GRANT NUMBER DAMD17-94-J-4513

TITLE: Breast Cancer Metastasis: Prognosis and Monitoring of Metastatic Disease

PRINCIPAL INVESTIGATOR: Douglas Boyd, Ph.D.

CONTRACTING ORGANIZATION: University of Texas at Houston
M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: October 1996

TYPE OF REPORT: Annual

19970421 028

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
**REPORT DOCUMENTATION PAGE**

**Agency Use Only (Leave blank)**

**Title and Subtitle**
Breast Cancer Metastasis: Prognosis and Monitoring of Metastatic Disease

**Author(s)**
Douglas Boyd, Ph.D.

**Performing Organization Name(s) and Address(es)**
University of Texas, at Houston  
M.D. Anderson Cancer Center  
Houston, TX 77030

**Sponsoring/Monitoring Agency Name(s) and Address(es)**
Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Frederick, Maryland 21702-5012

**Supplementary Notes**

**Distribution/Availability Statement**
Approved for public release; distribution unlimited

**Abstract (Maximum 200)**
Using biopsies of primary breast cancers taken at the time of surgical resection and blood of breast cancer patients, we are developing tests that will allow us to predict risk of breast cancer recurrence and follow each patient that has had breast cancer. Biopsy tissues obtained from primary tumors are being used to assess risk of metastasis, and patient blood samples will be used to monitor appearance of metastasis or response to therapy. The technical objectives are to: (1) Develop prognostic tests for breast cancer invasion and metastasis-associated degradative enzymes. In patients treated previously recurrence is associated with overexpression of three metastasis-associated enzymes. We are expanding these studies to include additional breast cancer patients, other malignancies and benign lesions. (2) Develop assays to monitor the appearance of metastatic disease in breast cancer patients by detecting the appearance of metastasis-associated degradative enzymes in blood. We found that blood enzyme activity correlates with the onset of breast metastasis and have been collecting blood samples to test this. (3) Develop assays to monitor the treatment of metastatic disease in breast cancer patients by following the levels of metastasis-associated degradative enzymes in blood. In patients with stage IV disease we found that response to therapy resulted in lowered blood levels of the metastasis-associated enzymes. We are collecting plasma samples from breast cancer patients undergoing chemotherapy, and we will determine if the blood levels of the three metastasis-associated enzymes correlate with response to therapy.

**Subject Terms**
Breast Cancer

**Security Classification of Report**
Unclassified

**Security Classification of This Page**
Unclassified

**Security Classification of Abstract**
Unclassified

**Limitation of Abstract**
Unlimited

**Number of Pages**
11

**Price Code**

NSN 7540-01-280-5500
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

P. Boyce 11/21/96

PI - Signature  Date
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF298</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>The Purposes</td>
<td>6</td>
</tr>
<tr>
<td>Body</td>
<td>6</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
</tbody>
</table>
A. INTRODUCTION

During the past several years we have conducted extensive studies on the role of degradative enzymes in the invasion and metastasis of breast cancers. We found that only a few enzymes were implicated in the invasion of human breast cancer cells, such as heparanase (active against basement membrane proteoglycans) and type IV collagenolytic metalloproteinases (active against basement membrane-type collagen) (1-4). We succeeded in the purification and partial sequencing, and more recently gene cloning of the human heparanase, and we have identified unique amino acid sequences in the type IV collagenolytic gelatinases of $M_r \sim 72,000$ and $\sim 92,000$ that can be used as immunogens for antipeptide reagents. These immunological tools can be used to monitor highly metastatic cells, since the metastatic cells secrete high amounts of these enzymes in order to invade adjacent tissues and basement membranes. We recently demonstrated that serum heparanase and type IV collagenolytic metalloproteinases are potential markers for metastasis of human melanoma and breast cancer, and these enzymes may be possible targets to block metastasis (3). Since these enzymes are functionally involved in cancer invasion through basement membranes, they should serve as excellent markers to monitor the metastatic phenotype as well as markers for breast cancer prognosis.

