

Positive Correlation between Estradiol and Vascular Endothelial Growth Factor but not Fibroblast Growth Factor-2 in Normal Human Breast Tissue *In vivo*

Charlotta Dabrosin

Abstract Purpose: Angiogenesis is crucial in tumor development and progression. Ovarian hormones regulate angiogenesis in the reproductive tract but very little is known about its regulation in the normal breast. Sex steroids play an important role in breast cancer development by poorly understood mechanisms. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are potent stimulators of angiogenesis. Both VEGF and FGF-2 function in autocrine/paracrine pathways and there is a major contribution of bioactive proteins by a posttranslational activation of sequestered molecules in the extracellular space. A direct measurement of these molecules in the extracellular compartment is, therefore, needed.

Experimental Design: In this study, microdialysis was used to measure extracellular VEGF and FGF-2 in normal human breast tissue *in situ* in 11 premenopausal and 5 postmenopausal women.

Results: Significantly higher level of VEGF in breast tissue of premenopausal women was found. Plasma as well as local estradiol and breast tissue VEGF exhibited significant correlations, whereas progesterone had no correlation with breast VEGF. FGF-2 did not correlate with either estradiol or progesterone.

Conclusion: The result suggests that estradiol is a more potent regulator of free VEGF levels than progesterone in the normal breast. The control of free FGF-2 seems to be independent of sex steroids in the breast. Estrogen induction of free extracellular VEGF may be one mechanism involved in sex steroid – dependent breast carcinogenesis.

The breast is a sex steroid-dependent organ and long-term exposure to these hormones increases the risk of breast cancer (1–4). The pathophysiologic mechanisms behind this increase are still not fully understood. Angiogenesis is a key factor in tumor development and progression (5). In the adult organism, angiogenesis is virtually absent except in the female reproductive organs, which are dependent on angiogenesis for normal function. This suggests a potential role for sex steroids on the regulation of angiogenesis.

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors and several isoforms of VEGF exist as a result of alternative splicing from a single gene, which differ in their binding to extracellular compounds (6). The freely diffusible VEGF in the extracellular space available to the endothelial cells is the bioactive fraction of VEGF (7–9). Very little is known about the regulation of VEGF in normal human

breast although sex steroids have been shown to increase VEGF and participate in the regulation of angiogenesis in experimental breast cancer (10–14). Our previous studies have revealed a cyclic variation of VEGF in normal human breast tissue *in vivo* during the menstrual cycle in healthy women (15). This suggests that sex steroids affect VEGF production in normal breast tissue but it is uncertain whether this is an effect of estradiol only or if progesterone also contributes to the regulation of VEGF.

Another potent angiogenic molecule is fibroblast growth factor-2 (FGF-2)/basic FGF, which has been associated with tumor growth and metastasis in several human cancers (16). In breast cancer, FGF-2 has been shown to be elevated compared with adjacent normal breast tissue (17). In endometrial tissue, sex steroids seem to regulate FGF-2 mRNA levels but it is not known if FGF-2 is regulated in the same manner in breast tissue (18).

Both VEGF and FGF-2 are sequestered in the extracellular matrix and the levels of bioactive free molecules are dependent of a posttranslational activation by enzymatic cleavage. To investigate the regulation of these compounds, it is, therefore, necessary to use a technique for sampling of the extracellular space. With the microdialysis technique, it is possible to mimic a blood vessel in a tissue and by diffusion of extracellular molecules over the microdialysis membrane, it is possible to mirror events in extracellular compartment. We have previously shown that microdialysis is applicable for investigations of the normal human breast (15, 19–22).

In the present study, microdialysis was used to determine VEGF and FGF-2 in normal human breast tissue *in vivo* in

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premenopausal and postmenopausal women. Results of these studies show that estradiol may play a critical role in the regulation of VEGF but not FGF-2 in normal human breast tissue.

Materials and Methods

Subjects. The local ethical committee approved the study and all women gave their informed consents.

Volunteers for this explorative study were recruited over a period of 1 year. A total of 16 women participated in the microdialysis study, 11 premenopausal (ages 22-30 years) and 5 postmenopausal (ages 60-65 years). All women had been off sex steroid containing medication, such as contraceptive methods or hormone replacement therapy, for >3 months. All premenopausal women had a history of regular menstrual cycles (cycle length 27-34 days). Seven of the premenopausal women were investigated in the luteal phase of the menstrual cycle, whereas four were investigated in the follicular phase.

