Organization and Growth of Mammary Epithelia in the Mammary Gland Fat Pad

LEWIS G. SHEFFIELD
Dairy Science Department
University of Wisconsin
Madison 53706

ABSTRACT

Mammary gland development consists of a series of very highly ordered events involving interactions among a number of distinct cell types. An important aspect of mammary gland development is that the mammary gland consists of a fat pad of mesodermal origin into which epithelial cells of ectodermal origin proliferate. This proliferation of epithelial cells into the mammary fat pad is the subject of this review. The nature of the stroma into which epithelial cells proliferate is of considerable importance in determining the structure of the resulting gland. In mice, white adipose tissue appears to be required for normal mammary development. Transplantation of mammary epithelia to other types of stroma does not support epithelial growth or result in abnormal growth. To date, a synthetic substratum capable of mimicking white adipose tissue has not been developed. Although collagen gel cultures are generally considered superior to glass or plastic substratum in supporting near normal epithelial growth, the technique has not advanced to the point that the in vivo growth pattern is duplicated. Recent research on the generation of chimeric mammary tissue (by transplanting mammary epithelia from rats, cows, and women to the mammary fat pads of athymic nude mice) suggests that there are important species differences in the stromal requirements for mammary gland development. In particular, extensive and expansive growth of rat mammary tissue is observed in mouse mammary fat pads. However, the mouse mammary fat pad appears incapable of supporting expansive growth of bovine or human mammary epithelia. The reason for this difference is not clear. However, human and bovine mammary epithelia may require the presence of more fibrous (collagenous) tissue than rodent mammary epithelia for normal proliferation.

INTRODUCTION

Even a cursory examination of mammary gland anatomy and development is sufficient to determine that the mammary gland is a highly organized organ consisting of a variety of cell types. The manner in which these various cell types are organized and the mechanisms controlling the development of this organization are important areas of research for mammary gland biologists. The objective of this review is to describe pattern formation in the mammary gland and discuss recent research in the area. The review will first describe normal mammary development, including recent developments in the mechanisms controlling pattern formation in the mammary gland. Next, the paper will describe pattern formation in the mechanisms controlling pattern formation in the mammary gland and conclude with a discussion of studies on species-chimeric mammary tissue (i.e., stroma and parenchyma from different species). These studies suggest a role of stroma in determining pattern formation in the mammary gland. In addition, they provide evidence that stromal requirements for proper mammary epithelial growth display a degree of species specificity.

QUANTITATION OF MAMMARY DEVELOPMENT

Numerous reviews have included descriptions of the major methods of quantitating mammary growth (3, 108). Early methods were largely
morphological (i.e., whole mounts, histological examination) and were primarily qualitative assessments of development. Semi-quantitative whole mount development scores, in which development was rated on a qualitative scale, were developed (109, 110). In addition, morphometric measurements (e.g., epithelial area, duct length, duct width, alveolar area, number of end buds, number of alveoli), either absolute or as a percent of total mammary area, were used as indices of mammary growth (100). These methods are very tedious and time consuming but can provide useful information.

About 1950, researchers began to recognize that the chromosomal content of DNA was constant and could be used as an index of cell numbers in tissue (39, 88). By the late 1950's, mammary DNA was widely used as an index of growth (64). This measure of growth has not been without controversy, however. Although some researchers found the DNA per nucleus to be constant among various physiological states, others have reported that DNA per cell is somewhat variable among states, particularly during lactation and mammary involution (167). Another limitation of using DNA as an index of mammary growth is that it does not distinguish between various cell types present in the mammary gland. It has been argued that the majority of the increase in mammary cell numbers during mamrnogenesis is due to increased epithelial cell number in the mammary gland (180). Although this finding may validate mammary DNA as a measure of growth for many purposes, there are still many instances in which knowledge of the numbers or spatial distribution of various cell types is important. For these applications, morphometric analysis is essential.

Another useful method of assessing mammary gland growth is to measure DNA synthesis rate by \[^3\text{H}\]thymidine autoradiography (21). This measure provides a useful measure of growth rate at a given time. In addition, measures can be made of the duration of DNA synthesis (20). One advantage of this method is that DNA synthesis of a particular cell type can be determined. However, it is a near instantaneous rate measurement and does not quantitate the amount of growth over a prolonged time. In addition, DNA synthesis can occur in the absence of cell division in mammary tissue (11).

Recent advances in image analysis may improve the measurement of mammary gland development. Welsch et al. (185) provides a recent example of the use of computer assisted image analysis to assess mammary epithelial growth. At the present time, only epithelial area (total or a percent of gland) can be readily determined without manual intervention. Such measures as duct length, end bud area, or penetration of ducts into the fat pad can be determined but require the use of a stylus to trace the areas to be measured, as computer programs have not been developed capable of recognizing, for example, a mammary end bud. However, the field of artificial intelligence is progressing rapidly, and expert systems capable of performing many morphometric measurements now performed manually or with minimal computer assistance may eventually be automated.

