Mitogenic Growth Factors in Breast Fluid Obtained from Healthy Women: Evaluation of Biological and Extraneous Sources of Variability

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Abstract

Peptide growth factors (GFs), including epidermal GF (EGF) and transforming GF-α (TGF-α), are presumed to play an important role in the local regulation of breast cell proliferation. Breast fluid collected by nipple aspiration provides a potential means to assess the concentration of these factors in contact with the ductal epithelium. Although identification of immunoreactive EGF-like GFs in breast fluid has been reported previously, we performed this study to evaluate the sensitivity and reliability of newer RIA methods and to characterize the sources and amounts of both intra- and intersubject variability. We also evaluated the relationship of hormone concentrations in breast fluid (9, 10). Other studies have demonstrated that a small volume of breast fluid can be obtained with ductal epithelial cells. Several groups of investigators have reported immunoreactive EGF levels within an individual were significantly associated overall with both TGF-α (P = 0.02) and plasma estradiol levels (P = 0.01). These data, which are the first comprehensive results on the feasibility of measuring mitogenic GFs in breast fluid, support the conclusion that women secrete consistent and individually distinct levels of EGF and TGF-α and that, in at least some women, EGF secretion in vivo covaries with both TGF-α in breast fluid and circulating estradiol.

Introduction

The prevailing model of breast cancer development assigns an important role to locally acting autocrine/paracrine GFs (1). Peptides such as EGF and TGF-α, which have a 30–40% amino acid homology, demonstrate potent mitogenic effects on human breast cancer cells in vitro (2). In addition, the EGF receptor and the homologous product of the oncogene c-erbB2, which bind both EGF and TGF-α, are overexpressed in a significant proportion of breast cancers, particularly those with a poor prognosis (3, 4). Undoubtedly, these GFs, the structures of which are highly conserved across species, also play a role in control of normal breast cell proliferation. Current evidence indicates that estradiol and antiestrogens such as tamoxifen have direct and opposite effects on production of these GFs by epithelial or stromal cells in the breast (5). High levels of ovarian activity and of estrogen itself are related to increased breast cell proliferation and indeed provide the most cogent explanation for the increased breast cancer risk attributable to diverse factors such as age at menarche, age at menopause, and obesity. Overexpression of EGF-type GFs is strongly associated with mammary cancer in transgenic mice and the early stages of spontaneous mammary tumor development in normal mice (6, 7). It is plausible, therefore, to hypothesize that healthy women with excessive production of these mitogenic GFs have an elevated risk of developing breast cancer.

Because EGF and TGF-α are produced locally and act locally, their concentrations in serum or urine are not necessarily relevant. On the other hand, nipple aspiration provides a noninvasive method for sampling fluid that is in close contact with ductal epithelial cells. Several groups of investigators have demonstrated that a small volume of breast fluid can be obtained from 40–70% of nonlactating women by using a simple pump-like device (8). On the basis of numerous biochemical analyses, this fluid appears to provide reasonable insight into the hormonal and metabolic microenvironment of the breast.

One group of investigators has reported immunoreactive EGF and TGF-α concentrations in breast fluid (9, 10). Other studies

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1 The abbreviations used are: GF, growth factor; EGF, epidermal GF; TGF-α, transforming GF-α; CV, coefficient of variation; QC, quality control; ICC, intraclass correlation coefficient.
have reported on these or similar GFs in human milk or breast cyst fluid, although the comparability of milk or cyst fluid to nipple aspirate fluid is questionable (11-13).

The studies described in this report were designed to address basic methodological questions concerning the assay of EGF and TGF-α in breast fluid. We evaluated the sensitivity and reproducibility of these assays and the effect of specimen handling and storage. We then explored the variation in levels within women between breasts and within the same breast over time, comparing these within-woman variations to the amount of variation seen between women. For a biomarker to be useful in clinical or epidemiological research, it is critical that there be a substantial amount of variation between individuals relative to the variation within individuals (14). Finally, we determined whether breast fluid EGF and TGF-α levels were related to the menstrual cycle phase, to plasma estradiol or progesterone levels, or to each other. By repeat sampling of individual women, we were able to assess these relationships within individual women, as well as in the group as a whole.