Breast tissue biopsies. Biopsies from human breast tissue were obtained from premenopausal women, without ongoing hormonal treatment, undergoing routine reduction mammoplasty. Tissue biopsies containing epithelium, stroma, and adipose tissue were produced by using an 8 mm biopsy punch (Kai Europe GmbH, Solingen, Germany) and placed in a 12-well plate (Costar, Cambridge, MA). Serum-free medium was used consisting of a 1:1 mixture of nutrient mixture F-12 (Life Technologies, Paisley, United Kingdom) and DMEM without phenol red (Life Technologies) supplemented with transferrin (10 µg/mL; Sigma), insulin (1 µg/mL; Sigma), and bovine serum albumin (0.2 mg/mL; Sigma) with or without physiologic levels of 10^{-9} mol/L estradiol (17β-estradiol, E2; Sigma) or a combination of 10^{-9} mol/L estradiol and 10^{-8} mol/L progesterone (Sigma). The control group was incubated in medium supplemented with the vehicle, ethanol, equivalent to the hormone-treated groups (0.001%). The biopsies were treated for 7 days at 37°C in a humidified atmosphere containing 5% CO₂ and the medium was changed every day. After the 7th day of incubation, the medium from each biopsy was collected and stored at -70°C for subsequent analysis. The tissue biopsies were weighed, formalin-fixed, and paraffin-embedded and subjected to H&E staining and immunohistochemistry.

Microdialysis device. A microdialysis catheter (CMA 65, CMA/Microdialysis AB, Solna, Sweden), which consists of a tubular dialysis membrane (10 mm length × 0.52 mm diameter, 100,000 atomic mass cutoff) glued to the end of a double-lumen tube (80 mm length × 0.8 mm diameter), was used. The catheters were inserted, guided by a catheter, for i.v. use (Venflon 1.4 mm, BOC Ohmeda AB, Helsingborg, Sweden). The catheters were connected to a microinfusion pump (CMA 107, CMA/Microdialysis) and perfused with 154 mmol/L NaCl and 40 g/L dextran-70 at a perfusion rate of 0.5 µL/min. The solution entered the catheter through the outer tube and left it through the inner tube from which it was collected. After a 30-minute equilibration period, the outgoing perfusate was collected and stored at -70°C for subsequent analysis.

Experimental design. Mepivacaine (5 mg/mL) was administered intracutaneously as a local anesthetic before the insertion of the microdialysis catheters. The microdialysis catheter was placed in the upper lateral quadrant of the left breast and directed toward the nipple as previously described (15, 19, 21, 23). Microdialysis is a technique that allows continuous sampling of the extracellular fluid by passive diffusion of substances over a semipermeable membrane. The recovery (i.e., the amount of substances that diffuse into the perfusion fluid) depends on the membrane properties, the flow rate, and the size of the compound of interest (24). Diffusion of low molecular substances over the dialysis membrane is almost complete at low flow rates using a 30-mm-long dialysis membrane (25). However, for larger molecules, the recovery over the membrane decreases and the measured levels in the microdialysis sample will not be absolute concentrations in the tissue.

The recovery of certain substance may be measured *in vitro* by putting a microdialysis in a vial containing the compound of interest, perfuse the catheter, and divide the concentration of the substance in the dialysate by the concentration in the vial. This *in vitro* recovery can only be an estimate of the *in vivo* recovery because other factors, such as tissue pressure and temperature, will affect the diffusion of substances. Therefore, all microdialysis values are given as original raw data without any recalculations.

Determinations of estradiol, progesterone, vascular endothelial growth factor, and fibroblast growth factor-2. Plasma was collected using a glass tube containing 3.8% sodium citrate as an anticoagulant and were spun down and frozen at -70°C within 20 minutes of collection.

