Radionuclide Imaging with Site Specific Labeled 99mTc-HYNIC-VEGF

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Purpose: Growth of primary tumor and metastatic lesions requires neovascularization that combines angiogenesis and vasculogenesis. Endothelial cells in tumor vasculature overexpress VEGFR-2, a major receptor for vascular endothelial growth factor (VEGF). In contrast, endothelial cells in normal vasculature express relatively low levels of VEGFR-2 and therefore, VEGF might be used as a targeting protein for selective imaging of tumor neovascularization and its responses to various anti-cancer treatments.

The major obstacle to this approach is "loading" of contrast agents onto VEGF because direct modifications of VEGF dramatically decrease its functional activity. Here we describe functionally active VEGF-driven constructs for optical imaging that are assembled using a novel adapter/docking tag technology for "loading" targeting proteins. In this approach, a standardized "adapter" protein with a unique cysteine residue for site-specific conjugation of contrast agents is docked to a "docking tag" fused to a targeting protein. To avoid dissociation of the complex, adapter protein and docking tag are engineered to form an intermolecular disulfide bond upon complex formation.

We developed a novel standardized adapter protein with a unique cysteine residue for site-specific conjugation of radionuclides docked to a tag fused to a targeting protein. To avoid dissociation of the complex, adapter protein and docking tag was engineered to form an intermolecular disulfide bond upon complex formation. We wished to test this form of Tc99m-VEGF(A, 121 isoform) for the imaging of tumor vasculature in vivo.

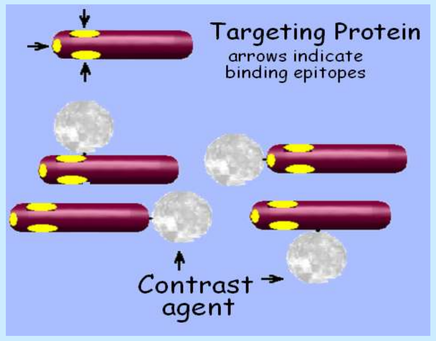
Methods: We first generated thiol groups for cross-linking to HYNIC (hydrazine nicotinamide), on the C4-VEGF dimer containing 20 cysteine residues per VEGF dimer, via treatment with DTT under mildly denaturing conditions (1.5-fold molar excess DTT for 2hrs at 34oC in the presence of 0.5 M urea and 0.5 M NDSB-221 to prevent protein aggregation). This treatment yielded two free thiol groups per VEGF monomer as ascertained by the reaction with N-(1-pyrene)-maleimide and HYNIC-maleimide. Treatment did not affect interactions of VEGF with cellular receptors, as judged by in vitro assays. Tc99m-HYNIC-VEGF was then prepared using tin/tricine as an exchange reagent and injected via tail vein (200-300 uCi, 1-2 ug protein) followed one hour later by microSPECT imaging (1mm pin hole, 64 steps, 360 degree rotation).

Results: Mass spectroscopic location of thiol groups indicated that under selected reducing conditions cysteine residues in the C4 tag at position -5 at the C-terminus of VEGF monomer become available for site specific cross-linking. Blood clearance of the tracer was rapid (t1/2 = 15 minutes). We were able to visualize tumor vascularity in Balb/c mice with 4T1 murine mammary carcinoma 10 days after implantation into the left axillary fat pad in controls (12.3 5.0 tumor/bkg, n=27) and following treatment with high (150 mg/kg/d x 4; 1.14 0.48 tumor/bkg, n=9) or low 25 mg/kg/d x 6; 1.03 0.18 tumor/bkg, n=9) dose cyclophosphamide.

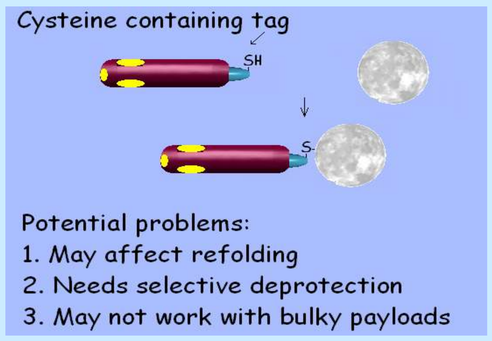
Conclusions: Tc99m and other contrast agents can be non-invasively loaded on C4-VEGF in a site specific fashion. This compound can be readily used to image and monitor tumor angiogenesis in vivo.

