Vascular Morphogenesis in the Female Reproductive System

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CHAPTER 2

Growth Factor Regulation of Physiologic Angiogenesis in the Mammary Gland

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During embryogenesis, the formation of new blood vessels occurs via two processes: vasculogenesis and angiogenesis. Vasculogenesis involves the de novo differentation of endothelial cells from mesoderm-derived precursors called angioblasts, which then cluster and reorganize to form capillary-like tubes (Risau and Flamme, 1995). Once the primary vascular plexus is formed, new capillaries form by sprouting or by splitting (intussusception) from preexisting capillaries in the processes called sprouting or nonsprouting angiogenesis, respectively (Risau, 1997). In postnatal life, the growth of normal as well as neoplastic tissues depends on angiogenesis. Angiogenesis is particularly important for normal reproductive function, including the cyclical growth of capillaries within the ovary (required for ovulation and corpus luteum formation) and the endometrium (required for regeneration following menstruation). Angiogenesis also occurs following implantation of the blastocyst, and is required for the formation of the placenta (Findlay, 1986). This chapter discusses the role of physiologic angiogenesis and angiogenic growth factors in relation to mammary gland function.

The mammary gland is one of the few organs that undergo hormonally regulated cycles of growth and morphogenesis during adult life. This makes it a particularly attractive model for studying angiogenesis (during pregnancy), vascular permeability (during lactation), and endothelial cell apoptosis (during postweaning involution). However, surprisingly few studies have been done, and thus very little is known about the molecular mechanisms of physiologic angiogenesis in the mammary gland.

Various cytokine families have been implicated in the neovascularization process. These include, but are not limited to, the vascular endothelial growth factor (VEGF) and angiopoietin families. The former is comprised of structurally related members, including VEGF (also known as VEGF-A), placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and Orf virus VEGFs (also called VEGF-E) (PlGF). VEGFs bind to three transmembrane receptors (VEGFR-1 to -3), which possess intrinsic tyrosine kinase activity and are mainly expressed in

the endothelial cells. Two angiopoietins have been well characterized, namely Ang-1 and Ang-2, both of which bind with similar affinity to the Tie-2 (Tek) receptor. The VEGFRs and Tie-2 are expressed almost exclusively by endothelial cells.

While angiogenesis is required for growth of normal and neoplastic tissues during adult life, active lymphangiogenesis has not been detected in tumors or in normal adult tissues. However, the function of lymphatics is crucial both for the normal physiology of the mammary gland as well as for the understanding of the mechanisms of lymphatic metastasis in breast cancer. VEGF-C is a growth factor for both lymphatic and blood vessels, and the expression of its receptor VEGFR-3 becomes restricted mainly to the lymphatic endothelium during development (Kaipainen et al, 1995).

VEGF FAMILY AND RECEPTORS

All VEGF family members possess a homology domain containing eight distinctly spaced cysteine residues. In addition, VEGF-C and VEGF-D have long N- and C-terminal extensions, of which the N-terminal parts do not display sequence homology to any known protein, whereas their C-terminal portions contain motifs similar to the Balbiani ring 3 protein, a major component of silk produced by the midge *Chironomus tentans* (Joukov et al, 1996; Lee et al, 1996; Orlandini et al, 1996; Achen et al, 1998). Each of the VEGFs binds selectively to one or two members of the VEGF receptor family (Table 2.1).

VEGF is considered to be the most important stimulator of vascular endothelial cell proliferation and migration, and it has a role in the maintenance of newly formed, immature vessels (Ferrara and Henzel, 1989; Plouet et al, 1989; Alon et al, 1995; Benjamin and Keshet, 1997). VEGF is expressed as several splice isoforms

TABLE 2.1. Summary of the interactions between vascular endothelial growth factor (VEGF) family ligands and their receptors.

	VEGFR-1	VEGFR-2	VEGFR-3	NP-1	NP-2	Tie-1	Tie-2
VEGF165	×	×		×	×		
VEGF121	×	×					
VEGF145	×	×			×		
VEGF189	×	×					
VEGF206	×	×					
VEGF-B 167	×			×			
VEGF-B 186	×						
VEGF-C		×	×				
VEGF-D		×	×				
VEGF-E (NZ-7 VEGF)		×					
VEGF-E (NZ-2 VEGF)		×		×			
PlGF-1	×						
PlGF-2	×			×	×		
PlGF/VEGF heterodimer		×					
Ang-1							×
Ang-2							×*

* A receptor antagonist.