Heparanase is released from metastatic tumor cells, and this enzyme can circulate in the body fluids of tumor-bearing animals. For example, heparanase and type IV collagenase activity in sera increased with time after subcutaneous injection of highly metastatic mammary adenocarcinoma cells into the fat pads of female F344 rats (3). In contrast, sera from rats bearing mammary adenocarcinomas of low metastatic potential possessed low levels of heparanase, even 30 days after tumor cell injection. Rats with large numbers of metastases in the lung and/or lymph nodes had much higher heparanase activities than sera from rats with few or no metastases. The levels of serum heparanase remained low (<2 mg HS degraded/ml/hr) until approximately 1-2 weeks before the detection of small (<1 mm) lung metastases when the levels in individual animals began to rise. As the metastases increased in size, the serum content of heparanase and collagenase IV increased further (3).

The data from the serum enzyme assays using solid-phase assay substrates demonstrated that heparanase activities in patient sera were related to the stage of disease and the presence of metastases (2). The sera from breast cancer patients of various stages were assayed for heparanase by HPLC using [3H] acetylated heparan sulfate (HS) as substrate (5). The mean of serum heparanase activities (in mg HS degraded/ml/hr) of malignant breast cancer patients ($n=20$) with documented disease and normal adults ($n=18$) were 49.4 and 6.3, respectively. The highest and lowest activities in the breast cancer patient sera were 69.6 and 6.6, and those in normal adult sera were 2.4 and 12.5, respectively. The source of HS-degrading activity in the normal adult serum includes platelets and other blood cells, because heparin-degrading activity was also detected. We have found that heparin is an excellent competitive inhibitor of tumor cell heparanase (6), but heparin can be degraded by platelet heparinase. Therefore, the difference in serum heparanase may be much greater in patients with metastatic disease than we have estimated. Using the degradation of heparin compared to heparan sulfate the relative levels of platelet and other normal host cell enzymes can be estimated. Unfortunately, due to the technical expertise, facilities and time required for running these assays, they are unsuitable for large scale clinical use.

Alternatively, the enzyme amounts in plasma can be determined using immunological detection methods. In preliminary experiments we have detected heparanase and the 92kDa type IV collagenase gelatinase in the sera and plasma of patients with known breast cancer metastases. Our assays are now being extended to detect the three enzymes in blood plasma and sera of patients before and after surgical removal of their breast cancers as well as before, during and after chemotherapy or radiotherapy.
B. THE PURPOSES

The major purposes of the proposal are:

B.1. Develop prognostic tests for breast cancer invasion and metastasis-associated degradative enzymes, Months 1-18:
   a. Preparation of antibodies against heparanase, 92 kDa type IV collagenase, 72 kDa type IV collagenase.
   b. Continue retrospective clinical trial on breast cancer.
   c. Initiate retrospective clinical trial on other cancers, normal tissues, trauma, infections, pregnancy, and other states.
   d. Begin prospective trial on breast cancer.

B.2. Develop assays to monitor the appearance of metastatic disease in breast cancer patients by detecting the appearance of metastasis-associated degradative enzymes in blood, Months 6-48:
   a. Develop a sensitive ELISA assay for assaying serum/plasma heparanase, 92 kDa type IV collagenase, 72 kDa type IV collagenase.
   b. Examine serum and plasma of breast cancer patients before and after primary treatment and thereafter each 4-6 months.
   c. Examine serum and plasma of other cancer patients, normals, and noncancer patients with infections, trauma, pregnancy and other states.

B.3. Develop assays to monitor the treatment of metastatic disease in breast cancer patients by following the levels of metastasis-associated degradative enzymes in blood, Months 6-48:
   a. Examine serum and plasma of advanced breast cancer patients before, during and after chemotherapy or radiotherapy treatment and thereafter each 4-6 months.
   b. Examine serum and plasma of other advanced cancer patients before, during and after chemotherapy or radiotherapy treatment and thereafter each 4-6 months.