**PATTERNS OF MAMMARY DEVELOPMENT**

**Embryonic Development**

The embryonic development of the mammary gland was reviewed extensively by Anderson (4). Because embryonic events are important in determining later development, mammary gland embryology will be reviewed briefly. For more detailed information, the reader is referred to one of the more extensive discussions published elsewhere (4, 8, 9, 128, 176, 183). The mammary gland begins as a proliferation of ectoderm lateral to the midline (mammary band). This develops into the mammary streak and eventually the mammary line. Local thickenings develop (mammary crest) corresponding to the eventual location of mature mammae. In addition to cell proliferation, cell migration is involved in the development of the embryonic mammary rudiment, at least in the rabbit (125).

The mammary crest evolves into the mammary bud, which gives rise to the primary sprout. The primary sprout then gives rise to the secondary sprouts. These structures first develop as solid cords of cells and are subsequently canalized. The exact mechanism of canalization is not known. Anderson (4) suggested that canalization occurred by cell death in the interior
of the sprout. However, other authors have suggested that cell death is not involved in initial canalization of the primary sprout. Recent studies (75) indicated that initial canalization of developing mammary epithelial structures did not involve cell death but was due to cells growing apart, probably due to loss of adhesive properties. The loss of adhesive properties was evidenced by the presence of microvilli on cell surfaces, which has been shown to be indicative of a nonadhesive surface (175). However, the nonadhesive surfaces may be a result, not a cause, of lumen formation. In addition, canalization did not have a distinct origin but began with the formation of several lumen which later fused. Only during later stages of canalization did cell death play an appreciable role. In the cow, the primary sprout eventually becomes the gland and teat cisterns, whereas the secondary sprout becomes the primary mammary ducts.

Early in the developmental process, mammary epithelia proliferate into relatively undifferentiated embryonic mesenchyme (4). Recently, Kimata et al. (87) have shown that this mesenchyme is actually two different mesenchymes. The fat pad precursors develop posterior to the mammary bud by about 14 d of gestation (in the mouse). Fibroblasts [referred to as mammary mesenchyme by Kimata et al. (87) and by Sakakura et al. (139)] develop in close association with the mammary bud. In most species (e.g., cows, rats, mice), the mesenchyme differentiates into a well-defined fat pad, consisting mostly of adipose cells. Associated stromal components (fibroblasts, blood vessels, nerves) are also present. The earliest signs of definitive fat pad formation are about 80 d of gestation in cows and 18 to 20 d in rats and mice, about the time of secondary sprout formation.

Although a distinct fat pad forms in most species studied, this is not the case with a few species, most notably humans. Although the adult female breast contains considerable adipose tissue, it is not closely associated with mammary ducts and does not develop until puberty. Normally, human mammary ducts proliferate in a stroma containing considerably more fibrous tissue than rodents. Although the cow does develop an extensive fat pad, histological observations of bovine mammary tissue also suggest that bovine mammary epithelia does not grow in close association with adipose cells but in association with a more fibrous stroma (personal observations, exact distances not determined).

The endocrine control of fetal mammary gland development is poorly understood, largely because of the technical difficulties involved in mammalian embryology. Studies to date suggest that the classical mammogenic hormones (e.g., estrogens, progesterone, prolactin) are not required for normal fetal mammary gland development (9, 68). However, fetal mammary tissue does have the ability to respond to a variety of hormones, as evidenced by its ability to produce milk proteins in response to appropriate lactogens (insulin, hydrocortisone, prolactin) (22, 23, 24, 26, 94). Apparently, the only requirements for fetal mammary development are the so-called metabolic hormones (insulin, growth hormone, thyroid hormones, glucocorticoids) and possibly growth factors (epidermal growth factor, fibroblast growth factor, insulin-like growth factors). In support of this, in vitro studies have shown that the timing of mammary bud formation is intrinsic to the developing mammary rudiment and does not depend on external endocrine signals (9, 68).

An important determinant of embryonic mammary development is the nature of the mesenchyme. In a series of studies involving combining mammary epithelia and salivary stroma (and vice versa), Sakakura et al. (136, 137, 138) demonstrated that the pattern of duct growth and branching was determined by the mesenchymal portion of the developing gland. That is, when mammary epithelia were grown in association with salivary mesenchyme, a pattern similar to the salivary gland was observed. However, hormone-induced cytodifferentiation of the epithelial cells was still similar to that observed in mammary epithelia (i.e., the cells were capable of producing milk components under appropriate hormonal stimulation). In addition, androgen-induced picnization of the mammary bud in mouse and rat embryos is also mediated through the mesenchymal portion of the gland (41, 42, 43, 89, 90, 91, 92, 93). Thus, the embryonic mammary epithelia has very specific stromal requirements for normal development.
Birth Through Puberty

From birth until shortly before puberty, the mammary gland grows isometrically with the rest of the body in rodents (29, 53, 166, 168) and cows (171). In the cow, mammary growth becomes positively allometric to body weight at about 3 mo of age and remains so for about 6 mo, or until about 9 mo of age. In the mouse, positive allometric growth begins at about 3 wk of age and continues for about 3 wk (until 6 wk of age) (with some variations among strains) (53). Similar results have also been observed in rats (166). At 3 wk of age, the mouse mammary gland consists mostly of a fat pad devoid of epithelia. Only a few ducts are present and they are located in the nipple region. Under the hormonal influences associated with puberty, ducts proliferate into the mammary fat pad. This proliferation is not random but is highly ordered. The ducts grow in such a way that, in the mature virgin animal the mammary ducts are approximately .25 mm apart (52). Mammary ducts do not entangle or cross. Furthermore, duct elongation does not occur at random points but at highly specialized, transient structures known as end buds (193).