Ang, angiopoietin; NP-1, neuropilin-1; PlGF, placenta growth factor.

consisting of polypeptides with 121, 145, 165, 189, and 206 amino acid residues. These isoforms differ in their ability to interact with heparan sulfate proteoglycans in the extracellular matrix (reviewed in Neufeld et al, 1999). The VEGF molecule is an antiparallel disulfide-linked dimer and it mediates its effects via endothelial cell–specific tyrosine kinase receptors VEGFR-1 and VEGFR-2. The most abundant and presumably major biologically active form is $VEGF_{165}$, which binds VEGFR-1 and VEGFR-2 with dissociation constants (K_d's) of 10 to 20 pM and 75 to 125 pM, respectively (De Vries et al, 1992; Terman et al, 1992). $VEGF_{165}$ also binds with high affinity to nonreceptor tyrosine kinase neuropilin-1 (NP-1) and NP-2 (Soker et al, 1998; Gluzman-Poltoraz et al, 2000). The crucial role of VEGF-mediated signaling during embryogenesis has been demonstrated in knockout mice, where the inactivation of a single *Vegf* allele results in aberrant vessel formation and lethality *in utero* (Carmeliet et al, 1996; Ferrara et al, 1996). This phenotype appears to be due to decreased gene dosage, and it is the first case where the loss of a single autosomal allele is lethal, indicating that a tight dose-dependent regulation of embryonic vessel development is mediated by VEGF. Among the members of the VEGF family, VEGF is the only one that is strongly upregulated by hypoxia and low glucose, conditions that occur in poorly vascularized tissues, such as tumors. In addition, VEGF is more potent than histamine in increasing capillary permeability to plasma proteins (Dvorak et al, 1995).

Recently, PlGF has also been found to bind to NP-2 (Gluzman-Poltorak et al, 2000) PlGF and VEGF-B are two members of the VEGF family that interact exclusively with VEGFR-1 and NP-1 (Migdal et al, 1998; Olofsson et al, 1998; Soker et al, 1998; Makinen et al, 1999). In normal tissues PlGF is expressed mainly in the placenta, but also in some malignant tumors such as renal cancer, suggesting that in addition to VEGF, it might regulate tumor angiogenesis in some settings (Takahashi et al, 1994). As a result of alternative splicing of the gene, PlGF exists as isoforms of 131, and 152 amino acid residues, the latter containing a stretch of 21 basic residues, which confers heparin- and neuropilin-1–binding ability (Maglione et al, 1991; Hauser and Weich, 1993). VEGF-B resembles PlGF in several aspects: there are two currently known splice isoforms of VEGF-B, and VEGF-B also binds selectively VEGFR-1 and NP-1. Of the splice isoforms, $VEGF\text{-}B_{167}$ remains cell associated via C-terminal basic sequences. $VEGF\text{-}B_{186}$, which lacks the highly basic region, is secreted from cells in a freely soluble form. Both forms of VEGF-B are produced as disulfide-linked homodimers, but they can also form heterodimers with VEGF (Olofsson et al, 1996b). VEGF-B–containing conditioned medium was able to stimulate thymidine incorporation into DNA in human umbilical vein endothelial cells (ECs) and bovine capillary endothelial cells, suggesting that it is mitogenic for ECs (Olofsson et al, 1996a). However, so far this has not been reproduced using recombinant VEGF-B, suggesting that the activity could be due to VEGF-B/VEGF heterodimers in transfected cells. VEGF-B is expressed in heart during development, and deletion of the *Vegfb* gene results in vascular dysfunction after coronary occlusion, and impaired recovery from experimentally induced myocardial ischemia (Bellomo et al, 2000).

VEGF-C and VEGF-D are produced as long precursor proteins that undergo proteolytic processing to generate several forms that differ in their receptor binding properties (Lee et al, 1996; Orlandini et al, 1996; Joukov et al, 1997; Yamada et al, 1997; Achen et al, 1998). Partially processed VEGF-C and VEGF-

