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Research Article

Expression of Shiga-Like Toxin Fused to Vascular Endothelial Growth Factor (VEGF/SLT) in *E. coli* for Targeting Angiogenesis

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Abstract

Angiogenesis is a highly controlled process of growing new blood vessels under normal circumstances. However, in a large number of pathologies, such as solid tumor growth, angiogenesis is a crucial component of the disease process. Therefore, inhibitors of angiogenesis are being investigated as potential therapeutics for tumor growth. During angiogenesis endothelial cells of existing blood vessels undergo a complex process of reshaping, migration, growth and organizing into new vessels. Vascular Endothelial Growth Factor (VEGF) is a central mediator of this process and acts via receptors whose expression is restricted almost exclusively to endothelial cells. Because of its selectivity, VEGF represents a unique vehicle for delivery inhibitors of angiogenesis to endothelial cells. Among potential inhibitors of angiogenesis, the Shiga-like toxin-1 (SLT-I) produced by *E. coli* O157:H7 has the advantage that endothelial cells appear to be particularly sensitive to its action. The hypothesis that combining a SLT-I toxin with VEGF as a delivery vehicle would serve as a highly selective and active inhibitor of angiogenesis. To this end, fusion proteins containing $VEGF_{121}$ and two forms of Shiga-like toxin-I (SLT-I) were developed and tested *in vitro* for activities that have the potential to inhibit angiogenesis *in vivo.* Plasmids encoding the fusion proteins $VEGF_{121}/A_1$ containing the catalytically active A_1 fragment of the SLT-1 A-subunit and $VEGF_{121}$/A containing the full length A-subunit of SLT-1 were constructed in plasmids pET-29a and pET-32a systems. *Escherichia coli* BL21 (DE3) pLysS bacteria were transformed with the plasmid constructs for the expression of these two fusion proteins. Both purified fusion proteins inhibited the translation of luciferase mRNA as a reporter gene *in vitro* translation system, indicating that both fusion proteins retain the *N*-glycosidase activity of SLT-I. However, only $VEGF_{121}/A_1$ fusion proteins displayed the ability to induce auto-phosphorylation of the VEGF receptor KDR/FLK-1 and displayed a strong, selective growth inhibition of cultured cells expressing KDR/FLK-1 receptors. These results indicated that VEGF/SLT fusion proteins are promising therapeutic agents that can be developed into powerful and selective inhibitors of angiogenesis.

Keywords: *VEGF/SLT; E. coli; Angiogenesis; Wegner's granuloma; pET-32a/$VEGF_{121}$*

Abbreviation: VEGFs: Vascular Endothelial Growth Factor; VEGFR: Vascular Endothelial Growth Factor and its Receptor; mKDR/Flk-1: Kinase insert domain receptor; SLT-1, Gb_3; furine; pJB144, pET-29a/$VEGF_{121}$; pET-32a/$VEGF_{121}$; S-tag; His tag; Luciferase; HEK-293/KDR

Introduction

Angiogenesis is a multiple process in the formation of new blood vessels from pre-existing blood vessel [1]. It occurs in pathological conditions such as cancer, retinopathies, wegner's granuloma, Takayasu's arthritis, systematic lupus erythematosus and autoimmune diseases conditions. Angiogenesis involves enzymatic degradation of basement membrane of a local venule, chemotactic migration and proliferation of endothelial cells, synthesis of new basement membrane and recruitment of auxiliary cells.

Various growth factors have been identified that promote angiogenesis in vivo. One of these factors that act directly on endothelial cells to induce cell proliferation are vascular endothelial growth factors (VEGFs). Molecular cloning of VEGF cDNA revealed at least four

different molecular isoforms having 121, 165, 189 and 206 amino acids respectively. All four isoforms are encoded by a single gene and generated by alternative splicing from single pre-mRNA [2] as shown in Figure (1).

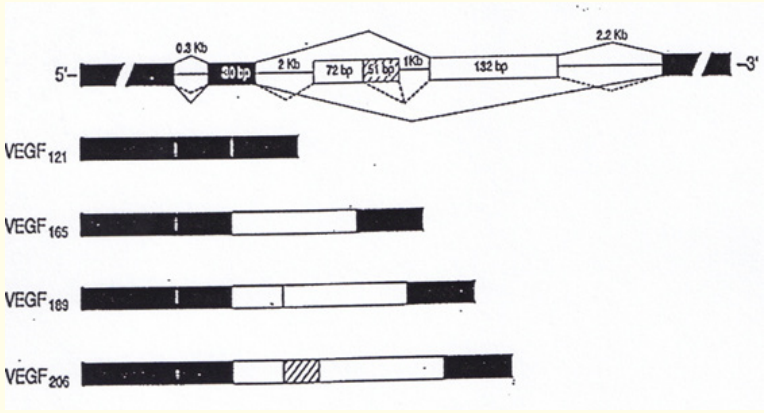


Figure 1: *Vascular Endothelial Growth Factors (VEGFs).*

Isoforms $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ have been detected in the majority of tumor cells and tumor tissues, in contrast $VEGF_{206}$ is a very rare form [3]. The two shorter forms $VEGF_{121}$ and $VEGF_{165}$ are efficiently secreted as free soluble proteins, while the longer forms $VEGF_{189}$ and $VEGF_{206}$ are mostly extracellular matrix associated. $VEGF_{121}$ differs from the other isoforms by the absence of the nonspecific binding of heparin-binding domain and bind to the specific binding of vascular endothelia growth factor receptor (VEGFRs). VEGFRs are a member of the tyrosine kinase family that includes three members of VEGFRs family flt-1 [4,5] and Tie2/Tek [6]. The vascular endothelial growth factor receptor KDR/Flk-1 [7] from a human endothelial cell cDNA library amplified by polymerase chain reaction (PCR) using degenerated oligonucleotide primer demonstrated a complementary to the conserved tyrosine kinase domain flank. The structure of KDR/Flk-1 is a characteristic type II receptor tyrosine kinase [8]. It is a large glycosylated extracellular domain that contains a single hydrophobic transmembrane region and cytoplasmic domain that contains a tyrosine kinase catalytic domain. The kinase domain is interrupted by a hydrophobic insertion sequence of approximately 70 amino acids as shown in Figure (2).

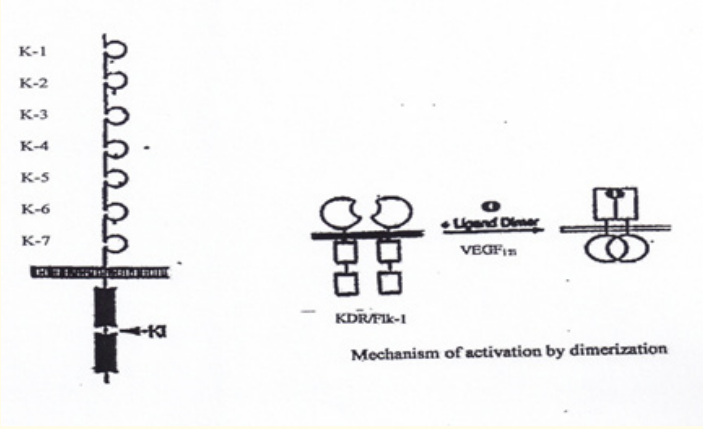


Figure 2: *Structure and Dimerization of the VEGF Receptor (KDR/Flk-1).*

KDR/Flk-1 dimerization and activation is mediated by specific homodimer ligand VEGF. Following VEGF/KDR binding, an array of cel-

lular responses occur which include the stimulation of Na^+/H^+ exchange, Ca^{2+} influx, and activation of phosphatidyl-inositol metabolites and the generation of Diaglycerol, the natural activator of protein kinase C (PKC). Phosphorylation of cellular substrates, together with alteration of ionic content of the cell provides an internal simulation for cell growth. In general, $VEGF_{121}$ is a specific mitogen for endothelial cells and induces endothelial cell migration, formation of tube like structure and vascular permeability as shown in Figure (3). Its binding to KDR/Flk-1 receptor is a major stimulus of angiogenesis [9].

