence of carcinogen-induced SCE is consistent with our earlier observation of a markedly enhanced DNA repair activity induced by NMU in the mammary cells in organ culture (21a). These findings are also consistent with the ability of NMU to induce transformation of the mammary epithelial cells in the same organ culture model. Diethylnitrosoamine (DENA), a relatively less potent mammary carcinogen than DMBA, also induced a low, but significant, increase in SCE.

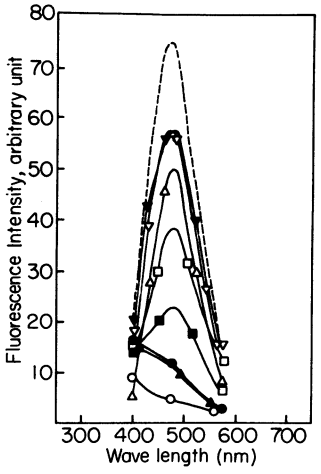

Figure 6. Fluorescence emission spectra of mammary gland extracts. Excitation was set at 340 nm (uncorrected). O——O, reagent blank; Δ——Δ, standard retinyl acetate (10^{-6} M); ●——●, extract of mammary gland after 10 days of culture; ▲---▲, extract of mammary gland after 10 days of culture in presence of 10^{-5} M β-carotene; ∇——∇, mixture of standard vitamin A and extract of mammary glands after 10 days of culture; ∇——∇, mixture of standard vitamin A and extract of mammary glands after 10 days of culture in presence of β-carotene; □——□ and ■——■, spectra of freshly excised virgin mammary gland extract, 0.5 μg to 0.25 μg retinol was added per 500 mg tissue, respectively, before extraction. The dotted line is the characteristic spectrum of an extract of murine liver (10 mg of liver per ml extract). The amount of mammary tissue taken was 500 mg in each case and the final volume of the extract before fluorimetry was 3 ml. (from S. Som, M. Chatterjee and M. R. Banerjee, 1984, Carcinogenesis 5, 937-940)

Table 3

Frequency of Sister-Chromatid Exchanges in Mouse Mammary Gland *In Vitro*

Treatment	Number of Metaphases	SCE/chromosome ± S.E.M.	't' vs. control
None (control)	30	0.238 ± 0.01	-
DENA	30	0.366 ± 0.02	2.99
DMBA	30	0.549 ± 0.04	11.17
NMU	30	0.756 ± 0.05	9.87
DMSO	30	0.278 ± 0.001	2.60

(from K. Manoharan and M. R. Banerjee, 1985, Mutation Res. 147, 165-169)

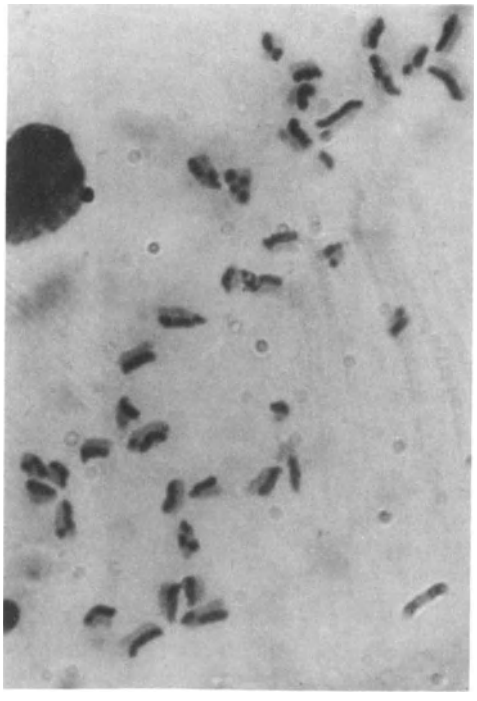

Figure 7. Sister-chromatid exchanges (arrows) in mammary epithelial cells in NMU-group. (from K. Manoharan and M. R. Banerjee, 1985, Mutation Res. 147: 165-169)

As indicated above, DMBA, NMU and DENA are capable of inducing a significant enhancement of SCE in the mammary cells in organ culture. Studies also showed that the presence of β-carotene in culture medium caused a pronounced reduction of SCE, acting at the initiation stage (74). A significant inhibition of SCE was also present in the DENA treated glands. The inhibition in the NMU-treated glands was 93%. In contrast, the retinoid (4-HPR), added in medium during DMBA treatment, failed to show a significantly inhibitive SCE transformation during the initiation stage. The virtual lack of an inhibitory action of the retinoid is consistent with its antipromotional action. β-Carotene, acting at the initiation stage, is capable of preventing carcinogen-induced DNA alteration in the mammary cells, as measured by the reduced SCE incidence. Although the biochemical pathways of the inhibitory action of β-carotene is unclear at this time, in cell-free assay β-carotene is an efficient quencher of singlet oxygen (75). In fact, in plants β-carotene may be the chief quenching agent for singlet oxygen, which is a product of photosynthesis (61). Singlet oxygen can activate the harmful effects of chemical carcinogens and consequently initiate the reaction such as lipid peroxidation. Also the free radical trapping property of β-carotene has been demonstrated (76). β-Carotene also exhibit antioxidant behavior at low oxygen pressure, such as exists in living tissue. β-carotene may also, because of its free radical trapping properties, modulate the cellular concentrations of the reactive electrophilic molecules derived from procarcinogen metabolism, thus reducing SCE. While the findings discussed above may not provide a complete explanation of the mode of action of the carotenoid, the important fact is that β-carotene has the potential to significantly modify DNA alterations induced by carcinogenic chemicals at the initiation stage. It is also of interest to note that β-carotene can modify the DNA damaging action of NMU, a direct acting carcinogen. Thus the results of the studies in the mammary glands done in a hormonally defined serum-free medium provide some important clues about possible pathways of action β-carotene in prevention of the neoplastic process.

XI. MECHANISM OF ACTION OF THE CHEMOPREVENTIVE AGENTS

Although a number of chemicals apparently can prevent DMBA-induced transformation of the mammary cells *in vivo* or in organ culture, the knowledge to date is largely confined to the phenomenology of tumor prevention and little is known about the mechanism of action of the preventive chemicals. This knowledge is essential before any of these chemicals can be endorsed as a prophylactic agent.

It is now general knowledge (77,78) that a variety of carcinogenic chemicals, including DMBA, are metabolized by the cellular microsomal monooxygenase system through a multistep process. The reactive electrophilic metabolites then alkylate DNA, forming covalently bound adducts of dihydrodiol epoxides. These DNA lesions then "initiate" mutagenesis, resulting in the formation of mutant cells which are promoted by a variety of nonelectrophilic chemicals, including hormones, to clonal proliferation. The transformed cells in the clonal population then are expressed as neoplasms. Thus it is reasonable to postulate that intervention with chemopreventive agents may occur at any one of the above multistep chemical events conceivably could modify the carcinogenic pathway with a resultant inhibition and/or reduction of tumor formation.

Se is one of the chemicals reported to prevent DMBA-induced transformation of mouse mammary cells in the animal and in organ culture by acting at the initiation and the promotional stages. Se as an antioxidant, may act on P-450 and thus modify the transforming ability of a carcinogen. Se, as a component of one of the glutathione peroxidases, may also by its intervention protect cellular membranes from aberrant oxidation, and thereby influence metabolism of the procarcinogen DMBA. Like Se, β-carotene, acting

as an antioxidant, may reduce (i) the activation of DMBA through the P450 pathway and reducing the cellular concentration of electrophilic reactants, (ii) may reduce DNA alkylation because of its strong affinity for reactive singlet oxygen, (iii) reduce DNA carcinogen adduct formation and consequently, mutagenesis. Thus β-carotene, by its intervention in any one of the multistep carcinogenic processes may act as a potent preventive agent. These possible pathways of action of Se and β-carotene has been discussed in recent reviews (61,63). Also the observations of the ability of β-carotene in reducing DMBA-induced SCE appear to provide cytological evidence of its modifying action on DNA alteration. However, knowledge about the mechanism of the chemopreventive actions of Se and β-carotene is rather fragmentary and the interpretations of the data are confusing. A dose-dependent, inhibitory action of Se on general metabolism of DMBA was observed in two earlier studies (79-81). In contrast, another study reported a total lack of an influence of Se on P-450 metabolism of DMBA, and DNA adduct formation assayed in a rat liver P-450 monooxygenase system (82). In this context it is pertinent to mention that the cell-free rat liver microsomal assay system may not reflect events occurring in vivo (83).

Recently Milner et al. (84) have reported that Se caused a dose-dependent inhibition of DMBA metabolism in mouse fibroblasts in culture. The dose dependent action of Se also caused a selective inhibition of anti-dihydrodiol-epoxide deoxyguanosine adduct formation in mouse fibroblasts, while formation of syn-dihydrodiol epoxide deoxyguanosine adduct remained unaltered in presence of selenium. The authors postulate that selective inhibition of a specific adduct formation may relate to the chemopreventive properties of Se. Virtually nothing is known about the pathway(s) of action of β-carotene in prevention of the neoplastic process in the mammary gland.

XII. ATTEMPTS TO MEASURE DMBA METABOLISM IN MAMMARY MICROSOMES

In consideration of the possible actions of Se and β-carotene on the multistep metabolic activation of DMBA, studies were undertaken in our laboratory to determine the influence of Se and β-carotene on DMBA metabolism in the mammary gland. Although binding of the hydrocarbon carcinogens including DMBA to mouse and rat mammary gland DNA was observed (85-88), we were astonished that no previous record of studies on DMBA metabolism in the mouse mammary gland, even though DMBA is the classical mammary carcinogen. Thus experiments were designed to measure DMBA metabolism in the mammary gland. An in vitro mouse mammary microsomal assay in a NADPH regenerating system containing ^{3}H-DMBA was developed. Virtually no diol derivatives of DMBA were produced in the assay, although phenol products were detectable in HPLC eluants. In contrast, a similar assay using liver microsomes from the same animals showed of both the diol and phenols were made in the reaction. Subsequently extraction of the DMBA diol metabolites directly from the mammary gland, was accomplished using formic acid digestion, followed by extraction of the digest with ethylacetate:acetone mixture. HPLC chromotography of the ethylacetate extract then resolved the characteristic diol derivatives of DMBA along with the usual phenols. TLC analysis of the DMBA treated tissue revealed that most of the metabolites remain entrapped in the mammary adipose material, resisting ethylacetate extraction. Thus the DMBA metabolites now can be obtained directly from the mammary glands in organ culture.

As indicated above, DMBA metabolites were obtained by ethylacetate extraction of ^{3}H-DMBA treated mammary gland from organ culture. HPLC analysis of the ethylacetate extract showed (Figure 8) that a prominant radioactive peak eluted slightly ahead of the cis-5,6-diol DMBA marker. Based on its close association with cis-5,6-diol DMBA marker, the material in this peak very likely represents the trans-5,6-diol DMBA. Trans-8,9-diol DMBA and trans-3,4-diol DMBA were also present, eluting with their respective

markers. Thus DMBA is metabolized in the mammary gland cultured in hormonally defined, serum-free medium to yield the 5,6-; 8,9-; and 3,4-diol derivatives. HPLC chromatography further revealed that, in addition to the diols, 2, 3 and 4 phenols along with the 12-hydroxymethyl- and 7-hydroxymethyl derivatives are also present, as identified using the appropriate markers. Thus the carcinogenic metabolites of DMBA are formed in the mammary glands in vitro. This finding is consistent with the requirement for formation of these products from DMBA for transformation of the glands in organ culture.

Quantitative analysis of the data further revealed that, of the total organo-soluble radioactive material recovered from the glands cultures, 54% was DMBA and the remaining 46% were the metabolites of DMBA. The metabolic derivatives of DMBA in the mammary gland, however, do not contain any water soluble material. Metabolites from the glands treated with ^{3}H-DMBA for 24 hr between 3rd and 4th day of culture was similar to that obtained at 16 hr. Analysis of ^{3}H-DMBA radioactivity in culture medium showed 95% of the DMBA added to the medium remains associated with the mammary gland in vitro and 99% of the remaining radioactivity in the medium is organo-soluble material. Of the organo-soluble material in the medium 99% was DMBA. The remaining 1% of the radioactivity, 22,000 cpm, (3.9 pmol) in the medium was water soluble conjugates of DMBA. However, analysis of the water soluble fraction failed to resolve any conjugate, presumably due to its extremely low concentration. Analysis of the metabolites obtained from collagenase-dissociated glands also revealed some interesting information. About 61% of the organo-soluble material isolated from the collagenase dissociated mammary cells was DMBA and its metabolites. Quatitative analysis of the material showed that ^{3}H-DMBA-exposed mammary gland produced the same metabolites after collagenase

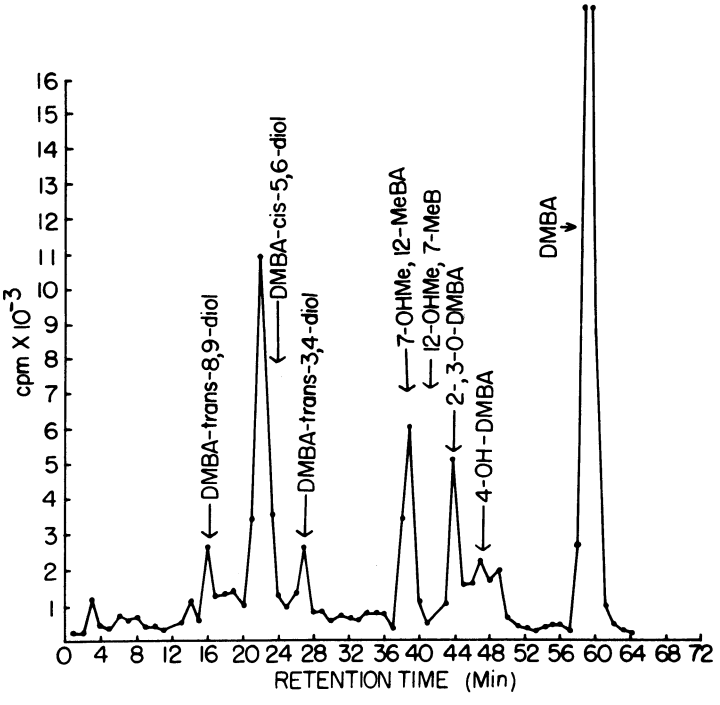

Figure 8. HPLC profile of ethylacetate:acetone (2:1) extractable metabolites from formic acid digests of whole mammary glands exposed to ^{3}H-DMBA for 16 hr in organ culture. 2 x 10^{-5} cpm was injected for HPLC analysis. The metabolites were analyzed on an ODS column (4.6 x 150 mm) using a methanol:water gradient.

dissociation. These metabolites, obtained from 20 collagenase treated glands amounted to only 613 nmol, whereas the yield of the metabolites from 4 whole glands was 1363 nmol. Thus the yield of the metabolites from the whole glands is 110-fold greater than the level obtained from collagenase dissociated cells. The markedly reduced yield from dissociated cells presumably is due to the loss of the entrapped radioactive material during the collagenase digestion procedure.

Since there is no previous report on recovering DMBA metabolites directly from the mammary glands, nothing is known about the characteristics of the dihydrodiol epoxide adducts formed with endogenous mammary cells DNA. Our studies, however, have shown that levels of the hydrocarbon bound to DNA is 24 pm/mg DNA after 16-hr exposure of the glands with ^{3}H-DMBA. Thus metabolites of DMBA do react to form DNA adducts in the mammary glands. These adducts are likely to result in mutagenesis. Additional analysis of the

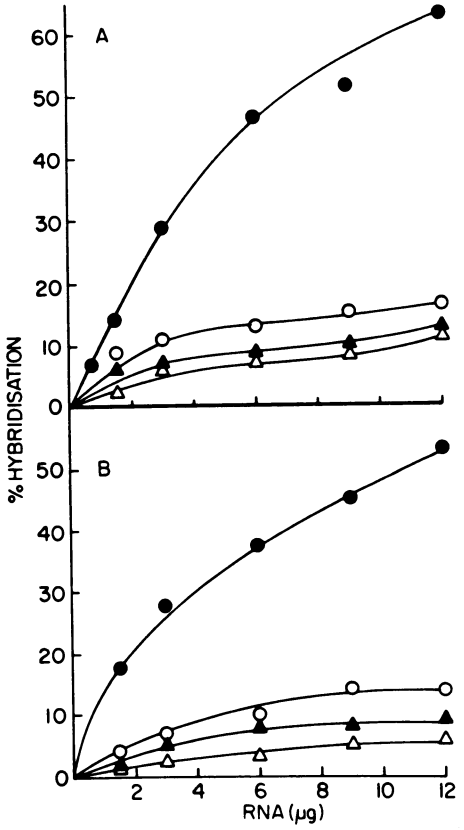

Figure 9. Influence of selenium on accumulation of WAP [A] and ϵ-casein [B] mRNA in murine mammary glands in vitro. [^{32}P] Labeled cDNAs were prepared from M13mWAP-719/1 and M13mϵ378/13 and hybridized to total RNA from cultured mammary gland subjected to different treatments. A RNA titration assay was employed whereby a constant amount of [^{32}P]cDNA (50 pg, ~10,000 hybridizable cpm) was hybridized with increasing amounts of RNA for 24 hrs. Data obtained from hybridization of total RNA (5 µg) from lactating mammary tissue was used as the 100% value. E. coli tRNA was used to measure non-specific hybridization. Glands were cultured for 9 days in medium containing O—O insulin (I), prolactin (Prl), aldosterone (A) and hydrocortisone (F); IPrlAF and different concentrations of selenium: O—O 100 nM, ▲—▲ 1 µM and Δ—Δ 10 µM.

adducts, including their identification, using appropriate markers are now underway.

XIII. POSSIBLE ADVERSE EFFECTS OF THE CHEMOPREVENTIVE AGENTS

Considerable evidence, from both in vivo and in vitro, experiments indicates that selenium, β-carotene and the retinoids are capable of preventing chemical carcinogen-induced tumorigenesis in different organs, including the mammary gland. This phenomenon is under intense investigation, knowledge about the possible adverse effect(s) of these agents is of particular importance, since they are being considered as potential prophylactic agents for MTN. The influence of the dietary, chemopreventive agents was tested by monitoring their effects on functional differentiation of the mouse mammary cells. The hormone-induced expression of the milk protein genes, β-casein, ε-casein and the whey acidic protein (WAP) was used as a marker of differentiation. The results, obtained by the molecular hybridization procedure, (88) indicated that selenium and the retinoid, 4-HPR, can cause a dose-dependent inhibition of accumulation of these mRNAs (Figures 9 and 10). In contrast, the concentrations of the three mRNAs in β-carotene treated glands were similar to those of the glands cultured in control medium containing the hormones and n-hexane, the solvent for β-carotene (Figure 11). However, the antagonistic action of Se and 4-HPR on mRNA accumulation was

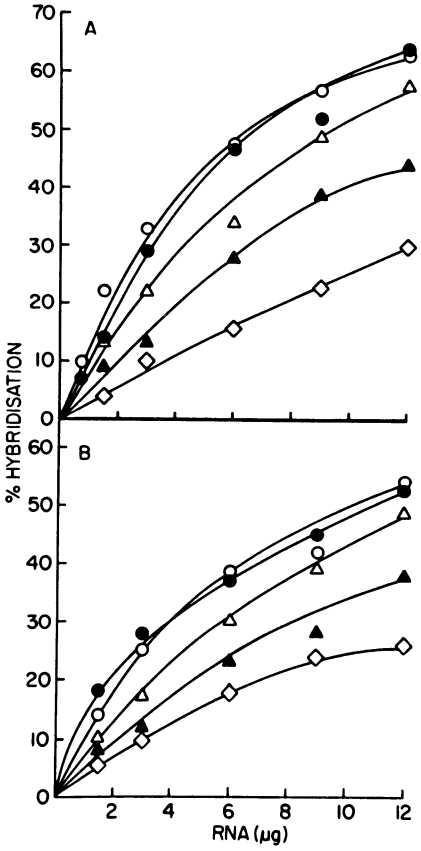

Figure 10. Influence of different concentration of 4-HPR on WAP [A] and ε-casein [B] mRNA accumulation. ●—● only IPrlAF; O—O IPrlAF and DMSO; IPrlAF and different concentrations of 4-HPR: Δ---Δ 10 nM; ▲—▲ 100 nM and ◆—◆ 1 μM. For details see figure 9.

reversible. Removal of these chemicals from the culture medium restored accumulation of the respective mRNAs in the hormone supplemented medium.

XIV. CONCLUDING COMMENTS

Thus in conclusion, the in vitro transformation model of mouse mammary cells in organ culture in an hormonally defined serum-free medium is ideally suited for studies on the elucidation of the mechanism of Se or β-carotene action in prevention of the mammary cell neoplastic transformation. We anticipate that future studies will provide important insight into the mechanism of action of the dietary chemicals in prevention of the neoplastic process. The knowledge then may make it feasible to develop some clinical approach for prevention of the disease.

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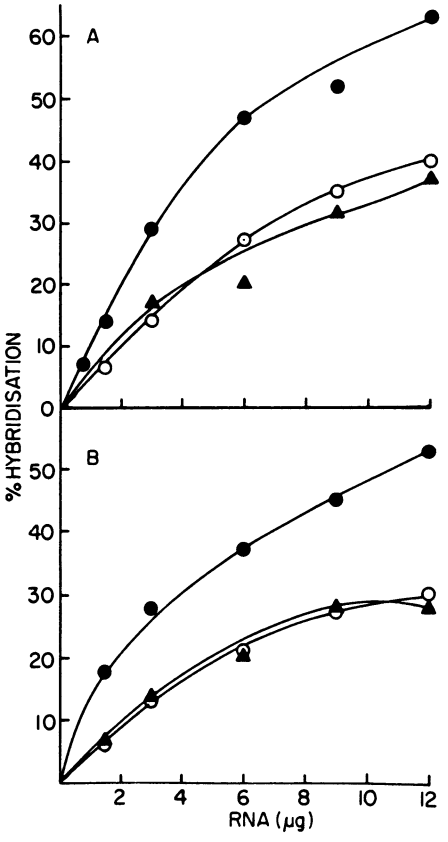

Figure 11. Influence of β-carotene on WAP [A] and ϵ-casein [B] mRNA accumulation. ●—● only IPrlAF; O—O hexane control for 1 μM β-carotene and▲---▲1 μM β-carotene. For details see figure 9.

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INVOLVEMENT OF ONCOGENES IN CARCINOGENESIS

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I. INTRODUCTION

Recent advances in cancer research have established the involvement of transforming genes (oncogenes) in the multistep process of carcinogenesis. Over the span of several decades, epidemiological studies have drawn attention to the fact that there is a high incidence of cancer among individuals exposed to mutagens in the workplace. The effect of many of these carcinogens, both physical and chemical, has been shown to result in direct alteration of the DNA in target cells by induction of point mutations or chromosomal translocations, suggesting a crucial role for genes in carcinogenesis (1,2). The observation that human tumors contain oncogenes activated by these mechanisms has led to the speculation that the direct mutagenic effect of the carcinogen occurs at the level of activation of the proto-oncogene, bestowing upon it the ability to cause phenotypic transformation.

The thirty or so proto-oncogenes characterized thus far have been shown to be constituents of the normal genetic repertoire of all cells (3,4). Several cellular proto-oncogenes show a tissue specific pattern of transcriptional activity and expression (5,6). Differential expression of cellular oncogenes during embryonic development (7,8,9) and liver regeneration (10) support the hypothesis that these genes are involved in normal growth and differentiation processes. This concept is further strengthened by the discovery that oncogene proteins can function as growth factors (e.g., one of the chains of platelet-derived growth factor is encoded by

c-sis) or growth factor receptors (e.g., epidermal growth factor receptor is encoded by c-erb B, and colony stimulating factor-1 receptor is presumably encoded by c-fms) (11,12,13,14).

The involvement of oncogenes in tumorigenesis became clear from several lines of experimentation. Studies on the transforming principle of acute transforming retroviruses revealed that the segments of the viral genome responsible for transformation had arisen by recombination between genomes of non-transforming viruses and transduced cellular proto-oncogenes. Since retroviral oncogenes were shown to be of cellular origin, it was reasoned that other related dominant genes may be involved in the development of cancers of nonviral origin. Activation of proto-oncogenes in human and animal tumors could occur by a) introduction of point mutation (ras-gene family, neu); b) chromosomal translocation (c-myc,c-abl, bcl-1, bcl-2, bcr); c) enhancer activation when the oncogene is placed under abnormal transcriptional controls (c-myc, c-erb-B, c-myb, int-1, int-2, pim, c-mos); or d) by gene amplification leading to appropriately high levels of expression of the oncogene (myc gene family, K-ras, c-abl, c-myb, c-erb B-1, c-erb B-2) (3,4). It is quite possible that cellular proto-oncogenes are activated by somatic mutations as a result of exposure to physical or chemical carcinogens, in a manner similar to those which occur in these loci during retroviral transduction.

The demonstration that the genomic DNA could be transferred to mammalian cells when introduced as a calcium phosphate precipitate was a major breakthrough in this field, as it set the stage for inquiry into the existence of transforming genes in naturally occurring tumors of man (15). In the next few years, using the transfection technique involving the ability of NIH/3T3 mouse fibroblasts to accept and express genes from donor tumor DNA, a wide variety of human as well as animal tumors were shown to contain genes capable of transforming these cells. As a result of the screening of a large number of human tumors of diverse origin and histological types, oncogenes have been detected in 10-15% of solid tumors and as many as 25% of leukemias tested (4,16).

Isolation and characterization of transforming genes from human tumors revealed that the majority of them belonged to the ras gene family, H-ras-1 and K-ras-2, identified previously as the transforming principle of Harvey and Kirsten strains of murine sarcoma virus (17,18,19), and the third member, N-ras (20,21). N-ras was discovered as a transforming gene present in the genome of a human neuroblastoma cell line and does not appear to have a retroviral counterpart. These results provided the first evidence for the presence of dominant genes in human tumors, analogous to those responsible for the transforming properties of acute transforming retroviruses.

In recent years, we have focused our research efforts on oncogenes in carcinogen-induced animal tumors. Carcinogen-induced animal tumors frequently contain activated oncogenes, and exhibit a specificity in terms of the type of tumor that result due to the carcinogen exposure and subsequent activation of endogenous proto-oncogenes (22,23,24,25,26). These models, as we shall see in this review, have provided us with a powerful tool for study of the role of oncogene activation in carcinogenesis.

II. MALIGNANT ACTIVATION OF RAS GENES

The conservation of the structure and expression of ras genes through the evolutionary ladder from yeast to man is strongly suggestive of the involvement of ras genes in normal housekeeping functions in the cell (27,28,29,30). All three mammalian ras genes code for highly related proteins termed p21 consisting of 188 or 189 amino acid residues. They bind guanine nucleotides (31,32), possess intrinsic GTPase activity (33,34), have been

localized to the inner surface of the plasma membrane (35,36) and share significant homology with the G-proteins (37,38). These properties suggest that ras proteins may participate in the process of signal transduction across the cellular membrane (39).

Molecular cloning and characterization of ras genes from a number of human tumor cell lines as well as from biopsy samples indicated that molecular alteration leading to the acquisition of tumorigenic potential by the ras gene could be attributed to a simple change in its coding sequence. The ras genes were shown to acquire malignant properties by single point mutations in two domains of their coding sequences, most commonly in codons 12 and 61 (40-44). Thus, substitution at position 12 of glycine by several other amino acids such as valine, cysteine, arginine, lysine and aspartic acid has been observed in transforming ras genes including H-ras-1, K-ras-2 and N-ras genes (16). In an attempt to determine what other amino acid substitutions would confer transforming properties to H-ras p21, Seeburg et al. demonstrated that all H-ras p21 proteins carrying amino acid residues other than glycine or proline at position 12 retain the ability to transform NIH/3T3 cells (45). These results fit very well with the prediction, based on computer assisted models, that the mutational activation of p21 may occur as a result of a conformational change due to the generation of a continuous α-helix with a rigid tertiary structure in the absence of glycine which normally provides breaks in the α-helical structure of proteins (46). In vitro mutagenesis of cloned human H-ras-1 gene has revealed that codons 13, 59 and 63 are also possible targets for mutational activation (47).

Although not frequently observed, a quantitative alteration in the ras protein by increased transcription or gene amplification has been shown to be involved in malignant transformation. Introduction of the normal H-ras-1 proto-oncogene under the influence of promotion by retroviral LTRs or integration of multiple copies of the normal human H-ras-1 gene, with resultant expression of abnormally high levels of cellular p21, promotes phenotypic and tumorigenic transformation of NIH/3T3 cells (48,49). In human and animal tumors there is no evidence for loss of transcriptional control of proto-oncogenes by mutations in their regulatory elements. On the other hand, more than a 10-fold amplification of ras genes has been observed in a variety of human tumors (49,50-52). Amplification of K-ras-2 up to 50-fold was observed in a mouse adrenocortical cell line associated with the presence of double minute chromosomes (53). Moderately elevated levels of p21 expression (2-10-fold higher than normal) has been seen in both premalignant and malignant tissues (54-56). Moreover, considering the fact that ras gene transcripts increase up to 8-fold in rapidly proliferating tissue such as regenerating rat liver (10), there is no experimental evidence to implicate moderate increases in ras gene expression in the development of neoplasia.

III. SUITABILITY OF ANIMAL MODELS TO STUDY THE ROLE OF RAS ACTIVATION

The overall low detection frequency of activated oncogenes in human tumors has raised questions regarding the significance of oncogene activation in the development of human cancer (57). The argument revolves around the central issue of whether oncogene activation is the cause or the consequence of malignant transformation, i.e., a mere manifestation of the genetic disarray characteristic of a tumor cell. The resolution of this argument requires the establishment of a clear link between oncogene activation and tumorigenesis. We believe that studies with human tumors, with the low detectable frequency of activated oncogenes, an etiology that is difficult to define, and a variable genetic background would not yield reliable answers. Well chosen animal model systems on the other hand, might provide clues into the role of activated oncogenes in carcinogenesis.

Animal models with well-defined etiological agents, reproducible induction and experimental manipulation of a specific tumor-type have provided the means of studying the significance of oncogene activation in the multistep process of carcinogenesis. How is oncogene activation related to the etiology? At what stage in the carcinogenic process are oncogenes activated? What are the effects of initiating or promotional influences on expression of the malignant phenotype of the activated oncogene? Is oncogene activation determined by the stage of differentiation of the cell, or by the etiological agent alone? In this review, we shall see how studies using experimental model systems have allowed us to make some progress in defining molecular events involved in the onset and progression of mammary neoplasia. The system we have chosen involves the induction of mammary cancer in rats by the chemical carcinogens N-nitrosomethyl urea (NMU) and 7,12,-dimethylbenz(a)anthracene (DMBA).

IV. RAS ONCOGENES IN ANIMAL TUMORS

The criteria that were important in the choice of animal models were that oncogenes be reproducibly activated under controlled experimental conditions. Mammary carcinomas are reproducibly induced in female rats by a single injection of NMU at puberty (58,59). The intravenous mode of injection of the carcinogen ensures distribution throughout the body and the age at injection provides target cells in the breast that are differentiating and proliferating under the influence of the sex hormones. In addition, mammary tumor development is hormone dependent. Ovariectomy prior to, or following NMU administration lowers tumor incidence to negligible levels. NMU is a direct acting carcinogen with a half life of about twenty minutes in the rat (58), which implies that tumor development occurs as a result of a single carcinogenic insult. These unique features make this model particularly suitable for studying the involvement of oncogenes in tumor development.

In our initial studies we demonstrated that DNA extracted from each of nine primary NMU-induced mammary carcinomas from Buf/N rats caused the appearance of morphologically altered foci in NIH/3T3 mouse cells due to the presence of transforming H-ras-1 oncogenes (22). In contrast, in the same assay, no transforming activity was observed using DNA from ten normal breast tissues obtained from age-matched female Buf/N rats. Molecular cloning and sequence analysis of the H-ras-1 oncogene from one of these tumors revealed that this carcinogen-induced tumor contained the same type of activating mutation as seen in human tumors. This observation validates the choice of this system as an adequate model for studying the role of ras gene activation in human neoplasia.

We have further extended and generalized these observations. Sixty-one of seventy-one (86%) NMU-induced mammary tumors in three separate strains of rats contained transforming H-ras-1 genes as detected by gene transfer assays (60). We have also utilized the DMBA-induced rat mammary tumor model in our studies. The mammary tumors induced by a single intragastric dose of this indirect-acting carcinogen are similar to those induced by NMU both in terms of histology and hormone responsiveness, but transforming H-ras-1 genes were detected in only 25% of the tumors tested, suggesting the existence of alternate pathways to tumorigenesis with this carcinogen (60).

Extensions of these studies have shown reproducible activation of H-, K-, and N-ras genes in a variety of carcinogen-induced animal models. These results are summarized in Table 1. Activation of the K-ras-2 locus has been observed in 40% of rat kidney mesenchymal tumors induced by single dose of DMN(OMe) (26) and 74% of lung tumors that arose following chronic exposure by inhalation to tetranitromethane (TNM) (S. Reynolds and M.W. Anderson,

TABLE 1. Transforming genes in Carcinogen-Induced Animal Tumors

Species	Carcinogen	Tumor	Oncogene	Oncogenes/No. Tumors Tested	Reference
Rat	NMU	Mammary ca.	H-ras-1	61/70	22,60
	DMBA	Mammary ca.	H-ras-1	6/29	60
	DMN(OMe)	Renal ca.	K-ras-2	10/35	26
	Radiation	Skin tumors	K-ras-2	6/12	a
	TNM	Lung ca.	K-ras-2	14/19	b
	ENU	Neuroblastomas	neu	3/3	25
	NMU	Schwannomas	neu	10/13	c
	MMS	Nasal ca.	?	8/8	88
Mouse	DMBA	Skin ca.	H-ras-1	33/37	23,62
	DBACR	Skin pap/ca.	H-ras-1	4/5	63
	DMBA	Mammary ca.	H-ras-1	3/4	64
	Several	Hepatomas	H-ras-1	25/25	67
	None	Hepatomas	H-ras-1	11/13	66
	X-rays	Lymphomas	K-ras-1	4/7	24
	3-MCA	Fibrosarcomas	K-ras-1	2/4	87
	TNM	Lung ca.	K-ras-2	9/9	d
	NMU	Lymphomas	N-ras-1	5/6	24

a S. Garte, personal communication
b S. Reynolds and M.W. Anderson, personal communication
c Our unpublished observations
d J. Stowers and M.W. Anderson, personal communication

personal communication). Whereas NMU-induced rat mammary tumors contain H-ras-1 oncogenes, consistent activation of the neu oncogene has been observed in tumors of the peripheral nervous system that arise following transplacental exposure to the same carcinogen (our unpublished observations). This transforming gene, first identified in three neuroblastomas cell lines derived from tumors induced by ENU, codes for a transmembrane receptor protein closely related to EGF (25,61).