D are able to bind and activate exclusively VEGFR-3, whereas their processed forms stimulate both VEGFR-2 and VEGFR-3. The mature form of VEGF-C, containing only the VEGF homology domain, binds to VEGFR-2 and VEGFR-3 with K_d's of 410 pM and 135 pM, respectively. Presumably through its interaction with VEGFR-2, VEGF-C shares the major functions of VEGF, including induction of angiogenesis and vascular permeability. In embryos, targeted disruption of the *Vegfr3* gene leads to failure of remodeling of the primary vascular network, and death after embryonic day 9.5 (Dumont et al, 1998). This indicates that VEGFR-3 has an essential role in the development of the embryonic vasculature before the emergence of lymphatic vessels. However, during further development, the expression of VEGFR-3 becomes restricted mainly to lymphatic vessels (Kaipainen et al, 1995; Kukk et al, 1996; Dumont et al, 1998). Overexpression of VEGF-C in transgenic mice using a basal keratin promoter induced a hyperplastic lymphatic vessel network in the skin of adult mice (Jeltsch et al, 1997). Lymphatic growth, therefore, is thought to be mediated predominantly via VEGFR-3.

Recently, viral homologs of VEGF were found to be encoded by two strains (NZ2 and NZ7) of parapox Orf viruses (Ogawa et al, 1998; Meyer et al, 1999). The resulting polypeptides carry the characteristic dimer cysteine knot motif present in all mammalian VEGFs. These factors are collectively termed VEGF-E, they exclusively activate VEGFR-2, and at least NZ2-VEGF-E can use neuropilin-1 as a co-receptor (Wise et al, 1999). The Orf virus produces a pustular dermatitis in sheep, goats, and humans. The induced lesions show extensive proliferation of vascular endothelial cells, dilation of blood vessels, and dermal swelling.

All the above-mentioned members of the VEGF family transmit angiogenic signals to the vascular endothelium via high-affinity VEGFRs, currently designated VEGFR-1 (originally named *flt*: *fms*-like tyrosine kinase), VEGFR-2 (KDR: kinase insert-domain containing receptor in humans; or *flk-1*: fetal liver kinase-1 in mice), and VEGFR-3 (*FLT4*) (Shibuya et al, 1990; Aprelikova et al, 1992; Galland et al, 1992; Terman et al, 1992). All of these receptors have seven immunoglobulin (Ig) homology domains in their extracellular region, and an intracellular tyrosine kinase signaling domain. In adults, VEGFR-1 and VEGFR-2 are expressed mainly in the blood vascular endothelium, while VEGFR-3 is mostly restricted to the lymphatic endothelium. VEGFR-1 is a 180-kd transmembrane glycoprotein, but due to alternative splicing, a shorter soluble protein consisting of only the six first Ig homology domains is also produced (De Vries et al, 1992; Kendall and Thomas, 1993). VEGFR-2 is a 230-kd protein, with no reported splice variants. In human VEGFR-3, alternative 3′ polyadenylation signals result in a 4.5-kilobase (kb) transcript and a more prevalent 5.8-kb transcript (Pajusola et al, 1993). The latter encodes 65 additional amino acid residues and is the major form detected in tissues. After biosynthesis, the glycosylated 195-kd VEGFR-3 is proteolytically cleaved in the fifth Ig homology domain, but the resulting 125- and 75-kd chains remain linked by a disulfide bond (Pajusola et al, 1993).

Like other receptor tyrosine kinases, the VEGFRs are thought to dimerize and to undergo transautophosphorylation upon ligand binding. Both VEGFR-2 and VEGFR-3 are tyrosine phosphorylated when stimulated with their respective ligands (Waltenberger et al, 1994; Joukov et al, 1996), but VEGFR-1 autophosphorylation is less obvious and has been studied mostly in receptor overexpressing transfected cells (Waltenberger et al, 1994; Seetharam et al, 1995). Phosphorylated tyrosine residues serve to control the kinase activity of the recep-

tor and to create docking sites for cytoplasmic signaling molecules, which provide substrates for the kinase. These molecules, either adapters or enzymes themselves, link VEGFRs to the signaling pathways leading to specific responses like proliferation, migration, survival, tube formation, or increased permeability of target endothelial cells.