BODY:

B.1. Preparation of immunological reagents. Polyclonal antibodies against purified heparanases and synthetic peptides against type IV collagenolytic (gelatinases) of M_r~72,000 (MMP-2) and ~92,000 (MMP-9) as well as carbohydrate moieties of heparanases were produced using goats, rabbits and sheep. We synthesized peptides according to the unique amino acid sequences of amino termini (heparanase) or against specific hydrophilic sequences found in metalloproteinases and their cyanogen bromide cleavage products in order to produce highly sensitive and specific antibodies. We have found that the following peptide sequences will produce specific antisera:

Heparanase-EEDLGKSREGSRSTDD-C
Heparanase-EVDVDDTVEEDLGKSREGSRSTDD-C
92kDa Type IV Collagenase-LRTNLTDRLAEVLYR-C
92kDa Type IV Collagenase-LGRFQTFEGDLKWHH-C
72kDa Type IV Collagenase- ΔVAPKKEVQNTAF-C
72kDa Type IV Collagenase-VANYNFPRKPD-C

We established an immunization program using goats and collected the following sera:

Volumes of polyclonal goat sera as of 11/20/96
against H96 - peptide sequence from heparanase:
   goat 5 - 11.3 liters
   goat 6 - 15.7 liters
   goat 7 - 16.3 liters

against C72 - peptide sequence from MMP-2:
   goat 8 - 8.2 liters
   goat 9 - 12.4 liters
   goat 10 - 8.2 liters
against C92 - peptide sequence from MMP-9:
  goat 11 - 3.4 liters
  goat 12 - 2.0 liters
  goat 13 - 15.2 liters

The high-affinity IgG fraction of anti-heparanase peptides detected bands of \( M_r \sim 97\text{K} \) and \( \sim 35\text{K} \) on Western blots of total cell lysates from both human and murine melanoma, breast cancer and lung cancer cells. A single band of \( M_r \sim 97\text{K} \) was also detected by fluorography of the immunoprecipitates of \( [\text{35S}] \)methionine labeled cellular proteins. We feel that the 35 kDa component is a degradation product of the high \( M_r \) band, because this component was active in enzymatic assays. Since there was a good correlation between the intensity of the \( M_r \sim 97\text{K} \) band of melanoma cells and their lung colonization potentials, this is further evidence that the antibodies were specific and against heparanase. The anti-peptide reagents against type IV collagenolytic gelatinases of \( M_r \sim 72,000 \) and \( \sim 92,000 \) were analyzed by Western blotting. These reagents detected collagenases of the appropriate size in lysates of MDA-MB-435 breast cancer cells, and the amounts of IgG precipitated corresponded to the relative amounts of these enzymes after zymography.

We have also produced monoclonal antibodies (MAB) against the human melanoma cell heparanase using murine hybridoma cells. One of these MAB (10E5) is of sufficient titer and specificity for immunoprecipitation and Western blot analyses. This MAB was used for heparanase immunohistochemistry on breast cancer samples (see the Table).

We have used the polyclonal and MAB to begin developing quantitative competition ELISA assays to monitor the amounts of heparanase and the two collagenases in the blood plasma of patients with breast cancer. So far we have found that the antipeptide reagents react well with denatured enzyme but do not react well with native enzyme found in plasma. Over the past 12 months, a sandwich fluorescence immunosorbent assay has been examined for its ability to quantitate the two collagenases. Although many variations of the capturing or secondary antibody (to the peptide or whole molecule) were tried, none proved workable due to lack of sensitivity or specificity. The assay currently being optimized is a substrate capture fluorescence immunosorbent assay. In this assay, gelatin (denatured collagen) is used as the capture substrate and the bound molecules are denatured. These bound and denatured molecules are recognized by the goat anti-peptide MMP-9 and rabbit anti-peptide MMP-2 antibodies. Subsequently, an alkaline phosphatase-conjugated secondary antibody is added followed by the addition of 4 methyl umbelliser phosphoryl phosphate as fluorophore. Fluorescence is finally read using 360 and 460 nm as activation and emission wavelengths respectively.