Subjects and Methods

Study Population and Sample Collection. Following approval of the protocol and informed consent procedures by the Institutional Review Board, we recruited women from the Chicago area to participate in the Repeat Sample Study. Criteria for eligibility included: ages 25-45 years, no history of breast cancer, regular menstrual periods, no lactation within 6 months, no use of oral contraceptives or other exogenous hormones within 6 months, and no major concurrent illnesses. Sixty-five eligible women were scheduled for four outpatient appointments each, 1 week apart, at the Clinical Research Center at Northwestern Memorial Hospital. Participants were allowed to start their visits during any day of the menstrual cycle, and they arrived at the Clinical Research Center in the morning after an overnight fast. The position of each visit day in the menstrual cycle was determined by recording the dates of onset of all menstrual bleeding minus 14 days, the average length of the luteal phase. Dating, in which the midcycle day is defined as the first day of menstrual bleeding immediately prior to and after the four visits. This allowed cycle position to be estimated by reverse dating, in which the midcycle day is defined as the first day of bleeding minus 14 days, the average length of the luteal phase. At each visit, we collected plasma and breast fluid. In addition, we measured body size and fat composition, physical activity, and dietary intake, for analyses not presented here. The mean age of the participants was 33.7 years; 76% were white, 11% were African-American, and 4% were Asian.

Breast Fluid Collection. At each visit, a trained nurse attempted to aspirate breast fluid from both breasts of each participant. After lightly scrubbing the nipple with a water-moistened gauze pad, the nurse asked the participant to compress the breast at its base with both hands. A sterile suction device made from a 20-cc plastic syringe body was then applied to the nipple surface after 10 s. Droplets of breast fluid appearing at the duct openings were collected in 75-mm plastic-coated capillary tubes that were then clay-sealed at both ends and kept on ice until storage at −20°C, no more than 1 h after collection. We obtained at least 2 µl of breast fluid from 39 (60%) of the 65 women who attended at least one visit. For the assays in this report, we selected samples from 18 women, 15 of whom gave samples at three or four visits and 13 of whom gave samples from both breasts on at least one visit.
Table 3  Breast fluid GF levels: variation between and within Repeat Sample
Study participants (same breast, different days)

<table>
<thead>
<tr>
<th></th>
<th>No. of subjects</th>
<th>No. of samples</th>
<th>Mean</th>
<th>CV between</th>
<th>CV within</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>15</td>
<td>57*</td>
<td>494 ng/ml</td>
<td>1.20</td>
<td>0.26</td>
<td>0.83</td>
</tr>
<tr>
<td>TGF-α</td>
<td>15</td>
<td>58</td>
<td>2.68 ng/ml</td>
<td>0.78</td>
<td>0.42</td>
<td>0.37</td>
</tr>
<tr>
<td>EGF/protein</td>
<td>15</td>
<td>57</td>
<td>6.87 ng/mg</td>
<td>1.61</td>
<td>1.05</td>
<td>0.24</td>
</tr>
<tr>
<td>TGF-α/protein</td>
<td>15</td>
<td>58</td>
<td>73.2 pg/mg</td>
<td>3.81</td>
<td>2.58</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* This sample was not included in analysis of EGF levels due to a laboratory error.

Assays for Plasma Estradiol, Bioavailable Estradiol, and Progesterone. Plasma estradiol was measured using a RIA kit obtained from Diagnostic Systems Laboratories (Webster, TX). This double antibody assay has a sensitivity of 29 pmol/liter and low cross-reactivity with forms of estrogen other than estradiol. The mean intra- and interassay CVs based on blinded QC samples were 0.08 and 0.04, respectively. Sex hormone binding globulin-bound estradiol was measured using the concanavalin A-Sepharose method described by Bonfrer et al. (16). This assay gave intra- and interassay CVs of 0.04 and 0.09, respectively, in our QC samples. Plasma progesterone concentrations were measured by RIA using antibodies prepared by Dr. R. Chatterton (17). The intra- and interassay CVs were 0.09 and 0.10, respectively, in our QC samples.