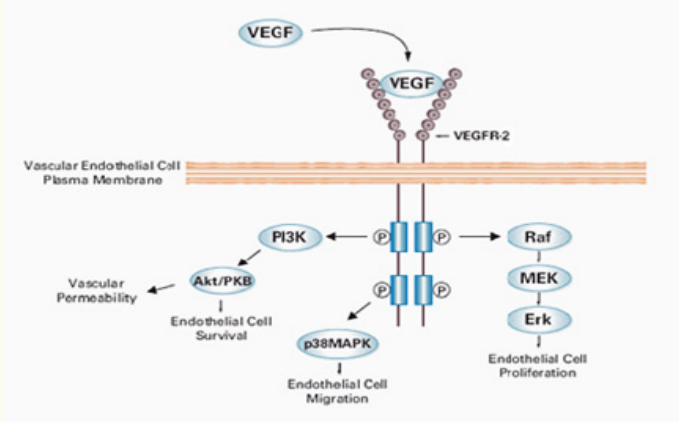


Figure 3: *Activation of the VEGF Receptor (KDR/Flk-1).*

Several strategies to inhibit either the production of angiogenic factors or their action on endothelial cells have been tested to inhibit neovascularization and tumor growth. For example, neutralization of VEGF with polyclonal VEGF antiserum generated against human recombinant $VEGF_{165}$ [10] inhibits new blood vessel growth and as consequence, inhibits tumor growth.

The high expression of VEGF receptor in endothelial cells of tumor vasculature offers selective advantages for targeting cytotoxic reagents to tumor vasculature [11]. Among cytotoxic reagents that are potential inhibitors of angiogenesis is the Shiga-like toxin-I (SLT-I) produced by *E. coli* O157:H7. Since the beginning of the 1980's several studies demonstrated that Shiga-like toxins from entero-hemorrhagic *E. coli* (EHEC) are associated with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The damage of endothelial cells of the glomerulus and kidney arterioles appears to play a pivotal role in the pathogenesis of (HUS). Histopathologic studies of kidney in HUS patients reveal swollen and detached endothelial cells and deposits of fibrin, all with glomerulus.

Shiga-like toxin as shown in Figure (4) is composed of a single 32-kDa A-subunit that associates with 5 receptors-binding 7-kDa B-subunits to form a ring shaped holotoxin [12]. Shiga-like toxin can damage cells after interaction with its cellular receptor globotriosyceramide (GbOse.cer) known by the name Gb_3. This receptor as shown in Figure (5) has been found in human kidney endothelial cells. The B-subunits of SLT's are responsible for binding to Gb_3 receptors on endothelial cells. Bound SLT is endocytosed via the clathrin pit pathway. The A-subunit of SLT contains two cysteines that are linked by disulfide bond. The loop between these two cysteines contains the sequence Arg^{348}-Val-Ala-Arg^{251} in the C-terminal, which is recognized by the membrane-anchored protease fuine. After SLT binds to Gb_3 cell surface receptors, the toxin is endocytosed, form clathrin-coated pits and transported to the *trans*-Golgi network where furine recognized the sequence between the disulfide bond in Shiga-like toxin A-subunit and cleaves it yielding 27.5-kDa A_1 fragment and 4.5-kDa A_2 fragment [13] The released A_1 fragment is a specific enzymatic activity of *N*-glycosidase that cleaves off a single adenine residue in position 4323 from 5' terminus of 28S rRNA of 60 S ribosome subunit. This cleavage inhibits binding of the elongation factor-1 (EF-1)/aminocyl tRNA complex to the ribosome, resulting in the inhibition of protein synthesis and triggers apoptosis in the endothelial cells [14] as shown in Figure (6).

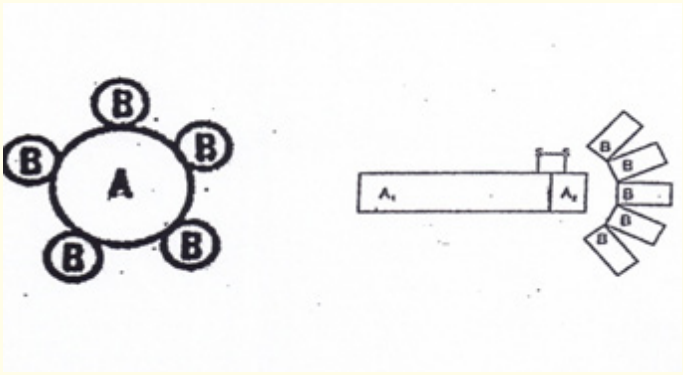


Figure 4: *Structure of Shiga-like Toxin (SLT-I).*

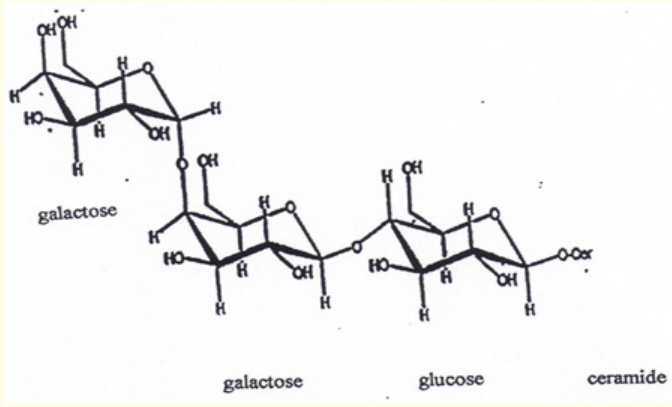


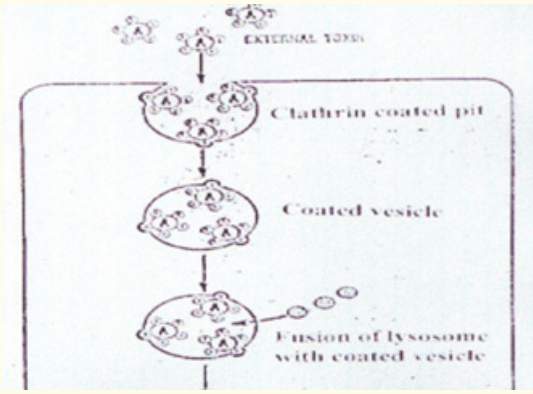
Figure 5: *Chemical Structure of the SLT-I Receptor (Gb_3).*

Since tumor growth is critically dependent on VEGF for angiogenesis and VEGF receptors (KDR/Flk-1) are over-expressed in endothelial cells of tumor vasculature and are almost non-detectable in the vascular endothelial cells adjoining normal tissue. These differential expressions of VEGF receptor offers selective advantage as a target VEGF-coupled cytotoxic such as Shiga-like toxin to inhibit angiogenesis. This is an attractive therapeutic approach for oncological diseases. The strategy adopted in this investigation is to eliminate the 5B-subunits (Gb_3 receptors) of Shiga-like toxin (SLT-1) and fused the A-subunit or A_1 fragment of Shiga-like toxin (SLT-1) to $VEGF_{121}$ to form fusion proteins of $VEGF_{121}$/A and $VEGF_{121}/A_1$ as therapeutic proteins for targeting angiogenesis.

