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Surface immobilization of active vascular endothelial growth factor via a cysteine-containing tag

Marina V. Backer[a,*], Vimal Patel[a], Brian T. Jehning[a], Kevin P. Claffey[b], Joseph M. Backer[a]

[a]SibTech, Inc., Newington, CT 06111, USA
[b]University of Connecticut, Health Center, Farmington, CT 06030, USA

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Abstract

Developing tissue engineering scaffolds with immobilized growth factors requires facile and reliable methods for the covalent attachment of functionally active proteins. We describe here a new approach to immobilize recombinant proteins based on expression of the protein of interest with a 15-aa long fusion tag (Cys-tag), which avails a free sulfhydryl group for site-specific conjugation. To validate this approach, we conjugated a single-chain vascular endothelial growth factor expressed with an N-terminal Cys-tag (scVEGF) to fibronectin (FN) using a common thiol-directed bi-functional cross-linking agent. We found that the FN-scVEGF conjugate retains VEGF activity similar to that of free scVEGF when used as a soluble ligand. Cells expressing VEGF receptor VEGFR-2 grown on plates coated with FN-scVEGF displayed morphological phenotypes similar to those observed for cells grown on FN in the presence of equivalent amounts of free scVEGF. In addition, 293/KDR cell growth stimulation was observed in the same concentration range with either immobilized or free scVEGF. The effects of immobilized scVEGF, and soluble scVEGF were blocked by NVP-AAD777-NX, a VEGF receptor tyrosine kinase inhibitor. These data indicate that site-specific immobilization via Cys-tag provides a facile and reliable method for permanent deposition of functionally active growth factors on synthetic or protein scaffolds with applications for advanced tissue engineering.

Keywords: Site-specific conjugation; Immobilization; Growth factors; VEGF; Tissue engineering

1. Introduction

One of the goals of tissue engineering is to design cell and organ scaffolds that facilitate cell survival, growth, and differentiation. Cell viability and differentiation in vivo is determined by signals initiated by soluble, matrix-bound, and cell surface ligands or by matrix itself, through specific interactions with cellular receptors. To model this environment, it would be beneficial to develop tissue scaffolds with covalently immobilized protein ligands, which would provide a controlled and sustainable influence on cell behavior over that of soluble or slow-released proteins [1]. Furthermore, covalent immobilization of soluble protein ligands might provide extended signaling since the ligand will not be internalized as a ligand/receptor complex.

The progress in developing scaffolds with covalently immobilized growth factors is rather limited. Several groups used amino-reactive bifuctional crosslinking reagents to immobilize epidermal growth factor [2–4], nerve growth factor [5], transforming growth factor β2 [6], and transforming growth factor β1 [7]. However, random conjugation via lysine ε-amino groups is hardly an acceptable approach, because it generates highly heterogeneous products with regard to functional activity and orientation of individual protein molecules. The use of a fusion tag comprising a substrate sequence for Factor XIIIa transglutaminase mediated conjugation to fibrin was a very elegant solution for site-specific immobilization of vascular endothelial growth factor ($VEGF_{121}$) on fibrin [8], but it lacks general applicability. Another possible solution was proposed for single-chain antibody fragments, namely, insertion of a cysteine suitable for site-specific conjugation at or near single-chain Fv (scFv) C-terminus

*Corresponding author. Tel.: +1 860 953 1753; fax: +1 860 953 1317.
E-mail address: mbacker@sibtech.com (M.V. Backer).

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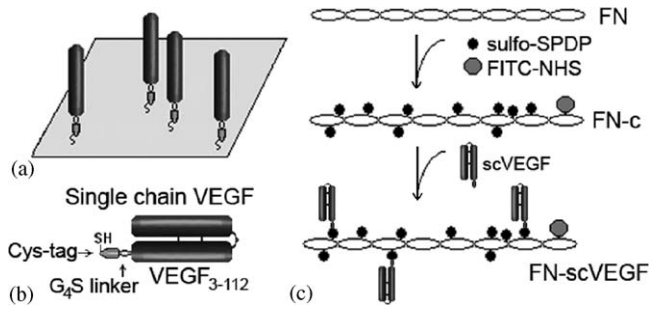

Fig. 1. Schematic representation of (a) surface coated with uniformly oriented Cys-tagged protein, (b) Cys-tagged single chain VEGF and (c) its site-specific conjugation to FN.

[9,10]. However, expression of such proteins appeared to be problematic in regards to protein yield and proper folding [11].

We have recently developed a cysteine-containing peptide tag, named here Cys-tag, that can be fused to either N- or C-terminus of a recombinant protein and used for site-specific conjugation of therapeutic and/or diagnostic payloads via thiol-directed chemistry [12–15]. In our experience, the Cys-tag peptide did not affect either yield or activity of several proteins. Importantly, site-specific conjugation of various payloads to Cys-tagged proteins did not significantly affect their functional activities [12–14]. We hypothesized that a similar approach might be used to conjugate functionally active Cys-tagged proteins to various surfaces, as shown on Fig. 1a. To test this hypothesis, we constructed a scVEGF with an N-terminal Cys-tag (Fig. 1b), conjugated it to fibronectin (FN) and used the FN-scVEGF conjugate as a substrate for cells expressing the VEGF receptor, VEGFR-2 (KDR/Flk-1). Our choice of scVEGF for covalent immobilization was stimulated by its potential use in engineering of vascular grafts, scaffolds for expansion of endothelial progenitor cells, and matrices for bone regeneration [15–17]. We report here that covalently immobilized scVEGF retains the functional activity of free scVEGF, as demonstrated by phenotypic changes and growth stimulation of VEGFR-2 overexpressing cells.