ANGIOPOIETINS AND TIE RECEPTORS

Angiopoietins are a second growth factor family with specificity for vascular endothelial cells (Table 2.1). Like VEGFs, angiopoietins have also been shown to play a role in the formation of the blood vascular system. Ang-1 is a ligand for Tie-2 (tyrosine kinase with Ig and epidermal growth factor [EGF] homology domains receptor tyrosine kinase-2, also called Tek: tunica interna endothelial cell kinase), and a related growth factor termed Ang-2 functions as a naturally occurring antagonist for Ang-1 and Tie-2 signaling (Maisonpierre et al, 1997; Suri et al, 1997). The receptors of the Tie family (Tie-1 and Tie-2) are structurally closely related and contain two immunoglobulin homology (IgH) domains and three epidermal growth factor and fibronectin type III homology domains in the extracellular part of the receptor. Trans- and juxtamembrane domains are followed by a tyrosine kinase domain and a carboxyterminal tail (Partanen et al, 1992). No ligand has been identified to date for the Tie-1 receptor. However, Tie-1 deficient mice die in utero between embryo day (E) 13.5 and birth mainly as a consequence of a decrease in vascular integrity (Puri et al, 1995). As a result, edema and localized hemorrhage occur in embryos deficient in this receptor.

Like VEGFRs, Tie-1 and Tie-2 are specifically expressed in endothelial cells, and these receptors have been shown to have distinct roles during vascular development. The phenotype of Tie-2– and Ang-1–deficient mice suggests a role for this ligand-receptor pair in maintaining communication between endothelial cells and the surrounding mesenchyme to establish stable cellular and biochemical interactions between endothelial cells and pericytes/smooth muscle cells (Dumont et al, 1994; Suri et al, 1997). On the other hand, overexpression of Ang-1 in the skin of transgenic mice demonstrated that Ang-1 can reduce vascular leakage due to inflammatory mediators even in the presence of excess VEGF (Thurston et al, 1999). This result suggests that Ang-1 can be used for reducing microvascular leakage in diseases in which the leakage results from chronic inflammation or elevated VEGF, or, alternatively, Ang-1 may be used, in combination with VEGF, for promoting growth of nonleaky vessels. In contrast to Ang-1, the expression of Ang-2 is mainly detected in sites of active angiogenesis (Maisonpierre et al, 1997). Analysis of the Ang-2 function in normal adults suggests that it plays a crucial role in destabilizing quiescent adult vessels, and thus seems to be involved in the initiation of normal vascular remodeling.

DEVELOPMENT AND MORPHOGENESIS OF THE MAMMARY GLAND

The mammary gland is one of the few organs that undergo cycles of growth, morphogenesis, differentiation, functional activity, and involution. It arises from an epidermal thickening on the ventral surface of the body (the milk line) at approximately the sixth week of fetal development. At birth, the mammary gland consists of branching ducts connected to the nipple by a single primary duct.

Development of the mammary gland is incomplete at the time of birth. Although growth and branching of the mammary ducts occur slowly during prepubertal life, during puberty, with the progressive increase in estrogen and progesterone levels, ductal growth and ramification are rapid, resulting in the formation of the mammary gland ductal tree. Following the onset of pregnancy, in response to sustained elevated levels of estrogens and progesterone, ductal elongation and branching resumes, and clusters of alveoli bud off from the growing ducts (Figure 2.1; see color insert). During the second half of gestation, alveolar morphogenesis is followed by the structural and functional differentiation of alveolar epithelial cells in preparation for milk fat and protein secretion, which occurs during lactation (Figure 2.1). After weaning, the mammary gland involutes rapidly, ultimately leaving only a rudimentary ductal system and a few remaining alveoli (reviewed by Daniel and Silberstein, 1987).

ALTERATIONS IN MAMMARY GLAND VASCULATURE DURING PREGNANCY, LACTATION, AND INVOLUTION

The arteries that supply the breast are derived from the thoracic branches of the axillary artery, the internal thoracic artery, and anterior intercostal arteries. Ramifications of these vessels accompany ductal structures, ultimately reaching the capillary network, which surrounds alveoli.

Previous descriptive studies have revealed that the mammary gland vascular bed is a highly dynamic structure that undergoes profound qualitatitive and quantitative alterations during the mammary cycle (Wahl, 1915; Turner and Gomez, 1933; Soemarwoto and Bern, 1958; Yasugi et al, 1989; Matsumoto et al, 1992). Thus, in the virgin mammary gland, the presence of a periductal capillary plexus has been described, which becomes richly developed with advancing pregnancy in association with epithelial growth. This is accompanied by concomitant growth of arterioles and venules. During lactation, there is a progressive increase in capillary size due to dilatation of existing capillaries in association with increased milk secretory function. During involution, the capillary bed progressively disappears, so that relatively thick-walled venules and arterioles appear disproportionately large compared with the capillary field that they supply. Gradually, however, the arterioles and venules also begin to involute, and ultimately the vascular bed resembles that of the virgin mammary gland.