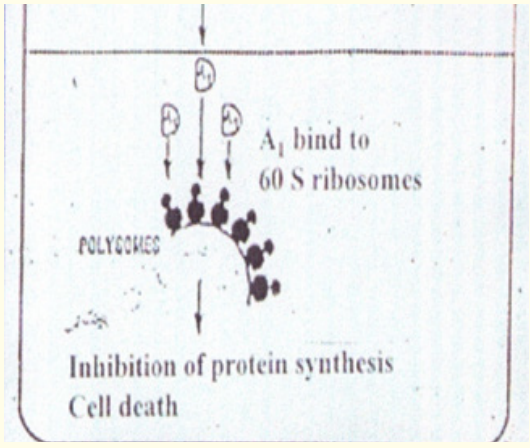
Material and Methods

Plasmids, Bacterial Strains and Growth Media

Plasmid pJB144 as shown in figure (7) encoding the A and B subunits holotoxin of Shiga-like toxin (SLT-1) was a kind gift from Dr. J Burunto, University of Tornato, Canada. PET-29a/$VEGF_{121}$ and pET-32a/$VEGF_{121}$ shown in figure (9) are encoding human $VEGF_{121}$ were constructed by Dr. Marina Backer of Sebtic Inc, U.S (unpublished data). *Escherichia coli* DH5α for constructed plasmids propagation was purchased from Life Technologies, Grand Island NY and *E. coli* BL2 (DE3) pLyS for fusion proteins expression was purchased from Novagen, Madison WI. Both recombinant bacteria strains were grown at 37^0C in Luria-Bertani (LB) supplemented with 30 µg/ml kanamycin and 34 µg/ml chloramphenical for inserted plasmid pET-29a/$VEGF_{21}$ and 100 µg/ml carbenicillin and 34 µg/ml chloramphenicol for inserted plasmid pET-32a/$VEGF_{121}$.



Step 1: *Activation in Early Endosome.*



Step 2: *Inhibition of Protein Synthesis.*

Figure 6: *Mechanism of Shiga-Like Toxin on Endothelial Cells.*

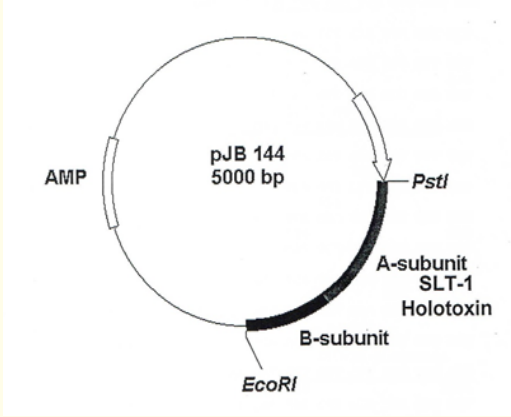


Figure 7: *pJB 144 Plasmid Containing the Holotoxin Gene of Shiga-Like Toxin.*

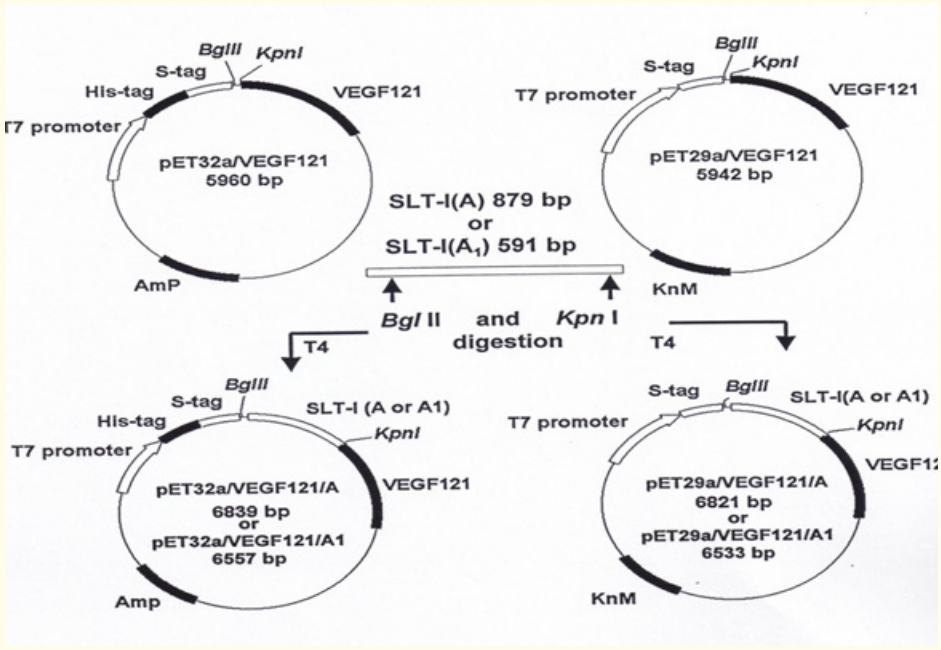


Figure 9: *Construction of $VEGF_{121}$/A, and $VEGF_{121}$/A_1 in pET-29a and pET-32a Host Plasmids.*

Construction of the Recombinants

The plasmid pJB144 was used as a main template for PCR amplifications of Shiga-like toxin DNA sequences encoding the full length of A-subunit and the short length A_1 fragment. Oligonucleotide primers sequence for both A and A_1 including in the 5'prime end restriction site AGATCT for *BgI* II restriction enzyme and the restriction site GGTACC for *Kpn* I restriction enzyme as shown below:

SLT (A) sense: 5'-CCG-AGATCTG-AAG GAA TTT ACC TTA GAC-3'
SLT (A_1) sense: 5'-CCC-AGATCTG-CTA CGG CTT ATT GTT GAA CG-3'
SLT (A and A_1) antisense: 5'-ATA GGTACC-ATC TCG CGG ACA CAT AGA AG-3T

The PCR amplification was performed for 25 cycles at 94^0C for 1 minute 58^0C for 2 minutes and 72^0C for 1 minute. Amplified DNA of A subunit and A_1 fragments of Shiga-like toxin (SLT1) were purified using PCR-DNA purification kit from QLAGEN, Valencia, CA and yielded 0.87kb for the full length A subunit and 0.6 kb for the short fragment A_1.

Following the purification from 1% agarose, gel electrophoresis, amplified DNA of A subunit and A_1 fragment were treated with *kpn* I and *BgI* II restriction enzymes before the insertion into extracted plasmids pET-29a/$VEGF_{121}$ and pET-32/$VEGF_{121}$ from the propagated host *E. coli* DHα cells using DNA kit from QIAGEN, Valencia, CA.

Twenty micrograms of purified host plasmids were digested with 8 units of *kpn* I restriction enzyme in REACT #4 restriction enzyme buffer for 2 hours at 37^0C. After the complete linearization of both plasmids was confirmed by 1% agarose gel electrophoresis, 8 units of *BgI* II in REACT #3 restriction enzyme buffer were added to each reaction mixture and incubated at 37^0C for 2 more hours. Double restricted host plasmids were purified from the 1% gel with gel extraction kit from QIAGEN, Valencia, CA for the insertion and ligation of the amplified A subunit or A_1 fragment DNA of Shiga-like toxin (SLT-I).

Restriction enzymes treated A-subunit, A_1 fragment of SLT-I and host plasmids pET-29a/$VEGF_{121}$ or pET-32-a/$VEGF_{121}$ were mixed at a

molar ratio of 3:1 respectively and ligated with 1 unit of T4 DNA ligase enzyme from Life Technology in 20 µl reaction volumes for overnight at 16^0C. The constructed plasmids pET-29a/$VEGF_{121}$/A, pET-29a/$VEGF_{121}/A_1$, pET-32a/$VEGF_{121}$/A and pET-32/$VEGF_{121}/A_1$ Figure (9) were diluted with TE buffer and 1 µl of each diluted solution was mixed on ice with 100 µl aliquot of component *E. coli* DH5α. After 30 minutes incubation in ice, cells were heated at 42^0C for 90 seconds, diluted with S.O.C media from Life Technologies to final volume of 1 ml and incubated at 37^0C for I hour with shaking.

Transformed *E. coli* DH5α cells with inserted constructed plasmids were placed on LB media containing 30 ug/ml kanamycin for the selection of constructed plasmids pET-29a/$VEGF_{121}$/A and pET-29a/$VEGF_{121}/A_1$ and LB media containing 100 ug/ml carbenicillin for the selection of constructed plasmids pET-32a/$VEGF_{121}$/A and pET-32a/$VEGF_{121}/A_1$. E. coli DH5α colonies containing constructed plasmids were extracted for the detection of inserted DNA sequence of $VEGF_{121}$/A and $VEGF_{121}/A_1$ using PCR.

