Regulation of VEGF and VEGF Receptor Expression in the Rodent Mammary Gland During Pregnancy, Lactation, and Involution

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ABSTRACT  Vascular endothelial growth factors (VEGFs) are endothelial cell-specific mitogens with potent angiogenic and vascular permeability-inducing properties. VEGF, VEGF-C, and VEGFRs -1, -2, and -3 were found to be expressed in post-pubertal (virgin) rodent mammary glands. VEGF was increased during pregnancy (5-fold) and lactation (15–19-fold). VEGF-C was moderately increased during pregnancy and lactation (2- and 3-fold respectively). VEGF levels were reduced by approximately 75% in cleared mouse mammary glands devoid of epithelial components, demonstrating that although the epithelial component is the major source of VEGF, approximately 25% is derived from stroma. This was confirmed by the findings (a) that VEGF transcripts were expressed predominantly in ductal and alveolar epithelial cells, and (b) that VEGF protein was localized to ductal epithelial cells as well as to the stromal compartment including vascular structures. VEGF was detected in human milk. Finally, transcripts for VEGFRs -2 and -3 were increased 2–3-fold during pregnancy, VEGFRs -1, -2 and -3 were increased 2-4-fold during lactation, and VEGFRs -2 and -3 were decreased by 20-50% during involution. These results point to a causal role for the VEGF ligand-receptor pairs in pregnancy-associated angiogenesis in the mammary gland, and suggest that they may also regulate vascular permeability during lactation. Dev Dyn 2000;218:507–524. © 2000 Wiley-Liss, Inc.

Key words: angiogenesis; vascular permeability; breast; growth factor

INTRODUCTION

Angiogenesis is required for the growth of normal and neoplastic tissues, and plays an important role in the maintenance of the functional and structural integrity of the organism. 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).

The mammary gland is one of the few organs that undergo cycles of growth, morphogenesis, differentiation, functional activity, and involution in postnatal life. At birth, the rodent mammary gland consists of 15–20 branching ducts connected to the nipple by a single primary duct. From about four weeks post-partum, the ducts grow and ramify under the influence of ovarian hormones, forming 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. 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. 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).

Early whole-mount and light microscopic studies on the vascularization of the virgin mammary gland described the presence of a periductal capillary plexus, 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 pro-
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 which they supply. Gradually however, the latter also begin to involute, and ultimately the vascular bed resembles that of the virgin mammary gland (Wahl, 1915; Turner and Gomez, 1933; Soemarwoto and Bern, 1958). Despite these well described alterations in mammary gland vasculature, surprisingly little is known about mammary endothelial cell function or the molecular mechanisms which regulate angiogenesis, permeability, and endothelial cell death in the mammary gland.

Fig. 1. Vascular architecture of mouse mammary gland. Whole-mount staining with BSL at various stages of the mammary gland cycle (stages indicated in each panel). Scale bars = 150μm.
Several polypeptide growth factors have been shown to promote angiogenesis in vivo, one of the most important of which is vascular endothelial growth factor (VEGF). VEGF is an endothelial cell-specific mitogen with potent angiogenic and vascular permeability-inducing properties, both of which may be important for mammary gland function. High affinity VEGF-cell surface interactions occur via two transmembrane tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). VEGF-C is a recently described member of the VEGF family, which interacts with VEGFR-2 and VEGFR-3 (Flt-4). VEGF-C shares the major functions of VEGF including induction of angiogenesis and vascular permeability, presumably through its interaction with VEGFR-2. However, by virtue of its interaction with VEGFR-3, VEGF-C also induces growth of the lymphatic vascular system, a process referred to as lymphangiogenesis (reviewed by Dvorak et al., 1995; Ferrara, 1999; Stacker and Achen, 1999; Veikkola and Alitalo, 1999).

The studies reported in this paper have focussed on the rodent mammary gland. Our first objective was to quantitate vascularization during pregnancy, lactation and involution following weaning. We next assessed the expression of VEGF and VEGF-C mRNA and protein. Experiments were performed to determine the importance of lactation, the relative contributions of epithelial and stromal components and the possible contribution of hormonal and polypeptide factors to VEGF synthesis. Levels of VEGF were also determined in human milk. Finally, the expression of VEGFRs was assessed at various stages of the mammary cycle. Our findings demonstrate that VEGF, VEGF-C, and VEGFR’s -1, -2, and 3 are expressed in the postpubertal mammary gland, and that alterations in expression during pregnancy, lactation and involution are compatible with a role for these ligand-receptor pairs during pregnancy-associated vascular growth and permeability.