2. Materials and methods

2.1. Materials

FN from bovine plasma (0.1% solution, 200–250 kDa for monomer), Gelatin–Agarose, monoclonal mouse anti-FN antibody clone IST-3, monoclonal anti-phosphotyrosine antibody clone PT-66, and other reagents, if not specified, were from Sigma. Sulfosuccinimidyl 6-[3′(2-pyridyldithio)-propion-amido] hexanoate (Sulfo-LC-SPDP, FW 527.57) and microBCA protein assay kit were from Pierce. *N*-hydroxysuccinimidyl (NHS) of fluorescein isothiocyanate (FITC), PD-10 disposable desalting columns, anti-mouse IgG-HRP conjugate, and ECL Plus kit were from GE Healthcare. NVP-AAD777-NX (Nx, FW 448.37) VEGF receptor tyrosine kinase inhibitor was kindly provided by Dr. P. Traxler (Novartis, Basel, Switzerland). Nx was stored at −20 °C as 2 mM solution in DMSO in small aliquots and diluted with complete culture medium immediately before the experiments. Recombinant human $VEGF_{165}$ was from R&D Systems. Mouse anti-human VEGF antibody was from Pharmingen.

2.2. Construction of scVEGF

The pET/Hu-R4C(G_4S) vector was constructed as described [18]. Human $VEGF_{121}$ was amplified from the pET32/VEGF plasmid [19] and cloned into *Bam* HI site of the pET/Hu-R4C(G_4S). The resulting plasmid containing one *Nco*I site immediately downstream of a (G_4S)-linker and another one in VEGF ORF (5–10 nucleotide region) was digested with *Nco*I and used for further cloning. DNA encoding a 3-112 amino acid fragment of $VEGF_{121}$ was amplified by PCR with CACCCATGGCAGAAGGAGGA (sense) and CCATGGCTCTTGCTCTATCTTTCTTTGGTCTGC (antisense) primers introducing *Nco*I sites to both termini. To compensate an ORF shift due to cloning into *Nco*I site, the antisense primer contained an inserted C (bolded), which reconstructed an alanine codon GCC between two VEGF fragments after ligation. Cys-tagged scVEGF was confirmed by sequencing and expressed in BL21(DE3) *Escherichia coli* as described [18].

2.3. Synthesis of FN-scVEGF conjugate

FN (1 nmol) was mixed with a bi-functional cross-linking reagent Sulfo-LC-SPDP (75 nmol) and FITC-NHS (20 nmol) in 0.8 ml of 20 mM carbonate buffer (pH 9.0), and incubated for 1 h at RT. The intermediate FN-SPDP-FITC conjugate, named FN-c, was desalted on a PD-10 column and analyzed for SPDP, FITC, and protein concentration. SPDP concentration was determined by optical density at $\lambda = 343$ nm, upon complete oxidation with 10 mM DTT for 1 h at 37 °C. FITC concentration was determined fluorometrically ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 520$ nm) using free FITC as a standard. Protein concentration was determined by microBCA assay according to the manufacturer's protocol. A typical FN-c preparation contained 450 ± 50 nM FN, 20 ± 4 μM SPDP, and 700 ± 60 nM FITC. scVEGF was incubated with equimolar DTT in 0.1 M Tris-HCl pH 8.0 for 30 min at 25 °C in order to obtain free C4 SH-group, and then mixed with FN-c at a VEGF to SPDP molar ratio of 2:1. After a 60-min incubation, the resulting FN-scVEGF conjugate was purified from unreacted scVEGF by affinity chromatography on Gelatin–Agarose as described [20]. Non-specifically bound protein was removed from the column by extensive washing with 0.1 M Tris-HCl pH 7.2, 150 mM NaCl. FN-scVEGF was eluted with 8 M urea and dialyzed in 100 V of 20 mM NaOAc pH 6.5, 0.15 M NaCl, for 16 h. Concentration of scVEGF was determined by Western blotting after complete reduction of FN-scVEGF conjugate in 0.1 M DTT for 15 min at 37 °C with free scVEGF serving as a standard. Concentration of FN was determined by FITC fluorescence as described above. An average FN-scVEGF preparation contained 5–6 scVEGFs per FN molecule.

2.4. Coating plates with FN-scVEGF

FN-scVEGF (or FN-c for control growth) was serially diluted with sterile PBS and added to duplicate wells of 96- or 24-well plates, 50 and 200 μl per well, respectively. After 18 h of incubation at 4 °C, solutions were aspirated; wells were washed twice with PBS and used for cell seeding. The amount of scVEGF per well was determined as follows: after PBS washing, plates were incubated at 37 °C for 15 min with 0.1 M DTT for complete scVEGF detachment, samples were collected, serially diluted and analyzed by Western blotting alongside unbound FN-scVEGF solution from the same well and scVEGF standard, using mouse anti-human VEGF monoclonal antibody (Pharmingen) diluted 1:2000 followed by anti-mouse IgG:HRP conjugate (GE Healthcare) diluted 1:10,000. Protein bands were visualized by ECL Plus chemiluminescence detection kit (GE Healthcare). Quantitative analysis of VEGF immunoblots indicated that the selected coating conditions yielded the range from 3.4×10^2 to 3.4×10^4 scVEGF molecules per μm^2 of tissue culture well.