Assays for Breast Fluid EGF, TGF-α, and Protein. Breast fluid was removed from the capillary tube while it was in a semi-frozen state, and, except when undiluted sample was needed for sensitivity studies, it was diluted with a Tris-saline buffer (pH 8.0) prior to assay. The initial dilution was made without BSA in the buffer, an aliquot was taken for protein assay, and the final dilutions were made with BSA to a concentration of 0.15%. We used competitive binding RIA kits purchased from BioMedical Technologies (Stoughton, MA) for both EGF and TGF-α. The EGF assay has an estimated cross-reactivity of less than 0.1% with human TGF-α and undetectable binding with other human peptides tested. The TGF-α assay has cross-reactivities of less than 0.1% with human EGF and undetectable binding with other human peptides tested. To evaluate assay sensitivity, dilutions of breast fluid ranging from undiluted to 1:200 were prepared. The intra-assay CV at each dilution was evaluated by analyzing replicates in the same assay run to determine the dilution level at which assay reliability became unacceptable. Total protein in breast fluid was measured by the Bradford method.

Data Analysis. We calculated CVs and ICCs to assess intra- and interassay variability and the amount of variation within versus between individuals. Interassay CV was calculated from the variance between assays with the intra-assay variance removed. The ICC is defined as the between-person variance divided by the total variance (Ref. 18). To determine which method minimized extraneous variation, we compared within-person CVs for GF concentrations expressed per unit breast fluid volume to those expressed per weight of total protein. To compare right versus left breast results and to compare EGF versus TGF-α or either GF versus plasma hormone levels, we computed the nonparametric Spearman correlation coefficients (r). These coefficients are unbiased, but because there were multiple measurements from the same person, the conventional variance estimate of the coefficient estimates was too low. To obtain correct variance estimates and compute Ps for r, we used a permutation method, which generates an approximate distribution of r under the null hypothesis (19).

Growth factor and hormone concentrations were not normally distributed. To facilitate parametric analyses, we evaluated several data transformations and concluded that the square-root transformation provided the best normalization for the key variables as a group. Therefore, to compute mean GF concentration plus estimated 95% confidence intervals for each phase of the menstrual cycle, we obtained SEs and confidence intervals from the transformed data and then converted back to the original units for reporting purposes. The menstrual cycle was divided into the following six phases, with 0 designated as the midcycle day: early (days < -10), mid-(days -10 to -6), and late (days -5 to -1) follicular and early (days 0–4), mid- (days 5–9), and late (days 10–13) luteal. Ps for comparison of GF levels by cycle phase were obtained by random effects modeling using PROC GLM in SAS (SAS Institute, Inc., Cary, NC). We also used random effects models, with EGF as the dependent variable, to determine the degree of linear association between EGF and TGF-α and plasma estradiol (20). An inter-
action term consisting of a binary dummy variable for each subject multiplied by the predictor level (TGF-α or plasma estradiol) allowed us to evaluate the biomarker associations within individuals. Similar models were developed with TGF-α as the dependent variable and EGF and plasma estradiol as the predictors. From these models, we obtained estimates of the total variance and within-woman variance explained by each model term. Although we had enough data to estimate the contribution of within-woman correlation to overall model fit, the small number of samples available per woman precluded testing hypotheses about correlation in specific individuals.

Results

Table 1 shows results indicating the sensitivity and reliability of the EGF and TGF-α measurements in breast fluid. For EGF, analysis of samples diluted 1:100 with assay buffer gave acceptable intra-assay reliability. Thus, we were able to obtain reliable results using only 1 μl of breast fluid, which contained concentrations in the range of 4–6 ng/ml in diluted samples from various QC pools. For TGF-α, both intra-assay and interassay reliability were acceptable at dilutions of 1:25, but not at 1:50. We were therefore able to reliably measure TGF-α in only 4 μl of breast fluid, with measured concentrations in pooled, diluted samples of about 0.1 ng/ml. EGF and TGF-α
concentrations in pools prepared from women with abundant versus scant volumes of breast fluid were indistinguishable. In one experiment, the number of freeze-thaw cycles (ranging from two to six) was not associated with any trends in measured GF concentrations.

