Original Article

In Vitro Evaluation of Vegf-Pseudomonas Exotoxin: A Conjugated on Tumor Cells

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Abstract

Background: Angiogenesis which occurs mandatory in solid tumors, is a critical step in malignancy progression. Vascular endothelial growth factor (VEGF) is mainly responsible for angiogenesis process and facilitates the formation of new vessels. Distribution of monoclonal antibodies against VEGF or VEGF receptor (VEGFR) into the solid tumors is limited because of their huge dimensions. Moreover, many investigations have demonstrated the usefulness of immunotoxins to halt angiogenesis in solid tumors. **Materials and Methods:** We designed, expressed and evaluated the cytotoxicity of a novel nano-immunotoxin composed of VEGF splice variant containing 121 amino acids (VEGF121) and truncated the exotoxin A of *Pseudomonas aeruginosa* (PE38-KDEL). The fusion protein VEGF121-PE38 was successfully cloned and expressed in *Escherichia coli*, purified by Ni^{+2} affinity chromatography. The fusion protein was subsequently subjected to refolding using the reduced and oxidized glutathione. **Results:** The expression level of the fusion protein reached to 1 mg/ml. The VEGF121-PE38 immunotoxin showed a 59 KDa MW which had cytotoxic effect on HUVEC and 293/KDR cells as low and high expressing VEGFR2 cells, respectively. But the cytotoxicity on 293/KDR was 100 folds more than that of VEGFR2 low expressing cell HUVEC. **Conclusion:** The designed immunotoxin showed more selectivity for higher VEGFR2 expressing cells *in vitro*.

Keywords: *Immunotoxin, pseudomonas exotoxin A, solid tumor, vascular endothelial growth factor*

Introduction

Vascular endothelial growth factor A (VEGF-A) plays a critical role in the growth and progression of various solid tumors.[1] VEGF-A gene consisting eight exons, is located on chromosome 6p21.3.[2] Oxygen tension in solid tumors larger than 2 mm^3 induces VEGF mRNA expression,[3] which induces endothelial cell proliferation and enhances vascular permeability.[4] After RNA splicing, different isoforms of VEGF-A are secreted as disulphide-linked dimeric glycoproteins, such as VEGF 206, 189, 165, 145 and 121.[5] In contrast to other isoforms that are highly basic, VEGF121 is an acidic polypeptide unable to bind to heparin.[6] In solid tumors, VEGFR1 and VEGFR2 are expressed significantly on endothelial cells at angiogenesis site compared to quiescent endothelial cells.[7] Inhibition of angiogenesis is a potential target for reduction of tumor growth and progression.[8] Angiogenesis would be inhibited by blocking of VEGF effects on the endothelial cells, which could be done either by VEGFR2 antagonists or by neutralizing antibodies against VEGF molecule.[9] Another approach for angiogenesis inhibition is delivery of potent toxin compounds to tumor endothelial cells via VEGF. In this regard, many toxic compounds such as chemicals, fungal or bacterial toxins can be used. Among the bacterial toxins, diphtheria and pseudomonas exotoxins are more known and popularly used.[10]

Exotoxin A of *Pseudomonas aeruginosa* (PE), a 613-amino acid protein, is composed of three domains and a disulfide bond.[11] The domains include two receptor binding domains: Ia (residues 1-252) and Ib (residues 365-404), the translocation domain II (residues 253-364) and the enzymatic domain III (residues 405-613).[12] PE binds to CD91 molecule on the cell surface of eukaryotes and enters into the cells by endocytosis.[13] At the endosomes, furin enzyme cleaves PE at furin site (residues 274-280) and consequent conformational change promotes internalization of the translocation and enzymatic domains into

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How to cite this article: Langari J, Karimipoor M, Golkar M, Khanahmad H, Zeinali S, Omidinia S, *et al.* *In Vitro* Evaluation of Vegf-Pseudomonas Exotoxin: A Conjugated on Tumor Cells. Adv Biomed Res 2017;6:144.

Received: July, 2013. **Accepted:** December, 2013.