In mice, H-ras-1 oncogenes were found to be reproducibly activated in skin papillomas and carcinomas of Sencar mice initiated by DMBA or dibenz-(c,h)acridine (DBACR) painting followed by promotion with TPA (23,62,63). Similarly, H-ras-1 oncogenes have been found in mammary carcinomas arising in mice treated with DMBA following implantation of the hyperplastic alveolar nodule line D1/UCD (64). Mouse thymomas induced by X-rays had transforming K-ras-2 oncogenes while the same type of tumor induced by chronic NMU treatment contained activated N-ras genes (65). K-ras-2 oncogenes have also been observed in lung tumors induced by TNM (J. Stowers and M.W. Anderson, personal communication). In the $B6C3F_1$ strain of mice, used very extensively for the screening of carcinogenic potential of chemicals, H-ras-1 oncogenes have been consistently detected in hepatocellular carcinomas arising spontaneously (66) or after treatment with carcinogens such as N-hydroxy-2-acetylaminofluorene (HOAAF) (67), vinyl carbamate (67), 1'-hydroxy-2'-3'-dehydroestragole (HODE) (67). The frequency of detection as well as the reproducible activation of ras genes in animal tumors strongly support the concept that ras oncogenes play a causative role in tumor development.

V. MODE OF ACTIVATION OF RAS ONCOGENE IN RAT MAMMARY TUMORS

Molecular cloning and sequencing of one of the H-ras-1 oncogenes isolated from a mammary tumor induced by NMU revealed that the mechanism of malignant activation of this gene was by single point mutation (22). The activating mutation was identified as a G → A transition in the second nucleotide of codon 12 (Figure 1). Such single point mutations in the oncogene alter sequences specifically recognized by restriction endonucleases, thus leading to generation of diagnostic restriction fragment length polymorphism (RFLP).

The $G^{35} \rightarrow A^{35}$ transition in the 12th codon of the H-ras-1 gene in mammary tumors induced by NMU eliminates a GAGG sequence (spanning residues 35-38) that is specifically recognized by the restriction endonuclease MnlI, creating a RFLP. Whereas the normal allele of H-ras-1 gene is contained in two MnlI DNA fragments of 206 and 74 base pairs, elimination of the MnlI restriction site by point mutation yields a single 280 bp MnlI DNA fragment (Figure 2). A total of 61 NMU-induced mammary tumors out of 71 (86%) in three different strains of rat showed the presence of a polymorphic MnlI site indicative of a malignant activation of the H-ras-1 locus (60). None of the 26 normal breast tissues obtained from age-matched normal Buf/N or Sprague Dawley rats, including four adjoining normal breasts from tumor bearers, exhibited the RFLP. This polymorphism was not detected in any of the mammary tumors induced by DMBA in spite of the fact that some of them contain H-ras-1 transforming genes as seen in gene transfer experiments. These results indicted that malignant activation of the H-ras-1 oncogene in NMU-induced tumors was the result of mutations specifically located in a region spanning four nucleotides suggesting a close association between mutagenesis by NMU in this specific domain of the H-ras-1 locus and the development of tumors.

In order to define the exact nature of the mutation in each of the H-ras-1 oncogenes present in NMU-induced mammary tumors, we used oligonucleotide probes capable of identifying specific point mutations in genomic DNA. As seen in Figure 3, using nonadecamers capable of identifying substitutions in position 35 of the 12th codon of H-ras-1 gene, each of 61 NIH/3T3

```
                    +1
                     met thr glu tyr lys leu val val val gly
H-ras-1              ATG ACA GAA TAC AAG CTT GTG GTG GTG GGC
NMU-H-ras            --- --- --- --- --- --- --- --- --- ---
                   +31
                     ala gly gly val gly lys ser ala leu thr
H-ras-1              GCT GGA GGC GTG GGA AAG AGT GCC CTG ACC
NMU-H-ras            --- -A- --- --- --- --- --- --- --- ---
                         glu
                   +61
                     ile gln leu ile gln asn his phe val asp
H-ras-1              ATC CAG CTG ATC CAG AAC CAT TTT GTG GAC
NMU-H-ras            --- --- --- --- --- --- --- --- --- ---
                   +91
                     glu tyr asp pro thr ile glu
H-ras-1              GAG TAT GAT CCC ACT ATA GAG
NMU-H-ras            --- --- --- --- --- --- ---
```

Figure 1: Comparative analysis of the nucleotide sequence of the first exon of rat H-ras-1 gene and its transforming allele, the NMU-H-ras oncogene. Nucleotide sequence identity in both genes is indicated by (-). The deduced amino acid sequence is also indicated. The single base change of G → A at +35 resulting in substitution of glycine to glutamic acid in p21 is shown.

transformants derived from NMU-induced mammary tumors hybridized to Ha19-A^{35} but not to Ha19-G^{35} or HA19-T^{35}, indicating that their H-ras-1 oncogene carried identical G → A transitions in position 35. These results demonstrate that each of the H-ras-1 genes present in NMU-induced mammary carcinomas became activated by the same mutation, a G → A transition in second nucleotide of the critical 12th codon. Taking into account that the preferred mutations induced by NMU are G → A transitions (68,69), these results strongly implicate the mutagenic activity of NMU in the generation of these oncogenes.

The striking specificity of the above findings suggest that the activating mutations in the H-ras-1 gene were a direct consequence of the mutagenic activity of NMU. However, other explanations are possible. It is possible that this mutation may confer a selective growth advantage to the neoplastic mammary cells carrying the mutated H-ras-1 gene. Alternatively, mammary cells may have repair systems that preferentially introduce deoxyadenosine residues. To address this fundamental question, we examined the mutations responsible for the activation of H-ras-1 oncogenes in mammary

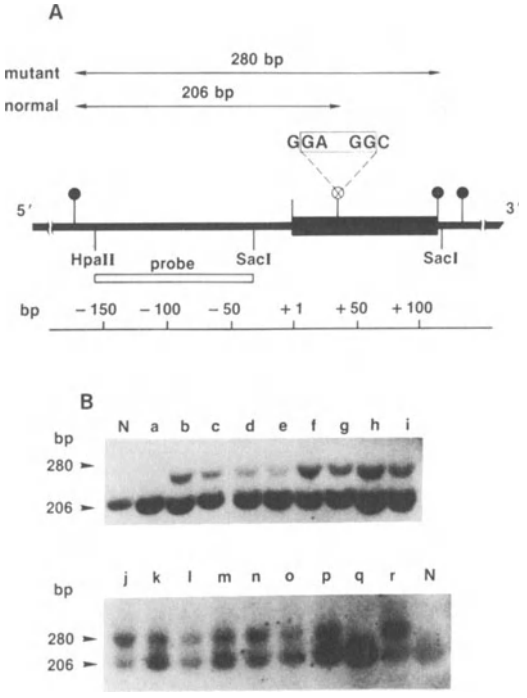

Figure 2: Detection of H-ras oncogenes in NMU-induced rat mammary carcinomas by molecular polymorphisms. Panel A: Wild type (●) and polymorphic (⊗) Mnl I cleavage sites either within or in the vicinity of the first exon (solid box, nucleotides +1 to +111) or the rat H-ras-1 locus are indicated. Southern blot analysis of Mnl I-cleaved normal rat DNA yields a fragment of 206 bp that can be identified with the indicated 120-bp Hpa II-Sac I [^{32}p]-labeled DNA probe. DNAs containing mutations within nucleotides +35 to +38 (GAGG) exhibit a 280-bp DNA fragment because of the elimination of the polymorphic Mnl I cleavage site (60). Panel B: DNAs isolated from normal rat mammary gland (N), and representative NMU-induced mammary carcinomas (a-r).

carcinomas induced by DMBA. This carcinogen forms large adducts with deoxyguanosine and deoxyadenosine residues leading to the induction of excision repair mechanisms, which occasionally generate point mutations of undefined specificity (70).

Each of the H-ras-1 oncogenes from DMBA-induced mammary tumors exhibited a normal 12th codon. It was, therefore, plausible that H-ras-1 oncogenes present in DMBA-induced mammary tumors were activated as a result of point mutations within the 61st codon, the other hot spot for activation of ras genes. Using mixed sequence oligonucleotides, activating mutations were localized in the two adenosine residues of codon 61 (CAA) (60). These findings rule out the possibility that the $G^{35} \rightarrow A^{35}$ mutations present in each of the NMU-induced H-ras-1 oncogenes are the result of either positive growth selection or specific repair systems. Instead they indicate that malignant activation of the H-ras-1 locus in NMU-induced mammary carcinomas is the result of the direct mutagenic effect of NMU on this locus.

VI. RAS GENE ACTIVATION AND INITIATION OF CARCINOGENESIS

The results reported thus far are highly indicative of an important role for ras activation in carcinogenesis. To understand the contribution of ras activation to the multistep process of carcinogenesis one must define the stage at which its activation occurs. Transforming H-ras-1 genes have been found in papillomas, a premalignant stage of mouse skin carcinomas induced by DMBA, followed by chronic application of a tumor promoter (62,71). This points to ras activation being an early event in skin carcinogenesis. It is difficult to define the stage at which ras activation occurs in the NMU-induced mammary tumor because tumor induction does not proceed through

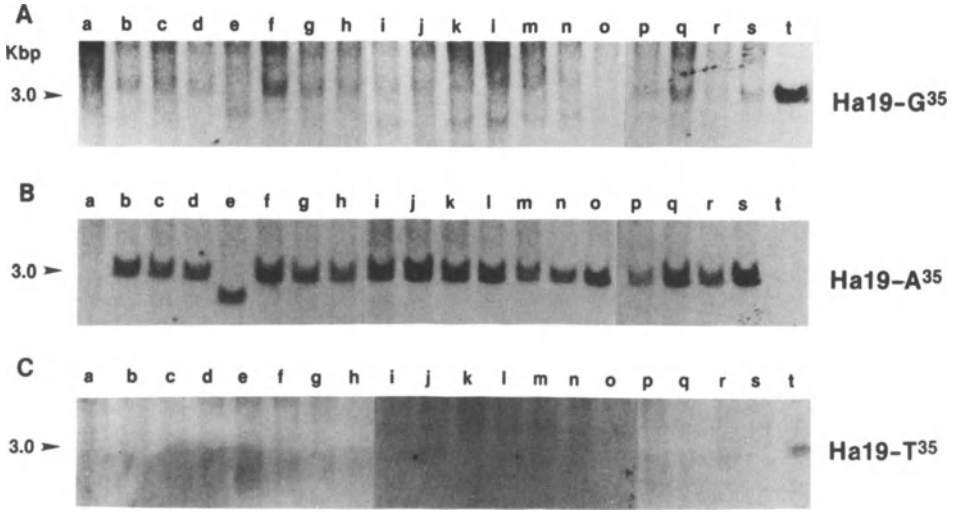

Figure 3: Use of synthetic oligonucleotide probes to determine the specific point mutation responsible for malignant activation of the H-ras-1 locus in NMU-induced mammary carcinomas. DNAs were isolated from NIH/3T3 cells (a), representative NIH/3T3 transformants derived from NMU-induced mammary carcinomas (b to s), and NIH/3T3 cells cotransfected with the normal H-ras-1 gene and pSV2-neo and selected for growth in the presence of G418 (t). A) Hybridization with probe Ha19-G^{35} (5'TGGGCGCTGGAGGCGTGGG 3'); B) Hybridization with probe Ha19-A^{35} (5'TGGGCGCTGAAGGCGTGGG 3'); C) Hybridization with probe Ha19-T^{35} (5'TGGGCGCTGTAGGCGTGGG 3'). Arrowheads indicate the expected 3.0 kpb Hind III DNA fragment of H-ras-1, which contains all coding sequences except the first 12 nucleotides.

identifiable preneoplastic stages. However, this model has other properties that made it possible to study whether ras oncogenes play a role in initiation of carcinogenesis. NMU is a potent alkylating agent that preferentially induces G → A mutations due to the generation of O^6-methyl-guanine adducts (68,69). Our results using oligonucleotide technique have shown that each of the 61 NMU-induced H-ras-1 genes carried the same activating G^{35} → A mutation. These findings strongly support the concept that these mutations are the result of NMU-directed methylation of the O^6 position of G^{35} in the critical codon 12. This direct acting carcinogen is very labile and must exert its mutagenic activity within hours after administration. Considering these two facts together, our results imply that the activating G → A mutation in the H-ras-1 gene must occur during initiation of carcinogenesis in this system.

VII. RAS GENES AS DIRECT TARGETS OF CARCINOGENS

The mutagenic action of most carcinogens is mediated via the formation of adducts with DNA bases (68). Some of these adducts are highly mutagenic due to their miscoding properties (69,72), while others lead to mutations due to generation of apurinic sites or because of the limited fidelity of repair polymerases (73). Although it was well recognized that a small number of these had the potential to trigger neoplastic transformation, identification of the genes in which these critical mutations occur proved elusive for many years.

The opportunity to correlate the known mutagenic effect of carcinogens with the activating point mutations in ras oncogenes arose following the detection of reproducibly activated oncogenes in chemically-induced animal tumors. The detection of a G → A transition in the H-ras-1 gene cloned from NMU-induced mammary tumors was a provocative finding in that one of the preferred mutations induced by NMU had occurred in an oncogene, resulting in its malignant activation. The O^6-methylguanosine adducts generated by the methylating activity of NMU are highly mutagenic as they are not removed efficiently by excision repair, mispair with thymidine residues and lead to G → A transitions (69,72). In contrast, DMBA forms large adducts with adenine and guanine residues whose repair rarely leads to generation of G → A transitions (68). These observations led to the proposal that NMU is directly responsible for the malignant activation of H-ras-1 oncogenes in this animal tumor system (60).

That oncogenes may be direct targets of chemical carcinogens is corroborated by the results obtained in other model systems (Table 2). Induction of mouse skin carcinomas by DMBA (62,63) and mouse mammary carcinomas induced by DMBA (64), specifically involve activation of H-ras-1 oncogenes by an A → T transition in the second base of codon 61. In spontaneous hepatomas arising in aged $B6C3F_1$ male mice H-ras-1 oncogenes are activated by random mutations in the first two nucleotides of codon 61 (S. Reynolds and M.W. Anderson, personal communication). However, when these tumors are induced by exposure to a carcinogen, specific mutational activations of the H-ras-1 oncogenes were observed, depending on the carcinogen used in the induction of hepatomas (67). Thus, hepatomas induced by a single dose of HO-AAF contained H-ras-1 oncogenes activated by C → A transversions in their 61st codon. The observed lesions arising from the formation of N-(deoxyguanosin-8-yl)-2-aminofluorene, the major DNA adducts of HO-AAF in mouse liver are G-C → T-A transversions (74,75). On the other hand, in hepatocellular tumors initiated by vinyl carbamate, H-ras-1 oncogenes were frequently activated by A → T transversions in the A^{182} residue (67). These results strongly support the concept that H-ras-1 genes are the direct targets of initiating carcinogens.

TABLE 2. Activation of H-ras Oncogenes by Specific Mutations in Carcinogen-Induced Animal Tumors

Species	Type of Tumor	Carcinogen	Activation Mutation*	Incidence	Ref.
Rat	Mammary ca.	NMU	$G^{35} \rightarrow A$	61/61	60
	Mammary ca.	DMBA	$A^{182}/A^{183} \rightarrow N$	5/5	60
	Lung ca.	TNM	$G^{35} \rightarrow A$	14/14	a
Mouse	Skin papil./ca.	DMBA	$A^{182} \rightarrow T$	33/34	62
	Skin ca.	DMBA	$A^{182} \rightarrow T$	3/3	63
	Skin papil./ca.	DBACR	$A^{182} \rightarrow T$	4/4	63
	Mammary ca.	DMBA	$A^{182} \rightarrow T$	3/3	64
	Hepatoca.	HODE	$A^{182} \rightarrow T/G$	10/10	67
	Hepatoca.	HO-AFF	$C^{181} \rightarrow A$	7/7	67
	Hepatoca.	VC	$A^{182} \rightarrow T$	6/7	67
	Hepatoca.	None	$C^{181}/A^{182} \rightarrow T/G$	10/10	a
	Lung ca.	TNM	$G^{35} \rightarrow A$	9/9	b

* Residues 35 corresponds to the second nucleotide of codon 12. Residues 181-183 correspond to codon 61.
a S.W. Reynolds and M.W. Anderson, personal communication.
b J. Stowers and M.W. Anderson, personal communication.

VIII. SECONDARY EVENTS IN MULTI-STEP CARCINOGENESIS.

The latency period of carcinogen induced tumors usually varies from a few weeks to several months, depending upon several factors including mutagenicity of the carcinogen, dose of carcinogen, and the species used forits induction. It is obvious that multiple steps are involved between the time of the infliction of genetic lesions by the carcinogen and the commencement of unrestrained growth characteristic of a tumor (76,77). Studies by Balmain and co-workers using the mouse skin model have provided a direct demonstration of secondary events involved in carcinogenesis following ras gene activation during initiation (23,62,71). Transforming H-ras-1 genes were present in the premalignant papillomas induced by painting the skin with DMBA followed by promotion with phorbol esters, a procedure that among other things, stimulates cellular proliferation. Most of these skin papillomas regress, but a select few progress to form carcinomas. Thus, in this system, one is able to distinguish at least three steps; initiating genetic lesions by the carcinogen, cellular proliferation induced by the tumor promoter, and an additional genetic lesion that commits a proportion of the papillomas to form carcinomas.

In the rat mammary tumor model, it is not possible to define the intermediate steps between initiation by the carcinogen and the appearance of a malignancy, since tumor development does not proceed through well defined, morphologically distinguishable, preneoplastic stages. Even so, there are at least two events that we can perceive as necessary for mammary carcinogenesis in this system; 1) activation of H-ras-1 during initiation and 2) cellular proliferation under the influence of the sex hormones during puberty. The proliferative state of the developing mammary gland at the time of the carcinogenic insult has been shown to play a fundamental role in mammary tumor development. Our recent results indicate that the two events, ras activation and mammary gland development do not have to occur concomitantly (unpublished observations). Newborn female rats treated with a single dose of NMU develop mammary carcinomas 2-3 months following sexual maturity. Most of these tumors carry H-ras-1 oncogenes, each activated by G → A transition diagnostic of NMU-induced mutagenesis. Interfering with

sexual maturation by treatment with anti-estrogens followed by ovariectomy completely prevents tumor development (unpublished observations). These results suggest that whereas H-ras-1 oncogenes might be activated early in life, hormone-mediated proliferation and/or differentiation is essential for the manifestation of its malignant properties.

In mouse skin carcinogenesis, it has been possible to demonstrate that initiated cells are capable of remaining dormant for long periods of time until they are treated with tumor promoters. Since most of the papillomas and carcinomas initiated by DMBA contain activated H-ras-1 genes (23,62,68) it is likely that initiated cells also carry activated ras oncogenes. Recently, experiments using Harvey murine sarcoma virus (78) have lent support to this hypothesis. Infection of mouse skin with Harvey MSV does not result in any overt changes unless it is treated with phorbol ester, following which tumor development occurs. Here again, cellular proliferation is an essential cooperating event for expression of the malignant phenotype of the ras oncogene.

Although there is sufficient evidence for the involvement of oncogenes in initiation of carcinogenesis, ras gene activation could also occur in later stages. In our own studies using guinea pig cell lines derived by treatment of embryo fibroblasts with a variety of chemical carcinogens, transforming ras genes were detectable in the late tumorigenic passages but not in the early non-tumorigenic ones (79). Primary rat embryo fibroblasts are refractory to transformation by ras genes unless cotransfected with nuclear oncogenes such as c-myc, N-myc, p53 or adenovirus E1A (80), or by pretreatment with chemical carcinogens (81). Here acquisition of immortality conferred by the nuclear oncogene or chemical carcinogen exposure was stated to be a necessary prerequisite to subsequent transformation by ras. The infrequent detection of activated H- and K-ras oncogenes in rat liver carcinomas initiated by the methyl(acetoxymethyl)nitrosamine followed by promotion with phenobarbital, and failure to detect them in the precursor lesions suggests that such activation is a late event in carcinogenesis (J.M. Rice, personal communication).

Considered together, these results show that ras oncogenes can participate at various stages of tumor development. ras gene activation can be involved in initiation of carcinogenesis; tumors develop only when the cells harboring the lesion are induced to proliferate either by application of phorbol esters in the DMBA-induced mouse skin tumors or hormonal influences during mammary gland differentiation in NMU-induced rat mammary tumors. In addition, ras oncogenes can also be involved in tumor promotion or progression in proliferating cells that have been initiated by mechanisms other than those involving activation of oncogenes (82,83).

IX. ROLE OF NORMAL DEVELOPMENT IN ACTIVATION OF SPECIFIC ONCOGENES BY CARCINOGENS

Our observation that there is exclusive induction of mammary tumors in rats treated with NMU at puberty is at odds with the fact that the carcinogen is administered intravenously and must therefore have access to all the organs of the body. Additionally, the only transforming gene observed in the NIH/3T3 gene transfer assay in each of these tumors is the H-ras-1 gene which is activated in each case by a G → A mutation. It is possible that NMU can exclusively activate only H-ras-1 genes. On the other hand, other oncogenes may become activated in various organs, but the phenotypic expression of their malignant properties is possible only under controlled physiological conditions. Since the mammary gland is undergoing differentiation and cell division at sexual maturity, the developmental stage of this organ might be playing a crucial role in determination of specific oncogene targets of carcinogens. It is probable that the H-ras-1 gene plays an

important role in the normal development of the mammary gland. Thus, in the presence of activated ras genes, the developmental stage of the organ with the accompanying cellular proliferation provides the physiological conditions necessary for the phenotypic expression of the transformed phenotype.

In order to determine if normal developmental factors influence oncogene activation, we changed the timing of the carcinogenic insult from puberty to the late stages of embryonal development, when several organs are undergoing active differentiation and development. NMU was given to pregnant rats at the 17th or 18th day of gestation. About 30% of the offspring that survive the transplacental treatment developed tumors of various types including those of neuroectodermal, epidermal, mesenchymal, and occasionally tumors of embryonic origin. DNAs extracted from these tumors were tested for transforming activity in the NIH/3T3 assays. Four different oncogenes were found to be activated in the tumors tested. For instance, tumors of the peripheral nervous system contained neu oncogenes, kidney mesenchymal tumors contained activated K-ras-2 genes, the three mammary tumors that arose in this protocol contained H-ras-1 oncogenes, and a neurofibrosarcoma contained a transforming gene distinct from 21 known oncogenes (unpublished observations). These results clearly ruled out the possibility that NMU could only activate H-ras-1 genes, and suggests that specific oncogenes can be activated in certain tumors depending on their developmental origin.

Each of the three mammary carcinomas were found to carry H-ras-1 oncogenes, adding further support to the concept that the nature of the target cell plays a fundamental role in determining the type of oncogene that participates in tumor development. Interestingly, the H-ras-1 gene present in these breast carcinomas became activated by G → A transitions, suggesting that their activation occurred as a result of direct mutagenic effect of NMU in the fetal stage (unpublished observations). If this is the case, it offers the opportunity to design experimental protocols in which initiation events involving ras oncogene activation in mammary carcinomas can be dissected from endogenous hormonal promotion occurring during sexual maturation and mandatory for the development of these tumors.

In summary, chemical carcinogens can target multiple proto-oncogenes; however, it appears that only one oncogene is allowed to phenotypically express its malignant properties leading to clonal expansion of these initiated cells to form a tumor. The phenotypic expression of the malignant properties of the activated oncogene appears to be, in a large part, determined by the developmental stage of the organ.

X. FUTURE TRENDS

There is substantial evidence, direct as well as indirect, that oncogenes are involved in the genesis of cancer. In their normal guise, as proto-oncogenes, they are concerned with the growth and differentiation of the cell. Mutations in their coding sequences change the function of the encoded product, resulting in the generation of a cancer cell.

The combined use of gene transfer and molecular cloning techniques have allowed the seminal discoveries in the past five years of the existence of dominant transforming genes in human and animal tumors and how they differ from their normal counterparts. Of particular importance have been studies using animal models with relevance to human cancer, which are providing insights into how and when oncogenes are mutationally activated. The reproducibility of activation of the oncogene by specific point mutations strongly argues against oncogene activation being a random event. In fact, this reproducibility casts them in a causative role in carcinogenesis. The model systems, in which it is possible to dissect various stages of neoplastic development, offer us the tools to study the nature of the cellular

factors that govern the phenotypic expression of activated oncogenes, and the role of the activated oncogene in the malignant progression of mammary tumors.

Although great strides have been taken in understanding the relevance of oncogenes in tumor development in carcinogen-induced animal tumors, and in neoplastic transformation of cells in tissue culture, attempts to extrapolate these findings to human cancer must be done with caution. The etiology of most human cancers is not known. It is possible that human tumors could be induced by chronic exposure to subcarcinogenic doses of a variety of carcinogens. Such exposures might cause the activating lesion in the oncogene; however, the expression of the neoplastic phenotype will depend on the existence of highly defined physiological conditions in the host, which in most cases, may not occur during a lifetime. Oncogenes could also be involved in later stages of tumor development and get activated as a result of an error in the replication machinery.

One must also remember that oncogenes have been discovered in a small proportion of human tumors. This may mean that oncogene activation is a minor pathway for neoplastic transformation in humans or that our detection techniques need improvement. The knowledge we have presently has been derived using primarily the ability of NIH/3T3 mouse cells to take up and express transforming genes. Nucleic acid hybridization techniques to identify ras oncogenes containing single point mutations, such as the use of oligonucleotide probes (84,85), and abnormal electrophoretic migration of DNA heteroduplexes containing single base mismatches in denaturing gradient gels (86) are proving to be useful in diagnosis, but are still quite cumbersome since they require at least six different probes to cover the two "hot spots" in the three ras loci and enough tissue to obtain sufficient DNA and RNA. Development of fast and reliable diagnostic tests will allow rapid screening of ras oncogenes in human biopsies. This data may help us find an association between activation of oncogenes and the etiology and pathology of human cancer, opening up the possibility of new strategies for early diagnosis and perhaps, treatment of cancer.

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ROLE OF DIFFERENTIATION ON TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS

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I. INTRODUCTION

There is increasing evidence that the fate of tumor development is determined by a conglomerate of circumstances acting at the time of initiation of the carcinogenic event (1). Tumors developed as a response to either a chemical, physical or biological insult do not arise at random, but require specific conditions of the host, of the target organ and of a specific target structure within that organ for the neoplastic phenomenon to take place (1,2).

Induction of breast cancer with chemical carcinogens administered to experimental animals emphasizes the importance of the conditions of the host at the time of carcinogen administration on the ultimate number and type of tumors developed. Successful tumor induction occurs when the carcinogenic agent is administered to young virgin animals, as long as they are postpuberal. Tumor incidence decreases with aging of the host, and administration of the carcinogen to parous animals almost completely fails to induce carcinomas (3-11).

Although the human population has not been experimentally examined, epidemiological evidence suggests that a trend similar to that observed in experimental systems also exists in human breast tumor development. Breast cancer arises more frequently in nulliparous women, or in women exposed to ionizing radiations at a young age, as was observed in the bombing of Hiroshima and Nagasaki (12), or in patients receiving chest radiations for tuberculosis (13).

The determination of whether carcinogenic initiation can take place only at a specific age, stage of development of the gland, or a specific condition of the target cell or target organ is of extraordinary importance from both a biological and an epidemiological point of view. If the above statement is true, then it would be easier to isolate the conditions that render an organ susceptible to neoplastic transformation, to identify the factors that are influencing such specific organ when it is at its peak of susceptibility, and as a consequence, to protect it from noxious stimuli at that time and to prevent cancer development.

Mammary carcinomas induced in rats by administration of the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) requires for their development that the mammary gland is still undifferentiated, meaning that its ductal structures end in dilated bulbous projections, the terminal end buds (TEB) (7-10). The presence of these TEBs that are highly susceptible to transformation by a chemical carcinogen is regulated by factors such as sex, age, ovarian function and gland topography, among others (2). Summation of these factors determines that the epithelium lining these structures shows the characteristics that make cells susceptible to neoplastic transformation: rapid cell division, which is associated with a greater ability of the cell to bind a carcinogen, and short cell cycle, which gives the cells a greater chance to fix and lesser chance to repair the damage inflicted in the DNA by a carcinogen (14-18).

The demonstration in the DMBA-induced rat mammary carcinoma model that there is a direct correlation between the presence of TEBs and mammary cancer incidence, and the fact that human breast develops in a pattern similar to that demonstrated for the rat mammary gland, suggests that young perimenarchal and nulliparous women between the ages of 12 and 24 years of age might represent a group of females at high risk of undergoing initiation of the carcinogenic process. This group, on the other hand, has a short period of time for exposure to any given carcinogenic agent. Therefore, pregnancy occurring at a young age would result in a shift of a high risk group to a low risk group through the protection offered by full differentiation of the mammary gland. Differentiation plays a key role in carcinogenesis because it reduces the high cell proliferative activity occurring in the immature gland of young females, and shifts the cells from a proliferative to a Go compartment (9), The differentiated cells produce less polar metabolites (18); the DMBA-DNA binding is significantly reduced in these cells, and the DNA repair is more efficient (17). All of these biological parameters favor reduction in susceptibility of the mammary gland to carcinogenesis.

Therefore, in the study of human mammary gland carcinogenesis, two main factors should be considered: (a) what are the biological parameters that determine the susceptibility of the mammary gland to carcinogenic initiation, and (b) the availability of an *in vitro* model that allows us to study the transformation of human breast epithelial cells as an end point in carcinogenesis.

In an *in vitro* system it is required that human breast epithelial cells are able to metabolize the carcinogen, to maintain their breast epithelial nature, and to show phenotypical manifestations of cell transformation when treated with carcinogens (18). The result of these changes should be related to the biological characteristics of the host from where they are derived. In the present work, we are reporting our results on the three major biological parameters that determine susceptibility of the breast to neoplastic transformation: (a) morphology of the gland; (b) rate of proliferation of the mammary epithelium; and (c) binding of the carcinogen to epithelial DNA.

II. BIOLOGICAL PARAMETERS OF SUSCEPTIBILITY TO CARCINOGENESIS

A. Morphology of the Human Breast

The breast is an organ characterized by marked heterogeneity, which is the result of the different phases of growth of the organism: from birth to puberty, at puberty, and finally during pregnancy (1,2,20-22). Besides changes associated with body growth and development, the topography of the gland is extremely heterogeneous due to its development from an invagination of the superficial ectoderm in the embryo, with the downward growth of the epithelium into the mammary fat pad, forming solid bands and chords of epithelial cells passing into the subjacent connective tissue. These epithelial chords become cannalized and convert into ducts by desquamation and lysis of the central epithelial cells. When fetal life ends, the breast in both males and females appears morphologically equal. An elementary system of ducts has been developed but no lobular structure or true secreting acini is present. During infancy the mammary ducts grow in length and only with the approach of puberty, their rudimentary mamma begins to show growth activity both in the glandular tissue and in the surrounding stroma. Glandular increase is seen initially in the formation of solid epithelial buds from a considerable length of terminal part of the ducts already laid down. These sprouts in turn form other short tube-like segments thus repeating on a smaller scale the type of growth observed at an earlier stage of development. Structural groups of small ducts or ductules are gradually formed and surrounded by loose connective tissue which is more cellular than the general supporting stroma of the breast. Before puberty, the ducts grow and divide in a dichotomous and sympodial basis. New branches and twigs develop from the terminal and lateral end buds. Lobule formation occurs only after menarche. The number of lobules increases with age, but some portions of the gland do not develop further if pregnancy does not supervene. Complete differentiation takes place only through pregnancy and lactation. From the histopathogenetic point of view, the functionally most important areas of the mammary gland are the terminal structures of the ductal system, either as terminal sprouts or lateral buds, and depending upon the degree of development of the gland, the terminal end buds, or lobules (2,22).

The morphological study of breast tissue obtained from reduction mammoplasty of 22 women allowed us to determine that the adult breast is composed of at least 4 different types of lobular structures according to the degree of alveolar development. Virginal or Type 1 lobules, which are branches that have lagged behind the general degree of development, although they may be found even through pregnancy or in fully lactating glands or in post-lactational glands (Figs. 1, 2). These virginal lobules may be either richly branched, with a strong tendency to form long sprouts or buds, or less extended with shorter internodes, and a tendency to form numerous clusters of short branches. These lobules are composed of approximately 11 large alveolar buds each (Table 1). The physiological significance of these two patterns of branching as well as the influence on further development of the gland have not been assessed yet. More developed lobules, composed of numerous alveolar buds of smaller size, are classified as either Type 2 (Figs. 3, 4 and Table 1) or Type 3, in which alveoli are even smaller and more numerous (Figs. 5, 6 and Table 1). Type 4 lobules are found in pregnancy and are composed of alveoli that are similar in size to Type 2, but the number per lobule is much higher, approximately 180 per structure (Table 1).

The characteristics of the lobular structures present in the mammary gland allowed us to classify these patients into three groups. All the patients with mammary glands composed exclusively of Type 1 lobules were classified in Group A (Fig. 7A and Table 2). The characteristics of the

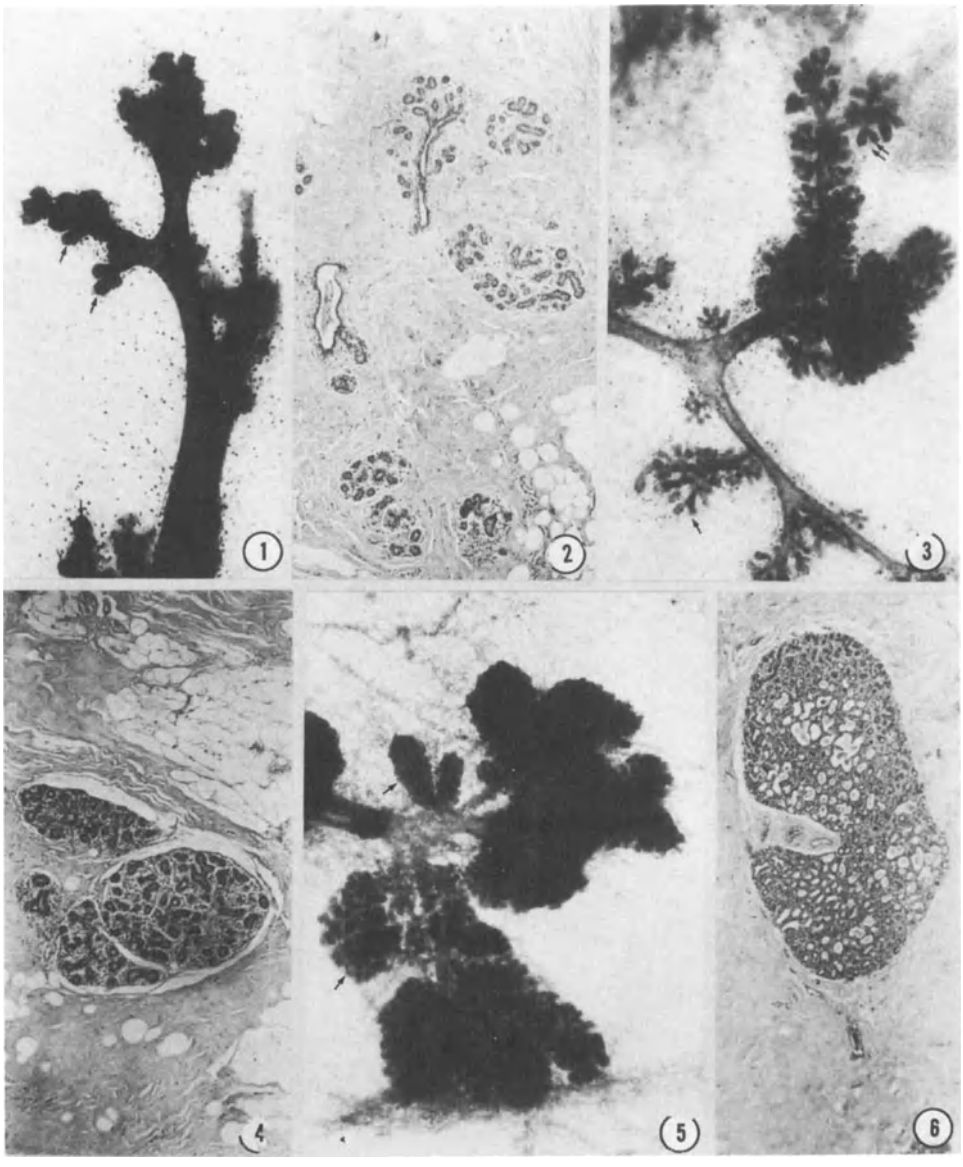

Figure 1: Whole mount of human breast (Sample #4) containing type 1 lobules are composed of alveolar buds (arrows). Toluidine blue, x 250.