RESULTS

Alterations in Vascular Density During Pregnancy, Lactation, and Involution

To ascertain whether vascular density is altered during the mammary gland cycle, mouse mammary glands were stained with Bandeiraea simplicifolia lectin (BSL) which recognizes mouse endothelium (Alroy et al., 1987) (Fig. 1 and Table 1). At 7–8 weeks the mature virgin mammary gland shows a fully developed capillary system (Fig. 1A), which increases significantly in density during early pregnancy (Fig. 1B). An increase in capillary loops can be seen as early as 3 days after pregnancy. Vascular networks surround the expanding parenchyma and developing alveolar units (Fig. 1C). During lactation, expansion of terminal alveoli distends the capillary networks; however this stage is not associated with increased vascular growth (Fig. 1D); in fact, the vascular density appears to decrease slightly. This is probably artifactual and due to alveolar distension during lactation. Regression of parenchyma upon weaning 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 (data not shown). A 7-day regressing gland is shown in Fig. 1E. Note dark epithelial units and thin vascular networks in between the involuting alveoli. Complete regression of the parenchyma is seen 21 days after weaning (compare Fig. 1A, F). At this stage, capillary density is comparable with that seen in the early stages of pregnancy (Table 1).

Cloning of Partial Rat VEGF-C, VEGFR-3, and CD31 cDNAs

Partial cDNAs were cloned from adult rat lung as described below. Nucleotide and amino acid sequences for VEGF-C, VEGFR-3, and CD31/PECAM-1 are shown in Figure 2. For rat VEGF-C (GenBank accession no. AF010302), identity at the nucleotide level was 97%, 88%, and 80% compared to mouse, human, and quail VEGF-C respectively. Identity at the amino acid level was 97%, 88%, and 80% compared to mouse, human, and quail. For rat VEGF-3 (GenBank accession no. AF010131), identity at the nucleotide level was 93% and 82% compared to mouse and human VEGF-3 respectively. Identity at the amino acid level was 90% and 74% for mouse and human. For rat CD31/PECAM-1 (GenBank accession no. U77697), identity at the nucleotide level was 88%, 73%, 68%, and 73% compared to mouse, human, bovine, and porcine CD31/PECAM-1 respectively (data not shown). Identity at the amino acid level was 84%, 66%, 59%, and 63% for mouse, human, bovine, and porcine CD31/PECAM-1.

### Table 1. Quantitation of Capillary Density in the Mouse Mammary Gland During Pregnancy, Lactation, and Involution

<table>
<thead>
<tr>
<th>Stage</th>
<th>Capillary density (per mm²) (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>27 ± 4.6</td>
</tr>
<tr>
<td>5 weeks</td>
<td>32 ± 8.9</td>
</tr>
<tr>
<td>10 weeks</td>
<td>39 ± 4.5</td>
</tr>
<tr>
<td>Pregnant</td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td>67 ± 6.5</td>
</tr>
<tr>
<td>14 days</td>
<td>110 ± 3.4</td>
</tr>
<tr>
<td>19 days</td>
<td>198 ± 7.6</td>
</tr>
<tr>
<td>Lactation</td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>119 ± 5.7</td>
</tr>
<tr>
<td>4 days</td>
<td>105 ± 7.5</td>
</tr>
<tr>
<td>7 days</td>
<td>109 ± 6.5</td>
</tr>
<tr>
<td>Involution</td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>93 ± 7.6</td>
</tr>
<tr>
<td>4 days</td>
<td>72 ± 8.7</td>
</tr>
<tr>
<td>7 days</td>
<td>52 ± 5.7</td>
</tr>
<tr>
<td>21 days</td>
<td>69 ± 6.4</td>
</tr>
</tbody>
</table>

Partial cDNAs were cloned from adult rat lung as described below. Nucleotide and amino acid sequences for VEGF-C, VEGFR-3, and CD31/PECAM-1 are shown in Figure 2. For rat VEGF-C (GenBank accession no. AF010302), identity at the nucleotide level was 97%, 88%, and 80% compared to mouse, human, and quail VEGF-C respectively. Identity at the amino acid level was 97%, 88%, and 80% compared to mouse, human, and quail. For rat VEGF-3 (GenBank accession no. AF010131), identity at the nucleotide level was 93% and 82% compared to mouse and human VEGF-3 respectively. Identity at the amino acid level was 90% and 74% for mouse and human. For rat CD31/PECAM-1 (GenBank accession no. U77697), identity at the nucleotide level was 88%, 73%, 68%, and 73% compared to mouse, human, bovine, and porcine CD31/PECAM-1 respectively (data not shown). Identity at the amino acid level was 84%, 66%, 59%, and 63% for mouse, human, bovine, and porcine CD31/PECAM-1.
Figure 2.
VEGF But Not VEGF-C Is Increased During Pregnancy and Lactation

RNase protection analysis using a cRNA probe that includes the common and alternatively spliced regions of the rat 164-amino acid VEGF isoform (VEGF$_{164}$), revealed the presence of VEGF transcripts in the virgin rat mammary gland (Fig. 3A). This was confirmed by RT-PCR, in which three bands were detected, which based on their size, represent mRNAs which code for the rodent 120, 164, and 188 amino acid VEGF isoforms (Breier et al., 1992) (data not shown).