The Mammary End Bud

The mammary end bud is not simply the termination of a mammary duct but is a specialized, club-shaped structure at which duct elongation occurs. The morphology of the end bud was described in detail by Williams and Daniel (193). In histological section, the end bud is seen to contain several cell types. The leading edge of the end bud consists of undifferentiated cap cells that appear to be stem cells, which give rise to other cell types, including myoepithelial cells (112). Immediately beneath the cap cells are the proliferating epithelial cells. These cells undergo progressive differentiation as they become distal to the proliferating end of the end bud. Eventually, the single layered duct typical of the mature gland is formed. One potential marker of differentiation of the ductal cells is that the basal lamina of the mature duct is quite different from the end bud. The basal lamina of the end bud area contains relatively little sulfated glycosaminoglycans, while the glycosaminoglycans of the more differentiated duct is more highly sulfated (162). Digestion of the glycosaminoglycans of developing ducts (using hyaluronidase administered with local release implants) results in abnormal end bud and duct development (163). These results suggest a role of glycosaminoglycans in regulating mammary duct development, although the exact role is not known. Some researchers have suggested that basal lamina glycosaminoglycans can regulate intracellular cyclic 3',5'-adenosine monophosphate (cAMP) concentrations, which may be causally related to mammary gland growth (164). According to this model, nonsulfated glycosaminoglycans in the basement membrane allow for high intracellular cAMP concentrations, which are permissive to or increase mammary epithelial growth. Sulfation of the glycosaminoglycans reduces intracellular cAMP. The reduced cAMP reduces epithelial cell proliferation. In support of these observations, pregnancy and mammogenic hormones increase intramammary cAMP concentrations and the activity of cAMP-dependent protein kinase (97, 98, 101, 102, 130, 141, 142, 146, 156, 158). In addition, agents that increase intracellular cAMP (dibutyryl cAMP, choler toxin) increase mammary epithelial growth in vitro and in vivo (154, 160, 164, 170, 174, 198). Also, Silberstein et al. (164) observed that plastic local release implants containing choler toxin increased end bud development in immature mice. Finally, glycosaminoglycans modulate adenylate cyclase activity (1, 30, 140). Thus, a model of end bud development and duct growth involving cAMP as an intracellular mediator of stromal influences appears plausible.

Very little work has been done to develop a mathematical model describing puberal mammary growth in a quantitative manner. Some workers have used total mammary DNA as an index of growth and developed regression equations to describe changes in mammary DNA with respect to time or body weight (6, 168). However, these equations do not consider the highly topological nature of mammary growth in the puberal animal. A useful method of describing mammary growth would be more abstract models, in which total mammary cells are divided into various classes (e.g., stromal cells, mature duct cells, proliferating duct cells), and growth described as multiplication or death of cells within a class as well as transition of cells from one class to another (e.g., differentiation). However, acquiring data on which to base
such a model is difficult and subject to considerable error because of the local nature of DNA synthesis in the developing mammary gland (21, 126, 127) and the difficulties of determining whether an increase in a particular population of cells is due to division of cells within the population or to differentiation of cells from another population.

**Mammary Glands in the Mature Virgin**

As discussed earlier, the mammary gland grows positively allometrically with body weight until shortly after puberty. From this time until the first pregnancy the mammary gland again grows isometrically with body weight. In mice, which have been most extensively studied, the mammary gland of the postpuberal, nonpregnant animal consists of ducts, with only a few end buds and very few alveoli. In animals with a luteal phase of the estrous cycle (e.g., cows), the mammary gland is usually more highly developed with some alveoli being present. However, the development is substantially less than observed during pregnancy. The reason for this dramatic deceleration in growth rate of the mammary gland has been a subject of considerable speculation and research. It is generally thought that the mammary ducts produce some growth-inhibiting factors (chalones) that are responsible for decreasing the sensitivity of the mammary tissue to the hormonal milieu of the postpuberal animal. Such factors were identified in the mammary gland some years ago (60, 61) but have received little attention in recent years.

**Pregnancy**

By the time sexual maturity is reached, the mammary duct system is fully developed. The mammary gland is in a state of relative quiescence, which approximates a steady state condition. That is, relatively little new mammary development is occurring. Under the influence of the hormones of pregnancy, mammary gland growth is reinitiated and continues until the mammary gland is fully developed.

