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Breast Cancer Microvesicles as a Novel Plasma Biomarker and Therapeutic Target (IDEA)

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14. ABSTRACT

We have developed a bead capture assay for quantitation of breast cancer tumor microvesicles (MV) to predict clinical outcomes in breast cancer patients. Flow cytometry, as originally proposed, and as used by numerous investigators, is inadequate for this task. Anti-MUC1 coated beads are used to bind and isolate tumor MV from patient plasma. We have introduced a 100 nm filtration step of plasma prior to bead-capture in order to eliminate the contribution of soluble antigens and smaller exosomes. This allows to specifically assay MV of epithelial origin in the blood. We have adapted this platform to assay MUC1 MV for total protein, tissue factor, MMP-9, and total DNA. We have banked over 200 clinical samples involving 40 breast cancer patients and 18 controls and are assaying these samples to determine if breast cancer MV may represent a biomarker associated with more aggressive disease, and a potential therapeutic target.

15. Subject Terms Microvesicles, MUC1, tumor assay

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Introduction

The current report is in the form of a progress report since we have received a no-cost extension on this project until 4/2010. This will enable us to complete Tasks 2 and 3 (see below).

This project proposes to develop a clinical assay for tumor microvesicles (MV) from the blood of patients with breast cancer. This assay will be applied to test the hypothesis that the level of plasma tumor MV predict response to treatment and subsequent relapse in patients with locally advanced breast cancer. This pilot clinical project will enroll 60 patients and 10 controls. We and others have shown that tumor MV can express HER-2 protein. Thus, we also will use this assay to determine if trastuzumab is able to clear tumor MV from the blood of breast cancer patients.

It is important to note that the overall interest of our laboratory is translational research in tumor metastasis. As part of this goal we are studying tumor microvesicles. These are produced by dead and dying cancer cells and are identical to "apopotic bodies". MV range in size from 100 to up to 500 nm. We are not proposing to study tumor exosomes. These are much smaller (30-100 nm) cellular fragments that are produced by healthy cells and are a form of cell-cell communication. Tumor exosomes are felt to be important in immunologic tolerance of the tumor. Both kinds of tumor cellular fragments are important in tumor pathophysiology, however tumor MV are pro-inflammatory, procoagulant, and full of proteolytic enzymes and are thus more directly involved with tumor metastasis.

Body

Task 1. Develop reliable flow cytometry and immunochemical techniques to measure and analyze breast cancer microvesicles from the blood of animals and humans.

This Task was completed by the time of the submission of last year's progress report. We reproduce below the previous response.

In our application we proposed to use a flow cytometry platform to develop a clinical tumor MV assay. This was based on our preliminary experience (as described in the application) and previously published work from other groups (1, 2). However, after about a year of work with both cell lines and clinical samples, it became apparent that tumor MV cannot be reproducibly and robustly assayed using flow cytometry. We struggled with wildly varying results using MV obtained from the same cell line or patient (even from the same sample). The problem involves the drawing of the gates on the forward and side scattergrams by the operator for subsequent immunochemical

analysis (see Figure 2B of our original grant application- the gate is marked R3). Even with the use of sizing beads, the drawing of the gate is very subjective. As one can see from the scattergram, there is not a discrete population of particles to gate on. The problem is that MV are too small for analysis with flow cytometry. These particles are roughly the same size as viruses. MV are close to the size of the wavelength of the laser light used by the flow cytometry and thus discrete particles can not even be accurately detected. Flow cytometry is essentially useless for a reliable clinical assay of these particle, in our opinion. This problem is now widely accepted by experts in the field (3, see section entitled "Current problems with measurement of microparticles" in this reference). We of course were not aware of this problem when we submitted our proposal in May 2005.

In our last report we described a new assay for quantitation of tumor MV. The main problem is to detect and/or isolate the tumor MV from the complex mixture of MV from the many tissue types found in blood. (see Figure 2 in our previous annual report). Our goal was to develop and assay that is 1) quantitative, 2) does not involve flow cytometry, and 3) avoids centrifugal sedimentation of the MV. The last requirement is to avoid MV fusion during sedimentation.

We elected to use antibody-coated beads to capture and isolate tumor MV. Figure 1 represents an updated cartoon of our current assay. MUC1 was chosen as a well-accepted breast cancer tumor antigen for which numerous well-characterized immunochemical reagents are available. We previously used derivatized latex beads for this assay, however they proved to be cumbersome to wash and centrifuge. We now have adapted our assay to incorporate derivatized magnetic beads (manufactured by Dynal). The beads are irreversibly coated with anti-MUC1 followed by bovine serum albumin (BSA). The BSA is used to completely coat and block all remaining binding sites.

