Near-infrared Fluorescent Imaging of Tumor Neovascularization with VEGF-driven Conjugates

Marina V. Backer, Vimal Patel, Brian T. Jehning and Joseph M. Backer
SibTech, Inc., Newington, CT 06111

Abstract

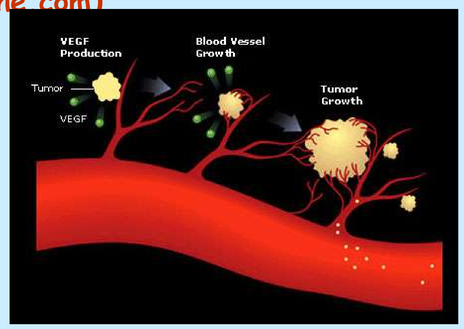
Endothelial cells in tumor vasculature overexpress VEGFR-2, a major receptor for vascular endothelial growth factor (VEGF). Since VEGF/VEGFR-2 pathway is crucial for tumor angiogenesis, it is a primary target for potential anti-cancer therapeutics. However, little is known about patterns of VEGFR-2 expression at various stages of tumor development or therapeutic treatments. In part, it is due to the paucity of targeted constructs that can selectively bind to VEGFR-2 in tumor vasculature. We reasoned that VEGF could be used as a targeting protein for selective imaging of tumor neovascularization and its responses to various anti-cancer treatments.

To optimize VEGF for molecular targeting, we cloned two fragments encoding $VEGF_{121}$ monomers as a single-chain VEGF (scVEGF) that was expressed as a single polypeptide and refolded to mimic a VEGF dimer. To optimize construction of imaging and/or therapeutic conjugates, scVEGF was armed with an N-terminal peptide tag, containing a unique cysteine residue for site-specific conjugation. Near infrared dyes Cy5 or Cy5.5 were conjugated to either VEGF or scVEGF. The resulting VEGF/Cy conjugates retained functional activity comparable to that of parental VEGF in tissue culture assays, such as induction of VEGFR-2 tyrosine autophosphorylation and competition with a VEGF-toxin for binding to cellular VEGFR-2.

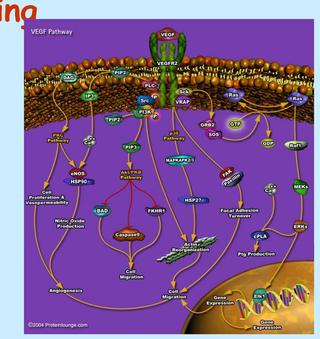
In several mouse tumor models, VEGF/Cy constructs selectively accumulated in the tumor area, as visualized by near-infrared fluorescent imaging. VEGFR-2 overexpressing cells are implicated in this effect, since it was dramatically decreased in mice pretreated with a VEGF-toxin that selectively eliminates such cells. Selective accumulation was detectable as early as four days after subcutaneous implantation of 10,000 mammary mouse adenocarcinoma luciferase-expressing 4T1luc cells and persisted for 4-5 days after VEGF/Cy injection providing opportunities for detection of vascular remodeling.

We expect that VEGF/Cy conjugates will be useful in development of anti-angiogenic compound in animal models and, perhaps, in monitoring "close-to-surface" angiogenesis in humans.

1. VEGF Drives Angiogenesis (www.gene.com)

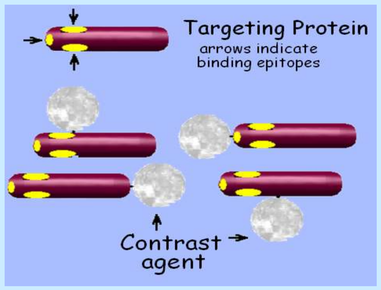


2. VEGF/VEGFR-2 Signaling

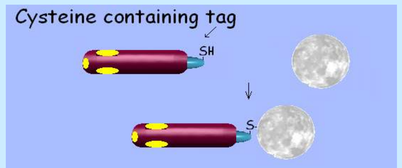


2. Site-specific Crosslinking of Contrast Agents to Targeting Proteins

Random crosslinking damages proteins

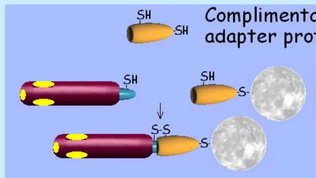


Fusion C-Tag for site- specific modification



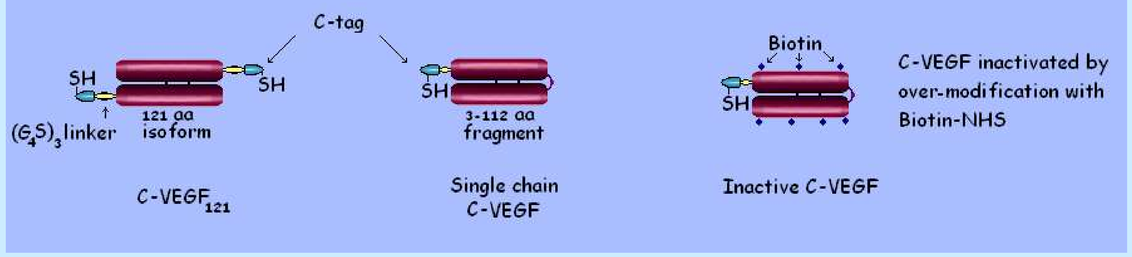
Potential problems:
1. May affect refolding
2. Needs selective deprotection
3. May not work with bulky payloads

Complimentary Adapter protein for standardized payloads



Advantages:
1. Self-assembly
2. Does not need selective deprotection
3. Works with bulky payloads