Mean GF levels and results on the variation between the right and left breast are shown in Table 2. EGF levels were unobtainable from both breasts in one woman due to a laboratory error. For EGF, the within-woman variation (between breasts) was considerably less than the variation in EGF levels between women. The ICC implies that 48% of the total variance in EGF could be attributed to between-woman differences. For TGF-α, the within-woman variation between breasts was even lower, and the ratio of between-woman to within-woman variation was even higher. Eighty-eight% of the total variance in TGF-α was attributable to between-woman differences. Table 2 also shows that expressing GF levels per weight of total protein rather than per unit volume did not improve, and in fact even lower, and the ratio of between-woman to within-woman variation was far greater than the variation between breasts. Total breast fluid protein levels were correlated between breasts (data not shown).

Fig. 1 displays the agreement in EGF and TGF-α levels between breasts for individual women sampled in both breasts on the same date. Right and left breast levels were well correlated: EGF, $r = 0.78$ and $P = 0.003$; and TGF-α, $r = 0.89$ and $P = 0.001$.

Table 3 shows the variation in GF levels between and within women in the same breast over time. Once again, the variation between women was far greater than the variation within individual women over time for both GFs. The range of breast fluid EGF concentrations between women was extremely high (over 100-fold differences), and thus, the between-women variance for EGF was by far the dominant component of total variance (ICC = 0.83). Variances over time were not reduced by expressing results per weight of total protein rather than per volume. Fig. 2 shows the EGF (Fig. 2A) and TGF-α (Fig. 2B) results for each woman over time. This graph provides visual evidence that women tend to have highly distinct levels of EGF that are relatively consistent over time. TGF-α levels for individual women (Fig. 2B) also tend to remain stable over time, although the decreased variation between women, compared to EGF, is evident.

To more closely examine whether GF levels in breast fluid vary in conjunction with the menstrual cycle, we plotted the mean EGF and TGF-α concentrations for six cycle phases, as shown in Figs. 3 and 4. We found no significant differences for either GF across cycle phases ($P = 0.23$ and 0.32, respectively, based on a random effects model accounting for repeated measures). For EGF, there is the appearance of an increase during the luteal phase, but direct comparison of, for example, midfollicular EGF indicated that the differences were highly compatible with chance ($P = 0.61$).

In Fig. 5, EGF (Fig. 5A) and TGF-α (Fig. 5B) levels are plotted against concurrent total plasma estradiol levels. Neither GF was meaningfully correlated with plasma estradiol ($r = 0.15$ for EGF and $r = 0.02$ for TGF-α by the permutation method). We obtained similar results using plasma bioavailable (i.e., non-sex hormone binding globulin bound) estradiol or progesterone instead of total estradiol.

The results shown in Fig. 6 indicate that, when all samples from all women were considered, EGF and TGF-α concentrations in the same sample were not well correlated ($r = 0.17$, $P = 0.50$). However, when we examined the EGF and TGF-α relationship for individual women, some striking covariation was apparent. Table 4 shows results from a random effects model that evaluates the EGF-TGF-α association within women. This model includes a universal coefficient reflecting the common relationship of EGF to TGF-α, as well as a term reflecting the relationship for each individual woman. The universal coefficient was very small, consistent with the low $r$ seen in Fig. 6. However, the association within individual women was statistically significant ($P = 0.02$) and explained nearly 56% of the variance in EGF within women. The high percentage (93.5%) of variance explained by simply specifying the individual subject corroborates the large amount of variation for EGF between women that was seen in the earlier analysis.