Access this article online

Website: www.advbiores.net

DOI: 10.4103/2277-9175.218691

Quick Response Code:

the vesicle.[14] Finally, enzymatic domain is released into the cytosol, where it inhibits protein synthesis.[15] Cell death is occurred by ADP-ribosylation of diphthamide in elongation factor 2.[15] PE has been fully characterized by using mutagenesis methods in many investigations. Two variants of PE utilized as immunotoxins against cancer cells are called PE38 and PE40.[16] Both variants have a common deletion in domain Ia (residues 1-252). Moreover, PE38 has a small deletion in domain Ib (residues 365-380) as well as replacement in residues 609-613 (REDLK) with "KDEL" sequence to increase its potency.[12] In this respect, many different PE-based immunotoxins have been undertaken in phase I, II and III clinical trials.[17]

This study aimed to design and evaluate a novel immunotoxin composed of VEGF121 and PE38 against VEGF receptors (VEGFR1 and VEGFR2). After expression, the cytotoxic effect was assayed in two different cell lines, HUVEC and 293/KDR, which vary in VEGFR2 density.

Materials and Methods

Cell lines and bacterial strain

E. coli strain top10F′ was used for cloning and *E. coli* strain BL21 (DE3) and Rosette 1 was used for protein expression. 293KDR and HUVECs cells were used in this study. 293KDR is a stably transfected with VEGFR2 cell line (Backer and Backer, 2001). 293KDR and HUVECs were grown in DMEM medium supplemented with 10% FBS. Cultures were maintained on plastic flask and incubated at 37˚C in 5% CO_2.

Design and construction of recombinant VEGF121-PE38 expression vector

The designed construct had the following fragment order; 5`-SacI-His.tag-Entrokinase site-VEGF121-$(G_4S)_4$ linker-PE38-KDEL-stop codon-*Hind*III-3` and was synthesized by Biomatik Corporation (www.biomatik.com). The construct was digested by SacI and HindIII restriction enzymes (Thermo Scientific, Lithuania) and subcloned into pET-28a expression vector (MilliporeMillipore, USA) by T4 DNA ligase (Thermo Scientific, Lithuania). The pVEGF121-PE38 was propagated in *E. coli* TOP10 strain (Millipore, USA), extracted by miniprep plasmid extraction kit (Thermo Scientific, Lithuania) and then stored at −20°C for next steps.

Protein expression in *E. coli*

The VEGF121-PE38-pET28a plasmid was transformed into BL21 (DE3), BL21 (DE3) pLysS Rosetta (Millipore, USA) *E. coli* strains by $CaCl_2$ method. After colony selection, a single colony containing the recombinant expression vector were cultured at 37°C in 2XTY medium (5 ml) containing 50 µg/ml kanamycin. In early log phase (OD = 0.5 at 600 nm), the protein expression was induced by adding 0.3 mM IPTG (Sigma-Aldrich, Germany) and incubated at 37°C overnight. At the end of the incubation, the cells were collected by centrifugation at 10,000 rpm for 5 minutes and stored at −20°C for further analysis.

Purification of expressed immunotoxin from inclusion bodies

Frozen bacterial pellet was suspended in binding buffer (8 M urea, 500 mM NaCl, l50 mM Tris-HCl and 30 mM imidazole, pH 7.4) and then stirred gently for 2 h. The solution was consecutively subjected to freeze-thaw process (11 times), sonication (20 sec ON and 25 sec OFF for 20 min) and centrifugation at 12,000 rpm at 4°C for 30 minutes. The lysate was filtered through a 0.22 µm filter (Millipore, USA) and loaded on a 5 ml of Ni-NTA column (Qiagen, USA) with 1 ml/min flow rate. The column was washed with washing buffer (4 M urea, 500 mM NaCl, 50 mM Tris-HCl and 60 mM imidazole, pH 7.4) and the anchored protein was eluted by elution buffer (2 M urea, 500 mM NaCl, 50 mM Tris-HCl and 60 mM imidazole, pH 7.4). The protein containing the fractions were pooled and dialyzed against the dialysis buffer (0.5 M urea, 500 mM NaCl, 50 mM Tris-HCl and 60 mM imidazole, pH 7.4) via a dialysis bag (14 kDa cut off).

