Vascular Morphogenesis in the Female Reproductive System

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CHAPTER 1

Vascular Morphogenesis in the Mammary Gland: Introduction and Overview

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The mammary gland is unique, it undergoes most of its development well after birth. Although organ immaturity is a constant feature of most tissues after birth, no other structure is associated with the significant changes in size, shape, and function that occur in the breast during puberty, pregnancy, lactation, and involution.

Much of mammary gland research has focused on understanding the growth, differentiation and apoptosis of epithelial cells, which compose the parenchyma of the gland. The contribution of the stroma has been acknowledged as an important modulator of epithelial growth and differentiation. In contrast, the vascular component of the mammary gland has received little attention. The mammary gland offers an important platform for studying blood vessel function including permeability, modulation of angiogenesis, and regulation of vascular regression following weaning. Chapters 1 to 5 illustrate several intrinsic aspects of mammary gland biology, summarize the current knowledge of vascular regulation associated with the development, function, and pathology of this organ, report the available data related to the vascular component of the mammary gland, and emphasize the importance of organ physiology in the regulation of vascular function.

Because of the importance of the angiogenic switch for tumor growth, Chapters 3 to 5 are devoted to this issue. Chapters 3 and 4 provide a summary of the current knowledge of angiogenesis in breast cancer and discuss some of the discrepancies present in the field. Chapter 5 summarizes the current mouse models used to study mammary carcinogenesis and discusses the information gathered related to the development of a vascular supply in these models.

The mammary gland consists of two primary components: the parenchyma, which forms a system of branching ducts from which alveolar secretory units develop, and the adipose stroma, which provides a substrate within which the parenchyma expands and differentiates.

Given the importance of lactation for nurturing offspring and the high incidence of breast cancer, the mammary gland has been the focus of intense research. Most of the interest has concentrated on the epithelial components. In some cases,

the stroma, in particular the role of the mesenchyme in the development of the mammary gland, has also been assessed. However, little attention has been devoted to the vascular network. This chapter summarizes the basic information related to the biology of the murine mammary gland in concert with the growth and regression of vessels in this organ.

Recently, several investigators in the vascular field have concentrated their attention on the mammary gland, and the results from these efforts are beginning to emerge. There is increasing awareness of certain experimentally useful features of this organ:

Late development and accessibility: The mammary gland parenchyma undergoes most of its growth during puberty. Thus, experiments on development can be performed in the adolescent or adult, and there are opportunities for experimental manipulation that are not as available in other organs.

Understanding of the biology of the organ: Much is now understood about the development of the parenchyma and the physiology of the gland. This knowledge can be used to understand the influence of the parenchyma and stroma on the regulation of vascular growth and involution.

Existence of well-developed animal models: Several tumor models have been generated using the mammary gland. In addition, the utilization of "mammary-specific" promoters (including the mammary tumor virus and the whey acidic protein promoters) to target the mammary epithelium offers the opportunity to generate transgenic animal models for overexpression of a variety of gene products. This is discussed at length in Chapter 5. In addition, a large number of techniques, including transplantation, in vitro growth and manipulation of parenchyma, and retroviral and adenoviral infections, have been well established in the literature.

Postembryonic angiogenesis: The mammary gland is one of the few organs that undergoes extensive physiologic angiogenesis in the adult. In addition, events related to alterations in vascular permeability during lactation make this an excellent site to study differentiation and endothelial function including vascular permeability.

Physiological remodeling: During involution (following lactation), a great number of vessels undergo regression, making this organ an excellent site to evaluate remodeling and apoptosis of endothelial cells. The effects of vascular remodeling can also be extended to other cells, particularly pericytes and smooth muscle cells, which contribute to the morphogenesis of blood vessels.

DEVELOPMENT OF THE DUCTAL EPITHELIAL TREE

The development of the mammary gland can be divided into four phases: fetal, postnatal, pubertal, and adult. During fetal life, mammary gland development mirrors the morphogenesis of other glands. The incipient mammary bud grows as an invagination of epithelial cells from the overlying ectoderm. In the mouse fetus this occurs at days 10 to 11 and in the human fetus between weeks 4 and 5. At that time, the initial indications of mammary gland primordia are two parallel lines of ectodermal thickening that extend from the anterior to the posterior limb bud on each side of the embryo (Turner and Gomez, 1933). In the mouse, these lines, referred to as mammary streaks, subdivide into five pairs of mammary gland pri-

mordia at day 12, three of which are thoracic and two inguinal. These lens-shaped epithelial primordia grow into bulb-shaped structures with a narrow neck by day 14, a time when the sexual phenotype in the mammary gland is determined. By day 16 in female embryos, the bud elongates and sprouts into the fatty stroma. Further growth and branching continues until each gland develops 15 to 20 branching ducts at the time of birth. In male mice, further development of the mammary primordia is suppressed by fetal androgens around day 14. The process involves detachment of the mammary bud from the surface epidermis by rupture of the epithelial stalk. This is mediated by testosterone receptor–positive stromal cells that in response to this hormone grow around the epithelial stalk and promote detachment (reviewed in Sakakura, 1987).

In newborn female mice, the parenchyma is organized into small cords of epithelial cells that branch from the nipple (Figure 1.1A). The mammary ducts invade the subdermal mammary fat pad and are highly vascularized by a profuse rete of capillaries that surrounds the epithelial branches. The postnatal phase is characterized by elongation of mammary ducts and further ramification. Growth, however, is slow and is dependent on the presence of the ovary since it can be suppressed by castration even though sexual maturation is not reached until 4 to 5 weeks in the mouse (reviewed by Knight and Peaker, 1982; Daniel and Silberstein, 1987).