Figure 2: Histological section of Sample #4, showing type 1 lobules composed of scarce alveolar buds. H&E, x 250.

Figure 3: Whole mount of human breast sample #17 in which type 1 lobules (double arrows) and Type 2 (arrow) are present. Toluidine blue, x 250.

Figure 4: Histological sections of sample #17 through a type 2 lobule. H&E, x 250.

Figure 5: Whole mount of sample #9 in which type 3 lobules composed of smaller alveoli are observed (arrows). Toluidine blue, x 250.

Figure 6: Histological section of sample # 9 in which a type 3 lobule is shown. It contains secretory material. H&E, x 250.

Table 1 Profile of the Compartments of Human Breast

Lobular Structure	Surface area[a]	Number of Alveolar Buds/Structure
Type 1	$0.232 \times 10^{-2} \pm 0.090 \times 10^{-2}$	11.2 ± 6.34
Type 2	$0.167 \times 10^{-2} \pm 0.035 \times 10^{-2}$	47.0 ± 17.0
Type 3	$0.125 \times 10^{-2} \pm 0.029 \times 10^{-2}$	81.0 ± 16.6
Type 4	$0.120 \times 10^{-2} \pm 0.050 \times 10^{-2}$	180.0 ± 20.0

[a]Surface area expressed in mm. The area was measured in a Zeiss Video plan. Statistical difference was determined by the t test. Type 1 versus Type 2, 3 and 4 lobules, $p < 0.001$. Type 2 versus 1, 3 and 4 lobules, $p < 0.001$.

Table 2 Profile of the Population Under Study

Group	Sample #	Age[a]	Parity History		Gland Development[f]
	1	30	G0[b]	P0[c]	Lob Type 1
	2	34	G0	P0	Lob Type 1, AB,ducts
	3	48	G0	P0	Lob Type 1, ducts
A	4	20	G0	P0	Lob Type 1
	5	23	G0	P0	Ducts only
	6	36	G0	P0	Lob Type 1
	7	53	G0	P0	Lob Type 1 and ducts
	8	24	G0	P0	Lob Type 1 and ducts
	9	52	G1	P1	Lob Type 2 & 3
	10	33	G1	P1	Lob Type 3
	11	31	G3	P3	Lob Type 3
B	12	30	G3	P3	Lob Type 3
	13	39	G2	P2	Lob Type 3
	14	18	G0	P0	Lob Type 3
	15	50	G2	P2	Lob Type 2 & 3
	16	60	G1	P1 + Ca[d]	Lob Type 1 & ducts
	17	30	G1	P1	Lob Type 1, 2 & ducts
	18	63	G4	P4 + Ca	Lob Type 1 & ducts
C	19	31	G3	P1 + Ab[e]	Lob Type 1 & ducts
	20	44	G5	P5	Lob Type 1 & 2
	21	31	G3	P3 + Ab	Lob Type 1
	22	36	G3	P3 + Ab	Lob Type 1

[a]age at the time of surgery in years
[b]Gravidity: Number of pregnancies
[c]Parity: Number of deliveries
[d]Ca: Infiltrating ductal carcinoma in contralateral breast
[e]Ab: Pregnancies ending in abortion in addition to full-term pregnancies (P)
[f]Lob = lobule, AB = alveolar bud

gland were correlated with the patient's age and parity history. It was observed that members of this group ranged in age from 20-53 years and they had no prior pregnancies. It is important to note here that there was no correlation between the age of the patient and the degree of development of the mammary gland. The morphology of the gland of a 20-year-old woman appeared almost identical to that of a 53-year-old woman. Those patients whose mammary glands were composed of Type 2 and 3 lobules were classified as Group B (Fig. 7B and Table 2). In this group, all the patients except one have had at least one pregnancy. They ranged in age from 18-52 years. In Group C were classified all those patients with glands that were quite heterogeneous, being composed of Type 1 and Type 2 lobules, or only ductal structures but no Type 3 lobules were present (Fig. 7C). In this group, the age range was from 30-63 years, and the most important feature was the fact that although they had a history of full-term pregnancy, some of them had abortions and two of the patients had developed a ductal carcinoma in the contralateral breast (Samples #16 and #18).

In summary, we conclude that human breast is a heterogeneous organ in which development is not related to age but to the reproductive history of the host. Therefore the reproductive history has to be considered as a normalizer in order to establish a baseline of mammary gland development,

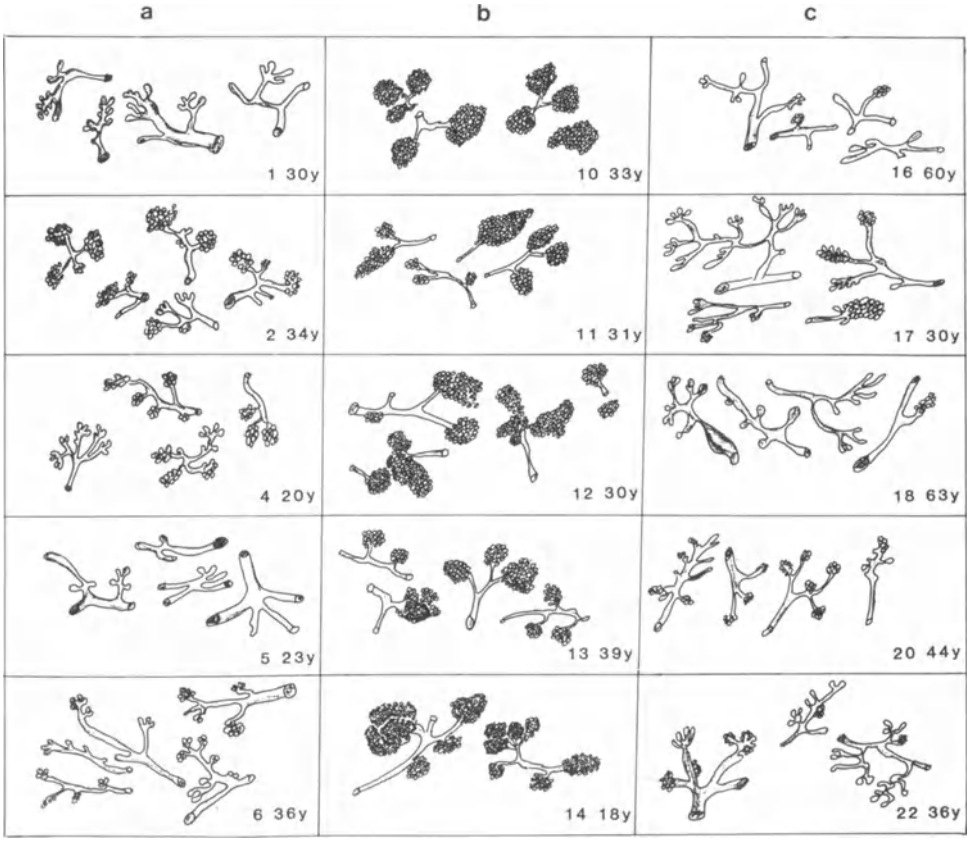

Figure 7: Schematic representation of human breast samples classified in groups A, B, and C. The first digit(s) indicate(s) the sample number, and the second two digits are the age of the patient. (See table 2 for comparison).

since this criterion seems to be more reliable than age or other parameters for the evaluation of mammary gland differentiation.

B. DNA Synthesis in Human Breast Epithelium

As it was described above, the development of the mammary gland is heterogeneous in the human population, but it is universally accepted that parity promotes gland development, as manifested by the presence of mature lobules or Type 3 lobules in this organ, and causes a diminution in proliferative activity in the different compartments of the gland (1, 2). This proliferative activity is modulated by the degree of differentiation of each specific structure (1,23,24). We have measured the proliferative activity of human mammary glands using the organ culture system. This method allows us to keep organ fragments in culture up to 5 weeks without loss of the relationship among the different topographic compartments of the gland and the surrounding stroma. The level of DNA synthesis (DNA-LI) was measured by one hour pulse of ^{3}H-thymidine and expressed as the number of labeled cells per 100 cells. This study led to the conclusion that the DNA-LI is higher in the more undifferentiated structures, namely Type 1 lobules, in which it is about 5.45 ± 2.5%. The more differentiated lobules, Type 2 and Type 3, have a statistically significant lower DNA-LI, 5 and 20 times lower than that of the Type 1 lobules (Fig. 8). The cell cycle (Tc) in the less differentiated structures (Type 1 lobules) is shorter than in the more differentiated ones, Type 2 and Type 3 lobules (Fig. 8). The lengthening of Tc is due to a lengthening of G1. These observations indicate that in the human breast, there are foci of high cell proliferation that are closely

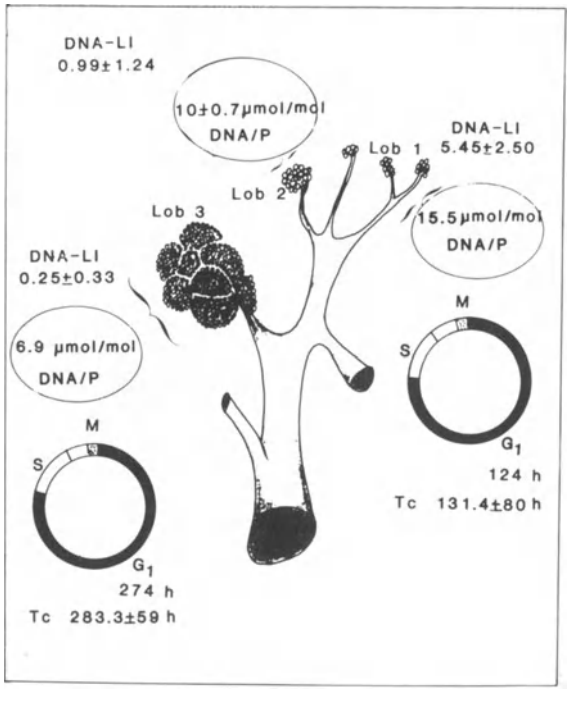

Figure 8: The values in the ovoid indicate binding activity of DMBA to DNA. The proliferative activity is represented by the DNA-LI and the length of the cell cycle (Tc) is expressed in hours (h); the length of G1 phase is also expressed in hours. The length of M and S phases do not change.

associated with the degree of differentiation of the gland, as it has been previously reported in experimental animal models (Fig. 8) (1,2).

C. DMBA-DNA Binding in Human Breast Epithelium

In our studies with the rat experimental model, we have shown that the incidence of carcinomas and binding of DMBA to DNA is inversely related to the age of the animal and the degree of differentiation of the mammary gland (17). Since covalent binding of the carcinogen to cellular DNA is accepted as a prerequisite for tumor initiation (25), it was of interest to initiate preliminary studies to determine whether DMBA also binds to human breast epithelial cell DNA and whether this binding is influenced by the degree of gland differentiation of the patient.

Primary epithelial cell aggregates prepared from normal human breast tissue (18) were treated with ^{3}H-DMBA (0.4 μg/ml) for 24 hrs after 4-5 days in culture (log phase). Cells were then harvested, DNA isolated and the amount of DMBA bound to DNA was determined. Binding was then correlated to the type of structures present in the mammary gland. The results of this correlation are summarized in Figure 8. Three distinct levels of DMBA-DNA binding which correlated with the degree of development of the structures of the mammary gland were observed. A higher level of binding (15.5 μmol/mol DNA-P) occurred in cultures from more undifferentiated breasts containing Type 1 or virginal lobules; an intermediate level of binding (10 μmol/mol DNA-P) was observed in cultures which were derived from breast tissues composed predominantly of Type 2 lobules, i.e., from more differentiated glands and a low level of binding (6-9 μmol/mol DNA-P) was observed in cultures from completely differentiated glands, exclusively composed of Type 3 lobules and with no evidence of terminal ductal structures. The DMBA-DNA binding levels for the less differentiated tissues were significantly higher than the values found in the more differentiated tissues ($P<0.0001$).

These results demonstrate that human mammary epithelial cells in culture not only possess the ability to metabolize DMBA to products that bind DNA, but also show that binding to DNA is dependent on the degree of differentiation of the mammary gland. They are also consistent with binding data obtained in our rat experimental model (17) which show a higher level of binding in the undifferentiated cells from young virgin rats and a lower binding in the more differentiated cells in the mammary gland of parous animals.

III. CELL TRANSFORMATION AS AN END POINT OF CARCINOGENESIS

Environmental carcinogens have been estimated to be the cause of 80-90% of human cancers (26-33). Carcinogenic compounds such as benzo(a)pyrene are found in the environment, food, cigarette smoke, in the milk of nursing mothers who smoke (41), and are secreted and concentrated by the mammary alveolar ductal system (34-38). Nitrosamides are found in food preservatives and food color enhancers, and aromatic amines and nitroquinolines are widely used for both industrial, medicinal and consumer products (39-42). In addition polycyclic hydrocarbons such as 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene and methylcholanthrene (8,27,43), nitrosamides such as N-methyl-N-nitrosourea (MNU) (44), aromatic amine derivatives (40,42), and nitroquinolines (41,45) induce breast cancer in animals. These observations emphasize the significance of environmentally derived chemicals as potential factors in the causation of human breast cancer. However, direct evidence that these chemical carcinogens are involved in the human disease is lacking. In order to determine whether chemical carcinogens are etiological agents in human breast cancer, it is necessary to establish an <u>in vitro</u> system in which a given etiological agent is capable of causing transformation from normal to a neoplastic state. The following studies were

Table 3 Characterization of Human Breast Epithelial Cells in Primary Culture

1. Growth Pattern	Flat monolayer of cuboidal cells
2. Ultrastructure	Numerous short microvilli at the free surface, Junctional complexes present, Tonofilaments are common both in association with desmosomes and elsewhere
3. Expression of mammary epithelial cell characteristics	Milk fat globule antigen (Monoclonal MC5)
4. Expression of epithelial cell characteristics	keratin
5. Histochemical marker	Positive for Mg^{++} ATPase Negative for $NA^{+}K^{+}$ ATPase

undertaken with the purpose of determining whether human breast epithelial cells can be transformed in vitro by chemical carcinogens, and whether their transformation depends upon certain biological properties of the host that may regulate their susceptibility to transformation.

A. Human Breast Epithelial Cells (HBEC) in Primary Culture

Assessment of in vitro transformation of human breast epithelial cells requires proper characterization of the cells, the identification of phenotypic changes of transformation, and confirmation by induction of tumorigenesis in response to carcinogen treatment. We have grown and subpassaged in a defined medium (46-47) normal human breast epithelial cells (HBEC) obtained from reduction mammoplasties. The cells, obtained as organoids, form relatively flat monolayers which spread from the original cell aggregate, exhibiting all the characteristics of epithelial cells at ultrastructural level (48) (Table 3). The monolayers of cells, both in primary cultures and in subsequent passages, are made up of three morphologically distinct cell populations: (a) small cells, measuring around 15 μm^2, with a round to slightly indented nucleus and scanty acidophilic cytoplasm, these cells comprise 56% of the total population; (b) large cells, measuring 30 μm or more in surface area, with an eccentric leptochromatic nucleus and abundant cytoplasm, they represent about 6% of the population; (c) intermediate cells of a size of 21 μm^2, in which the nucleus is round, uniform with homogeneous chromatin and contains one nucleolus, these cells represent 38% of the population. A summary is shown in Table 4. The three cell types are present in all passages, but a decrease of large and intermediate cells is observed in later passages.

The mammary origin of the epithelial cells was confirmed by reacting them with a specific monoclonal antibody against the milk fat globule antigen (MC5, supplied by Dr. R. Ceriani; Bruce Lyon Memorial Res. Lab., Children's Hospital, Oakland California, USA) (49-51), using the immunoperoxidase technique (PAP) (52). Twenty eight percent of HBEC react positively with this antibody; although the reaction is more frequently observed in intermediate (80%) than in small cells (20%). One hundred percent of the HBEC react positively with anti-keratin antibody, confirming their epithelial nature (52). HBEC show a positive Mg^{++} dependent ATPase, the histochemical reaction characteristic of epithelial cells; and they are negative for NA^{+}-

Table 4 Cell Type Distribution In HBEC In Culture

	Small Cells	Intermediate Cells	Large Cells
% of cells	56.0 ± 9.3[a]	37.6 ± 6.5	6.4 ÷ 5.4
Size[b]	15 ÷ 3	21 ÷ 1.3	30 ÷ 2.5

[a]Mean + Standard deviation
[b]Surface area in μm^2

K^+-ATPase, the histochemical reaction characteristic of myoepithelial cells (48,53). Karyotype analysis showed a human diploid configuration.

B. Transformation of HBEC in Culture

Experimentally it has been shown that the process of neoplastic transformation is accompanied by an array of in vitro phenotypic changes. These changes include altered cellular morphology, loss of density-dependent inhibition of growth, reduced requirement for serum growth factors, enhanced proteolytic activity, altered metabolic rates, expression of new products and surface antigens, anchorage independent growth and alteration of cytoskeletal and membrane architecture (54-56). The most consistent marker of epithelial cell transformation seems to be growth in agar (54-56).

Our first objective was to determine whether the carcinogens DMBA, or MNU, induce detectable phenotypic changes in HBEC in vitro. Primary cultures of HBEC, obtained and cultured in defined medium, or cells in their first passage were treated at 48 hrs post plating with 0.5 ug/ml DMBA for 24 hrs or with 0.5 ug/ml MNU for 3 hrs (19); these doses were shown not to be toxic for the cells. When the cells reached confluence, generally 12 to 13 days post plating, monolayers were subcultured with a split of 1:8 in defined medium. At the 4th passage, the cells were monitored for the following phenotypic changes of cell transformation: survival in agar-methocel, colony efficiency and multinucleation assay.

Survival and colony efficiency of HBEC in agar-methocel. The method has been described elsewhere (57). Basically, trypsinized monolayers were replated in growth medium containing 10% fetal calf serum plus 1.2% agar-methocel at a concentration of 1×10^4 cells in an agar-methocel layered 60 mm Petri dish. The plates were incubated at 37°C without subsequent feeding. The number of isolated cells and cell clumps was evaluated 24 hrs post-plating and so was the number and size of colonies that developed at 3 weeks post-plating. Colony size was determined according to the number of cells in each (8-100 cells = small; more than 100 cells = large). The values were expressed as colony efficiency (number of colonies formed/number of cells plated) x 100. The survival efficiency in agar-methocel is the expression of the ability of the cells to remain alive 2-3 weeks post-plating, as evaluated by the avidity for neutral red, and expressed as the number of cells that stain with neutral red/number of cells plated x 100.

As shown in Table 5, HBEC samples exhibited increased survival in agar-methocel at a similar rate whether they were treated with DMBA or MNU. Some of the cells that survived in agar were able to grow and form colonies of up to 100 cells each (Table 6). Colony efficiency was higher in DMBA and MNU treated cells than in controls. However, not all the cells showed increased survival; only those samples containing immature lobules (groups A and C) were the ones that showed a highly significant difference in survival. Cells coming from glands with higher lobular development did not show increased survival after carcinogenic treatment.

Table 5 Survival Efficiency In Soft Agar As An Early Phenotypical Change in Cell Transformation[a]

Patient Group	Sample No.	Cell Treatment Control	DMBA	MNU
	1	0.04	0.20	0.50
	2	0.30	1.30	1.10
	3	0.08	0.20	N.D.
	4	0.20	0.70	1.0
A	5	1.00	1.90	3.6
	6	0.25	0.00	2.40
	7	1.17	3.27	1.24
	8	1.52	1.78	2.29
	9	0.20	0.20	0.00
	10	0.42	0.09	0.00
	11	0.10	0.00	0.06
B	12	3.40	3.20	3.40
	13	1.62	1.53	2.28
	14	1.72	0.10	0.86
	15	1.15	0.93	0.94
	16	0.60	2.20	4.20
	17	0.70	1.30	1.40
	18	0.50	0.40	1.00
C	19	0.70	0.60	1.40
	20	0.94	1.58	4.02
	21	0.09	0.14	0.32
	22	0.58	1.11	1.05

[a]Survival efficiency (SE) is measured by the number of cells that are stained with neutral red after 21 days of being plated in agar methocel and expressed as a percentage of plated cells.

$$S.E. = \frac{\text{No. of cells stained with neutral red} \times 100}{\text{No. of cells plated}}$$

Multinucleation assay. It has been shown that cytochalasin B (CB)-induced multinucleation can be used as a reliable method to discriminate neoplastic from normal and preneoplastic mouse mammary epithelial cell cultures (58). We used this method as another parameter to monitor the DMBA and MNU induced changes in HBEC. We have standardized the assay after showing that exposure to 1.5 μg/ml of CB for 72 hrs is the optimal concentration for use with human epithelial cells.

Figure 9 shows that multinucleation, expressed either as 2 or 3-5 nuclei per cell, was highly significant in carcinogen treated cells from groups A and C patients (whose glands contained immature lobules), but no difference was observed in the cells coming from the more differentiated glands of the patients in group B, which are composed of Type 3 lobules.

C. Karyotyping.

The number of chromosomes was determined in metaphase chromosomes stained with Giemsa according to the method of Moorehead et al. (59). Samples of control and carcinogen-treated HBEC were examined in duplicate and coded before interpretation. The first karyotype was done at the 4th-5th passage. Subsequent ones were performed any time that flow cytometric changes or phenotypic evidences of transformation were observed. Karyotyping did not reveal changes or chromosomal aberrations and flow cytometry

Table 6 Colony Efficiency in Agar-Methocel[a]

Patient Group	Sample No.	Control	Cell Treatment DMBA	MNU
A	6	0.01	5.00	7.00
	7	0.20	0.30	0.51
	8	0.14	0.16	0.25
B	13	0.12	0.11	0.17
	14	0.08	0.08	0.09
	15	0.06	0.04	0.09
C	20	0.14	0.19	0.40
	21	0.02	0.83	19.25
	22	0.40	0.61	0.73

[a]Colony efficiency (CE) is expressed as the percentage of cells that form colonies after 21 days in agar methocel.

$$CE = \frac{\text{Total number of colonies} \times 100}{\text{Total number of cells plated}}$$

(19) did not show ploidy differences between treated or control cells. These observations, however, did not invalidate the possibility that the cells have been transformed, since it is known that visible alterations in chromosome structure are not essential to the initial change, and transformation can take place in tissue culture and certain tumors develop in vivo without such cytogenetic abnormalities (60).

D. Transforming activity of DNA of Human Breast Epithelial Cells treated with Chemical Carcinogens In Vitro

Transfection of cellular DNA has recently been used to investigate the biological activity in both normal and neoplastic cells, of genes that are capable of inducing oncogenic transformation (61-63). The presence of activated cellular transforming genes in neoplastic cells was initially demonstrated by the finding that high molecular weight DNA of 5 out of 15 chemically transformed mouse cell lines efficiently transformed NIH/3T3 cells (61). Activated cellular transforming genes detectable by efficient transformation of NIH/3T3 cells with high molecular weight DNA also have been found in neoplasms induced by viruses (62).

In the present work, we have investigated the transforming activity of DNA of HBEC that have been treated with chemical carcinogens in vitro. For this specific experiment, primary cultures of HBEC were treated with DMBA or MNU as described in section IIIB and in addition, one sample was treated with 0.2 μg/ml 4-nitro-quinoline N-oxide (NQO) for 3 hours. At the fourth passage the cells showed increased survival and colony formation in agar in the three groups of treated cells. Eighty micrograms of genomic DNA of each one of these groups of cells were used to transfect NIH/3T3 cells using the calcium phosphate precipitation method (61). The only DNA that showed transforming activity was that derived from NQO treated cells, which had a transforming efficiency (TE) of 0.012, against 0.000 in the control (DNA of nontreated HBEC). The foci were isolated and expanded (Rf) and after the second passage, they were called NQO-3T3-Rf. These recultured foci of NQO transformed fibroblasts were inoculated into nude mice. Fibrosarcomas developed at the site of injection in 3 out of 3 animals injected. Control

NIH/3T3 cells did not induce tumors in nude mice. Genomic DNA of NQO-3T3-Rf and tumors were used for secondary transfection assays (Table 7). The TE was 0.053 for NQO-3T3-Rf, and 0.093 for NQO-3T3-Rf induced tumors (Table 7).

DNA from these 3 sources was used for the analysis of the presence of human DNA sequences. The DNA was digested with endonuclease EcoR1, electrophoresed in agarose, transferred by the Southern technique in nylon membrane and hybridized with total human DNA. This hybridization produced two bands in both NQO-3T3-Rf and NQO Tumors at 11kb and 9.5kb, which were absent from the DNA control.

These findings suggest that chemical carcinogens, in this case NQO, resulted in the activation of cellular transforming genes in HBEC. It is known at the present time that at least 30 oncogenes are derived from the cellular genome and 10 or more from DNA tumor viruses (64). In a recent publication, Weinberg (65) pointed out that these 40 oncogene products can be localized either in the cytoplasm or in the nucleus of the cell. Among the nuclear oncogene products, of special interest are those of the myc family which confer immortalizing ability. In contrast, cytoplasmic oncogene products of the ras family are generally weak in their ability to immortalize cells (65-66) but strong in their ability to promote anchorage independence of fibroblasts.

In our study, there is a significant trend indicating that the cells treated with carcinogens are able to survive and grow in agar and our preliminary data indicate that an oncogene may be associated with this ability. We have been unable to immortalize our treated HBEC or to induce tumors in nude mice with carcinogens. It is possible that a second carcinogenic event has to occur and/or another oncogene needs to be activated in order to induce expression of chromosomal changes, tumorigenesis and metastasis (Fig. 10). This is the subject of our present research.

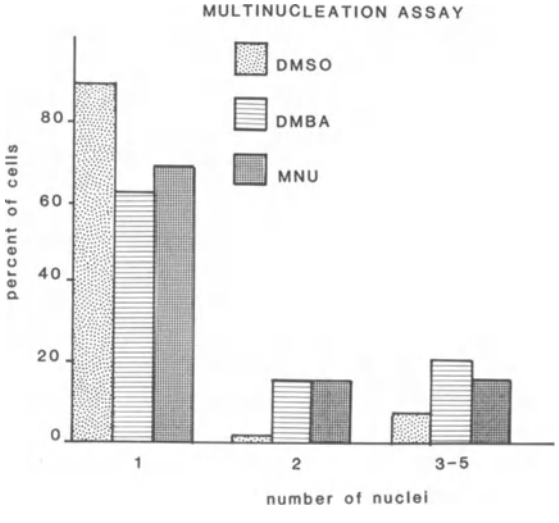

Figure 9: Histogram of Multinucleation Assay results shows an average of the sample studied. Only Group A showed sufficient changes in multinucleation after treatment with carcinogen. DMBA: 7,12- Dimethylbenz(a)anthracene; MNU: N-Methyl-N-nitrosourea, DMSO: Dimethyl sulfoxide.

Table 7 Transforming Activity of Human Breast Epithelial Cell DNA

Donor cells	Donor DNA[f]	Total No. foci/ Total recipient culture	No. foci./μg DNA
None	--	0/3	0.00
HBEC[a]	80	0/3	0.00
NQO-HBEC[b]	80	1/3	0.012
Rf NQO[c]	50	8/3	0.053
NQO Tumor[d]	50	14/3	0.093
pT24 C3[e]	50	2/3	0.020

[a]Untreated human breast epithelial cells (HBEC)
[b]HBEC treated with 4-nitroquinoline N-oxide
[c]Replated foci of HBEC treated with 4-nitroquinoline N-oxide
[d]Tumors induced in nude mice by HBEC treated with 4-nitroquinoline N-oxide
[e]pT24 C3 DNA
[f]DNA of donor cells in μg/dish

NIH/3T3 cells were obtained from Dr. Barbacid's laboratory.

IV. SUMMARY AND CONCLUSIONS

The objective of these studies is to elucidate the relationship between cell differentiation and cell proliferation and how these two parameters affect cell transformation. We have found that in the DMBA-induced rat mammary carcinoma model, cell proliferation and susceptibility to carcinogenesis are a function of gland differentiation, which in turn is modulated by both age and reproductive history (2,67). This is due to the fact that the proliferative compartment of the mammary gland is maximal at young age in nulliparous animals and decreases with time and parity (68), as does the number of undifferentiated glandular structures (2,68). The study of the proliferative compartment of normal human breast samples by measuring DNA-LI reveals in humans a similar trend to that shown in the experimental model. We have observed that in human breast tissue, there are topographic differences in DNA-LI of intralobular terminal ducts and alveolar buds (23, 24). The Type I lobules in general have a significantly higher DNA-LI than Type 2 and 3 lobules.

It has been shown that the incidence of DMBA-induced rat breast carcinomas and the binding of DMBA to DNA is inversely related to the age of the animal and the degree of gland differentiation (16-18, 67). Covalent binding of the carcinogen to cellular DNA is an accepted prerequisite for tumor initiation in experimental animals, and it seems to be similar for the human breast (18).

We have found three distinct levels of DMBA-DNA binding, which correlate with the lobular development of the mammary gland. A higher DMBA-DNA binding is observed in undifferentiated breasts, which are composed of numerous virginal lobules; an intermediate binding occurs in cultures derived from breast tissues with a lower number of virginal lobules and the lowest binding is observed in cultures from completely differentiated glands, the latter showing an abundant lobular formation. The DMBA-DNA binding levels

of the less differentiated tissues are significantly higher than those of intermediate differentiation. These results demonstrate that human mammary epithelial cells in culture not only possess the ability to metabolize DMBA to products that bind DNA, but also show that binding to DNA is dependent on the degree of differentiation of the mammary gland. These results suggest the possibility that susceptibility to carcinogenesis may be due to a greater capacity of the highly proliferating structures present in the breast of young women to bind carcinogenic compounds, supporting the hypothesis that although breast cancer in humans is diagnosed at middle or old age, the neoplastic process might have been initiated at a young age, as epidemiologic evidence seems to suggest (12).

Our studies have indicated that polycyclic aromatic hydrocarbons are metabolized by HBEC in vitro and that the metabolites are bound to cellular DNA, resulting in phenotypic changes in the cells, such as the acquisition of uniform active growth (19), growth in anchorage independent medium, shifting cells from G_1 compartment to S, expression or loss of specific surface antigens (19). This array of phenotypic changes is the expression of a multistep progression which is probably under multigenic control (69-71). This has been clearly shown in studies of human cell hybrids (69) which indicate that the transformed phenotype is different from the tumorigenic phenotype (69), requiring more time for the latter than for the former to be expressed in vitro (69-72). The fact that the cells survive in agar, form microcolonies, and that the DNA of HBEC treated with carcinogens transfects 3T3 cells is an indication that activation of an oncogene has taken place.

The identification of phenotypical changes of cell transformation induced in breast epithelium by chemical carcinogens allowed us to conclude that the human breast epithelial cell is susceptible to transformation by chemical carcinogens, and furthermore, to determine that this susceptibility is regulated by the degree of development of the mammary gland.

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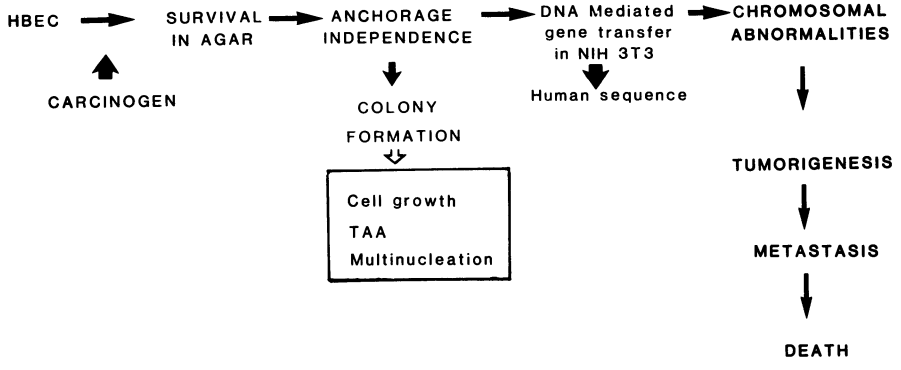

Figure 10: Schematic representation of the different steps in carcinogenesis. Data shown in this page indicate the sequence in which the events have been studied. It is possible that genetic changes may occur before the survival and anchorage independence phenotypes are expressed.

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GROWTH AND TRANSFORMATION OF HUMAN MAMMARY EPITHELIAL CELLS IN CULTURE

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I. INTRODUCTION

The culture systems available for the study of normal human mammary epithelial cells (HMEC) have advanced dramatically in the past 10 years. HMEC can now be serially cultivated in serum-free media, as monolayers, on prepared substrates, and in collagen gels (1-6). This capacity to obtain active growth of the epithelial cell component of the breast has made it possible to examine a variety of aspects of HMEC physiology and carcinogenesis, including transformation in vitro by chemical carcinogens and oncogenic viruses.

Mammary systems are particularly valuable for the study of human cellular carcinogenesis because: (a) large quantities of normal, atypical, and malignant tissues are readily available from individuals of all ages as discard material from commonly performed surgical procedures (reduction mammoplasties, biopsies, mastectomies; (b) functionally differentiated cells are available from lactation fluids; (c) the mammary epithelial cell is a major site of malignant transformation in vivo (approximately 1 out of 11 women in the USA develop breast cancer); (d) the breast glandular epithelial cells perform a variety of specialized functions in response to hormonal stimuli, and thus many good markers exist for both identification of HMEC in culture, and examination of the effects of transformation on expression of cell specific functions.

