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Validation of Different Systems for Tumstatin Expression and its *in-vitro and in-vivo* Activities

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Abstract

Research Article

The aim of the present study is to identify an effective and efficient expression system for purification of recombinant antiangiogenic protein tumstatin. The sequence encoding carboxy-terminal non-collagenous domain of $\alpha 3$ chain Type IV collagen, $\alpha 3$ (IV)NC1 (tumstatin) was isolated from human placental tissue and cloned in three different expression vectors pET22b, pcBFT and pAcHLT-A to express it in bacteria, mammalian and Sf-9 insect cells respectively. Expression and purification profiles of tumstatin were evaluated by coomassie staining and immunoblotting, and the efficiency was determined based on the yields of soluble protein. Our results indicate that, baculovirus expression system was efficient for scalable yields of soluble protein that could be purified in its biologically active form. This baculovirus expressed tumstatin was used to evaluate its anti-angiogenic and anti-tumarogenic functions such as inhibition of endothelial cell proliferation, cell viability, migration, tube formation, cap dependent protein translation and the associated signaling mechanism including in-vivo tumor study. Our evaluated approaches using a modified baculovirus expression system shows high expression and high yield of biologically active tumstatin, as compared to two expression systems, indicating baculovirus expression system to be an ideal method for bulk production of soluble tumstatin that needed for preclinical and clinical trails.

Abbreviations

Tumstatin: non-collagenous α 3 chain of type IV collagen; AcNPV: Autographa californica nuclear polyhedrosis virus; *Sf-9*: Spodoptera frugiperda; HEK-293: Human Embryonic Kidney cells; mTOR: mammalian Target of Rapamycin and LLC lung lewis carcinoma.

Introduction

Vascular basement membrane (VBM) is an important constituent of blood vessels providing structural support (Darland and D'Amore, 1999). VBM was also speculated to modulate capillary endothelial cell behavior especially during sprouting of new capillaries (Darland and D'Amore, 1999). During matrix re-organization, several short protein fragments are generated from VBM by proteases. Some of these fragments were identified as inhibitors of angiogenesis (Boosani et al., 2007; Kalluri, 2003; O'Reilly et al., 1997; Petitclerc et al., 2000; Reynolds et al., 2002; Sudhakar, 2009; Sudhakar and Boosani, 2007; Sudhakar and Boosani, 2008; Sudhakar et al., 2005). At present there are about 25 endogenous angioinhibitors in clinical trials and many more in preclinical studies for the treatment of cancer. These angioinhibitors fall into two general categories: (a) antibodies or small molecules that target pro-angiogenic factors of tumor cells such as VEGF, bFGF or PDGF, and (b) endogenous angioinhibitors such as thrombopondin-1, angiostatin, interferons, endostatin and some of the non collagenous (NC1) domains of Type IV collagen that target vascular endothelial cells (Boosani et al., 2007; O'Reilly et al., 1997; O'Reilly et al., 1994; Petitclerc et al., 2000; Qian et al., 1997; Sudhakar and Boosani, 2007; Sudhakar and Boosani, 2008; Sudhakar et al., 2005). This novel discovery of NC1 domains from human Type IV collagen as angioinhibitors initiated a new line of research in several laboratories and identified their significance for the treatment of cancer (Boosani et al., 2007; Boosani and Sudhakar, 2006; Borza et al., 2006; Maeshima et al., 2002; Marneros and Olsen, 2001; Petitclerc et al., 2000; Roth et al., 2005; Sudhakar and Boosani, 2007; Sudhakar et al., 2005; Sudhakar et al., 2003).