Table 5 shows a similar random effects analysis for the relationship of EGF to plasma estradiol. Again, although the overall correlation between EGF and estradiol was poor, the results indicate a significant correlation within individual women ($P = 0.01$). Fifty-eight% of the within-woman variance in EGF was explained by the plasma estradiol values. The within-woman association between breast fluid TGF-α and plasma estradiol was not substantial. Fig. 7 includes graphs of selected participants showing strong covariation between EGF.
and TGF-α levels (Fig. 7A) and covariation between EGF and plasma estradiol (Fig. 7B). These participants were not atypical, although the extent of covariation in some individuals appeared to be lower. The small number of replicates per woman, however, prevents us from drawing reliable conclusions about correlation in specific individuals.

Discussion

Peptide GFs such as EGF and TGF-α are potent signaling molecules for regulating the growth and perhaps differentiation of breast epithelial cells. Abnormal expression or activity of these factors could result from mutations of proto-oncogenes transcribing the GFs themselves or their receptors. Alternatively, because these GFs have a role in normal growth and therefore must be regulated by endogenous signals, abnormal expression could occur as a result of up- or down-regulation of gene transcription by compounds such as steroid hormones.

The data in this report, although preliminary to the study of any relationships in vivo between breast cancer and GF expression, indicate that immunoactive EGF and TGF-α can both be detected reliably in breast fluid from healthy premenopausal women and that individual women secrete distinctive amounts of these factors, amounts that are consistent both over time and between breasts. This study also provides evidence that levels of breast fluid EGF tend to covary over time with TGF-α and plasma estradiol, within individual women.

The presence of EGF in human milk has been demonstrated previously. In fact, Carpenter (11) reported that 90% of the mitogenic activity of human milk in cell culture systems was negated by a neutralizing antibody for human EGF. Connolly and Rose (9, 10) have reported previously the detection of EGF and TGF-α in breast fluid from healthy premenopausal women. They found concentrations of TGF-α in single samples from 21 women ranging from 0 to 50 ng/ml, with a median of 5.1 ng/ml, levels somewhat higher than we found. They also found EGF levels in samples from 17 women similar to ours; intriguingly, however, 9 of these women with unspecified biopsy-confirmed benign breast disease appeared to have higher EGF levels than the 8 women designated as controls (9). Although the results published previously probably required higher volumes of breast fluid for analysis, we found no difference in GF concentrations between women with abundant versus scanty breast fluid volume. We used highly sensitive RIAs that require only 1 and 4 p1 of breast fluid for EGF and TGF-α, respectively, and therefore make it possible to obtain measurements even on women with scanty breast fluid samples. The median volume of breast fluid we obtain is approximately 25 μl, but the frequency distribution for sample volume is highly skewed, and many women have samples under 10 μl. Highly sensitive assay methods are therefore important. We found no evidence for an effect of thaw-refreeze cycles on GF concentrations, nor did we find any evidence for a decay in measured GF during 18 months of storage at temperatures of -15 to -20°C.

Several aspects of our findings require further elaboration. In looking at variation over time, we found that be-

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Table 4: Random effects model evaluating the associations within individual women between breast fluid EGF and TGF-α

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Sum of squares</th>
<th>% variance explained within-woman</th>
<th>% variance explained</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual subject</td>
<td>3808.17</td>
<td>93.49</td>
<td>93.49</td>
<td>0.99</td>
</tr>
<tr>
<td>TGF-α</td>
<td>0.12</td>
<td>0.003</td>
<td>0.003</td>
<td>0.99</td>
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<tr>
<td>Error</td>
<td>116.87</td>
<td>2.87</td>
<td>2.87</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Model: dependent variable, EGF; n = 60 samples from 18 women.