To determine whether quantitative differences occur in expression of VEGF$_{164}$, RNase protection analysis was performed on total cellular mRNA from various stages of the mammary cycle (Fig. 3A). To overcome the problem of transcript dilution which occurs as a consequence of milk protein synthesis, an appropriate housekeeping gene was sought for normalization of mRNA levels. It has previously been reported that expression of the GAPDH gene is not uniform during the mammary gland cycle, but that it decreases progressively as one proceeds through pregnancy and into lactation (Gavin and McMahon, 1992). A reciprocal increase was seen in $\beta$-casein, reflecting the large increase in milk protein mRNAs during this period (Nakahasi and Qasba, 1979; Fig. 3A). Levels of a second housekeeping gene, $\beta$-actin, paralleled those of GAPDH (Gavin and McMahon, 1992; Fig. 3A). To correct for the relative decrease in non-milk protein mRNAs, VEGF was therefore normalized relative to GAPDH. Figure 3B shows that mRNA for VEGF$_{164}$ is increased during pregnancy (5.0-fold increase on day 12 with a subsequent decrease on day 18) and during lactation (18.5-fold increase on day 7). Levels of VEGF appear to be minimally altered during involution. This experiment has been repeated four times with similar results, and the quantitative data in Figure 3B, which represent the means ± s.d. of these four experiments, indicate minimal variation between experiments. The samples analyzed were pools of total cellular RNA from 3–7 animals per stage. Similar results were obtained when VEGF mRNA levels were determined by northern blot analysis and expressed relative to GAPDH on the same filter (data not shown). RT-PCR performed on the same samples revealed that VEGF$_{164}$ is expressed during pregnancy (5.0-fold increase on day 12 with a subsequent decrease on day 18) and during lactation (18.5-fold increase on day 7). Levels of VEGF appear to be minimally altered during involution. This experiment has been repeated four times with similar results, and the quantitative data in Figure 3B, which represent the means ± s.d. of these four experiments, indicate minimal variation between experiments. The samples analyzed were pools of total cellular RNA from 3–7 animals per stage. Similar results were obtained when VEGF mRNA levels were determined by northern blot analysis and expressed relative to GAPDH on the same filter (data not shown). RT-PCR performed on the same samples revealed that VEGF$_{164}$ is expressed during pregnancy (5.0-fold increase on day 12 with a subsequent decrease on day 18) and during lactation (18.5-fold increase on day 7). Levels of VEGF appear to be minimally altered during involution. This experiment has been repeated four times with similar results, and the quantitative data in Figure 3B, which represent the means ± s.d. of these four experiments, indicate minimal variation between experiments. The samples analyzed were pools of total cellular RNA from 3–7 animals per stage. Similar results were obtained when VEGF mRNA levels were determined by northern blot analysis and expressed relative to GAPDH on the same filter (data not shown).
Results obtained in rat mammary glands prompted us to extend our studies to the mouse. Northern blot analysis of RNA isolated at various stages of the mammary cycle (Fig. 4A) demonstrated 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 (Fig. 4B). The results shown in Fig. 4B were obtained from 5 animals per time point. Samples from each animal were analyzed separately, and were normalized to GAPDH to account for the dilution factor. These data confirm the increase seen in the rat during pregnancy and lactation.

RNAse protection analysis also revealed the presence of VEGF-C transcripts in the virgin rat mammary gland (Fig. 3A). However, in contrast to VEGF, the levels of VEGF-C mRNA, when expressed relative to GAPDH, were only modestly increased during pregnancy (2.8-fold increase on day 4) and lactation (1.9-fold increase on day 2) (Fig. 3A, B).

To further establish the relationship between increased levels of VEGF mRNA and lactation, rat pups were removed from lactating mothers, and after two days were reintroduced for a further three days. In a parallel (control) group, pups were removed for a period of five days. Analysis of VEGF mRNA expression (relative to GAPDH) revealed a 4.1-fold increase in the experimental group relative to controls (Fig. 5A and data not shown), 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) (Fig. 5A), previously observed under the same conditions (Pepper et al., 1995).

Mammary tissue is comprised 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 versus stroma by examining VEGF mRNA expression in cleared murine mammary glands devoid of epithelial components. For these experiments, the nipple and epithelial primordia were surgically removed from 3-week-old female mice. After an additional 7 weeks,
RNA was isolated from operated and control glands (contra-lateral sham-operated glands). Northern blots were hybridized with probes for VEGF and keratin 8, the latter of which was included to assess for the presence and relative amount of epithelium in operated and sham-operated glands. A weak signal for VEGF mRNA was detected in the operated glands (Fig. 5B). Quantitation revealed that 15–35% of VEGF mRNA is synthesized by stroma (operated glands), indicating that mammary epithelium is the major source of VEGF mRNA.