The clinical blood sample is immediately processed to platelet free plasma (PFP) following collection as follows:

1. Whole blood- 1,200 x g for 15 minutes yields plasma.
2. Plasma- 12,000 x g for 12 minutes yields platelet free plasma (PFP).

An aliquot of the PFP is filtered through 100 nm syringe push filters (see below for further explanation). The filtered and unfiltered PFP is then frozen at -80^{o} for batch assay at a late date.

For bead-capture the PFP (thawed on wet ice) is incubated with MUC1 beads overnight at 4^{o} C. The beads are collected via a magnet and washed twice. The MV are then eluted from the beads using routine detergent lysis buffer and assayed (see

below). In Figure 4 of our annual report last year we demonstrated quantitative recovery of MUC1 antigen from clinical samples. Note, however, that both tumor exosomes and soluble tumor antigens will also bind to our beads. We wish to specifically study tumor microvesicles as a tumor biomarker. Therefore, an aliquot of the PFP is filtered through a 100 nm filter prior to bead capture. This filtration step removes the tumor MV. Thus the arithmetic difference of assay results between unfiltered and filtered PFP represents the specific quantitation of tumor MV. Figure 2 demonstrates effects of 100 nm filtration on PFP from a breast cancer patient. MUC1 antigen is unchanged in plasma compared with PFP. However, 100 nm filtration results in removal of significant MUC1. The removed MUC1 is contained in subcellular fragments larger than 100 nm and thus by definition derived from MV.

We have utilized several simple clinically applicable assays with this bead-capture platform. Figure 3 gives an example showing a much larger amount of total protein in MUC1 MV in one breast cancer patient compared to two normal controls. The total protein is largely removed by filtration and thus is primarily in the MV fraction.

Figures 4 and 5 demonstrate the result of a commercial ELISA for tissue factor (TF, Figure 4) and matrix metalloproteinase-9 (MMP-9, Figure 5). Several different patterns are noted with the patient samples- marked TF in MUC1 MV (patient #1 in Figure 4), detectable TF but none in the MV fraction (patient #2 in Figure 4), and no detectable TF (patient #3 in Figure 4).

Figures 6-8 represent the results of analysis of DNA contained in tumor MV. Figure 6 demonstrates the results of a fluorescent DNA assay of DNA extracted from MUC1 MV. Patient #2 has a high amount of total DNA that is completely restricted to the MV fraction. Figure 7 demonstrates that total DNA from MUC1 MV from the MDA-MB-231 cell line is in is a DNA laddering pattern (apoptotic fragments) following serum-free or doxorubicin treatment. We are currently analyzing clinical samples with large amounts of MV DNA to see if a similar apoptotic pattern occurs.

Figure 8 represents the interesting finding that detectable HER2 DNA is present in clinical MUC1 MV samples from breast cancer patients. It has previously been demonstrated that apoptotic bodies can horizontally transfer DNA to surrounding cells (4). This suggests a mechanism for rapid propagation of oncogenic mutations from a tumor cell to surrounding tumor cells and normal stroma. It also suggests the possibility that pathogenic mutations in tumors may be detected in patients in “real-time” during therapy via analysis of tumor MV in plasma.

The above results allow us to now proceed to the systematic analysis of clinical samples.

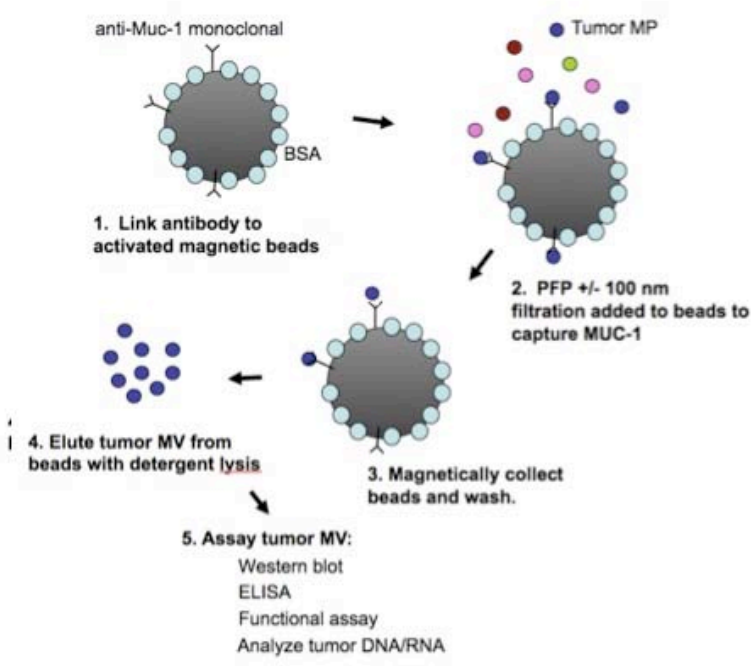

Figure 1. Schematic of bead capture procedure.