Microdialysate and plasma samples were analyzed for VEGF, FGF-2, estradiol, and progesterone using a commercial quantitative immunoassay kits, and VEGF and FGF-2 using the Fluorokine mitogen-activated protein cytokine multiplex kits (R&D Systems, Abingdon, United Kingdom) analyzed with the Luminex 100 analyzer. The sensitivity of VEGF (165 isoform) was 0.81 pg/mL and intra-assay variation was from 3.7% to 6.7%. The sensitivity of FGF-2 was 1.81 pg/mL and intra-assay variation was from 3.4% to 5.5%. Estradiol and progesterone were analyzed using ELISA kits (DRG Instruments GmbH, Marburg, Germany). The sensitivity of the assay was 16 pmol/L and the intra-assay variation was 5%. For progesterone, the cutoff level for luteal phase was 13 nmol/L, the sensitivity was 0.15 nmol/L, and the intra-assay variation was 5%. The intra-assay variation of the kits was confirmed during the experiments. All samples were assayed in duplicate.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue biopsies were cut in 3 µm sections, deparaffinized, and subjected to antihuman immunohistochemistry [mouse anti-human VEGF, dilution 1:20, or goat anti-human FGF-2 dilution 1:40 (R&D Systems) with Envision detection (DAKO, Solna, Sweden)] Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. For VEGF and FGF-2 scoring, the whole material was scanned to identify the range of intensity of the staining. Thereafter, the staining on each biopsy section was scored either as weakly or strongly positive. In a blinded manner, 10 high power fields (×200) were examined per section.

Statistics. Data are expressed as mean ± SE. Student's *t* test, Pearson's correlation coefficient with Fisher's *r* to *z* test for the corresponding *P* values, and Fisher's exact test were used where appropriate. *P* < 0.05 was considered as statistically significant.

Results

Estradiol and progesterone. There were no subsequent complications after the microdialysis experiments.

The plasma level of estradiol in the postmenopausal women was 77 ± 30 pmol/L and the progesterone level was 0.7 ± 0.1 nmol/L. In the premenopausal women, the estradiol level was 237 ± 18 pmol/L. The progesterone level of women in the follicular phase was 2.8 ± 1.6 nmol/L. For women in the luteal phase, the progesterone level was 38 ± 2 nmol/L, indicating that they had ovulated during the cycle.

Vascular endothelial growth factor. The postmenopausal women had a significant lower level of extracellular VEGF in the breast compared with the premenopausal women (2.3 ± 0.4 versus 4.8 ± 0.7 pg/mL, *P* < 0.05; Fig. 1). In all 16 women, there was a positive significant correlation between plasma estradiol levels and VEGF in breast tissue (*r* = 0.814, *P* < 0.0001, *n* = 16; Fig. 2A). There was also a significant correlation between extracellular local estradiol levels and local VEGF levels in the breast (*r* = 0.67, *P* = 0.004, *n* = 16; Fig. 2B). The significant correlation between estradiol and breast VEGF was maintained when the postmenopausal women were excluded from the calculation (*r* = 0.804, *P* = 0.0028, *n* = 11). There was

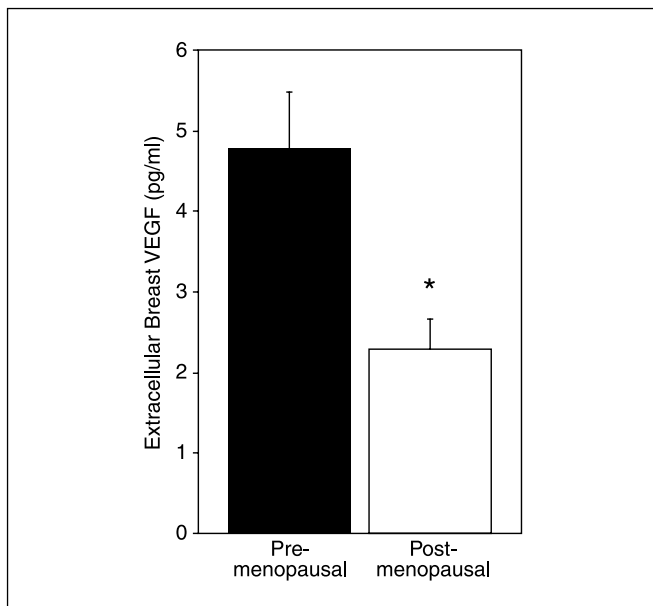


Fig. 1. Extracellular levels of VEGF in normal breast tissue *in vivo* were measured using microdialysis in 11 premenopausal and 5 postmenopausal women. There was a significantly higher level of VEGF in the premenopausal women. *, $P = 0.03$.

no correlation between plasma or local progesterone levels and VEGF either in the whole study group of 16 women [$r = 0.328$, $P = 0.24$, $n = 16$ for plasma progesterone (Fig. 2C) and $r = 0.34$, $P = 0.18$ for local progesterone] or for the premenopausal women ($r = 0.012$, $P = 0.97$, $n = 11$ for plasma progesterone and $r = 0.034$, $P = 0.92$ for local progesterone).