The use of human cell culture systems provides the advantages of studying directly the behavior of the cells responsible for human cancer. This is particularly important since human epithelial cells in culture display many characteristics different from those of the commonly used rodent model systems, e.g., in karyotypic stability and rates of spontaneous

transformation, in response to specific carcinogens and promoters, in presence and expression of tumor viruses and known oncogenes. Human systems also allow examination of interindividual differences. However, the human mammary systems are limited by the lack of available tissue from a variety of differentiated states, and the lack of recourse to in vivo, whole animal experiments. Consequently, well defined culture systems are especially important. Continued refinement to make the in vitro systems as accurate reflections of the in vivo situation as possible is necessary for the optimal elucidation of normal and pathologic human cellular processes.

With human cells, transformation in vitro provides the only experimental means to observe systematically progressive changes occurring as a result of exposure to potential carcinogenic agents (chemical carcinogens, radiation, oncogenes), and thus, which of these agents, and in what combinations, is capable of inducing phenotypic and genotypic aberrancies. On the other hand, it is difficult to know how accurately transformation observed in culture relates to malignant transformation in vivo. Correlating experimentally induced aberrancies with the properties found in cells derived from human breast tumors offers one approach to determining if similar transformation pathways are being utilized. In practice, obtaining and identifying transformed human epithelial cells in culture have been extremely difficult. Unlike many of the rodent model systems, human cells in culture show little or no spontaneous or induced transformation to either immortality or malignancy. Human mesenchymal cells have been induced to express anchorage independent growth, at a low frequency, by oncogenic viruses, chemical carcinogens, and radiation (7-13). Immortality, and malignant transformation (assayed by tumorigenicity in nude mice) have rarely been achieved (7-10). Human epithelial cells have been even more refractile to transformation in culture. Immortality has been obtained in some organ systems, including breast, after exposure to oncogenic viruses or oncogenes (14-18). Rare tumorigenicity has also been induced (16-18). Chemical carcinogens or radiation alone have thus far failed to yield malignant transformation, but immortal transformation of breast cells (19), and malignant transformation of breast and keratinocyte cells after exposure to both oncogenic viruses and chemical carcinogens have been achieved (20,21). Possible explanations for this low rate of human cell transformation in vitro include the observed karyotypic stability of the human cells in culture, the heterogeneity of human populations, and possible suppressor gene expression (22).

The successful demonstration of in vitro transformation of HMEC required several prior developments: (a) actively proliferating populations of pure epithelial cells; (b) transformation agents applicable to HMEC; and (c) methods to identify transformed cells. The following describes how these developments have led to transformation in vitro of HMEC, and the nature of the transformed cells produced.

II. GROWTH OF NORMAL HUMAN MAMMARY EPITHELIAL CELLS (HMEC) IN CULTURE

Reduction mammoplasties provide a large source of normal human mammary tissue. The epithelial cell content of this tissue appears similar to that seen in smaller breasts, although the fat content may be greatly increased. Pathology evaluation of these tissues indicates no abnormalities in the majority of cases, but mild or moderate fibrocystic disease is present in a significant fraction of specimens (similar to its incidence in the population in general). Mastectomies also provide a common source of human breast tissue - both tumor and non-tumor. However the non-tumor tissue cannot be considered normal as it usually contains some atypias, and breast cancer is often multifocal. The large quantities of surgical discard material available make possible the frozen storage of multiple ampoules of epithelial tissue from each individual. Thus repetition of experiments with the same person's cell pool can be conducted over an indefinite time span. The

processing of the surgical material to nearly pure epithelial clumps involves coarse dissection followed by enzymatic digestion (collagenase, hyaluronidase), and then filtration to separate the epithelial clumps (termed organoids) from the single cells and small clumps of the digested stromal tissue (1,23).

HMEC from reduction mammoplasties can display active growth in culture (doubling times of 18-30 hours) when placed in appropriate media (Figure 1). Our laboratory has utilized two main media (Table 1): MM, which contains fetal calf serum and conditioned media from other cell lines, and MCDB170, which is serum free and has been optimized for normal HMEC growth (2,3,23). In MM, cells proliferate rapidly for 3-5 passages (about 15-25 pd) maintaining a cobblestone epithelial morphology. At senescence, they become larger and vacuolated. In MCDB170, after rapid growth for 2-3 passages, most cells undergo what appears to be terminal differentiation. The cells gradually change from a cobblestone epithelial morphology to larger, flatter, striated cells with irregular edges. The small subpopulation displaying the typical epithelial morphology maintains active growth, becoming the uniform cell type within 1-2 passages. Thereafter, the cells grow for another 5-15 passages, depending upon the individual. For some individuals, a cAMP stimulator is required to maintain the actively growing population during this "self-selection" period. Figure 2 shows the growth capacity of the different individual HMEC specimens which have been cultured in MCDB170. In general, cells from the same individual show consistency in the passage level at which they senesce, despite the uncontrollable vagaries present in tissue culture. No correlation between the age of the specimen donor and population doubling capacity in culture has been observed. Consistent individual variations in morphology are also apparent (Figure 3).

The other main source of HMEC is from lactation fluids. These cells provide the potential of representing functionally differentiated cell populations, but large quantities of cells per individual are not possible to obtain. In contrast to organoids, milk cells can generally be subcultured only a few times before they senesce (4), perhaps as a result of their more differentiated phenotype.

The human mammary epithelial nature of HMEC has been demonstrated by a number of methods in addition to morphology. They display epithelial specific keratins (24,25) and patterns of cell associated fibronectin (26), human mammary specific milk fat globule antigens (27-29), the mammary specific enzyme thioesterase II (30), and synthesis of milk products (Bartley, J.C., Levine, G., Stampfer, M.R., submitted). Ultrastructurally, they show the epithelial elements of microvilli, tonofilaments, and desmosomes (1).

III. AGENTS FOR HMEC TRANSFORMATION IN CULTURE

Thus far, two main agents have been used to transform HMEC <u>in vitro</u>, chemical carcinogens and oncogenic viruses. Our laboratory initially demonstrated that HMEC could readily metabolize the pro-carcinogen, benzo(a)-pyrene (BaP) to the active diol-epoxide product, which forms adducts with the DNA (31,32). Fibroblastic cells from the same individual's breast showed a greatly reduced capability to metabolize BaP to its active form. We chose BaP for study since it belongs to the class of compounds (polycyclic aromatic hydrocarbons) known to be excellent inducers of mammary cancer in rodents, much was already known about its metabolic path, and large quantities of it are released into the atmosphere as the result of incomplete combustion of organic materials, e.g. fossil fuels and wood. Another polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene, has also been shown to be metabolized by HMEC (33).

Table 1 Contents of Cell Culture Media

Ham's F12	30%
Dulbecco's Modified Eagles Medium	30%
Fetal Calf Serum	0.5%
Conditioned Media from*	
Hs767B1 and/or fHs74Int	30%
Hs578Bst	9%
Insulin	10 μg/ml
Hydrocortisone	0.1 μg/ml
Epidermal Growth Factor	5 ng/ml
Triiodothyronine	10^{-8} M
Estradiol	10^{-9} M
Cholera toxin	1 ng/ml

Basal MCDB170	100%
Insulin	5 μg/ml
Hydrocortisone	0.5 μg/ml
Epidermal Growth Factor	5 ng/ml
Ethanolamine	10^{-4} M
Phosphoethanolamine	10^{-4} M
Transferrin	10 μg/ml
Bovine Pituitary Extract	70 μg/ml
(Isoproterenol)	10^{-5} M

*Cells fed with 1:1 Ham's F12/Dulbecco's MEM, 5% fetal calf serum, 5 μg/ml insulin.

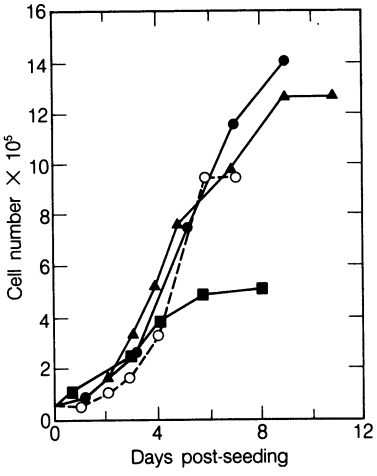

Figure 1: Growth curves of normal HMEC in culture. Cells (5 x 10^4/35 mm dish) were seeded in either MM or MCDB170, and duplicate dishes assayed at the indicated times by removal of the total cell population with trypsin and counting in the hemocytometer. Cells in MM (—) typically grow rapidly in 2nd (●—●) and 3rd (▲—▲) passage, slow by 4th (■—■) passage, and show little or no net growth in 5th passage. Cells in MCDB170 (0--0, 5th passage) grow rapidly for 10 to 20 passages.

Transformation of HMEC via exogenous oncogenes has been accomplished using SV40 virus infection, calcium phosphate mediated transfection with SV40 DNA, infection with murine sarcoma viruses (Kirsten and Harvey sarcoma virus), and retrovirus vector constructs containing known oncogenes (14,21, 34).

IV. METHODS TO IDENTIFY TRANSFORMED HMEC

A major difficulty in establishing in vitro transformation systems for human epithelial cells lies in defining markers to identify the transformed cell, if and when it appears. Ideally, markers that permit easy selection (e.g. anchorage independent growth, altered growth requirements, escape from senescence) or detection (e.g. antigenic alterations) of small numbers of transformed cells within a large cell population are most useful. More complicated assays can be utilized to determine what other changes are correlated with the isolated selected cells.

Comparing the in vitro behavior of cells obtained from normal and tumor tissues provides one means of determining differences in phenotype (see Smith et al., this volume). Unlike fibroblast cells, obvious changes in morphology, such as those that lead to distinguishable foci formation,

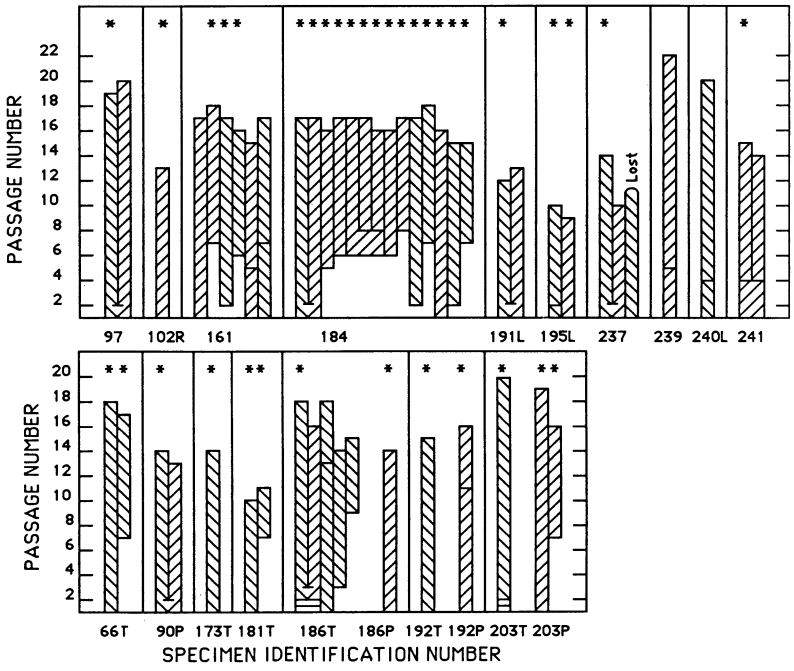

Figure 2: Growth capacity of different HMEC specimens in MCDB170. Primary cultures were initiated and subcultured as described (23), with about 8-10 fold amplification per passage. Bottom horizontal lines indicate passage level of initiation of frozen ampules. Top horizontal line indicates passage level of no net increase in cell number. Continuous hatched lines indicate cells derived from the same "selection". Asterisks indicate cells exposed to a cAMP stimulator (cholera toxin or isoproterenol). Top row; cells obtained from reduction mammoplasties. A few of these specimens were grown in MCDB202, a closely related medium (3). Bottom row; cells obtained from mastectomies (T = tumor tissue, P = non-tumor tissue of tumor bearing breast). In a few cases, indicated by ▭, the tumor cultures were grown in MM in primary culture.

are not necessarily characteristic of tumor derived epithelial cells. Neither is anchorage independent growth (AIG) invariably associated with these cells (22). With human cells, testing for tumorigenicity in a syngeneic animal is not possible, and tumor derived cells are not always tumorigenic in nude mice (22). Consequently, each organ systems has been examined to determine if there are markers of transformation specific to those cells in culture. For example, in some keratinocyte and bronchial epithelial cell systems, sensitivity to Ca^{++} or TGF-β induced terminal differentiation provides useful markers for transformed cells (35-37). With breast cells, invasiveness and AIG have been associated with tumor derived cells (25,38). There have also been extensive studies of monoclonal antibodies which may distinguish between tumor and normal cells (39,40). Although some of these look promising with in situ material, they have not all been shown to distinguish the same cell populations actively growing in culture. There are some data that antibodies raised against the human MFGA may be useful in differentiating normal vs. transformed cells (41, 42). Genotypic differences, i.e., non-diploid karyotypes, are also found in most tumor derived human cells and cell lines, although primary tumor specimens do contain diploid cells (43).

Another property associated with tumor derived human cells is the ability to generate established cell lines in culture. Spontaneous transformation to immortality is not seen with normal human epithelial cells, but various tumor tissues produce cells of indefinite lifespan at frequencies from rare to frequent. In the case of breast tissue, established cell lines have occurred very rarely with primary tumor tissue, whereas about 1 out of 10 metastatic tissues yield a rare cell displaying immorality. Thus, although it is not a property of most breast tumor cells, the capacity to display immortality is correlated with breast tumor tissues in culture. Immortality is also a very selectable marker of transformation, and is therefore a convenient, if not necessarily elegant method to detect carcinogen induced aberrancies. Similarly, extended life in culture is easily selectable, and is associated with transformation of many cultured epithelial cells (14,15,18,44). It is possible that this extended proliferative potential in vitro may be related to the hyperplasia commonly seen in abnormal breast tissue in vivo, which is likely a result of some alterations in normal growth control, senescence, and thus proliferative capacity.

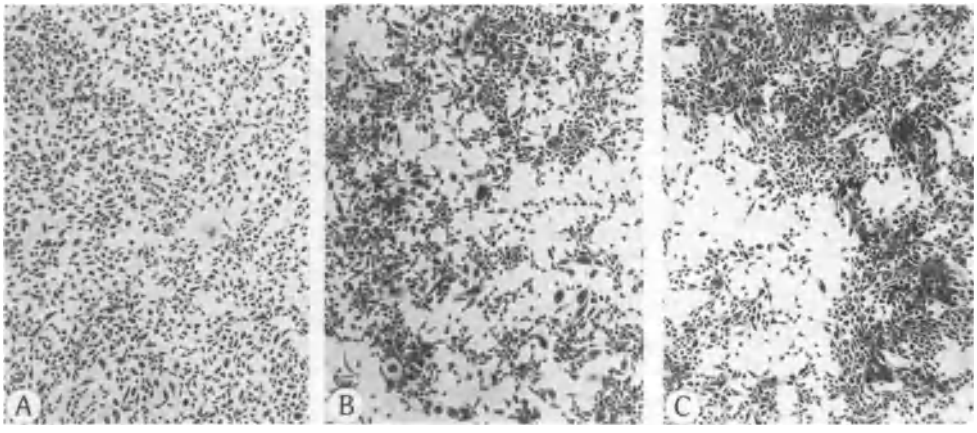

Figure 3: Morphology of normal HMEC grown in MCDB170. Giemsa stained cultures of A; specimen 184, 7th passage, B; specimen 172, 13th passage, C; specimen 161, 9th passage, all show a typical epithelial cobblestone morphology. However, considerable variability is present, e.g. #184 shows least cell-cell contact, #172 often has prominent large cells, #161 grows as patches with a swirling pattern.

Therefore, the studies conducted thus far with HMEC transformation in vitro have used these markers of altered growth capacity to detect transformed cells. However, the cells thereby selected will of necessity reflect only the method of selection, and may represent only a limited spectrum of possible transformation pathways.

V. TRANSFORMATION AFTER EXPOSURE TO BENZO(a)PYRENE

Initial studies (19) involved BaP exposure of rapidly growing primary cultures of HMEC in MM; the 15-25pd lifespan of HMEC in MM would permit early detection of cells displaying an extended lifespan. Three different experiments were performed with cells from specimen 184, normal reduction mammoplasty tissue of a 21 yr. old individual. In all cases, two T 25 flasks received 2 or 3 exposures of 1 μg/ml of BaP (equivalent to 20% survival), while two control flasks received the DMSO carrier alone. The primary cultures were subjected to repeated partial trypsinization (PT); i.e., 50% of the cells were removed and the cells remaining in the flask were allowed to regrow. After some PT, the removed cells were seeded into secondary culture ($5 \times 10^3/cm^2$), and were thereafter observed for growth potential, morphology, and AIG in methocel.

No AIG was seen in the control or treated cultures, but changes in morphology and an extended life (EL) were consistently found in the cells exposed to BaP. Figure 4 illustrates these results. The treated cells grew both for more subcultures, and for longer times in primary culture. Unlike the control cells, in which rapid growth was followed by a relatively uniform slowdown to senescence, the BaP treated cultures displayed widespread heterogeneity in growth and morphology. The EL cultures could display uniform growth, mixed growing and senescent cells, or patchy cell growth. Subculture of dishes containing any one of these growth patterns could lead to appearance of any of these patterns at the next passage. Various morphologies not seen in the control cultures were also observed (Figure 5). This heterogeneity was found not only among the three different experiments, but also among cells seeded from the same or different PT within one experiment.

Although EL cultures were a common outcome of the BaP treatment, eventually these cells also senesced. From the many subcultures seeded from the three experiments, only two instances were observed of continued cell growth, leading to two established immortal cell lines. Figure 6 outlines the emergence of these two lines, 184A1 and 184B5, from the EL cultures.

In the case of 184A1, a single EL patch at passage 5 (similar in morphology to figure 5c) gave rise to a uniformly growing cell population designated 184Aa, with a swirly, patchy growth pattern. When the 184Aa cells senesced around passage 9, a population of cells with a distinctive morphology (small cells growing as singlets), and an indefinite lifespan, emerged. These 184A1 cells are likely the result of a clonal event, since karyotypic analysis shows the same few chromosomal markers in all 184A1 cells at passage 11 (Walen, K., and Stampfer, M.R., in preparation), whereas the 184Aa cells appear normally diploid, Also, on two subsequent occasions when frozen stocks of seventh passage 184Aa cell were placed in culture, no immortal cell population emerged. The 184A1 cells have been carried to passage 101 in MCDB170, with approximately 20-fold amplification per passage, and 24-36 hour doubling time. At early passages (up to around 30), a heterogenous population of small and large cells is present (Figure 7b). By later passages, the larger cells have mostly disappeared (Figure 7c), and the extent of singlet versus patchier growth varies among different subpopulations. It might be noted that the normal 184 parental cells display the least patchy

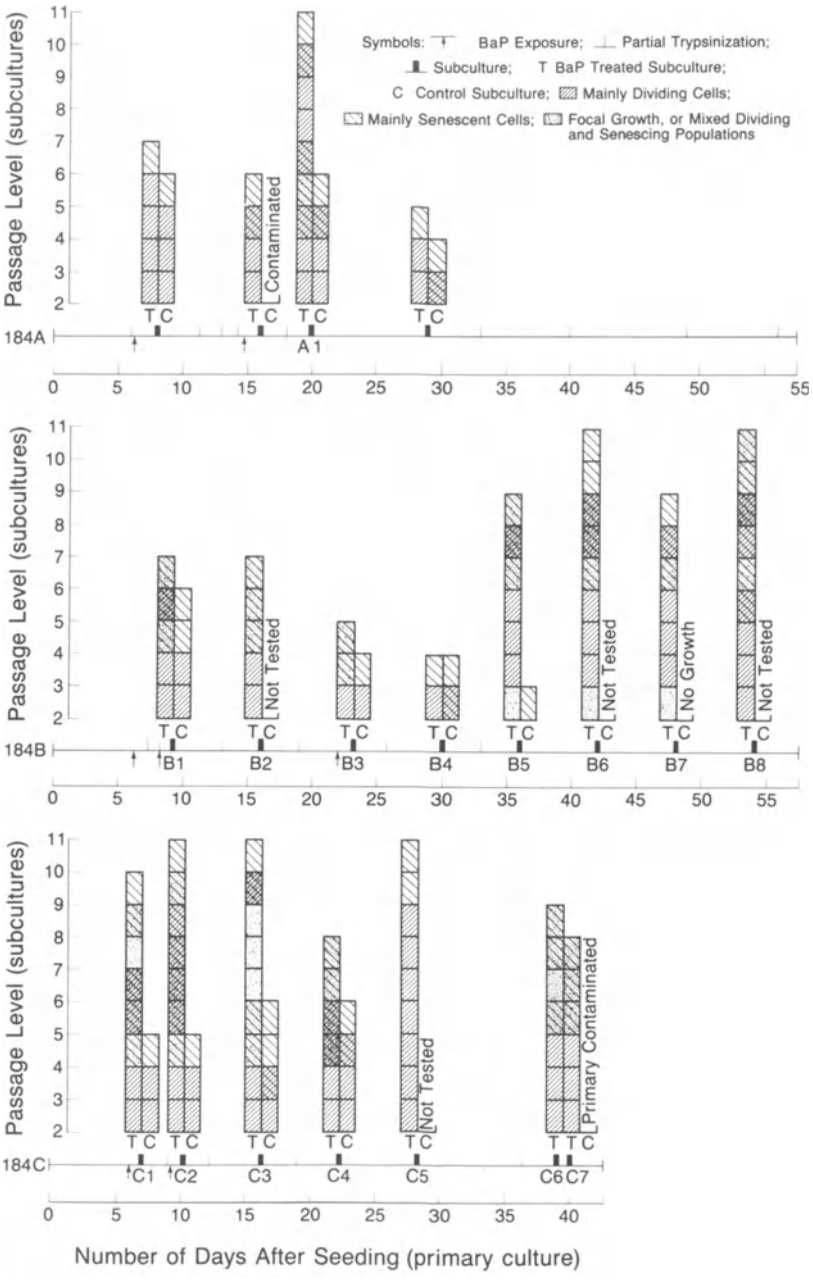

Figure 4: Growth of HMEC after exposure to BaP. Cells from specimen #184 were exposed to 1 μg/ml of BaP at the indicated times, as previously described (19), in 3 separate experiments (designated A,B and C). Primary flasks were subjected to repeated partial trypsinization, and the fate of both the subcultured cells, and the cells in the primary flask observed. Since several dishes were plated at each subculture, and if growing, their lineages often followed independently, more than one kind of growth pattern could be observed at a given passage level. In experiment 184C, cholera toxin was inadvertently omitted from the medium until 22 days after seeding (passage 5 of subculture C1, 4 of C2, 3 of C3).

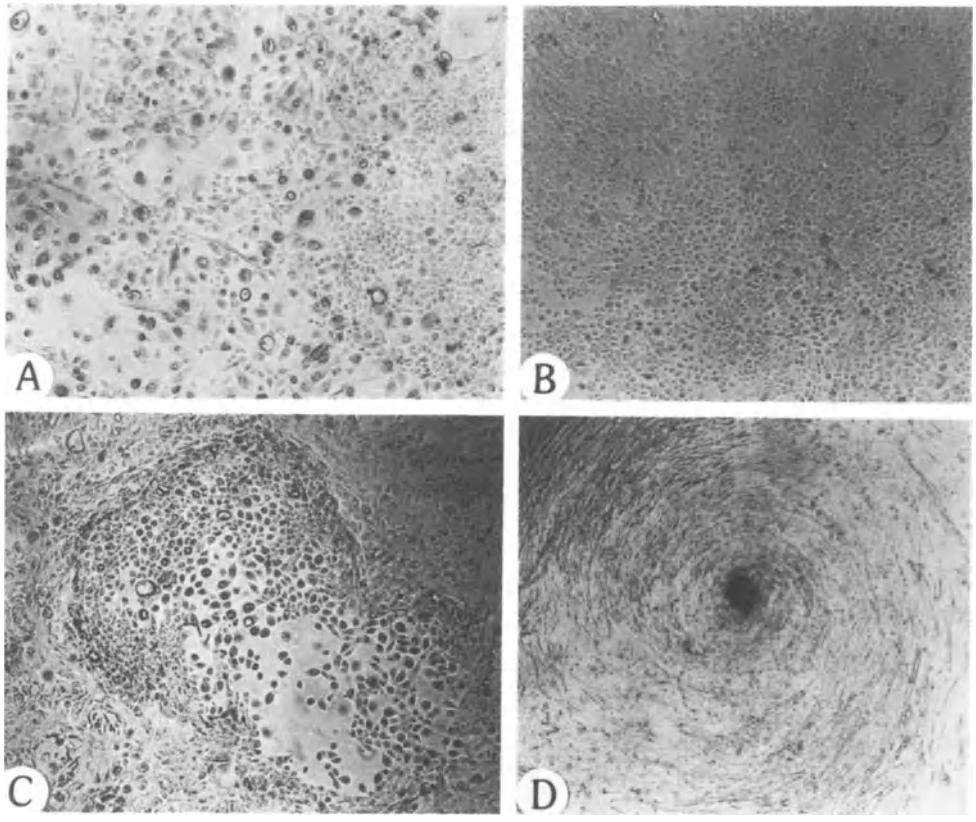

Figure 5: Extended life cultures of BaP treated HMEC grown in MM. A; experiment 184C, subculture C1 8th passage, showing a mixed growing and senescing population throughout the dish. B; experiment 184C, subculture C6, 6th passage, showing one actively growing focal area with normal morphology. This dish contained one other area of focal growth with a morphology like 5A. C; experiment 184B, subculture B6, 6th passage, showing a "hyperplastic" morphology of cell growth within pockets of surrounding senescent cells. D; experiment 184C, subculture C4, 7th passage, showing focal growth with a swirly, thumbprint morphology.

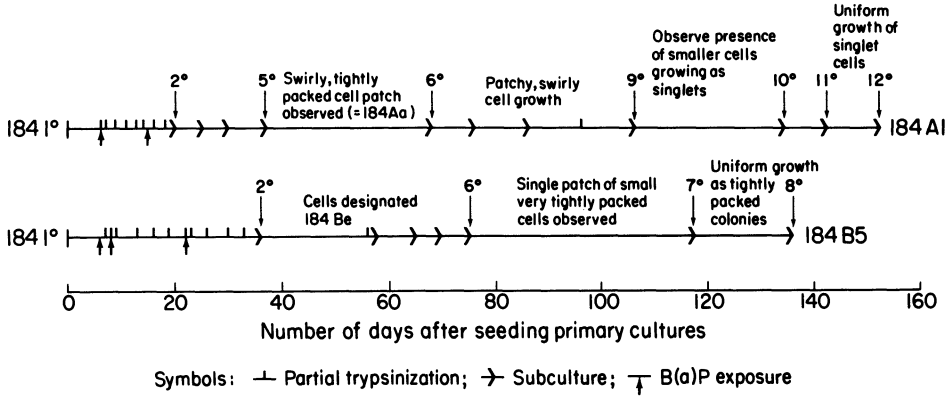

Figure 6: Development of established cell lines following BaP exposure of HMEC specimen 184.

growth pattern in MCDB170 of all the 11 reduction mammoplasty specimens we have followed (see also figure 3).

In the emergence of the 184B5 cells, an EL culture (184Be) displaying a few areas of focal growth at second passage, grew uniformly actively to passage 6, when focal growth reappeared. One small patch with a very distinctive tightly packed morphology was noticed, and has displayed an indefinite growth potential. The 184B5 cells have been carried to passage 100 in MCDB170, with about 30-fold amplification per passage and 24-hour doubling time. Subculture may have selected for a more rapidly growing population since this doubling time is greatly decreased from that seen originally (passages 6-15), 184B5 cells have maintained their distinctive tightly packed colonial growth pattern (Figure 7d), although some variability is observed among the colonies and different subcultures. The 184B5 line is likely also the result of a clonal event since, in addition to its first appearance as a single patch, the cells all contain several chromosomal markers.

VI. CHARACTERIZATION OF THE IMMORTAL CELL LINES

The 184A1 and 184B5 lines have been compared to their normal parental 184 cells to determine what other properties may have been altered in the transformation to immortality (19). First, the derivation of these lines from 184 was shown by comparing their profile of seven different polymorphic isozymes. All three cell types had an identical profile; the probability that such a result would occur by chance is <0.01%. The mammary epithelial nature of the cell lines has been demonstrated by several methods. Immunofluorescent assays have shown that the cell lines are virtually 100% positive for the mammary specific enzyme thioesterase II, for epithelial specific keratin fibrils, and for an epithelial pattern of powdery cell associated fibronectin. No grossly obvious differences were found between the cell lines and normal 184 cells by indirect immunofluorescence, however 2-dimensional gel electrophoresis of material secreted into the medium has shown that the immortal lines secrete much less fibronectin (Bartley, J.C., and Stampfer, M.R. in preparation).

Another marker largely specific to human mammary cells is the human MFGA. Expression of specific MFGA has been correlated with both the state of differentiation, and with transformation (41). We employed 6 monoclonal antibodies developed by Gordon Parry (45,46) to screen the cell lines. Table 2 summarizes some of our preliminary results with three of these antibodies. Although all three cell substrates are recognized by some of the antibodies, differences in expression are present. Further experiments

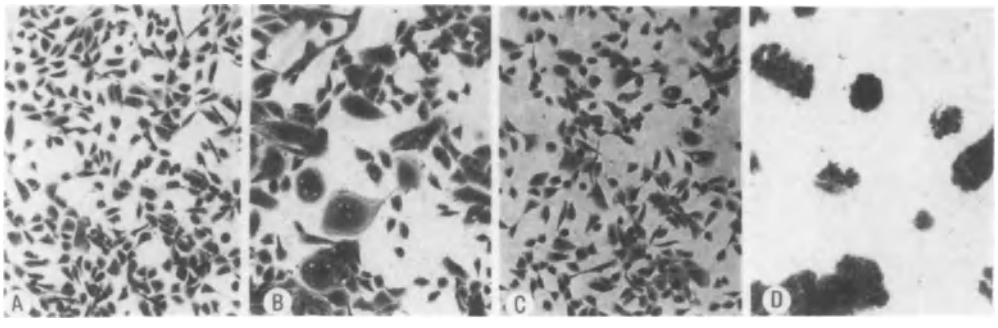

Figure 7: Giemsa stained normal and immortalized cultures of specimen 184 grown in MCDB170. A; untreated normal, 9th passage. B; 184A1, 15th passage. C; 184A1 42nd passage. D; 184B5 11th passage.

Table 2 Binding of Monoclonal Antibodies to Human Milk Fat Globule Antigens by Normal and Immortalized HMEC

Specimen	Medium	% of Cells Binding Antibody* P_2A_1	P_3B_1	P_2C_1
184	MCDB170	++	0/+	0/+
	MM	++++	+	0/+
184A1	MCDB170	+	+	+
	MM	+	+	+
184B5	MCDB170	++++	++	+
	MM	++++	++	++

*0/+=<1% +=1-25% ++=25-50% +++=50-75% ++++=75-100%

Cells were grown in the indicated medium on glass cover slips, fixed in 3.5% formaldehyde, and assayed by indirect immunofluorescence.

are in progress to determine the nature of the antigens recognized. It is possible that P_2A_1 is detecting a differentiation related antigen, whereas P_3B_2 and P_2C_1 may recognize antigens associated with the transformed state.

These cells are also being examined for other properties which might be associated with differentiation. Glycogen and lactate synthesis have been identified as markers of the differentiated, prelactating state in murine mammary epithelial cells (47). In HMEC, synthesis of glycogen and lactate is induced by growth in MM, suggesting that this medium may produce a functionally more differentiated state than MCDB170 (48). Table 3 indicates the amount of glycogen and lactate production by normal 184 and the BaP treated cells in MM and MCDB170. Glycogen production is greatly decreased even in the EL cultures examined, whereas lactate synthesis is less affected. This result is similar to that found in tumor derived cells (n=6), which exhibited lower rates of glycogen synthesis (11-50 nm/mg protein) but similar rates of lactate synthesis as HMEC from normal specimens. Normal HMEC grown in MM may also be induced to secrete proteins found in human milk, but thus far neither of the cell lines has been demonstrated to secrete identifiable milk-related proteins.

As mentioned earlier, both 184A1 and 184B5 have a few karyotypic abnormalities, whereas the parental 184 cells, 184Aa, and 184Be appear normal diploid. Karyotypes of both early (11th passage) and later passage cultures have been obtained to determine the karyotypic stability of these cells upon repeated subculture in MCDB170. All the early passage 184A1 cells examined (n=50) contain monosomy for chromosome 6 and deletions of chromosomes 3 and 12. Later passage (47th) cells contain these markers as well as a few others, with most cell showing the same 5-7 markers. Thus relatively little chromosomal changes have occurred after around 150 pd in vitro. Early passage 184B5 cells all contain 6 stem-cell markers, but a few other markers are also present in different cells. The later (41st) passage cells show even more heterogeneity, with around 10 additional markers observed (n=100). Therefore, 184B5 appears less stable than 184A1, but still relatively stable compared to most human tumor derived or virally induced established cell lines. One of the 184B5 stem-cell markers (breakage and translocation of the long arm of chromosome 1) is also commonly found in breast tumor cells and cell lines (49,50).

Table 3 Glucose metabolites of normal and BaP treated HMEC

Specimen	184			184Aa			184A1				184Be		184Bh	184B5		184Cc
Medium	MM		170	MM	170		MM			170	MM		MM	MM	170	MM
Passage	2	8*	8	8	8	17	27	42	13	42	4		4	10	11	7
Metabolite (nmol/mg protein)																
Glycogen	70	142	12	6	5	8	10	8	2	4	11	3		20	1	5
Lactate	1075	995	358	855	374	420	472	917	157	150	1275	NT		1184	85	495

*Indicates cells grown in MCDB170 and transferred at eighth passage to MM for 72hr prior to addition of labelled glucose. NT = not tested. Bh are extended life cells from subculture B8; Cc are from subculture C3 (see figure 4).

Cells were grown in the indicated medium and analyzed for glucose metabolites by two dimensional chromatography as previously described (19).

Table 4 Anchorage independent growth of normal and immortalized HMEC

	SPECIMEN		
	Parental 184	184A1	184B5
% colony formation	≤0.001	≤0.001	0.001-0.008

Cells grown in MCDB170 were suspended in 1.25% methocel (5×10^5 cells/5 ml) made up in MCDB170 plus 0.5% FCS and layered over 5 ml of 1.0% bacto agar in MCDB170. Plates were fed once a week and colonies >100 μM in diameter were counted after 3 weeks. Normal 184 (9-12 passage) and 184A1 (54 passage) showed rare (≤ 1/dish) if any colonies. 184B5 (46-80 passage) displayed a variable but consistent low level of colony formation.

