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TITLE: Early Detection Based On Angiogenic Growth Factors in Nipple Aspirate Fluid

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Early Detection Based on Angiogenic Growth Factors in Nipple Aspirate Fluid

The objective of this proposal is to develop new methods of early breast cancer detection by identifying increases in angiogenic growth factor secretion in nipple aspirate fluid (NAF). Specifically, the study is examining FGF-2 (basic fibroblast growth factor) and VEGF (vascular endothelial growth factor), two of the most potent angiogenic molecules whose expression is thought to increase as an early event in breast carcinogenesis. By comparing levels of these growth factors in NAF samples from 40 women in each of three groups, i.e., those with normal breasts, DCIS (ductal carcinoma in situ), and early invasive breast cancer, we will determine whether increases in either of these molecules heralds the transition to the pre-invasive and/or invasive phenotype.

In this first year of the study efforts have focused on methods and infrastructure. We have determined the most appropriate means of handling and processing the samples to prevent degradation of FGF-2 and VEGF. Accrual of patients has been slower than expected, primarily due to staffing changes at the three clinics providing subjects for this study, and a greater proportion of patients than found in our previous experience whose concern for their health status precluded interest in participating in the study. To increase accrual we have increased the percentage effort of the Research Assistant (using institutional funds) and have also engaged the efforts of additional surgeons to refer patients to the study.
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PI - Signature

Date
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INTRODUCTION

The objective of this proposal is to develop new methods of early breast cancer detection by identifying increases in angiogenic growth factor secretion in nipple aspirate fluid (NAF). Specifically, the study is examining FGF-2 (basic fibroblast growth factor) and VEGF (vascular endothelial growth factor), two of the most potent angiogenic molecules whose expression is thought to increase as an early event in breast carcinogenesis. By comparing levels of these growth factors in NAF samples from women from three groups, i.e. those with normal breasts, DCIS (ductal carcinoma in situ), and early invasive breast cancer, we will determine whether increases in either of these molecules heralds the transition to the pre-invasive and/or invasive phenotype.

This report covers essentially startup activities during the first year of the project. These activities include putting into place the clinical protocol for identifying, enrolling, and obtaining samples from women in the three groups, optimizing sample processing protocol, and beginning enrollment of subjects and analysis of samples. Unanticipated problems and proposed solutions to the first and third activities are discussed below, as are the results of the sample protocol optimization.
BODY

Progress in the study during the first year of funding will be described below with respect to each of the tasks in the original Statement of Work (only those tasks expected to begin during Year 1 will be described).

Task 1: Coordinate with physicians, nurses and scheduling secretaries to receive schedules of patients who will undergo breast surgery (Surgery Clinic), and patients who will attend the Comprehensive Breast Center (CBC), or the Breast Cancer Consultation Group (BCCG).

This aspect of the study has progressed more slowly than anticipated. There has been considerable turnover of ancillary staff in all three clinics, and the staff in the CBC has been reduced to a single nurse practitioner and part-time clerical help. This has made it difficult for the Research Assistant (who enrolls patients and obtains NAF samples) to be notified of patients on a timely basis. It has also reduced the time that clinicians have available to introduce the study to patients and determine their interest in participating. Our original strategy (Section 4a of the grant proposal) called for clinic schedules to be faxed in advance to the Research Assistant, who, in turn, would notify the respective clinicians about patients who fit the eligibility criteria for the study. The clinician would then discuss the study with each eligible patient; for interested patients the Research Assistant would be paged to come to the clinic for enrollment and NAF sampling. To enhance the efficiency of patient accrual, we have arranged for the Research Assistant to call or visit each clinic at the end of each day to pick up the clinic schedule for the following day. She will then arrange to be at the clinics during the time period when eligible patients are scheduled (rather than depending on being paged, which clinic staff are frequently too busy to do in a timely fashion). Having her stationed more regularly in the clinics also serves as a reminder to the clinicians of the need to enroll patients. Because this requires a greater time commitment for the Research Assistant than the original budget request (which was 25% FTE), we are using institutional funds to cover the additional time that she must spend in the clinics. Because this arrangement may result in patients needing to be enrolled and aspirated in two separate clinics during the same time period, we have arranged for the nurse practitioner who runs the CBC to obtain samples from her patients as time permits. This nurse practitioner has extensive experience in clinical breast management and has performed nipple aspiration for other studies that we have conducted. Because the BCCG clinic only operates one morning per week, conflicts between this clinic and the other two are less likely.

Task 2: Develop appropriate quality control methods for VEGF and FGF-2 assays.

Before conducting ELISA assays on NAF samples, we conducted preliminary studies to optimize the sampling and assay conditions. Samples were prepared from human serum obtained from
healthy volunteers in Dr. McLeskey’s lab. The samples were spiked with increasing amounts of FGF-2. The objective of the experiment was to determine whether addition of protease inhibitors could prevent degradation of FGF-2 in samples that were left at room temperature, i.e. could protease inhibitors substitute for immediate freezing.

The following three groups of samples were prepared at each of six concentrations of added FGF-2 (0, 1, 3, 10,30, 100 pg/ml FGF-2):

1. Control (no protease inhibitor)
2. aprotinin (2 µg/ml)
3. phenylmethyl sulfonyl fluoride (100 uM)

Duplicate samples of each combination of FGF-2 concentration and protease inhibitor were prepared. One sample of each duplicate was frozen immediately at -70°C overnight and the second sample was left sitting at room temperature overnight. The following day, the ELISA was done using the R&D Systems “Quantikine” kit. The samples for the standard curve (reagents supplied with kit) were generated at that time.

