Targeted Optical Imaging of Tumor Vasculature with VEGF/Cy5.5 Conjugates

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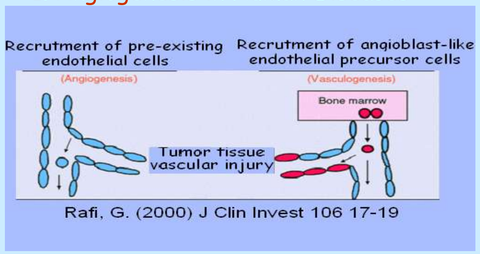
Abstract

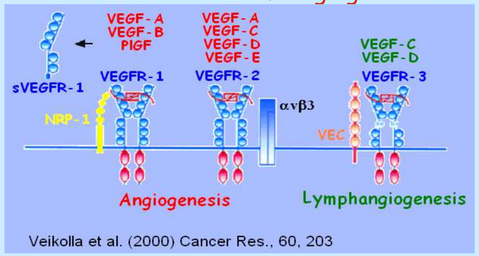
The VEGF/VEGFR-2 pathway is the crucial regulator of tumor angiogenesis and therefore it is a primary target for potential anti-cancer therapeutics. However, little is known about patterns of VEGFR-2 expression at various stages of tumor development or therapeutic treatments. In part, it is due to the paucity of targeted contrast agents that selectively image VEGFR-2 positive cells in tumor vasculature. We report here development of a VEGF/Cy5.5 conjugate for optical near-infrared imaging of VEGFR-2 positive cells in vivo. The conjugate retains VEGF functional activity *in vitro* and provide near-infrared imaging of specific regions of tumor vasculature *in vivo*. Excess unlabeled VEGF, as well as targeted depletion of VEGFR-2 positive cells, decreases Cy5.5 signal in the region of interest ~10-fold indicating VEGF receptor mediated mechanism of uptake. Cy5.5 is retained in specific regions of tumor vasculature for days, allowing for long-term observation of changes induced by various therapeutic agents. Direct modification of VEGF with Cy5.5 resulted in significant loss of VEGF functional activities even with conjugates containing only one Cy5.5 dye per VEGF dimer. To avoid VEGF damaging, VEGF/Cy5.5 conjugate was prepared using a "dock-and-lock" technology for site-specific modification of targeting proteins. Briefly, VEGF was expressed as a fusion protein with a cysteine-containing 15-aa tag. A complimentary adapter protein was designed to have two free cysteines: one for forming a disulfide bond with a cysteine of the tag, and the second one for site-specific conjugation of Cy5.5 via maleimide chemistry. The adapter was "docked" to the tagged VEGF, and the complex was "locked" by a disulfide bond between the tag and the adapter protein. The adapter protein in the resulting conjugate was then labeled site-specifically with Cy5.5-maleimide. VEGF/Cy5.5 functional activity tested in two tissue culture assays, induction of tyrosine autophosphorylation of VEGFR-2 in 293/KDR cells, and protection of 293/KDR cells from cytotoxicity of VEGF-toxin was comparable to that of parental VEGF. For near-infrared imaging of tumors, 4T1luc mouse mammary adenocarcinoma cells expressing luciferase for bioluminescent imaging were grown on the backs or in the mammary fatpads of Balb/c mice. Tumor bearing mice were injected intravenously with VEGF/Cy5.5 conjugates and imaged on KODAK 2000 image station. Bioluminescent imaging of tumors and near-infrared imaging of VEGF/Cy5.5 conjugates revealed preferential accumulation of VEGF/Cy5.5 at the edges of the growing tumors. In addition, accumulation of VEGF/Cy5.5 in the nearby areas of host vasculature was detected as early as four days after implantation of 2,000 4T1luc cells. Co-injection of excess unlabeled VEGF, or depletion of VEGFR-2 positive cells with toxin-VEGF protein dramatically decreased accumulation of VEGF/Cy5.5 in tumor vasculature indicating a receptor-mediated mechanism of uptake.