PCR was performed in 40 amplification cycles at 94^0C for 1 minute 59^0C for minute and 72^0C for 32minutes, using the sense primer --5'-CCG-AGATCTG-AAG GAA TTT ACC TTA GAC-3-- for both A subunit or A_1 fragment of Shiga like toxin and the antisense primer --5'- TAC TCG AGT TCA CCG CCT CGG CTT TGT TCA-3'--, for the C-terminal of $VEGF_{121}$ DNA.

PCR products were resolved on a 1% agarose gel for the detection of 1.25kb of long chain $VEGF_{121}$/A DNA and 0.95kb of short chain $VEGF_{121}/A_1$ DNA.

DNA Sequence Analysis

Constructed plasmids pET-29a/$VEGF_{121}$/A, pET-29a/$VEGF_{121}/A_1$, pET-32a-$VEGF_{121}$/A and pET-32a $VEGF_{121}/A_1$ were analyzed for correct sequences using sense primer corresponding to a non-translated DNA region of T7 promote (5'-TAATACGACTAATA-3') and antisense primer encoding the internal $VEGF_{121}$ DNA (5'-TAC TCG AGT TCA CCG CCT CGG CTT TGT TCA-3'). Colonies selected for constructed plasmids showed the correct nucleotides sequence.

Expression the Recombinants $VEGF_{121}$/A, and $VEGF_{121}/A_1$ Fusion Proteins

E .coli BL21 (DE3) PLysS is a lysogen of bacteriophage DE3 that carries the *LacI* gene and the gene for T7 RNA polymerase. The T7 RNA polymerase contains *LacUV5* promoter, which is educable by isopropyl-B-D-thiogalactopyranoside (IPTG) as illustrated in figure (11). A relatively small amount of T7RNA polymerase provided from a cloned copy T7 gene in the lysogenic *E. coli* (DE3) is sufficient to direct high level of transcription of VEGF/SLT fusion proteins from a T7 promoter in pET plasmids [15].

Using the following protocol, competent *E. coli* BL21 (DE3) PLysS were transformed with constructed plasmids carrying DNA encoding $VEGF_{121}$/A and/or $VEGF_{121}$/A fusion proteins.

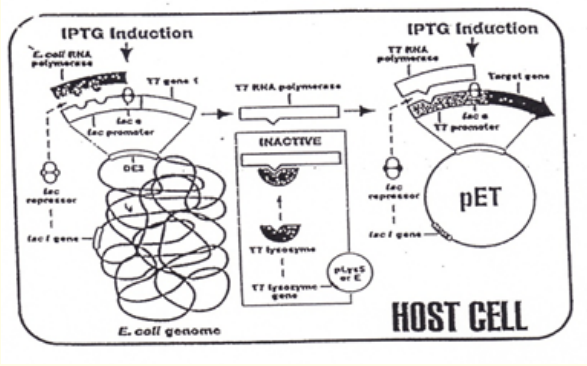


Figure 11: *Mechanism of Protein Expression by pET Vectors in E. coli BL21 (DE3) pLysS Host Cells.*

100 µl aliquots of component *E. coli* BL21 (DE3) PLysS cells were mixed on ice with 5 µl aliquot containing 0.1 µg DNA of one of constructed plasmid. After 30 minutes incubation on ice, cells were heated at 42^{0}C for 2 minutes, then diluted with S.O.C media for the final volumes of 1 ml and incubated for 37^{0}C for one hour with shaking. Transformed *E. coli* BL21 (DE3) PLysS cells were placed on LB agar containing 30 µg/ml kanamycin and 34 µg/ml chloramphenicol for the selection of bacteria cells transformed with plasmid pET-29a/VEGF$_{121}$/A or pET-29a/VEGF$_{121}$/A$_1$ and 100µg/ml carbencillin and 34 µg/ml chloramphenicol for the selection of bacteria cells transformed with plasmid pET-32a/VEGF$_{121}$/A or pET-32a/VEGF$_{121}$/A1. After overnight incubation at 37^{0}C individual colonies were picked, suspended in 1 ml of LB medium and incubated at 37^{0}C until an optical density (OD$_{600}$) of 0.4-0.6 was reached (exponential growth phase). Expression of VEGF$_{121}$/A and VEGF$_{121}$/A$_1$ fusion proteins as shown in figure (12) as a diagram, were induced by adding to bacteria culture IPTG to a final concentration of 1mm and incubated for 2.5-3.0 hours at 37^{0}C.

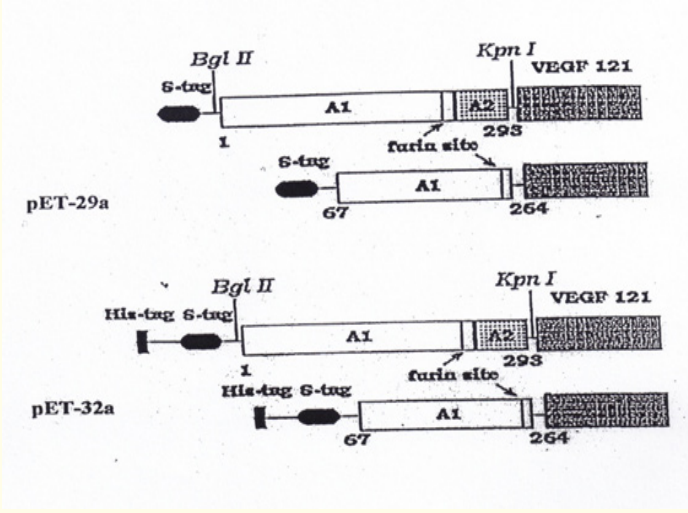


Figure 12: *Structure of VEGF$_{121}$/A and VEGF$_{121}$/A$_1$ fusion proteins expressed in pET-29a and pET-32a plasmids.*

Harvested bacteria cells we suspended in 0.1 ml water, mixed, boiled for 5 minutes and analyzed by SDS-PAGE on 15% agarose gel. Colonies that expressing strong protein band of the expected molecular weight of 51-kDa for the long chain VEGF$_{121}$/A fusion protein or 42-kDa for the short chain VEGF$_{121}$/A$_1$ fusion proteins were maintained and propagated on large scale fermenter for the expression and purification of a high quantity of these fusion proteins.

Fusion Proteins Assay

Purified fusion proteins concentration were analyzed using S-tag assay kit purchased from Novagen, Madison, WI. The S-tag assay method is based on the interaction of the 15 amino acids S-tag encoded in the fusion protein with the 104 amino acid S-protein derived from pancreatic ribonuclease-A. Neither S-tag in fusion protein nor S-protein alone has ribonuclease. An enzymatic activity, but the S-tag/S-protein complex forms active ribonuclease-A enzyme.

The fusion protein was assayed by adding to a buffer containing 5 ng/ml S-protein and 0.1 mg/ml the substrate Poly C. After incubation at 37^{0}C for 5 minutes the reaction was terminated by adding ice cold trichloro acetic acid (TCA) to the final concentration of 5% and placing in ice cold for 5 minutes followed by centrifuge at 14,000xg for 10 minutes. The activity of S-tag/S-protein complex in supernatant was measured by O.D$_{280}$ which increases as the substrate poly C is broken into acid-soluble nucleotides. The fusion protein concentration was calculated based on the molar concentration of S-tag in fusion proteins, using standard known concentration of S-tag (0.1 pmol) and the following calculation:

(Sample$O.D_{280}$)/(sample volume) X (0.1 pmol S-tag)/(S-protein $O.D_{280}$)

N-Glycosidase Enzyme Activity Assay

The *N*-glycosidase enzymatic activity for fusion proteins was assayed indirectly by measuring the inhibition *in vitro* translation of firefly luciferase mRNA in rabbit reticulocytes lysate purchased from Promega Corporation, Madison, WI.

