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Detection of Breast Cancer

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14. ABSTRACT Despite the widespread use of X-ray mammography for routine breast cancer (BC) screening, the technique suffers from a number of major limitations including a false-negative rate of at least 10-15% and a positive predictive value (PPV2) of only 25-40%. Accordingly, a large number of patients either do not receive treatment when they should, or are subjected to needless physical and emotional trauma. The weakness of X-ray mammography stems from its poor ability to a) image small or noncalcified lesions, particularly in patients with high breast density, and b) to distinguish between benign and malignant microcalcifications. A tumor-selective X-ray contrast agent would mitigate both of these limitations. This proposal was focused on developing radiopaque gold nanoparticles targeted to HER2, a BC-relevant antigen. Administered intravenously, these conjugates should accumulate preferentially at tumors, enhancing visualization due to the presence of the gold. Interpretation could be aided by subtraction imaging or comparison to a baseline mammogram performed prior to contrast injection. The major objective were 1) to determine the ability of nanogold clusters to detectably alter X-ray mammograms performed using plastic phantoms, 2) to develop nanogold conjugates using small engineered antibody fragments and 3) to perform initial in vivo targeting studies with these conjugates.					
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INTRODUCTION:

Despite the demonstrated utility of X-ray mammography for breast cancer screening, there is limited ability to image small or noncalcified lesions and to distinguish between benign and malignant microcalcifications. The sensitivity and specificity of mammography could be greatly improved if the X-ray attenuation of breast cancer tissue could be enhanced selectively using tumor-specific antibodies labeled with gold nanoparticles (AuNPs). After i.v. administration, these conjugates would clear rapidly from normal tissue but accumulate at breast tumors. The tumors could then be visualized by X-ray imaging due to the comparatively strong attenuation effect of the gold.

The objective of this Concept Award project was to perform preliminary studies towards developing gold nanoparticle immunoconjugates and determining their potential use in vivo.

BODY:

Our accomplishments to date against the objectives outlined in our Statement of Work are described below.

Task 1: To determine the amount of gold that must be accumulated at a tumor for improved contrast resolution on a clinical mammography camera.

Studies were performed to determine if it would be possible to localize sufficient quantities of gold in tumors to alter the attenuation of X-rays in mammography studies.

In lieu of fabricating our own phantom, we used a standard 96 well acrylic tissue culture plate to represent an array of virtual “tumors”. Each cylindrical well was then filled with a prescribed volume and concentration of AuNP suspension, thereby varying both attenuation length and tumor uptake. The entire apparatus was imaged by a conventional film mammography unit.

Nanoparticle suspensions were prepared by dilution of a stock solution of citrate-stabilized, 10 nm diameter AuNPs (Meliorum Technologies, Rochester, NY) with tissue culture grade water. Based on our preliminary X-ray absorption model calculations for expected tumor uptake (data not shown), we prepared ten concentrations of 5, 1, 0.5, 0.1, 0.08, 0.06, 0.04, 0.03, 0.02, and 0.01 wt% gold; and two duplicate controls comprising only water. Wells were filled with 50, 100, 150, 200, 250, or 300 μ L of suspension such that concentration was varied along each row of the plate and volume was varied along each column. Thus, (12 concentrations) x (6 volumes) = 72 scenarios were imaged in parallel.

Numerous attempts were made to optimize the imaging conditions by varying exposure time, peak kV, etc. Initially, this was determined visually according to the opinion of a trained mammography technician, as would be the case in a routine clinical exam. Under conditions gauged to be optimal, no obvious difference in contrast was detected as a function of nanoparticle concentration.

However, subsequent quantitative examination by densitometry revealed a detectable increase in attenuation relative to baseline for higher concentrations of nanoparticles. These results suggest that the quantity of gold that must be deposited in a tumor to increase conspicuity should be attainable using conjugates between AuNPs and antibody fragments. This is particularly true if repeat dosing were employed, allowing for active internalization and sequestering of the gold particles by breast cancer cells.

Task 2: To validate that HER2+ tumors can be visualized in immunodeficient mice using targeted gold nanoparticles.

Numerous attempts were made to modify gold nanoparticles and render them reactive for conjugation to our anti-HER2 C6.5 diabody. Our initial approach was to perform a ligand substitution reaction in the presence of a stabilizing surfactant, e.g. Tween 20. This involved the displacement of labile citrate ligands with thiol bearing molecules, which would self-assemble on the gold nanoparticles via dative bonding. Attempts were made with PEG-2k diamine modified with 2-iminothiolane, 16-mercaptohexadecanoic acid, and other similar molecules. We then intended to activate the derivatized nanoparticles, for example via an NHS/carbodiimide activation scheme, to facilitate conjugation to C6.5.

Surprisingly, and for reasons we do not yet understand, the ligand substitution reactions resulted in visible aggregation (as determined by a colorimetric change) and/or sedimentation of the gold nanoparticles. Attempts to redisperse the nanoparticles, for example by vortex mixing, were not successful. Analysis by dynamic light scattering (NanoS particle size analyzer, Malvern Instruments, Malvern, UK) showed a time progression of increasing size followed by decreasing scattering intensity, a characteristic signature of aggregation and sedimentation.