In the breast biopsy experiments ($n = 6$ in each group), estradiol exposure induced a significant increase of VEGF secretion into the medium (430 ± 62 pg/mg tissue) compared with 142 ± 20 pg/mg tissue in the control group ($P < 0.01$). Estradiol + progesterone treatment to the tissue increased the VEGF levels in a similar manner as estradiol exposure only (443 ± 46 pg/mg tissue, $P < 0.01$ compared with controls). Immunohistochemical staining [10 high power fields ($\times 200$) were examined per section, $n = 6$ in each group] of the sections did not reveal any differences of intensity between the groups.

Fibroblast growth factor-2. Extracellular FGF-2 in the breast exhibited no difference between premenopausal and postmenopausal women; premenopausal levels were 12.7 ± 2 pg/mL and postmenopausal levels were 14.7 ± 4.7 pg/mL ($P = 0.66$; Fig. 3). There were no correlation between plasma estradiol and FGF-2 either for the whole study group ($r = 0.113$, $P = 0.67$, $n = 16$; Fig. 4A) or for the premenopausal women only ($r = 0.029$, $P = 0.93$, $n = 11$). There was no correlation between local extracellular estradiol and FGF-2 ($r = 0.27$, $P = 0.32$, for the whole study group, $n = 16$; Fig. 4B) or for the premenopausal women ($r = 0.058$, $P = 0.57$, $n = 11$).

Plasma progesterone levels exhibited no correlation either when the whole study group ($r = -0.359$, $P = 0.17$) was included (Fig. 4C) or for the premenopausal women only ($r = -0.521$, $P = 0.1$, $n = 11$). Local progesterone did not show any correlation with FGF-2 for the whole study group ($r = -0.325$, $P = 0.22$, $n = 16$) or for premenopausal women ($r = -0.458$, $P = 0.16$, $n = 11$).