To determine the localization of VEGF transcripts, in situ hybridization was performed on rat mammary glands at various stages of the cycle. This revealed expression of VEGF in the ductal epithelial cells of the virgin mammary gland (data not shown), and in the ductal and alveolar epithelial cells during pregnancy (Fig. 6B, D), confirming the data obtained in operated mouse mammary glands described above. A positive signal was also seen over alveolar and ductal cells during lactation (data not shown), although the precise localization was more difficult to determine due to the gross morphological distortion which occurs during this phase. Epithelial localization of VEGF mRNA was also observed during involution (data not shown).

Localization of VEGF protein was achieved using an affinity purified antibody against a synthetic peptide corresponding to amino acids 1–20 of the amino terminus of human VEGF. In the rat, positive cytoplasmic immunoreactivity was observed in ductal epithelial cells of the virgin mammary gland (Fig. 7A), in ductal and alveolar epithelial cells throughout the whole pe-
period of gestational evolution (Fig. 7B and data not shown) as well as during lactation (Fig. 7C), and in epithelial cells during involution (Fig. 7D). At all stages, DAB reaction product was also seen in microvascular profiles as well as in the wall of medium and large sized blood vessels. Immunostaining was completely abolished after incubation of the sections with the anti-VEGF antibody preabsorbed with its corresponding peptide at a concentration of \(0.05 \text{ mg/ml}\) (Fig. 7E, F). Incubation without primary antibody or with non-immune serum as well as use of the second antibodies or DAB alone failed to stain any structure

To assess whether hormonal or other factors might increase VEGF mRNA in cultured mammary epithelial cells, we exposed cells from a clonal mouse mammary epithelial cell line (called TAC-2, Soriano et al., 1995) to a variety of mammotrophic factors for 18 hours in medium containing 10% serum. Levels of VEGF mRNA were determined by Northern blot hybridization and expressed relative to GAPDH. With the exception of HGF, which decreased levels of VEGF mRNA, none of the other factors analyzed significantly altered VEGF mRNA levels in these cells (Fig. 5C). The factors assessed included 17-\(\beta\) estradiol (10–100 nM), progesterone (1 \(\mu\)g/ml), prolactin (5 \(\mu\)g/ml), hydrocortisone (0.1–10 \(\mu\)g/ml), aldosterone (1 \(\mu\)g/ml), insulin (10 \(\mu\)g/ml), and heregulin-\(\alpha\) (20 ng/ml), as well as the inactive steroids tetrahydrocortisone (1 \(\mu\)g/ml) and 17-\(\alpha\) hydroxyprogesterone (1 \(\mu\)g/ml) (Fig. 5C and data not shown).

Finally, to determine whether VEGF might be secreted into human milk, levels were measured by ELISA in milk obtained from lactating mothers during the first week post-partum. VEGF levels ranged from 38–220 ng/ml using a sandwich ELISA, or from 17–100 ng/ml using a competitive ELISA (Fig. 8). The reasons for the approximately two-fold difference in detection levels using the two ELISA methods are not known. However, it is important to note that differences between the different samples were reproducible with the two ELISA kits. Attempts at isolating milk from the rat mammary gland were repeatedly unsuccessful.

Fig. 5. (A) Induction of VEGF during lactation—RNase protection. Samples analyzed were as follows: 5 days post-lactation (5d post-lact.), 2 animals; replacement of pups after 2 days of weaning for a further 3 days (2 d.p.l. + 3d pups), 3 animals. All values were normalized to GAPDH, and expressed relative to the first 5-day post-lactation animal. Results are mean ± s.d.. Values for HGF and c-Met are from data described in Pepper et al. (1995), and are also expressed relative to GAPDH. (B) Contribution of epithelial versus stromal components to VEGF expression. Fifteen (15) \(\mu\)g of total cellular RNA were resolved on 1% denaturing gels and transferred to nylon membranes. Blots were probed with cDNA fragments corresponding to VEGF and K8. To normalize for loading and transfer efficiency, blots were also probed with GAPDH. (C) regulation of VEGF expression in cultured murine mammary epithelial cells. Confluent monolayers of TAC-2 cells were exposed to HGF (10 ng/ml) or heregulin-\(\alpha\) (HRG\(\alpha\) - 20 ng/ml) for 18 hr, and levels of VEGF mRNA determined by Northern blot analysis. The results of three independent experiments are shown, in which VEGF and HRG-\(\alpha\) were normalized to GAPDH. Arrows in the left hand panel indicate mean values.
VEGFR Expression During Pregnancy, Lactation, and Involution