The cell lines have been tested for two properties associated with malignant transformation, AIG and tumorigenicity in nude mice. No tumors have been seen in either adult or newborn mice injected intraperitoneally with 5-10 x 10^6 cells. Table 4 indicates the results found for AIG in methocel. Normal 184 and 184A1 show almost no AIG; 184B5 displays a very low level of AIG, <0.01%. It is thus likely that these immortally transformed cells are not also malignantly transformed. However, their low level of genetic instability could possibly lead to the generation of some AIG or malignant cell variants. The absence of a malignant phenotype makes these cell lines convenient substrates to determine what further steps are required to confer malignancy, but the relationship of the steps occurring in these cultured cells to those occurring during malignant transformation in vivo is difficult to determine.

VII. TRANSFORMATION TO MALIGNANCY

Three approaches have been initiated to derive malignant HMEC (defined by tumorigenicity in nude mice) from the immortalized cell lines: exposure to oncogenic viruses or oncogenes, further exposure to chemical carcinogens, and selection of possible rare malignant variants. The latter two approaches have only recently been started and no results are yet available. The general procedure in these cases involves treatment with the direct acting carcinogen MNU followed by testing for AIG and tumorigenicity, and repeated isolation and expansion of the rare AIG colonies to obtain an AIG variant.

Two oncogenic viruses have been used to infect 184A1 and 184B5, Harvey sarcoma virus (HSV) and Kirsten sarcoma virus (KSV) with a baboon pseudotype. The results thus far (Arnstein, P., and Stampfer, M.R. in preparation) indicate that KSV infection of 184B5 causes 100% tumor formation in all injected nude mice. These tumors are classified as poorly differentiated epidermoid carcinoma, and grown to 2-5 cm in 1-3 months. However the tumors then remain with no further growth. The 184B5-KSV cells have been reisolated from these tumors, and show the same or less AIG than the uninfected 184B5 cells.

Both KSV and HSV have been used to infect a subclone of 184A1 (21). In this case, some AIG is induced (0.004-0.025% colonies >75 μ), but nude mice injected with these cells show only a low frequency of small (<1 cm) undifferentiated tumors. Exposure of 184A1 to a retrovirus vector containing the SV40 T antigen prior to HSV infection, renders these cells 100% tumorigenic, with 2-3 cm undifferentiated carcinomas appearing by 1 month. The SV40 T antigen alone does not confer any tumorigenicity.

Transformation after exposure to SV40

Chang et al. (14) infected suspensions of primary cultures of pooled milk derived HMEC with SV40. The cells were allowed to grow for 3-4 weeks in monolayer culture in RPMI 1640 with 15% fetal calf serum, 10% human serum, 10 μg/ml insulin, 5 μg/ml hydrocortisone, and 50 ng/ml cholera toxin, and then tested for AIG in soft agar. Control cultures produced no colonies, whereas from 4 out of 10 experiments, the infected cells produced 12 clones. Isolation of these clones yielded 12 EL cell strains capable of another 10-15 population doublings, at least a 2-fold increased life span compared to the control populations. One strain survived crisis, and after a 6 week quiescent period, gave rise to viable foci. These cells were recloned in soft agar, yielding the fR series of cell lines with indefinite lifespan. These lines are morphologically heterogeneous, having either a cuboidal (majority) or elongate morphology (similar to the morphology of cell types found in primary milk culture).

These immortally transformed cells have been characterized by indirect immunofluorescence, and shown to be HMEC by the presence of epithelial keratins and human MFGA. They also express the SV40 T antigen. Heterogeneity in keratin and MFGA expression was found among the different fR lines, with the cuboidal cells showing greater expression of certain keratins. The MFGA strongly expressed by differentiated HMEC was found on many of the fR lines. Although growth of the fR lines is anchorage independent, they do not form tumors in nude mice. Cytogenetic analysis shows mainly hypotetraploid cells with several specific chromosomal markers in all the fR cells, including rearrangement of chromosome 1 (51).

These milk cell cultures have also been successfully transfected with SV40 DNA via the calcium-phosphate mediated technique (34). Results thus far indicate that EL strains are produced by this procedure.

It should be noted that another SV40 immortally transformed HMEC line exists, i.e. HBL-100 (52) which has recently been shown to express the SV40 T antigen (53), most likely as a result of in vivo exposure. Continued passage of HBL-100 in culture produces tumorigeneic cells (54).

VIII. CONCLUSIONS

Chemical carcinogens, oncogenic viruses, and oncogenes are capable of transforming HMEC in vitro. Similar to other human cells, transformation to immortality or malignancy appears to be a very rare, multi-stage process, whereas generation of EL cell strains is a more common early response. Similar to other human epithelial cells, infection with oncogenic viruses produced AIG, and less frequently immortality, but not tumorigenicity. Since HMEC are the only reported chemical carcinogen induced human epithelial immortal cell lines, comparisons are not possible. In our hands, this was an extremely rare event, and yielded lines that were largely non-anchorage independent, non-tumorigenic, with a relatively stable, near diploid karyotype.

Morphology, and the expression of mammary epithelial properties, varied widely among the carcinogen induced EL strains and immortal cell lines. Morphologies similar to, as well as greatly altered from normal HMEC were seen. Expression of differentiated properties ranged from less than, to greater than that seen in the normal HMEC. Thus, there are likely many

pathways to immortal transformation in vitro, and although abnormalities or differentiated functions exist, they can manifest in many different forms.

Malignant transformation of HMEC in vitro was possible when the chemical carcinogen treated immortalized cell lines were further exposed to oncogenic viruses and oncogenes, demonstrating the multi-step progression to neoplastic transformation. These lines represent good substrates for experimentation in what other agents, alone or in combination, can also yield malignancy, and further, comparisons of the properties of cells malignantly transformed by different processes.

As discussed earlier, with human cell systems, definitive conclusions about the relationship between changes observed in vitro with those occurring in vivo are not possible, yet cultured systems are the only means to study HMEC carcinogenesis under controlled conditions. The multi-step progression to malignancy described here, including the early expression of EL cells with enhanced proliferative capacity, and the range of aberrancies in differentiation, are consistent with observed in vivo development of breast cancer. However, the importance in vivo of immortal transformation, or known oncogenes, is not clear. Further development of HMEC culture systems, markers of HMEC transformation, and carcinogen transformed cells should help elucidate the factors contributing to breast cancer, and the processes involved in carcinogenesis.

Acknowledgements

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CELLULAR MANIFESTATIONS OF HUMAN BREAST CANCER

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Clonal etiology is a central tenet of current oncology. It is supported by cytogenetic and biochemical studies of human cancers and by studies in animal model systems. We do not question this tenet as it applies to the late and lethal manifestations of cancer. We do, however, question its validity when applied to the premalignant processes associated with the development of human breast cancer. In this chapter, we present our field hypothesis of carcinogenesis and review studies from our own laboratory and work of others that bear on this hypothesis.

I. FIELD HYPOTHESIS OF CARCINOGENESIS

We hypothesize that the earliest events in carcinogenesis involve a field effect causing subtle changes in a region of an organ such as the human breast. The changes may best be described as a deterioration of the

A. Evidence from fibroblast studies

Two laboratories have described abnormalities in the properties of cultured skin fibroblasts from patients with breast cancer (1-5). Azzarone, et al. (1) found, as expected, an inverse correlation between the doubling potential of fibroblasts in vitro and the donor age for cells from patients with benign lesions; however, no correlation was found with cultures from cancer patients. Moreover, cultured skin fibroblasts from cancer patients, unlike fibroblasts from normal donors, formed colonies in soft agar and on monolayers of normal human epithelial cells; they showed that these cells increased saturation densities in overcrowded culture conditions and also invaded embryonic heart tissue. Skin fibroblasts from a benign lesion of one patient whose mother had developed breast cancer displayed all the abnormal growth properties characteristic of fibroblasts from breast cancer patients. Close follow up of this patient resulted in the early detection of a carcinoma three years after the operation for the benign lesion. This is the first report that expression of an abnormal fibroblast phenotype can precede the appearance of clinically detectable invasive breast cancer and hence could potentially be an important indication of high cancer risk. The patient was not in a recognized high risk category (i.e., multiple relatives with bilateral premenopausal breast cancer). A woman whose mother had a single breast cancer has only a slightly elevated risk (6). Hence, the abnormal skin phenotype may have wide applicability as a screening test among the general population as well as confirming susceptibility in women with known high risk. The major criticism of Azzarone et al. work is that the sample size was small (7 skin specimens each from patients with carcinoma vs. benign disease). Clearly, additional studies are necessary to confirm and extend these important observations.

Schor and his colleagues have also been studying skin fibroblasts from breast cancer patients (2-5). They have devised an assay based on migratory response to cell density. Skin fibroblasts from normal adults migrate into collagen gels more readily when plated at low density rather than high density. Fetal fibroblasts display the opposite behavior, migrating more readily at high plating densities rather than low density. In studies involving skin fibroblasts derived from 37 breast cancer patients, 70% displayed fetal-like migratory behavior. In contrast, only 7% (14 cases) of normal foreskin fibroblast and 8% (24 cases) of adult skins displayed fetal behavior. Of those breast cancer patients with a familial history of breast cancer, 90% (10/11) were fetal-like. In a pilot study, they observed that 2/2 unaffected first degree relatives of patients with a family history of breast cancer also had fibroblasts which displayed fetal like behavior (5).

When fetal fibroblasts were passaged in culture they underwent a transition to expression of adult migratory phenotype after approximately 50 population doublings. Fibroblasts from breast cancer patients never acquired the adult phenotype after passage in culture. Schor and his colleagues hypothesized that fetal fibroblasts normally undergo a transition during development from the fetal to adult migratory pattern. This transition does not occur in certain individuals which appears to put them at an elevated risk of developing breast cancer.

Abnormalities of cultured human fibroblasts have also been described for a number of other malignancies. Most recently, Parshad et al. (7) found that skin fibroblasts cultured from individuals with a variety of neoplasms who were from families with a history of neoplastic disease showed increased G-2 chromosomal radiosensitivity. A number of years ago, we made the observation that cultured skin fibroblasts from osteosarcoma patients could form small nodules in nude mice, unlike similarly grown fibroblasts from normal individuals (8). Skin fibroblasts obtained from individuals suffering from

entire tissue environment with many cells being involved. The changes may affect not only epithelial cells, which ultimately are the cells that undergo malignant transformation, but also the stromal elements of fibroblasts and connective tissue matrix. We suggest that there are subtle losses of cell to cell communication which manifest as imperfect social behavior. For example, cellular differentiation may be arrested; cells may proliferate inappropriately; inappropriate (fetal) proteins may be synthesized; and there may be unusual biochemical and metabolic activity. The important concept is that these are synergistic phenomena involving many cells and their interactions. The net effect is to produce an increasingly disorganized and aberrant environment. Superficially, the resultant environment resembles, in many respects, the environment of the developing embryo; however, it lacks the exquisitely controlled organization which is the essence of embryogenesis.

We suggest that this anarchistic milieu of early oncogenesis results in genetic instability. When cells are no longer under the strict scrutiny and control of their neighbors, slight deviations are tolerated. This leads to a situation in which all the cells in the affected tissue region may both differ from normal and show marked variability among themselves. At this time, genetic instability may be manifested by the sporadic appearance of biochemical, cytochemical and morphologic markers without any detectable change in the karyotype. It also may be manifested by grossly detectable yet apparently random chromosomal rearrangements. We postulate that during this early phase of oncogenesis, that great majority of cells are destined to die, and that these gross aneuploidies are almost invariably lethal within a few generations. The corollary to this postulate therefore is that most of the cells, and in particular detectable aneuploidies, are not central to the oncogenic process but rather are almost irrelevant by-products. We further suggest that it is at this stage that most primary breast cancers are diagnosed.

The observed histopathologic heterogeneity of human breast cancer is the manifestation of the underlying anarchistic milieu. Much of what is seen is irrelevant to the final outcome of the disease. But this abnormal tissue field provides the appropriate environment for the evolution of abnormal but relatively stable surviving clone(s). The cells in the clone are likely to express an abnormal phenotype and to have a detectably aneuploid genotype. Cells from such clones may metastasize and may exhibit clonal evolution leading to all the later manifestations of progressive and ultimately lethal malignancy.

We now will review, in the light of our hypothesis, the early events in the malignant transformation and the malignant progression of human breast cancer.

II. EARLY EVENTS IN MALIGNANT TRANSFORMATION OF BREAST CANCER

There is a large body of literature using various model systems to address early events in neoplastic transformation. These studies (which encompass various suggested etiologic agents such as viruses, carcinogens, hormones and growth factors, oncogenes, radiation, etc.) all focus on the target cell itself. However, carcinomas arise in organized tissues where there is a close association with mesenchyme. Hence, it is reasonable to consider the possibility that abnormal stromal tissue may initiate the malignant process by presenting inappropriate signals to otherwise normal epithelial cells. A number of recent studies suggest that this view may be particularly relevant for induction of breast cancer.

carcinoma of the bronchus have been reported to undergo spontaneous transformation in vitro as assessed by focus formation, ability to form colonies in semi-solid medium, karyotypic abnormalities and reduced capacity to compact a fibrin clot when these cells were cultured under crowded conditions (9). Kopelovich and his colleagues have utilized cultured skin fibroblasts to identify high risk individuals in cancer-prone families. They have shown that cultured skin fibroblasts from individuals afflicted with hereditary adenomatosis of the colon and rectum show many properties characteristic of partially transformed cells when grown in culture (10-13). These properties include increased saturation density, increased ability to be transformed, increased response to tumor promoters, the ability to proliferate in the presence of low serum concentrations (10), colony formation in semi-solid medium (11), production of elevated levels of plasminogen activator (12), and disruption of actin-containing cytoskeletal cables (13).

B. Evidence from epithelial cell culture

If an abnormal environment causes malignancy in otherwise normal epithelial cells, one might be able to induce the malignant phenotype by altering the environment in culture. In fact, just growing cells in culture seems to create an environment in which normal cells can become malignant.

There are numerous reports in the literature that rodent epithelial cells (as well as fibroblasts) undergo neoplastic transformation after cell culture. For example, we showed that mouse epithelial cell lines derived from normal liver and mammary gland produced benign cysts at low passage, but progressed to carcinomas after additional passages in culture (14).

For normal human mammary epithelial cells, we find that merely placing the cells in culture totally alters morphology so that the cells appear malignant to a cytopathologist. Furthermore, we find that normal cells in culture also express antigens that are tumor specific in vivo. One example is carcinoembryonic antigen (CEA). Fig. 1 illustrates how this antigen is turned on in second passage normal mammary epithelial cells. Another example is the expression of the intermediate filament, vimentin (15-17), a phenotype displayed by epithelial tumor cells upon metastasis to human ascites and pleural fluids (18).

We hypothesize that the abnormal environment of cell culture simulates events occurring in vivo during malignancy. Nonmalignant breast epithelial cells may find themselves in an abnormal stromal environment which allows expression of at least some aspects of the malignant phenotype.

III. MALIGNANT PROGRESSION OF BREAST CANCER

It has been hypothesized by Nowell (19) that increased capacity for genetic instability is a fundamental aspect of malignancy. He suggests that the gross chromosomal abnormalities observed in most cancers are the result of this genetic instability occurring in conjunction with selective pressures over a long period of time. In this model, the initial malignant changes occur without detectable cytogenetic abnormalities except in occasional instances where the same cytogenetic abnormality is present in nearly all cases of a given malignancy (i.e., the Philadelphia chromosome in chronic granulocytic leukemia). In most cases, diploid neoplastic cells generate nonviable cells with grossly abnormal chromosomal contents. Only occasionally does this process generate a viable aneuploid cell with growth advantage allowing it to selectively populate the original tumor or subsequent metastases.

There may be marked differences in the time course of these events in tumors of different tissues and organs. In some instances, the sequence

occurs in a compressed time frame so that only a population with a grossly abnormal karyotype is present by the time of initial diagnosis. In other cases this evolutionary process may be slower so that the original diploid cells, the nonviable aneuploid cells, and the viable aneuploid subpopulations with growth advantages (i.e., at metastatic sites) all coexist in the primary lesion. We suggest that the latter situation occurs for breast cancers. This model makes predictions which have been verified by work reported from a number of laboratories as well as our own. These predictions are summarized and discussed below.

A. Prediction 1: Diploid cells will be found in primary breast cancers.

Evidence from cytometric studies

The majority of studies on the DNA of breast cancers have utilized either flow cytometry or microspectrophotometry on in situ material (for reviews, see refs. 20-22, and chapter in this book by Dr. S. Wolman). In these studies, the majority of breast cancers contain at least some cells with diploid DNA content. In many cases, all of the tumor cells are indistinguishable from true diploidy (23-28). The frequency of breast cancers that are apparently diploid in DNA content varies from 8 to 52%, however,

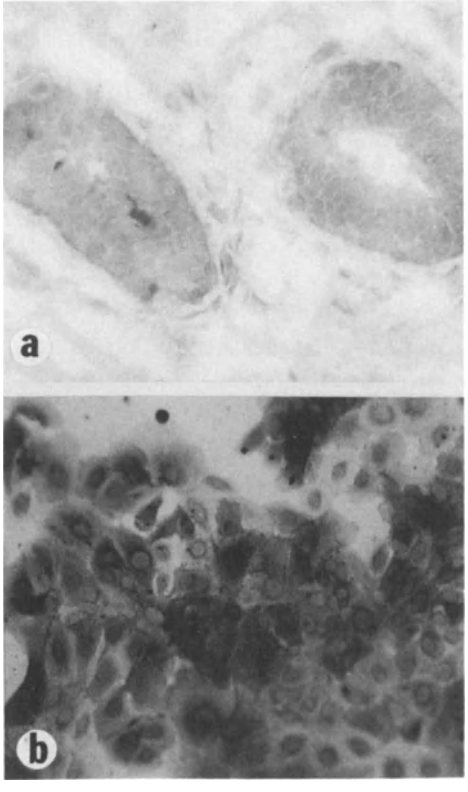

Figure 1: Immunoperoxidase localization of anti-CEA on (a) mature normal mammary ducts showing general lack of reactivity with epithelial and stromal components, (b) secondary cultures of normal mammary epithelium. Note strong reactivity of the antibody with most cells in the field. Mag. (a) 440x, (b) 140x.

the majority of reports indicate that approximately one-third of the specimens are of this type. Auer et al. (25), using microspectrophotometry, described three additional types of primary breast cancer specimens. In addition to diploid malignancies, a second type of cancer shows either a distinct modal value in the tetraploid region or has two well-defined peaks around the 2C and 4C regions. Whether those cells in the 4C regions are truly tetraploid cells or diploid cells in the G_2 phase of the cell cycle cannot be distinguished. The third type contains both 2C and 4C peaks but also includes cells with intermediate values for DNA content. The cells with intermediate DNA content may be either aneuploid or in the S phase of the cell cycle. The fourth group of cancers, which comprise only 30% of specimens, shows a pronounced and irregular aneuploidy with DNA content per cell ranging from levels near 2C up to values beyond 6C. All of the other studies on DNA content of breast cancer also describe specimens containing 2C, 4C, intermediate and polyploid values for DNA content within individual specimens. Studies using flow cytometry may tend to overestimate the size of the diploid peaks because they cannot distinguish normal cells (e.g., lymophocytes or stromal cells) within the tumor specimen from the tumor cells. Alternatively, the suggestion has been made that preparations for flow cytometry result in selective loss of diploid tumor cells (24).

Evidence from karyotypic studies

Cytometric analysis is limited by the inability to detect small deletions, rearrangements or other subtle deviations from diploidy. Because of its slow growth rate, direct karyotypic analysis of breast cancers has been difficult. Most of the earlier studies on breast cancer karyotypes describe a single breast cancer; hence, it is difficult to evaluate how representative these are of most breast cancers (detailed in the chapter by S. Wolman). In two recent studies (29,30), approximately 40% of primary breast cancers were karyotyped. Both of these studies detected aneuploid cells in the tumors. However, it is most notable that, in the vast majority of cases, from 2 to 75% (median 9%) of the observed spreads were diploid. While the authors interpret these diploid cells as being of normal lymphocytic origin, they present no evidence that excludes the possibility that they are bonafide tumor cells. If lymophocytes were indeed proliferating, we would expect to see them in S or G-2 phase by microspectrophotometry. In our studies, only 4 lymphocytes were seen with S phase DNA contents among over 800 lymphocytes examined from 10 consecutive breast cancer cases; and none had G-2/M DNA contents (unpublished observations). Furthermore, there is no evidence in the literature that lymphocytes continue proliferation within tumors.

Evidence from cell culture

Other evidence for diploidy of primary carcinomas comes from cell culture studies. We have found that the majority of cells in short-term cultures from 15 different primary carcinomas were diploid. Only occasional cells within these cultures showed minimal and non-clonal karyotypic deviations from normality (31). A detailed description of the methods that we used for dissociating and culturing breast carcinomas is presented in the chapter by Stampfer. Briefly, the tumors are digested with collagenase and hyaluronidase to degrade the stromal matrix. With such treatment, part of the tumor is dissociated to single cells but most remains as tightly associated cell clumps. These conditions result in dissociation of the stromal fibroblasts and blood vessels to single cells; the tumor clumps can then easily be isolated free from fibroblasts by sedimentation at unit gravity (32) or by filtration through nylon mesh filters (33). The tumor cells proliferate to some degree in a variety of culture conditions (32-41); however, recent media formulations have resulted in considerably greater proliferation in mass cultures (41) and have permitted tumor cell growth in a highly

efficient clonogenic assay when cells are plated sparsely on a fibroblast feeder layer (42). Most recently, a serum-free medium has been described which allows clonal proliferation without fibroblast feeder cells (43).

A number of criteria have been used to demonstrate the epithelial nature of these tumor cells. The cultured cells possess a typical cuboidal morphology with the formation of secretory domes and duct-like, three-dimensional ridges at confluence. Ultrastructurally, the cells show junctional complexes and evidence of secretory activity (33). The cells are positive for cytokeratins (44,45), have a diminished punctate pattern of cell-associated fibronectin (44,45,46) typical of epithelium and express epithelial membrane antigens as defined by antibodies raised to milk-fat globule membrane (47).

There is some controversy as to whether the carcinoma-derived cultures are bona fide tumor cells or nonmalignant cells originating from tissue peripheral to the malignancy (48,49). It is unlikely that cellular clumps dissociated from tumors originate from nonmalignant peripheral

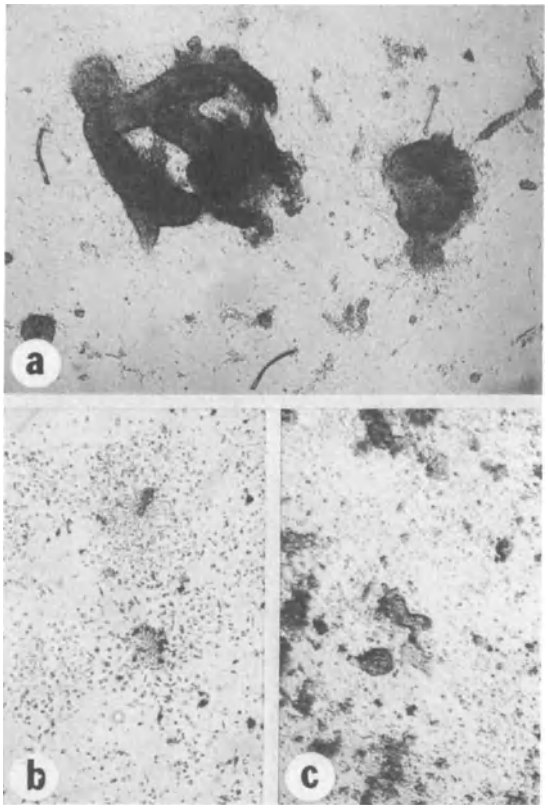

Figure 2: Digested breast carcinomas have smaller and less structured clumps than those digested from nonmalignant tissue.

a) Nonmalignant peripheral tissue (typical field)
b) Tumor from same patient (typical field)
c) Tumor from same patient (field with largest clumps)

(photomicrographs taken after two days in culture, original magnification x 25)

tissue since they tend to be much smaller and less structured than clumps digested from nonmalignant tissue (Fig. 2). Additional justification for the belief that primary breast cancer cells are, in fact, being cultured stems from reports of consistent differences between cultures derived from malignant and nonmalignant tissues. Asaga et al., (49) reported a significant increase in multinucleated cells after incubation of human mammary carcinoma cultures with cytochalasin when compared with cultures derived from various benign tissues. Similar results were reported for cultured rodent mammary tissues (50). Carcinoma-derived cultures also showed increased variability in surface antigen expression when compared to nonmalignant tissue from the same donor (51). Many of the tumor-derived cultures also show increased ability to form diol-epoxide adducts in their DNA after incubation with benzo(a)pyrene (52). We have compared malignant and nonmalignant cultures using a polyvalent antiserum prepared by Edgington and colleagues (53,54) to a 19.5 kilodalton polyglycoprotein reported to be tumor-specific. All tumor-derived cultures were positive while all nonmalignant cultures tested were negative (44). More recently, to obtain further evidence that the cells cultured from primary carcinomas indeed represent malignant cells, we investigated the phenotype of invasiveness, probably the most important single criterion by which human solid malignancies can be presently characterized (55). We employed an in vitro assay for invasion utilizing denuded human amnions (56) and we found that the tumor cells retained their malignant phenotype in culture by being capable of invasive growth.

In earlier studies, the malignant nature of the tumor derived cells had been questioned (47,48). It had been observed that most of the carcinoma-derived cultures were morphologically indistinguishable from nonmalignant cultured mammary cells. In 15 to 20% of cultures from primary carcinomas, a cell type with unusually abnormal morphology was also observed. The abnormal cell type, designated E', was thought to resemble cells from metastatic lesions, but unlike the cells from metastases, it was unable to grow in culture. It was hypothesized the E' cells were the tumor cells and that only nonmalignant cells associated with cancers and not the actual tumor cells were growing out in culture from the majority of primary carcinomas. However, we have found that the normal as well as the tumor cultures were reported as malignant, when coded samples were examined by a cytopathologist (Fig. 3). Since normal cells in culture can appear malignant, any morphological observation inferring normality or malignancy is likely to be invalid when considering cultured cells.

B. Prediction 2: Many aneuploid cells in primary breast cancers are nonviable

Some breast cancer cells are only loosely attached to the stromal matrix so that they can be easily dissociated by mechanical manipulation. These cells represent a population within the tumors, characterized by low viability using dye exclusion tests. Mechanically separated populations are enriched in aneuploid cells, while cells released after enzymatic digestion of minced tumor tissue are predominantly viable and diploid (57). These observations are predicted if the proposed model is correct since one would expect populations enriched in aneuploid cells to be largely nonviable. However, it is important to be certain that the nonviable cells were present in vivo and not the result of artifacts arising during tissue processing. In the published studies, the mechanically dissociated cells were subjected to sheer stress in order to create a single cell population. More recently, we have mechanically dissociated tumors in a very gentle manner making no attempt to dissociate tumor cell clumps. Even under these conditions, many preparations contained a large percentage of nonviable cells. We also ruled out the possibility that the mechanically dissociated cells are viable in vivo, but die during the period from initiation of surgery to dissociation in the laboratory. To approach this question, we examined cells immediately

after needle aspiration of the lesion prior to commencement of surgery. We found a similar proportion of nonviable cells as with gentle mechanical dissociation of the tumor biopsy (unpublished observation in collaboration with Drs. C. Lodish, C. Boyer and their surgical colleagues at Duke University, Durham, NC). It is noteworthy that the mechanical dispersion techniques are the ones that are frequently used for analyzing DNA contents of tumor specimen; hence many reports of aneuploidy within breast cancers may overestimate the percentage of aneuploid cells within the tumor.

C. Prediction 3: Aneuploidy is associated with malignant progression.

In contrast to the primary carcinomas, the malignant effusion-derived cultures were all aneuploid. We utilized flow cytometry to determine the DNA content of the slowly proliferating malignant effusion cultures. When

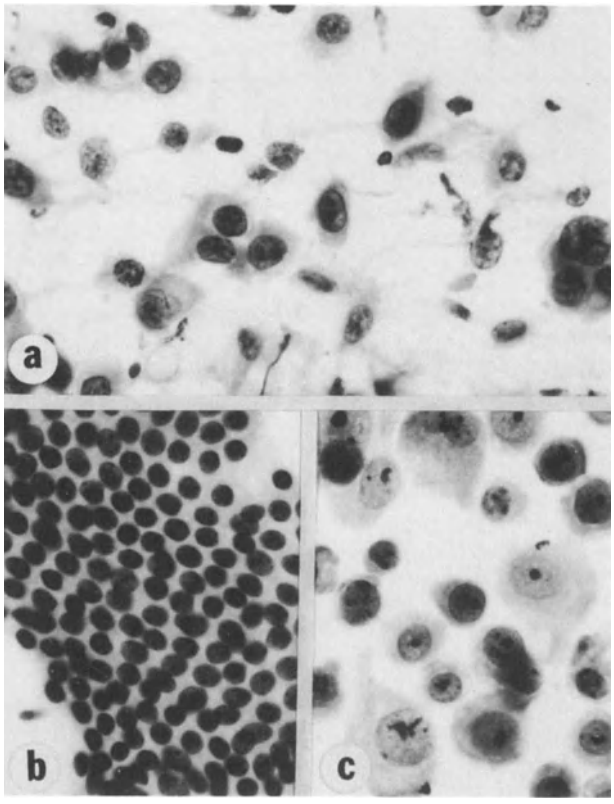

Figure 3: The morphology of normal mammary epithelium appears malignant in culture.

a) Benign cultured breast cells. There is marked polymorphism, including coarse chromatin pattern, prominent nucleoli, and variation in cell size.
b) Benign breast epithelium obtained by fine needle aspiration. Monomorphous small cells in orderly cohesive monolayered sheet.
c) Breast cancer cells obtained by fine needle aspiration. The cells are markedly pleomorphic with coarse chromatin, prominent nucleoli, variation in cell size, and mitotic figures (original magnification x 400)

aneuploidy was also verified karyotypically, the tumor cells had markers indicative of clonal origin. All of the specimens which appeared diploid by flow cytometry were karyotypically abnormal with several marker chromosomes.

All of the studies on ploidy levels in breast cancer agree that the tumors with more aneuploid and aberrant ploidy levels tend to be more dedifferentiated, negative for estrogen receptors and indicate a poor prognosis for the patient (25). Auer et al. (58) undertook a retrospective study of fine-needle aspiration biopsy material from 112 patients with primary mammary carcinoma. They divided the specimens into those from patients who were alive after 10 years and those who were dead after 2 years. The vast majority of patients with long survival times had cancers with DNA values within the limits of normal mammary epithelial cells, while the majority of patients with apparently highly malignant breast carcinomas exhibited DNA profiles with significant deviation from those of normal mammary epithelium.

D. Prediction 4: Metastases will be highly variable because they arise after accumulation of many random genetic alterations.

To gain insight into the properties of cells representing later stages of malignant progression, we have begun to study malignant effusions in culture. The first two specimens grew initially, but failed to develop into cell lines. Each specimen was also unique in morphology and growth properties, although karyotypic markers indicated a common origin (59). These observations also suggest that the ability to develop cell lines from a human breast cancer is not random, either in relation to culture technique or to tumor progression. Furthermore, the capacity for infinite life <u>in vitro</u> is not a characteristic of all malignant breast cancer cells, rather it is an example of another phenotype which is sometimes acquired by some breast cancer cells at a late stage of malignant progression. As expected, pleural effusions are much less predictable than primary carcinomas even when they are handled in an identical manner (60,21). For example, one effusion metastatic population will attach well to the plastic substrate, proliferate until confluence and then form domes and ridges characteristic of normal mammary epithelium. In contrast, a second effusion will not attach to the plastic, but will proliferate slowly in suspension (illustrated in ref 60). Cellular morphology and growth properties differ dramatically among pleural effusion specimens. In the samples we have studied, the short-term cultures of malignant effusions often grew more slowly than primary cancer cultures and very few were able to clone on irradiated fibroblasts. Many malignant effusions grew better with medium M199 plus 10% fetal calf serum than with the medium formulation derived for normal mammary epithelium, although sometimes the reverse was true. Thus, each pleural effusion seems to have a combination of properties in culture that deviate in a unique way from the uniform and predictable behavior of most primary carcinomas. These observations suggest that effusions have diverged from primary carcinomas via differing pathways, as would be predicted by our hypothesis.

Another example of the variability of metastatic specimens is seen in the derivation of established cell lines. Almost all breast cancer cell lines in existence have been derived from effusion metastases (for reviews, see 61-63). Although the majority of metastatic specimens from pleural effusions can be cultured for a few population doublings, even under the best conditions, only approximately 5 percent of these cultures develop into cell lines. In one case, we examined the properties in culture of three breast cancer effusions from the same patient. Despite repeated attempts with cryopreserved cells, only the last specimen reproducibly exhibited immortality in culture.

E. What causes genetic instability?

One possible mechanism for generating chromosome instability is spontaneous somatic cell fusion. It is know that somatic hybridization causes karyotypic instability resulting in chromosome losses and rearrangements (64). A number of investigators have reported spontaneous fusions between experimental cells and host cells in vivo (65-73) including xenografted tumors, such as human tumors growing in the cheek pouch of hamsters. Kovacks (74) and others (76-79) provide evidence that somatic cell hybridization occurs in a number of human cancers, including breast. They observed premature condensed chromatin, an indicator of cell fusion, in chromosome spreads taken directly from human tumor material. One of the breast cancers in Kovacks''s study clearly indicated a fusion between two tumor cells had occurred because the same marker was found in the prematurely condensed G-1 chromosomes, as in the appropriately condensed chromosomes of the fusion partner. Direct evidence for tumor x host cell fusion was obtained by Atkin (77) for a bladder carcinoma. These cell fusions may result in the wildly aneuploid, often nonviable cells found in most cancers. It has been suggested that such fusions, perhaps with lymphoid cells which normally express a migratory and invasive phenotype, could confer on neoplastic cells the capacity for metastatic spread.

Cell fusion is probably not the whole story. Gene amplification is another mechanism to explain the increased capacity of tumor cells for genetic alteration. Double minute chromosomes have been observed in direct chromosome spreads in a number of primary breast cancers from untreated patients (29). There is now a large literature associating double minutes with gene amplification (30). In most cases, the double minutes are the result of selecting for drug resistance. However, there is no reason to assume that they would not occur when other chromosomal regions are amplified. In addition to gene amplifications, more subtle genetic rearrangements also may be occurring which are not visible at a gross karyotypic level.

IV. CONCLUSION

We hypothesize that early events in the development of human breast cancer involve complex interaction between stromal and epithelial cells which can be described as a "field effect". Thus, early stages in the etiology of this disease may be monoclonal in origin. Evidence for mesenchymal involvement in breast cancer include studies that fibroblasts from many breast cancer patients differ in culture from firbroblasts of normal donors. Further support for this hypothesis comes from studies showing that normal epithelial cells placed in an abnormal environment acquire some properties associated with malignancy.

