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Shiga-like toxin-VEGF fusion proteins are selectively cytotoxic to endothelial cells overexpressing VEGFR-2

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Abstract

Growing endothelial cells at sites of angiogenesis may be more sensitive than quiescent endothelial cells to toxin-VEGF fusion proteins, because they express higher numbers of VEGF receptors. We have constructed, expressed and purified a protein containing the catalytic A-subunit of Shiga-like toxin I fused to $VEGF_{121}$ (SLT-VEGF/L). SLT-VEGF/L inhibits protein synthesis in a cell-free translation system and induces VEGFR-2 tyrosine autophosphorylation in cells overexpressing VEGFR-2 indicating that both SLT and VEGF moieties are properly folded in the fusion protein. SLT-VEGF/L selectively inhibits growth of porcine endothelial cells expressing $2–3\times10^5$ VEGFR-2/cell with an IC_{50} of 0.1 nM, and rapidly induces apoptosis at concentrations >1 nM. Similar results are observed with human transformed embryonic kidney cells, 293, engineered to express 2.5×10^6 VEGFR-2/cell. In contrast, SLT-VEGF/L does not affect three different types of endothelial cells (PAE/KDR_{low}, HUVE, MS1) expressing between 5×10^3 and 5×10^4 VEGFR-2/cell, and quiescent endothelial cells overexpressing VEGFR-2. Growth inhibition and induction of apoptosis by SLT-VEGF/L require intrinsic *N*-glycosidase activity of the SLT moiety, but occur without significant inhibition of protein synthesis. The selective cytotoxicity of SLT-VEGF proteins against growing endothelial cells overexpressing VEGFR-2 suggests that they may be useful in targeting similar cells at sites of angiogenesis.

Keywords: Angiogenesis; Endothelial cells; VEGF; Shiga-like toxin; Fusion protein

1. Introduction

Angiogenesis is a tightly controlled process of growing new blood vessels (reviewed in Ref. [1]). During angiogenesis endothelial cells of existing blood vessels undergo a complex process of reshaping, migration, growth, and organization into new vessels. Under normal circumstances, angiogenesis occurs only during embryonic development, development of the corpus luteum, and during muscle or weight gain. However, angiogenesis occurs in a large number of pathologies, such as solid tumor growth, various eye diseases, wound healing, chronic inflammatory states, and ischemic injuries (reviewed in Ref. [2]). Thus, growing endothelial cells present unique targets for treatment of several major pathologies. It is widely believed that inhibitors of angiogenesis may be effective therapeutics for these pathologies and a number of compounds targeting angiogenesis are currently at various stages of development.

Several positive and negative regulators control

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the process of angiogenesis. The crucial positive regulator of angiogenesis is vascular endothelial growth factor (VEGF) also known as vascular permeability factor (reviewed in Ref. [3]). The action of VEGF on endothelial cells is mediated by tyrosine kinase Flt-1 and KDR/Flk-1 receptors, also known as VEGFR-1 and VEGFR-2, with the latter playing the dominant role in angiogenesis (reviewed in Refs. [4,5]). These receptors are preferentially expressed on endothelial cells and there is evidence that endothelial cells at the sites of angiogenesis express significantly higher numbers of VEGFR-2 than quiescent endothelial cells [6–10].

Since VEGF binds specifically to endothelial cells, this growth factor provides a unique vehicle for targeted drug delivery to the sites of angiogenesis. Indeed, it has been shown that conjugation or fusion of the catalytically active fragments of diphtheria toxin (DT) to VEGF yielded proteins that inhibited growth of endothelial cells and displayed anti-angiogenic activity in in vivo models of angiogenesis [11–13]. In order to inhibit angiogenesis without damaging quiescent endothelium, toxin-VEGF fusion proteins should selectively target endothelial cells at sites of angiogenesis that overexpress VEGFR-2. We report here preliminary evidence that toxin-VEGF fusion proteins containing the catalytically active A-subunit of Shiga-like toxin 1 (SLT-1) may possess such selectivity.