Using a novel whole-mount lectin-based technique to quantitate vascularization in the murine mammary gland, we have observed that the mature virgin mammary gland contains a fully developed capillary system, which increases significantly in density during early pregnancy (Table 2.2.) with vascular networks surrounding the expanding parenchyma and developing alveolar units (Pepper et al, 2000). During lactation, expansion of terminal alveoli distends the capillary networks; however, this stage is not associated with increased vascular growth. In fact, the vascular density appears to decrease slightly. This is probably artifactual and due to alveolar distention during lactation. Upon weaning, regression of parenchyma results in involution of capillary loops. Vessels appear to be thinner and further apart; eventually many of these vessels regress. These events are also associated with loss of pericytes and complete obliteration of the lumen. Thus,

TABLE 2.2. Quantitation of capillary density in the mouse mammary gland during pregnancy, lactation and involution.

Stage	Capillary density (per mm^2) (mean ± SD)
Virgin	
5 weeks	27 ± 4.6
7 weeks	32 ± 8.9
10 weeks	39 ± 4.5
Pregnant	
5 days	67 ± 6.5
14 days	110 ± 3.4
19 days	198 ± 7.6
Lactation	
2 days	119 ± 5.7
4 days	105 ± 7.5
7 days	109 ± 6.5
Involution	
2 days	93 ± 7.6
4 days	72 ± 8.7
7 days	52 ± 5.7
21 days	69 ± 6.4

Quantification was performed using whole-mount preparations stained with *Bandeiraea simplicifonia* lectin (Vector Labs, Burlingame, CA). Images were scanned using a Toshiba cool CCD camera and quantified with ImagePro software analysis program. Four independent animals were evaluated per stage.

vascular density increases progressively during pregnancy and subsequently decreases during lactation, to levels observed during midpregnancy; during involution, there is a further reduction in vessel density. These findings are consistent with a study in the rat using India ink injection (Yasugi et al, 1989), in which vessel density increased progressively during pregnancy and decreased thereafter through late pregnancy, lactation, and involution. Alterations in vascular volume have been discussed in Chapter 1.

VEGF AND VEGFRS IN THE MAMMARY GLAND DURING PREGNANCY, LACTATION, AND INVOLUTION

Despite the long-known and well-characterized morphologic alterations in mammary gland vasculature described above, surprisingly little is known about mammary endothelial cell function or the molecular mechanisms that regulate angiogenesis, permeability, and endothelial cell death in the mammary gland.

Studies in the virgin rodent mammary gland have revealed that VEGF (188, 164, and 120 amino acid isoforms), VEGF-C, and VEGFR-1, -2, and -3 are expressed, with VEGF messenger RNA (mRNA) and protein being localized predominantly in mammary epithelium (Figures 2.2A and 2.3) (Pepper et al, 2000). Low levels of VEGF mRNA have also been observed in terminal duct epithelium of normal