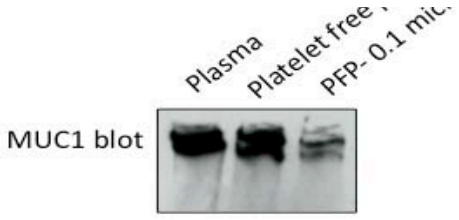

Figure 2. MUC1 western blot of fractionated plasma.

Whole plasma from a breast cancer patient was fractionated by differential centrifugation and filtration as described in the text. Samples were immunoblotted with MUC1 antibody.

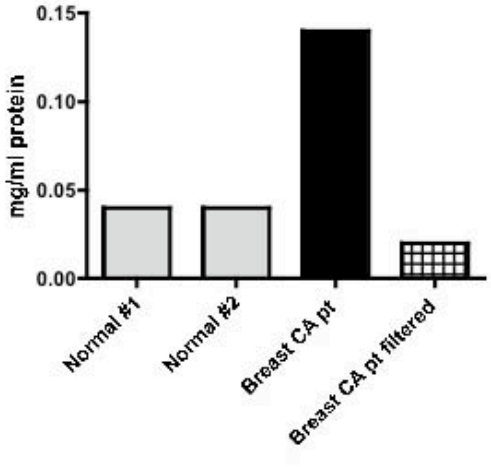

Figure 3. Total protein assay of MUC1 MV.

PFP and MUC1 MV were prepared as described in the text. Two normals and one breast cancer patient were studied. The normals are only unfiltered plasma; the breast cancer patient is both filtered and unfiltered plasma. Total protein assay was performed with a Bio-Rad protein assay kit using the microassay procedure in 96 well format.

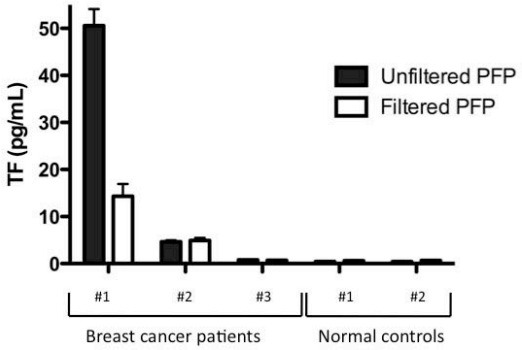

Figure 4. Tissue factor ELISA of MUC1 MV.

PFP and MUC1 MV were prepared as described in the text. Samples were assayed in triplicate with a tissue factor ELISA (American Diagnostica) following the manufacturer's instructions. Standard error bars are shown.

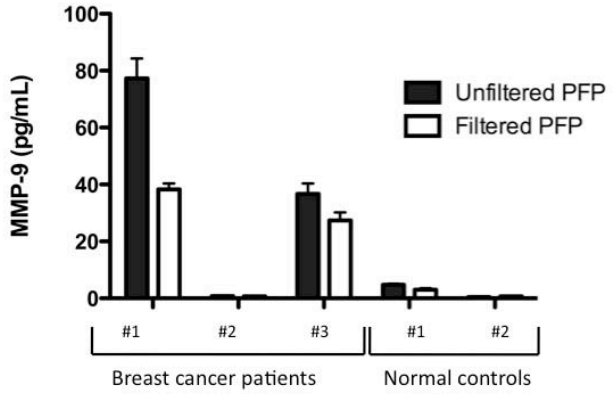

Figure 5. MMP-9 ELISA of MUC1 MV

PFP and MUC1 MV were prepared from as described in the text. Samples were assayed in triplicate with a tissue factor ELISA (Ray-Biotech) following the manufacturer's instructions. Standard error bars are shown.