Protein refolding and lipopolysaccharide removal

Fusin protein was dialyzed by refolding buffer (PBS, 0.2 mM oxidized glutathione and 2 mM reduced glutathione adjusted to pH 7.4) at 4-8°C overnight. For LPS removal, Triton X-114 was added to the protein solution (1% V/V) and incubated by gentle stirring at 4°C for 30 minutes and then at 37°C for 20 minutes. Finally, the solution was centrifuged at 12,000 rpm at room temperature for 30 minutes and upper aqueous phase was collected and stored at −20°C for further analysis. In addition, limulus amebocyte lysate (LAL) test was performed for determining residual LPS in samples treated either by Triton X-114 method as in manufacturers manual (Sigma-Aldrich, Germany).

Immunotoxin characterization

Purified protein was analyzed by SDS-PAGE in a 12% resolving gel at 100 V for 50 min using Mini-PROTEIN® Tetra Cell system (Bio-Rad, USA) and visualized by Coomassie brilliant blue staining method. For immunoblotting, the protein was transferred onto nitrocellulose membrane (1.5 h at 300 V) using Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad, USA). Subsequently, the membrane was blocked by 2% BSA in tris-buffered saline (0.05 M Tris and 0.15 M NaCl) at 4-8°C overnight. Then, the membrane was incubated with Avastin® (primary antibody in TBS/BSA [12.5 µg/ml]) (Genentech, USA). After washing, the membrane was incubated with horseradish peroxidase (HRP) conjugated goat-anti human IgG (1:10,000 dilution in TBS/BSA) (Bethyl, USA) for 2 h and soaked in diaminobenzidine (DAB)(Sigma-Alderich) solution at room temperature for 15 minutes.

Evaluation of refolding process by CD spectroscopy

CD spectra in the far UV regions (start 350 - end 190) was performed using 0.1 cm path cell, scanning speed 200 nm/min at room temperature. To evaluate the refolding process, 400 µl of samples (0.5 mg/ml of each refolded and unfolded protein) was injected into a CD apparatus (Jasco, J-810) and the percentage of each secondary structure (α-helix, β-sheet, random coil and turn) was determined. Protein content of all the samples was determined by Bradford's method.

Evaluation of refolding process by ELISA

In addition CD spectroscopy, to evaluate the refolding process, a 96-well plate was coated with unfolded and refolded fusion protein (1 µg per well) in PBS at 4°C overnight. The same concentration of BSA was used as a non-specific binding protein. The excess of fusion protein was removed, and the plate was washed with PBST (0.1% V/V Tween 20 in PBS) and then blocked with BSA at 37°C for 1 h. The blocking reagent was discarded and Avastin®, diluted in blocking buffer, added to the wells and incubated while gently shaking at 37°C for 2 h. The plate was washed four times with PBST and PBS separately. After washing, HRP-conjugated goat-anti human IgG diluted in the blocking buffer was added to each well and incubated while gently shaking at 37°C for 1 h. The wells were then washed with PBST and PBS, respectively. Afterward, 100 µl TMB was added to each well and incubated at room temperature for 15 minutes. The reaction was stopped by addition of 1 M HCl and the absorbance was measured at 450 nm.

VEGFR2 expressing cell inhibition assay

Log-phase HUVEC cells (ATCC No. CRL-1730) and 293/KDR cells (SBT021-293, USA) in DMEM medium (10% [V/V] FBS [Gibco-BRL, Germany]) were diluted to 8,000 and 10,000 cells per 200 µl, respectively. Aliquots (200 µl) were added to 96-well flat-bottomed tissue culture plates (Orange Scientific, Belgium) and incubated in 5% CO_2 at 37°C for 24 h. After 24 h, different concentrations of the purified fusion protein (diluted in 100 µl medium) were added to the wells and incubated for 72 h. Then, 30 µl MTT solution ([5 mg/ml] PBS) was added to the wells and incubated in 5% CO_2 at 37°C for 4 h. The medium was removed from the plates and formazan crystals were dissolved in 100 µl DMSO. The absorbance was measured at 450 nm.

