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

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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

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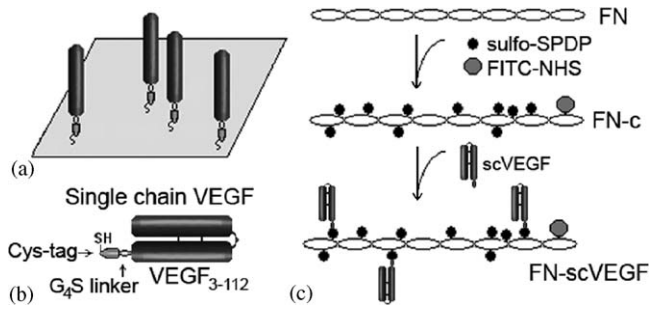

Fig. 1. Schematic representation of (a) surface coated with uniformly oriented Cys-tagged protein, (b) Cys-tagged single chain VEGF and (c) its site-specific conjugation to FN.

[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.

Amount of FN bound to wells was independently estimated by a decrease of FITC fluorescence in solutions collected from wells after coating. This analysis indicated that bound FN varied within 13–27% of initial FN used for coating.

2.5. Cells

HEK293 human transformed embryonic kidney cells (CRL-1573) were from American Type Culture Collection (Rockville, MD). 293/KDR cells expressing 2.5×10^6 VEGFR-2/cell have been developed in SibTech, Inc. [19]. PAE/KDR porcine aortic endothelial cells expressing human VEGFR-2 were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, Bronx, NY, USA). All cells were grown in DMEM with 10% FBS, 2 mM L-glutamine and antibiotics at 37 °C, 5% CO_2.

2.6. Cell growth assays

Cells were plated on 96-well plates coated with either FN-scVEGF or FN-c, 1000 cells/well. Nx was added to appropriate wells 3 h later to a final concentration of 1 μM. For free scVEGF control, varying amounts of scVEGF, alone or in a mixture with Nx, were added to cell culture medium 3 h after plating cells on FN-coated wells. Cells were allowed to grow for 96 h, and then quantitated by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega). All cell growth assays were done in triplicate. For microscopy, cells were plated on FN-scVEGF (or FN-c) coated 24-well plates, 7×10^3 cell/well. Control cells were grown in non-coated wells. Nx, scVEGF, or their mixtures were added to cells 3 h after plating. After 48 h of incubation under normal culture conditions, cells were washed twice with PBS, fixed with fresh 4% glutaraldehyde for 20 min at RT, washed twice with deionized water, and stained for 1 min with Giemza stain. Images were acquired with an AxioCam HRc Cooled CCD Camera attached to a Zeiss Stemi SVII disecting microscope.

3. Results

3.1. Derivatization of FN

FN is the major component of extracellular matrix that controls many facets of cell physiology via integrin signaling [21]. It is widely accepted as an excellent coating for growing cells, and therefore we used it as a scaffold for site-specific conjugation with scVEGF. First, FN was derivatized with a bi-functional cross-linking reagent Sulfo-LC-SPDP on lysine ε-amino groups. Under conditions of reducing SDS-PAGE, unmodified FN migrates as a major band with an apparent molecular weight of 250 kDa and a minor ~120-kDa band, as visualized by Western blotting with anti-FN monoclonal antibody (Fig. 2a). Surprisingly, after 10 min of modification with sulfo-LC-SPDP, FN bands disappeared from the Western blot (Fig. 2a). Samples taken at 0, 10, 20 and 30 min of FN-SPDP modification showed the same protein content by microBCA analysis (data not shown), indicating that the absence of FN signal was not due to protein degradation, but rather due to conjugation of SPDP, which, most likely, destroyed a recognition epitope of the monoclonal antibody. Therefore, in order to quantitate derivatized FN, we tagged it with FITC (approximately one FITC per FN molecule).

3.2. Conjugation of scVEGF to FN

We have recently developed several versions of Cys-tagged VEGF for site-specific conjugation of therapeutic and diagnostic payloads [12–14]. For this work we developed scVEGF with a single N-terminal Cys-tag (Fig. 1b). We reasoned that scVEGF immobilized on the surface via a single Cys-tag would be more exposed to interactions with cellular receptors than dimeric VEGF anchored via both Cys-tags, since dimeric VEGF has a head-to-tail conformation that would lead to only partial exposure from the surface.