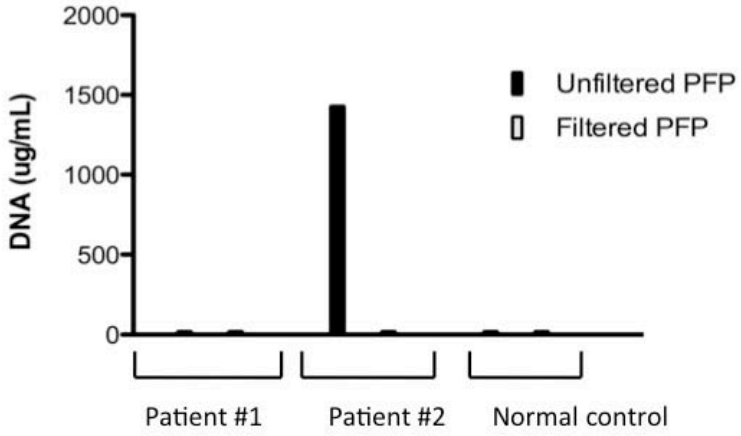

Figure 6. Total DNA assay of MUC1 MV

PFP and MUC1 MV were prepared as described in the text. DNA was extracted with a DNA purification kit (Qiagen). Samples were assayed in triplicate with a fluorescent DNA assay (Invitrogen) following the manufacturer's instructions.

Figure 7. Apoptotic DNA in MDA-MB-231 MUC1 MV.

MUC1 MV were prepared from conditioned media of MDA-MB-231 breast cancer cells following the indicated treatments. Samples were electrophoresed in agarose gels and stained with ethidium bromide.

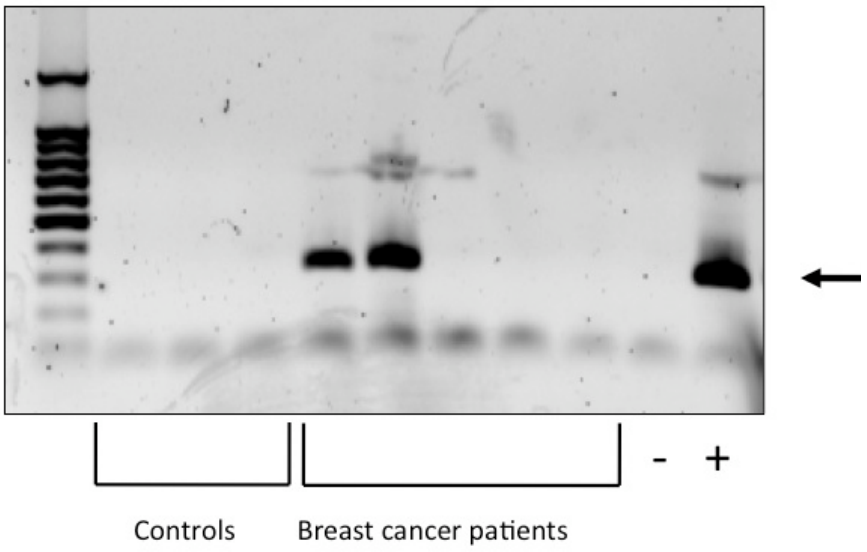

Figure 8. Apoptotic DNA in MDA-MB-231 MUC1 MV.

MUC1 MV were prepared from patients and controls. DNA was extracted as described above. PCR was used to amplify exon 19 of HER2. Products were electrophoresed in agarose gels and stained with ethidium bromide.

Task 2. Test the ability of plasma tumor MV to predict response to treatment and subsequent relapse in locally advanced breast cancer.

Over the course of this project we have enrolling patients with breast cancer in order to perform the studies described in Tasks 2 and 3. We currently have about 240 plasma samples from 43 patients and 10 controls.

We have been unable to make any progress on this aim since the last report. This is because our technician gave 2 weeks notice in June 2008 of her resignation in order to move with her family to Texas. We identified a suitable replacement in September 2008. She began work and was in the process of being trained when she delivered a baby in October. After 6 weeks of family leave she returned. It became clear by mid-winter 2009 that this technician did not have the skills or intellect to be able to perform at the level we needed for this work. Although she did receive IRB training, I did not allow her to analyze any of the human samples. She was let go in March of 2009. In April 2009 when we got word that we had received a one year no-cost extension, we hired an excellent technician from here at UAB with considerable experience. She has proven to be excellent. We will be able to analyze the human samples in the coming year to complete this task

We have obtained up to 5 samples at various times from each participant. We will assay these samples for MUC1 MV total protein, TF and MMP-9 by ELISA, and total DNA as described above. We are particularly interested in the longitudinal change in these parameters with treatment in individual patients, and correlation with outcome.