VEGFR-1, -2, and -3 transcript levels were measured in rat mammary glands by RNase protection analysis (Fig. 9A). Levels of VEGFR mRNA were normalized to CD31 mRNA as a means of accounting for alterations in endothelial cell number. In these experiments, we have assumed that CD31 mRNA expression is not altered in mammary endothelial cells under the conditions we have analyzed, as has been assumed and published by others (Thomasset et al., 1998). A quantitative analysis revealed an increase in VEGFR-2 (1.6-fold at 4 days) and VEGFR-3 (2.2-fold at 4 days) during pregnancy. During lactation, VEGFR-1 (2.7-fold at 7 days), VEGFR-2 (3.8-fold at 7 days), and VEGFR-3 (1.5-fold at 21 days) were increased. Both VEGFR-2 (45%, 50% and 34% on days 1, 2, and 3 respectively) and VEGFR-3 (33%, 21%, and 45% on days 1, 2, and 3 respectively)
Fig. 7. Immunolocalization of VEGF in rat and human mammary gland. Bouin-fixed paraffin sections were incubated with an affinity purified rabbit polyclonal antibody prepared against a synthetic peptide corresponding to amino acids 1–20 of the amino terminus of human VEGF. 
(A–F) Rat mammary gland: (A) 3-month virgin rat; (B) 12-day pregnant rat; (C and E) 7-day lactating rat; (D and F) 21 days post-lactation, rat. In (E) and (F) the antibody was preincubated with the corresponding antigen at the following concentrations: E \(=\) 2 \(\mu\)g/ml; F \(=\) 0.5 \(\mu\)g/ml. Scale bar \(=\) 100 \(\mu\)m. (G,H) Non-pregnant human mammary gland: (G) Hematoxylin and eosin; (H) anti-VEGF antibody. Scale bar \(=\) 150 \(\mu\)m.
respectively) were decreased in the early phases of involution (Fig. 9B). To confirm the results obtained with VEGFR-2, the RNase protection analysis was repeated using cRNA probes generated from non-overlapping cDNAs for both VEGFR-2 (430 bp fragment) and CD31 (260 bp C' fragment). The quantitative data shown in Figure 9B represent the means ± s.d. for the two experiments. All samples analyzed were pools of total cellular RNA from 3–7 animals per stage.

To assess whether VEGFR-2 expression was modulated in the mouse, Northern blot hybridization was performed (Fig. 10A). 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) (Fig. 11B). The results shown in Fig. 10B were obtained from 7 animals per time point. Samples from each animal were analyzed separately, and as in the rat, VEGFR-2 levels were normalized to CD31 in the same samples. These results confirm the observations in the rat mammary gland, namely that VEGFR-2 mRNA levels, when normalized to CD31 (and presumably therefore for endothelial cell abundance), are increased in the pregnant, and more significantly in the lactating mammary gland.

Attempts at localizing VEGFR-1 and -2 transcripts in rat mammary glands by in situ hybridization were repeatedly unsuccessful. Similarly, localization of VEGFR-2 by immunohistochemistry using antibodies from numerous sources did not produce acceptable results. In contrast, antibodies to VEGFR-1 clearly detected immunoreactivity on endothelial cells, which were identified by staining with antibodies to RECA-1 (a rat endothelial cell-specific antibody; Duijvestijn et al., 1992) and type IV collagen (basement membranes) (Fig. 11).

**DISCUSSION**

The existence of a precise sequence of tightly-scheduled, hormonally-regulated changes in tissue function and architecture makes the mammary gland an attractive model for studying the mechanisms of angiogenesis (i.e., during pregnancy), vascular permeability (i.e., during lactation), and endothelial cell death (i.e., during post-weaning involution). Previous descriptive studies have revealed that the vascular bed is a highly dynamic structure which undergoes profound qualitative and quantitative alterations during the mammary cycle (Wahl, 1915; Soemarwoto and Bern, 1958; Yasugi et al., 1989; Matsumoto et al., 1992). We have developed a novel whole-mount technique based on the affinity of BSL for mouse endothelium (Alroy et al., 1987) in order to quantitate vascularization in the mammary gland. We demonstrate that vascular density increases progressively during pregnancy, reaching a maximum on day 19, and subsequently decreases during lactation, to levels observed during mid-pregnancy. During involution, there is a further reduction in vessel density. Our findings in the mouse are consistent with a study in the rat using India ink injection (Yasugi et al., 1989). These authors found that vessel density increased progressively during pregnancy, reached a peak at day 10, and decreased progressively thereafter through late pregnancy, lactation, and involution.

In addition, we have found that VEGF (188, 164, and 120 amino acid isoforms (Breier et al., 1992)), VEGF-C and VEGFRs -1, -2, and -3 are expressed in the virgin rodent mammary gland, with VEGF mRNA and protein being localized predominantly in the cytoplasm of mammary epithelial cells. Low levels of VEGF mRNA have previously been observed in terminal duct epithelium of normal human breast tissue (Brown et al., 1995), and VEGF immunoreactivity has been observed in these cells in normal human and non-human primate mammary gland (Nakamura et al., 1999, and our own observations). Low levels of VEGF protein (as determined by ELISA) and VEGF mRNA (189, 165, and 121 amino acid isoforms) have been reported in non-neoplastic 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.