Different concentrations of $VEGF_{121}/A$, $VEGF_{121}/A_1$, fusion proteins and standard $sVEGF_{121}$ were added separately to *in vitro* translation test tubes containing 18 µl of rabbit reticulocytes lysate, 0.5 µl complete amino acids mixture, 0.7 µl 2.5Kcl and 1 µg of firefly luciferase mRNA. The mixture with a total volume 25 µl was incubated at 30^0C for 10 minutes and luciferase activity was measured in LUMI-ONE luminomater from BioScan, Inc, Washington DC.

KDR/FLk-1 Auto-Phosphorylation Assay

The biological activity of $VEGF_{121}/A$ and $VEGF_{121}/A_1$ fusion proteins were assayed using HEK-293 human embryonic kidney cells transfected with VEGF Receptor KDR/FLK1. HEK-293/KDR cells expressing the VEGF receptor KDR/Flk-1 were plated into 24 wells at the concentration of 1.0 to $1.5x10^5$ cells/well and incubated for overnight at 37^0C. Cells were washed once with phosphate buffer and incubated in serum free DMEM at 37^0C for 4 hours, followed by placing the serum-free DMED with serum free DMED containing 0.5 mm sodium vanadate and 25mm HEPES at PH-7 and incubated for 20 minutes at 37^0C then for 20 minutes at 40C. Fusion proteins of $VEGF_{121}/A$ or $VEGF_{121}/A_1$ were added at different concentrations and incubated for 1 hour at 37^0C. Harvested cells were rinsed with ice cold phosphate buffer solution (PBS) containing 0.5 mm sodium vanadate and lysed in 2x Laemmli buffer. Cells lysates were heated at 100^0C for 5 minutes, separated by SDS-PAGE on 7.5% agarose gel and plotted onto Hybond C membrane purchased from Amersham, Arlington Height-Illinois. Tyrosine phosphorylated proteins were detected on immunoblots with horseradish peroxidase conjugated monoclonal anti-phosphotyrosine antibody purchased from Transduction Laboratories, Lexington, KY was diluted 1:2000 and according to the manufacturer's instruction the ECL, detection system from Amersham was used to visualize the phosphorylated tyrosine bands.

Growth Inhibition Assay

HEK-293/KDR and HEK-293 parental cells were plated at a density of $2.5X10^3$ cells/wells in 24 wells plates for 24 hours. Cells were washed once with PBS and incubated with serum-free DMEM at 37^0C for 4 hours, the medium was replaced with serum-free DMEM containing 25mm HEPES at PH-7.2 and incubated for 20 minutes at 37^0C followed by 20 minutes at 4^0C. Fusion proteins $VEGF_{121}/A$ or $VEGF_{121}/A_1$ or the control soluble $VEGF_{16}5$ ($sVEGF_{165}$) at the concentration of 10 nm were added to each well and fetal bovine serum (FBS) was added to final concentration of 10% per well. Plates were incubated in $C0_2$ incubator for 5days and cells were harvested by trypsinization and counted using hemocytometer.

Results and Discussion

Genes Cloning and Construction of Expression Vectors

DNA size for amplified A-subunit and A_1 fragment of Shiga like toxin generated by PCR yielded as expected the 0.87kb for the full length of A-subunit and 0.6kb for the short fragment of A_1 as shown in figure (8).

PCR screening for $VEGF_{121}/A$ and $VEGF_{121}/A_1$ DNA inserted in pET-32a/$VEGF_{121}$ plasmid extracted from 20 transformed *E. coli* DH5α colonies resistant to carbencillin resulted in 4 colonies #2, 4, 8 and 16 with the expected 1.25kb DNA for long chain $VEGF_{121}/A$ fusion protein and resulted in 3 colonies #4, 9 and 10 with the expected 0.95 kb DNA for the short chain $VEGF_{121}/A_1$ $VEGF_{121}/A_1$ as shown in figure (10).

PCR screening for $VEGF_{121}/A$ and $VEGF_{121}/A_1$ DNA inserted in pET-29a/$VEGF_{121}$ plasmid extracted from 10 colonies of transformed *E. coli* DH5α resistant to kanamycin resulted in only one colony #9 showed the expected 1.25kb DNA for the long chain $VEGF_{121}/A$ fusion protein and two colonies #13 and 18 showed the expected 0.95kb DNA for the short chain $VEGF_{121}/A_1$ fusion protein as shown in figure (10).

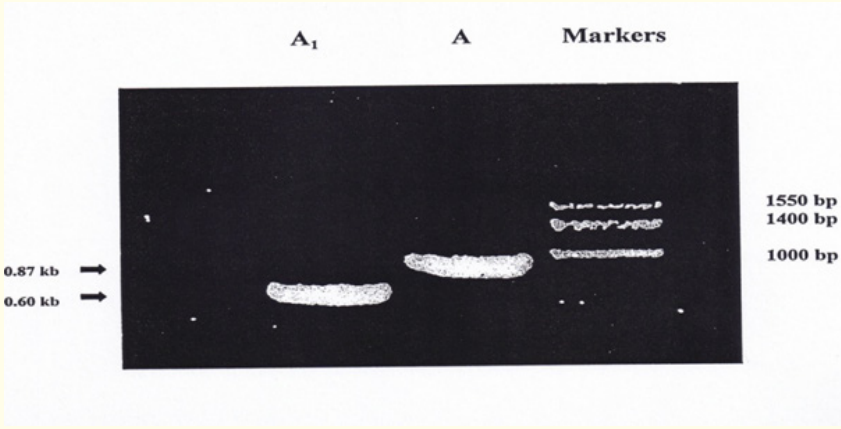


Figure 8: *PCR Amplification of DNA Encoding SLT-A Subunit and A_1 Fragment.*

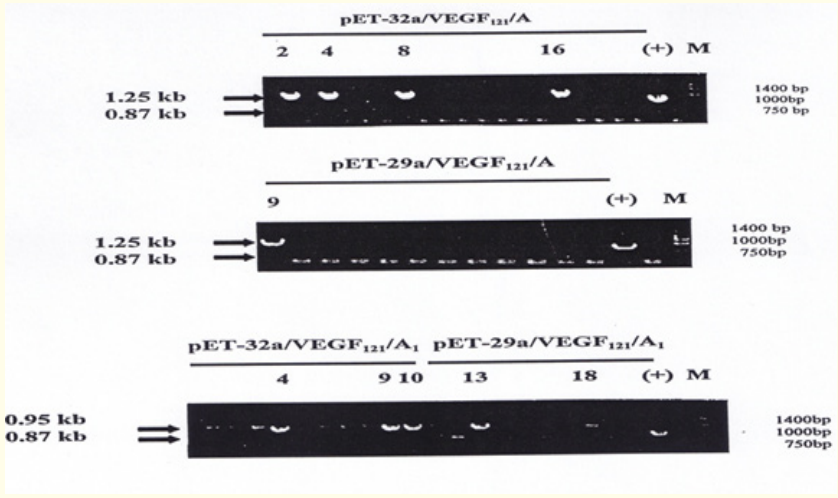


Figure 10: *PCR Screening of Colonies for VEGF/SLT Constructs.*

Expression of VEGF/SLT Fusion Proteins from Constructed Plasmids

Constructed plasmids pET-29a/$VEGF_{121}$/A, pET-29a/$VEGF_{121}$/A_1, pET-32a /$VEGF_{121}$/A and pET-32a/$VEGF_{121}$/A_1 were extracted from 8 selected colonies of *E. coli* DH5α and transformed into competent *E. coli* BL21 (DE3) pLysS cells for the expression of $VEGF_{121}$/A and $VEGF_{121}$/A_1 fusion proteins as described in material and methods.

