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14. ABSTRACT The majority of cancer mortalities occur not from the primary tumor but rather from distant metastases. Since the lymph system provides a route for the spread of metastatic cancer cells, it is not surprising that lymph node status serves as the primary prognostic indicator in most cancers. This work aims to improve the methodology for and accuracy of diagnosing cancer positive lymph nodes. Better diagnoses could increase survival by detecting more true positive nodes which can then be removed while reducing adverse affects by correctly identifying the negative nodes which can be retained and should contribute to improved overall lymph function. To date, metastatic tumors have been generated in the lymph nodes of mice and the IR800 labeled anti-Ep-CAM antibody injected intradermally to detect the tumors. To reduce possible complications associated with the large antibody molecule, we are developing a smaller, single chain binding fragment (scFv) against Ep-CAM.					
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Introduction

The majority of cancer mortalities occur not from the primary tumor but rather from distant metastases. Since the lymph system provides a route for the spread of metastatic cancer cells, it is not surprising that lymph node status serves as the primary prognostic indicator in most cancers. This work aims to improve the methodology for and accuracy of diagnosing cancer positive lymph nodes. Better diagnoses could increase survival by detecting more true positive nodes which can then be removed while reducing adverse affects by correctly identifying the negative nodes which can be retained and should contribute to improved overall lymph function. To date, metastatic tumors have been generated in the lymph nodes of mice and the IR800 labeled anti-Ep-CAM antibody injected intradermally to detect the tumors. To reduce possible complications associated with the large antibody molecule, we are generating smaller portions of the antibody against Ep-CAM.

Body

A unique imaging agent was developed to sensitively identify metastatic tumor cells within the lymph nodes of cancer patients, including breast cancer patients. This agent is an established humanized antibody against the epithelial cell adhesion molecule (Ep-CAM) that is dual-labeled with a near infrared fluorescent dye and a radiotracer for optical and nuclear imaging of epithelial cell based cancers. This agent has been used to identify epithelial cancer cells *in vitro* and *in vivo* in the lymph nodes of mice. Breast cancer cells were inoculated into the left mammary fat pads of mice and allowed to develop tumors for two weeks. Then the imaging agent was delivered intradermally and mice imaged over time to determine if the tumors spread to the left axillary node, with the right axillary lymph node as a contralateral control. A pseudocolor image of the 24 hour imaging time point is shown for one mouse in Figure 1.

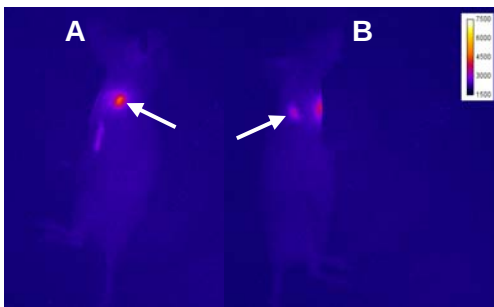


Figure 1:
Pseudocolor image of left (A) and right (B) sides of a mouse with the axillary node locations indicated with arrows. The right lymph node appears swollen and more fluorescent than the left, possibly indicating the presence of tumor cells.

Although higher intensity is present in the right (tumor positive) lymph node than the left (tumor negative) lymph node, there is still significant intensity in the negative node. This retention could be due to the Fc receptor present on immune cells in the lymph nodes or due to the large size of the antibody increasing the antibody clearance time. To increase the clearance speed of the Ep-CAM binding agent, we are developing smaller binding molecules based upon the antibody.

Although whole antibodies, such as trastuzumab (Herceptin) for Her-2 positive cancers, have been used clinically as an anti-cancer therapy, as reviewed by Lennon, et al., 2009, Ma et al., 2009, and Seidenfeld, et al., 2008,[1-3] it may be necessary to utilize a smaller antibody fragment that does not contain the Fc domain to detect metastatic cancer in the lymph nodes. Certain cells within the lymph system such as lymphocytes and NK cells have Fc receptors (FcR) on their surface, and therefore may bind and clear the anti-Ep-CAM antibody before it has a chance to reach the tumor nodules. When these cells bind to the Fc domain of an antibody, they can generate an immune response. Therefore, the removal of the Fc domain from the anti-Ep-CAM agent could also reduce any immunogenicity that could result due to direct injection of the agent into the lymphatic space.