Tumstatin (α 3(IV)NC1) a 28 kDa cryptic domain is liberated from the carboxy terminal region of α 3 chain type IV collagen by matrix metalloproteases was shown to inhibit proliferation of melanoma and epithelial tumor cell lines *in-vitro* by binding to CD47/ α V β 3/ α 3 β 1 integrin complex (Boosani et al., 2007; Borza et al., 2006; Han et al., 1997; Hemmings, 1997; Maeshima et al., 2000; Monboisse et al., 1994; Petitclerc et al., 2000; Qian et al., 1997; Shahan et al., 1999; Sudhakar and Boosani, 2007; Sudhakar et al., 2003). We previously reported that tumstatin binds to α V β 3/ α 3 β 1 integrins and inhibits PI3K/Akt/mTOR/ 4E-BP1/COX-2 signaling leading to the inhibition of protein synthesis and hypoxic tumor angiogenesis (Boosani et al., 2007; Maeshima et al., 2002; Sudhakar et al., 2003). Tumstatin

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was also reported to inhibit tumor angiogenesis and tumor growth in mice by up-regulating apoptosis in endothelial cells (Petitclerc et al., 2000). These studies indicate that tumstatin has complex signaling mechanisms in regulating tumor angiogenesis which are to be explored, and for such studies a suitable expression system is needed to over express and purify the protein in its biologically active form.

In the present study, we isolated the coding sequence of tumstatin from human placenta, expressed it in three different expression systems and compared the expression and purification strategies in obtaining the recombinant protein in a biologically active suitable form for *in-vitro* and *in-vivo* studies. Among the three systems tested, baculovirus expression system was found effective for production of soluble tumstatin protein. This expression system provides ambient conditions for proper folding, disulfide bond formation and oligomerization of the over expressed recombinant proteins besides correct post-translational modifications. Baculovirus expressed purified tumstatin protein when tested showed its characteristic angioinhibitory functions such as inhibition of endothelial cell proliferation, translation, tube formation and the associated signaling pathway including inhibition of tumorangiogenesis in LLC tumors. Our studies demonstrate that the baculovirus expression system is ideal for bulk production of tumstatin in its biologically active form, and this method may be suitable for bulk production of this angioinhibitor for pre-clinical and future clinical studies.

Materials and Methods

Baculovirus transfer vector pAcHLT-A, transfecting agent lipofectin, Affinity matrix (Ni-NTA agarose) were obtained from PharMingen, USA. The plasmid pBlueScriptII-SKplus (pBSIISKP) was purchased form Stratagene. Plasmid pET22b expression vector was purchased from Novagen. Recombinant human bFGF and VEGF were obtained from R&D systems. Horseradish peroxidase (HRP)-labeled secondary antibodies, penicillin/streptomycin, fibronectin (FN) low melting Agarose and neutral red staining solution and sepharose beads were obtained from Sigma-Aldrich. Primary HUVECs were purchased from ClonetechTM Inc and LLC tumor cells were purchased from ATCC (CRL-1642). BD Martigel™ (14.6 mg/ml) was purchased from BD Biosciences Discovery lab. Recombinant human endostatin was obtained from R&D Systems (Minneapolis, MN). Graces insect cell culture medium, cell fixer and staining H&E and X-Ray films (Kodak) were purchased from Fisher Scientific Inc. Protease inhibitor solutions were purchased from Boehringer and Mannheim, GmbH. FCS, ECL Kit, Random primer labeling kit, hybond N⁺ membrane, $[\gamma^{32}P]dCTP$, DNA ladder, competent cells, DNA polymerase I, klenow fragment and Multiprime DNA labeling system were purchased from Amersham Biosciences. Trizol, ThermoScript RT-PCR system, Lipofectamine Plus, E. coli strain BL21(DE3)AI and Pfx polymerase reagent were obtained from Invitrogen. Restriction enzymes were obtained from New England Biolabs.

Cell culture

Primary HUVECs were maintained in EGM-2 medium at 37°C in a humidified 5% CO₂ incubator. HUVECs passages 3-5, were used for studies. LLC cells were maintained in DMEM medium supplemented with 10% FCS with 100 µg/ml of antibiotic and antimycotic solution. *Sf-9* cells were grown as monolayer cultures in TNM-FH medium supplemented with 10% FCS and with 100 µg/ml of antibiotic and antimycotic solution. *Sf-9* cell viability was tested and cell cultures with above 95% cell viability were used for transfection and expression studies. *Sf-9* ells were fed with fresh culture medium for every two days and were passed routinely at 80% confluence.