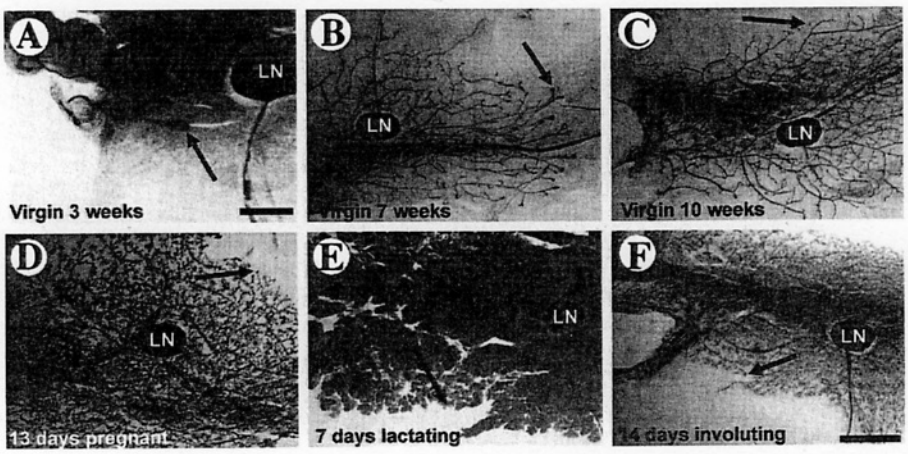

FIGURE 1.1. Growth of murine mammary gland parenchyma. At the specified times, the fourth inguinal murine mammary gland was removed and mounted on a microscope slide, briefly dried, and fixed overnight in 3.7% formaldehyde. Fat was removed by several washes in acetone. Hydration was achieved by serial washes in decreasing concentrations of ethanol. The glands were then stained overnight with carmine solution, washed, dehydrated, and mounted in permount. Carmine strongly stains the parenchyma. The lymphonode (LN) is clearly detected and is a landmark of this gland (fourth pair). A: Ductal invasion into the stroma can be detected by 3 weeks. Note the distended appearance of the terminal end bud (arrow). B: Growth continues during the pubertal stage (7 weeks) by proliferation of the terminal epithelial end-buds (arrow). C: By week 10, parenchymal growth has ceased and the distended end-buds are no longer visible (arrow). D: Additional and profuse epithelial branching (arrow) and growth occurs during pregnancy. E: The terminal alveolar units are distended and occupy most of the available space leaving little room for the fatty stroma. F: Significant remodeling and reduction in the epithelial tree (arrow) are associated with involution of the gland. Bar in A = 2 mm; Bar in B–F = 6 mm.

At about 4 weeks in mice, the ductal tips begin to proliferate, elongate, and invade the mammary fat pad. This is associated with and dependent on the presence of sexual steroid hormones, and marks the pubertal phase. In mice, the extent of epithelial invasion of the fat pad is genetically controlled and varies from strain to strain. Full expansion of the gland is completed between 8 and 10 weeks, again depending on genetic background.

In the parenchyma, the terminal end bud, a distended and highly cellular bulb-like structure (arrows in Figure 1.1A,B) is responsible for morphogenesis of the epithelial tree. The bud is the source of ductal and myoepithelial cells. It has been demonstrated that the end bud is controlled by systemic hormones and by local environmental signals that provide the direction of growth and also lead to regression and involution of the epithelial tree (Faulkin and DeOme, 1960). The final pattern of the mammary ducts is established by an interaction between epithelial and stromal factors.

As the invasion of the ductal tree progressess, blood vessels surround the terminal bud and provide nutrients and oxygen to the rapidly dividing epithelial cells. (A more detailed discussion of vascular growth is provided below.) Concomitant with epithelial invasion there is intense stromal reorganization near the end bud. This area becomes more cellular. Fibroblasts orient themselves along the growing ducts and fill the interstitial spaces with fibrillar collagen (Williams and Daniel, 1983). Secretion of glycosaminoglycans also takes place. In fact, these extracellular matrix components have been shown to be important in the regulation of branching morphogenesis and are likely to be determinants of mammary gland patterning (Bernfield et al, 1984).

Complete growth and maturation is achieved at the point when the mammary parenchyma has grown to the limits of the fat pad and the gland reaches "stasis." Subsequently, during pregnancy, the ductal system increases in complexity by further growth of side branches that sprout from preexisting ducts (Figure 1.1D) (Daniel and Silberstein, 1987). The process can be divided into two stages: the early stage is characterized by rapid growth and branching, resulting in the neoformation of terminal structures called acini or alveolar units. This is clearly seen by day 5 of pregnancy in the mouse. The epithelial cells continue to grow in number and size mainly because of cytoplasmic enlargement. In the second stage, by day 16 to 18 of pregnancy, the structure of the acini becomes more complex with evident myoepithelial cells, distended lumina, and accumulation of secretory material. During lactation, the acini become fully distended as acinar cells secrete milk (Figure 1.1E).

Although the development of the murine, human, and rat glands are very similar, there are some important differences that should be considered when studying each individual model. Figure 1.2 shows the normal histologic alterations in the rat mammary gland during pregnancy, lactation, and involution. Similar alterations are seen in human and mouse glands, with an important exception being that the myoepithelial cells undergo apoptosis in the rat but not in the human or murine mammary gland (Zena Werb, personal communication).

THE MAMMARY GLAND STROMA

As previously alluded to, the mammary gland grows into and is maintained by the underlying and surrounding mesenchyme. Epithelial-stromal interactions appear to be important both for the growth of the mammary epithelium during the