One method for generating smaller antibody fragments is papain digestion. Anti-Ep-CAM will be digested using papain, a specific thiol-endopeptidase that cleaves an antibody on the amino-terminal side of the disulfide bonds that link the two heavy chains of the molecule. After papain treatment, the original whole antibody will be broken into two Fab fragments and one Fc domain fragment. The Fc fragment can then be removed from the desired Fab product by binding to immobilized Protein A, as schematically described in Figure 2.

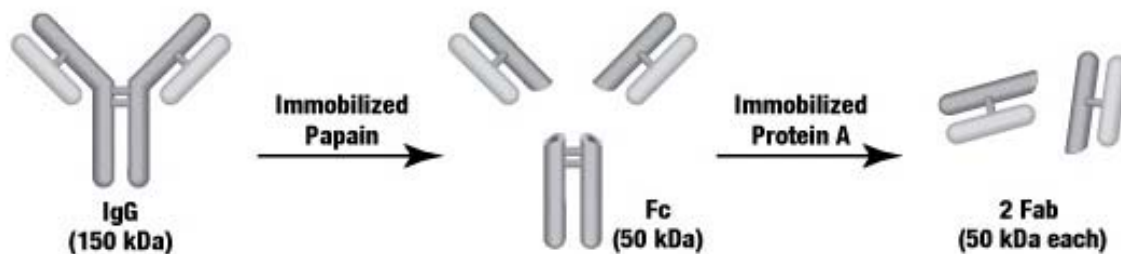


Figure 2: Schematic diagram of Fab generation and purification, image from www.piercenet.com

After papain digestion and purification of the Fab fragments, we will test that the binding functionalities were retained in the Fab after the processing and then the fragments will be dual-labeled and used as Ep-CAM imaging agents in the subsequent experiments. Generating Fab fragments allows testing of an Ep-CAM binding protein with the specific binding ability of the full antibody, but at a third of the size and without the nonspecific Fc domain.

The necessity of removing the Fc domain has not been established, but I believe it will improve the agent's ability to bind specifically to tumor cells in the lymph nodes. The Fc receptors (FcR) could bind the full anti-Ep-CAM antibody before it is able to bind the tumor cells, reducing the efficiency of the agent. Binding of the FcR could signal to the immune system to start an immune response, which could have deleterious side effects.

In addition to antibody fragmentation, the binding domain of an antibody can be grafted into a single chain formation called a single chain binding domain (scFv). Initially not all scFvs were stable and functional, but Jung, et al., 1997 reported the ability to graft the binding domain of an antibody onto a known functional single chain variable fragment (scFv) to form a stable scFv directed against any protein of interest.[4] Willuda, et al., 1999, grafted binding domains specific for human Ep-CAM into a stable scFv framework.[5] The amino acid sequences reported in each paper were combined to form the amino acid sequence for the stable anti-hEp-CAM scFv. The resultant amino acid sequence was reverse-translated into a DNA sequence. To the DNA sequence, I added a purification tag (histidine 6) and a cleavage site (enterokinase) to remove the purification tag. The DNA sequence was then codon-optimized for bacterial expression. The resultant DNA sequence was generated and cloned into bacterial expression vectors by DNA2.0 (Menlo Park, CA). The bacterial expression vector will be used to quickly generate large amounts of protein for initial binding and labeling studies.

I anticipate cloning the scFv into a mammalian expression vector once the *in vitro* labeling and binding experiments are proven successful. This is because proteins expressed in bacteria contain bacterial endotoxins even after purification of the protein of interest. Endotoxin removal will be another possible solution, if the mammalian expression vectors are unable to generate sufficient protein. As reviewed by Magalhaes et al., in 2007, many different endotoxin removal or reduction procedures can be used for specific applications. The biggest drawback from each being the loss of protein of interest after the additional processing steps.[6] A two-phase method of separating endotoxins from recombinant histidine 6-labeled proteins has been successful at removing endotoxins without reducing the biological activity of the protein of interest;[7] we will use this method first, if endotoxin removal is needed.