We propose that one result of the abnormal field is that tumor cells acquire increased capacity for genetic instability. As suggested by Nowell (19), diploid neoplastic cells generate nonviable cells with grossly abnormal chromosomal contents. Only occasionally does this process generate a viable aneuploid cell with growth advantage allowing it to selectively populate the original tumor or subsequently to metastasize. We suggest that this evolutionary process is quite slow in breast cancer so that the original diploid cells and the nonviable and viable aneuploid populations all coexist in the primary lesion. This model makes the following four predictions: 1) diploid cells will be found in the primary tumors, 2) aneuploidy is associated with malignant progression, 3) metastases will be highly variable because they arise from multiple random genetic changes and 4) many aneuploid cells are nonviable. Evidence for each of the above predictions is discussed.

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CYTOKINETICS OF MAMMARY TUMOR MODELS AND THE EFFECT OF THERAPEUTIC INTERVENTION

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INTRODUCTION

Using ^{32}P labelling and autoradiography, Howard and Pelc (1) demonstrated that DNA synthesis was relegated to a discrete phase (S-phase), temporally separated from mitosis by a post-synthetic gap (G2), and a post mitotic gap (G1). The synthesis of [^{3}H]-thymidine in 1957 (2) provided a specific radiolabeled DNA precursor suitable for detailed studies of cell cycle kinetics. The fraction labelled mitosis (FLM) and autoradiography methods were used by many early workers (3-5) to assess cell cycle transit. Mendlesohn and coworkers (6-9) were the first to use this method to study cell cycle dynamics of autochthonous C3H mammary tumors and their finding that non-proliferating cells can comprise a large fraction of the cell population had a major impact on tumor kinetics and the formulation of therapeutic strategies. Although the development of computer simulations added precision (10-13), the FLM method is subject to a number of artifacts which can result in the underestimation of cell cycle transit times (14-17).

Stathmokinetic techniques employing metaphase arresting agents such as vincristine (VCR) have also been used to monitor cell proliferation (18). Although the results may be affected by the nature and dose of the drug, Wright's (19) review indicates that carefully performed experiments can provide reliable and useful data.

<u>In vitro</u> labelling techniques were developed to provide cell kinetic parameters for individual tumor samples (20-23). [^{3}H]-thymidine labelling indices (TLI's) obtained for C3H/HeJ autochthonous mammary tumors by these methods were similar to those obtained after <u>in vivo</u> labelling (20). The

[^{3}H]dThd - [^{14}C]dThd double labelling technique (21) for the determination of the DNA synthesis time (Ts) was adapted for in vitro assay of Ts in solid tumors (20) and an in vitro autoradiographic assay (PDP assay) was developed (22-27) to assess the tumor growth fraction (GF). The methodology for this assay and other methods to measure tumor GF were recently reviewed (27).

My intent in this chapter is to update previous reviews (28) on the cytokinetics of mammary tumor models, review the relationships between cytokinetics and tumor physiology, describe cytokinetic perturbations induced by therapy and illustrate new approaches which have translated the conceptual aspects of studies in model systems to the clinical level.

II. CELL CYCLE PARAMETERS IN MAMMARY TUMOR MODELS

Mendlesohn (29) found that volume doubling times (Td's) for C3H tumors ranged from 2-50 days (n=155) while in our series (24), Td's ranged from 3.6 to 73.2 days with a mean of 17 and a median of 12.4 days. These differences are reflected in the cytokinetic data (Table 1). In our series TLI's and GF's were about 50% of those seen by Mendlesohn (8). The mean cell cycle times (Tc) in our series were not different however, from that seen after further analysis of Mendlesohn's data (30). The metaphase arrest method also provided similar Tc estimates (~32 hrs) for these tumors (31).

In collaboration with Dr. D. Martin, we obtained cell kinetic profiles for 33, CD8F1 autochthonous mammary tumor (Table 1). Intertumor variation was high. Furthermore, TLI and PDP indices were higher and mean Tc's longer than in C3H/He tumors. Cell cycle parameters in a small series of spontaneously arising mammary adenocarcinomas, adenomas and fibroadenomas in Sprague-Dawley rats were quite similar. In spontaneous lung metastases, from adenocarcinomas, TLI's were higher and mean Tc's shorter in metastases than in the primary tumors. FLM studies in rat (SD) mammary tumors induced by dimethylbenz(a)anthracene (DMBA), indicated Tc's of approximately 24 hours and GF's of approximately 35% (32,33). Using in vitro methods (20,-23,24,27) our studies with DMBA-induced mammary tumors (SD/ZM rats) showed longer Tc's and lower GF's than obtained by FLM analysis (32,33). We analyzed the data with respect to histology, tumor size and estrous cycle (Tables 2,3). TLI's in type B and C tumors were similar, but TLI's in well differentiated type A tumors were significantly lower. The Ts in type A tumors was longer than in type B or C tumors and the GF, for type A (PDP assay) was lower than for type B and C tumors. The Tc's for poorly differentiated type C tumors were significantly shorter than in type A. Size did not significantly impact on the cell cycle parameters for moderately differentiated type B tumors but in type C tumors, the shorter cell cycle times were seen in small tumors.

Although no overall influence of estrous cycle was detected (Table 3), type B tumors in proestrous rats had shorter Ts's than type B tumors in diestrous rats. The Tc and potential doubling time (Tpot's) also tended to be shorter in proestrous than in diestrous. Such an observation is consistent with the known hormone dependency of DMBA-induced mammary tumors (34-36) and high serum estradiol, progesterone and prolactin during proestrous (37).

Growth rates of early transplant generation tumors are generally more rapid than for autochthonous donors (38,39). Increased growth rates in early passage tumors are reflected by increased TLI's and GF's and shortened Td's, Ts's and Tc's (Table 4).

Spontaneous and artificially induced lung metastases from autochthonous C3H/He mammary tumors may also be considered first generation tumors. TLI's were obtained after in vivo labelling, DNA synthesis times were

Table 1. Cell Kinetic Parameters in Autochthanous Mammary Tumor Models

Tumor	Td[a]	TLI[b]	TG1	Ts	TG2	Tc	GF	Ref.
CeH/He	204	14.0	19.4	11.7	1.9	34.6	40.0	6
C3H/He			14.0	10.0	3.0	25.0	45.0	11
C3H/He	400	7.4	15.2	10.9	2.0	28.6	17.0	24
CD8F1		9.3		10.1	2.0	35	35.9	28
Rat								
AdenoCa		5.1		11.0	~2.0	45	22.0	43
Sarcoma		20.8		10.0	~2.0	22	48.1	
Fibrosarcoma		10.6		10.7	~2.0	29	32.6	
Fibroadenoma		4.5		11.1	~2.0	38	16.1	
Adenoma		4.2		9.8	~2.0	45	20.2	
Metastases		10.4		9.8		25	27.6	
DMBA								
SD rat	168-384	6.1-7.1	5.5-7.1	8.1-11.4	0.9-1.9	17.5-18.4	10-20	32
SD rat	432	10.0	11.5	8.5	1.0	22	31	35
SD rat			14.0	9.0	1.0	24	35	41
SD/ZM		6.1		9.4		39	21.1	43

a., Td, TG1, Ts, TG2, Tc in hours
b., TLI, GF in percent

Table 2. Cell Kinetic Parameters for DMBA-Induced Mammary Tumors in SD/ZM Rats

	TLI[a]	Ts[b]	PDPI	Tc
A < 3 cm[d]	4.0 ± 1.5 (6)[c]	10.3 ± 1.6 (6)	16.7 ± 6.4 (13)	48 ± 12 (6)
B < 1	7.2 ± 3.5 (23)	9.0 ± 1.1 (23)	22.0 ± 10.9 (32)	35 ± 11 (18)
B 1 - 3	6.4 ± 3.1 (19)	9.1 ± 1.0 (17)	22.7 ± 13.8 (18)	35 ± 25 (17)
B > 3	7.3 ± 3.1 (13)	9.9 ± 1.6 (16)	21.2 ± 8.8 (12)	35 ± 23 (10)
All Type B	6.7 ± 3.3 (58)	9.3 ± 1.4 (56)	21.9 ± 11.2 (62)	35 ± 21 (45)
C < 3	7.8 ± 4 (9)	8.6 ± 1.0 (9)	24.4 ± 1.7 (19)	25 ± 10 (7)
C > 3	7.5 ± 3.9 (6)	9.8 ± 1.7 (6)	27.4 ± 6.0 (6)	49 ± 24 (5)
All Type C	7.7 ± 3.7 (15)	9.2 ± 1.3 (15)	25.1 ± 8.1 (25)	33 ± 14 (12)

a., TLI, PDPI in percent
b., Ts, Tc in hrs.
c., mean ± SD (n)
d., A, well differentiated, B, moderately differentiated, C, poorly differentiated

Table 3. The Influence of the Estrous Cycle on Cell Kinetic Parameters from DMBA-Induced Mammary Tumors SD/ZM Rats

	TLI[a]	TS[b]	PDPI	Tpot	Tc
All Tumors					
PE[d]	6.8 ± 3.7 (18)[c]	9.0 ± 1.1 (17)	24.4 ± 12.1 (16)	118 ± 63 (17)	36 ± 2.6 (16)
E	6.1 ± 2.8 (29)	9.9 ± 1.4 (29)	20.8 ± 9.5 (43)	134 ± 60 (28)	44 ± 32 (22)
DE	5.8 ± 3.2 (50)	9.3 ± 1.3 (49)	20.7 ± 10.2 (50)	140 ± 67 (49)	38 ± 26 (47)
Type B					
PE	7.9 ± 3.8 (13)	8.7 ± 1.0 (12)	25.5 ± 14.1 (11)	88 ± 32 (10)	27 ± 8 (10)
E	6.8 ± 3.0 (15)	9.6 ± 1.4 (17)	21.9 ± 10.3 (21)	116 ± 43 (12)	31 ± 25 (11)
DE	6.6 ± 3.1 (22)	9.3 ± 1.5 (23)	21.8 ± 11.6 (24)	122 ± 56 (21)	35 ± 23 (21)

[a], TLI, PDPI in percent
[b], Ts, Tpot, Tc in hours
[c], mean ± SD (n)
[d], PE, proesterous, E, esterous, DE, diesterous

Table 4. Cell Kinetic Parameters for First Generation Mammary Tumors and Lung Metastases

Tumor	Td[a]	TLI[b]	TG1	Ts	TG2	Tc	GF	Ref.
First Generation								
C3H	108	15.9	6.5	6.5	2.0	15	37.0	41
"			7.8	6.5	1.5	16	46.0	41
CD8F1			9.3	9.5	1.5	20.3	45.0	42
DMBA rat	43	15	6.2	7.7	1.8	16.0	32.0	42
Metastasis								
C3H								
Spontaneous		10.3		11.6		33	32.1	43
Artificial								
	46	16.9	6.8	8.7	2.2	17.2	38.3	44
	94	13.3	7.7	9.0	2.1	18.4	30.5	44
	211	11.8	7.1	8.9	2.0	18.6	26.0	44
Artificial								
LNS14		15.1		8.9		31	61.1	45,43
LNS15		14.0		9.4		21	30.3	43
LNS16		7.1		10.6		31	22.3	43
LNS17		15.8		9.2		16	26.5	43

[a], Td, TG1, Ts, TG2, Tc in hours
[b], TLI, GF in percent

determined by an in vivo [^{3}H]dThd-[^{14}C]dThd double labelling technique and the GF was determined by PDP assay of individual lung nodules. TLI's and PDPI's in spontaneous lung metastases were higher than in primary tumors, however, Tc's were not different. Artificial lung metastases were induced by intravenous injection of a mixture of single cells and tumor cell aggregates prepared from autochthonous mammary tumors. At four weeks after passage (LNS14), 169 tumor nodules from twelve mice ranged from less than 1 mm to approximately 4 mm in diameter (mean $1.8^{+}1.1$mm;$^{+}$SD). Cell proliferation was markedly increased relative to that in the three donor tumors; TL, 10.7 $^{+}3.4$ (n=3); PDPI; 24.6 $^{+}6,2$ (n=3). At five (LNS15) and seven (LNS16) week the mean tumor nodule diameter was 2.3 $^{+}1.3$ and 3.3 $^{+}1.4$ mm respectively. Although TLI and GF did not correlate well with tumor size in any of the 3 studies, TLI's and GF's seemed to decrease with tumor age.

In LNS17, mammary tumor cells were isolated from artificially induced metastases and injected into C3H/He mice via the lateral tail vein. Calculated Tc's in this second generation transplant were about one half that for spontaneous metastases and first generation lung nodules. Gunduz (44) found that TLI's and GF's for artificial C3H/He lung metastases decreased with increasing Td. The FLM method and computer simulation (13) provided shorter cell cycle durations than we observed in our studies.

In 4th generation C3H tumors TLI's were approximately 10% near necrotic areas and nearly 50% for cells adjacent to capillaries (46). FLM analysis indicated similar Tc's in all areas suggesting that the lower TLI's were due to decreases in the GF. In similar studies with the KHH tumor model, Hirst et al. (48) divided the tumor cords into microenvironmental zones, the inner and outer zones corresponding to 2 cell layers from capillaries and necrosis respectively. The intermediate zone contained 3-4 cell layers between the inner and outer zones. TLI's decreased with increasing distance from the central capillary, but the GF in the intermediate zone (100%) was similar to that for cells closest to the vasculature. The GF in the outer zone was 58%. The Tc, assessed by FLM, increased with increasing distance from the capillary and reflected increases in G1 transit time (TG1). Subsequent studies (49) showed that the TLI for endothelial cells was high (~18%) in rapidly growing mammary tumors (KHH,KHU), somewhat lower (~4.5%) in a slow growing mammary tumor model (CaRH) and the growth rates were most closely related to tumor GF's. Since FLM methods usually require at least 36 hours of sampling and migration times across the tumor cord were 36-50 hours, the FLM results for population in various zones may be suspect. Jones and Camplejohn (50) used a stathmokinetic technique which required only 3 hours of sampling in their studies with a transplantable, corded, C3H mammary tumor. Cell birth rates and tumor GF's decreased with increasing distance from the central capillary, thus, confirming previous work in C3H (46) and other mammary tumor models (51). In artificial C3H/He mammary tumor lung metastases, decreases in tumor growth rate with increasing tumor size correlated with a decrease in the tumor GF (44). Endothelial cell proliferation was similar in small and large lung nodules, however the capillary component of the tumor vascular volume decreased from 98% in micrometastases to 38% in large macrometastases (44).

Several other authors have described size dependent changes in tumor cell proliferation (Table 5). In HB mammary tumors, the median Tc (FLM method) increased from 14.6 hours, at 7 days, to 23 hours in 30 day tumors reflecting increases in the Ts and TG1. The median GF decreased from 53% at 7 days to 39% in 30 day tumors (52,53). In another serially passaged C3H mammary tumor, TLI's and PDP indices (GF) decreased with increasing tumor size (54). Increases in Ts and Tc were also observed during tumor growth. Cell loss was markedly increased in larger tumors.

Table 5. Cell Kinetic Parameters for Transplantable Mammary Tumors in Mice

Tumor/Host	Td	TLI	TG1	Ts	TG2	Tm	Tc	GF	Ref.
C3H Mouse									
4th Generation		35.1	3.0	7.2	3.0		13.0	50	46
S102F			6.5	7.7	2.5	0.6	17.2	55	43
S102F		17.2		9.5			25.8	54.2	47
S102S			6.7	11.0	1.3	1.1	20.1	30	47
A67			17.3	12.5	2.1	1.6	33.5	23.0	47
A67		7.8		11.5			36.4	26.8	43
Line 284		12.5	4.7	6.8			13.6	25	55
C3HBA	62	27.4	10.4	8.7	3.2		20.3	70	56
H2712	34	33	0.9	5.8	2.1		9.0	51-67	28
HB		5.9	5.9	7.0	1.4		15.5	47	52
HB	36	6.8	6.8	6.8	1.9		14.6	53	53
HB	178	7.9	7.9	7.6	1.2		15.3	46.4	53
HB	20	15.9	15.9	8.4	3.0		23.0	38.6	53
HeB/Fe	36			7.2			12-14	45	54
	178-250			8.3			15-18	21-37	54
KHH	120	10	10	8	1.5		21	83	48
KHU	144	6.5	6.5	7	2.0		15.5	63	49
DBA Mouse									
DBAH			7.5	6.0	2.5		16		60
DBAG			3.0	12.0	1.0		16		60
DBA Mouse									
T1699	55	13.5	5.4	5.8	3.7		13.1	40	23
T1699	52	18.6	5.7	6.7	3.9		18.3	49	57
T1699	85	15.6	7.2	6.8	3.9		18.9	46	
T1699	134	13.5	8.6	6.6	3.9		20.1	43	
T1699	72	25.4		6.9			18	71	58
T1699	67	23.8		6.1			15.3	63	59
T1699	149	22.5		5.8			15.5	64	

Tumor/Host	Td	TLI	TG1	Ts	TG2	Tm	Tc	GF	Ref.
Balb/C Mice									
KHJJ	38	35.8	2.4	8.4	2.0		13.7	52	63
KHJJ		22.3	4.6	9.4	2.2		16.1	38	63
EMT-6		29.3	7.3	11.9	1.9		20.7	48	63
EMT-6		23.9	9.4	11.6	2.6		22.6	51	63
EMT6/M/AC	31	32.0	5.6	7.3	1.5		14.1	68	64
	120	24.1	7.5	9.9	1.8		18.5	51	
IX			11.0	10.4	0.8	1.5	23.7	43	80
C57B1 Mouse									
Ca775	20	27.0	3.5	7.0	0.5		12.0	51	65
Ca775	38	18.0	4.5	6.0	2.5		14.0	45	66
Ca775	233	9.0	7.5	6.0	1.5		16.0	28	66
WHT Mouse									
CaRH	320	11.0	11.5	8.0	2.0		21.5	29	49
CBA Mouse									
CaNT	64	17	6.3	8.7	2.1		21.0	40	69
CaNT	94	37.1	11.8	8.9	5.1		26.8	80	15,16
BICR/SA1	74	40	4.7	9.1	2.5		16	88	51
BICR/SA1		62	3	10.7	1.3		16	100	51
BICR/SA1		42	4	10.7	1.3		17	>80	51
BICR/SA1		30	4	10.7	1.3		17	50	51

[a], TD, TG1, Ts, TG2, Tc in hours
[b], TLI, GF in percent

Several transplanted mammary tumors in the DBA mice have been used as model systems (Table 5). Cell kinetic parameters for the poorly differentiated T1699 mammary tumor were reported in studies from our laboratory between 1976 and 1980. In the earliest report (23) the TLI was 13.5% and FLM studies indicated Ts of 5.8 hrs, Tc of 13 hrs and a GF of 40%. In 1978, cell kinetic parameters were determined by in vitro labelling techniques (20,23,24) during tumor progression (57). Although TLI's and GF's decreased with increasing tumor size, there was little change in Tc. GF's estimated by PDP assay were greater than those observed in the FLM study. Baseline cell cycle parameters determined again in 1979 (58) and 1980 (59) indicate that the proliferative characteristics of this model changed considerably during 5 years of serial passage.

Cell cycle parameters were also determined for the DBAH and DBAG tumor models (60) and subsequent studies showed that nucleolar morphology could provide a means to recognize proliferating and non-proliferating cell populations (61). [^{3}H]-Thymidine labelling studies indicated that cells with dense nucleoli comprised the GF in these tumors. Cells exhibiting ringed shaped nucleoli or nucleolar fragments were more deeply G1-confined than cells with tabeculate nucleoli (62).

The KHJJ tumor model was derived from a hyperplastic alveolar nodule arising in the BALB/cCrgl mouse and subsequently passaged in BALB/c mice. FLM studies (63) and computer simulation (11) showed that cells at the periphery of the tumor proliferated more rapidly than cells in the center of the tumor mass where reduced GF's and prolonged Ts, TG1 and Tc's were seen. The KHJJ tumor was adapted to tissue culture, passaged in BALB/c mice and renamed EMT-6 (63). TLI's for cells at the periphery were higher than for cells near necrotic regions. FLM studies indicated this to be due to increases in TG1 as the GF's for the two populations were similar. In an EMT-6 variant, designated EMT6/M/AC, reduced TLI's in large tumors were a manifestation of reduced GF and increased Tc's (64).

In the Ca775 mammary tumor in C57Bl/6 mice, a cell kinetic basis for decreasing tumor growth rates during tumor progression was also demonstrated (65,66). In experiments employing [^{3}H]-thymidine labelling, autoradiography and cytophotometric techniques (67), most of the cells outside the GF in 8-day Ca 755 tumors had a G1 DNA content, while in larger tumors most of the cells with a G2 DNA content could be shown to be non-proliferating. As reviewed by Gelfant (68), G_2 phase arrest is not an unusual finding in normal and some neoplastic tissues, but, it remains to be seen whether such G_2 arrested tumor cells are reproductively dead or whether they can be recruited back into active proliferation.

Hamilton and Dobbin (15,16) reported that in CaNT tumors (CBA mice) about 50% of the S-phase cells do not incorporate [^{3}H]-thymidine, but can incorporate [^{3}H]-deoxyuridine into DNA. Using[^{3}H]-thymidine, the FLM method indicated a Ts (~9 hours) that was nearly half that deduced from repeated [^{3}H]-thymidine labelling studies. Serial labelling and stathmokinetic studies indicate a turnover time of about 40-50 hours while turnover times computed from the FLM curve were only about 24 hrs. Using [^{3}H]-deoxyuridine, the FLM method provided a Tc estimate of 16 hrs, but grain count halving studies indicated a Ts of 19.2 hrs and a Tc of approximately 40 hrs. Thus, the FLM method clearly underestimated cell cycle durations, providing estimates of phase durations for only the most rapidly proliferating cells.

The cell kinetics of several rat mammary tumor models have been reported (33,41,70-77) (Table 6). Cell kinetic parameters for subcutaneous 13762 tumors (74) in Fisher 344 rats were obtained by FLM analysis and by in vitro labelling procedures (70,71,72). When 13762 tumor cells were innoculated into rats, previously cured of 13762 tumor by chemotherapy, Td's in 3 and 5

Table 6. Cell Kinetic Parameters in Transplanted Rat Mammary Tumors

Tumor	Td[a]	TLI[b]	TG1	Ts	TG2	Tm	Tc	GF	Ref.
BICR/M1	23	34.2	8.0	8.0	3.0		19.0	95	73
BICR/A4	120	12.0	6.0	11.0	2.0		18.0	26	74,41
	720	5.2	19.0	17.0	2.5		41.0	13	
BICR/A9	108	15.8	13.0	14.0	2.5		30.0	38	75
BICR/A9	41	31	3.5	9.0	2.5		16.0	69	75
BICR/A7	432	5.0	48	18.2	2.6		74	25	74
BICR/A10	113	9	4.1	10.3	1.3		16	16	41
BICR/A12	90	28	9.4	8.9	2.4		21	87	76
BICR/A14	182	16	21.0	13.2	0.7		36	48	41
AdCa 1/C	43	15	6.3	8.6	2.4		17.3	30	33
								70-80	
13762	108	31.8	5.2	5.6	1.8	0.4	12.7	69.8	70,71
Control	38	36.0		6.3			12.7	74.4	72
Treated	96	30.9		7.0			13.3	61.0	72
Control	158	21.7		6.1			16.1	63.6	72
Treated	372	15.6		7.3			21.1	55.2	72
R3230AC		13.3		10.9			41	55.9	77

[a], Td, TG1, Ts, TG2, Tc in hours
[b], TLI, GF in percent

cm tumors could be ascribed to reduced cell production with little change in cell loss (72).

Xenograft tumor models have been widely used to study the growth and therapeutic sensitivity of primary human tumors and human tumor cell lines, but very little cell kinetic data exists for mammary tumor models. Tsuyuki's (78) FLM study with MX-1 tumor xenografts indicated a rapidly growing, high growth fraction tumor with a Tc of approximately 96 hours (Table 7).

In unpublished studies we compared the cell kinetics for a transplantable C3H/He mammary tumor line implanted in athymic nude mice with a C3H background (nu/nu), normal litter mates (nu/+) and in athymic nudes, 30 days after thymic reconstitution. Using in vitro techniques (20,23,24) we detected little difference in the tumors in the 3 types of mice. Similarly, cell kinetic parameters determined for R3230AC rat mammary tumors growing in female Fisher 344 rats and in female athymic nude (BALB/c) mice were not different, implying T-cell function has little effect on the proliferation of these cells (77).

III. CYTOKINETIC PERTURBATIONS RELATED TO THERAPEUTIC INTERVENTION

A. Diet

The pioneering studies of Rous (79) showed that dietary manipulation can influence the development and growth of malignant cells. In the T1699 tumor model, 48-hour fasting resulted in decreased TLI's, increased Tc, TG1 and Ts with little change in the tumor GF (58). In the autochthonous C3H/He mammary tumors, a 60-hour fast also produced decreased TLI's, a five-fold lengthening of Tc, but no significant change in GF (58). The refeeding response in this model was characterized by increases in the tumor GF approaching 3 times that for controls and increased TLI's.

Gabor et al. (80) recently reported FLM data for a BALB/c mammary adenocarcinoma designated "tumor IX". Cell kinetic parameters in tumors from mice on high and low polyunsaturated fat diets were not different. Slower tumor growth in the low fat group was ascribed to increased cell loss rates.

B. Hormones

The pioneering work of Huggins (34) demonstrated the hormonal dependency of chemically induced rat mammary tumors and later studies described the effects of steroid hormone manipulations on cell cycle progression. In one of the first studies, Combs (81,82) reported that although Tc's for DMBA-induced mammary tumors did not change during the estrous cycle, the proliferative fraction (GF) was lowest during proestrous and reached a maximum during early diestrous. Such studies are complicated by the fact that the tumors may have progressed 1 or 2 stages during FLM data collection. Using in vitro labelling techniques which provided point in time assessment of cell kinetic parameters we observed the longest and shortest Tc's during diestrous and proestrous respectively. There was no significant change in the tumor GF as measured by the PDP method.

Estrogen ablation has marked effects on cell proliferation in this model as marked reductions in TLI's were sen 3 days after ovariectomy (32,-33). By 2 weeks, TLI's in regressing tumors were reduced by over 95%. When ovariectomized rats with regressing tumors were reconstituted with estradiol and progesterone, TLI's were 8.2% at 4 days and nearly 12% after 7 days of hormonal stimulation. It was subsequently shown that the estrogenic response of these tumors was mediated by stimulation of prolactin (36).

Table 7. Cell Kinetic Parameters for Mammary Tumor Models Grown as Xenografts in Athymic "Nude" Mice

Tumor/Host	Td	TLI	TG1	Ts	TG2	Tc	GF	Ref.
C3H								
Nu/Nu		9.1 ± 1.6	10.4 ± 0.7	10.4 ± 0.7		28 ± 7	26.6 ± 6.1	43
Nu/+		11.8 ± 2.0	10.1 ± 0.7	10.1 ± 0.7		20 ± 6	23.8 ± 2.4	43
Nu/Nu + T		10.8 ± 3.5	10.5 ± 0.9	10.5 ± 0.9		25 ± 4	26.6 ± 6.0	43
R3230AC								
Fisher rat		13.3 ± 1.1	10.9 ± 0.6	41.0		41.0	55.9 ± 4.0	77
Nu/Nu		10.5 ± 0.8	12.8 ± 1.0	46.0		46.0	46.0 ± 2.9	77
MX-1	112	23.9	21.5	9.5	~5	36.0	96.0	78

[a], Td, TG1, Ts, TG2, Tc in hours
[b], TLI, GF in percent
[c], mean ± 1SD (n)

Several murine mammary tumors are known to be hormone dependent (Table 8). The GR tumor model has a high GF (~60%), high TLI (27%) and short Tc (15.5 hours) in intact mice, but after oophorectomy, the tumor GF is reduced to 17%. Prolonged Tc's were due to increases in both TG1 and TG2 (83). Similar responses were reported for HB mammary tumors (52).

In male mice FLM derived cell kinetic parameters for CS-1, a subline of the androgen dependent Shinogi (SC-115) tumor were similar to those of the SC-115 parent line (84). In female mice the Cs-1 Tc was markedly increased, reflecting increased G1 and S-phase transit times. The GF was increased from ~50% to ~85% and cell loss decreased by 70% When testosterone was given to CS-1 bearing female mice, the inhibitory effects of estrogen on tumor growth were partially reversed, but Tc's remained prolonged. The GF was also higher than for tumors in male mice and little or no cell loss was seen (85).

In castrated (BD2f)F_1 female mice a single progesterone treatment resulted in increases in the TLI of MXT-Tumors within 12 hours. TLI's at 24 hours after progesterone or estradiol reconstitution were about twice that seen prior to stimulation. By 48 hours, TLI's were similar to that in the unstimulated controls (86).

Dao et al. (87) demonstrated that estrogen and progesterone treatments could stimulate proliferation in human breast cancer in vivo, as 7/10 patients showed increased TLI's within 3 days after hormonal stimulation. Interestingly, 3/7 responders were estrogen receptor negative. Conte et al. (88) demonstrated that a 3 day course of DES would also stimulate cell proliferation in human breast cancer in vivo. 8/16 patients demonstrated TLI increases after DES, while 13/16 patients demonstrated an increase in the PDP index. As in Dao's study, receptor status did not predict for response as 4/8 TLI responders and 7/13 PDP index responders were receptor negative.

The effects of antiestrogens on mammary tumor cell proliferation have been studied in several model systems. [^{3}H]-Thymidine and flow cytometry studies with MCF-7, ZR75-1, T47D and 47DN human mammary tumor cell lines indicated in vitro that the major cell cycle effect of tamoxifen is to block cell cycle progression in G1 (89-95). In the short term, this cytostasis was nontoxic and reversible as estrogen stimulation promoted synchronous cell cycle progression (89,92). Although estrogen rescue in vitro led to an increased chemoresponse (95) and tamoxifen inhibited proliferation in MCF-7 xenografts (96,97), estradiol "rescue" in vivo and in vivo response kinetics have not been reported. Several clinical trials evolved from the in vitro synchronization studies with the intent of exploiting cell kinetic changes in human breast cancer after estrogen stimulation (88,98,99). Stimulation was achieved (87,88) and high complete remission rates for stage IV breast cancer have been reported with this strategy (99).

The effects of corticosteroid hormones on cell proliferation were also studied in several mammary tumor models (100-102). Profound antiproliferative effective were noted in C3H/He autochthonous mammary tumors (100,101), their first generation transplants (102) and in the R3230AC rat mammary tumor (77) after a short treatment course. Proliferation inhibition was drug and dose dependent (101), mediated through specific corticosteroid receptors (77) and expressed as a reversible G1 block (77,100-103). Corticosteroid treatments produced little change in GF or Ts (11-101). Recovery in vivo was characterized by partial synchronous progression through S-phase (100-101). The recovery time course was shown to be drug and dose dependent (77,101). In the R3230AC tumors, resumption of cell proliferation after dexamethasone was preceded by recovery of saturable cytosolic dexamethasone receptor sites. Responses in R3230AC tumors in Fisher rats and athymic nude mice were similar (77).

Table 8. Cell Kinetic Parameters in Hormone Dependent Mouse Mammary Tumors

Tumor	Td[a]	TLI[b]	TG1	Ts	TG2	Tc	GF	Ref.
GR								
Intact	67	27.0	5.1	8.6	1.8	15.5	60.0	83
Castrate		7.5	9.9	9.1	2.6	21.6	17.0	83
SC115	140	14.4	9.3	8.5	1.1	20.5	39.1	85
CS-1								
Male	86	17.4	10.6	9.1	1.1	22.7	49.8	85
Female	127	14.7	51.8	14.8	0.4	71.0	85.3	85
Female, plus testosterone	78	19.4	36.0	14.9	1.0	54.4	78.5	85

[a], Td, TG1, Ts, TG2, Tc in hours
[b], TLI, GF in percent

The increased S-phase cellularity (TLI) after corticosteroid treatments was temporally correlated with intervals of increased chemosensitivity to 5-FUra and methotrexate (100,102), VCR and 5-FUra (100) and cyclophosphamide (CP) (101,103). We also showed that in first generation C3H/He mammary tumors corticosteroids could inhibit the recruitment of noncycling cells after cytotoxic chemotherapy (102). The results from these and other studies indicated that in receptor positive tumors, the time for resumption of proliferation after dexamethasone was dose dependent, dependent on pretreatment proliferative characteristics and strongly correlated with the pretherapy cytosol receptor content (77).

Gross et al. (104) described the kinetic response of insulin-deprived MCF-7 cells to insulin reconstitution. Flow cytometry and thymidine labelling studies showed dose dependent increases in S-phase cellularity after reconstitution and suggested that the major cell kinetic effect of insulin in this line was to promote G1 transit.

C. Surgery

Accelerated growth of metastatic tumors after primary excision has been recognized for many years and several studies have shown that the attendent kinetic changes are exploitable in an adjuvant setting. Simpson-Herron et al. (105) showed increases in the TLI for Lewis lung tumor metastases soon after the excision of primary tumors and Braunschweiger et al. (106) subsequently showed that such changes could be exploited for a therapeutic gain. In a rapidly proliferating C3H/He mammary tumor line, Gunduz et al. (107) and Fisher et al. (54,108) showed that amputation of a large tumor on the hind limb stimulated the TLI and GF in distant small tumor foci but the response to chemotherapy was not sequence dependent (54,107,108).

In autochthonous C3H/HeJ mammary tumors the response to surgical debulking was characterized by a 40-50% decrease in the TLI for the residual tumor at 24 hours and by expansion of the S-phase population, 24-48 hours later (109). The time course of the response and the maximal TLI correlated with the extent of debulking. In first generation C3H mammary tumors, regrowth delays after debulking and sequential chemotherapy with VCR, Cp or 5-FUra were clearly sequence dependent. The most effective schedules were

those in which chemotherapy was given at intervals to coincide with maximal proliferation in the residual tumor. In R3230AC rat mammary tumors, TLI's in the residual tumors were markedly subnormal for up to 36 hours after tumor debulking and proliferative recovery was noted at 48 hours (110). Similarly, in animals with artificially induced lung metastases the proliferation of clonogenic cells was initially inhibited after primary tumor excision but increased proliferation in this population was noted at 72 hours. The best median survivals were obtained when chemotherapy was given 3 days after primary tumor excision (110). Bilateral adrenalectomy abbrogated the interval of decreased TLI after surgery and TLI increases were seen within 24 hours of debulking (110). This response and the response in a low receptor tumor line (SaD2) were not unlike those seen by Fisher and coworkers (107,108). These and other studies (110,111) showed that the time course of the proliferative response after surgery is mediated by changes in endogenous adrenal hormone levels and that increased chemoresponsiveness may be due to recruitment. Although tumor vascular function can markedly influence the tumor GF and corticosteroids can have profound effects on tumor vascular function (112), vascular function in the residual tumor after surgical cytoreduction has not been studied.