Task 3. Determine if breast cancer MV can be cleared from the blood *in vivo* by the use of immunotherapy.

We have not made progress on this Task since the last report for the reasons stated above. We previously obtained serial samples from 5 patients during Herceptin treatment in order to test the hypothesis that this targeted therapy efficiently clears HER2 expressing tumor MV from circulation. In addition, we will perform HER2 immunoblots on MUC1 MV on all these samples to follow expression of this protein with treatment. Now that we have competent technical help we will be able to perform these assays in order to complete this Task.

Key Research Accomplishments

1. Development of a novel breast cancer MV capture protocol.

2. Application of simple clinical assays for total protein, TF and MMP-9 ELISA, and total DNA to breast cancer MV.

Reportable Outcomes

1. Development and characterization of a novel breast cancer MV capture protocol.

2. No manuscripts have been published from DOD-funded research in the second year of our grant. We could have submitted a paper describing the bead-capture assay. However, we decided to include this with the results of ongoing assays of all of our clinical samples because this would be a much more complete paper. However our evolving expertise in tumor biology afforded by the DOD support contributed to our efforts on the following work published in the last 2 years:

Merrell MA, Wakchoure S, Ilvesaro JM, Zinn K, Gehrs B, Lehenkari PP, **Harris KW**, and Selander KS. Differential effects of Ca^{2+} on bisphosphonate-induced growth inhibition in breast cancer and mesothelioma cells. European Journal of Pharmacology, 559:21-31, 2007.

Wang D, Liu Z, Li Q, Cao H, Dronadula N, Rizvi F, Kundumani-Sridharan V, Bajpai A, Zhang C, Muller-Newen G, **Harris KW**, and Rao GN. An essential role for gp130 in neointima formation following arterial injury. Circulation Research, 100: p. 807-816, 2007.

Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, **Harris KW**, Selander KS. Toll like receptor -9 agonists stimulate prostate cancer invasion *in vitro*. The Prostate, 67:774-81, 2007.

Merrell MA, Wakchoure S, Lehenkari PP, **Harris KW**, Selander KS. Inhibition of the mevalonate pathway and activation of p38 MAP kinase are independently regulated by nitrogen-containing bisphosphonates in breast cancer cells. European Journal of Pharmacology, 570:27-37, 2007.

Jukkola-Vuorinen A, Rahko E, Vuopala KS, Desmond R, Wakchoure S, Lehenkari

PP, **Harris KW**, and Selander KS.Toll like receptor -9 expression is inversely correlated with estrogen receptor status in breast cancer. In press, Journal of Innate Immunity, 2008

Ilvesaro JM, Merrell MA, Graves D, Rahko E, Jukkola-Vuorinen A, Vuopala KS, **Harris KW**, and Selander KS. TLR9 mediates CpG-oligonucleotide-induced cellular invasion. Molecular Cancer Research, 6:1534-43, 2008.

Wakchoure S, Swain TM, Hentunen TA, Bauskin AR, Brown DA, Breit SN, Vuopala KS, **Harris KW**, Selander KS. Expression of macrophage inhibitory cytokine-1 in prostate cancer bone metastases induces osteoclast activation and weight loss. Prostate 69:652-61, 2009.

Conclusion

We have developed a novel bead capture procedure to isolate and analyze tumor MV from the blood of breast cancer patients. The implications of an easy, precise, and robust clinical assay for circulating tumor MV could include improved detection of early relapse and prediction of metastatic potential of newly diagnosed disease. The assay will also allow quantification of tumor MV in animal studies of breast cancer and improve our understanding of the activity of these particles in patients.

So What Section: This assay could be more clinically useful than the soluble MUC1 assay since the target of the assay (tumor MV) are much more likely to be involved in the process of metastasis than soluble MUC1 (this is because tumor MV are loaded with pro-angiogenic and pro-metastatic proteins). Also, this assay could be more clinically useful than the circulating tumor cell (CTC) assay since the dynamic range of the assay represents thousands of tumor MV per ml, rather than a small number of CTC in 10 ml of blood. A better understanding of tumor MV could result in therapeutic approaches (such as antibody therapy) to clear these malignant particles from the blood of patients.

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3. Furie B, and Furie BC. Cancer-associated thrombosis. Blood Cells, Molecules, and Diseases. 36:177, 2006.

4. Bergsmedh A, Szeles A, Henriksson M, Bratt A, M. Folkman MJ, Spetz AL, and Holmgren L. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proceedings of the National Academy of Sciences 98:6407– 6411, 2001.