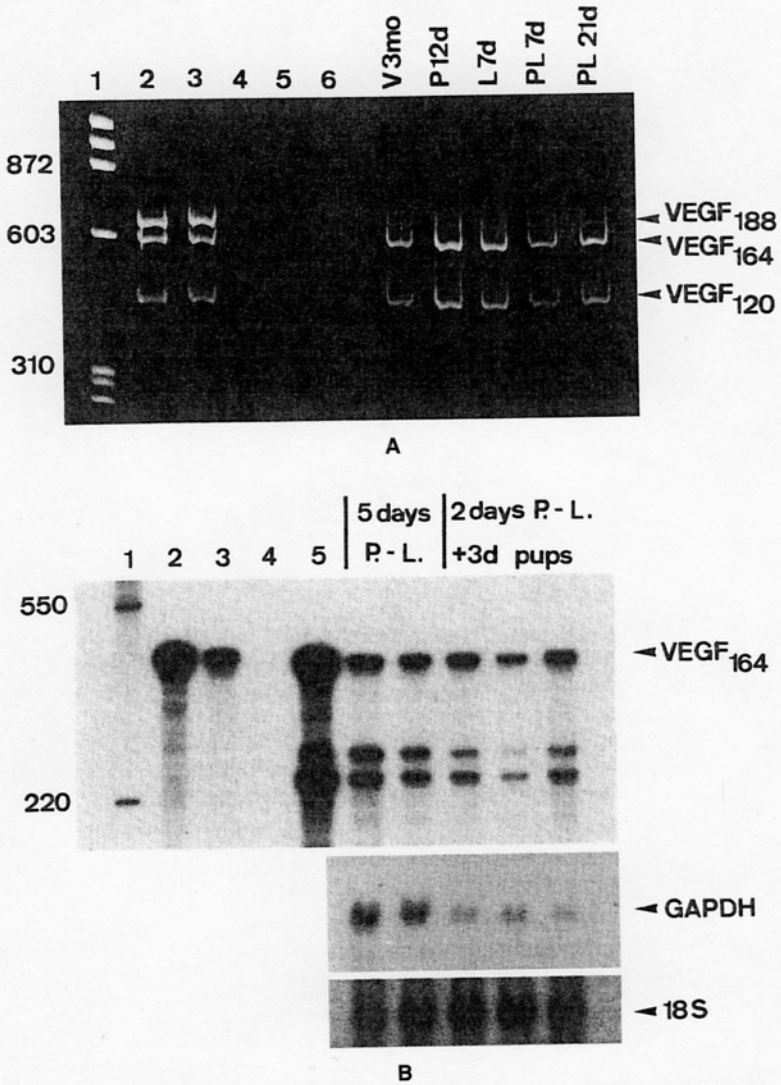

FIGURE 2.2. A: Vascular endothelial growth factor (VEGF) isoforms expressed in rat mammary gland. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of VEGF mRNA expression at various stages including 3-month virgin (V 3mo), 12-day pregnant (P 12d), 7-day lactating (L 7d), 7-day postlactating (PL 7d), and 21-day postlactating (PL 21d). Lane 1: molecular size markers; lane 2: mouse lung; lane 3: rat lung; lane 4: H_2O; lane 5: mouse lung—reverse transcriptase; lane 6: rat lung—reverse transcriptase. B: Induction of $VEGF_{164}$ during lactation. Samples from rat mammary glands were analyzed by RNase protection as follows: 5 days postlactation/weaning (5 days P.-L.), two animals; 2 days of weaning/postlactation followed by replacement of pups for a further 3 days (2 days P.L. + 3d pups), three animals. Lane 1: molecular size markers; lane 2: chromosomal RNA (cRNA) probe; lane 3: cRNA probe + hybridization mixture; lane 4: yeast transfer RNA (tRNA); lane 5: rat lung. 18S ribosomal RNA is shown as an indicator of RNA loading and integrity. When normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), $VEGF_{164}$ is increased 4.1-fold during lactation.

human breast tissue (Brown et al, 1995), and VEGF immunoreactivity has been observed in these cells in normal human and nonhuman primate mammary gland (Nakamura et al, 1999, and our own observations). Low levels of VEGF protein, as determined by enzyme-linked immunosorbent assay (ELISA), have been observed in nonneoplastic human breast tissue (Obermair et al, 1997; Greb et al, 1999), and weak VEGF-C immunoreactivity has been reported in normal human mammary epithelium (Valtola et al, 1999). Using a dorsal skinfold chamber model in nude mice, Lichtenbeld et al (1998) have reported that in contrast to tissue samples from breast cancer, samples from normal healthy breasts had no angiogenic activity. The precise role of the VEGF ligand-receptor system in the normal mammary gland is not known.

In the rat, mRNA for $VEGF_{164}$ is increased during pregnancy (5.0-fold increase on day 12) and during lactation (18.5-fold increase on day 7) (Pepper et al, 2000). Levels of VEGF appear to be minimally altered during involution. VEGF 120, 164, and 188 amino acid isoforms are expressed during pregnacy, lactation, and involution, and their abundance relative to one another does not alter during the mammary cycle (Figure 2.2A). In the mouse, there is an increase in VEGF mRNA levels throughout pregnancy (maximum 5.5-fold at 5 days) and a more marked increase during lactation (maximal 9.7-fold at 7 days); levels of VEGF decreased progressively during the phase of involution. These findings point to a causal role for VEGF-VEGFR interactions in the increase in vascularization, which occurs during pregnancy-associated mammary growth, as well as endothelial cell function during lactation. Whether the downregulation of VEGFRs observed following weaning is causally related to involution of the vasculature is not known.