Isolation and characterization of recombinant tumstatin clones

Total RNA was isolated from human placental tissue using Trizol, and cDNA synthesis was carried out using ThermoScript RT-PCR system following manufacturer's guidelines. The coding sequence corresponding to α 3 Type IV NC1 (720 bp, GenBank accession number EU268882) was PCR amplified from

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1	-cg	tgg	aga	cag	tgg	atc	acc	tgc	aac	ctg	gac	aac	gag	agg	ctt	tgt	ctt	cac	ccg	acac
21	S	-Q-	T	T	_A_	I	P	S	-C-	P	E	G	T	V	P	L	¥.	S	G	F
61	ag	tca	aae	cae	age	aat	tee	tte	atg	tee	aga	ddd	gac	agt	gee	act	cta	eag	tgg	gttt
41	S	F	L	F	V	Q	G	N	Q	R	A	H	G	Q	D	Ŀ	G	T	L	G
121	tc	ttt	tct	ttt	tgt	aca	agg	aaa	tca	acg	agc	cca	egg	aca	aga	eet	tgg	aac	tct	tggc-
<u>61</u>	S			Q	R	F	<u> </u>	<u> </u>	<u>M</u>	<u>P</u>	F	<u> </u>	F		N	<u>V</u>	N			
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101	N	M	A	P	I	T	G	R	A	L	E	P	Y	I	S	R	C	y ac T	V	C
301	aa	cat	aac	tcc	cat	tac	taa	caq	age	cct	tga	acc	tta	tat	aaq	caq	atq	cac	tqt	ttqt
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361	ga	agg	tcc	tgc	gat	ege	cat	agc	cgt	tca	cag	cca	aac	cac	tga	cat	tcc	tcc	atg	tcct
141	H	G	W	I	S	L	W	K	G	F	S	F	I	M	F	T	S	A	G	S
421	са	egg	ctg	gat	ttc	tct	ctg	gaa	agg	att	ttc	att	cat	cat	gtt	cac	aag	tge	agg	ttct
161	E	G	T	G	Q	-A-	<u> </u>	A	S	P	-G-	_ <u>S</u>		<u> </u>	E	E	F	R	A	S
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Table 1: Human tumstatin coding sequence and translation map.

the above cDNA using Platinum *Pfx* polymerase and Forward primer 5'-CCGTGGAGACAGTGGATC-3' and Reverse primer 5'-GTGTCTTTTCTTCATGCACA-3' sequences. PCR amplification was performed in PTC-100 Programmable Thermal Controller from MJ Research Inc, with an initial denaturation at 94°C for 2 mins, followed by 35 cycles of "94°C/1min-58°C/ 1min-68°C/1min", and with final extension at 68°C/5mins. The resulting amplicon was cloned at EcoRV site in pBlueScriptII-SKplus vector and the recombinant clones "pBSIISK/ tumstatin" were identified by blue white selection. The recombinant clones "pBSIISKP/tumstatin" were sequenced using high-throughput DNA sequencing facility at University of Nebraska Medical Centre (UNMC) and the sequence was confirmed following a BLAST search. The translation map of tumstatin coding sequence was shown in Table 1.