Results

Design and synthesis of VEGF121-PE38 construct

The DNA construct (5`-SacI-His.tag-Entrokinase site-VEGF121-$(G_4S)_4$ linker-PE38-KDEL-stop codon-HindIII-3`) encoding the immunotoxin was designed according to the Figure 1 and manufactured by Biomatik Corporation (Canada). The construct was Thermo Scientific cloned into the pBAD/gIII and pET-28a and confirmed by restriction map analysis and sequencing [Figure 1].

Expression and purification of the fusion protein

Running the induced lysate on 12% SDS-PAGE showed an expected molecular weight of 59 kDa expect to the fusion protein [Figure 2a]. The purified protein was also analyzed on 12% SDS-PAGE and a 59 kDa band was also observed on the gel [Figure 2b]. Based on LAL test, the LPS level of purified fusion protein was <0.125 following the treatment with Triton X-114. In immunoblot, Avastin® was able to recognize the fusion protein in lysate of induced bacteria, while no specific protein was detected in lysate of uninduced bacteria [Figure 3].

Evaluation of refolding process by CD and ELISA

The folded and unfolded forms of the fusion protein were analyzed by CD spectroscopy to see if the secondary structure had been formed. As shown in Table 1, secondary structure compositions of refolded and unfolded fusion protein were different due to refolding process. In ELISA test, the folded and unfolded fusion proteins were attached to the bottom of the plate according to the standard protocol. Then, the first and second antibodies were added. Results showed different absorbance levels in 450 nm [Table 2].

Cytotoxicity of the fusion protein on VEGFR2 expressing cells *in vitro*

To determine the sensitivity of the cells to VEGF121-PE38-KDEL, the MTT assay was used, a technique based on the reduction of MTT to formazan by viable cells. HUVECs and 293/KDR cells were incubated with different concentrations of fusion protein for different time lengths and treated with different concentrations of VEGF121-PE38-KDEL. The IC_{50}sIC50s of fusion protein which are required for 50% inhibition of the protein synthesis were 1.27 and 130 nM for 293/KDR and HUVEC cells, respectively [Figure 4].

Discussion

Chemotherapy, radiation and surgery methods in cancer treatments have some obstacles. Although surgery is only applied for non-metastatic cancers, metastatic risks will still remain. The main barrier for chemotherapy and radiation is damage and subsequent mutations in genomic DNA of both normal and abnormal cells.[18] These mutations may also raise risks of new cancer formation. The promising approach to overcome these flaws is the employment of antibodies against the tumor specific antigens.[19] But, low penetration property of antibodies into the solid tumors practically limits their application as an efficient approach in cancer treatment.[20]

Angiogenesis plays a critical role in cancer cell survival. Therefore, many researchers have focused on the angiogenesis inhibition in the solid tumors. In addition,

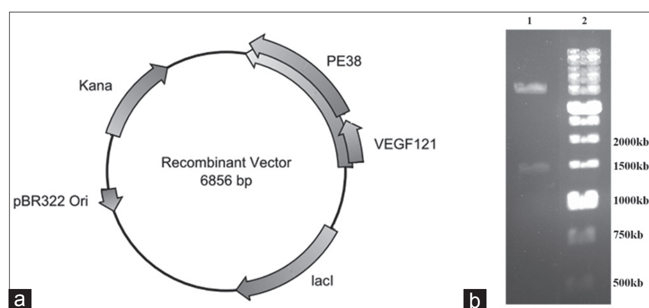

Figure 1: (a) Schematic representation of recombinant vector containing designed construct and (b) 1% agarose gel electrophoresis of recombinant vector [Lane 1: recombinant vector digested by HindIII and SacI and Lane 2: 1 kb DNA ladder SM0331 (Fermentas, Lithuania)]