Mammary gland growth patterns during pregnancy have been studied in a variety of species. Munford (107) proposed that mammary gland growth during pregnancy could be described by an exponential equation of the form: \( Y = a \exp (bt) \) where \( Y \) = mammary size, \( t \) = day of gestation, and \( a \) and \( b \) are constants. Such equations were reported for mammary growth in cows (171), goats (5), and guinea pigs (6). The a term of this equation can be considered mammary gland size at the beginning of pregnancy and \( b \) is the first order rate constant. The time for mammary size to double is equal to \( \ln 2/b \). This was calculated by Sheffield and Anderson (151) using DNA as an index of growth.

Although mammary DNA is a useful method of describing mammary growth, it does not consider the differences in mammary cell type and the way mammary cells are organized. Recognizing differences among growth rates of various classes of mammary cells, a more descriptive model of mammary growth during pregnancy can be developed. If mammary growth occurs topologically, the growth rate can be described using the type of abstract model discussed for duct growth. However, difficulties in obtaining adequate data for the development of such an extensive model have limited the application of these techniques.

**EXPERIMENTAL MANIPULATION OF DUCT DEVELOPMENT**

**Hormone Requirements**

The hormonal requirements for duct development in the postnatal mammary gland have been described in numerous publications and review articles. A brief review of these studies, emphasizing recent results, will be included. For more detailed information, the reader is referred to reviews (3, 25, 123, 178, 179, 182).

Ovariectomy abolishes the allometric growth of mammary glands seen around the time of puberty (29). The effects of ovariectomy can be reversed and allometric growth initiated by treatment with estrogens (29, 165). More recent studies have indicated that other factors may also be involved in regulating mammary gland development during the puberal stage. Silberstein et al. (164) found the cholera toxin (a potent stimulator of adenylate cyclase and hence, intracellular cyclic AMP (74) increased duct growth in mice. As discussed earlier, cAMP may be a mediator of stromal or hormonal effects on mammogenesis. More recently, another nucleotide, cyclic cytidine monophosphate (cCMP), has been implicated in controlling mammary growth (147). Relaxin has also
been shown to increase duct length and end bud area in ovariectomized mice (Figure 1; unpublished data). Several researchers have shown that relaxin increased mammary growth in rats and mice (14, 70, 71, 153). Wright and Anderson (196) postulated that relaxin acted to remodel mammary connective tissue during duct and alveolar development. Sheffield and Anderson (149) observed that relaxin increased thymidine and uridine uptake by fibroblasts isolated from guinea pig mammary glands. Also, Sheffield and Anderson (150) observed that relaxin altered the net synthesis of collagen and noncollagenous proteins by guinea pig mammary gland fibroblasts, although the effects were relatively small. Thus, relaxin may be related to or control production of the mammary gland spreading factor discussed by Elliott and Turner (46, 47, 48, 49). This factor was an extract of mammary tissue that increased proliferation of mammary epithelia into the fat pad when injected locally into rabbit mammary glands. Elliott and Turner proposed that this factor was an enzyme that acted to break down connective tissue, thus allowing duct elongation. Using plastic, local release implants, Silberstein and Daniel (163) administered hyaluronidase in the vicinity of developing mammary ducts. They found that the hyaluronidase partly digested the glycosaminoglycans of the basement membrane and resulted in dysplastic growth of bud ends, suggesting a role of the basement membrane in maintaining normal ductal morphology.

Epidermal growth factor has also been implicated as a factor controlling mammary gland development. In vitro studies have clearly shown that epidermal growth factor is capable of inducing mammary growth (122, 172, 173), 177, 181, 197, 198). Recently, Okamoto and Oka (111) reported that sialoadenectomy reduced offspring survival in mice. They proposed that this effect was due to decreased milk production, which was due to decreased mammary size. They also observed that injections of epidermal growth factor restored offspring survival to control levels. Sheffield and Welsch (155) reported that sialoadenectomy reduced the ability of mouse mammary tissue to respond to estradiol plus progesterone treatment. However, studies on the epidermal growth factor receptor concentration in mammary tissue found that it was highest in young animals and in early pregnant animals (45). These data suggested that epidermal growth factor may be more important in regulating development of mammary ducts than alveolar development. To explore this hypothesis, Sheffield and Welsch (Figure 2) used mice ovariectomized at 5 wk of age and treated for 2 d with estradiol. They found that sialoadenectomy reduced the ability of the mammary epithelia to initiate DNA synthesis in response to estradiol. The responsiveness of the mammary gland to estradiol could be restored with epidermal growth factor injections. Thus, the early stages of mammary duct development may require epidermal growth factor.
SYMPOSIUM: ROLE OF THE EXTRACELLULAR MATRIX IN MAMMARY DEVELOPMENT

Figure 2. Effect of epidermal growth factor (EGF) on DNA synthesis by mammary tissue in sham operated or sialoadenectomized mice after 2 d treatment with estradiol (1 μg/d) (Sheffield, unpublished data).