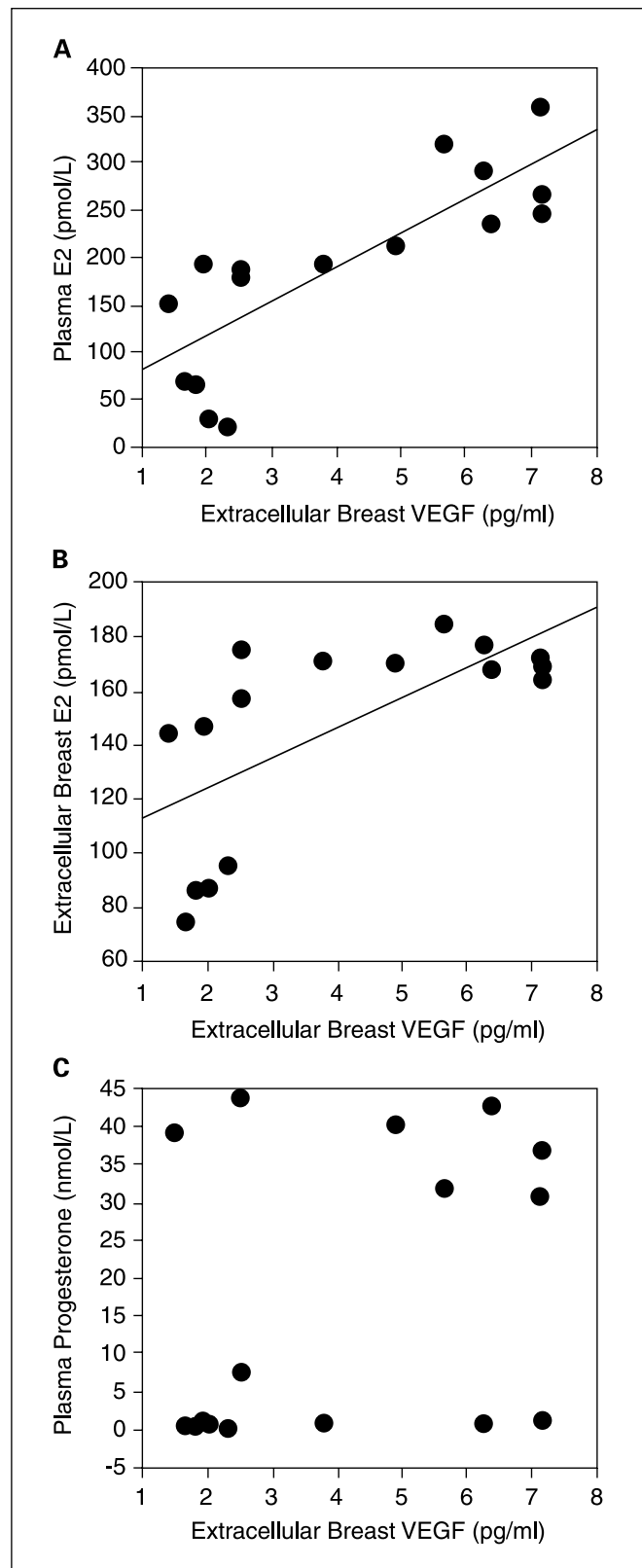


Fig. 2. Extracellular VEGF in normal breast tissue *in vivo* was measured as described in Fig. 1. A, there was a significant correlation between extracellular breast VEGF and plasma estradiol (E2) in premenopausal and postmenopausal women ($r = 0.814$, $P < 0.0001$, $n = 16$). B, there was a significant correlation between breast VEGF and extracellular breast estradiol ($r = 0.67$, $P = 0.004$, $n = 16$). C, there was no correlation between plasma progesterone and breast VEGF ($r = 0.328$, $P = 0.24$, $n = 16$).

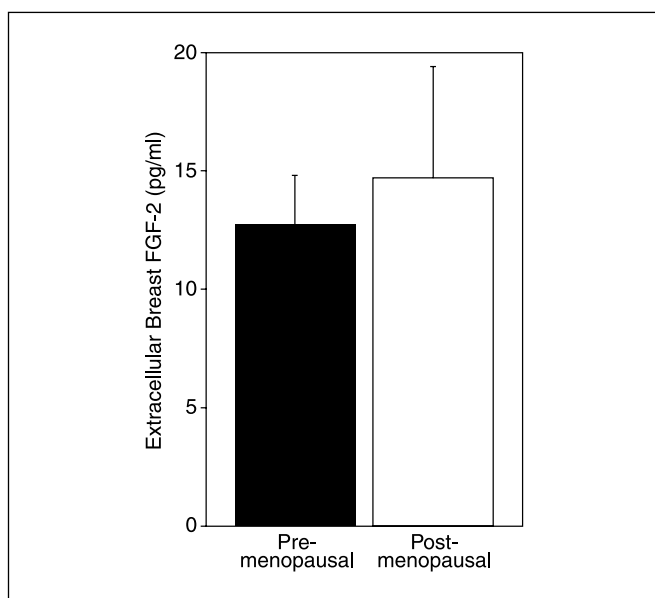


Fig. 3. Extracellular levels of FGF-2 in normal breast tissue *in vivo* were measured using microdialysis in 11 premenopausal and 5 postmenopausal women. There were no significant difference between the levels in premenopausal and postmenopausal women ($P = 0.66$).

In the breast biopsy experiments ($n = 6$ in each group), hormone exposure did not affect secreted FGF-2 into the medium— 146 ± 23 pg/mg tissue in the control group, 147 ± 12 pg/mg tissue in the estradiol exposed group, and 139 ± 10 pg/mg tissue in the estradiol + progesterone exposed group. Immunohistochemical staining (10 high power fields, $\times 200$, were examined per section, $n = 6$ in each group) of the sections did not reveal any differences of intensity between the groups.

Discussion

This study shows a positive correlation between plasma estradiol as well as local breast estradiol and extracellular VEGF in normal human breast tissue *in vivo*, whereas progesterone and VEGF had no correlation. There were no correlations between estradiol or progesterone and extracellular FGF-2 in the breast. Estradiol exposure of human breast tissue biopsies in culture increased secreted VEGF levels compared with controls with no additional effect of estradiol + progesterone treatment compared with estradiol only, whereas FGF-2 was unaffected by the hormone treatment. These results suggest that estradiol plays an important role in the regulation of secreted VEGF in normal breast tissue and that the regulation of FGF-2 is unaffected by sex steroids.