As reported elsewhere, the C4 thiol group of Cys-tag forms mixed disulfides with glutathione during red-ox refolding of bacterially expressed Cys-tagged VEGF, therefore, prior to conjugation to FN it was "deprotected" under mild reducing conditions that do not affect intramolecular disulfide bonds [12,13]. FN-scVEGF conjugate was eluted from Gelatin–Agarose with 8 M urea, while free scVEGF was found exclusively in the flow-through fraction (Fig. 2b). The absence of FN in the flow-though fraction was confirmed by FITC fluorescence that

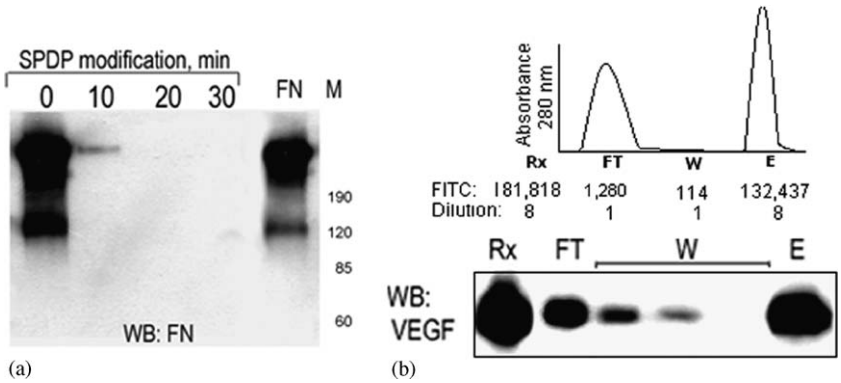

Fig. 2. Conjugation of scVEGF to FN. Reducing SDS-PAGE was done on 4–20% gradient gels. M, pre-stained molecular weight markers. (a) Aliquots of FN-SPDP modification reaction were taken at indicated time points and analyzed by Western blotting with anti-FN antibody. FN, unmodified FN. (b) Separation of free and FN-conjugated scVEGF on Gelatin–Agarose (upper panel). FT, flow-through fraction; W, wash; E, elution with 8 M urea. FITC fluorescence was measured after appropriate dilution of the fractions. Rx, conjugation reaction mixture. Samples were separated on 17.5% gels and analyzed by Western blotting with anti-VEGF antibody (lower panel).

was observed only in the initial conjugation mixture and in the urea-eluted fraction, while the presence of scVEGF in both flow-through and elution fractions was confirmed by Western blotting (Fig. 2b).

3.3. Functional activity of conjugated scVEGF

Functional activity of scVEGF moiety in FN-scVEGF conjugate was tested by its ability to induce VEGFR-2 tyrosine autophosphorylation in 293/KDR cells [19]. In these cells, VEGF induces readily detectable VEGFR-2 tyrosine phosphorylation, which can be inhibited in a dose-dependent manner by Nx, a low-molecular weight inhibitor of VEGFR-2 tyrosine kinase activity (Fig. 3). We found that FN-scVEGF was somewhat less active than equimolar amounts of free scVEGF or dimeric human $VEGF_{165}$ at the lowest tested concentration (Fig. 3). However, activity of FN-scVEGF was similar to that of both control proteins at higher concentrations, indicating that cross-linking to FN and harsh conditions of conjugate purification did not significantly affect the ability of scVEGF to interact with and activate cellular VEGFR-2.

3.4. Immobilization of scVEGF

FN is attached to the tissue plastic surfaces by passive absorption, and is widely used as a substrate for growing cells. Therefore, to explore the effects of immobilized scVEGF, we simply coated tissue culture plates with serially diluted FN-scVEGF. Western blot analysis (Fig. 4a) demonstrated that only 10–20% of FN-scVEGF was immobilized in the wells at every point in the selected serial dilution range. The absence of saturation (Fig. 4a, DTT-released) provided an opportunity to explore dose-dependent effects of immobilized scVEGF. As shown on Fig. 4b, the amount of scVEGF released from duplicate

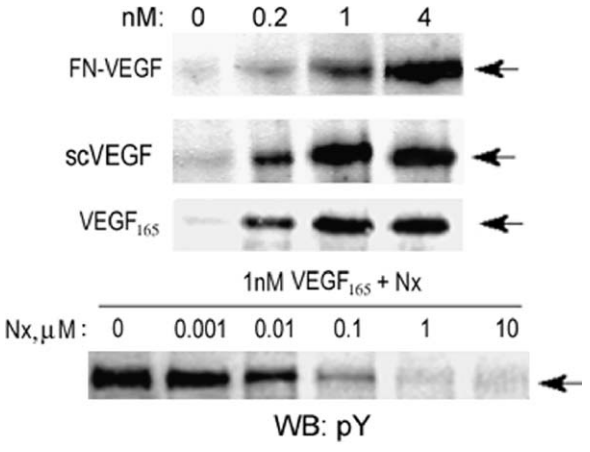

Fig. 3. scVEGF conjugated to FN activates cellular VEGFR-2. Near confluent 293/KDR cells after overnight starvation were stimulated with varying amounts of FN-conjugated or free scVEGF, or control $VEGF_{165}$ for 10 min at 37 °C; then lyzed and analyzed by Western blotting using anti-phosphotyrosine antibody. Nx tyrosine kinase inhibitor was tested on cells stimulated with 1 nM $VEGF_{165}$.