We have observed that VEGF and VEGFR-2 (which mediates VEGF signal transduction and induces endothelial cell mitogenesis) are increased during pregnancy. These findings, which were observed in both mouse and rat, point to a causal role for VEGF-VEGFR-2 interactions in the increase in vascularization which occurs during pregnancy-associated mammary growth. Whether the downregulation of VEGFRs observed following weaning is causally related to involution of the vasculature, is not known. Expression of VEGF-C and VEGFR-3 was only modestly increased during pregnancy and lactation. VEGFR-3, which is known to be specific for lymphatic vessels in most adult tissues, is also expressed in the blood capillary endothelial cells in the resting mammary gland (Valtola et al., 1999). The function of VEGF-C in the mammary gland is still unknown.

The greatest increase in VEGF and VEGFRs -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 30–55 nm (Stirling and Chandler, 1976; Matsumoto et al., 1992). During lactation, there is a marked increase in permeability as well as 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 pre-pregnancy state. VEGF, which was first identified as a result of its capacity to increase vascular permeability (reviewed by Dvorak et al., 1995), induces morphological alterations in endothelial cells, including the appearance of vesiculo-vacuolar 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. The function, if any, of VEGF in human milk (Siafakis et al., 1999; this paper), is not known.

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 immu-
nohistochemistry data suggest that although VEGF is expressed predominantly by epithelial cells, there is also a significant contribution from the stroma. To further explore this issue, we have studied VEGF expression in cleared murine mammary glands of 10-week-old animals, in which the epithelial component had been surgically removed 7 weeks earlier. This allowed us to compare epithelial- and non-epithelial-containing compartments. We found that, although VEGF is expressed predominantly by epithelial cells, the stromal component accounts for approximately 25% of VEGF expression in operated glands. VEGF has been detected in early passage mammary fibroblast cultures, in which its expression is dramatically increased by hypoxia (Hlatky et al., 1994).

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 been unable to identify hormonal factors which might be implicated in the regulation of VEGF expression in vitro. However, VEGF was downregulated by HGF in these cells. 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.

Working with the mammary gland presents a number of challenges. First, the changing cellular composition during pregnancy, lactation, and involution requires separate consideration of the epithelial and stromal components. Indeed, we have found that both epithelial and stromal components contribute to VEGF expression. Second, we have used CD31 as an internal control for endothelial cell number when assessing levels of VEGFR expression. This was prompted by a previous report in which the authors expressed mammary gland vascularization in terms of CD31 expression (Thomasset et al., 1998). However, like these authors, we have had to assume that endothelial CD31 expression is not modulated during the various phases we have studied. The third problem concerns dilution of the mRNAs of interest by milk protein mRNAs, whose production is massively increased during the late stages of pregnancy and lactation (Nakahasi and Qasba, 1979). To overcome this problem we have normalized all our samples to the housekeeping gene GAPDH, although very similar results were obtained when β-actin was used in the same way. This approach had previously been reported by others (Gavin and McMahon, 1992).

Finally, 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 physiological 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).

Fig. 11. Immunolocalization of VEGFR-1 in pregnant rat mammary gland. Cryostat sections of 18-day pregnant rat mammary gland were immunostained with (A) RECA-1 antibody, (B) anti-VEGFR-1 and (C) anti-type IV collagen. The section in (A) was counterstained with 0.06% Evans Blue for 20 seconds. Scale bar = 50μm.
In summary, the results obtained in this study point to a causal role of VEGF and its receptors in the increase in vascularization that occurs during pregnancy. They also support a role for VEGF in increasing vascular permeability during lactation, when increased transport of molecules from the blood is required for efficient milk protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Animals and Tissues**

Mammary glands were obtained from adult female Sprague-Dawley rats or Swiss Webster mice. Tissue samples were frozen in liquid-nitrogen-cooled isopentane, and stored at −80°C until use. The staging of most animals was confirmed on conventional hematoxylin and eosin-stained sections from Bouin-fixed paraffin-embedded tissues. In one series of experiments, surgical ablation of the epithelial components of the mammary gland was performed on 3-week-old mice, which were then allowed to mature to 10 weeks before isolation of mammary tissue. Verification of epithelial ablation was performed in parallel experiments by staining with Carmine red and by evaluating levels of keratin 8 mRNA in the operated glands. Contralateral sham-operated glands were used as a control. Human mammary tissue obtained from a 44 year old woman, who died suddenly of a cerebral vascular accident, was fixed in Bouin’s solution and embedded in paraffin. Details of fertility and parity were not available.