Breast Cancer Protocols Used for Collection of Blood Samples

To monitor breast cancer therapy and recurrence we collected blood samples under the following clinical protocols. These protocols are approved clinical research projects on a variety of new therapies or modifications of existing therapies. We hope to be able to evaluate breast cancer response (or lack of response) earlier in each of these patients than the usual evaluation periods which can be many months after therapy is terminated. If we can determine if a given therapy is working or not early in the protocol, this approach will be extremely useful in allowing oncologists to change or discontinue therapy or continue therapy and for how long. We will also be comparing breast cancer with other cancers.

**DM 90-107**
Bone complication prevention study
Drugs: Pamidronate (Aredia) vs placebo + chemo (variety of agents)
Sample No.: 188

**DM 90-113**
Bone complication prevention study
Drugs: Pamidronate (Aredia) vs placebo + hormonal therapy
Sample No.: 183

**DM 91-094**
Metastatic breast cancer
Drug: oral Etoposide (VP-16)
Sample No.: 91

**DM 92-014**
Metastatic breast cancer
Drug: TLC D99 (liposomal doxorubicin) + 5 FU (fluourouricil) + Cytoxan (cyclophosphamide)
Sample No.: 113

DM 91-022
Patients without previous chemo for breast metastasis
Drug: Taxol with Doxorubicin (adriamycin) and G-CSF
Sample No.: 120

DM 92-044
patients who failed at least 3 prior chemo regimens
drug: Taxol
sample No.: 175

DM 92-058
Metastatic breast cancer resistant to anthracycline chemo
Drug: RP56976 (Taxotere)
Sample No.: 163

DM 92-110
Post menopausal women with advanced breast cancer
Drug: IDIC1033 (Arimidex) vs Megestrol (megace)
Sample No.: 351

ID 91-015
Patients with T2-4, N0-3, M0 without prior chemo, surgery or XRT
Drug: 4-7 wks chemo (FAC with/without G-CSF); surgery; 6-8 wks chemo (variety of agents); radiation therapy (some patients)
Sample No.: 353

DM 93-125
Patients without previous chemo for breast metastasis
Drug: Navelbine with Paclitaxel
Sample No.: 15

DM 93-142
Inflammatory carcinoma of the breast treated with combined modality approach
Regimen: FAC - surgery - Paclitaxel - XRT
Sample No.: 8

TUB1/tub4 (General Breast Surgery)
Breast surgery
Sample No.: 174

Genitourinary Protocols

DM 92-107
Hormone refractory prostate cancer
Drug: TNP-470 (analogue of fumagillin)
Sample No.: 286

DM 93-039
Hormone refractory prostate cancer
Drug: Taxol
Sample No.: 66

Radiotherapy
Sample No.: 6

Samples without protocol number
Sample No.: 818
TOTAL NUMBER OF SAMPLES: 3110

B.2. Prognosis of Metastatic Breast Cancers: Development of New Clinical Assays. A sizeable percentage of patients presenting with early stage breast cancers will recur with metastatic disease. For example, 25-35% of patients with node-negative stage I/II breast cancer will eventually recur with metastatic disease (7). We are developing new assays for assessing prognosis based on the overexpression of metastasis-associated degradative enzymes, such as 92 and 72 kDa type IV collagenases and heparanase, and we are testing their clinical usefulness. The reason that we will assay three enzymes instead of one is that errors due to intra- and inter-tumor heterogeneity and instability should be minimized.

During the past three years we have conducted extensive studies on over-expression of degradative enzymes in metastatic breast cancer. We succeeded in the purification and partial sequencing of human heparanase (4), and we have identified unique amino acid sequences in gelatinases of 72 and 92 kDa, and we have generated specific antipeptide antibodies against these enzymes. The antibodies have been used in a retrospective blinded study on the expression of type IV collagenases and heparanase in >200 paraffin embedded specimens of stage I/II breast cancers removed surgically 5-10 years ago at the M. D. Anderson Cancer Center. Recently we have concentrated on only node-negative patients, and we have been able to add some additional node-negative patients to our study (see the Table).