SLT-1 was chosen as a potential 'natural killer' of endothelial cells. Indeed, damage to endothelial cells caused by SLT-1 plays a causative role in the pathogenesis of hemorrhagic colitis and hemolytic uremic syndrome induced by *E. coli* O157:H7 [14–17]. SLT-1 is composed of a single copy of a 32-kDa A-subunit associated with a ring shaped pentamer of 7-kDa cell-binding B-subunits. The A-subunit is a site-specific *N*-glycosidase that cleaves off A_{4324} in the 5′ terminus of 28S rRNA [18,19]. Depurination of A_{4324} inhibits binding of the elongation factor EF-2/aminocyl-tRNA complex to ribosomes, resulting in the inhibition of protein synthesis and initiation of apoptosis. Fusion proteins containing the full length A-subunit as well as various truncated A-subunits fused to the N-terminus of CD4 retain *N*-glycosidase activity and are cytotoxic for cells expressing the HIV-1 gp120/gp41 complex [20–22].

2. Materials and methods

2.1. SLT-VEGF fusion proteins

SLT-VEGF fusion proteins were constructed, expressed in *E. coli* strain Origami(DE3)pLysS (Novagen) and purified as described elsewhere (Backer and Backer, submitted).

2.2. Cells

Porcine aortic endothelial (PAE) cells and 293 human primary embryonic kidney cells were obtained from American Type Culture Collection (Rockville, MD). PAE cells expressing $2–3\times10^5$ VEGFR-2 per cell (PAE/KDR), PAE cells transfected with the empty vector (PAE/V), human umbilical vein endothelial (HUVE) cells and MS1 mouse endothelial cells were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, New York). 293/KDR cells expressing 2.5×10^6 VEGFR-2/cell, and PAE/KDR_{low} cells expressing $\sim5\times10^3$ VEGFR-2/cell were constructed from parental 293 and PAE cells as described elsewhere (Backer and Backer, submitted). All cells were grown at 37°C with 5% CO_2. Low passage number HUVE cells (3rd–7th passages) were grown in gelatin-coated flasks in DMEM with 20% FBS, 50 ng/ml basic fibroblast growth factor, 100 μg/ml heparin, 2 mM L-glutamine and antibiotics. MS1 cells were grown in DMEM with 5% FBS, 4 mM L-glutamine and antibiotics. All other cells were grown in DMEM with 10% FBS, 2 mM L-glutamine and antibiotics.

2.3. VEGFR-2 autophosphorylation and Western blot analysis

VEGFR-2 autophosphorylation in PAE/KDR cells was assayed as described [23]. For Western blot analysis, cell lysates were separated by SDS–PAGE on 7.5% gels and transferred to nitrocellulose membranes (Life Technologies). After incubation with primary antibodies against phosphotyrosine (RC20H, PharMingen, San Diego, CA) or α-fodrin (Chemicon), immune complexes were visualized by the *femto*Lucent Chemiluminescence System (Genotechnology).

2.4. Cytotoxicity assay

Cells were plated in 24-well plates at densities of $5–10\times10^3$ cells/well. Twenty hours later various amounts of SLT-VEGF fusion proteins in fresh culture medium were added to duplicate wells, 1 ml/well. After 72-h treatments, cells were detached with trypsin–EDTA and counted in a Coulter Counter.

2.5. Caspase-6 assay

Caspase-6 activity was assayed with a colorimetric substrate according to the manufacturer's instructions (Calbiochem).

2.6. Naked DNA delivery in rat model

A 4-cm long abdominal midline excision was performed in 150–200-g adult Sprague–Dawley rats anesthetized with 80 mg/kg ketamine and 40 mg/kg xylazine. Microvessel clips (Edward Weck, Research Triangle Park, NC) were placed on external iliac, caudal epigastric, internal iliac, and deferent duct arteries and veins to block both outflow and inflow of the blood to the rat leg. A 27-g butterfly needle was inserted into the external iliac artery and 5 ml of a 10-nM SLT-VEGF/L solution in 0.15 M NaCl was injected within 30 s. The animals were exposed to SLT-VEGF for 30 min, following clip removal and suturing the wound. Twenty-four hours later the same surgery was performed and 475 μg of pCILux (Promega) in 9.5 ml of 0.15 M NaCl were injected unilaterally into the iliac artery within 30 s. Two days after injection luciferase activity was measured in six hindlimb muscle groups (anterior upper leg, medial upper leg, posterior upper leg, anterior lower leg, posterior lower leg and foot).