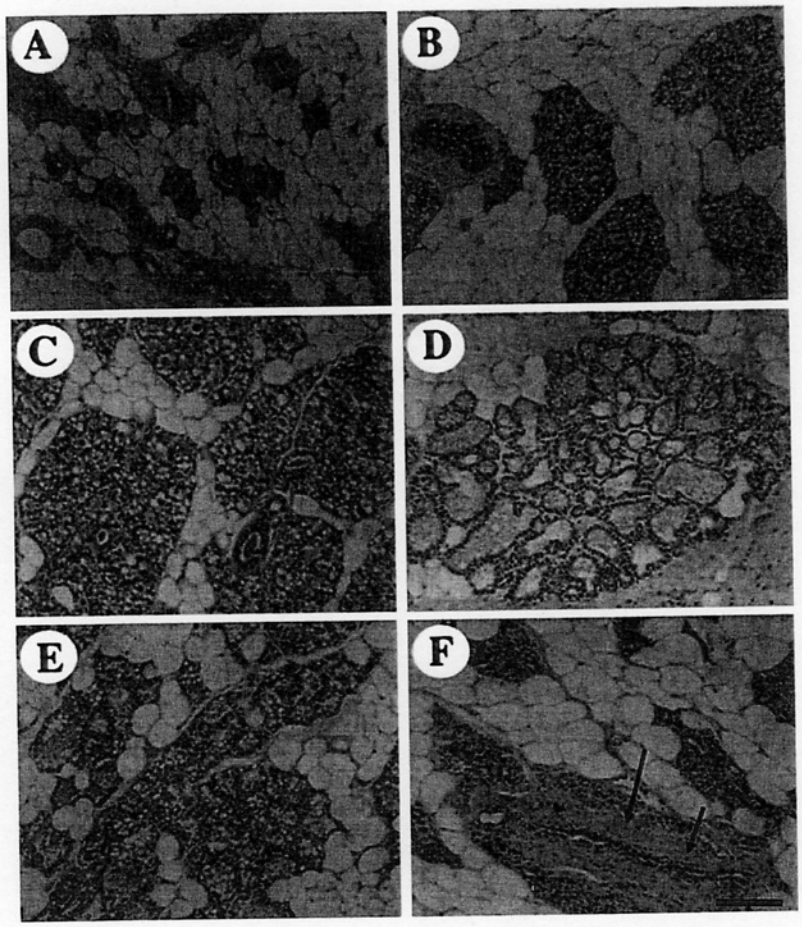

FIGURE 1.2. Histologic changes in rat mammary gland parenchyma and stroma. Rat mammary gland during pregnancy, lactation, and involution. Hematoxylin- and eosin-stained paraffin-embedded sections of (A) 3-month virgin, (B) 12-day pregnant, (C) 18-day pregnant, (D) 2-day lactating (E) 7-day postlactating, and (F) 21-day postlactating mammary gland. Bar = 100 μm.

embryo and for further growth and differentiation in the adult animal. It has also been recognized that the nature of the stroma is important. If mammary epithelium is separated from the surrounding tissue and recombined with mesenchyme derived from other organs, the mammary epithelium makes a typical mammary gland with elongated ducts and end bud formation only if fat pad precursors are present (Sakakura et al, 1982). During development, growth of the adipose stroma occurs normally in the absence of parenchyma. However, mammary epithelial cells cannot grow in the absence of adipose stroma. Furthermore, the stroma can fully support subsequent mammary parenchyma development. Studies by Kratochwil (1987), Sakakura (1991), Lin and Bissel (1993), and Zangani et al (1999a,b) suggest that the stroma plays an inductive role in mammary gland development and function.

Genetic evidence for the influence of the stroma in parenchymal morphogenesis has come from the inhibin null mouse (Robinson and Hennighausen, 1997). These animals are unable to nurse their pups due to aberrant and incomplete devel-

opment of the mammary epithelium. The results clearly demonstrate that ductal elongation and epithelial cell differentiation during puberty require activin/inhibin signaling from the stroma.

Additional important genetic evidence that underscores the relevance of stromal-epithelial interactions has been elucidated through the use of tissue recombinants and transplantation experiments (Wiesen et al, 1999). Ductal growth and branching morphogenesis was normal in transplants of mammary epithelium from Egfr –/– mice into wild-type fat pads. However, epithelial development (growth and branching) was impaired in tissue recombinants prepared with Egfr –/– stroma regardless of the genomic nature of the epithelium (stroma –/– Epi^{wt}, stroma –/– Epi –/–) (Wiesen et al, 1999).

The complete pattern of reciprocal stromal-epithelial interactions is still far from being elucidated, but a few steps are known. First, mammary mesenchyme "determines" mammary epithelium and fixes the ability of embryonic mammary epithelium to interact with the fatty stroma (Propper, 1970; Sakakura et al, 1982; Zangani et al, 1999a). Second, the mammary epithelium induces androgen receptors in the mammary mesenchyme by direct cell-cell contact and thereby controls the development of androgen responsiveness in this tissue. If androgen is present at this stage, the mammary mesenchyme responds to the hormone and condenses around the mammary epithelium, causing detachment of the mammary bud from the ectodermal surface and subsequent suppression of epithelial growth (Kratochwil, 1971). From these studies, it is apparent that the mammary mesenchyme is both the target and the mediator of the morphogenetic effect of androgens on mammary epithelium during development. Interestingly, only mesenchyme from the mammary gland is able to respond to androgens and regulate epithelial morphogenesis. Fibroblasts from other organs were unable to condensate around the epithelium in response to androgens. It is clear that fibroblasts from mammary glands have androgen receptors and that there is a strong degree of specificity as to the ability of other cells to affect mammary epithelium.

Third, a very important contribution of the stroma is the synthesis and secretion of growth factors, such as transforming growth factors α and β (TGF-α, -β), among many others (Daniel and Robinson, 1992) as well as extracellular matrix proteins and proteases that clearly modulate epithelial function.

Through a series of very elegant studies, Bissell and colleagues (Howlett and Bissell, 1993; Boudreau et al, 1995; Pujuguet et al, 2000) have provided proof that specific extracellular matrix components are essential for the induction of mammary-specific genes, maintenance of the epithelial differentiated phenotype, and prevention of apoptosis.

An equally important contribution of the stroma is the expression of matrix metalloproteinases and their inhibitors. A clear example has been provided by experiments in which overexpression of stromelysin-1, also known as matrix metalloproteinase-3 (MMP3) was targeted to the mammary epithelium. In these animals, the glands develop more branches, show early differentiation of alveoli, and undergo precocious apoptosis during pregnancy. This is also associated with formation of an abnormal stroma and development of neoplasia (Sympson et al, 1994; Sternlicht et al, 1999). In summary, these data support the notion that disruption of the basement membrane can regulate branching morphogenesis during development, apoptosis, stromal organization, as well as induction and progression of cancer.

KINETICS OF VASCULAR GROWTH

Early studies indicated that the mammary gland vasculature is a dynamic and highly specialized structure that undergoes alterations associated with the physiologic state of the gland (Wahl, 1915; Turner and Gomez, 1933; Soemarwoto and Bern, 1958). For example, functional changes in permeability have been reported in late pregnancy and during lactation (Matsumoto et al, 1992a,b, 1994a, 1995a,b; Yasugi et al, 1989). However, with the exception of these studies, little has been documented on the growth and involution of capillaries in the mammary gland, despite a large amount of interest in angiogenesis in mammary tumors (see Chapters 3 and 4).