VEGF is a key regulator of angiogenesis and several VEGF isoforms can be generated by alternative splicing of a single gene (6). Up to date, six isoforms have been reported that vary in their ability to bind to heparin and heparin-like molecules (8). The long isoforms are almost completely sequestered in the extracellular matrix (26). It has been suggested that it is the soluble forms of VEGFs that are biologically active and available to endothelial cells (26). The soluble free VEGF may either be released as freely diffusible proteins from the cells or released as a cleaved fragment from the longer isoforms by

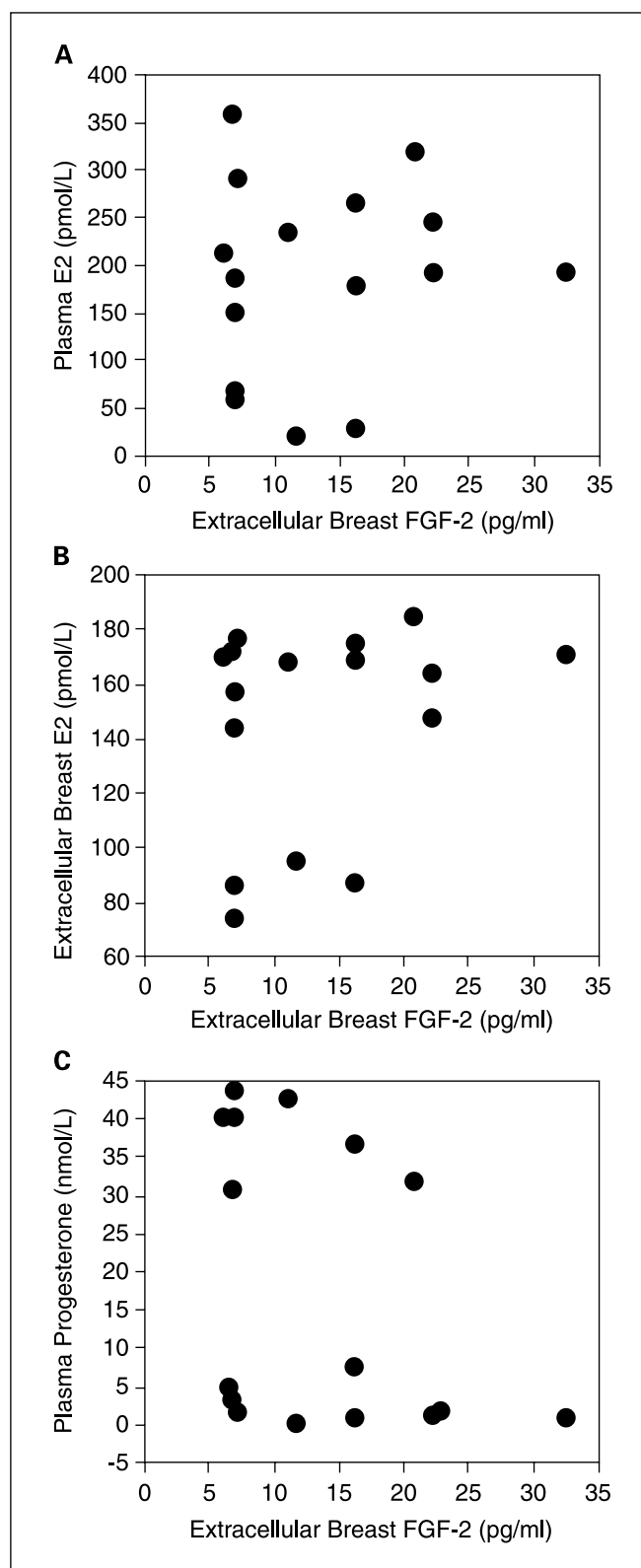


Fig. 4. Extracellular FGF-2 in normal breast tissue *in vivo* was measured as described in Fig 3. *A*, there was no correlation between breast FGF-2 and plasma estradiol in premenopausal and postmenopausal women ($r = 0.113$, $P = 0.67$, $n = 16$). *B*, there was no correlation between breast FGF-2 and local extracellular breast estradiol ($r = 0.27$, $P = 0.32$, $n = 16$). *C*, there was no correlation between breast FGF-2 and plasma progesterone ($r = -0.359$, $P = 0.17$, $n = 16$).