Therefore, it was not possible to perform a conjugation reaction using our planned protocol. We elected to pursue the alternate approach provided in the original proposal. Specifically, we expressed C6.5 diabody with C-terminal cys residues on each monomer, intending to attach these directly to AuNPs using known gold-thiol chemistry. In this scheme, no intermediate step of activating the AuNPs would be required.

The gene encoding the C6.5 scFv was modified by PCR to incorporate a cysteine residue on its carboxy-terminus (C6.5-cys). The gene encoding C6.5-cys was successfully cloned and used to transform TG1 E. coli. The C6.5-cys protein was expressed from the E. coli in shake flasks, purified by Immobilized Metal Affinity Chromatography and size exclusion chromatography on the HPLC as described [1]. The resulting molecule was found to retain its specificity for the HER2 target antigen in flow cytometry against HER2 expressing SK-OV-3 cells and in BIAcore surface plasmon resonance studies against immobilized recombinant HER2 extracellular domain.

Initial assays have been performed to attempt to directly conjugate the C6.5-cys to the AuNPs, but we have not yet evaluated these data. We expect to perform the remaining in vitro and in vivo assays as soon as we can verify successful conjugation of the scFv/diabody molecules.

1. Adams, G.P., Schier, R., McCall, A.M., Crawford, R.S., Wolf, E.J., Weiner, L.M. and

Marks, J.D. Prolonged *in vivo* tumor retention of a human diabody targeting the extracellular domain of human HER2/*neu*. British J. Cancer, 77:1405-1412, 1998.

KEY RESEARCH ACCOMPLISHMENTS:

- Using a phantom, determined that gold nanoparticles can be detected with a clinical mammography unit at concentrations equal to those expected for tumor uptake *in vivo*
- Designed, cloned and expressed an engineered antibody fragment with a carboxy-terminal cysteine residue to facilitate site-directed conjugation to gold nanoparticles

REPORTABLE OUTCOMES and BIBLIOGRAPHY: None to date.

CONCLUSION:

We have invested a significant effort to determine the feasibility of developing antibody-AuNP conjugates for X-ray mammography contrast. We have successfully demonstrated that AuNPs can enhance contrast on images obtained on a clinical mammography instrument. We encountered unanticipated difficulties in conjugating the C6.5 diabody to the AuNPs using our proposed methodology and were therefore forced to develop an alternate conjugation strategy, which we continue to evaluate. We are now in position to initiate comprehensive studies that will validate this approach for X-ray mammography contrast enhancement.

The major impact of our work to date is (1) demonstration that the amounts of gold that can be reasonably targeted to breast tumors in the preclinical and clinical setting should be sufficient to alter images acquired using clinical mammography instruments and (2) the development of novel molecules that can be used for the production of immunogold conjugates

REFERENCES:

C6.5 affinity maturation manuscript: Schier et al.

LIST OF PERSONNEL:

Gregory P. Adams, Ph.D., Principal Investigator
Calvin C. Shaller, Ph.D., Scientific Associate
Kathryn Hodge, Scientific Technician

APPENDICES: N/A

SUPPORTING DATA:

Sequence of C6.5-cys

5'-

ATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGCAGAGGTGAAAAAG
CCCGGGGAGTCTCTGAAGATCTCCTGTAAGGGTTCTGGATACAGCT
TTACCAGCTACTGGATCGCCTGGGTGCGCCAGATGCCCGGGAAAGG
CCTGGAGTACATGGGGCTCATCTATCCTGGTGACTCTGACACCAAA
TACAGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGTCGACAAGT
CCGTCAGCACTGCCTACTTGCAATGGAGCAGTCTGAAGCCCTCGGA
CAGCGCCGTGTATTTTTGTGCGAGACATGACGTGGGATATTGCAGT
AGTTCCAAGTGCGCAAAGTGGCCTGAATACTTCCAGCATTGGGGCC
AGGGCACCTGGTCACCGTCTCCTCAGGTGGAGGCGGTTCAAGCGG
AGGTGGCTCTGGCGGTGGCGGATCGCAGTCTGTGTTGACGCAGCCG
CCCTCAGTGTCTGCGGCCCCAGGACAGAAGGTCACCATCTCCTGCT
CTGGAAGCAGCTCCAACATTGGGAATAATTATGTATCCTGGTACCA
GCAGCTCCCAGGAACAGCCCCCAAACCTCCTCATCTATGGTCACACC
AATCGGCCCCGCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTG
GCACCTCAGCCTCCCTGGCCATCAGTGGGTTCCGGTCCGAGGATGA
GGCTGATTATTACTGTGCAGCATGGGATGACAGCCTGAGTGGTTGG
GTGTTGCGCGGAGGGACCAAGCTGACCGTCCTAGGTGCGGCCGCTG
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