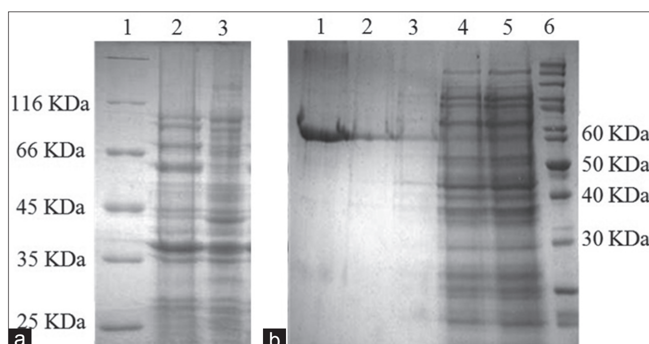

Figure 2: (a) 12% SDS-PAGE of lysate extracts; [Lane 1: Protein marker SM0431(Fermentas, Lithuania), Lane 2: Induced sample (TB medium), Lane 3: Uninduced sample (TB medium), Lane 4: Induced sample (2XTY medium), Lane 5: Uninduced sample (2XTY medium)] and (b) 12% SDS-PAGE of fusion protein after purification by Ni-NTA column chromatography; [Lane 1: Elution (500 mM imidazole), Lanes 2, 3 and 4: Wash (70 mM imidazole), Lane 5: Flow through, Lane 6: Protein marker SM0661(Fermentas, Lithuania)]

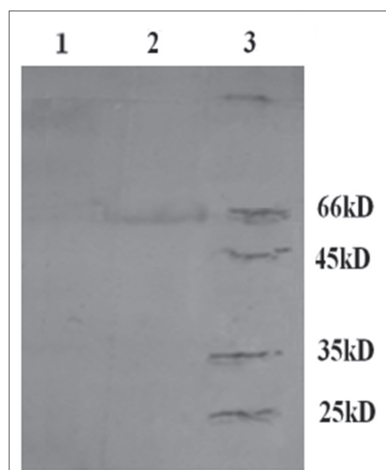

Figure 3: Western blotting of fusion protein using HRP conjugated goat-anti human IgG; [Lane 1: Uninduced sample, Lane 2: Induced sample, Lane 3: Unstained protein marker SM0431 (Fermentas, Lithuania)]. As expected, a band around 59 kDa was observed in IPTG-induced sample but not in uninduced sample

different strategies have been applied for this purpose, including retrovirus-delivered dominant negative Flk-1 mutant,[21] VEGF antibodies, VEGFR2 signaling inhibitors,[22] antisense oligonucleotides,[23] anti-VEGFR2 antibodies[24] and soluble VEGF receptors.[25] Furthermore, VEGF isoforms have been conjugated to plant, bacterial and fungal toxins to target VEGF receptors.[26] Among VEGF isoforms, VEGF121 binds to VEGFR1 and VEGFR2 due to the lack of exon 7 that facilitates only its binding to NP-1 and heparin.[27] It has been also reported that the absence of exon 7 does not affect VEGF121 binding affinity to VEGFR1 and VEGFR2 compared to other VEGF isoforms.[28]

Table 1: CD of the fusion protein shows secondary structure compositions of each sample. The differences between folded and unfolded protein samples were confirmed the alteration of fusion protein conformation under refolding condition

Protein	Alpha helix (%)	Beta sheet (%)	Turn(%)	Random coil (%)
Fold	0.2	64.7	0.0	35.1
Unfold	38.1	0.0	0.0	61.9

CD: Circular dichroism

Table 2: The same amount of the folded and unfolded fusion protein showed different absorbance in 450 nm

Fold	Unfold	Blank
2.333	0.714	0.284
2.564	0.687	0.3

PE, a 613-residue toxic protein, is widely used in immunotoxin preparation, in which the exotoxin binding domain is replaced by a ligand or by an antibody.[29] Nowadays, different immunotoxins composed of PE truncated forms, such as PE38 and PE40, are in phase I, II and III clinical trials.[30] Both variants have common deletion in domain Ia. Moreover, PE38 has a small deletion in domain Ib and a replacement in residues 609-613 (REDLK) with "KDEL" sequence to increase its potency.[12]

In this study, a novel nano-sized recombinant immunotoxin including VEGF121 and PE38 against VEGF receptors was designed and expressed. Furthermore, its ability to inhibit the proliferation of VEGFRs expressing cells was evaluated. The VEGF121 sequence is located at the N-terminal of the construct, because it has been reported that the C-terminal sequence of PE38 must be free to do its catalytic activity.[31] REDLK sequence at the end of the catalytic domain was replaced by KDEL sequence. Another study showed that REDLK sequence increased toxin potency via extended the intraendoplasmic reticulum retention time.[32] Moreover, Seetharam revealed the replacement of REDLK by KDEL sequence produced a more potent toxin by the same mechanism.[33] A histidine tag followed by enterokinase site was inserted at the N-terminal of the immunotoxin to facilitate the protein purification by affinity chromatography and subsequent his-tag removal. The immunotoxin moieties were attached