Cloning and expression of tumstatin in Sf-9 insect cells

The sequence encoding human tumstatin was PCR amplified from the plasmid vector pBSIISK/tumstatin using Pfx polymerase and forward primer (5'-CGCCATATGCCGTGGAGACA GTGGATC-3') and reverse primer (5'-GCGAGATCTTCA GTGTCTTTTCTTCATGCACA-3') sequences that were modified to incorporate Nde I and Bgl II restriction sites. The resulting amplicon was digested with Nde I and Bgl II restriction enzymes and ligated into baculovirus transfer vector pAcHLT-A (BD PharMingen) predigested with the same restriction enzymes. The recombinant baculovirus transfer vector harboring Tumstatin, pAcHLT-A/tumstatin with 6xHis tag located Nterminal to tumstatin (enables simple one-step protein purification using Ni-NTA agarose affinity chromatography) were screened through colony hybridization and autoradiography as reported earlier by us (Boosani et al., 2007; Boosani and Sudhakar, 2006; Sudhakar et al., 1999; Sudhakar et al., 2000). The recombinant transfer vector pAcHLT-A/tumstatin was cotransfected with Bsu361 digested linearized BaculogoldTM(Cat No: 21100D) viral DNA into Sf-9 cells using Bacfectin to obtain an infectious complete viral genome (Boosani et al., 2007; Boosani and Sudhakar, 2006; Sudhakar et al., 1999; Sudhakar et al., 2000). After 72 hr of co-transfection the medium supernatant containing recombinant viral particles harboring tumstatin was collected and used in plaque assay, where six out of 18 plaques tested were found positive. The recombinant virus harboring tumstatin was confirmed by dot-blot hybridization using the radioactive probe corresponding to 720 bp tumstatin cDNA, similarly to our earlier reports (Boosani et al., 2007; Boosani and Sudhakar, 2006; Sudhakar et al., 1999; Sudhakar et al., 2000). Positive recombinant virus identified from dot blot hybridization was used for further amplification of virus and a high viral titer (MOI-10) was used to infect Sf-9 cells for expression studies as reported previously (Boosani et al., 2007; Boosani et al., 2009b; Boosani and Sudhakar, 2006; Sudhakar et al., 1999; Sudhakar et al., 2005; Sudhakar et al., 2000). Sf-9 cells after 72 hrs of post infection were washed twice with ice cold PBS (pH 6.2) and lysed in buffer containing 20 mM tris-Hcl pH 7.8, 250 mM NaCl, 1 mM MgCl, 1 mM DDT, 0.2% TritonX-100 and 1x complete protease inhibitor cocktail (Roche). The lysate was centrifuged at 13000 rpm for 30 min at 4°C and the clear supernatant containing recombinant protein was quantified and analyzed by SDS-PAGE before being used for protein purification.

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Metal affinity purification of tumstatin

Lysate containing about 40 mg of total protein was mixed with Ni-NTA agarose affinity matrix (1ml bed volume, binding capacity 20 mg/ml), and incubated for 1 hr at 4°C on a rocker and centrifuged at 500xg for 5 min at 4°C. The supernatant was discarded and the matrix with bound protein was washed once with lysis buffer containing 1x complete protease inhibitor cocktail, and then thrice with wash buffer containing 100 mM NaH₂PO₄, 10 mM Tris HCl, 250 mM NaCl and 50mM Imidazole and 1x complete protease inhibitor cocktail (Roche), final pH 8.0. The 6xHis-tagged tumstatin protein bound to the matrix was eluted in elution buffer containing 100 mM NaH₂PO₄, 250 mM NaCl and 250mM Imidazole and 1x complete protease inhibitor cocktail (Roche) final pH 8.0. For purification of E. coli expressed tumstatin protein, the above wash and elution buffers were supplemented with 3M GuHCl and 4M Urea. Tumstatin expressed in Sf-9 cells has an N-terminal 6xHis tag and a downstream thrombin cleavage site to remove 6xHis tag by Thrombin digestion. The same protein expressed in E. coli has 6xHis tag at C-terminal end without any thrombin cleavage site. The protein eluted from the affinity matrix was dialyzed and concentrated by 0-80% ammonium sulphate precipitation. Both soluble and insoluble protein was suspended in equal volume of PBS and stored at -80°C. Concentration of soluble and insoluble protein was quantified using Biorad protein assay kit and analyzed on SDS-PAGE.