The relationship between increased levels of VEGF mRNA and lactation was confirmed by removing rat pups from their lactating mothers, and after 2 days reintroducing the pups for a further 3 days. In a parallel (control) group, pups were removed for a period of 5 days (Figure 2.2B). Analysis of VEGF mRNA expression, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), revealed a 4.1-fold increase in the experimental group relative to controls, thereby establishing a clear relationship between lactation and increased VEGF expression. The increase in VEGF was in contrast to the decrease in hepatocyte growth factor (HGF) and its receptor c-Met (71% and 86%, respectively, when normalized to GAPDH), previously observed under the same conditions (Pepper et al, 1995).

With respect to VEGFR-1 and -2, a quantitative analysis in the rat mammary gland revealed an increase in VEGFR-2 during pregnancy (1.6-fold at 4 days). During lactation, VEGFR-1 (2.7-fold at 7 days) and VEGFR-2 (3.8-fold at 7 days) were increased. In contrast, VEGFR-2 was decreased in the early phases of involution (45%, 50%, and 34% on days 1, 2, and 3, respectively). In the mouse, VEGFR-2 mRNA levels were moderately increased during pregnancy (maximum 2.7-fold at 19 days), and increased further during lactation (maximum 3.7-fold increase on day 7).

As can be appreciated from the above, the greatest increase in VEGF and VEGFR-1 and -2 was seen during lactation. What is the relevance of these findings? The capillary is the primary site of transport between the blood and alveolar epithelium, and constitutes the so-called blood–milk barrier. In the virgin mammary gland, the capillary wall is usually continuous with rare fenestrations of 30 to 55 nm (Stirling and Chandler, 1976; Matsumoto et al, 1992). During lac-

tation, there is a marked increase in permeability as well as in the number of cytoplasmic vesicles in endothelial cells surrounding alveoli (Matsumoto et al, 1992, 1994). These vesicles tend to fuse with one another to form clusters. These authors also describe a striking increase in the number and length of microvillous processes emanating from the surface of parenchyma-associated capillary endothelial cells. Following weaning, these features progressively disappear, with the endothelium ultimately returning to its prepregnancy state. VEGF, which was first identified as a result of its capacity to increase vascular permeability (reviewed by Dvorak et al, 1995), induces morphologic alterations in endothelial cells, including the appearance of vesiculovacuolar organelles (reviewed by Feng et al, 1999), which are entirely consistent with this function. We suggest that during lactation, the marked increase in VEGF induces functional alterations in endothelial cells, which lead to the increase in vascular permeability.

Mammary tissue is composed of parenchymal (epithelial structures) and stromal (connective tissue, blood and lymphatic vessels, and nerves) compartments. Since fibroblasts, epithelium, endothelial, and smooth muscle cells have been reported to synthesize VEGF in vitro and in several organs in vivo, we evaluated the relative contribution of parenchyma vs. stroma by examining VEGF mRNA expression in cleared murine mammary glands devoid of epithelial components. Quantitation revealed that approximately 25% of VEGF mRNA is synthesized by stroma, indicating that mammary epithelium is the major source of VEGF mRNA. It has previously been demonstrated that mammary gland fat pads devoid of parenchyma fail to develop the vascular pattern associated with pregnancy (Soemarwoto and Bern, 1958). The authors concluded that the various changes observed in the vascular bed occur in association with alterations in the epithelial structures (ducts and alveoli), and that these changes do not occur in the absence of parenchyma. Our in situ and immunohistochemistry data suggest that although VEGF is expressed predominantly by epithelial cells (Figure 2.3; see color insert), there is also a significant contribution from the stroma. VEGF has been detected in early passage mammary fibroblast cultures, in which its expression is dramatically increased by hypoxia (Hlatky et al, 1994). These findings indicate that rapidly growing epithelial cells are the major source of VEGF in the mammary gland. VEGF has also been detected in human milk (Siafakas et al, 1999, Pepper et al, 2000).

What are the factors that regulate VEGF expression during pregnancy and lactation? Using a clonal mouse mammary epithelial cell line (Soriano et al, 1995), we have observed that VEGF is downregulated by HGF. Interestingly, the period of maximal VEGF expression (i.e., lactation) is also the period during which HGF and c-Met are markedly downregulated (Pepper et al, 1995; Yang et al, 1995). It has been demonstrated that HGF, which regulates many epithelial cell functions (Matsumoto and Nakamura, 1996) including the induction of mammary ductal morphogenesis (Soriano et al, 1995), is downregulated during lactation (Pepper et al, 1995; Yang et al, 1995), when the HGF-dependent tubulogenic process is essentially completed. It is not possible at this point to ascertain whether there is causality in the inverse relationship between VEGF and HGF levels. In contrast to HGF, mammotrophic hormones did not directly alter VEGF mRNA levels in cultured mammary epithelial cells (Pepper et al, 2000).