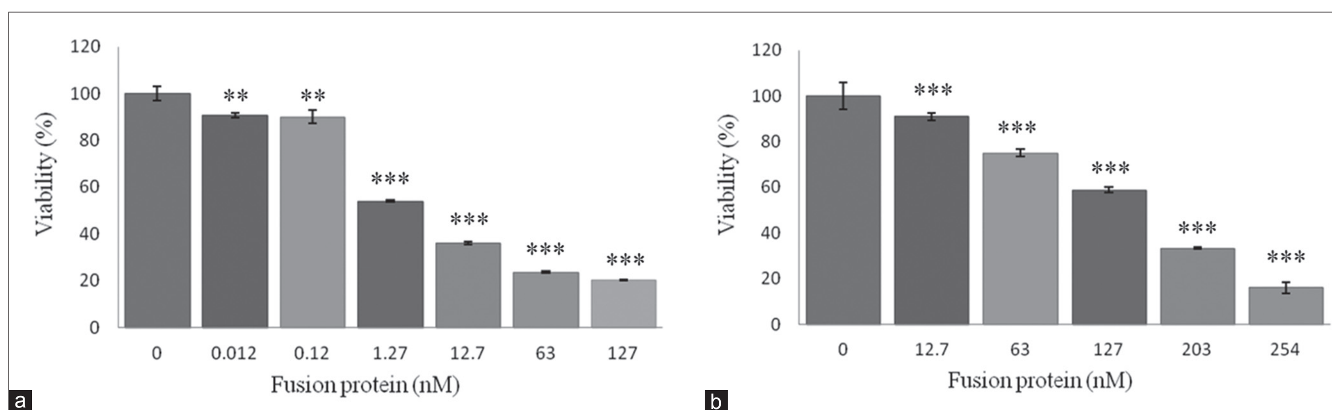

Figure 4: The cytotoxicity effect of different concentrations of VEGF121-PE38-KDEL on (a) 293/KDR and (b) HUVEC cell lines compared to the control group treated with PBS. (Mean ± SD; **: *P* <0.01; *: *P* <0.001)**

to each other by a $(Gly_4Ser)_4$ linker. Many studies demonstrated that conformational change occurs when the moieties are linked directly, resulting in less or no toxicity. Glycine and serine are suitable residues because of high degree of freedom. Therefore, the linker has a positive effect on stability, dynamics and binding properties of the immunotoxin.[34]

At first, pBAD/gIIIa expression vector (Invitogen, USA), was employed for the immunotoxin expression in the periplasmic space of BL21 (DE3). Heterologous protein expression in preplasmic space abolishes the need for refolding process, an additional step in downstream process. Unfortunately, the expression of pBAD/gIIIa vector was not successful (data not shown). Therefore, the constructed sequence was subcloned into pET-28a vector and expressed in an inclusion body form.

The expression was evaluated in various hosts, IPTG concentrations and culture media and also a time schedule was applied to achieve the high-yield expression. BL21 (DE3) and BL21 (DE3) pLysS are the most widely used strains for recombinant protein expression in *E. coli*. Furthermore, the pET vector systems are commercially optimized for these strains. Therefore, these host systems were employed for protein expression. Unfortunately, the expression level in both BL21 and BL21 plysS were not detectable (data not shown). Low-level expression observed in the mentioned strains would be interpreted by 9 rare codons in the immunotoxin sequence. It is proved that the existence of rare codon in a sequence can dramatically affects the expression level in various systems.[35] Many studies showed that Rosetta strain could be successfully used for expression of rare codon containing proteins because of a compatible plasmid which transcribes tRNAs for rare codons so, we used this host.[36]

The comparison of different time periods after IPTG induction showed that 4 h after induction, the level of the protein expression equals to that of the overnight induction in LB media. In various IPTG concentrations, the expression reached the maximum at 0.1 mM IPTG. Although the expression levels in 2XTY and TB was higher than LB media, the maximum expression of immunotoxin was observed in 2XTY, a nutrient-rich medium as illustrated in Figure 2a.