Cloning and expression of tumstatin in E. coli BL21(DE3)Al

The coding sequence corresponding to tumstatin was amplified from "pBSIISK/tumstatin" using Pfx polymerase, and the following primer sequences that were modified to incorporate NdeI and XhoI restriction sites; forward primer (5'-CGCCATATGCGTGGAGACAGTGGATC-3') and reverse primer (5'-GCGCTCGAGGTGTCTTTTCTTCATGCACA-3'). The amplicon was digested and cloned at the same sites in pET22b, upstream to 6xHis tag. The recombinant plasmid "pET22b/ tumstatin" was used to transform E. coli BL21(DE3)AI for expression. A 1:100 dilution of the overnight starter culture was used to inoculate 1 liter culture in LB (Laura Bertani) medium and the culture was grown for 2 hr at 220 rpm and 37°C, where the cells reached an O.D A_{600} of 0.421. The expression of the recombinant protein was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 1% L-Arabinose with further incubation for 4 hr at 220 rpm and 37°C. The induced culture was then centrifuged at 3000 rpm for 10 mins and the bacterial cell pellet was suspended in buffer containing 100 mM NaH₂PO₄, 10 mM Tris HCl and 250 mM NaCl (pH 7.6). The bacterial suspension was sonicated on ice and centrifuged at 13000 rpm for 30 min at 4°C. The resulting bacterial pellet containing recombinant protein was suspended in lysis buffer containing 3 M Guanidine, 4 M urea, 100 mM NaH₂PO₄, 10 mM Tris HCl and 250 mM NaCl (pH 7.6) and centrifuged at 13K for 30 mins at 4°C. The clear supernatant obtained after centrifugation was used for protein purification using metal affinity purification as stated above.

Cloning, expression and purification of tumstatin in HEK-293 cells

The coding sequence of tumstatin was amplified from pBSIISK/tumstatin using forward primer (5'-CGCGGATCCCGT

GGAGACAGTGGATC-3') and reverse primer (5'-GCGCTCG AGGTGTCTTTTCTTCATGCACA-3') sequences, and cloned in frame at BamHI and XhoI sites of mammalian expression vector pcBFT downstream to BM40 secretion tag, Flag tag and Thrombin cleavage sites. The recombinant vector pcBFT/ tumstatin was used to transfect HEK-293 (human embryonic kidney) cells using lipofectamine plus reagent. The transfected cells were selected using G418 (500 μ g/ml) and the resistant colonies were cultured in 150 mm culture plates containing DMEM-F12 medium. When the cells reached about 90% confluence they were fed with serum free medium and after every 48 hrs medium supernatant was collected, pooled and stored at 4°C. About 2 liters of medium supernatant containing the secreted recombinant tumstatin protein was concentrated by 0-80% ammonium sulphate precipitation and the resulting pellet was dissolved in 4 ml of buffer containing 20 mM Tris-Hcl pH 7.8, 2 mM Mg(OAc), 50 mM KCl, 5% glycerol and 100 µM EDTA, and subjected to sepharose immunoaffinity chromatography using anti-tumstatin antibody. The affinity column was washed twice with 50 mM KCl buffer and the bound tumstatin protein was eluted with 0.4 M KCl buffer as described previously (Gunwar et al., 1991; Neilson et al., 1993). The total eluted protein was digested with Thrombin to remove BFT sequence, dialyzed and concentrated by 0-80% Ammonium sulphate precipitation and both insoluble and soluble protein was suspended in equal volume of PBS as described above.

Proliferation assay

A suspension of about 5.0×10^4 HUVECs/well in a 24 well plate were serum starved, trypsinized and transferred to a fresh 24-well plate pre-coated with fibronectin and cultured for 24 hr in EGM-2 medium containing various concentrations of recombinant baculovirus expressed tumstatin. After 24 hr of treatment 1µl of [³H]-thymidine was added into each well and incubated further for 72 hr at 37°C. [³H]-thymidine incorporation as a direct means of cell proliferation was measured using scintillation counter as described previously by us (Boosani and Sudhakar, 2006; Sudhakar et al., 2005; Sudhakar et al., 2003).