Our interest in understanding the physiologic changes in mammary vasculature was born from the need to interpret the effect of overexpression of angiogenesis inhitors in this organ (Iruela-Arispe et al, 1999a,b; Ortega and Iruela-Arispe, 2000). While comparing the vascular changes in the transgenic animals, we performed a systematic morphologic and quantitative analysis of the vasculature during fetal and pubertal growth, pregnancy, lactation, and involution, which we summarize below.

During midembryonic stages in the mouse (day 10), differentiation of vascular structures with progressive angiogenic growth occurs in concert with the development of the mammary fat pad stroma and prior to the differentiation of the parenchyma. As epithelial buds invade the underlying mesenchymal tissue (embryonic day 14), capillary sprouts grow toward and surround the incipient epithelial structures. A second wave of angiogenesis progresses in concert with epithelial expansion during puberty by recruitment of new vessels from the highly vascularized fat pad (Figure 1.3A,B). Although interconnected, parenchymal vessels are subject to greater changes (angiogenesis and regression) than those in the fatty stroma. These vessels are more stable, as revealed by proliferation (data not shown) and apoptosis assays (discussed below).

The association between parenchyma and new vessels becomes clearly apparent in whole-mount preparations using specific labeling to visualize the vasculature. We found that whole-mount analysis is a very effective method for examining the extent of parenchymal-vascular interactions. For example, the topologic distribution of new vessels to the ingrowing alveoli during pregnancy (Figure 1.3C), as well as the embracement of epithelial ducts by capillaries, has only become apparent through this technique. Also variations in the capillary plexus, such as distention in the absence of proliferation, can be observed in whole-mount preparations of lactating glands. Note the distended thin capillaries in Figure 1.3D.

As a complement for visualization of epithelial-capillary interactions, a more detailed cellular resolution can be achieved by confocal microscopy. This technique provides, in a single specimen, cytologic evaluation within a visual three-dimensional framework (Figure 1.4; see color insert). It is also prone to quantitative applications through computer programs that enable assessment of several morphometric parameters. Using this approach, we have undertaken a systematic study of mammary gland vasculature and have gathered morphometric information related to the changes associated with growth and involution of the gland. As presented in Table 1.1, expansion of the vascular component follows the expansion of epithelial structures during puberty and pregnancy. To reveal vascular architecture, animals were injected with fluorescein isothiocyanate (FITC)

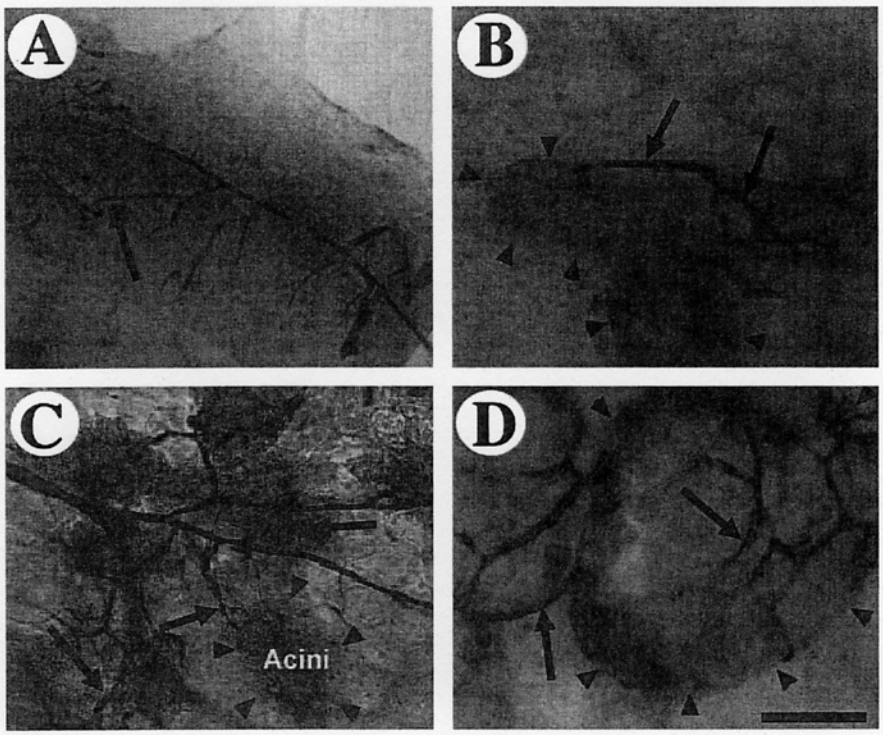

FIGURE 1.3. Capillary growth and expansion in the murine mammary gland. A: During pubertal growth (week 6) ingrowing parenchyma recruits vessels (arrow) from the previously vascularized fatty stroma as can be seen in this carmine-stained whole-mount preparation. B: High magnification of a terminal end bud (arrowheads) at week 6 of postnatal development. Note the intimal association of epithelial cells with the newly angiogenic vessels (arrows). C: By midpregnancy, significant angiogenesis has occurred and results in vascularization (arrows) of the differentiating lobuloalveolar units (acini, arrowheads). D: In the 2-day lactating gland, distended capillaries (arrows) embraced the milk-producing alveolar units (arrowheads). Bar in B and D = 50 μm.

conjugated *Lycopersicon esculentum* lectin followed by perfusion fixation. The *L. esculentum* lectin has been shown to bind to the luminal surface of murine endothelial cells and it binds equivalently to all categories of normal vessels (arterial, venous, and capillaries) (Thurston et al, 1998). Precautions were taken to allow fair comparison between different animals and mammary gland stages. The following criteria were used for analysis: (1) evaluations were carried out using the same scanning parameters (magnification, thickness, laser intensity, reconstruction analysis); (2) for pregnant and lactating animals, only females pregnant with eight embryos and suckling offspring were included; (3) for involuting glands the females were allowed to feed eight pups for 7 days, at which time the animals were removed (time of removal = day 0 of involution); (4) several females (three to five) were evaluated per stage to ensure appropriate sampling and to enable statistical analysis; (5) the area scanned was maintained as X (width) = 2.5 mm by Y (length) = 2.5 mm by Z (depth) = 250 μm; and (6) four areas were evaluated from each gland; the location used to perform the scan was exactly 500 μm from the lymph node (north, south, east, and west). The gland studied was always the fourth inguinal left gland. Volume measurements were obtained using ImagePro software algo-

TABLE 1.1. Morphometric changes of parenchymal and capillary structures during mammary gland growth and involution.