3. Results and discussion

3.1. SLT-VEGF proteins

Three SLT-VEGF fusion proteins containing $VEGF_{121}$ isoform (Fig. 1A) were constructed, expressed and purified as described elsewhere (Backer and Backer, submitted). The $VEGF_{121}$ isoform was chosen for construction of these proteins because it has a selective affinity for VEGFR-2 [24]. Full-length and truncated SLT-1 A-subunits were used to provide fusion proteins with different *N*-glycosidase activities [20–22]. A double mutant (Y114S and R170L) catalytically inactive A-subunit [25,26] was constructed and fused to VEGF as a control for possible non-catalytic effects of SLT-VEGF.

In a cell-free translation system programmed with a luciferase mRNA, SLT-VEGF/L and SLT-VEGF/S, but not SLT-VEGF/Lci or $VEGF_{121}$, inhibited protein synthesis (Table 1). All SLT-VEGF fusion proteins induced VEGFR-2 tyrosine autophosphorylation in PAE/KDR cells engineered to overexpress VEGFR-2 (see, Fig. 1B for VEGF-SLT/L and VEGF-SLT/S). These results indicated that both SLT and VEGF moieties are folded into functionally active conformations in the fusion proteins. Native VEGF proteins are homodimers with an anti-parallel arrangement of subunits whose N-termini are oriented outward in mature proteins and in VEGF-receptor complexes [27]. The ability of SLT-VEGF proteins to induce VEGFR-2 tyrosine autophosphorylation indicates that this three-dimensional structure can accommodate as long as 329 aa N-terminal extension without significant disruptions.

3.2. SLT-VEGF/L enhances transendothelium DNA delivery in animal model

To test whether SLT-VEGF/L may target endothelial cells in vivo, we have employed a procedure developed for delivery of naked DNA to muscle cells from blood stream. According to current models, naked DNA extravasates from blood stream into surrounding tissue through pores in endothelium [28]. We reasoned that even minor damage to endothelium might enlarge these pores and enhance DNA extravasation. Preliminary experiments in a rat model indicated that injection of 10 nM SLT-VEGF/L solution for 30 min into clamped iliac artery provided a small but significant enhancement in DNA delivery to hindlimb muscle, when naked DNA was injected into the same artery 24 h later (Fig. 2).

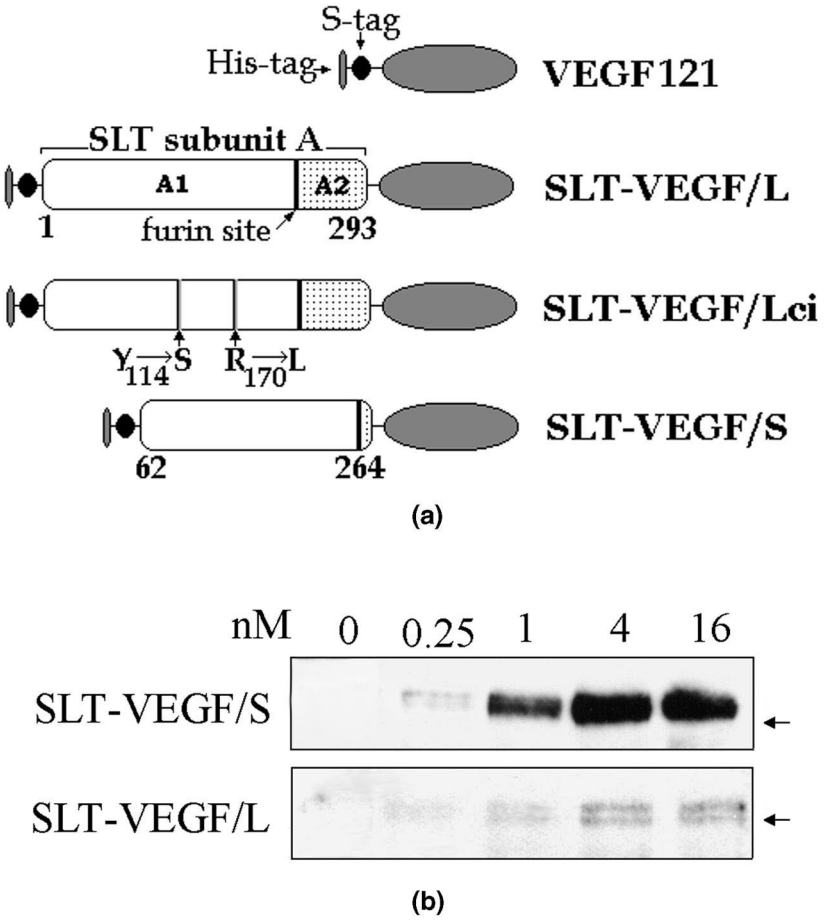

Fig. 1. VEGF121 and SLT-VEGF fusion proteins. (A) Schematic representation of the proteins. (B) Dose-dependence of VEGFR-2 tyrosine autophosphorylation in PAE/KDR cells induced by SLT-VEGF fusion proteins. Positions of a 205-kDa marker are indicated by arrows.