3. Engineering VEGF for in vivo Molecular Targeting



4. Testing VEGF/Cy Probes in Tissue Culture

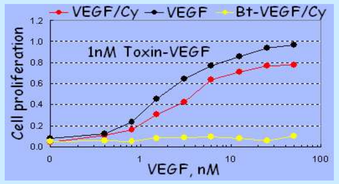
Tools for studying VEGF/VEGFR-2 interaction

- VEGFR-2 expressing cell lines
- Toxin-VEGF: Shiga-like toxin subunit A fused to $VEGF_{121}$

cell line	VEGFR-2 per cell	Toxin-VEGF IC_{50}(nM)
293/KDR	2×10^6	0.12 + 0.09
PAE/KDR	10^5	0.19 + 0.13
PAE/0.2-KDR	0.2×10^5	0.85 + 0.35
PAE/0.1-KDR	10^4	2.85 + 0.43
PAE/V	none	3.95 + 0.95

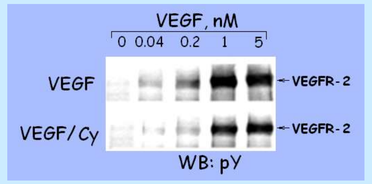
Backer M. & Backer J. Bioconj Chem 2001, 12, 1006

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



VEGF competitor was serially diluted in toxin-VEGF containing culture medium and added to 293/KDR cells to a final toxin-VEGF concentration of 1 nM. Cell proliferation was determined 96h later.

Dose-dependent induction of VEGFR-2 autophosphorylation



Near-confluent 293/KDR cells after o/n starvation were stimulated with VEGF or VEGF/Cy for 10 min at 37°C and analyzed by Western blotting

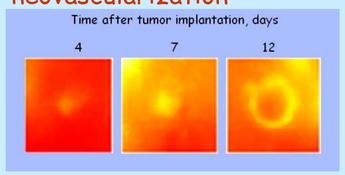
5. VEGF/Cy Imaging of 4T1 Tumors Grown in Balb/c Mice

SQ (subcuteneous) 4T1 tumor

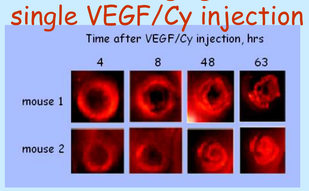
MFP (mouse fat pad) 4T1 tumor

Bioluminescent (BLI) or near infra-red fluorescent (NIRF) images of mice bearing 4T1 tumors were taken after a single i.v. injection of VEGF/Cy (10-20 µg/mouse). The same dose of inactivated Bt-VEGF/Cy was injected to control mice to determine level of non-specific imaging.

Various stages of tumor neovascularization



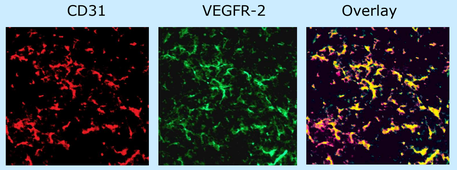
Serial re-imaging after a single VEGF/Cy injection



Inactivated Bt-VEGF/Cy fails to imaae SQ tumor

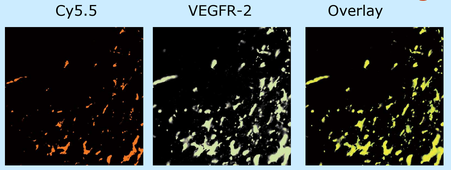
6. VEGF/Cy Binds to VEGF Receptors in Growing Tumor Neovasculature

VEGFR-2 positive cells are 85-95% endothelial



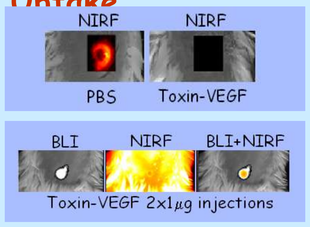
The majority of VEGFR-2 positive cells in 4T1 tumor (more than 89%) co-localizes with CD31 pan-endothelial marker

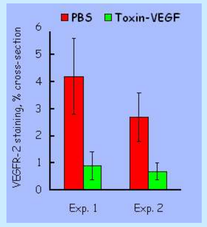
Cy5.5 fluorescence is associated with VEGFR-2 immunostaining



4T1 tumor bearing mice were sacrificed 4 hrs after VEGF/Cy injection. Tumor cryosections were stained for CD31 and VEGFR-2 and analyzed by confocal microscopy.

7. Depletion of VEGFR-2 Overexpressing Cells Dramatically Decreases VEGF/Cy Uptake





Mice bearing 1 week old 4T1 SQ tumors received two injections of Toxin-VEGF (1 µg/mouse, each), and then were imaged with VEGF/Cy. For quantitative analysis, microscopic images of VEGFR-2 stained tumor cryosections were converted to grayscale, binarized to black and white with the threshold values that preserve immunostaining visible by the eye. The staining in the same central area of the image (~250,000 pixels) was quantitated by a histogram analysis that yielded the percentage of the area occupied by black pixels.

Conclusions

2. **Functionally active VEGF/Cy conjugates for near-infrared fluorescent imaging are prepared, using a 15-aa cysteine-containing tag fused to VEGF and a novel single-chain VEGF**
3. **VEGF/Cy accumulates in tumor vasculature in VEGFR-2 overexpressing cells providing near-infrared images of tumor neovascularization.**
4. **Imaging with VEGF/Cy provides information about early stages of tumor angiogenesis, remodeling of tumor vasculature, and response of tumor vasculature to VEGFR-2 targeted therapeutics.**