Stage	Vascular-epithelial volume (mm^3)	Parenchymal volume (mm^3)	Vascular-stromal volume (mm^3)	Intercapillary volume (mm^3)
Virgin				
7 weeks	53 (± 7)	354 (± 12)	194 (± 21)	87 (± 7)
10 weeks	297 (± 58)	427 (± 21)	173 (± 19)	98 (± 3.5)
13 weeks	288 (± 39)	431 (± 19)	187 (± 17)	85 (± 8.2)
Pregnant				
2 days	284 (± 28)	529 (± 22)	199 (± 25)	99 (± 10.5)
4 days	527 (± 59)	927 (± 31)	252 (± 22)	52 (± 15)
6 days	993 (± 108)	1,227 (± 47)	203 (± 38)	39 (± 11)
12 days	1,127 (± 87)	2,096 (± 52)	195 (± 19)	43 (± 7)
18 days	1,183 (± 77)	2,772 (± 51)	184 (± 20)	74 (± 12)
Lactating				
2 days	1,034 (± 83)	3,455 (± 47)	174 (± 23)	201 (± 25)
4 days	1,187 (± 128)	6,470 (± 42)	168 (± 27)	527 (± 45)
7 days	1,274 (± 144)	7,273 (± 59)	162 (± 19)	558 (± 25)
Involuting				
2 days	1,179 (± 98)	4,274 (± 39)	177 (± 21)	374 (± 42)
4 days	1,034 (± 92)	2,478 (± 29)	172 (± 17)	281 (± 38)
7 days	743 (± 34)	1,567 (± 33)	194 (± 19)	134 (± 25)
14 days	493 (± 52)	674 (± 21)	187 (± 22)	157 (± 19)

Evaluation of volume was performed by scanning fluorescently labeled specimens (Figure 1.4) and following specific parameters (described in text). Numbers represent the average of three independent specimens (± SD). Volume obtained from one specimen is the mean of four scans, as indicated in text.

rithms. Quantitation was performed at two levels within the gland: (1) at the same level as the parenchyma, and (2) in the fatty stoma located above the parenchyma. These evaluations provided the first indication that the vascular component in the stroma varies little in comparison to the vessels associated with the parenchyma (Table 1.1).

Capillary growth is associated with the invasion of parenchyma during the pubertal phase. A significant increase in capillary volume is detected by week 10 in comparison to week 7. After week 10, there are no significant alterations in volume. At this time, the vasculature reaches "stasis." A second wave of postnatal angiogenesis occurs during pregnancy and can be detected as early as day 4. Little to no expansion was observed late in pregnancy or during lactation. No statistically significant vascular expansion was seen after day 12 postconception.

Evaluation of intercapillary distance was also revealing. In early pregnancy, capillaries are closer to one another (Figure 1.4B and Table 1.1). During lactation, however, a 10-fold or greater intercapillary distance can be observed (Figure 1.3D and Table 1.1). This is most likely due to the distention associated with alveolar secretion. Variations in intercapillary distance are an important consideration, since quantitation of vascular profiles by histologic means alone would most likely indicate a reduction in the vascular density during this time, which is artifactual. No apoptosis in vessels was detected during this stage (data not shown).

An important conclusion also reached from these analyses is that regression of capillaries during involution of the mammary gland occurs subsequent to the remodeling events in the parenchyma. While parenchymal volume decreased

significantly by days 2 to 7, vascular volume was only reduced from 7 to 14 days postweaning (Table 1.1). Vascular regression is discussed in detail below.

Studies using mammary-derived endothelial cell cultures are scant (Gumkowski et al, 1987; Hewett et al, 1993). However a clear protocol for their isolation has been reported (Hewett et al, 1993). The heterogeneous response of parenchymal versus stromal-associated vessels is intriguing, and further understanding of the molecular basis of this diversity is warranted.

GROWTH REGULATION IN THE MAMMARY GLAND

Systemic Growth Regulators of the Mammary Gland

The initial understanding of the stimuli responsible for mammary gland growth resulted from classic endocrine ablation and replacement studies (Lyons, 1958; Nandi, 1958). More recently, the use of homologous recombination and the generation of null mice that lack hormone receptors have confirmed and expanded these original observations (Lubahn et al, 1993; Lydon et al, 1995), but fundamentally the major conclusions reached at that time stand.

Early endocrine studies performed with ovariectomized and hypophysectomized rodents showed that treatment with estrogen and growth hormone stimulated vigorous end bud growth in areas where ducts faced noninvaded stroma (Lyons, 1958). Interestingly, increased branching was not seen, and little to no effect was detected if mature glands were used (Nandi, 1958). These findings support the concept that local environmental clues are in place to further regulate the growth of the gland independently of systemic signals.

Estrogen

Administration of estrogen to ovariectomized females leads to expansion and branching of the ductal parenchymal system. Nonetheless, in vitro studies using mammary epithelium have mostly failed to demonstrate a direct proliferative effect with estradiol (Yang et al, 1980a,b; Imagawa et al, 1982). However, mixed cultures of mammary epithelial cells and stromal cells have resulted in increased epithelial proliferation in the presence of estradiol (McGrath, 1983; Haslam and Levely, 1985; Haslam, 1986). Together these results indicate that the influence of estrogens on mammary gland morphogenesis is likely to be indirect, and dependent on stromal cells.

The generation of animals that lack the estrogen α-receptor support the essential role of estrogen signaling for mammary epithelial expansion (Lubahn et al, 1993). Although ductal invasion and proliferation occurred during fetal development, estrogen receptor null mice showed poor ductal expansion during puberty and upon exogenous administration of estrogen and progesterone.

Endothelial cells express the estrogen receptor (Kim-Schulze et al, 1996). The effects of estradiol on endothelial cells include increased angiogenesis, nitric oxide production, and suppression of apoptosis (Morales et al, 1995; Rubanyi et al, 1997; Spyridopoulos et al, 1997). Although no systematic studies have been performed on mammary vasculature in estrogen receptor (ER)-null mouse, the compromised nature of the epithelium is likely to be a caveat in the interpretation of results. The generation of cell-specific ER-null animals (particularly endothelial cells with

no ER) through the cre-lox system could add important information as to the role of estrogen signaling in mammary gland angiogenesis, particularly during pregnancy.