Although the role of epidermal growth factor in mammary gland development is not clear, it may act to increase the synthesis of type IV collagen by mammary epithelia. That collagen synthesis is required for normal mammary development has been shown both in vitro and in vivo (189, 190). Epidermal growth factor has been shown to increase type IV collagen synthesis by epithelial cells and to increase their proliferation (184). However, it cannot be determined unequivocally whether the increase in collagen synthesis is a cause or result of cell proliferation.

Growth In Vitro

Mammary tissue has been successfully grown in vitro by numerous researchers using a variety of systems. These systems can be divided into three main categories: 1) plastic or glass sub-stratum, 2) collagen or other biological sub-stratum (e.g., adipocytes, fibroblasts, irradiated fibroblasts feeder layers), and 3) organ or explant culture in which epithelium is allowed to remain associated with native stroma.

Using organ cultures consisting of either pieces of mammary tissue or intact mammary glands, growth and differentiation of mammary epithelia has been demonstrated in vitro (2, 12, 13, 67, 194). However, an organ culture consists of a number of cell types. Thus, in order to determine hormonal effects on a specific cell type one of the first two methods, which allows isolation and culture of a single cell type, must be used.

Recently, collagen gel cultures of mammary epithelia has become a popular in vitro model (33, 50, 54, 129, 191, 199). Mammary epithelia grown on collagen gels have a more normal morphology than cells grown on glass or plastic. In addition, cells in collagen gel culture appear to retain more normal hormone responsiveness than cells grown on glass or plastic substratum. However, the morphology and hormone responsiveness is not entirely normal. In particular, structures analogous to end buds do not seem to form in collagen gel cultures. In a recent study, Daniel (34) transplanted pieces of collagen gel containing mammary epithelia into gland free fat pads of mice. The epithelia retained their in vitro morphology while in the collagen gel that was within the fat pad. However, when the epithelia grew out of the collagen gel and into the fat pad, a morphologically normal end bud was formed. These observations suggested that collagen gel culture does not entirely duplicate mammary growth in vivo.

More recently, researchers have studied cocultures of mammary epithelial cells and other cell types. Levine and Stockdale (96) observed enhanced differentiation of mammary epithelia cultured with adipocytes. In addition, Haslam and Levely (72) found that culture of mouse mammary epithelia with mouse mammary fibroblasts greatly enhanced estrogen responsiveness of the epithelial cells. These studies clearly indicate the importance of stroma in mammary epithelial development. Such improvements in cell culture methods should make in vitro studies more representative of in vivo physiology.

Mammary Gland Free Fat Pads

DeOme et al. (40) developed a useful technique of studying mammary gland development known as the mammary gland free fat pad. This method is based on the observation that the epithelial portions of the mammary gland of a 3-wk-old mouse are located in the nipple region, with the remainder of the gland being a fat pad devoid of epithelia. The nipple region can be removed surgically, leaving a fat pad devoid of epithelia. Mammary epithelia can then be transplanted into this gland free fat pad and development monitored. Although this technique predates most in vitro research on mammary epithelia, it remains a viable alternative for some applications.
Early research on transplantation of mammary epithelia into gland free fat pads found that mammary epithelia possessed a degree of totipotency (76, 113). That is, epithelia from one part of the mammary gland was usually capable of reforming all of the epithelial structures of the mammary gland. The major exception to this is tissue from the nipple, which often degenerates and is not formed from other tissue elements (78).

Very extensive studies have been reported on mouse mammary epithelia transplanted to mouse mammary fat pads. Hoshino (77) developed a method to transplant quantitatively pieces of mammary duct to the gland free fat pad. This method was based on vital staining of the mammary ducts, which allowed the researchers to remove defined duct segments easily. Shortly after transplantation of a duct segment to the mammary gland free fat pad of a mouse, the basement membrane surrounding the duct degenerated (27). The duct segment was not visible in a whole mount preparation, although individual epithelial cells were still present. Subsequently, the epithelial cells were found to reorganize into a new ductal structure (82, 192). The reorganized epithelia were morphologically indistinguishable from a normal mammary gland except for lacking a nipple. Treatment of mice with mammogenic or lactogenic hormones induced substantial growth and differentiation of the epithelial cells. In addition, ultrastructure of the transplanted cells appeared normal (145). Similar results have also been observed by injecting dissociated mammary cells into the mammary gland free fat pad (63), with male mammary tissue grafted to female mice (18) and with fetal mammary tissue transplanted to gland free mammary fat pads of adult mice (80). Thus, the mammary epithelia are capable of organizing into ductal structures if given the appropriate stroma.

Additional studies were conducted to determine the stromal requirements for epithelial growth (76, 79, 169). Ducts were placed in various locations, including subcutaneously, in the pararenal fat pad, the intrascapular fat pad, and the mammary fat pad. These studies indicated that the amount of available stroma limited growth of the grafted mammary tissue. In addition, they indicated that the preferred stroma for mouse mammary epithelial growth was white adipose tissue.