1. Angiogenesis in Human Diseases



Rafi, G. (2000) J Clin Invest 106 17-19

2. Molecular Markers of Angiogenesis



Veikolla et al. (2000) Cancer Res., 60, 203

3. Why Image VEGF Receptors

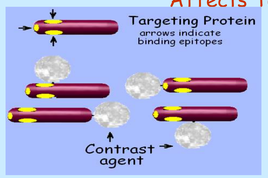
- endothelial cell specific
- overexpressed at the sites of angiogenesis
- internalizes upon binding VEGF

4. VEGF121 as a Targeting Protein

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

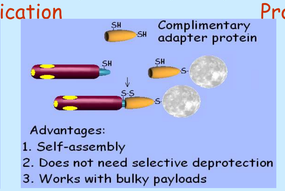
5. Platform Technology for Site-specific Crosslinking of Contrast Agents

Random Crosslinking Affects Targeting

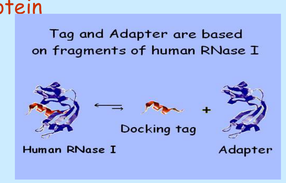


Fusion Tag for Site-Specific Modification

Complimentary Adapter Protein



Human Tag/Adapter pair



7. Testing Cy5.5-VEGF probes in tissue culture

VEGF-Cy5 Conjugates

Conjugate	Reagent	Modification
VEGF-Cy5 (NHS)	Cy5.5-NHS	NH-group in VEGF
VEGF-Cy5 (Mal)	Cy5.5-Mal	SH-group of VEGF Tag
VEGF/Cy5	Cy5.5-Mal	SH-group in VEGF-Adapter

Cy5.5to VEGF molar ratio in all cases 1 to 1

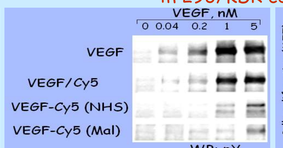
Tools for in vitro studies

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

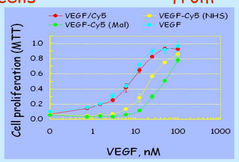
cell line	VEGFR-2 per cell	Toxin-VEGF IC_{50}(nM)
293/KDR	2×10^6	0.12 + 0.09
PAE/KDR	10^5	0.19 + 0.13
PAE/0.2-KDR	0.2×10^5	0.85 + 0.35
PAE/0.1-KDR	10^4	2.85 + 0.43
PAE/V	none	3.95 + 0.95

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

VEGFR-2 autophosphorylation in 293/KDR cells

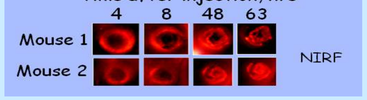


Protection of 293/KDR from



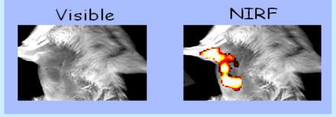
8. VEGF/Cy5.5 Imaging of SQ and MFP 4T1 Tumors

SQ (subcuteneous) 4T1 tumor



Mice bearing 10-days old s.q. 4T1 tumors were imaged repeatedly after receiving single 5-μg injection of VEGF/Cy5.5 in the tail vain

MFP (mouse fat pad) 4T1 tumor



Mouse bearing a 14-days old MFP 4T1 tumor was imaged with 5 μg VEGF/Cy5.5

9. Activation of Host Vasculature Imaged with VEGF/Cy5.5

Mouse carcinoma 4T1 cells transfected with firefly luciferase were injected sq in the backs of BALB/c mice. VEGF/Cy5.5 was injected via the tail vain 4 and 14 days later, 5 μg/mouse. For BLI, mice were injected intraperitoneally with aqueous luciferin, 0.5 mg/mouse

10. Anti-angiogenic Treatment Imaged with VEGF/Cy5.5

Mouse 1

Mice bearing subcutaneous 4T1-Luc tumors received two 1-μg injections of toxin-VEGF and were imaged 3 days later.

Mo use 2

Conclusions

2. **A novel technology for facile derivatization of targeting proteins with contrast agents is developed.**
3. **The technology allowed synthesis of functionally active VEGF/Cy5.5.**
4. **VEGF/Cy5.5 conjugate images VEGF receptors in tumor vasculature.**
5. **Imaging with VEGF/Cy5.5 reveals highly heterogeneous and tumor model dependent distribution of VEGF receptors.**
6. **Imaging with VEGF/Cy5.5 detects activation of host vasculature as an early event in tumor development.**
7. **Imaging with VEGF/Cy5.5 detects targeted depletion of VEGF receptors in tumor vasculature induced by anti-angiogenic treatment.**