Progesterone

Until recently the contribution of progesterone to mammary gland development has been difficult to ascertain, with studies supporting both suppressive and proliferative roles. However, the phenotype displayed by the progesterone receptor knockout (PRKO) mouse has recently shed light on this controversial issue (Lydon et al, 1995). Although there is no significant difference in ductal development between wild-type and PRKO mice at 6 weeks, hormonally treated glands were markedly different. Treated progesterone receptor (PR)-null animals showed poor ductal side-branching and lack of lobuloalveolar development in contrast to control littermates (Lydon et al, 1995; Humphreys et al, 1997; Brisken et al, 1998). These findings provided unquestionable proof that progesterone is required for complete mammary gland development, and it is likely that the effects of progesterone are indirect (Brisken et al, 1998). To this end, it has been demonstrated that the lack of branching displayed by the PRKO animals can be overcome by ectopic expression of the proto-oncogene *Wnt-1*. It has been proposed that Wnt proteins function as paracrine factors that operate downstream of progesterone to mediate the process of side-branching. In addition, Wnt-4 has an essential role in branching during early pregnancy. Progesterone has been shown to induce Wnt-4 expression during pregnancy. Thus, it appears that Wnt signaling is essential in mediating progesterone function during mammary gland morphogenesis (Brisken et al, 2000).

Endothelial cells also express the progesterone receptor (Vazquez et al, 1999). Treatment of culture endothelial cells with progesterone results in cell cycle arrest in early G1 (Vazquez et al, 1999). It is likely that this hormone suppresses angiogenesis, given its effects on endothelial growth suppression, yet these experiments have not been performed in vivo. As with estrogen, the role of progesterone in the regulation of mammary gland vasculature is unknown.

Prolactin

Mammary gland development is severely compromised in mice lacking prolactin (Brisken et al, 1999). Although growth of epithelial structures appears normal up to puberty, further expansion and alveolar differentiation is suppressed. In addition, heterozygous females showed significantly reduced mammary gland development during the first pregnancy (Ormandy et al, 1997). This was associated with almost complete failure to lactate. The effects of prolactin on mammary development during puberty appear to be indirect, since transplantation of epithelium from null mice into fat pads of wild-type animals showed normal developmental expansion and differentiation. In contrast, the effects on lobuloalveolar differentiation appear to be directly mediated by this hormone (Ormandy et al, 1997).

Interestingly, a 16 kD fragment of prolactin has been shown to suppress the mitogenic response of endothelial cells to vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) and to inhibit angiogenesis in vivo

(Clapp and Weiner, 1992; Clapp et al, 1993; Duenas et al, 1999). Although the specific mechanism of action of prolactin on endothelial cells is unclear, it appears that its effects are not mediated by the classic prolactin receptor. Treatment of endothelial cell cultures with 16 kD prolactin suppresses VEGF-induced Ras activation (D'Angelo et al, 1999). In addition, prolactin has been shown to stimulate plasminogen activator inhibitor-1 and suppress urokinase activity, which can also contribute to its antiangiogenic effects (Lee et al, 1998).

Local Regulators of Mammary Gland Growth

Epidermal Growth Factor

The lack of a more profound growth effect in vivo in hormonal experiments with mature mammary glands, or even with end buds in vitro, suggested that other factors act in conjunction with systemic hormones to achieve a well-balanced growth regulation. Using end buds implanted in collagen gels in vitro, Yang et al (1980b) demonstrated that epidermal growth factor (EGF), as well as agents that increase intracellular levels of cyclic adenosine monophosphate (cAMP), strongly stimulated mammary epithelial proliferation. Similar experiments performed later by Nandi et al (1984) demonstrated that a combination of insulin, EGF, transferrin, bovine serum albumin, and cholera toxin stimulated cellular proliferation up to 10-fold within 10 days. Additional experiments demonstrated that EGF at low concentrations (1 ng/mL) could synergize with prolactin and progesterone to stimulate growth (Imagawa et al, 1985).

Evaluation of EGF receptor activity by autoradiography in a hormonally intact, 5-week-old animal showed a heavy concentration of receptors in the stroma immediately adjacent to the end bud, with less dense labeling in stroma surrounding the cap region. Few, if any, receptors were seen in the luminal epithelium. These results were also supported by quantitative analysis of EGF receptor in vitro using fibroblast and epithelial cell lines from mammary glands. The evaluation revealed about a 100-fold more receptors in the fibroblast cell line (Danielson et al, 1984).

More mechanistic experiments showing the participation of EGF signaling were recently performed using EGF receptor (EGFR) null mammary tissue in combination with transplantation assays. Growth of neonatal EGFR null mammary glands under the renal capsule of wild-type animals showed that the participation of EGF is essential for mammary ductal growth and branching morphogenesis, but not for mammary lobuloalveolar development. The impairment in ductal growth is mediated by the stroma and not the epithelial components (Wiesen et al, 1999). These experiments provide additional support to the role of stromal-epithelial interactions in mammary mophogenesis.

Transforming growth factor-α is related to and mimics EGF by eliciting its effects through binding to the EGF receptor. TGF-α is known to be present in rapidly growing normal tissues including the mammary gland. Transgenic animals overexpressing TGF-α exhibit mammary hyperplasia and increased incidence of mammary carcinogenesis, and a shorter latency period for mouse mammary tumor formation after carcinogen treatment (Jhappan et al, 1990; Matsui et al, 1990; Sandgren et al, 1990).

Transforming growth factor-β has been demonstrated by several investigators to play an important role in mammary gland biology. Slow release pellets con-

taining TGF-β can inhibit ductal growth (Silberstein and Daniel, 1987). The other two mammalian isoforms, TGF-β_2 and -β_3, have also been examined and shown to cause disappearance of the proliferating mammary stem cell layer, involution of ductal end buds, and suppression of glandular growth (Daniel and Robinson, 1992). These results are consistent with growth inhibitory effects of TGF-β on mammary epithelial cells in vitro (Ethier and Van de Velde, 1990; Knabbe et al, 1987). Overexpression of TGF-β in transgenic mice elicits general mammary hypoplasia (Jhappan et al, 1993; Pierce et al, 1993). Reduction of mammary ductal branching was evident at 7 weeks of age and even more pronounced at 13 weeks (Jhappan et al, 1993). Lactation is not possible in TGF-β transgenic mice due to failure of lobuloalveolar development and suppression of milk protein production (Pierce et al, 1993).