In order to remove non-specific binding proteins in washing steps, various concentrations of imidazole (60, 70, 80 and 120 mM) were analyzed and optimized concentration was found to be 70 mM (data not shown).[37]

ELISA and CD spectroscopy were used for protein refolding assessment. The CD results showed significant differences in the α-helix and β-sheet percentage after refolding step. Since, CD pattern of every engineered protein should be compared to its native form and we had no native protein related to engineered protein, the results were confirmed by ELISA test. Avastin®, a humanized monoclonal antibody which specifically binds to VEGF, was used for ELISA test. The results showed three folds increase in the absorbance of folded fusion protein compared to unfolded form in binding assay [Table 2].

The VEGFR2-expressing cells, HUVEC and 293/KDR were used to determine the fusion protein cytotoxicity. HUVEC and 293/KDR cells express $3\text{-}5 \times 10^4$ and 2.5×10^6 VEGFR2 on the cell surface, respectively.[38] The designed immunotoxin inhibited 293/KDR as a high VEGFR2 expressing cell line, with IC50 value of 1.27 nM, whereas the value for HUVEC as a low VEGFR2 expressing cell line was 130 nM. The observed IC50s indicates that cells over-expressing VEGFR2 are nearly 100 fold more sensitive compared to those with lower receptor density. As the same result was found in the other study which VEGF 121-rGelonin immunotoxin inhibited PAE/KDR and HUVEC cells with IC50 values of 30 nM and 800 nM respectively.[39,40] Interestingly, only over-expressing VEGFR2 cells can be affected at the therapeutic doses, whereas the quiescent endothelial cells were not influenced.[41]