The mouse models proposed in this research project did not account for the background expression of Ep-CAM. Ep-CAM is expressed in many vertebrates such as humans, mice, rats, dogs and pigs, with very high homology between human and murine Ep-CAM amino acid sequences, which are 80% identical.[8] Human Ep-CAM expression is restricted to epithelial cells,[9] while murine Ep-CAM is additionally expressed in T cells, B cells and dendritic cells (DC).[8, 10, 11] McLaughlin et al. generated transgenic human Ep-CAM (hEp-CAM) FVB/N mice with hEp-CAM under the control of the human Ep-CAM promoter.[12] This transgenic mouse was back-crossed into a C57BL/6 mouse strain and the progeny displayed expression of hEp-CAM in an expression pattern similar to that in human, and murine Ep-CAM in a normal expression pattern.[12-14] Work is underway to secure this transgenic mouse for future use in the animal studies for this project.

For the transfer of this fellowship, I reworked the animal protocol to conform to the CLAMC guidelines and regulations at UTHSC. I also learned about the procedures involved in transferring grants and learning the rules and procedures at a new institution. During the period I was unable to make progress in the laboratory, I focused on literature searches and data review to develop ideas for furthering this fellowship and my education. This report covers a three month period, Aug 15, 2008 to Dec 29, 2008, during that time, due to transfer of the grant, effort was only billed to this project for one month, Aug 15, 2008 to September 19, 2008.

Key Research Accomplishments

Transfer of grant from Baylor College of Medicine, Houston, TX to University of Texas Health Science Center at Houston, Houston, TX

- Animal protocol submitted and approved by Animal Welfare Committee

- Obtained training (including animal, laboratory, radiation, and human subject research training) necessary to continue research plan at UTHSC

Development of smaller, single chain binding fragment (scFv) against Ep-CAM

- Research through literature to find stable scFv scaffold

- Research through literature to find binding domains for Ep-CAM

- Combined sequences to construct anti-Ep-CAM scFv

Discovery of human Ep-CAM transgenic mouse

- Transgenic mouse has hEp-CAM expressed under control of an epithelial promoter so that the expression pattern mimics that in humans, as mice have a different expression pattern; currently attempting to obtain this mouse model from the group who developed it.

Reportable Outcomes

Grant transfer complete with animal protocol approval

Development of anti-Ep-CAM scFv amino acid sequence

Conclusion

Increasing the speed and accuracy of nodal staging is vital because the majority of cancer mortalities occur due to distant metastases rather than the primary tumor. The lymph system provides a primary route for the spread of metastatic cancer cells, thus lymph node status serves as the primary prognostic indicator in most cancers. Currently, occult lymph node staging is extremely invasive and time consuming, requiring surgical removal of lymph nodes for biopsy. This surgical disruption of the lymphatic system followed by radiation has a significant morbidity. Specifically, for breast cancer, axillary node resection is associated with an elevated risk of breast cancer-related lymphedema. Work continues to develop a unique imaging agent to identify metastatic tumor cells within the lymph nodes of cancer patients, beginning initially with breast cancer patients.

Successful development of our agent would eventually include intraoperative nodal staging, allowing surgeons to determine during surgery which nodes must be removed due to metastatic cancer presence, and which nodes are cancer free and can be retained. Increasing the number of cancer free nodes retained, through more accurate detection of metastatic cancer in the lymph nodes, should reduce the risk of breast cancer-related lymphedema development.

This work is imperative because epithelial cancers account for 90% of all cancers; thus, the agent developed herein will have numerous applications in cancer imaging and nodal staging.

The ultimate goal of this project is a novel, non-invasive method for nodal staging of epithelial cancers such as breast, prostate, head and neck, and colon cancer, which could eventually become an intraoperative tool to improve nodal staging.

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Appendices

None