The data in the Table indicate that four-times as many node-negative breast cancer patients that had recurrence of distant metastatic disease had high expression of type IV collagenase and heparanase in their primary tumors at the time of resection of their cancers than patients with a low expression level of these enzymes. As additional patients recur, it is expected that some of the high expressors in the non-recurrence group will move into the high expression recurrence group. These preliminary studies suggest that we can identify many of the patients at high risk for future metastatic disease by the overexpression of basement membrane degradative enzymes. It is these patients that should be closely monitored for future metastatic disease.
Distribution of Patients Based on Expression of Collagenase IV and Heparanase in Tumor Tissue

<table>
<thead>
<tr>
<th>Node Negative Breast Cancer 5-10 year Recurrence of disease</th>
<th>Recurrence</th>
<th>Non-recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>High expression (grade 2-4)</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>Low expression (grade 0-1)</td>
<td>6</td>
<td>51</td>
</tr>
</tbody>
</table>

n = 87 patients

Table. Immunohistochemical scoring of 92 kDa and 72 kDa type IV collagenase and heparanase in stage I/II (node-negative) breast cancer patients based on the recurrence of disease at distant sites within 5-10 years after 1º surgery. The expression levels were confirmed by image analysis. Normal surrounding tissue cells and stroma = 0. Normal tumor-associated blood capillaries = 1-2. Normal leukocytes = 1-2. At least 20 sections were examined of each tumor, excluding H & E sections, and the data were read independently and blinded by two investigators, including a board-certified pathologist.

B.3. **Measurement of Degradative Enzymes in Blood.** Heparanase and collagenase IV will be measured in the blood serum and/or plasma of breast cancer patients of all stages, normal subjects and patients with benign breast adenomas, other carcinomas (with and without lymph node or distant disease), infections, autoimmune disorders, trauma, and other states, such as pregnancy, that could result in elevated levels of these enzymes. Preliminary results with our enzymatic assay for blood heparanase indicate that patients with malignant breast carcinoma have significantly (P <0.001) higher levels of heparanase in their blood, and that the patients with the highest levels of heparanase recur earlier with detectable metastatic disease than those breast carcinoma patients with lower levels of blood heparanase. We will be extending these preliminary random studies and following individual patients serially from before surgery, after surgery, before adjuvant therapy (chemotherapy or radiotherapy), after adjuvant therapy and thereafter at 3-6 month intervals.

Patients with elevated degradative enzymes in their blood plasma should be at high risk for clinical development of metastases. In preliminary studies on advanced (stage IV) breast cancer patients with metastatic disease, we followed the levels of heparanase in blood sera (or more recently plasma) before, during and after chemotherapy with an advanced FAC or taxol protocol. The initial levels of heparanase, measured by an enzymatic assay, were high. Thus these samples will be invaluable in the development of clinical assays that can quickly determine several metastasis-associated degradative enzymes.

CONCLUSIONS

1. We have successfully immunized goats to obtain large quantities of antibodies against heparanase, 72 kDa and 92 kDa type IV collagenases. These reagents are necessary to perform the large number of clinical tests with a uniform source of antibodies.

2. We have expanded our data base on the expression of degradative enzymes and recurrence of breast cancer in a retrospective study. In this study we only used node-negative patients and found that patients with high expression of the three enzymes were 4-times more likely to recur with metastases within 5-10 years. The patients with high expression levels of degradative enzymes should be considered at risk for development of metastatic disease.

3. We are developing quantitative immunoassays to determine the levels of degradative enzymes in the blood plasma of patients before and after surgery and before, during and after chemotherapy or radiotherapy.

4. We are collecting the necessary numbers of blood samples from breast cancer patients for analysis of their plasma for degradative enzymes and other markers.

REFERENCES