1. Platform Technology for Site-specific Crosslinking of Contrast Agents

Random crosslinking affects targeting

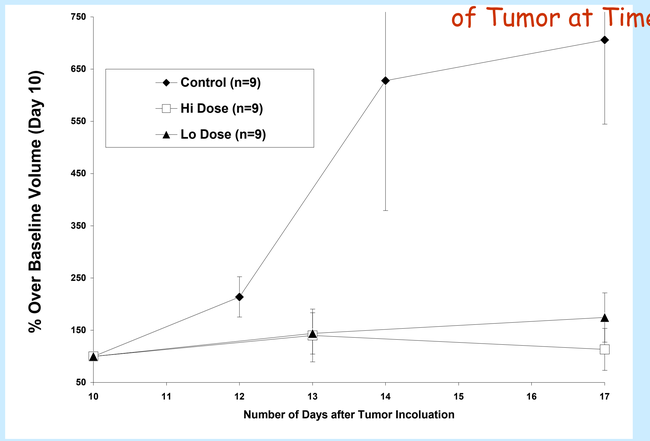


Fusion Tag for site- specific modification

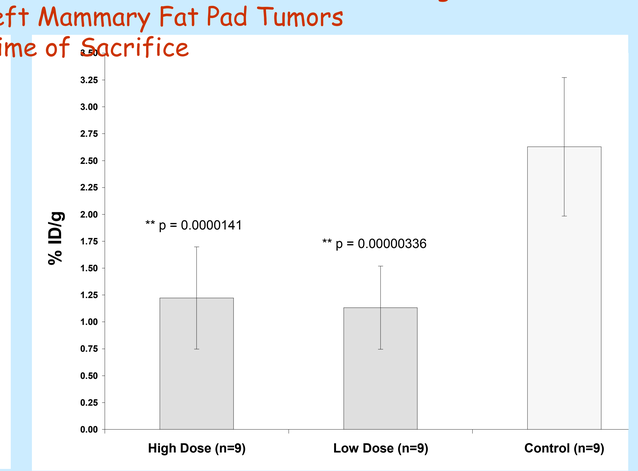


5. Results from High and Low Dose Cyclophosphamide Treatment in 4T1 Tumor Mice

Growth Curves of Left Mammary Fat Pad Tumors



Scintillation Well Counting of Tumor at Time of Sacrifice



2. Why Image VEGF Receptors

VEGFR-2 (KDR/Flk-1)Targeting

- endothelial cell specific
- overexpressed at the sites of angiogenesis
- internalize upon binding VEGF

$VEGF_{121}$ as a Targeting Protein

- highly specific
- lowest nonspecific binding
- retains activity as a fusion protein

3. $VEGF_{121}$ as a Targeting Protein

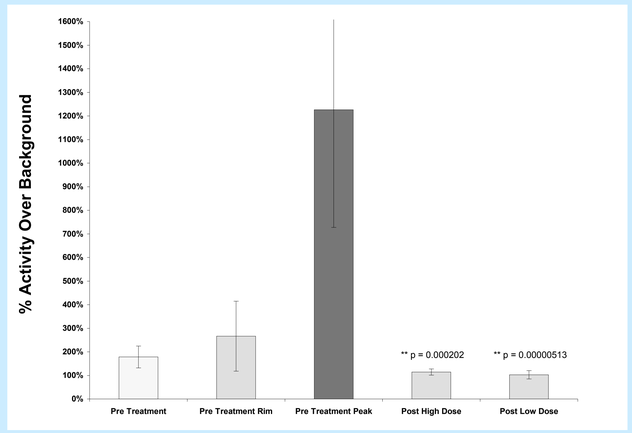
Tools for testing VEGF conjugates in vitro

1. 293/KDR cells: $2x10^6$ VEGFR-2 per cell
2. Toxin-VEGF: Shiga-like toxin subunit A fused to $VEGF_{121}$

Growing 293/KDR cells are highly sensitive to toxin-VEGF with IC_{50} of 0.12 ± 0.09 nM

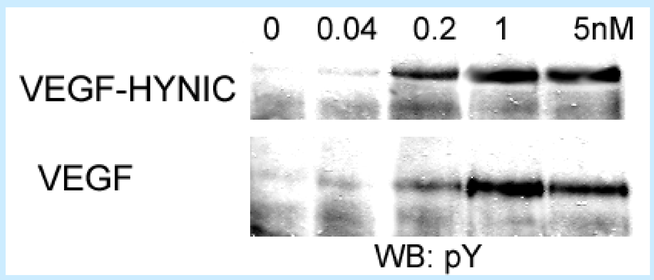
Backer M. & Backer J. Bioconj Chem 2001, 12, 1006

6. Region of Interest Analyses of Serial Radionuclide Images

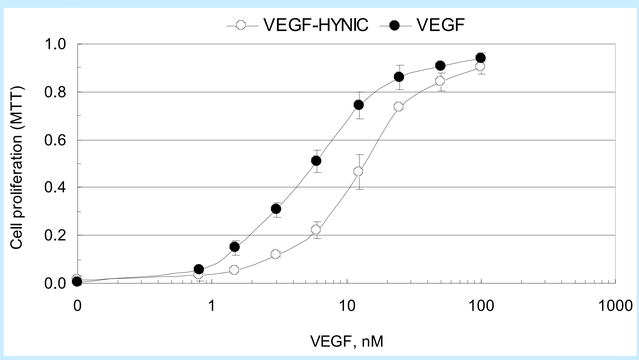


4. Testing HYNIC-VEG in tissue culture

VEGFR-2 autophosphorylation 293/KDR cells



Protection of 293/KDR cells from toxin-VEGF induced death in



7. Axial microSPECT Images Pre and Post Treatment