protease activation (8). The posttranslational activation process of VEGF clearly indicates that it is necessary to measure VEGF in the compartment where the protein is biologically active, namely in the extracellular space rather than at a gene or mRNA level. This is shown by a previous study that showed that although both estradiol and the antiestrogen tamoxifen, in a similar fashion, induced increased mRNA and intracellular VEGF protein, secreted VEGF was decreased by tamoxifen and, along with this decrease, angiogenesis was inhibited (27). Similar to these results, immunohistochemistry of hormone treatment of breast tissue biopsies in the present study did not reveal any differences, whereas the secretion of VEGF significantly increased after hormone exposure.

Estrogens have been shown to regulate angiogenesis and VEGF in experimental breast cancer but very few studies have investigated the regulation of angiogenesis in the normal human breast (13, 14, 28). In breast cancer cell lines, both estrogen and progesterone have been shown to increase VEGF levels (11, 27). In the few studies that exist of normal breast tissue, human and baboon, extracellular VEGF and mRNA levels were shown to be increased in the luteal phase of the menstrual cycle when both estradiol and progesterone were elevated (15, 29). However, in these studies, it was not possible to elucidate if this increase was dependent on estradiol and/or progesterone. In addition to estrogen synthesis in the ovaries, estradiol may be produced locally in tissues, especially in postmenopausal women. In the present study, estradiol was, therefore, measured both in plasma and locally in the microdialysis fluid from the breast. Both local and plasma estradiol exhibited a significant correlation with breast VEGF and these results strongly suggest that estradiol has a critical role in the regulation of VEGF in normal human breast tissue, whereas progesterone seems to be of minor importance.

Another well-defined proangiogenic molecule is FGF-2 (30). FGF-2 belongs to a family of polypeptide growth factors, which include at least 20 distinct FGFs (31). Although the secretion pathway(s) of FGF-2 is undefined, it is clear that this peptide acts extracellularly by high-affinity extracellular cell surface tyrosine kinase receptors (31). In the extracellular space, FGF-2 binds to heparin or heparan-like glycosaminoglycans and this binding functions as a local reservoir that protects the peptides from degradation (31). FGF-2 may be released from this

reservoir either by enzymatic cleavage or by binding to a carrier protein (31). The enzymatic cleavage may occur either by proteases or heparanases. It has been shown that increased expression of heparanases is associated with an invasive phenotype of breast cancers (32). Hence, similarly to VEGF, there is a major contribution of bioactive FGF-2 by a posttranslational activation by several pathways and clearly these growth factors need to be investigated at their bioactive site. As we previously showed, microdialysis provides an excellent tool for investigations of the extracellular compartment in breast tissue (13, 15, 19–22) and, to my knowledge, this is the first study that measured extracellular FGF-2 in human tissue.

In the case of FGF-2, much less is known regarding a possible sex steroid regulation in breast tissue. In the endometrium, FGF-2 mRNA has shown a cyclic variation (18, 33), suggesting that at least mRNA levels may be under the regulation of sex steroids. In breast cancer, FGF-2 levels seem to be up-regulated compared with normal adjacent tissue and related to high expression of the estrogen receptor, suggesting that there may be a correlation (17). However, data on direct regulation of FGF-2 levels by sex steroids in breast tissue is sparse. The present study does not support the concept that extracellular FGF-2 is under a regulation of estradiol or progesterone in normal human breast tissue either *in vivo* using microdialysis or *in vitro* using breast tissue biopsies in culture. FGF-2 may still be involved in the control of angiogenesis in the breast; however, factors other than sex steroids may be of greater importance for its regulation.

In summary, this study shows that extracellular levels of VEGF in normal human breast tissue *in vivo* exhibit a positive correlation with estradiol but not progesterone levels. This result suggests that estradiol is a more potent regulator of extracellular VEGF levels than progesterone in the normal breast. The control of extracellular FGF-2 seems to be independent of sex steroids in the breast. Tumor development may be facilitated by a dysregulation of angiogenic factors and future studies of these mechanisms will be required to elucidate the role of these proteins in sex steroid-dependent breast cancer development. Studies of the proteins in their bioactive compartment, the extracellular space, will substantially increase our knowledge of the control of these factors at their bioactive site.

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