D. Radiotherapy

Cell cycle phase dependent radiosensitivity is well recognized (113). Early studies showed that the most radiosensitive phases of the HeLa cell cycle were G_2 and the G1/S boundary (114), but later studies reviewed by Sinclair (115) indicated that this phenomenon was cell line dependent. In vitro, irradiated cells tended to accumulate in G_2 as prolongation of G1 and S transit was substantially less than that seen for G_2. This "mitotic delay" was shown to be dose and cell line dependent (113). In a mammary tumor cell line, Sakai et al. (116) demonstrated maximal G_2+M accumulation 6-8 hours after 2-10 Gy, but the duration of the block was dose dependent. Progression delays at the G1/S and G2/S boundaries were seen in the EMT-6 clonogenic population after 3.0 Gy (117). Synchronous cell cycle progression was also seen in this population (118). Tc's obtained by the FLM method at 24 hours after 3 Gy were not different than in unirradiated tumors (119).

Proliferation inhibition and the time to recovery after x-irradiation were also dose dependent in vivo (55,57,120). Expansion of the GF during proliferative recovery was accompanied by the loss of cells from the non-proliferating compartment (120). In T1699 mammary tumors, the duration of subnormal TLI's was dose dependent between 2 and 12 Gy suggesting a delay of 3 or 4 Tc's per Do dose. Kinetically based fractionation schemes provided better local control and longer survival than acute doses or schedules permitting proliferation between fractions (57). In first generation C3H/He mammary tumors fractionation studies suggested a proliferative delay equivalent to 1 Tc for each Do dose (121) but thymidine labelling studies in autochthonous C3H/He mammary tumors indicated delays of 24 and 96 hours after 6 and 12 Gy respectively (122). In small artificial lung metastases, 2.1 Gy produced a 25 hour proliferative delay (123). When radiation dose was expressed as a multiple of the tumor specific Do dose, a strong correlation with time to proliferative recovery was seen (122).

E. Chemotherapy

Although the results from a large study with DMBA-induced rat mammary tumors failed to show any simple relationships between pretreatment labelling indices (TLI, PDP Index) and regrowth delay after chemotherapy (43), studies in several models have shown that cytotoxic cytoreduction can produce profound cell kinetic perturbations in vivo (31,45,59,70,71,102,117, 119,124-130).

Hydroxyurea (HU) kills cells in S-phase, effectively imposes a block at the G1/S boundary, and was shown to induce partial synchrony in mammary tumors in vivo (117,119,128,129). Cell kill and synchronous progression of clonogenic cells was observed in EMT-6 tumors after HU treatment (119). Computer simulations indicated a G1/S block and the recruitment of non-cycling cells (117). HU was also shown to produce perturbations in T1699 mammary tumors (125).

The kinetic response to drugs such as VCR (31,124), CP (59,70,102, 127), ADR (59,71,126,130) and BCNU (45) is initially characterized by variable intervals of reduced TLI's and GF's. Prior to tumor regrowth, the surviving cell population may undergo an interval of hyperproliferation (proliferative recovery), characterized by increasing TLI's and GF's and perhaps shortened TC's. In 13762 rat tumors (70,71), C3H/He autochthonous (31,70) and first generation mammary tumors (102), and T1699 mammary tumors (59,124,126) the proliferative recovery interval was coincident with intervals of increased chemoresponsiveness.

The effect of ADR, with and without x-irradiation, on cell proliferation was studied in T1699 mammary tumors (126). ADR (1.0mg/kg) and x-ray (2.0GY) alone induced G_2 blocks but the G_2 block after x-rays was partially reversible. In combination, 1 mg/kg ADR + 2.0 Gy not only induced a G_2 block but inhibited S to G_2 transition with progression about 18 hours after treatment. When a second ADR + x-ray fraction was given at 15 hours, entry into S-phase continued but a longer S to G_2 transition delay was noted. The proliferative delay was increased from 18 hours after the first fraction to about 33 hours after the second fraction. Four combined fractions given at intervals, proscribed by the kinetic responses, to minimize proliferative recovery between fractions, produced regrowth delays that were twice the observed additive response (126).

In mammary and other tumor models the timing of proliferative recovery after CP, ADR and cis-diaminedichloroplatinum II (C-DDP) was dose dependent but highly variable. In these heterogeneous tumor models, the dose dependent timing for proliferative recovery, expressed as a multiple of the mean TC was strongly correlated with the pretreatment tumor GF (27,131,132). Such response models were predictive for effective sequence intervals in animal models.

Recent studies indicate that cytotoxic cytoreduction (133,134) and hormonal manipulations (112,135,136,137) which produce cell kinetic changes in vivo are often accompanied by profound changes in tumor vascular function and tissue water distribution. In one such study we showed that proliferative recovery after cyclophosphamide was coincident with marked increases in tumor blood flow and changes in tumor water distribution (133). Although kinetic responses usually cannot be followed in patients, vascular responses might conceivable be monitored by relatively non-invasive, repeatable nuclear medicine techniques.

Proton Nuclear Magnetic Resonance imaging and spectroscopy is an exciting new non-invasive tool for the study of physiologic changes associated with cancer therapy. Proton Spin-Lattice (T1) and Spin-Spin (T2) relaxation times have been shown to change during the cell cycle (138) and to correlate with Td in human breast cancer cell lines (139). Our studies showed that observed T1 and T2 for tumor tissue is also influenced by tissue water distribution (134,135,136). Furthermore, drugs and hormones (134,136,137) which produce kinetic and hydrodynamic perturbations are often accompanied by coincident changes in T1 and T2. Although the relationships between changing NMR relaxation times, tumor vascular function and proliferative

recovery are yet to be fully elucidated, there may be great potential for this noninvasive technique in therapy planning and evaluation.

In vivo (31) P-NMR spectroscopy may offer yet another way to noninvasively monitor responses to cytotoxic therapy in vivo. This new technology permits serial in vivo measurements of high energy phosphate metabolites such as inorganic phosphate (Pi), ATP, phosphocreatine (PCr), phosphomonoesters and phosphodiesters in tumor and normal tissues (140). Furthermore, it is possible to deduce intracellular pH from the relative chemical shifts of Pi and PCr. In as much as intracellular pH increases as cells progress through the cell cycle (141) and profound therapy induced (31) P-NMR spectral changes can be seen non-invasively (140), in vivo (31) P-NMR spectroscopy might provide a way to monitor proliferative recovery or associated changes during therapy. In RIF-1 tumors, pH, Pi: NTP and PCR:NTP ratios were strongly correlated with vascular patency (142). Previously, we showed that in RIF-1 tumors, an increase in intracellular pH, assessed by in vivo (31) P-NMR spectroscopy, after a single CP treatment occurred coincidently with proliferative recovery (143) and increased tumor blood flow (133).

IV. SUMMARY

In summary, the cell cycle kinetics of many breast cancer models have been described. Most studies have employed the FLM method to measure cell cycle phase transit times but in several systems this approach results in shorter Tc estimates than those obtained by stathmokinetic in vitro labelling methods. These later methods are also more appropriate in systems displaying widely heterogeneous cell cycle times and in studies of migrating or perturbed populations.

The utility of flow cytometry (FCM) for cell cycle analysis was recently reviewed by Gray et al. (144). Analysis of cell cycle phase distributions and in some systems quantitation of non-cycling cells is possible. Cell cycle phase distributions obtained by flow cytometry however do not always compare well with distributions obtained by labelling methods and polyploid populations present considerable problems in analysis. Furthermore, unless [^{3}H]-thymidine or bromodeoxyuridine (BrdU) labelling studies are also conducted, DNA distributions provide no information on cell cycle transit times. The development of monoclonal antibodies against BrdU has provided the means to rapidly assess functional S-phase fractions and cell cycle transit by FCM (145). Monoclonal antibodies to gamma-DNA polymerase (146,147) and specific proliferation or quiescence gene products (148,149) may greatly enhance the utility of FCM for characterizing cell populations and monitoring response to therapy.

The ethical and logistic considerations of obtaining appropriate and timely measurements together with the wide heterogeneity of human cancers with respect to cell kinetics and clinical therapy response has greatly diminished the enthusiasm for kinetically based strategies. However, except for hormone stimulation strategies, rigid clinical evaluation of the conceptual aspects of strategies found to be efficacious in animal models is lacking. In this regard the application of flow cytometry, nuclear medicine and/or in vivo multinuclear magnetic resonance techniques to monitor proliferation related biochemical and physiologic changes during therapy may have great potential for the implementation and timely assessment of therapeutic strategies.

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CURRENT CONCEPTS OF SELENIUM AND MAMMARY TUMORIGENESIS

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I. INTRODUCTION

The essentiality of selenium as a trace element was recognized in 1957 (1). On the biochemical level, selenium has long been known to have an antagonistic effect on lipid peroxidation. However, it was not until 1973 that the molecular basis of the antioxidant function of selenium was established. The milestone publication from Hoekstra's laboratory reported that glutathione peroxidase, an enzyme that metabolizes peroxides, contains selenium as a prosthetic group (2). The activity of glutathione peroxidase in tissues is directly related to the availability of dietary selenium; thus low enzyme activity is invariably associated with selenium deficiency (3). Although other selenium-containing proteins have since been identified in eukaryotic cells (4,5), glutathione peroxidase is the only selenoprotein to date that is functionally characterized.

Selenium is present in different chemical forms and at different levels in soil. The concentration of selenium in forage and crops is therefore dependent on the biological availability of selenium. A deficiency of selenium may result if vegetation with a low selenium content is consumed by humans or animals. Numerous cases have occurred in poultry and livestock

on farms in those areas of the world where the selenium content of soil is low. An example is found in the eastern half of the United States, where selenium deficiency diseases like cardiac myopathy in turkeys and muscular dystrophy in calves can now be controlled by supplementation of feeds with selenium.

There is currently no evidence of apparent selenium deficiency in the population of this country. However, certain epidemiological data have suggested an inverse relationship between blood selenium levels and cancer risk (6-9). Since blood selenium concentration depends on intake, it is not surprising to find that a similar inverse relationship exists between cancer mortalities and dietary selenium consumption in different countries (10). Despite the fact that epidemiological studies are confounded by many uncontrollable variables, the prophylactic effect of selenium is substantiated by an array of laboratory investigations which have been both consistent and promising. In the last decade, there is increasing evidence in the literature that selenium supplementation can protect experimental animals against preneoplastic lesion development or tumorigenesis induced by chemical carcinogens or viruses. For a comprehensive listing of these reports, the readers are referred to two recently published review articles, written by Ip (11) and Medina (12), respectively, which summarize the effect of selenium in several organ system models. This chapter, however, will only focus on the chemopreventive role of selenium on mammary gland neoplasia.

II. INHIBITION OF MAMMARY TUMORIGENESIS IN ANIMAL MODELS

There are no less than twenty studies showing that selenium is effective in suppressing the appearance of rodent mammary tumors induced by either 7,12-dimethylbenz(a)anthracene (DMBA), methylnitrosourea (MNU), or 2-acetylaminofluorene (AAF), and in several mouse strains that are positive for the murine mammary tumor virus (MMTV). Due to the fact that these reports are already referenced individually in the two reviews quoted above, they are briefly summarized here in Table 1. However, particular papers will be cited throughout the text where appropriate. Since adenocarcinoma is the prevalent type of mammary cancer developed in these models, it is assumed that selenium inhibits the expression of glandular malignancy. With the exception of only one case in which selenium supplementation increased mammary fibroadenoma formation induced by adenovirus in the rat (13), results from different laboratories have been in agreement with each other. In general, the inhibitory response is manifested by a lower tumor incidence, fewer number of tumors per animal, and/or a longer latency period. It is seldom to see all three parameters move in the desired direction in any given study, in part due to the constraint and sensitivity of the model. Multiple mammary tumors are rare in mice; consequently, a decrease in incidence combined with an increase in the time of tumor appearance are commonly observed in those receiving selenium supplementation. In the rat, mammary tumors develop within a relative short time following carcinogen administration. It is therefore more difficult to detect any significant difference in latency period between selenium-treated and control rats. Total tumor yield per group or tumor multiplicity is a more sensitive indicator in this respect. This is especially true if a high dose of carcinogen is used which may preclude any meaningful difference in tumor incidence. Thus consideration should be given to the limitation of the experimental model in data interpretation concerning the efficacy of selenium.

Selenium can be supplemented either in the diet or in the drinking water. Different concentrations have been examined, ranging from 0.5 to 6 ppm. As a matter of reference, the recommended requirement established by the National Research Council for animals is around 0.1 ppm. Several dose-response studies have suggested that the degree of inhibition in tumorigene

Table 1 Effect of selenium supplementation on murine mammary tumorigenesis

Species	Strain	Carcinogenic agent[a]	Route of Se administration	Response
rat	Brown	AAF	diet	decrease
rat	SD	DMBA	diet/water	decrease
rat	SD	MNU	diet	decrease
rat	WF	Ade-9	water	increase
mouse	$BD2F_1$	DMBA	diet/water	decrease
mouse	BALB/c	DMBA	diet/water	decrease
mouse	C3H/StWi	DMBA	water	decrease
mouse	C3H/St	MMTV-S	diet/water	decrease
mouse	BALB/cfC3H	MMTV-S	water	decrease
mouse	GR	MMTV-P	water	no effect

[a]AAf = 2-acetylaminofluorene; DMBA = 7,12,dimethylbenz(a)anthracene; MNU = N-methyl-N-nitrosourea; Ade-9 = adenovirus type 9; MMTV = mouse mammary tumor virus

sis is proportional to the level of selenium supplementation (14-17). A significant inhibition was observed in mice given only 0.5 ppm of selenium in both the DMBA- and MMTV-induced mammary tumor models (17,18). No systemic adverse effect was detected even when the level of selenium was increased to 6 ppm (20). In contrast, it was found that dietary selenium had to be raised to 1.5 to 2 ppm before its chemopreventive effect became noticeable in the rat (14, 16); and toxicity in the form of a slight reduction in body weight was evident at 5 ppm (14). Thus the window of a selenium-mediated response in mammary tumorigenesis is considerably wider in the mouse model than in the rat model.

The anticarcinogenic efficacy of selenium was diminished by a large dose of carcinogen, as demonstrated in the DMBA-induced mammary tumor model in the rat (14) and in the mouse (19). Moreover, studies with the rat model indicated that selenium was unable to counteract completely the enhancing effect of fat, since rats on a high fat diet developed more tumors than those on a normal fat diet even at comparable levels of selenium supplementation (14). The implication of these findings is that a greater amount of selenium is required to achieve a significant inhibition under conditions in which the carcinogenic process is enhanced by external agents.

At the other end of the dose-response spectrum, the effect of selenium deficiency on DMBA-induced mammary tumorigenesis has also been examined by Ip (20). Only in rats that were maintained on a high polyunsaturated fat diet (25% corn oil) did selenium depletion result in a further increase in mammary tumor development. Such an enhancement was not observed in rats given either a low fat diet (1% corn oil) or a diet rich in saturated fat (coconut oil). Further studies suggested that there was an association between susceptibility of the mammary gland to carcinogenesis and its lability to peroxidation; and that both parameters were intimately regulated by fat intake in conjunction with the selenium status of the animal (21).

Based on all these experimental data, it seems that breast cancer risk is higher with a low selenium intake and decreases proportionately with dietary selenium at levels above the recommended requirement. On the biochemical level, the mechanism of action of selenium in affecting tumor development may be quite different at the physiological versus pharmacological range. Quantitatively, the correlation between the amount of

supplementation and the degree of inhibition obviously depends on the species of the animals used, the composition of diet (to be discussed in more detail in later section) as well as the dose of the carcinogen.

III. STAGE-SPECIFICITY OF SELENIUM-MEDIATED INHIBITION OF MAMMARY TUMORIGENESIS

It has been extensively documented that mammary tumorigenesis proceeds through several discrete stages from normal to preneoplastic to neoplastic cells. Consequently, investigation has been focused on determining the responsiveness of these different cell populations to selenium. Under conditions where selenium inhibits MMTV- and DMBA-induced mammary tumorigenesis, Medina and Shepherd (19) have shown that it also markedly reduces the occurrence of primary hyperplastic alveolar nodules and ductal hyperplasia, the prominent preneoplastic populations in the mouse mammary gland. However, further results from Medina's laboratory (22) suggested that later stages of mammary tumorigenesis (preneoplastic to neoplastic transformation and tumor growth) are not as sensitive to selenium-mediated inhibition as the early stages (the induction and/or expression of mammary preneoplastic lesions). Thus selenium inhibits the occurrence of mammary preneoplasias by 75%, inhibits the tumor-producing capabilities of established mammary preneoplastic outgrowth lines in almost 40% of the lines, and inhibits the net growth of first and second generation transplantable mammary tumors in less that 10% of the samples. In conclusion, there seems to be an increase in resistance to selenium-mediated inhibition as cells progress from normal to preneoplastic to neoplastic stages.

The majority of studies in the literature have used selenium supplementation throughout the course of the experiment. In relation to how the time and duration of selenium supplementation affect chemical carcinogenesis, recent evidence has suggested that selenium can inhibit both the initiation and post-initiation phases independently. Thompson et al. (16) have demonstrated that dietary selenium supplementation around the time of DMBA administration (from 3 weeks before to 2 weeks after) was sufficient to inhibit mammary tumorigenesis in the rat. Short-term selenium supplementation, starting one month before to one month after DMBA administration, has also been reported by Welsch et al. to result in a protective effect (15). Data from Thompson and coworkers (23,24) showed that selenium significantly reduced mammary tumorigenesis when it was supplemented starting one week after administration of MNU or DMBA and continued until termination of the experiment, suggesting that selenium can also act at the post-initiation phase.

Since these experiments were performed by different investigators at different times and using different carcinogens, it is difficult to ascertain the time frame in which selenium is most effective in cancer chemoprevention. In order to answer this question, Ip conducted a series of experiments in which the efficacy of selenium supplementation during the initiation and post-initiation phases of DMBA-induced mammary carcinogenesis was examined (25). In this study, control rats received 0.1 ppm of selenium, and the experimental groups were supplemented with 5 ppm of selenium for various periods of time: -2 to +24, -2 to +2, +2 to +24, +2 to +12 and +12 to +24 weeks. The time of DMBA administration (at 50 days of age, 10 mg/rat) was taken as time 0; minus and plus signs represent the time in weeks before and after carcinogen treatment, respectively.

Analyses of the data show that selenium can inhibit the initiation and post-initiation phases of carcinogenesis. This is suggested by the observation that a decrease in tumorigenesis was evident when selenium was supplemented either around the time of DMBA administration (-2 to +2 weeks) or during the proliferation of tumor development (+2 to +24 weeks).

Furthermore, it seems that selenium given after carcinogen treatment is the more effective protocol. A continuous intake of selenium is necessary to achieve maximal inhibition of tumorigenesis, such as in rats that were supplemented with selenium for the longest period of time (-2 to +24 weeks). In a previous study, Schrauzer et al. (26) also noted that switching mice from a high- to a low-selenium diet nullified the suppression of spontaneous mammary tumorigenesis observed during high selenium supplementation, suggesting that selenium retards tumor development only as long as it is supplied in "adequate" amounts. The reversibility of the selenium effect was also confirmed in Ip's experiment, in which the chemopreventive response was found to be severely compromised when selenium supplementation was limited from +2 to +12 weeks. In rats that were supplemented with selenium from +12 to +24 weeks, there was only a slight but insignificant reduction in the number of tumors found, suggesting that the efficacy of selenium is much attenuated when it is given long after carcinogen injury.

A similar experiment was reported by Medina and Lane (22) in mice that were given DMBA once a week for 6 consecutive weeks starting when the mice were 8 weeks of age. Compared to controls that did not receive selenium supplementation and had a tumor incidence of 42%, mice that were exposed to selenium for the entire duration of the experiment (starting at 8 weeks of age and thereafter) or in the post-initiation phase (starting at 14 weeks of age and thereafter) showed an incidence of 13% and 8%, respectively. The tumor incidence in mice given selenium between 6 and 14 weeks was 25%. Results of this study again confirm the notion that selenium is an effective chemopreventive agent in both the initiation and post-initiation phases of chemical carcinogenesis.

IV. EFFICACY OF DIFFERENT SELENIUM COMPOUNDS

The bulk of the database on selenium chemoprevention has been compiled with the use of inorganic selenium. Selenite is most commonly tested in experimental carcinogenesis studies, probably because this is the form of selenium added to all mineral mixes sold commercially. Next to selenite, selenium dioxide has also been reported by several investigators to be effective. Both selenite and selenium dioxide have the same valence of 4+. Selenate, which has a higher oxidation state with a valence of 6+, has been shown by Milner and coworkers to suppress the growth of Ehrlich ascites tumor cells and L1210 leukemic cells (27,28). Preliminary results from Ip's laboratory comparing the efficacies of selenate, selenite and selenium dioxide have indicated that all three compounds were equally active in inhibiting DMBA-induced mammary tumorigenesis (unpublished data). These experiments suggest that some common metabolite(s) may be the active selenium species responsible for controlling neoplastic proliferation. Support for this hypothesis is in part provided by the recent finding of Poirier and Milner (29) that selenodiglutathione, an intermediate in the detoxification of selenate and selenite, has a greater antitumorigenic potency than products formed further down in the metabolic pathway.

Unlike the inorganic form of selenium in soil, selenium in plants and animal tissues is present largely in an organic form, some of which is associated with proteins and amino acids (30). There are only a few reports in the literature comparing the anticarcinogenic efficacy of inorganic versus organic forms of selenium. Using the DMBA-induced mammary tumor model in rats, Thompson et al. (24) showed that selenite was more potent than selenomethionine in inhibiting tumorigenesis. Selenomethionine at high levels of supplementation (6 ppm) caused liver damage, which was not noted in the selenite-treated rats. With the Ehrlich ascites tumor cell and the L1210 leukemic cell models, Milner and coworkers also found that selenomethionine and selenocystine were less effective than selenite in suppressing malignant growth when the selenium compounds were injected intraperitoneally to mice (27,28). Ip, on the other hand, found that selenomethionine was

comparable to selenite in prophylactic efficacy at different doses in rats treated with DMBA (31). At 3 ppm, selenomethionine did not cause hepatic toxicity.

Both selenite (or selenate) and selenomethionine are metabolized to dimethylselenide and trimethylselenonium ion as excretory products via hydrogen selenide (4). In addition, selenomethionine can be incorporated directly into proteins in place of methionine. On the other hand, selenide is postulated to be the precursor molecule for the insertion of selenium into specific selenoproteins. Thus, the sequential reduction and methylation reactions are important steps in the metabolism of selenium, but the understanding of the biochemical mechanisms that convert ingested selenium into biologically active form(s) is still incomplete. Future research should be directed towards elucidating and identifying the functionally operative selenium species that is instrumental in regulating cell transformation and neoplastic growth.

V. INTERACTIONS OF SELENIUM WITH VITAMINS A, C AND E

Two of the most promising agents for chemoprevention of cancer include vitamin A and selenium. Thompson et al. (32) were the first to demonstrate an additive inhibitory effect on MNU-induced mammary carcinogenesis resulting from combined treatment with retinyl acetate and selenium. Ip has also examined the combined use of these two compounds (33). By feeding rats a diet containing 4 ppm of selenium and 250 ppm of retinyl acetate, the yield of DMBA-induced mammary tumors was reduced to 8% of control, as compared to 51% and 36%, respectively, for selenium and retinyl acetate alone. This combined regimen, although very effective in inhibiting tumorigenesis, was not well tolerated by the animals, thus resulting in decreases in food consumption and weight gain (approximately 15% reduction compared to controls). Hence, the significance of this particular treatment cannot be properly evaluated until additional studies on the toxicological and pharmacological effects are clearly differentiated.

Prompted by the observation that vitamin C could lead to a reduction of selenite to a presumably unavailable form (34), Ip has investigated the effect of supplementing vitamin C with different forms of selenium on DMBA-induced mammary carcinogenesis (31). The interaction of 0.5% vitamin C with either selenite or selenomethionine (3 ppm) was studied. Results showed that the protective effect of selenite was nullified by vitamin C; whereas the chemopreventive action of selenomethionine was not affected. It is possible that selenite is reduced by vitamin C to elemental selenium, and is therefore not available for uptake by tissues. In contrast, selenomethionine is more stable in the presence of reducing agents, such as vitamin C, and can still express its biological action under the experimental condition.

This hypothesis was indirectly supported by tissue selenium measurements which showed that 0.5% or 0.25% of vitamin C in the diet completely negated the accumulation of selenium in blood, liver and mammary gland induced by 3 ppm of selenite supplementation. Lower levels of vitamin C (0.1% and below) were found to have no effect on tissue selenium concentrations. Furthermore, in parallel with the biochemical data, the presence of 0.1% vitamin C in the diet did not abolish the anticarcinogenic effect of selenite. Contrary to the selenite results, vitamin C in the diet (even at 0.5%) had no effect on the accumulation of selenium in the tissues of rats given selenomethionine. This study suggests that high levels of vitamin C can interfere with the concentration of tissue selenium when the latter is supplied as selenite and that an increased titer of this trace element in cells is essential for retarding tumor development.

The interaction of selenium and vitamin E was examined in a series of experiments reported by Ip using the DMBA-induced mammary tumor model in rats. Although vitamin E supplementation alone was found to have no prophylactic effect (35), it potentiated the anticarcinogenic action of selenium (36). It was postulated that the systemic suppression of lipid peroxidation resulting from vitamin E supplementation might be partly responsible for the enhanced efficacy of selenium in chemoprevention (36). This hypothesis is congruent with the findings in a subsequent report showing that the protective effect of selenium was much attenuated in animals with a low vitamin E intake (37). As expected, vitamin E deficiency also significantly increased lipid peroxidation in the mammary fat pad. The underlying mechanism behind the interaction of these two nutrients is not clear, but it appears that the effectiveness of selenium in chemoprevention can be modulated depending on the extent of oxidant stress in the cellular environment.

The purpose of this section is to highlight the message that the effect of single nutrient can be masked or enhanced, depending on the bioavailability of other dietary components. Thus the formulation of a purified ration with precise ingredient information is essential for animal carcinogenesis studies if reproducible results are to be attained. Emphasis on future research should be focused on defining a set of guidelines which optimizes the prophylactic action of selenium.

VI. MECHANISMS OF ACTION

A. Antioxidant

The only function characterized for selenium in mammalian cells is its antioxidant function (2,3,38). As indicated in the Introduction section, glutathione peroxidase (GSH-Px), which metabolizes both inorganic and organic peroxides, is a seleno-enzyme. The concerted action of vitamin E and selenium in protecting against endogenous peroxidation has been summarized by Hoekstra (3). One of the major functions of vitamin E is to restrict the formation of lipid hydroperoxides, presumably by neutralization of free radicals -- the so called "scavenger mechanism". Whereas vitamin E is located primarily in cell membranes, GSH-Px is a soluble enzyme which is present in the cytosol and mitochondrial matrix. The interrelationship between selenium and GSH-Px in mammary tumorigenesis has been examined by several investigators.

Lane et al. (39,40) examined the levels of selenium and GSH-Px in the normal, lactating and neoplastic states of the mammary gland. The enzyme in the mammary gland was inducible by dietary selenium concentrations from 0.02 to 0.10 ppm but was not further increased when dietary selenium was above 0.10 ppm. The biochemical characteristics of the mammary GSH-Px enzyme were similar to that reported for other tissues. In mice fed a chow diet without supplemental selenium (0.15 ppm Se), the levels of mammary GSH-Px and selenium increased in pregnant and lactating mice compared to virgin mice. This increase paralleled the increase in the epithelial component of the mammary gland. Interestingly, preneoplastic and neoplastic mammary tissues contained GSH-Px levels similar to that in mammary gland of pregnant mice, whereas selenium levels in the tumor tissues were 50% higher. As discussed in an earlier section, these tumor tissues were unresponsive to selenium-mediated inhibition of growth, even though the selenium concentration reached 0.5 ppm in these tissues. Whereas the selenium concentration in the mammary gland was responsive to the levels of dietary selenium at each stage of mammary gland development, the levels of GSH-Px were responsive only in those mammary tissues exhibiting a rapid growth rate (i.e., mammary GSH-Px in 10-week-old but not 26-week-old virgin mice was responsive to dietary selenium levels). Horvath and Ip reached a similar conclusion regarding GSH-Px inducibility in their studies on rat mammary tumorigenesis (36). The general

conclusion arising from experiments in both model systems is that the selenium-induced increase in GSH-Px activity in mammary glands of virgin rodents appears to be saturated at relatively low levels of dietary selenium (0.1 ppm Se for mice; 0.2 ppm Se for rats), even though the level of intracellular selenium increases with the level of dietary selenium. However, the actual GSH-Px activity is dependent also upon the age of the host, the differentiation state of the mammary gland, and the pathological stage of tissue.

In DMBA-treated mammary glands of mice, the levels of GSH-Px decreased significantly (50%) and selenium supplementation failed to increase the basal GSH-Px activity levels (41). The effects of dietary Se, DMBA treatment, and age of host were examined in 3-, 6- and 9-month-old mice by Lane and Medina (42). The gland GSH-Px activity followed a complex pattern that was statistically independent of dietary Se levels but varied with age and DMBA treatment. In controls as well as in DMBA-treated animals, GSH-Px activity decreased at 6 months of age then increased sharply at 9 months of age. Overall, the GSH-Px specific activity was higher in the glands of the control mice than that of DMBA-treated mice. The increased dietary levels of selenium counteracted the DMBA-induced decrease in GSH-Px activity only in the mammary glands of 9-month-old mice where the GSH-Px activity was very high in all groups. A similar result has been shown for the chemical carcinogens dimethylhydrazine (DMH) (43) and bis(2-oxopropyl)nitrosamine (44). Both carcinogens reduced GSH-Px activity in the liver which was not reversed by chemopreventive levels of selenium. In summary, the above results support the conclusion that the inhibitory effects of high levels of selenium are not related to its biochemical regulation of the selenium-dependent glutathione peroxidase.

A second approach to examine a related mechanism was to measure the effect of selenium on lipid peroxidation in the mammary gland. Ip and Sinha (45) found no correlation between the anticarcinogenic effect of selenium supplementation and its ability to suppress lipid peroxidation in mammary tissues, regardless of whether the rats were fed a diet high in saturated or unsaturated fat. Although the assay method (thiobarbituric acid) used in the study was an indirect measurement of lipid peroxidation, it was probably sufficiently sensitive to pick up major alterations. Additionally, Horvath and Ip (36) demonstrated that vitamin E suppressed lipid peroxidation in the rat mammary gland under conditions where it did not inhibit mammary tumorigenesis. Lipid peroxidation in mouse mammary gland membrane preparations was also measured by thiobarbituric acid reactants and decreased only 16% (an insignificant reduction) by chemopreventive levels of selenium (42). Thus, the data available for the mammary gland suggest that the chemopreventive effect of selenium is probably not mediated by inhibiting elevated levels of lipid peroxidation. However, before this mechanism is completely ruled out, the experiments of Wong et al. (46) should be considered. They provided evidence that N-hydroxyl-2-acetylaminofluorene was activated by a lipoxygenase-peroxidase route and that this enzyme was inhibited by selenium. Unfortunately, the details of the experiment were not provided. Moreover, the recent results of Borek et al. (47) demonstrated that in C3H/10T-1/2 cells transformed by X-rays, benz(a)pyrene or tryptophan pyrolysate, cellular pretreatment with chemopreventive levels of selenium, but not vitamin E, resulted in increased levels of cellular GSH-Px, catalase, and glutathione and in an enhanced destruction of peroxide. Therefore, although the experiments in the mammary gland so far indicate that the anticarcinogenic effects of selenium are not correlated with GSH-Px levels or its presumed antioxidant functions, it is important to examine other peroxidative pathways and also use more direct methods to measure lipid peroxidation before this potential pathway for selenium action can be eliminated from consideration.

B. Alterations in Carcinogen-DNA Interactions

In considering the possible mechanisms by which selenium can inhibit mammary tumorigenesis, several results emerge as significant. It has been well established that selenium inhibits viral and chemical carcinogen-induced tumors, that selenium inhibits post-initiation events, and that the effects are reversible. Moreover, selenium is effective in inhibiting tumors induced by a wide spectrum of carinogens including aromatic amine, aflatoxin, nitrosamine, polycyclic hydrocarbon, and methylnitrosourea (a direct alkylating agent). These observations suggest that the primary action of selenium is probably not mediated through interference of carcinogen metabolism. However, data in the literature document that selenium can inhibit chemical carcinogen-induced mutagenesis (48-50), can alter the metabolism of dimethylhydrazine, benz(a)pyrene and acetylaminofluorene (50-53) and that it has variable effects on carcinogen-DNA adduct formation (50,53,-54). One experiment has examined directly the effects of selenium on carcinogen metabolism in the mammary gland (55). Ip and Daniel reported that neither dietary selenium deficiency (<0.02 ppm) nor excess (2.5 ppm) had any significant effects on DMBA binding levels to DNA and on the qualitative or quantitative formation of DMBA:DNA adducts (55). This experiment supports the hypothesis that selenium modulation of carcinogen metabolism is not a critical mechanism in the mammary tumor model.

C. Inhibition of Cell Proliferation

Numerous experiments have implicated selenium as an important nutrient for the growth of cells in vitro (56-65). Conversely, Milner and co-workers demonstrated that selenium supplementation inhibited the growth of several neoplastic cell lines including canine and human mammary cells (27,28,66,-67). On the basis of his results, Milner suggested that selenium exerted a marked and specific effect on cell proliferation (28). Harbach and Swenberg (53) suggested that selenium inhibition of dimethylhydrazine-induced colonic tumorigenesis was attributable to a decrease in DNA synthesis since ^{3}H-thymidine incorporation was reduced by 65%. Gruenwedel and Gruickshank (68) demonstrated that 5 μM selenium inhibited the incorporation of labeled precursors into DNA and RNA of HeLa cells; protein synthesis was slightly less sensitive by a factor of two.

The effects of selenium on DNA synthesis in mouse mammary cell lines grown in vitro were examined by Medina et al. (65,69,70). Low doses of selenium stimulated cell growth, whereas high doses (5 x10^{-6} M) inhibited cell growth reversibly. A biphasic effect of selenium on DMBA-induced noduligenesis in mammary gland organ culture was reported also by Chatterjee and Banerjee (71). The decreased cell growth was reflected by a decreased cell number, decreased uptake of ^{3}H-thymidine into DNA, decreased DNA labeling index, and a decreased rate of DNA synthesis. The increased cell growth was reflected by an increase in all these parameters of cell growth kinetics. Flow cytometry analysis of selenium-treated mouse mammary cells demonstrated that the cells appeared to be delayed transiently in G_2 and delayed for a prolonged time in the S-phase of the cell cycle (41). In contrast, mammary cells exposed to 5 x 10^{-8} M Se continued to traverse the cell cycle.

The growth inhibitory effects of selenium were reversible. This reversibility was seen for mammary cells in organ and cell culture (69-71) as well as for other cell types grown in cell culture (68). The reversibility was dose-dependent since the growth of mammary cells exposed to 5 x 10^{-6} M Se, but not 5 x 10^{-5} M Se was reversible. The lag time preceding recovery was associated with the time necessary for selenium to be cleared from the cell (70,72). The observations in vitro coincided with observa-

tions in vivo which indicated that inhibition of mammary tumorigenesis was dependent upon the continued presence of selenium (25,26,33). The sum total of the above experiments indicated that one of the mechanisms of selenium modulation of carcinogenesis could be through inhibition of DNA synthesis. The molecular basis for this effect remains to be determined.