**Whole-Mount Staining and Quantitation of Vessel Density**

Dissected murine mammary glands were spread on histological slides and fixed in 4% paraformaldehyde for 1 hr at 4°C. The tissues were then digested with trypsin (50 μg/ml) at 37°C for 1 hr under agitation, incubated in 70% methanol containing 3% H₂O₂ for 30 min to remove endogenous peroxidases, and subsequently incubated overnight with 100 μg/ml of biotinylated *Bandeiraea simplicifolia* lectin B4 (Vector Laboratories, Burlingame, CA). Bound lectin was localized with an avidin-biotin-peroxidase amplification system (Vector), and mammary glands were mounted in Permount. Photographs were taken with a Zeiss Axiohot photomicroscope. For quantitation of vascular density, five random images of whole-mount glands were scanned into a quantitation program (Image-Pro) using a 3CCD Toshiba camera linked to a Dell computer. The algorithms used for conversion were based on a V constant value estimated after scanning a single capillary vessel (10 μm in diameter by 300 μm in length).

**Molecular Cloning of Rat VEGF-C, VEGFR-3,** and CD31/PECAM-1 Partial cDNAs

Partially degenerate oligonucleotides were designed from the amino acid sequences TFFKPPC and NKELED/E, SSQSSEE and QGRRRRP, or E/KAVYSVM and MSRPAA/VP, which are conserved in the human (Joukov et al., 1996) and mouse (Kukk et al., 1996) VEGF-C cDNAs, the human (Pajusola et al., 1993; Lee et al., 1996) and mouse (Finnerty et al., 1993) VEGFR-3 cDNAs, or the human (Newman et al., 1990), mouse (Xie and Muller, 1993) and bovine (Stewart et al., 1996) CD31 cDNAs, respectively. The primer sequences used were as follows: VEGF-C, forward: 5’-CCGGAATTCATGCTTCTTATACCCGTTGCTTG(C/T)T (containing an artificial EcoRI site at the 5’ end); reverse: 5’-CCGGAATTCAGCAGCTCTCTCAGCTGCTTG(A/G)GA(A/G)GA(A/G) (containing an artificial EcoRI site at the 5’ end); VEGFR-3, forward: 5’-CCGGAATTCAGCAGCTCTCTCAGCTGCTTG(A/G)GA(A/G)GA(A/G) (containing an artificial EcoRI site at the 5’ end); reverse: 5’-CCGGAATTCATGCTTCTTATACCCGTTGCTTG(C/T)T (containing a partial BamHI site at the 5’ end); CD31, forward: 5’-CCGGAATTCATGCTTCTTATACCCGTTGCTTG(A/G)GA(A/G)GA(A/G) (containing an artificial EcoRI site at the 5’ end); reverse: 5’-CCGGAATTCATGCTTCTTATACCCGTTGCTTG(A/G)GA(A/G)GA(A/G) (containing an artificial BamHI site at the 5’ end).

**Plasmid Construction**

Rat VEGF: a partial 393-bp cDNA fragment containing the common and alternatively spliced regions of the rat 164aa isoform (nucleotides 242–632 in Conn et al., 1990), was provided by Dr. B. Berse (Beth Israel Hospital, Boston, MA).

Mouse VEGF: a cDNA containing the entire coding region of murine VEGF (nucleotides -21 to 939 bp in Claffey et al., 1992) was provided by Dr. K. Claffey (Department of Physiology, University of Connecticut).

Rat VEGF-C: a partial 378-bp cDNA fragment was cloned as described above and in the Results section.

Rat VEGFR-1: a partial 600-bp cDNA (nucleotides 800–1,400; Yamane et al., 1994), was provided by Dr. D. Shibuya (University of Tokyo, Japan).
Rat VEGFR-2: non-overlapping partial 350-bp and 430-bp cDNAs were subcloned from a 1.3-kb cDNA obtained from a Fisher rat placenta cDNA library (nucleotides 2962–3313 and 3837–4256 in Wen et al., 1998), and were provided by Dr. M. Shibuya (University of Tokyo, Japan).

Mouse VEGFR-2: a partial 564-bp cDNA fragment (nucleotides 2345–2909 in Matthews et al., 1991) was obtained by RT-PCR from mouse placenta.

Rat VEGFR-3: a partial 357-bp cDNA fragment was cloned as described above and in the Results section.

Rat CD31 (C′): a partial 260-bp KpnI/FspI cDNA fragment (nucleotides 322–583) was subcloned from the 1026 cDNA described above and in the Results section. Rat CD31 (3′): the 1026 cDNA fragment described above and in the Results section was linearized with FspI; transcription from the T7 promoter produced a 450-bp 3′ cRNA probe.

Mouse CD31: a partial 1.6 kb cDNA fragment (nucleotides 639–2285 in Xie and Muller, 1993) cloned by RT-PCR from mouse placenta was provided by Dr. G. Wiedle (Basel Institute for Immunology, Switzerland).

Mouse keratin 8: a partial 670 bp cDNA fragment (nucleotides 12–682 in Baribault et al., 1993) was obtained by RT-PCR from mouse mammary gland.

RNA Extraction
Total cellular RNA was extracted from tissues and cells according to a modification of the acid guanidine-phenol-chloroform method of Chomczynsky and Sacchi (1987) as described by Chirgwin et al. (1979).