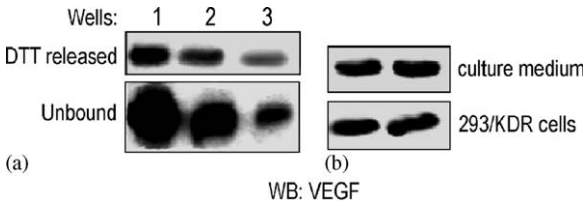

Fig. 4. Analysis of immobilized scVEGF before and after 293/KDR cell growth. (a) Serial 1:2 dilutions of FN-scVEGF in PBS were added to wells of a 96-well plate, 100 μl/well. After an 18-h incubation at 4 °C, DTT-released scVEGF from 3 consecutive wells, and unbound FN-scVEGF collected from the same wells, were separated on 17.5% gels and analyzed by Western blotting using anti-VEGF antibody. (b) Complete culture medium (100 μl/well) or 293/KDR cells (1000 cells/100 μl/well) were added to duplicate FN-scVEGF coated wells and incubated under normal culture conditions for 96 h. Then medium was aspirated, and DTT-released scVEGF was analyzed by Western blotting.

FN-scVEGF coated wells after 96-h cell growth was close to that from wells exposed to medium alone (compare upper and lower panel), and also to scVEGF released from wells prior to cell seeding (compare with Fig. 4a, well 1). No detectable scVEGF was released from FN-c coated wells incubated in the presence of soluble scVEGF (data not shown), indicating that only covalent attachment can stably immobilize scVEGF on FN.

3.5. Functional activity of immobilized scVEGF

Endothelial cell migration is enhanced by either VEGF or FN alone, as reported for human microvascular endothelial cells (HMVECs), and a combination of VEGF and FN increases migration by 2.5-fold over VEGF alone [22]. Therefore, to test functional activity of immobilized scVEGF, we first examined how it affects a pattern of cell growth that would reflect changes in cell motility. PAE/KDR grown on FN-c formed colonies, whose density was notably decreased in the presence of soluble scVEGF (Fig. 5a). This effect was not observed in the presence of Nx, a VEGFR-2 inhibitor, indicating a causative role for VEGFR-2 signaling in growth pattern alterations. We found that PAE/KDR grown on FN-scVEGF formed low-density colonies, similar to those formed in the presence of soluble scVEGF, suggesting that immobilized scVEGF retained its ability to affect cells through VEGFR-2 signaling. Indeed, Nx abrogated the effects of immobilized scVEGF, restoring the cells to dense colonies similar to those grown on FN-c (Fig. 5a).

Effects of FN-immobilized scVEGF were further analyzed using 293/KDR cells expressing an order of magnitude more VEGFR-2 molecules per cell compared to the PAE/KDR cells. Similar to the PAE/KDR cells, the 293/KDR cells formed cell–cell adherent colonies when seeded on FN-c coated wells (Fig. 5b). However, in the presence of soluble scVEGF, 293/KDR grew mostly as well-separated cells, suggesting that scVEGF-induced activation of VEGFR-2 resulted in increased cell motility and/or decreased cell–cell interactions. Again, in the