A comparison of the responses to selenium of primary D2 mammary tumors grown in vitro as primary cell cultures or in vivo as tumor implants provided paradoxical results. Primary D2 mammary tumors grown in primary cell cultures and exposed to 5×10^{-6} M Se appeared responsive to selective inhibition in vitro. However, primary D2 tumors, the same cell populations that were grown in vitro, were unresponsive to selenium-mediated inhibition when assayed by subcutaneous transplantation in syngeneic mice and treated with selenium (22) under the same conditions that Milner and co-workers (27,28,-62,67) had successfully shown inhibition of tumor growth. These results suggest caution in extending results generated from highly selected cell populations like L1210 leukemia or YN-4 mammary cells to conditions found in in situ neoplasms. Primary cultures of D2 tumors behave as partly synchronized cell populations, thus they proceed through 2 waves of cell division characterized by a high proliferative compartment. However, primary mammary tumors even when serially transplanted for 2-3 generations, are a heterogeneous population with a relatively small proliferative compartment. Thus, L1210 cells in vitro are more analogous to D2 and YN-4 mammary cells in vitro than to D2 tumor cells in vivo. It is important to recognize that L1210 leukemia cells and CMT-14B canine mammary cells in vivo and YN-4 mammary cells in vitro are useful model systems to examine the mechanisms of action of selenium, but the results cannot be used to support the hypothesis that selenium effectively inhibits the growth of primary tumors in situ.

The effects of selenium and its metabolic products on RNA and protein synthesis have been examined in several cell systems (68,69,73,75), however, the results have been inconsistent. Only two experiments have utilized mammary epithelial cells. In two mouse mammary epithelial cell lines, DNA synthesis was preferentially inhibited (69) whereas in canine mammary cell lines, RNA synthesis was preferentially inhibited by selenium (76). Further studies in these systems might clarify the biochemical and molecular basis for the ability of selenium to inhibit cell growth.

D. Other Modes

An intriguing mechanism for the effect of selenium was proposed by Spallholz et al. (77,78) who suggested that selenium acts as an immunoadjuvant. In support of this hypothesis were the observations that selenium enhanced the primary immune response in mice by increasing the number of plaque forming cells (PFC) and antibody titers as determined by hemagglutination, and by promoting immunoglobulin M synthesis. These effects were seen at relatively low levels of dietary selenium (0.75-2.0 ppm). A similar finding was reported by Shakelford and Martin (79) when selenium was given in the drinking water. So far, there does not appear to be any experiment where the immunoregulatory effects of selenium on mammary tumors or in a mammary carcinogenesis study have been examined.

Since mammary tumorigenesis is markedly influenced by the endocrine status of the host, it is worthwhile to know if selenium alters the circulating levels of hormones. Ip (14) reported that there was no difference in the estrus cycle of the rat, regardless of selenium intake (0.1-2.5 ppm). Furthermore, serum prolactin, estrone and estradiol levels determined at proestrus were the same for rats fed 0.1 or 2.5 ppm dietary Se. Ip (25) also showed that selenium delayed the regrowth of tumors that had regressed after ovariectomy. The above two approaches which examined the effects of selenium on host systemic events indicate that there is little evidence

available to suggest an indirect effect for selenium-mediated inhibition of tumorigenesis.

Although GSH-Px is the only characterized seleno-protein, there are other presumptive seleno-proteins in mammalian cells (4,80). The functions of these proteins are unknown, although they have been associated with selenium deficiency diseases, such as white muscle disease (81) and sperm immotility (82,83). They have been identified as transport proteins (84,85) or simply as selenium-labeled proteins in testis (86), kidney (87,88) and mammary gland (89). The selenoproteins in the mouse mammary gland, were analyzed by 2-dimensional polyacrylamide gel electrophoresis (89). Eleven selenoproteins ranging in molecular weight from 12,000 to 78,000 daltons were detected with major selenoproteins at 58,000, 26,000 (GSH-Px), 22,000, 18,000 and 14,000 daltons. Analysis of the total cellular protein extract and of each of the 5 major proteins indicated that selenium was present as selenocysteine in the proteins. Partial peptide mapping with N-chloro-succinamide indicated that the 58,000, 26,000 and 22,000 dalton proteins were dissimilar in the amino acid sequences containing the selenoamino acid. The pattern of selenoproteins of mammary gland cells in vivo was similar to that obtained for mammary cells in culture and a variety of organs in vivo (89). The existence of selenoproteins other than GSH-Px was also demonstrated by Hawkes et al. (90). In various rat tissues, seven selenoproteins ranging in molecular weight from 8,000 to 89,000 daltons were separated by column chromatography. Selenium in GSH-Px accounted for only one-third of particulate-associated selenium (5). The results warrant further study to determine if and under what conditions these proteins play a role in selenium-mediated inhibition of cell growth.

VII. CONCLUSION

To date, most of the experimental studies on selenium chemoprevention are descriptive in nature. Cumulatively, the evidence is quite convincing that selenium is effective in inhibiting not only viral- and chemical-induced mammary tumors, but also tumors of other organ sites. The majority of the reports have used supplementation levels which are far in excess of the recommended requirement. Thus it is critical that the pharmacological and the toxicological consequences of selenium be clearly differentiated. Further research should aim at delineating the conditions which optimize selenium chemoprevention, including the design of combination regimen protocol that may enhance the anticarcinogenic action of lower levels of selenium compounds. Another avenue is the formulation of novel selenium compounds which are more potent than that traditionally used selenite or selenomethione in inhibiting tumorigenesis. In practice, the use of nutritional levels of supplementation is obviously more attractive than that of pharmacological levels.

From a nutritional standpoint, the interaction among nutrients cannot be overemphasized. Examples have been provided above showing that the prophylactic efficacy of selenium in mammary carcinogenesis is influenced by the intake of fat as well as vitamins A, E and C. This type of study should be expanded to include other macro- and micro-nutrients in an attempt to define whether the interactions are additive, synergistic or even antagonistic. The ultimate test of selenium in cancer prevention can only be provided by controlled clinical intervention trials. There are already a few studies underway at the present time. Thus it is essential to understand how the action of selenium can be modulated by other dietary factors.

Before any systematic effort is directed towards determining the mechanisms of action of selenium in chemoprevention, perhaps the first and crucial step should be focused on the disposition and distribution of selenium under conditions in which selenium administration is shown to be

inhibitory to neoplastic development. Although much is known about selenium metabolism, there are significant gaps in current knowledge especially when the intake of selenium is high. Research in this area should encompass the quantitation of known intermediates in the selenium detoxification pathway, the identification of novel selenium metabolites and the characterization of specific selenoproteins in normal and neoplastic tissues. Thus selenium metabolism is a key domain of investigation which may provide valuable information in designing agents and strategies for chemoprevention.

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DIETARY RETINOIDS AND THE CHEMOPREVENTION OF MAMMARY GLAND TUMORIGENESIS

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I. INTRODUCTION

Over the years, the thrust of cancer therapy has been the destruction of malignant cells; comparatively little effort has been directed toward modifying the behavior of malignant cells toward normalcy. In recent years, considerable interest in the use of dietary retinoids (vitamin A and its naturally occurring and synthetic analogues) as potential mediators of this process has come to the fore. To date, the "chemopreventive" effects of dietary retinoids have been demonstrated in a variety of experimental tumor models; tumorigenesis of the bladder, cervix, colon, forestomach, integument, liver, lung, pancreas, vagina and mammary glands has been suppressed by the feeding of pharmacological levels of natural and synthetic retinoids. The purpose of this communication is to provide a succinct review of the pertinent information that has emerged in recent years documenting the use of retinoids in the chemoprevention of mammary tumorigenesis in experimental animals. More specifically, this review will examine the chemopreventive effects of dietary retinoids in the development of mammary tumors in rats and mice and lastly will focus on potential mechanisms by which these compounds may suppress tumorigenesis.

II. EFFECTIVENESS OF DIETARY RETINOIDS IN THE CHEMOPREVENTION OF MAMMARY TUMORIGENESIS IN RATS

The first direct evidence of a significant inhibitory effect of high levels of dietary retinoids on the development of mammary tumors in laboratory rats was reported by Moon et al. in 1976 (1). Female Sprague-Dawley rats were intubated with a single dose of 7,12-dimethylbenzanthracene (DMBA) and 7 days later fed a standard laboratory chow containing retinyl acetate. Daily consumption of retinyl acetate was adjusted to either 1.0 mg or 2.5 mg per rat. The incidence of carcinomatous and benign mammary tumors 7 months after carcinogen treatment was significantly reduced in animals fed the natural retinoid. This study has been confirmed in many laboratories using a variety of mammary gland carcinogens, i.e., DMBA, N-methyl-N-nitrosourea (MNU), benzopyrene (BP) and X-irradiation, and dietary retinoids, i.e., retinyl acetate, retinyl methyl ether and N-(4-hydroxyphenyl)-retinamide (4-HRP) (2-20).

McCormick et al. (2) initiated a series of studies to determine whether mammary tumorigenesis could be suppressed in DMBA-treated female rats when retinyl acetate feeding was limited to specific time periods during this neoplastic process. Feeding retinyl acetate at -2 to +1 weeks, +1 to +30 weeks, +1 to +12 weeks, +12 to +30 weeks and -2 to +3 weeks, where time 0 was the day of DMBA administration, resulted in a striking difference in tumorigenesis between these groups. Thirty weeks after DMBA treatment mammary carcinoma incidence was significantly reduced in all groups but one (+1 to +12 weeks) when compared with placebo fed controls. The greatest decrease in mammary tumor incidence was seen in the longest treatment group (-2 to +30 weeks) but a nearly equal reduction was seen in the group receiving a short retinyl acetate exposure (-2 to +1 weeks). In the +1 to +12 week group, the inhibition of tumor development was temporary; tumor incidence returned to that observed in placebo fed control rats by 30 weeks post carcinogen treatment. Nearly identical results were obtained in a subsequent study using a different carcinogen (BP) (14). However, as reported by Kelly and colleagues (17), when retinyl acetate feeding is delayed until 6 months after carcinogen treatment, the effectiveness of the retinoid may be reduced or absent.

In all of the above cited studies (1-20), large amounts of dietary retinoids (0.6 - 2.0 mM) were used for successful chemoprevention of rat mammary gland carcinogenesis. Dietary retinyl acetate levels as low as 0.2 mM do not appear to be effective in the chemoprevention of rat mammary gland carcinogenesis (5). This level of retinyl acetate (0.2 mM) still provides 20-100 times the daily requirement (I.U.) of retinol. Thus a narrow and continuous high level of the retinoid is prerequisite for successful chemoprevention of this neoplastic process. Retinyl acetate accumulates excessively in the liver, causing mild hepatic toxicity (13). Whether or not retinyl acetate-induced hepatic toxicity affects this neoplastic process remains to be determined. Despite the mild hepatic toxicity, rats fed dietary levels of retinyl acetate up to 1.0 mM have normal body weight gains, normal estrous cycles and in general appear as healthy as placebo fed control animals. Certain synthetic retinoids (e.g., 4-HPR) do not accumulate excessively in the liver therefore little or no hepatic toxicity is observed in these animals (13). Thus, retinoid-induced prophylaxis of rat mammary gland carcinogenesis can be observed in animals without concurrent hepatic toxicity. It should be pointed out, however, that the administration of certain retinoids (i.e., 4-HPR) to female rats fed low doses of certain carcinogens (i.e., MNU) does not appear to suppress mammary tumorigenesis (13,15). Furthermore, the reduction of dietary levels of retinyl acetate from 1.0 mM to 0.2 mM in carcinogen- treated rats may actually result in increased mammary tumorigenesis (5).

Recent studies have demonstrated that the chemoprevention activities of retinoids in rat mammary gland carcinogenesis can be significantly enhanced by other biological response modifiers, i.e., hormone antagonism, immune stimulation and the administration of pharmacological levels of selenium (3-5, 7, 9-11, 18). In comparing the effectiveness of retinoid feeding, selenium treatment and hormone antagonism in the prophylaxis of rat mammary gland carcinogenesis, it is clear that hormone antagonism is superior to retinoid feeding (5,18). Retinoid feeding, on the other hand, appears to be slightly superior to selenium treatment (3,10). Clearly, the effectiveness of retinoid feeding can be enhanced by either selenium treatment or hormone antagonism (3,5,9,10,18). Immune stimulation (cell particulate of mammary carcinoma plus Freund's complete adjuvant), although not effective in suppressing rat mammary gland carcinogenesis when given alone, did significantly reduce mammary carcinoma incidence in rats fed retinyl acetate (5). A lack of synergism between other immune stimulants (i.e., methanol extracted residue of Bacillus Calmette-Guerin, cell wall skeleton of Nocardia rubra and maleic anhydride-divinyl ether copolymer - MVE-2) and retinoid feeding in the chemoprevention of rat mammary gland carcinogenesis has been reported (5,19). The combined efficacies of retinoid feeding, hormone antagonism (inhibition) and immune stimulation in the chemoprevention of rat mammary gland carcinogenesis was recently reported (5,18). At termination of the study (20 weeks after carcinogen treatment), no mammary carcinomas were observed in rats treated with the combination of retinoid feeding, hormone antagonism and immune stimulation (5). In a subsequent study, by one year after carcinogen treatment, only 2 mammary carcinomas were observed in a group of 40 rats treated similarly (18).

Although the effects of dietary retinoids have been examined extensively in carcinogen treated rats, there are only two reports that have evaluated the effect of this dietary constituent on the development of spontaneous mammary carcinomas in this species. McCormick et al. (14) fed female Lew/Mai rats retinyl acetate for 92 weeks, commencing at 5 weeks of age. Mammary carcinoma incidence in retinyl acetate fed animals was 28%, while 30% of the control animals developed these tumors. Stone et al. (20) fed female Sprague-Dawley rats retinyl acetate for 1 1/2 years beginning at 2 months of age; mammary tumor incidence was reduced in the retinoid treated rats by approximately 50%, although tumor frequency in both control and experimental rats was too low to reach statistical significant levels. Thus, to date, it is uncertain whether or not dietary retinoids can effectively suppress the genesis of spontaneous rat mammary carcinomas.

There are only a few reports in which the effects of retinoids on growth of rat mammary carcinoma cells were examined in vitro. Retinoic acid or retinyl acetate inhibited growth of mammary tumor cells obtained from Fischer 344 rats (cell lines 13762NF, DMBA#8 and R-3230AC) by 39-88% (21). Retinoic acid was consistently superior to retinyl acetate in inhibiting cellular proliferation of the mammary carcinoma cell lines. Growth inhibition by the retinoids was most pronounced in lines 13762NF and DMBA#8, both derived form female rats treated with DMBA. In a second report, retinol, retinal, retinoic acid and retinyl acetate were effective in reducing proliferation of a rat mammary carcinoma cell line (Rama 25) isolated from a Sprague-Dawley rat bearing a DMBA-induced rat mammary carcinoma (22).

III. EFFECTIVENESS OF DIETARY RETINOIDS IN THE CHEMOPREVENTION OF MAMMARY TUMORIGENESIS IN MICE

Although there have been a number of reports documenting the chemopreventive activity of a variety of dietary retinoids in chemical carcinogenesis of the rat mammary gland, fewer laboratories have examined this activity in mice (23-29). It is clear that the response to dietary retinoids is quite varied, ranging from significant inhibition (24,29), to

no effect (23,24,26,28), to stimulation (25,27) of mouse mammary gland tumorigenesis. It should be pointed out, however, that mice are unable to tolerate the high dietary levels of natural retinoids (e.g., retinyl acetate) that are commonly fed to rats (0.6-2.0 mM) in chemoprevention studies. Dietary levels of retinyl acetate of 0.2 mM are maximally tolerated by this species, i.e., dietary levels of retinyl acetate in excess of this amount cause significant reductions in mouse body weight gains. 4-HPR, in contrast, can be fed to mice at higher dietary levels (e.g., 1.0 mM) without any adverse side effects. When 4-HPR is fed to nulliparous C3H mice, commencing at 2 months of age, a slight but significant reduction in the incidence of spontaneous mammary carcinomas is observed (24). The lack of effect of dietary 4-HPR in the prophylaxis of mammary tumorigenesis in multiparous C3H mice (24) and in $BD2F_1$ mice treated with DMBA (26) provides evidence, however, which supports the concept that mouse mammary gland tumorigenesis is less responsive to the inhibitory actions of dietary retinoids than is carcinogen-induced rat mammary tumorigenesis. Furthermore, it appears that elevated levels of dietary retinoids, under certain experimental conditions, may even enhance this neoplastic process; mammary tumor incidence was significantly increased in hormone-treated GR/A mice fed retinyl acetate (25) and mammary tumor pulmonary metastasis was increased in C3H/St/Ha mice fed retinyl palmitate (27). Indeed, slight increases in mammary adenocarcinoma incidence by hyperalimentation of certain retinoids (i.e., 4-HPR) may even occur in carcinogen treated $BD2F_1$ mice and in multiparous C3H mice (24,26).

A number of laboratories have examined the effect of various retinoids in vitro on the induction and progression of mouse mammary gland preneoplastic lesions. Sorof and colleagues (30) examined the effects of a synthetic retinoid (retinylidene dimedone) on DMBA-induced preneoplastic transformation of the BALB/c mouse mammary gland. The mammary glands were cultured for 10 days in a growth promoting media (containing mammotropic hormones) and for an additional 14 days in a media that lacked mammotropic hormones and caused mammary gland regression. DMBA was added to the culture media for only one day, i.e., on day 4. Retinylidene dimedone was added to the culture media at days 0-3, 3-4, 4-10, 7-12 or 10-24 of culture. The percent of mammary glands containing nodular-like alveolar lesions (NLAL) (transformed glands) in control cultures ranged from 30-45%. The percent of glands containing NLAL in retinylidene dimedone treated cultures, when the retinoid was added to culture media at 0-3, 3-4, 4-10, 7-12 or 10-24 days of culture, was 28%, 25%, 3%, 5%, and 15%, respectively. Thus, significant suppression of in vitro preneoplastic transformation of the mouse mammary gland was observed only when the retinoid was administered after the carcinogen. When the retinoid was administered either before or during carcinogen treatment, mammary gland transformation was comparable to control values. In subsequent studies, using different carcinogens [BP, N-2-fluorenylacetamide (FAA), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), N-nitrosodiethylamine (DENA)] and retinoids (retinylidene dimedone, 4-HPR), similar results were obtained, i.e., the retinoids were effective in suppressing the genesis of NLAL when administered after carcinogen treatment (31-33). In contrast, Banerjee and colleagues provided evidence that the in vitro genesis of NLAL can be inhibited by β-carotene, when β-carotene was administered either during or after carcinogen (DMBA) treatment (34). The in vitro development and/or maintenance of spontaneous mouse mammary gland preneoplastic alveolar lesions (MAL) were also influenced by retinoids. Teland and Sarkar (35) reported that 4-HPR suppressed the development and inhibited maintenance of MAL in organ cultures of mammary glands from RIII mice.

The addition of certain retinoids to the media of cultures of mouse mammary tumor cells has been reported to suppress proliferation of these cells (21,36,37). Retinoids such as retinyl acetate, retinoic acid and 4-

HPR suppressed proliferation in mouse mammary tumor cell lines Mm5mT (from C3H mice), M12 (from C3H mice), DD3 (from carcinogen treated BALB/c mice) and GR-3A (from GR mice). Thus, in vitro, retinoids have been reported to suppress not only the progression of mouse mammary preneoplastic cells but, in addition, suppress the proliferation of more advanced mammary tumor cells, i.e., cells from established mouse mammary tumor cell lines.

IV. MECHANISM OF ACTION OF DIETARY RETINOIDS IN THE CHEMOPREVENTION OF MAMMARY TUMORIGENESIS

A. Retinoid-Binding Proteins

Retinoids act through cellular binding proteins in a manner similar to that described for steroid hormones. A cellular protein which specifically binds retinol has been detected and characterized. A second protein which specifically binds retinoic acid has also been detected and has been partially characterized. It has been proposed that the retinoid-binding protein complex migrates from cytoplasm to nucleus where it influences gene expression (38-40).

The first evidence of retinoid-binding proteins in rodent mammary tissues was obtained by Ong and Cytil in 1976 (41). Cellular-retinol binding proteins (CRBP) and cellular retinoic acid binding proteins (CRABP) were observed in 2 transplanted mammary carcinoma cell lines, i.e., the Walker 256 carcinosarcoma maintained in Sprague-Dawley rats and MAC-1 carcinoma maintained in Fisher 344 rats. Subsequently, CRABP and/or CRBP have been reported in normal mammary glands of virgin, pregnant and lactating Sprague-Dawley rats and virgin C57BL mice and in C3H mouse mammary tumors and carcinogen-induced rat mammary tumors (42-45). In carcinogen-induced rat mammary carcinomas, CRABP has been reported to be found in nuclear and cytosolic cellular fractions (40). CARBP concentrations are lower in the normal rat mammary gland compared to mammary carcinomas mammae (44). CRABP concentrations are lower in hormone-dependent than in hormone-independent rat mammary carcinomas (45). Although experiments have demonstrated that CRABP or CRBP is present in higher concentrations in neoplastic than in normal mammary tissue, this difference may reflect the greater amount of extracellular stroma found in normal versus neoplastic mammary tissues. This problem was recently addressed by Bunk et al. (46) who reported higher concentrations of CRABP and CRBP in cell cultures of mouse mammary tumors (C57BLfRIII) than in cell cultures of normal mouse (C57BL) mammary tissue. Thus, it appear that the higher concentrations of CRABP and CRBP found in neoplastic mammary cells reflect increased intracellular levels of retinoid-binding proteins. It remains to be determined whether or not the intracellular metabolic effects of retinoids on normal or neoplastic mammary gland are exerted partially, primarily or solely via specific receptor proteins.

B. Initiation Stage of Mammary Gland Carcinogenesis

1. Modification of Carcinogen Metabolism

DMBA and a number of other polycyclic aromatic hydrocarbons exert their mutagenic and carcinogenic effect only after metabolic activation, which produces ultimate carcinogens, that bind covalently to biologically active macromolecules such as DNA, RNA and protein. Several studies have demonstrated that retinoids inhibit the binding of carcinogen to DNA of a variety of tissues (47) including the mammary gland (48), while retinol deficiency enhances such binding (49). A possible mechanism to explain the inhibition by retinoids of carcinogen binding to DNA involves competition with the activated or ultimate carcinogen. Retinoids readily form epoxides. Since ultimate carcinogens may be epoxides, retinoids could present themselves as another substrate for epoxide generating systems. It is well

known that 5,6-epoxyretinoic acid is formed in tissues in vivo (50). Thus, epoxidation of retinoids may be one of the biological mechanisms that interfere with carcinogen activation. Alternatively, the inhibiting effect of retinoids on carcinogen binding to DNA may be mediated via prostaglandins since prostaglandin production can be enhanced by retinoids (51) and prostaglandins have been reported to inhibit carcinogen binding to DNA (52). Additionally, several studies have demonstrated that retinoids can directly modulate metabolic activation of carcinogens that are catalyzed by microsomal monoxygenases such as aryl hydrocarbon hydroxylase. For example, retinoids were found to inhibit the induction of BP activating enzymes in mice (53), while in retinol-deficient guinea pig microsomes, the activity of carcinogen activating enzymes was high (54). These studies provide evidence that retinoids can prevent the formation of the ultimate carcinogen by a variety of mechanisms.

It is clear that the anticarcinogenic actions of retinoids at initiation cannot be explained entirely by an intervention in carcinogen activation, since retinoids can prevent tumor (colon) induction by carcinogens that do not require metabolic activation (55). Thus, additional mechanisms must be postulated for the prevention of initiation by retinoids of many carcinogens.

2. Modification of Cell Division

Any substance which inhibits normal mammary gland cell division would, in addition, have the capability of suppressing the initiating activities of chemical carcinogens since it is generally accepted that replicating cells are more vulnerable to carcinogen action than are non-replicating cells. In general, treatments that suppress normal rat mammary gland DNA synthesis when implemented before and during carcinogen treatment, reduce the incidence of mammary carcinomas (56,57). If it can be shown that retinoids inhibit mammary gland cell division in vivo (or in vitro), an attractive hypothesis explaining the inhibitory effects of dietary retinoids at initiation would be provided.

Mehta and Moon (58) reported that the mammary glands of carcinogen (DMBA and MNU)-treated female Sprague-Dawley rats fed retinyl acetate had lower DNA synthetic activity than placebo fed controls, as judged by ^{3}H-thymidine incorporation into chemically extracted DNA. McCormick et al. (14) reported that the mammary glands of female Lew/Mai rats fed retinyl acetate had decreased DNA synthesis as judged by radioautographic analysis of 3H-thymidine uptake. Morphological assessment of these glands, however, revealed only a slight reduction in development. Welsch et al. (5,9,18), Moon et al. (13) and Aylsworth et al. (16) examined whole-mounts of mammary glands from rats treated with pharmacological levels of retinoids and observed reduced cellular proliferation or altered morphology (reduced number of ducts and alveoli) of the epithelium within these glands. This was observed in rats fed either retinyl acetate or 4-HPR. Thus, it appears that pharmacological levels of retinoids are anti-proliferative to the normal rodent mammary gland in vivo.

C. Promotion Stage of Mammary Gland Carcinogenesis

1. Membrane Alteration

It is well know that when retinoids are added in excess to biological systems, they can act on cellular and subcellular membranes. Although this phenomenon has not been examined in normal or neoplastic mammary gland cells, it has been studied in other biological and experimental systems. High, non-physiological concentrations of retinol, for example, can labilize membranes causing leakage of intracellular components and finally, dis-

ruption of tissue (59). Thus, a hypothesis for the mechanism of the antipromotional activity of pharmacological doses of retinoids is the release of hydrolytic enzymes causing destruction of neoplastic cells (60). However, in several studies, no correlation was observed between the ability of compounds to stabilize or labilize lysosomal membranes and their antitumorigenic properties (61). It is probable, therefore, that labilization of membranes by retinoids is not a valid mechanism to explain the antipromotional activities of retinoids.

Considerable evidence, however, does point to membranes as a possible cellular site for the initiation of physiological as well as the antitumorigenic functions of retinoids. Among the mechanisms proposed for the physiological function of retinol, glycosylation of membrane proteins and lipids is currently being extensively examined (62). Membrane glycosylation is associated with cell surface recognition mechanisms that are crucial in the regulation of cellular growth and differentiation. Profound changes in cell surface properties, in addition to cytoplasmic changes, are also linked to neoplasia. Current evidence supports the concept that cell membrane glycoconjugates have an important role in the neoplastic transformation (63).

Since retinoids, when added to cells in culture, almost invariably alter cell surface associated phenomena, it is logical to seek an explanation for the antitumorigenic properties of these compounds by examining biochemical alterations in the membranes of carcinogen initiated transformed cells that have been exposed to retinoids. Modulation of cell surface functions (adhesion, spreading, ion transport, cell-cell communication) and composition (glycoproteins, glycolipids, phospholipids) by retinoids has been studied in numerous in vitro systems. Such studies have demonstrated that retinoids increase cellular adhesion and restore anchorage dependent growth to transformed cells, possibly by an effect on cell surface protein glycosylation. It is possible that the restoration of normal function (e.g., anchorage dependent growth, active cell-cell communication, etc.) to a transformed cell may be the basis for the antitumorigenic effects of pharmacological levels of dietary retinoids (61-65).

It should be pointed out that this postulated mechanism of retinoid action, i.e., modification of membrane glycosylation may or may not require binding of the retinoid to a specific receptor. Other actions of retinoids on cellular or subcellular membranes which could be important in the antipromotional activity of these compounds are alteration of conformation and/or charge of membrane proteins, alteration of membrane electron transfer and/or alteration of membrane fluidity, these activities may or may not involve a specific receptor mediated event (66,67).

2. Modification of Cell Division

Inhibition of the action of mitogenic peptides. An intriguing hypothesis, proposed by Todaro, Sporn and co-workers (68), is that retinoids may modulate the mitogenic effects of peptide hormones on specific epithelial target organ sites. This hypothesis was derived, in part, from the observation that retinoids antagonize the stimulatory effects of sarcoma growth factor (a peptide) on cell proliferation and anchorage independent growth of fibroblasts in culture. Recent results derived from the study of rat mammary gland carcinomas provides evidence in support of this hypothesis. Prolactin, a peptide hormone, is a well known rat mammary carcinoma mitogen in vivo (69). Retinyl acetate, fed to rats treated with a drug (haloperidol) to induce hyperprolactinemia, completely blocked the stimulatory effect of prolactin on mammary carcinoma development (18). Insulin, another peptide hormone, is a potent mitogen of rat mammary carcinomas in vitro (70). The addition of retinoic acid to media of organ cultures of rat

mammary gland carcinomas blocked the stimulatory effect of insulin on ^{3}H-thymidine incorporation into DNA (18). Retinoic acid added to the culture media alone did not effect ^{3}H-thymidine incorporation into DNA. Thus, both, in vivo and in vitro evidence demonstrated that retinoids inhibited the proliferative actions of peptide hormones on rat mammary gland carcinomas. These results and those of Todaro et al. (68) are similar in many respects to a recent report by Mehta et al. (71) which showed inhibition by retinoic acid of prolactin induced DNA synthesis and lobulo-alveolar development of organ cultures of normal mouse mammary gland.

Induction of differentiation. It has been known for many years that retinol modulates the differentiation of many mammalian tissues (72) although the possibility has been raised that the mammary gland may be an exception (73). Retinoids can also enhance differentiation of certain carcinomatous tissues. Perhaps the most striking example of this phenomenon is the conversion of murine embryonal carcinomas to benign teratomas by retinoids in vivo (74). Strain 129 mice bearing PCC4 embryonal carcinoma subcutaneously were treated daily (intratumor) with retinoic acid. The retinoid induced nearly complete morphological differentiation mainly into neuroepithelial and glandular derivatives. Differentiation was associated with a decreased tumor growth rate, decreased mitotic index and increased survival time of hosts. In 4 of 18 cases, long term survival of the hosts was correlated with complete differentiation of the malignant embryonal carcinoma tumors into benign teratomas. It is not known if such a phenomenon occurs in rat mammary carcinomas as a result of retinoid feeding. Histological assessment of mammary tumors from rats treated with pharmacological levels of retinoids provided no indication of retinoid-induced tumor cell differentiation, i.e., no morphological evidence of secretion in the tumors cells was observed (5,9,16,18). Furthermore, morphological examination of the normal mammary gland of these animals did not provide any indication of enhanced differentiation (secretion and/or alveoli formation). In contrast, when GR mice were fed large doses of retinyl acetate, a marked increase in mammary alveoli development was observed in these animals (25). This observation is consistent with the report by Rudland et al. (22) showing enhanced differentiation (casein and dome formation) of rat mammary carcinoma cells (Rama 25) by retinoids in vitro.

3. Immune Stimulation

There is evidence that retinoids are immunoregulatory, i.e., dietary retinoids may act as immune adjuvants (75-78). For example, the administration of certain retinoids to laboratory animals has been reported to cause thymus and lymph node enlargement (76), to enhance humoral antibody response to a variety of antigens (75), to enhance certain cell mediated responses (77) and to enhance tumoricidal macrophage activity (78).

There are three studies that have examined the interaction of retinoid feeding and immune stimulation on the genesis of rodent mammary tumors (5,18,19). In all three studies, pharmacological levels of retinoids were used. Concurrent treatment with an immunostimulant consisting of pooled cell particulates of carcinogen-induced rat mammary carcinomas enhanced retinyl acetate suppression of DMBA or MNU-induced rat mammary gland carcinogenesis (5,18). Other immunostimulants (e.g., BCG, Norcardia rubua, MVE-2) were not effective in enhancing the chemopreventive activities of retinoids in this carcinoma model (5,19). A significant synergism between retinoid treatment and various immune modifying therapies has been reported for carcinogen-induced murine fibrosarcomas (79). It should be stated, however, that it was not established that this synergism was a result of enhanced immune system reactivity.

The concept that the antitumorigenic effects of pharmacological levels of retinoids occurs via the immune system is attractive. Nevertheless, it has not been demonstrated that retinoids enhance immune effector cells or non-cellular immune activities which are specifically directed toward neoplastic mammary epithelium. Furthermore, the ability of retinoids to exhibit antitumorigenic effects in vitro, where immune processes are absent, provides evidence that immune stimulation can not be the sole mechanism by which retinoids influence tumorigenic process.

V. CONCLUSION

To date, the chemoprevention of mammary tumorigenesis by chronic feeding of high dietary levels of natural and synthetic retinoids has been repeatedly demonstrated in one experimental animal model, i.e., chemical carcinogen induced rat mammary carcinoma. The consistent and significant inhibitory effects of dietary retinoids in this laboratory animal model have provided the primary experimental basis for the projected utilization of retinoids in the chemoprevention of human breast cancer. While this is a seemingly logical extension and application of the results of laboratory animal studies, a number of concerns exist regarding the feasibility of dietary retinoids in the chemoprevention of human breast cancer. These concerns are summarized as follows. 1. Dietary retinoids are only definitive and consistent inhibitors of mammary tumorigenesis in one experimental animal model (chemical carcinogen induced rat mammary carcinoma model). 2. Effective inhibition in rats requires a sustained consumption of very large amounts of this dietary constituent. 3. When low levels of chemical carcinogens are administered to laboratory rats, the inhibitory effects of dietary retinoids in mammary tumorigenesis appear to be lost. 4. Attempts to inhibit the spontaneous development of mammary tumors in rats by chronic feeding of retinoids, to date, have not been entirely successful. 5. Although slight inhibition by dietary retinoids of mammary tumorigenesis has been demonstrated on one nulliparous mouse strain, dietary retinoids do not appear to inhibit mammary tumorigenesis in other nulliparous mouse strains, in multiparous mice or in chemical carcinogen treated mice. 6. Significant enhancement by dietary retinoids of hormone-induced mammary tumorigenesis in GR/A mice has been observed; evidence that dietary retinoids may increase the incidence of mammary adenocarcinomas in chemical carcinogen- treated mice has also been provided. 7. Significant enhancement of pulmonary metastasis of mouse mammary tumor cells in mice fed high levels of retinoids has also been reported. Thus, a consistent effect of dietary retinoids in the suppression of mammary tumorigenesis in rodents has not been observed; indeed, the danger of enhancement of the tumorigenic process by this dietary constituent appears to be omnipresent, not only in the mammary gland but possibly in other organ sites as well (80). Although increased efforts toward the elucidation of the underlying mechanism(s) in retinoid-induced inhibition of mammary tumorigenesis and the realization of the potential chemopreventive activities of dietary retinoids in an array of rodent mammary tumor models should certainly be pursued and encouraged, the concerns raised above should be incorporated into current decision making processes involving the utilization of dietary retinoids in the chemoprevention of human breast cancer.

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