Ribonuclease Protection Assay
Prior to use in RNase protection assays, RNA integrity was assessed by direct visualization of 18S and 28S ribosomal RNAs following electrophoresis in a 1.2% agarose gel, transfer to nylon membranes (Hybond, Amersham, Buckinghamshire, UK), and methylene blue staining. RNAse protection assays were performed according to the manufacturer's instructions and previously published procedures (Pepper et al., 1990). Dried gels were exposed to Kodak XAR-5 or MR film (Eastman Kodak) at room temperature or at −80°C between intensifying screens. Autoradiographic images were scanned and quantitated using a densitometric scanner or phosphoimager. To ensure linearity of signal, quantitations were frequently performed at two different exposure times.

Immunohistochemistry
For VEGF, tissues were fixed in Bouin’s solution and embedded in paraffin. Six (6)-μm thick sections were deparaffinized, transferred to 100% ethanol, and treated for 10 minutes in methanol containing 0.5% H2O2. Sections were hydrated and exposed to 0.1% trypsin for 10 min at 37°C. Sections were preincubated for 30 min with 0.5% bovine serum albumin and then incubated for 2 hr at room temperature with an affinity purified rabbit polyclonal antibody prepared against a synthetic peptide corresponding to amino acids 1–20 of the amino terminus of human VEGF (cat no. sc-152, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1/200 dilution (0.5 μg/ml). A biotinylated anti-rabbit IgG was then applied for 30 min, followed by a streptavidin-peroxidase complex for a further 30 min. The presence of VEGF immunoreactivity was revealed by diaminobenzidine tetrahydrochloride (DAB) (0.05 v/v) and 0.01% H2O2 for 10 min. Sections were counterstained with hematoxylin, dehydrated in alcohol and mounted with Eukitt. The following specificity controls were used: omission of the primary antibody or incubation with normal rabbit serum, incubation with biotinylated anti-rabbit IgG or streptavidin-peroxidase complex alone followed by DAB treatment, and adsorption of the specific anti-VEGF antibody with its homologous antigen at concentrations ranging from 0.05–1.50 μg/ml for 12 hr prior to incubation.

For RECA-1, VEGFR-1, and type IV collagen, cryostat sections were fixed in cold acetone (RECA-1, VEGFR-1) for 4 min or 4% paraformaldehyde (collagen IV) for 10 min. RECA-1 (purchased from MEDAC GmbH, Hamburg, Germany; Duijvestijn et al., 1992) was applied at a dilution of 1:30 followed by a goat anti-mouse fluorescent antibody at 1/600. For VEGFR-1, an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 1312–1328 of the carboxy terminus of human VEGFR-1 (cat. no. sc-316, Santa Cruz Biotechnology, Santa Cruz, CA) was applied at a dilution of 1/50 followed by a fluorescent goat anti-rabbit antibody at 1/400. Collagen IV was detected by a sheep polyclonal anti-human collagen IV antibody (provided by Drs. H. Kleinman and G. Martin, NIDR, Bethesda, MD) applied at a dilution of 1/400 followed by a rabbit anti-sheep fluorescent antibody at 1/400. All incubations with primary antibodies were for 2 hr at room temperature; secondary antibodies were applied for 1
hr. Sections were counterstained with 0.06% Evans Blue for 20 sec. The first antibody was omitted as a control of specificity.

In Situ Hybridization

Sections were prepared from rat mammary glands frozen as described above. Eight (8)-µm thick frozen sections were cut using a Leica Jung CM 3000 cryotome and fixed in 4% paraformaldehyde for 5 min. Prehybridization, hybridization, and post-hybridization washes were as described (Sappino et al., 1989). Sections were hybridized using [3H]-labeled rat VEGF cRNAs prepared from the 393-bp VEGF cDNA fragment described above. Following hybridization and dehydration in ethanol, sections were immersed in Kodak autoradiographic emulsion (Eastman Kodak) and stored at 4°C for 117 days. Slides were developed in Kodak D-19 developer, fixed in Tetenal (Ott and Wyss AG, Zofingen, Switzerland) and photographed under dark field illumination using a Zeiss Axioshot photomicroscope.

Cell Culture

TAC-2 cells (Soriano et al., 1995), a clonally-derived subpopulation of the NMuMG mammary gland epithelial cell line (ATCC CRL 1636) (Owens et al., 1974), were cultured in collagen-coated tissue culture flasks (Falcon, Becton-Dickinson and Co., San José, CA) in high glucose DMEM (Gibco). Total cellular RNA was prepared as described above.

VEGF ELISA

Human milk was obtained from 5 lactating mothers during the first week post-partum. VEGF levels were determined using ELISA kits from two different sources. The first, a sandwich ELISA, was purchased from R&D Systems Europe Ltd. (Abingdon, Oxford, UK). The second, a competitive ELISA, was purchased from Cyt Immune Sciences Inc. (College Park, MD). ELISAs were performed according to instructions in the enclosed manuals.

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