In addition to studies on mammary duct organization, the mammary gland free fat pad technique has been used to study a variety of other problems in developmental biology. Daniel et al. (31, 32, 35, 37, 38, 200) have used these methods to determine the in vivo lifespan of mammary epithelial cells and to conduct studies on cellular aging in vivo. By transplanting epithelia from older animals into fat pads of young animals, they have determined that aging of the mammary epithelia is intrinsic to the epithelial cell and not due to systemic factors. Similarly, the mammary gland free fat pad has been used to study growth of transplanted tumors (66).

Recently, we have been interested in studying the growth of mammary glands of species other than the mouse. However, ethical considerations preclude repeating much of the research previously conducted in mice on humans and economic considerations prevent repeating it in cows. In order to overcome these limitations, we have begun transplanting bovine and human mammary tissue to immune deficient laboratory animals. Early work with using immune compromised animals for growth of foreign tissue in laboratory animals utilized the hamster cheek pouch (28) or thymectomized mice (135). More recently, we have transplanted mammary tissue to congenitally athymic "nude" mice, particularly to the gland free fat pads of these animals. Thus, we have studied species-chimeric mammary tissue.

THE ATHYMIC NUDE MOUSE

General

Before discussing research on transplanting human and bovine mammary tissue to gland free fat pads of athymic nude mice, a few comments concerning the general nature of the athymic nude mouse are appropriate. An athymic mutation was described in mice by Pantelouris (117) in 1968. This mutation resulted in the lack of a functional thymus gland as well as hairlessness. The immunology of this athymic nude mouse has been studied and reviewed extensively (19, 99, 118, 131, 195). Briefly, this mouse lacks thymic lymphocytes but has natural killer cells and bursa-equivalent lymphocytes. Because it lacks thymic helper cells, it produces relatively little Ig G, but...
produces abnormally large amounts of Ig M. Lacking a complete immune system, the mice are normally maintained under germ-free conditions (44). In our laboratory, mice are maintained in a small animal isolator capable of maintaining class 100 conditions. All supplies, including food, water, and bedding, are sterilized prior to use. Under these conditions the mice remain healthy and the addition of antibiotics to drinking water is unnecessary as a general practice, although it may be advisable under some conditions (e.g., after surgery).

Grafts from a wide variety of donors, including different species and even different orders (e.g., reptiles and amphibians) have been maintained in athymic nude mice (103, 132, 133, 143). Athymic nude mice have been used extensively in cancer research (55). A wide variety of tumors have been grown in athymic mice (56, 57, 58, 59, 116, 124, 134, 144, 161). However, not all tumors are capable of growth in the athymic nude mouse. Among primary breast carcinomas for example, only about 10 to 15% will grow in athymic nude mice. The reasons for this are not clear but could be due to tumor antigenicity (85), natural killer cell activity (86), or hormone dependency of tumors (73, 95).

**Human Tissue Slices Subcutaneously**

The first reports of successful transplantation of normal mammary tissue to athymic nude mice was in 1974, when Outzen and Custer (115) reported the growth of human breast lobules in the gland free mammary fat pad of athymic nude mice. However, there is some question concerning whether the tissue they transplanted was normal. Jensen and Wellings (84) transplanted pieces of human breast tissue to athymic nude mice gland free mammary fat pads and did not observe the extensive proliferation reported by Outzen and Custer (115). Welsch et al. (186) reported successful transplantation of human breast tissue to athymic nude mice as subcutaneous slices of tissue. In subsequent reports, McManus and Welsch (104) found that human breast tissue transplanted to athymic nude mice retained the ability to respond to estradiol. In addition, they observed that a rat pituitary tumor (MT-tw10), which secretes large amounts of prolactin (83), increased growth of human breast tissue if exogenous estradiol were also administered. However, the mice in the study were not ovariectomized. Because prolactin is luteotropic in mice, the observed growth stimulation may have been due to progesterone and not a direct effect of rat pituitary hormones on human breast tissue. Similar considerations apply to other studies in which human placental lactogen increased growth of human breast tissue in athymic nude mice (105, 106). However, subsequent studies showed that progesterone injections failed to enhance DNA synthesis by human breast tissue maintained in athymic nude mice (105). Thus, the possibility cannot be dismissed of secretion of factors mammogenic to human breast epithelia by a rat pituitary tumor.

**Bovine Tissue Slices Subcutaneously**

Welsch et al. (187) reported that transplantation of bovine mammary tissue slices to athymic nude mice. They found that estradiol plus progesterone, bovine growth hormone plus bovine prolactin or estradiol plus progesterone plus growth hormone plus prolactin increased DNA synthesis of the transplanted tissue. Furthermore, they observed that ovariectomy and treatment with hydrocortisone plus prolactin induced α-lactalbumin production by bovine mammary tissue transplanted to athymic nude mice. Sheffield and Welsch (154) confirmed that bovine mammary tissue was maintained in athymic nude mice and that growth of the transplanted tissue was increased by estradiol, estradiol plus progesterone, estradiol plus progesterone plus somatotropin plus prolactin, cholera toxin or estradiol plus cholera toxin (Figure 3). In a subsequent study, Sheffield and Welsch (156) observed that estradiol and progesterone, alone or together, increased growth of bovine mammary tissue in ovariectomized athymic nude mice. Sheffield et al. (159) determined that α-lactalbumin production could be induced in bovine mammary tissue in athymic nude mice by priming mice with estradiol plus progesterone plus somatotropin plus prolactin, followed by ovariectomy and hydrocortisone treatment. Prolactin and rDNA-derived bovine somatotropin increased α-lactalbumin production. Other studies (156) confirmed the ability of the athymic nude mouse to support differentiation of bovine mammary
epithelia when given appropriate hormonal stimulation. In addition to these studies, we observed that patterns of protein synthesis, protein phosphorylation, and protein content (on SDS gel electrophoresis) are similar between tissue maintained in athymic nude mice and tissue freshly excised from midpregnant heifers.