Angiogenic Growth Factors

Little is known about the mechanisms that regulate growth and remodeling of mammary gland vasculature. Recently we have reported the expression of VEGF and VEGF-C, as well as VEGFR1, -2, and -3 during the cycle of pregnancy-associated mammary growth, lactation, and involution (Pepper et al, 2000). Angiogenic growth factors, and in particular the VEGF family of cytokines, are discussed in depth in Chapter 2.

Inhibitors of Angiogenesis

The 16 kD form of prolactin has already been discussed, although the role of this fragment in the regulation of the vasculature in the mammary gland is not known.

Another inhibitor of angiogenesis, thrombospondin-1 (TSP-1) (Iruela-Arispe et al, 1991, 1999b; Tolsma et al, 1993), has been more thoroughly studied in the mammary gland. TSP-1 messenger RNA (mRNA) is easily detected in the virgin postpubertal gland, yet it is rapidly downregulated during pregnancy and lactation. Increased transcript levels were detected in regressing glands, particularly 4 days after the removal of pups (Ortega and Iruela-Arispe, 2001). Overexpression of TSP-1 in the mammary epithelium using the mammary mouse tumor virus promoter results in a dramatic suppression of parenchymal-associated vessels during the first pregnancy (Ortega and Iruela-Arispe, 2001). Interestingly, mammary glands of TSP-1–null animals showed defective vascular regression during involution. In both cases, the vascular phenotypes were also associated with epithelial abnormalities. In the null animals, excessive epithelial branching was detected, while hypoplasia was noted in the TSP-1–overexpressing mice. Although direct effects of TSP-1 were also detected on mammary epithelial cells in culture, these results raise important questions related to the effects of abnormal vascularization on parenchymal growth and differentiation. A recent report utilizing an in vitro model that allows heterotypic cell interaction (endothelial and breast cancer cell tissue) has demonstrated that actively proliferating endothelial cells are essential for ductal-alveolar morphogenesis of preneoplastic epithelial cells (Shekhar et al, 2000). It is hoped that cell-targeting knockout models will enable us to discriminate between direct and indirect effects of TSP-1 in mammary epithelium.

VASCULAR REGRESSION DURING APOPTOSIS

The involuting mammary gland offers one of the most impressive examples of tissue remodeling, during which epithelial cells engaged in the production of milk die by apoptosis and the entire organ is restructured. The process appears to involve two phases (Lund et al, 1996):

1. Apoptotic phase, which is characterized by rapid induction of proapoptotic genes within the epithelium (days 1 to 5 postweaning).
2. Remodeling phase, which is characterized by the induction of genes encoding proteases within stromal cells that results in the remodeling of the gland (days 4 to 21 postweaning).

These phases, particularly the apoptotic phase, are significantly enhanced by loss or removal of the suckled offspring. Natural weaning differs greatly from forced weaning, as autocrine inhibition of milk secretion is likely to prevent the dramatic milk stasis that occurs if the pups are abruptly removed. During natural weaning, there is a progressive reduction in the milk secretion rate (controlled by galactopoietic hormones), which matches the lowering milking demand. Apoptosis is thus less abrupt and more spread out during late lactation stages. (For a review on epithelial mammary gland involution see the *Journal of Mammary Gland Biology and Neoplasia* 4(2) 1999.) The initial signals that lead to apoptosis in the mammary gland have not been completely defined, but major candidates include alterations in survival molecules such as insulin-like growth factor-1 (IGF-1) and glucocorticoids, availability and distribution of active metalloproteases, and presence of high levels of milk, which contains apoptosis-inducing factors (Strange et al, 1992; Feng et al, 1995; Lund et al, 1996; Tonner et al, 1997; Sudhakaran et al, 1999; and reviewed by Streuli et al, 1997; Furth, 1999).

Recently much of the intracellular pathway that leads to apoptosis has been elucidated, and it is known that the cysteine protease family of caspases partakes in the processes that result in program cell death. In addition, several members of the Bcl-2 family of proteins participate in the fine-tune regulation of the apoptotic process (Farrow and Brown, 1996; Jacobson, 1997; Metcalfe and Streuli, 1997; Green and Kroemer, 1998; Colitti et al, 1999). Members of the Bcl-2 family include proapoptotic proteins such as Bax and Bak, and death-suppressors such as Bcl-x and Bcl-w (Oltavai et al, 1993; Farrow et al, 1995; Gibson et al, 1996). All of these proteins have been found to regulate epithelial apoptosis during the involution of the mammary gland (Metcalfe et al, 1999). Although the specific mode of action of these proteins has not been completely elucidated, they appear to act as switches that determine the apoptotic cell fate. They form homo- and heterodimers with each other and are able to act in independent pathways to promote or repress apoptosis (Gross et al, 1998; Knudson and Korsmeyer, 1997). In a recent paper, Metcalfe and colleagues (1999) demonstrated that mammary parenchymal apoptosis, triggered during weaning, is regulated by de novo expression of Bak and Bad and repression of Bcl-x and Bcl-2. The timing was associated with maximal epithelial apoptosis during days 1 to 3 postweaning. This was also associated with increased levels of p53, another important player during mammary gland apoptosis (Jerry et al, 1999).

Currently there is no information in the literature regarding the remodeling of the vascular bed upon regression of mammary parenchyma. Recently, we have been able to reproduce the results published by Metcalfe and shed some light on capillary regression and postweaning apoptosis. Our findings indicate that regression of the capillary network occurs secondarily to that of the mammary epithelium (Figure 1.5; see color insert). These findings were initially obtained by morphometric analysis during mammary gland involution (Table 1.1) and were subsequently reinforced by gene expression analysis (Figure 1.6).