Table 1
SLT-VEGF fusion proteins inhibit in vitro translation

Protein	Inhibition of translation IC_{50} (nM)
SLT-VEGF/L	0.02
SLT-VEGF/S	1
SLT-VEGF/L_{ci}	nd[a]
$VEGF_{121}$	nd

Effects of SLT-VEGF fusion proteins on luciferase production in an in vitro translation system containing a rabbit reticulocyte lysate programmed with luciferase mRNA. Luciferase activity in the presence of 1 μM recombinant VEGF121 was taken as 100%.
[a] nd, not detected.

3.3. SLT-VEGF/L is selectively cytotoxic to endothelial cells overexpressing VEGFR-2

We have tested the effects of SLT-VEGF/L on several endothelial cell lines expressing different numbers of VEGFR-2 per cell. In addition, we have used 293/KDR cells overexpressing VEGFR-2 and MDA-MB-435 human breast carcinoma cells that do not express VEGFR-2. In dose-dependence experiments we found that SLT-VEGF/L inhibits growth of cells that express 2.5×10^5 to 2.5×10^6 VEGFR-2/cell with IC_{50} values of 100–150 pM (Table 2). In contrast, SLT-VEGF/L did not affect cells that express from none to $3–5\times10^4$ VEGFR-2/cell, even

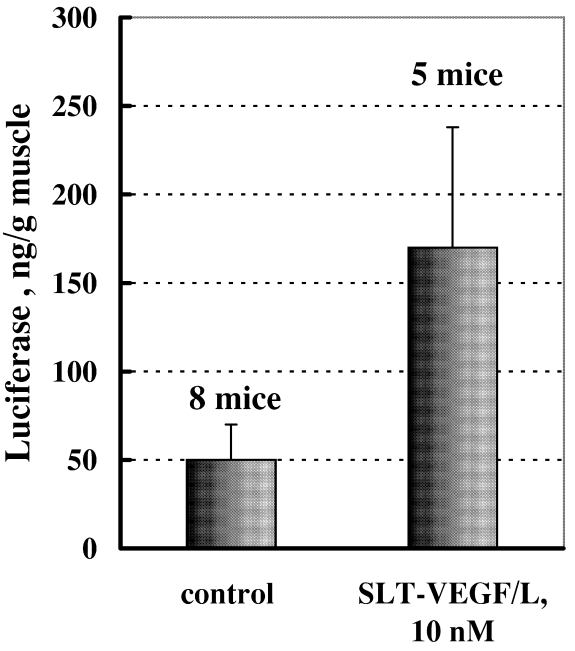

Fig. 2. Pretreatment with SLT-VEGF/L enhances naked DNA delivery across endothelium into rat hindlimb muscles Animals were treated as described in Section 2. Luciferase activity was measured as described before [28] and expressed as ng luciferase per g of muscle tissue. Additional experiments indicated that recombinant VEGF does not affect DNA delivery in this experimental model. Number of experimental animals per group indicated above bars.

at concentrations as high as 20 nM (Table 2). SLT-VEGF/S was less inhibitory to 293/KDR cells than SLT-VEGF/L, while SLT-VEGF/Lci did not affect PAE/KDR and 293/KDR cells (Backer and Backer, submitted). At concentrations ≥1 nM, SLT-VEGF/L was visibly cytotoxic to PAE/KDR as evidenced by a decrease in cell number below that initially plated (Fig. 3A). Interestingly, PAE/KDR cells that survived SLT-VEGF/L exposure to SLT-VEGF/L grew as rapidly as control cells (Fig. 3A). Activation of caspase-6 (Fig. 3B), DNA fragmentation, and cleavage of 240-kDa α-fodrin into 150- and 120-kDa fragments (Backer and Backer, submitted) were observed after 12–48-h exposures of PAE/KDR cells to SLT-VEGF/L.