Fig. 1 (frozen samples) shows that there is considerable FGF-2 immunoreactivity in the normal serum itself. At zero added FGF, we obtained an optical density corresponding to approximately 41 pg/ml FGF-2. Since our preliminary studies with FGF-2 in NAF (in original grant application) demonstrated that the basal level in healthy women is much higher than in serum (mean FGF-2 > 300 pg/ml), the samples will have to be diluted to get them down to the area of the standard curve. This figure also shows that PMSF introduces a significant artifact - it inflates the basal level of FGF-2 (zero added FGF-2) to 77 pg/ml. Thus, PMSF is not an appropriate protease inhibitor for our study. Aprotinin did not have the same effect (the curve from the aprotinin samples is the same as the no protease inhibitor samples).

Fig. 2 (room temperature samples) shows that leaving the samples overnight at room temperature results in considerable loss of FGF-2 immunoreactivity. PMSF appeared to perform the best in this assay, preserving more of the FGF-2 immunoreactivity. However, that is undoubtedly because of the artifactual elevation that was observed with the frozen samples and not because it actually preserved the FGF-2 protein.

Based on this experiment it does not appear that protease inhibitors will be an acceptable substitute for collecting the sample on ice and processing it and freezing it immediately after collection. Therefore, sample collection efforts will emphasize the need for immediate icing and rapid transport of the sample to the lab. Dr. McLeskey’s lab staff are instructed to go to the clinic as soon as they are informed that a sample is available for pickup. As a backup, Dr. Trock’s staff can also be contacted and transport the sample to the lab. In the unlikely event that
no one is available in Dr. McLeskey's lab to process the sample, the lab of Dr. Stephen Byers will also process and store the samples until they can be retrieved by Dr. McLeskey. Dr. Byers conducted the pilot assays (reported as preliminary data in the grant application) of FGF-2 and TGF-β in NAF, so he is well-acquainted with the protocol for processing the samples.

Because these results indicate that neither protease inhibitors nor prolonged exposure of samples to room temperature provide adequate results for FGF-2, it was not necessary to conduct a similar experiment for VEGF, as both growth factors will be assayed in the same samples.

**Task 3:** Implement patient accrual and NAF collection (Months 2-30).

Because of the staffing problems described above in reference to Task 1, accrual has been slower than expected. In addition, the breast surgeons who refer patients to the study have been encountering more patients than usual who are too emotionally distraught to be referred to the study. To date, we have collected samples from 20 breast cancer patients and 11 unaffected women. We continue to explore with the surgeons alternative ways to present the study to patients. One such approach is for the surgeon to tell the patient that we have a research study she may want to consider, and ask whether the patient is willing to have the Research Assistant call her at home in a few days to describe the study. For patients who agree, the nipple aspiration can be done on the day of surgery in the surgical suite. We are also discussing participation with three other surgeons who are on staff at Georgetown University Medical Center but who see their patients in private practice offices off of the Georgetown Campus. Again, if necessary, institutional funds will be used to provide additional staff time to obtain samples at the offices of these surgeons or in the surgical suite.

**Task 4:** Conduct ELISA assays for VEGF and FGF-2 (Months 4-30).

Because the ELISA kits use 96 well plates, we will wait until we have more samples before conducting assays. The number of kits included in the supply budget assumed that each ELISA would be performed with a full or nearly-full plate. All samples are run in duplicate. The standard curve (8 concentrations of FGF-2, including 0) uses 16 wells, and a positive control uses two wells. Therefore, a full plate can hold duplicate samples from 39 patients.
FIGURE 1.  FGF-2 immunoreactivity in normal human serum samples frozen overnight following addition of protease inhibitors aprotinin or PMSF.
FIGURE 2. FGF-2 immunoreactivity in normal human serum samples at room temperature overnight following addition of protease inhibitors aprotinin or PMSF.

FGF-2 ELISA

![Graph showing FGF-2 ELISA results](image-url)
CONCLUSION

Study efforts in this first year of the project have focussed on process issues. Unanticipated obstacles to accrual have slowed the progress of the study. These obstacles include staffing problems at the clinics that are the source of study subjects, and a larger than expected number of patients who were too distressed about their diagnosis to want to participate. To address these issues we have increased the percentage effort of the Research Assistant who enrolls patients so that she can spend more time in the clinic, and are expanding the number of surgeons who will contribute subjects to the study. These changes are expected to increase our accrual and put the study on course to completion within the three year time frame. Although our accrual is lower than anticipated, we are confident that we will be able to accrue the expected number of cases.

Interest among physicians, researchers and patients in the use of NAF for early detection has expanded considerably since we originally submitted our proposal.

We tested different methods for sample handling and processing and determined that the risk of sample artifacts is minimized if the samples are immediately iced and taken to the lab for immediate processing. Allowing the samples to sit at room temperature with or without protease inhibitors produces artifacts. Furthermore, by not using protease inhibitors the process is actually simplified. Backup systems have been put in place to ensure that someone will always be available to transport the samples from the clinic to lab within less than one hour from the time of aspiration, and to process the samples within two hours from the time of aspiration.
## LIST OF ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NAF</td>
<td>nipple aspirate fluid</td>
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<tr>
<td>FGF-2</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>CBC</td>
<td>Comprehensive Breast Center</td>
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<tr>
<td>BCCG</td>
<td>Breast Cancer Consultation Group</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunoabsorption assay</td>
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<tr>
<td>PMSF</td>
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Meeting abstracts during reporting period: None in connection with this project

Publications during reporting period: None in connection with this project

Manuscripts in preparation: None in connection with this project

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