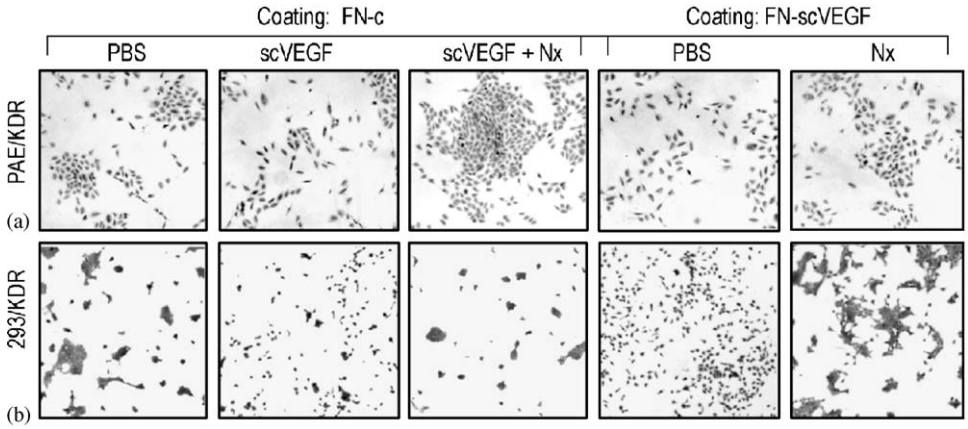

Fig. 5. Surface immobilized scVEGF changes cell growth pattern of VEGFR-2 expressing cells. (a) PAE/KDR cells, 5000 cells/well or (b) 293/KDR cells, 7500 cell/well, were plated on 24-well plates coated with FN-scVEGF or equivalent amounts of FN-c. Free scVEGF or Nx were added to control wells 3 h later to final concentrations of 10 nM and 1 μM, respectively. After 48 h of growth under normal culture conditions, cells were washed with PBS, fixed and stained with Giemsa stain.

presence of Nx, the pattern of growth reverted to tight colonies. We found that immobilized scVEGF induced similar, but somewhat more pronounced alterations in the pattern of growth of 293/KDR cells: cells grown on FN-scVEGF did not form colonies at all, while large cell–cell adherent colonies were formed in the presence of Nx (Fig. 5b).

PAE/KDR cells displayed the same ~15% growth stimulation when grown on either FN-scVEGF or FN-c control wells; and free scVEGF added to FN-c coated wells in the range of 0.1–120 fmoles/well did not promote further growth stimulation (data not shown). Since 293/KDR cells appeared to be more responsive to scVEGF (Fig. 5, compare panels a and b), we used them to quantitatively assess the effects of immobilized scVEGF on cell growth.

As shown in Fig. 6a, 293/KDR growth on FN-c (black squares) was not affected until the FN content for the well coating solution reached 0.4 pmol. In contrast, both FN-scVEGF and a combination of FN-c plus free scVEGF enhanced 293/KDR growth in a dose-dependent manner, ranging from 0.02 to 0.2 pmoles of FN used for coating (Fig. 5b, filled and open circles). Interestingly, higher doses of either FN-scVEGF or a combination of FN-c plus free scVEGF inhibited cell growth, suggesting antagonistic relationships between VEGF and FN signaling in this range (Fig. 6a). Importantly, these effects were observed only with 293/KDR, but not with parental HEK293 cells grown on FN-scVEGF coated plates, indicating their dependence on VEGFR-2 expression (data not shown).

Somewhat unexpectedly, 293/KDR cells grown on FN-scVEGF responded differently to the addition of Nx as compared to cells grown on FN-c and supplemented with free scVEGF (Fig. 6b). While the effects of free scVEGF were completely blocked with Nx treatment, the dose-dependent growth stimulation provided by immobilized scVEGF was not affected and, in fact, persisted through the full range of Nx dosing (compare Fig. 6a and b, filled circles). Although mechanistic analysis of these effects requires further experimentation, these results indicate subtle differences between the biological activity of immobilized and free scVEGF.

4. Discussion

We describe here a new technology for site-specific covalent immobilization of recombinant proteins, using immobilization of scVEGF as an example. It has been recently reported that FN promoted VEGF-induced differentiation of endothelial progenitor cells into endothelial cells [23]. Based on this natural synergism of FN and VEGF, we have chosen FN as a platform for covalent immobilization of VEGF.

Our technology for site-specific protein immobilization is based on genetic fusion of the protein of interest to a 15-aa long Cys-tag containing a cysteine in position 4, whose thiol group is utilized for site-specific modification. We have recently reported the use of Cys-tag for attachment of therapeutic and diagnostic payloads to several recombinant proteins [12–14]. We found that neither N- nor C-terminally fused Cys-tag interfere with functional activities of the fusion proteins; and, in addition, it was equally compatible with proteins expressed in a soluble form or recovered from inclusion bodies [12–14]. In part, this may be due to the fact that Cys-tag is an R4C mutant of the N-terminal fragment of human RNase I, which has a strong tendency to form α-helical structure [24] that might shield the C4 thiol group from interacting with native cysteines or free thiols.