The selective cytotoxicity of SLT-VEGF/L against endothelial cells overexpressing VEGFR-2 suggests that this protein might display lower side effects in vivo than other toxin-VEGF constructs. Indeed, unlike DT-VEGF fusion protein [13], SLT-VEGF/L does not affect endothelial cells with the lower number of VEGFR-2. This selectivity may be crucial since only endothelial cells at the sites of active angiogenesis overexpress VEGFR-2 [6–10], while quiescent endothelial cells express relatively low number of VEGFR-2. We expect that the ongoing in vivo experiments will establish whether SLT-VEGF/L can be used either alone or in adjunct therapy of angiogenesis-dependent pathologies.

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Table 2
SLT-VEGF/L is selectively cytotoxic for cells overexpressing VEGFR-2

Cells	Cell origin	VEGFR-2/cell	Growth inhibition IC_{50} (pM)
PAE/KDR	Porcine endothelial	~2.5×10^5	~100
293/KDR	Human embryonic kidney	~2.5×10^6	~150
HUVE	Human endothelial	$3–5\times10^4$	nd[a]
MSI	Mouse endothelial	$3–5\times10^4$	nd
PAE/KDR-low	Porcine endothelial	~5×10^3	nd
MDA-MB-435	Human breast carcinoma	nd	nd

Cells were plated onto 24-well plates at densities of $5–10\times10^3$ cells/well and exposed to various concentrations of SLT-VEGF/L 20 h later. After a 72-h exposure to SLT-VEGF/L cells were counted in a Coulter Counter.

[a] nd, not detected.

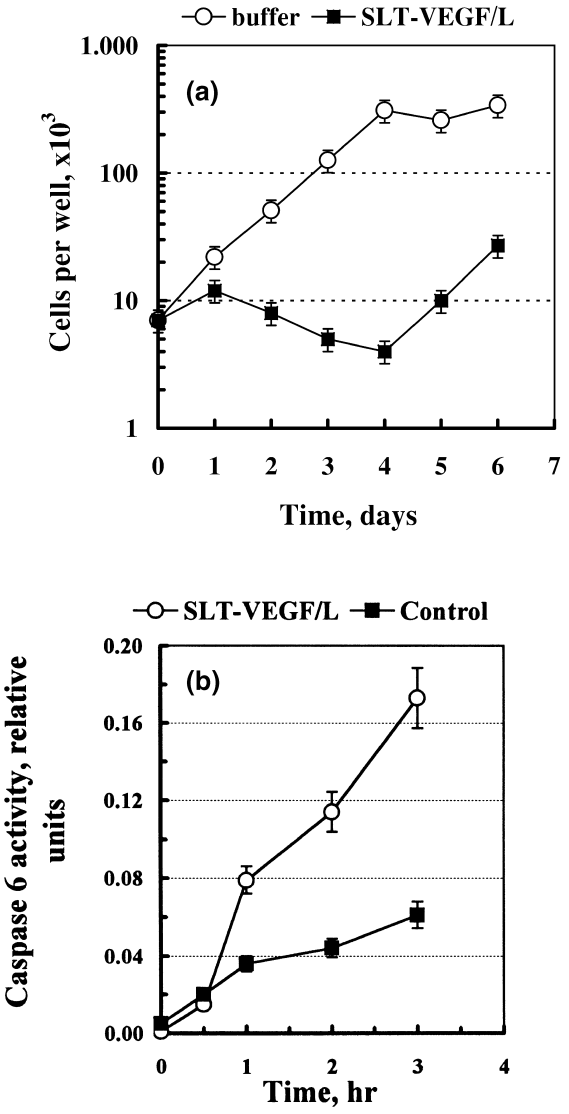

Fig. 3. SLT-VEGF/L inhibits growth and induces apoptosis in PAE/KDR cells. (A) Time-course of SLT-VEGF/L action. PAE/KDR cells were plated onto 24-well plates at a density of 7×10^3 cells/well. Twenty hours later cells were exposed to 1 nM SLT-VEGF/L or control buffer, and counted at indicated time points. (B) SLT-VEGF/L treatment activates caspase-6 in PAE/KDR cells. A total of 400 000 PAE/KDR cells was exposed to 5 nM SLT-VEGF/L for 48 h. Cells were lysed, solutions containing 160 μg of cleared lysates prepared from SLT-VEGF/L treated or control cells were mixed with calorimetric substrate specific for caspase-6 (Calbiochem), and assayed according to the manufacturer's instructions.

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