Protein synthesis assay

Cells were serum starved for 24 hr and then stimulated with 10% FCS in presence of baculovirus expressed tumstatin for 48 hr. Cells were pre-incubated in methionine-free medium for 1 hr and then incubated in culture medium containing radioactive methionine for 1 hr at 37°C. The incorporation of radioactivity into trichloroacetic acid precipitates was analyzed as described previously (Maeshima et al., 2002; Sudhakar et al., 2003). ANOVA with a one tailed student's *t* test was scored to identify significant differences in multiple comparisons in the present study. A level of *P*<0.05 was considered statistically significant.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide assay (MTT)

HUVECs, about 8 x 10³ cells/well, were plated on a 96-well plate in EGM-2 medium. After 24 hr, various concentrations of baculovirus expressed tumstatin was added, and after 24 hr of incubation cell viability was assessed by MTT assay, following our previously described protocols (Boosani and Sudhakar, 2006).

Migration assay

About 1 x 10⁴ HUVECs in 30 µl of incomplete medium were pre treated with and without baculovirus expressed recombinant tumstatin protein (1 µM) and seeded into each upper well of the Boyden chamber. In the lower wells of Boyden chamber, ICM containing 25 ng/ml bFGF was added and the cells were incubated for 24 hr at 37°C with 5% CO₂ ICM alone was used as negative control. α 1(IV)NC1 (1 µM) was used as control since it is a known inhibitor of endothelial cell migration. The number of cells that migrated and attached to the under side of the membrane were stained with H&E and observed through Olympus CK2 light microscope as reported (Boosani et al., 2009a; Sudhakar et al., 2005; Sudhakar et al., 2003).

Tube formation assay

Matrigel matrix was thawed overnight at 4°C and 250 µl of matrigel matrix added to each well of a chilled 24 well plate and allowed to polymerize for 30 min at 37°C. A suspension of 5x10⁴ HUVECs in EGM-2 without antibiotics was plated on top of the matrigel coated wells and the cells were treated with or without 1.0 mM baculovirus expressed tumstatin or arresten (control). After incubation for 48 hr at 37°C, tube formation was observed using Olympus CK2 light microscope as reported (Boosani et al., 2009a; Sudhakar et al., 2003).

Cell signaling experiments

About 10⁶ HUVECs were seeded on a 10 cm² petridish coated with fibronectin and the monolayer culture was pre-incubated with baculovirus expressed turnstatin $(1 \,\mu M)$ or endostatin $(1 \,\mu M)$ μ M) for 0-60 min. The cells were then lysed in 200 μ l of lysis buffer containing 20 mM tris-Hcl pH 7.8, 1 mM Mg²⁺, 1 mM DDT, 1X pepstatin A, 1X leupeptin, 1X aprotinin, 1 mM PMSF and 80 mM KCl. Cell extracts were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane by western blotting. The proteins involved in the anti-angiongenic signaling were evaluated following immunoblotting using phosphorylated and unphosphorylated antibodies specific to FAK (tyr397; BioSource International.), Akt (Ser473; New England Biolabs Inc.), mTOR (ser 2448; Cell Signaling), 4E-BP1 (thr 37/46; Cell Signaling) proteins, similar to our previous studies (Boosani et al., 2007; Maeshima et al., 2002; Sudhakar et al., 2005; Sudhakar et al., 2003).