**Rat Tissue in Gland Free Fat Pads**

Daniel et al. (36) transplanted pieces of rat mammary ducts to the mammary gland free fat pads of athymic nude mice and observed that they proliferated in association with mouse stroma. Proliferation of the rat mammary stromal cells was minimal. Welsch et al. (188) injected collagenase dissociated rat mammary cells into the mammary gland free fat pads of athymic nude mice. They found that rat mammary epithelia organized and grew to form patterns very similar to normal rat mammary tissue (the major exception being that no nipple or primary duct was present). The transplanted epithelia were able to respond to mammogenic hormones. These results suggested that the athymic nude mouse readily accepts foreign mammary tissue and that the mouse mammary stroma is able to support normal development of rat mammary epithelia.

**Bovine Tissue in Gland Free Fat Pads**

Sheffield and Welsch (154) injected dissociated bovine mammary tissue into the gland free mammary fat pads of athymic nude mice. Unlike results with xenografted rat tissue, bovine mammary epithelia were unable to proliferate into the mammary fat pad of the mouse (Figure 4). Instead, small, spherical epitheloid organoids formed in the fat pads. The size of the organoids was increased somewhat by estradiol, estradiol plus progesterone, estradiol plus prolactin, estradiol plus somatotropin plus prolactin, cholera toxin or estradiol plus cholera toxin (Figure 5), but they remained spherical. These organoids resembled mammary ducts in histological sections, but exhibited no tendency to elongate or form branched structures typical of mammary tissue. If mice were maintained longer than the 6 wk used by Sheffield and Welsch (154), (up to 6 mo, with and without estradiol plus progesterone treatment), no further development of bovine mammary epithelia was evident in the mouse fat pads (unpublished observations). Furthermore, the mammary organoids were present only if the original bovine mammary tissue was from a nonlactating cow. If tissue from a lactating cow was dissociated and injected into the mammary gland free fat pads of athymic nude mice, no epithelial structures were found (unpublished observations). These data could be explained in a number of ways, including that appropriate hormonal stimulation was not present in the athymic nude mice, epithelial cells were damaged during isolation or the cells became mitotically senescent before they could proliferate extensively. However, tissue transplanted as subcutaneous slices responded readily...
Figure 4. Whole mounts of athymic nude mouse mammary fat pads containing rat (top) or bovine (bottom) mammary epithelia. [From Sheffield and Welsch (154) and Welsch et al. (186).]
Figure 5. Effect of estradiol (E, 1 μg/d), progesterone (P, 1 mg/d), growth hormone (GH, 1 mg/d), prolactin (PRL, 1 mg/d), or cholera toxin (CT, 1 μg/d) for 10 d on area of bovine mammary organoids in mammary gland free fat pads of athymic nude mice. [From Sheffield and Welsch (154).]

to hormonal treatment. The tissue dissociated by the method used by Sheffield and Welsch (154) appears capable of growth. Mitotic senescence of mammary epithelia has been studied extensively in mice but not cows. However, it appears unlikely that this is the reason for the lack of proliferation of bovine mammary epithelia in the athymic nude mouse fat pad, since studies on rodent tissue indicate that mammary epithelia can be serially transplanted several times before growth is greatly diminished (81). However, an attractive hypothesis is that the mouse mammary fat pad does not provide the appropriate stromal elements for proliferation of bovine mammary epithelia. Thus, these observations provide preliminary evidence of a species dependent stromal requirement for mammary duct development.