SDS-PAGE analysis for 7 soluble extracts showed the following in Figure (13).

Expression of the short chain $VEGF_{121}$/A_1 fusion protein in *E. coli* BL21 (DE3) pLysS contain the constructed plasmid pET-29a/$VEGF_{121}$/A_1 showed two colonies #13 and 18 with the expected 42-kDa fusion protein.

Expression of long chain $VEGF_{121}$/A fusion protein in *E. coli* BL21 (DE3) pLysS contain the constructed plasmid pET-29a/$VEGF_{121}$/A showed one colony #9 with the expected 51-kDa fusion protein.

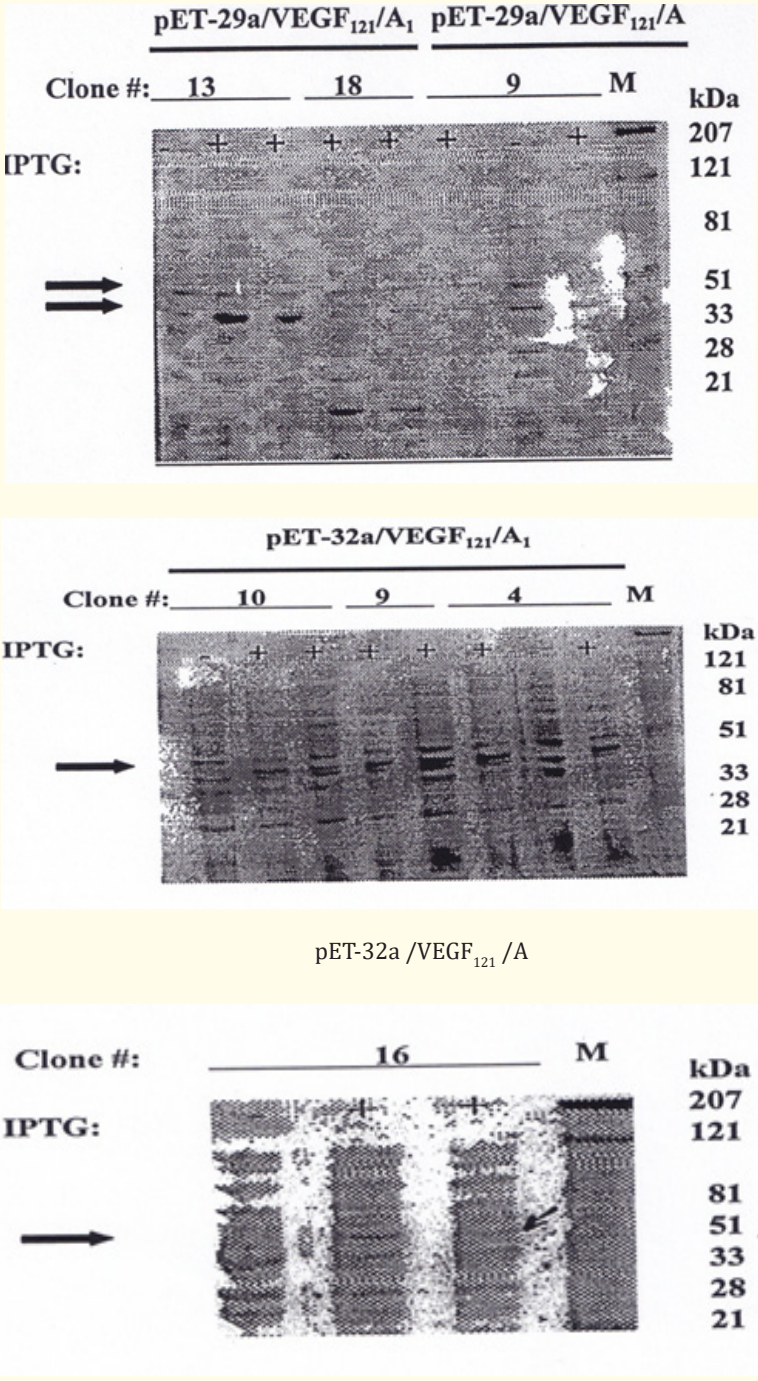


Figure 13: *Detection of $VEGF_{121}/A_1$ and $VEGF_{121}/A$ fusion Proteins in E. coli BL21 (DE3) pLysS Crude Cell Extract.*

Expression of short chain $VEGF_{121}/A_1$ fusion protein in *E. coli* BL21 (DE3) pLysS contain the constructed plasmid pET-32a/$VEGF_{121}/A_1$ showed three colonies #4, 9 and 10 with the expected 42-kDa fusion protein.

Expression of long chain $VEGF_{121}$/A fusion protein in *E. coli* BL21 (DE3) pLysS contain with constructed plasmid pET-32a/$VEGF_{121}$/A showed one colonies #16 with the expected 51-kDa fusion protein.

Distribution of VEGF/SLT Fusion Proteins (soluble and insoluble) in *E. coli* BL21 (DE3) pLysS Cells

Two short chain $VEGF_{121}/A_1$fusion proteins expressed in *E. coli* BL21 (DE3) pLysS cells from colonies #9 and 13 containing plasmids pET-29a/$VEGF_{121}/A_1$ and pET-32a/$VEGF_{121}/A_1$ respectively were analyzed by SDS-PAGE for the distribution of this fusion protein in the form of soluble and insoluble fractions from bacteria cell lysates. The insoluble fraction of cell lysate is comprised of inclusion bodies that were solubilized with 8M urea and dialyzed against buffer containing 10 mM Tris-HCL PH8, 150 mM Nacl and 0.01% NP-40. The dialyzed $VEGF_{121}/A_1$ fusion protein solutions from these two colonies were analyzed by SDS-PAGE, on 15% gels demonstrate equal distribution of 42-kDa $VEGF_{121}/A_1$ fusion protein in the form of soluble fraction located in the bacteria periplasmic space and insoluble fraction in the form of inclusion body inside the bacteria cell sat the ratio of 1:1 as shown in Figure (14).

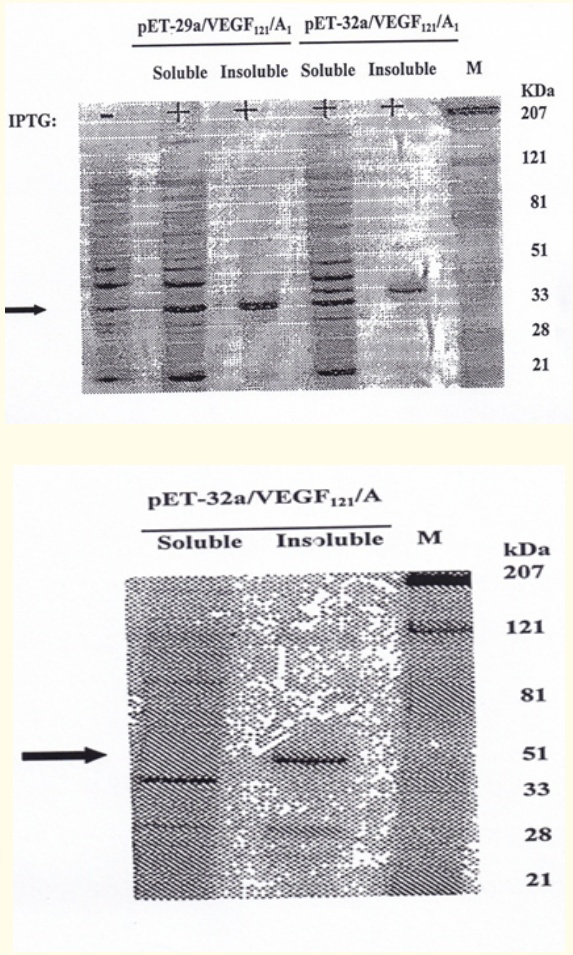


Figure 14: *Distribution of $VEGF_{121}/A$ and $VEGF_{121}/A_1$ in Soluble and Insoluble Forms after Dialysis.*

One long chain $VEGF_{121}$/A fusion protein expressed in *E. coli* BL21 (DE3) pLysS cells from colony #16 was analyzed by SDA-PAGE as described above for the distribution of fusion protein between soluble and insoluble fractions from bacteria cell lysate and demonstrate that the 51-kDa $VEGF_{121}$/A is 100% located in the form of inclusion bodies inside the bacteria cells as shown in Figure (14).