Conclusion

we designed a novel nano-sized immunotoxin that targets specifically high VEGFR2 expressing cells, which sounds promising for further *in vivo* assays.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931-4.
2. Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 1996;93:1493-5.
3. Safran M, Kaelin WG Jr. HIF hydroxylation and the mammalian oxygen-sensing pathway. J Clin Invest 2003;111:779-83.
4. Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res 2000;60:203-12.
5. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13:9-22.
6. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 1992;267:26031-7.
7. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, *et al*. Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol 1993;143:1255-62.
8. Brown JM, Giaccia AJ. The unique physiology of solid tumors: Opportunities (and problems) for cancer therapy. Cancer Res 1998;58:1408-16.
9. Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, *et al*. AZD2171: A highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. Cancer Res 2005;65:4389-400.
10. Kreitman RJ. Immunotoxins for targeted cancer therapy. AAPS J 2006;8:532-51.
11. Allured VS, Collier RJ, Carroll SF, McKay DB. Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution. Proc Natl Acad Sci U S A 1986;83:1320-4.
12. Kreitman RJ, Pastan I. Importance of the glutamate residue of KDEL in increasing the cytotoxicity of Pseudomonas exotoxin derivatives and for increased binding to the KDEL receptor. Biochem J 1995;307:29-37.
13. Kounnas MZ, Morris RE, Thompson MR, FitzGerald DJ, Strickland DK, Saelinger CB. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. J Biol Chem 1992;267:12420-3.
14. McKee ML, FitzGerald DJ. Reduction of furin-nicked Pseudomonas exotoxin A: An unfolding story. Biochemistry 1999;38:16507-13.
15. Carroll SF, Collier RJ. Active site of Pseudomonas aeruginosa exotoxin A. Glutamic acid 553 is photolabeled by NAD and shows functional homology with glutamic acid 148 of diphtheria toxin. J Biol Chem 1987;262:8707-11.
16. Kondo T, FitzGerald D, Chaudhary VK, Adhya S, Pastan I. Activity of immunotoxins constructed with modified Pseudomonas exotoxin A lacking the cell recognition domain. J Biol Chem 1988;263:9470-5.
17. Pastan I. Immunotoxins containing Pseudomonas exotoxin A: A short history. Cancer Immunol Immunother 2003;52:338-41.
18. Benedict WF, Baker MS, Haroun L, Choi E, Ames BN. Mutagenicity of cancer chemotherapeutic agents in the Salmonella/microsome test. Cancer Res 1977;37:2209-13.
19. Khandare JJ, Minko T. Antibodies and peptides in cancer therapy. Crit Rev Ther Drug Carrier Syst 2006;23:401-35.
20. Jain RK. Delivery of molecular and cellular medicine to solid tumors. J Control Release 1998;53:49-67.
21. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A. Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. Nature 1994;367:576-9.
22. Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Curwen JO, Hennequin LF, *et al*. ZD4190: An orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. Cancer Res 2000;60:970-5.
23. Oku T, Tjuvajev JG, Miyagawa T, Sasajima T, Joshi A, Joshi R, *et al*. Tumor growth modulation by sense and antisense vascular endothelial growth factor gene expression: Effects on angiogenesis, vascular permeability, blood volume, blood flow, fluorodeoxyglucose uptake, and proliferation of human melanoma intracerebral xenografts. Cancer Res 1998;58:4185-92.
24. Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, *et al*. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res 1999;59:5209-18.
25. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, *et al*. VEGF-Trap: A VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A 2002;99:11393-8.
26. Kreitman RJ. Immunotoxins for targeted cancer therapy. AAPS J 2006;8:E532-51.
27. Grünewald FS, Prota AE, Giese A, Ballmer-Hofer K. Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. Biochim Biophys Acta 2010;1804:567-80.
28. Wild R, Dhanabal M, Olson TA, Ramakrishnan S. Inhibition of angiogenesis and tumour growth by VEGF121-toxin conjugate: Differential effect on proliferating endothelial cells. Br J Cancer 2000;83:1077-83.
29. Pastan II, Kreitman RJ. Immunotoxins for targeted cancer therapy. Adv Drug Deliv Rev 1998;31:53-88.
30. Frankel AE, Neville DM, Bugge TA, Kreitman RJ, Leppla SH. Immunotoxin therapy of hematologic malignancies. Semin Oncol 2003;30:545-57.
31. Siegall CB, Xu YH, Chaudhary VK, Adhya S, Fitzgerald D, Pastan I. Cytotoxic activities of a fusion protein comprised of TGF alpha and Pseudomonas exotoxin. FASEB J 1989;3:2647-52.
32. Pelham HR. Control of protein exit from the endoplasmic reticulum. Annu Rev Cell Biol 1989;5:1-23.
33. Seetharam S, Chaudhary VK, FitzGerald D, Pastan I. Increased cytotoxic activity of Pseudomonas exotoxin and two chimeric toxins ending in KDEL. J Biol Chem 1991;266:17376-81.
34. Zhang J, Liu S, Shang Z, Shi L, Yun J. Analysis of the relationship between end-to-end distance and activity of single-chain antibody against colorectal carcinoma. Theor Biol Med Model 2012;9:38.

35. Angov E. Codon usage: Nature's roadmap to expression and folding of proteins. Biotechnol J 2011;6:650-9.
36. Tegel H, Tourle S, Ottosson J, Persson A. Increased levels of recombinant human proteins with the *Escherichia coli* strain Rosetta (DE3). Protein Expr Purif 2010;69:159-67.
37. Petsch D, Anspach FB. Endotoxin removal from protein solutions. J Biotechnol 2000;76:97-119.
38. Backer MV, Budker VG, Backer JM. Shiga-like toxin-VEGF fusion proteins are selectively cytotoxic to endothelial cells overexpressing VEGFR-2. J Control Release 2001;74:349-55.
39. Veenendaal LM, Jin H, Ran S, Cheung L, Navone N, Marks JW, *et al*. *In vitro* and *in vivo* studies of a VEGF121/rGelonin chimeric fusion toxin targeting the neovasculature of solid tumors. Proc Natl Acad Sci U S A 2002;99:7866-71.
40. Arora N, Masood R, Zheng T, Cai J, Smith DL, Gill PS. Vascular endothelial growth factor chimeric toxin is highly active against endothelial cells. Cancer Res 1999;59:183-8.
41. Couffinhal T, Kearney M, Witzenbichler B, Chen D, Murohara T, Losordo DW, *et al*. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in normal and atherosclerotic human arteries. Am J Pathol 1997;150:1673-85.