Table 5: Random effects model evaluating the associations within individual women between breast fluid EGF and total plasma estradiol

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Sum of squares</th>
<th>% variance explained within-woman</th>
<th>% variance explained</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual subject</td>
<td>3808.17</td>
<td>93.49</td>
<td>93.49</td>
<td>0.99</td>
</tr>
<tr>
<td>Plasma estradiol</td>
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<td>0.06</td>
<td>0.06</td>
<td>0.99</td>
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<tr>
<td>Subject × plasma estradiol</td>
<td>154.66</td>
<td>3.80</td>
<td>3.80</td>
<td>0.99</td>
</tr>
<tr>
<td>Error</td>
<td>107.95</td>
<td>2.65</td>
<td>2.65</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Model: dependent variable, EGF; n = 61 samples from 18 women.
Fig. 7. Breast fluid EGF versus TGF-α (A) and breast fluid EGF versus plasma estradiol (B) across time for selected individuals.
between versus within-woman variation was greater for EGF than TGF-α, largely because of a much greater difference between women for EGF. However, TGF-α variation between the right and left breasts was very low (r = 0.89, CV = 0.11), so that the between- versus within-woman variation was greater for TGF-α than for EGF, although the differences between women were once again greater for EGF. Taken together, these findings imply that breast fluid levels of TGF-α, perhaps the more potent of the two GFs, are regulated within more narrow ranges than EGF in healthy women. More data are needed on this question.

The lack of a correlation between EGF and TGF-α or plasma estradiol concentrations when samples from many women are considered and the presence of significant correlations within individual women can be puzzling at first. However, this type of result arises if the quantitative relationship of EGF to TGF-α and estradiol varies from one woman to another, whereas the levels of breast fluid EGF within an individual woman tend to change over time in proportion to changes in TGF-α and plasma estradiol. Correlation analyses composed of samples from multiple women, such as those shown in Figs. 3–6, can fail to reveal these relationships. We conclude that these data provide preliminary evidence that breast fluid EGF and TGF-α are coregulated in vivo and that, in the case of EGF, regulation could involve circulating levels of estradiol. Other compounds, including other steroid hormones related to estradiol, could be involved in regulating TGF-α levels, or alternatively, estradiol and TGF-α levels could be related but less well synchronized than estradiol and EGF.

One limitation of these studies is that we were able to obtain breast fluid from only 60% of the women on whom nipple aspiration was attempted. Factors related to success in obtaining breast fluid have been studied extensively and appear to include age, parity, lactation history, Asian ethnicity, and cerumen type (21). In this study, such factors did not strongly differentiate those who provided fluid and those who did not; however, the study population was small and relatively homogeneous. Although we cannot rule out the possibility that our findings would not apply to the nonsecretors, had breast fluid been available from them, we find that argument to be implausible. The distinctions between secretors and nonsecretors are more likely to involve differences in the volume of breast fluid secretion and/or the physical consistency of material that normally plugs the nipple ducts, which is in part genetically determined (22). We found no evidence for a relationship between GF concentration and the amount of breast fluid obtained. Another limitation is that we have not yet confirmed the precise immunoreactive species in each RIA. The kit manufacturer’s testing indicates only minimal cross-reactivity with other peptides for the antibodies used in the EGF and TGF-α assays; however, these cross-reactivities were determined for selected peptides that might or might not be present in breast fluid. We are currently conducting Western blot analyses to identify the immunoreactive species by molecular weight and ionic charge.

From these results, it appears that breast fluid EGF and TGF-α could eventually serve as useful biomarkers in studies of breast cancer etiology. Our next series of studies will examine whether GF concentrations are related to hyperproliferative states in the normal breast, to known or suspected breast cancer risk factors, and to the occurrence of breast cancer itself. If altered local GF secretion can be established as a link in the causal pathway of breast cancer development, breast fluid GF levels could provide novel intermediate end points for the evaluation of suspected risk factors or of interventions designed to reduce breast cancer risk.

Acknowledgments

The authors gratefully acknowledge Paulette O’Donnell, Yuppadee Sukosol, and the staff of the Clinical Research Center at Northwestern Memorial Hospital for assistance in collecting samples; Lynette May and Yu-Cai Lu for laboratory assistance; Susan Gaspur and Susan Colilla for study planning and coordination; Cheng-Fang Huang for statistical programming; and the women volunteers in the Repeat Sample Study for their patience and commitment.

References

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