Biological Activity of VEGF/SLT Fusion Proteins

Fusion Proteins $VEGF_{121}$/A and $VEGF_{121}/A_1$ Concentration Assay

Purified fusion proteins were analyzed using S-tag assay kit as described in materials and methods. Results demonstrated that the concentration of the short chain $VEGF_{121}/A_1$ is 0.87 pmole/µl and the concentration of the long chain $VEGF_{121}$/A is 1.65 pmole/µl as shown in Table (1).

Sample	Dilution Factor	O.D Reading at 280	Concentration
$VEGF_{121}/A_1$	10 X	0.502	0.87pmole/ µg/ml
	100 X	0.030	----
$VEGF_{121}$/A	10 X	0.920	1.65pmole/ µg/ml
	10 X	0.030	----
Standard (S-tag)	----	0.290	----
Blank (water)	----	0.000	----

Table 1: *S-Tag Assay for the Concentration of $VEGF_{121}$/A and $VEGF_{121}/A_1$ Fusion Proteins.*

Protein Synthesis Inhibition by Fusion Proteins

Catalytically active sites for *N*-glycosidase enzymatic activity are located in both Shiga-like toxin (SLT-1). A-subunit and A_1 fragment in fusion proteins. This enzymatic activity is responsible to depurinates a specific Adenine (A_{4324}) in 28S rRNA resulting in the inhibition of protein translation.

The ability of $VEGF_{121}$/A and $VEGF_{121}/A_1$ fusion proteins to inhibit protein translation was tested in a rabbit reticulocytes lysate translations assay using firefly luciferase mRNA as the reporter gene. Inhibitory activity was measured as a reduction in luciferase activity in the presence of the fusion protein in *in vitro* translation reaction using soluble $VEGF_{121}$ ($sVEGF_{121}$) as a control for non-specific inhibition of translation, as described in material and methods.

Results demonstrated that both $VEGF_{121}$/A and $VEGF_{121}/A_1$ inhibited in vitro translation of luciferase mRNA at the concentration of 10µM, indicating that both A-subunit and A_1 fragments of Shiga-like-toxin fused to $VEGF_{121}$ maintained its *N*-glycosidase enzymatic activity as shown in Figure (15).

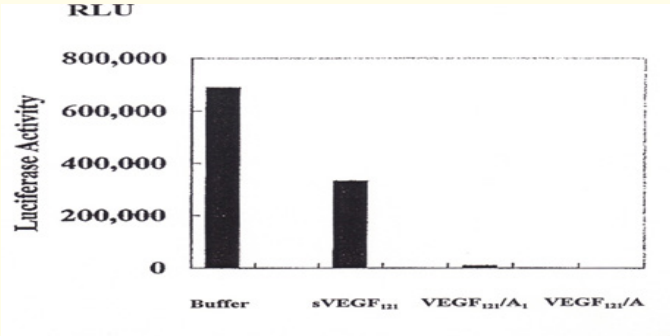


Figure 15: *N-glycosidase Activity of $VEGF_{121}$/A1 and $VEGF_{121}$/A Fusion Proteins.*

Evaluation of the effect of different concentrations of fusion protein sat the range of 0.8-8.0 nM demonstrated that $VEGF_{121}/A$ inhibited protein synthesis of luciferase in a dose dependent manner at a minimum concentration of 0.8 nM for the short chain $VEGF_{121}/A_1$ and at a minimum concentration of 40 nM for the long chain $VEGF_{121}/A$ as shown in Figure (16).

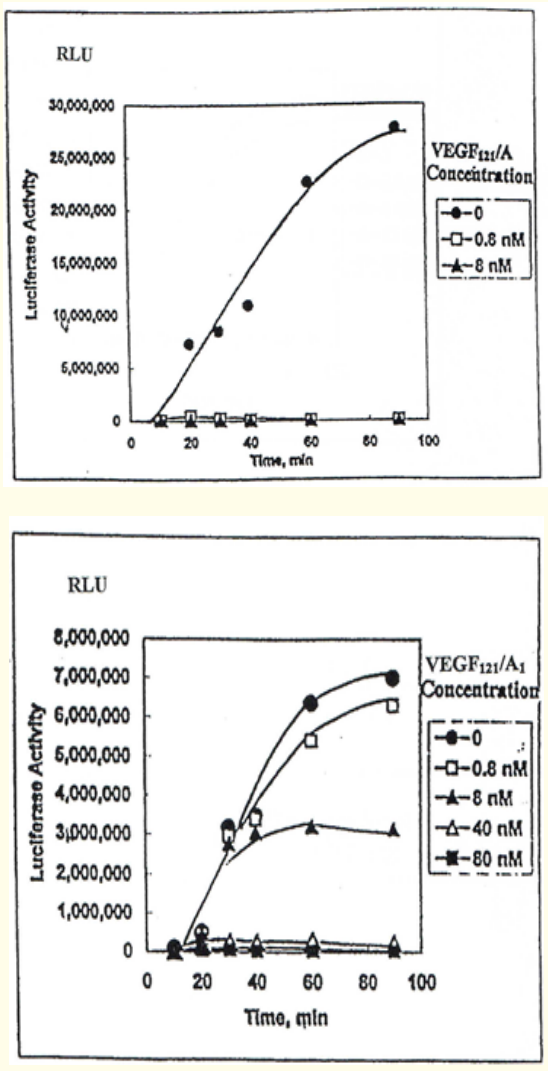


Figure 16: *Kinetics and Dose Dependence of Luciferase Synthesis Inhibition by $VEGF_{121}/A$, and $VEGF_{121}/A_1$ Fusion Proteins.*

VEGF receptor (KDR/Flk-1) Auto-phosphorylation by VEGF/SLT Fusion Proteins

This assay is to evaluate the functional activity of the $VEGF_{121}$ moieties of VEGF/SLT fusion proteins. This function is $VEGF_{121}$ in the fusion protein bind to its cellular receptor (KDR/FlK-1) causing the receptor dimerization and induce its auto-phosphorylation. Tyrosine phosphorylation can be detected in induced cell lysate by western blotting using anti-phosphotyrosine antibodies.

The transformed human embryonic kidney cell line HEK-293 transfected with full-length of KDR/Flk-1 (293/KDR cells) is used for this assay. Recombinant soluble $VEGF_{165}$ ($sVEGF_{165}$) was used as a positive control for KDR/Flk-1 auto-phosphorylation and lysate of untreated HEK-293/KDR cells with fusion proteins served as negative control.

Results demonstrated that the short chain of $VEGF_{121}/A_1$ fusion protein induced auto-phosphorylation of KFDR/Flk-1 receptor as well as the positive control $sVEGF_{165}$ in a dose-dependent manner. In the case of long chain of $VEGF_{121}/A$ fusion protein was failed to induce KDR/Flk-1 auto-phosphorylation, suggesting that the long chain form of $VEGF_{121}/A$ fusion protein did not bind or form the proper complex with KDR/Flk-1-receptor expressed on KEK-293 cell line (HEK-293/KDR) to induce to induce auto-phosphorylation as shown in Figure (17).