The mouse mammary tumor virus long terminal repeat (MMTV LTR) is widely used as a promoter in mouse models to study the role of different growth factors in mammary gland and in breast cancer. This promoter results in hormonally

regulated, mammary gland–specific expression during pregnancy and lactation. To study the effects of VEGF in mammary gland angiogenesis, transgenic mice were generated that express human $VEGF_{165}$ under the MMTV promoter (Karkkainen et al, 1998). Morphologic analysis of the mammary glands in these mice revealed edema and loss of the regular alveolar pattern (Figure 2.4; see color insert). During pregnancy, the mammary glands were hypervascularized, and there were abnormally large and malformed vessels surrounding the lobular structures, indicating that they might have developed in response to the expression of the transgene in the ductal epithelial cells, as detected by in situ hybridization. These remarkable alterations are likely to explain why the multiparous female founder mice had problems in fostering their pups after their third or fourth litters.

LYMPHATIC VESSELS IN THE BREAST

The lymphatics of the breast are especially numerous and well developed. Four principal lymphatic pathways drain the breast: cutaneous, axillary, internal thoracic, and posterior intercostal lymphatics (Haagensen, 1986). Most of the lymphatics (75%) drain chiefly to axillary nodes. Lymphatics play a crucial role in homeostasis of normal breast tissue, and also serve to disseminate tumor cells to regional lymph nodes in metastatic breast cancer.

As indicated previously, the VEGF-C/VEGFR-3 ligand-receptor pair is clearly implicated in the growth of lymphatic vessels (Kaipainen et al, 1995; Jeltsch et al, 1997; Dumont et al, 1998). In contrast to VEGF, the levels of VEGF-C mRNA are only modestly increased in the rat mammary gland during pregnancy (2.8-fold increase on day 4) and lactation (1.9-fold increase on day 2). Similarly, VEGFR-3 is modestly increased during pregnancy (2.2-fold at 4 days) and lactation (1.5-fold at 21 days). In contrast, VEGFR-3 is decreased in the early phases of involution (33%, 21%, and 45% on days 1, 2, and 3, respectively). VEGFR-3, which is shown to be specific for lymphatic vessels in most other tissues, is also expressed in the blood capillary endothelial cells in the resting mammary gland (Valtola et al, 1999).

The role of VEGF-C in the function of the lymphatic network of the mammary gland is still unknown. Recently, VEGFR-3 was found to be expressed in the endothelium of certain fenestrated and discontinuous capillaries in certain tissues such as nasal mucosa (Partanen et al, 2000; Saaristo et al, 2000). These results suggest that VEGFR-3 might play a role in discontinuous endothelia, which are known to display increased permeability to macromolecules. Regulation of vascular permeability is also crucial in the mammary gland during lactation. VEGF-C may therefore function as a growth factor for lymphatic vessels, and might also play a role in modifying the permeability of lymphatics as well as blood vessels during lactation.

CONCLUSION

The existence of a precise sequence of tightly scheduled, hormonally regulated changes in tissue structure and function makes the mammary gland an attractive model for studying the mechanisms of angiogenesis, vascular permeability, and endothelial cell death. Results obtained from our studies point to a causal role for VEGF and its receptors in the increase in vascularization, that occurs during preg-

nancy. They also support a role for VEGF in increasing vascular permeability during lactation, during which increased transport of molecules from the blood is required for efficient milk protein synthesis.

The relationship between microvessel density and breast cancer progression has previously been reported (Weidner et al, 1991). The controlled increase and subsequent decrease we have observed in VEGF/VEGFR expression in response to the physiologic requirements of the mammary gland contrast sharply with the sustained elevation in VEGF and its receptors reported in breast carcinoma (Brown et al, 1995; Gasparini et al, 1997; Obermair et al, 1997). Breast cancer cells also express VEGF-C, and there is upregulation of its receptor VEGFR-3 in the surrounding angiogenic blood vessels (Valtola et al, 1999). In an animal model VEGF-C stimulates the growth of tumor-associated lymphatic vessels and promotes the intralymphatic spread of breast cancer cells (Karpanen et al, unpublished observation). In the future anti-VEGF-C therapy might be used in preventing the formation of tumor metastasis.

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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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