As shown in this report, Cys-tagged scVEGF can be readily cross-linked to soluble FN via a commonly available bi-functional cross-linking agent. The VEGF moiety retains functional activity both as a soluble FN-scVEGF conjugate and after its deposition onto tissue culture plastic. In this respect, our results are in agreement with the report from Zisch et al. [15] that $VEGF_{121}$

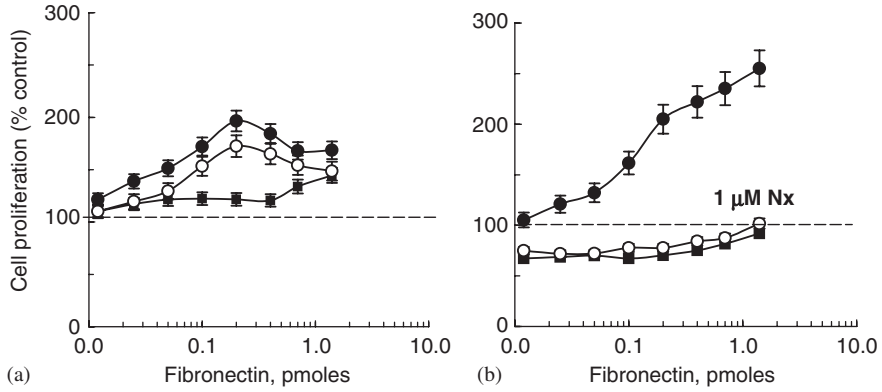

Fig. 6. Surface immobilized scVEGF stimulate 293/KDR cell growth. (a) 293/KDR cells were plated on duplicate FN-scVEGF or FN-c coated wells, 1000 cells/well. Free scVEGF was serially diluted and added to FN-c coated wells 3 h later, at a range matching bound scVEGF in FN-scVEGF coated wells. Well coating: (●) FN-scVEGF; (■) FN-c; (O) FN-c plus free scVEGF. After 96-h growth under normal culture conditions, cells were quantitated using CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega). Control cell growth on un-coated wells was taken as 100%. (b) Experiment described under (a) was done in the presence of Nx added to wells 3 h after cell plating to a final concentration of 1 μM.

covalently immobilized in fibrin matrices retained growth stimulation of human umbilical vein endothelial cells.

Interestingly, native VEGF is internalized upon binding to its cognate receptors [25], and there are suggestions that some of its functions might require internalization [26]. However, our data, as well as results from Zisch et al. [15], indicate that VEGF receptors stimulated by immobilized VEGF are fully capable of activating signal transduction pathways resulting in significant changes in cell behavior. Considering that the use of soluble or non-covalently immobilized VEGF for therapeutic applications is severely limited by potential health risks, rapid diffusion, and short in vivo half-life, we expect that covalent immobilization of VEGF on vascular grafts and other tissue scaffolds could provide significant therapeutic advantages. For example, site-specific immobilization of scVEGF might be useful in designing of small-caliber synthetic vascular graft suitable for effective endothelialization. Development of such grafts was described as "a search for the Holy Grail" [27], and it is possible that the presence of immobilized VEGF would stimulate colonization by host endothelial and/or endothelial precursor cells. The same logic might be applicable to other immobilized growth factors and prostetic devices in need of cellular development. Another important application of immobilized functionally active proteins would be development of scaffolds for maintenance of undifferentiated stem cells. One can envision scaffolds with several immobilized ligands for stem cells receptors, whose collective activity is responsible for maintenance of the pluripotent state.

In general, experience with various affinity tags that are used for protein detection, purification and non-covalent protein immobilization [28,29] indicates that functionality of the majority of proteins are not affected by fusion tags. We therefore expect that Cys-tag will be a useful tool for facile site-specific covalent immobilization for a wide variety of recombinant proteins.

5. Conclusion

In conclusion, we developed a new approach to uniform surface immobilization of recombinant proteins, which is based on expression of proteins with Cys-tag containing a unique cysteine residue for site-specific conjugation. Specifically, we conjugated Cys-tagged scVEGF to FN, coated tissue culture plates with FN-scVEGF, and validate functional activity of surface immobilized scVEGF in tissue culture experiments. VEGFR-2 expressing cells grown on plates coated with FN-scVEGF displayed pattern of growth similar to that observed for cells grown on FN in the presence of equivalent amounts of free scVEGF. VEGF receptor tyrosine kinase inhibitor Nx blocked the effects of immobilized scVEGF, confirming a VEGFR-2 mediated mechanism of the observed effects. Thus, site-specific immobilization via Cys-tag provides a facile and reliable method for functionalizing tissue engineering scaffolds and other biomedical surfaces.

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