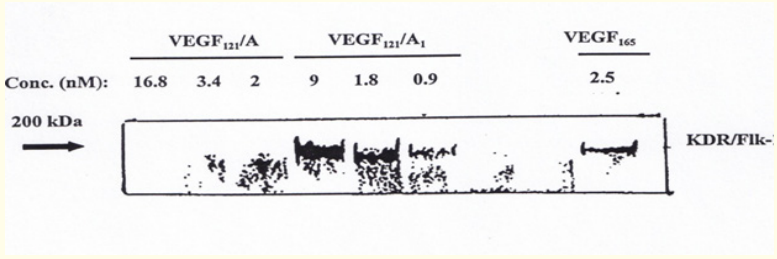


Figure 17: *Induction of KDR/Flk-1 Autophosphorylation in HEK 293/KDR Cells by $VEGF_{121}/A$, and $VEGF_{121}/A_1$ Fusion Proteins.*

Cell Growth Inhibition by VEGF/SLT Fusion Proteins

It is anticipated that, after $VEGF_{121}$ in VEGF/SLT fusion proteins bind to its receptor KDR/Flk-1, VEGF/SLT fusion proteins would be internalized into cells expressing KDR/Flk-1 by endocytosis, *N*-glycosidase of A-subunit or A_1 fragment of VEGF/SLT fusion proteins will be activated after cleavage by the intracellular enzyme furine and inhibit protein translation, causing selectively inhibit endothelial cells growth expressing VEGF receptor of KDR/flk-1.

HEK-293/KDR cells expressing a high density of cell surface KDR/flk-1 receptor at a concentration of $2.6/10^6$ cell were used versus HEK-293 parental cells to evaluate the growth inhibitory activities of VEGF/SLT fusion proteins.

HEK-293/KDR and HEK-293 parental cells were incubated in the presence of 10mM $VEGF_{121}/A$ or $VEGF_{121}/A_1$ fusion proteins for 6 days. In this experiment soluble $VEGF_{121}$ ($VEGFs_{121}$) was used a positive control.

Results demonstrated that short chain $VEGF_{121}/A_1$ fusion protein significantly (P= 0.0006) inhibited the growth of KEK-293/KDR cells, while the long chain $VEGF_{121}/A$ fusion protein failed significantly (P= 0.856) to inhibit the growth of HEK-293/KDR cells. Incubation of HEK-293/KDR cells with 10 mM of $sVEGF_{121}$ as a positive control showed a significant (P= 0.003) growth inhibition of HEK-293/KDR as shown in Figure (18).

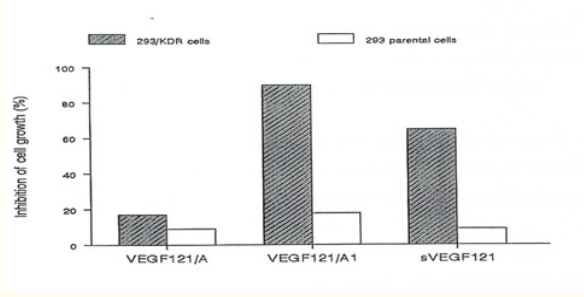


Figure 18: *Growth Inhibition of HEK 293 and HEK 293/KDR by $VEGF_{121}/A$, and $VEGF_{121}/A_1$ Fusion Proteins.*

Conclusion

Folkman [16] proposed over 40 years the hypothesis that growth of solid tumors and the formation of metastases are dependent on the formation of new blood vessels. This hypothesis has been largely supports by multiple discoveries that have been unraveling the molecular and cellular process involved tumors angiogenesis. These angiogenesis discoveries supported the hypothesis and demonstrated that many opportunities are exist for therapeutic interventions.

Among the various angiogenic factors, $VEGF_{121}$ play a pivotal role in tumor angiogenesis. The VEGF receptor (KFR/Flk-1) is expressed almost exclusively in endothelial cells in vessel lining and penetrating tumors. The high expression of KDR/Flk-1 receptor in endothelial cells of tumor vasculature offers window of opportunity to enable successful cancer therapy using VEGF-toxin without side effects associated with conventional cytotoxic approaches. This therapeutic strategy for targeting angiogenesis is being investigated as potential therapeutics for cancer treatment. During angiogenesis endothelial cells of existing blood vessels undergo a complex process of reshaping, migration, growth and organization into new vessels. Vascular endothelial growth factors (VEGFs) are central mediator for this process and acts via receptors whose expression is restricted almost exclusively of endothelial cells. Because of its selectivity, VEGFs represents are a unique vehicle for delivery angiogenesis inhibitors to endothelial cells.

Among potential inhibitors of angiogenesis is the Shiga-like toxin-I (SLT-I). Shiga like toxin (SLT-1) is produced by *E. coli* O157:H7 and appears to have the advantage to inhibit endothelial growth. The hypothesis for this research work is to combine Shiga-like toxin (SLT-I) with $VEGF_{121}$ as a delivery vehicle fusion protein that would serve as a highly selective to endothelial cells expressing VEGF receptor (KDR/Flk-1).

To this end, fusion proteins containing $VEGF_{121}$ and two forms of Shiga-like toxin-I (SLT-I) were developed and tested *in vitro* as the potential to inhibit angiogenesis.

Plasmids encoding the fusion proteins $VEGF_{121}$/A containing the full length A-subunit of Shiga like-toxin (SLT-I) and $VEGF_{121}/A_1$ containing only the catalytically active A_1 fragment of Shiga-like toxin (SLT-I) were constructed in pET-29a/$VEGF_{121}$ and pET-32a/$VEGF_{121}$ systems for the expression of these fusion protein in *E. coli.*

Many proteins secreted from mammalian cells require glycosylation and proper disulfide-cross linking for functional activity. The use of *E. coli* as an expression system was previously limited due to the difficulties in expressing heterologous proteins with functional activity in these organisms [17]. Induction experiments demonstrated that *E. coli BL21* (DH3) pLysS is the best system for the expression of non-glycosylated proteins [18] and also demonstrated that the glycosylation of VEGF/SLT fusion proteins does not play a vital role for this therapeutic strategy to inhibit angiogenesis.

Expression experiments of $VEGF_{121}$/A fusion protein in *E.coli BL21* (DH3) pLysS transformed with the constructed plasmids pET-29a/$VEGFr_{121}$/A and pET-32a/$VEGF_{121}$/A resulted in the expression of this long chain fusion protein in the form of inclusion bodies inside bacteria cells upon induction of *Lac I* operon with the inducer isopropyl-thiogalactopyranoside (IPTG).

While the expression of $VEGF_{121}/A_1$ fusion protein in *E. coli BL21* (DH3) pLysS transformed with the constructed plasmids pET-29a/$VEGFr_{121}/A_1$ and pET-32 a/$VEGF_{121}/A_1$ resulted in the expression this shorter chain fusion protein inside bacteria cells in periplasmic space and in the form of inclusion bodies at a ration 1:1 approximately upon induction of *Lac I* operon with the inducer isopropyl-thiogalactopyranoside (IPTG).

Both recovered and purified fusion proteins inhibit the translation of luciferase mRNA as a reporter gene in *in vitro* translation system, indicating that both fusion proteins retained the *N*-glycosidase activity of A_1 fragment of Shiga-like toxin (SLT-I). However, the shorter form $VEGF_{121}/A_1$ fusion protein only displayed the ability to induce auto-phosphorylation of VEGF receptor (KDR/Flk-1) and strong selective growth inhibition of endothelial cells expressing VEGF-receptor (KDR/Flk-1). The inability of the long form of $VEGF_{121}$/A fusion protein

to induce VEGF-receptor (KDR/Flk-1) auto-phosphorylation and inhibition of endothelial cells expressing the VEGF receptor (KDR/Flk-1) suggesting that the long form $VEGF_{121}$/A fusion protein did not fold properly or forming the proper complex with VEGF-receptor (KDR/Flk-1).

In summary this research work [19] indicated that VEGF/SLT fusion protein as a therapeutic agent to inhibit angiogenesis is promising strategy for cancer treatment. This processing discovery for targeting angiogenesis with VEGF/SLT protein was lately demonstrated *in vitro* by other researchers [20-22] and confirmed *in vivo* on animal experiments [23,24].

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