Since the initial report of bovine mammary organoids in the mouse mammary fat pad, we have examined the stromal elements associated with the mammary organoids in more detail. The bovine epithelial cells do not seem to grow in association with mouse adipose cells but in association with a fibrous tissue whose origin we have not been able to determine unequivocally. However, because this area is associated with the epithelial cells and is often larger than typical for mouse stroma, we believe that it is of bovine origin. Collagen appears to be present in the immediate area of the organoids in greater amounts than the remainder of the mouse fat pad. However, the amount of collagen immediately surrounding the organoids (i.e., the basal lamina) is reduced relative to that around normal bovine mammary ducts. Glycosaminoglycans were also detected by alcian blue staining, but in lesser amounts than typical of bovine mammary ducts. The glycosaminoglycans were more sulfated than typical of growing mammary tissue. The significance of these findings is not clear. However, it is possible that the ability to form an appropriate stroma is a limiting factor in mammary gland development. The collagen content of the mammary gland in the rat changes more slowly than mammary DNA (69, 119, 120, 121), even though collagen synthesis is required for mammary development (190). Sheffield and Anderson (148) found that, in goats, collagen initially comprised about half of the mammary protein. However, about 80% of the increase in total mammary protein that occurred during pregnancy was due to non-collagenous proteins, and collagen comprised only about 20% of the mammary protein by the end of pregnancy. Studies on the size and composition of mammary glands of guinea pigs over successive lactations indicated that all of the increase in mammary size observed over the first five lactations was due to parenchymal tissue (i.e., total collagen remained constant; percent collagen decreased) (7). A series of studies on the effect of hormones on the growth of fibroblasts from guinea pig mammary glands (149, 150, 152) suggested that the growth response of these cells to mammogenic hormones is fairly small, although significant responses
could be detected. Although fibroblasts synthesize only part of the mammary connective tissues, these data suggest that the rate of growth of mammary stroma may be a factor limiting mammary epithelial growth.

Human Tissue in Gland Free Fat Pads

Outzen and Custer (115) initially reported transplantation of human breast lobules to the gland free mammary fat pads of athymic nude mice. They reported outgrowth of epithelial tissue into the mouse fat pad. However, they did not study hormone response of the tissue and there is some question concerning whether or not the tissue they used should be considered normal. In a subsequent study, Jensen and Wellings (84) also transplanted human breast lobules to athymic nude mouse mammary fat pads. They failed to observe proliferation of mammary epithelia into the mouse fat pads, but did not examine whether the proliferation of human breast tissue could be increased by mammogenic hormones. More recently Gusterston et al. (65) transplanted lobules dissected from human breast tissue to mammary gland free fat pads of athymic nude mice. The mice were then bred and the effect of pregnancy on the transplanted tissue examined. They found that DNA synthesis in the transplanted tissue was increased. Also, they found histochemical evidence of induction of milk protein synthesis in human breast tissue in the mammary gland free fat pads of pregnant athymic nude mice. Sheffield and Welsch (157) conducted an extensive set of studies on the effect of hormonal manipulations on growth of collagenase dissociated human breast tissue in the gland free fat pads of athymic nude mice. As with the cow, they found that the cells organized to form hollow balls of cells (organoids) similar to those formed by bovine tissue. The size of the organoids could be increased by hormone treatments (estradiol, progesterone, somatotropin-releasing pituitary tumors, cholera toxin, and human placental lactogen producing choriocarcinomas), but there appeared to be a size limitation beyond which further growth was not possible. Also, elongation, or branching, or elongation plus branching of the organoids was observed only rarely.

Unpublished histochemical studies indicated a pattern of connective tissues similar to that observed in bovine mammary tissue transplanted to athymic nude mouse gland free fat pads. Collagen was associated with the human organoid, but not in amounts as high as in normal human breast ducts, which may explain the lack of growth of organoids. However, because type IV collagen is synthesized by mammary epithelia the lower amounts of this component may be due to the lack of epithelial growth rather than the cause of it.

Glycosaminoglycans were present in the organoid basal lamina. Selective staining for sulfated glycosaminoglycans indicated that the glycosaminoglycans associated with the organoid basal lamina were highly sulfated. Since this is a marker for differentiation into a mature duct, (i.e., differentiation of a proliferating end bud into a resting duct; as opposed to differentiating into lactating tissue) it may be related to the lack of organoid proliferation. The organoids apparently behave as mature mammary ducts. However, while normal mammary tissue is capable of overcoming the growth inhibition seen in mature mammary ducts and proliferate extensively under appropriate hormonal stimulation, this does not appear to be the case with human breast tissue in the athymic nude mouse mammary fat pad.

CONCLUSIONS

The differences observed between normal bovine and human basement membrane and that associated with organoids in mouse mammary gland free fat pads may well explain the lack of proliferation of human and bovine mammary epithelia in mouse fat pads. Numerous studies have demonstrated the importance of collagen and glycosaminoglycans in maintaining and inducing normal morphology in several organs, most notably the mammary gland and salivary gland (10, 15, 16, 17, 62, 114). In this regard, the salivary gland and mammary gland appear similar (i.e., both consist of branched epithelial ducts in a nonepithelial stroma). All studies to date indicate that basement membrane components of such structures are deposited in a regular, highly organized manner. Growth of the epithelia appears to require synthesis and remodeling of the extracellular matrix. At the present time, no information is available concerning why the human and bovine mammary organoids appear to lack the
proper extracellular components for normal growth. However, the observed differences in growth of rat, human, and bovine mammary tissue in mouse mammary fat pads, as well as apparent differences in growth of bovine mammary tissue as subcutaneous slices (in association with bovine stroma) and in mouse mammary fat pads, suggest important species differences in stromal requirements for mammary epithelial growth, which warrants further investigation.

REFERENCES


activities of bovine mammary tissue maintained in athymic nude mice: effects of mamnogenic and lactogenic hormones. J. Dairy Sci. 71:75.


2874 SHEFFIELD