To determine the progression of apoptosis in the capillary network independent from that of epithelial structures, we devised a rapid isolation procedure that separates endothelial from epithelial structures. The technique involves digestion with collagenase and differential filtration (Figure 1.5C). The preparations are

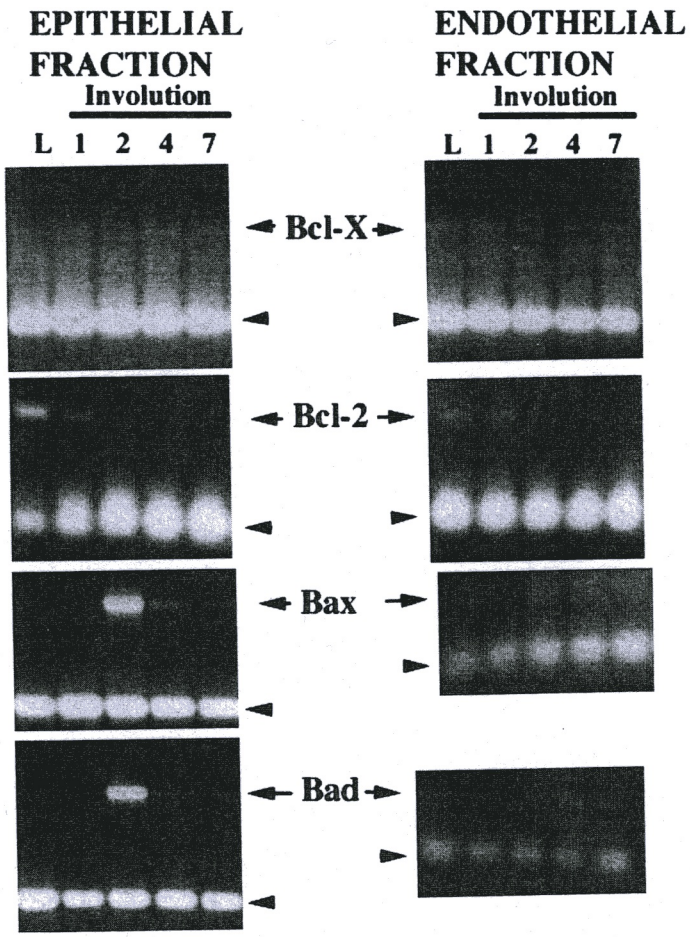

FIGURE 1.6. Evaluation of Bcl-2 and other family members during murine mammary gland apoptosis. RNA from parenchymal (epithelial) and vascular (endothelial) fractions (Fig. 1.5D,E) was subjected to RT-PCR for assessment of several members of the Bcl-2 family as indicated. L, lactation; 1, 2, 4, and 7 days after removal of pups. Arrowhead in each gel represents migration of L-32 product used as an internal control.

about 85% pure capillaries or epithelial alveoli and some ducts. Most of the fibroblasts are contained in the capillary fraction. The purity of the samples has been assessed by reverse-transcriptase polymerase chain reaction (RT-PCR) using keratin 18 as a marker for epithelial cells and CD-31 as indicator of endothelial cells (Figure 1.5D,E).

Initial assessment of apoptosis was performed by Southern blot analysis (Figure 1.5F,G). While epithelial apoptosis peaked at day 2, apoptosis of endothelial cells peaked at days 4 to 5. Another important distinction is that while most alveolar epithelial cells died postweaning by apoptosis, the extent of endothelial cell death did not appear to be as significant, as revealed by Southern blot (Figure 1.5F,G) and terminal transferase UTP nick end labeling (TUNEL) analysis (data not shown).

A more refined study of epithelial apoptosis was performed by RT-PCR analysis for expression of Bcl-2 family members; specifically, we examined Bcl-x, Bcl-2, Bac, and Bad (Figure 1.6). Our results confirmed the recent publications on epithelial apoptosis (Figure 1.6-epithelial fraction). We found constant levels of Bcl-x, reduction in Bcl-2, and upregulation of Bak and Bad. The Bak and Bad peak was seen 2 days after weaning, in contrast to day 1 in the published report (Metcalfe et al, 1999). Nonetheless, our animals were 129-BALB/c mice while the Metcalfe study used ICR mice. Both studies concur on the point that both Bak and Bad appear to play a significant role in triggering epithelial apoptosis.

The same evaluation for levels of Bcl-2 family members was applied to the isolated endothelial cells. Here the levels of Bak and Bad were only increased at days 4 to 5 during involution of the gland. Furthermore, Bak appeared to be more prevalent than Bad. In contrast to epithelial cells, the decreased levels of Bcl-2 were not as dramatic.

CONCLUSION

Little is understood about the biology of vascular alterations associated with the mammary gland. Yet this organ offers a significant opportunity to answer questions related to the mechanisms of angiogenic expansion and remodeling in a whole-organ system. Of great importance are the differences in the response of the parenchymal versus the stromal-associated vessels. It is clear that these two populations, although interconnected, are regulated by different mechanisms, some of which might be epithelial in nature. Additional and equally important questions include: What are the factors that mediate angiogenic growth and regression? Is there a direct role for estrogen and progesterone in vascular growth regulation? What is the participation of vascular mural cells (pericytes and smooth muscle) in the mammary vascular cycle?

The increasing number of animal models (including gene deletion studies), combined with the accessibility of the organ, should enable extensive mechanistic experimentation. It is clear that additional and more molecular studies are required to advance our current knowledge in this important area of vascular biology.

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Vascular Morphogenesis in the Female Reproductive System

The field of angiogenesis is one of the most exciting fields in contemporary biomedical research. Angiogenesis in the healthy adult is primarily associated with pathological conditions such as tumor growth, inflammation, ischemia, and retinopathies. Physiological angiogenesis in the healthy adult is restricted to the organs of the female reproductive system where it occurs cyclically as well as during pregnancy. The importance of vascular morphogenetic events in the female reproductive system is increasingly being recognized. For example, it has a vital role in normal ovarian function, endometriosis, and placental function as well as being important in reproductive tumors of the mammary gland, ovary, and uterus. The insights gained from studying this system have a more general interest too since they help shed light on the fundamental characteristics and components involved in the formation of the vascular system and blood vessels during development and tumorigenesis.

This is the first book that reviews and presents recent advances in the emerging field of reproductive vascular biology. It is divided into four parts reflecting the four female reproductive organs (breast, ovary, uterus, and placenta), and the authors cover the basic biological processes involved and also stress their clinical significance.

The book will interest vascular and reproductive biologists as well as cell and developmental biologists interested in angiogenesis and vasculogenesis.

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