In-vitro kinase assay for mTOR activity

Phosphorylation of mTOR and GST-4EBP-1 fusion protein (mTOR substrate) was evaluated in HUVECs transfected with HA-mTOR/FRAP expression vector. Briefly, HUVECs were serum-starved and transiently transfected with HA-mTOR/FRAP plasmid using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Transfected HUVECs ($4x10^6$ cells) were treated with equimolar concentrations of baculovirus expressed tumstatin or endostatin for 24 hr as explained earlier (Maeshima et al., 2002; Sudhakar et al., 2003). The cells were lysed and 200 µg of extracts was subjected to immunoprecipitation with anti-HA antibody (Boosani et al., 2007; Sudhakar et al., 2003). HAmTOR/Anti-HA complexes were incubated with recombinant GST-4E-BP1 fusion protein in presence of 10 µCi of [γ^{32} P]dCTP in kinase buffer. The reactions were terminated by boiling and the samples were subjected to 10% SDS-PAGE and western



Figure 1: Expression and purification of tumstatin in *Sf-9* insect cells.

(A) pAcHLT-A harboring tumstatin: The 720 bp coding sequence corresponding to tumstatin was cloned between Nde I and Bgl II sites of baculovirus transfer vector, pAcHLT-A. (B) Time course expression of tumstatin in *Sf-9* cells: Each lane contains about 30 μ g of total *Sf-9* cells extract. Lane 1 shows cell extracts at 0 hr time point (control). Lanes 2, 3, 4, 5 & 6 represent *Sf-9* cell extracts from 6, 12, 24, 48 & 72 hr of tumstatin protein expressed in *Sf-9* cells: Lane 1 shows molecular weight marker; lane 2 and 3 show 10 μ l of flow through; lanes 4 and 5 show insoluble and soluble tumstatin protein (total soluble and insoluble tumstatin protein expression of the gel. B and C are coomassie stained 10% SDS-PAGE gel. Arrows in B and C indicate tumstatin protein.

immunobloted as reported (Boosani et al., 2007; Maeshima et al., 2002; Sudhakar et al., 2005; Sudhakar et al., 2003). The phosphorylated proteins of mTOR-P and GST-4EBP1-P were detected by autoradiography as described previously (Gingras et al., 2001; Kumar et al., 2000; Maeshima et al., 2000; Maeshima et al., 2002; Sudhakar et al., 2003).

Immunohistochemical staining

Briefly, 4-mm frozen tumor sections were fixed in 100% acetone for 3 min at -20°C and air-dried. The tumor sections were incubated with primary rat anti-mouse CD31 antibody at room temperature for 1 hr. Subsequently, tumor sections were washed with PBS and incubated with tetramethyl rhodamine-conjugated secondary antibody at same conditions about 1hr. In each group, the numbers of CD31-positive blood vessels were counted in 10 fields in a blinded fashion as previously described (Sudhakar et al., 2005).

In-vivo tumor studies using C57BL/6 mice

Sixteen male four months old mice were used for this tumor study. Mice backs were shaved and about 1.0x10⁶ LLC cells

were injected subcutaneously into the back of each mouse. Ten days after tumor cells implantation, the mice were divided into 2 groups (8 each). For the experimental group, tumstatin was intravenously injected daily at 1mg/kg per body weight or 30 μ g per mouse, while only sterile PBS was injected into the control group mice. When control mice tumors reached about 2.0 cm³, mice were sacrificed and the tumors were frozen for histological analysis (Boosani et al., 2007; Martin and Evans, 1975; Sudhakar et al., 2005).

Measurement of circulating endothelial cells

About 400 μ l of control and tumstatin treated tumor bearing mice blood was collected before scarifying in EDTA/heparin containing microcentrifuge tubes. Blood plasma was separated and 300 μ l of DMEM supplemented with 10% FBS was added to each tube. Red blood cells were removed with RBC lysis solution and the mixture was placed on 8-chamber slides about 6hr and incubated at 37°C. The attached endothelial cells were stained with anti-VEGFR2 antibody as reported (Boosani et al., 2007). The positive cells were counted under the fluorescence microscope in 10 fields at a magnification of 200 X as reported (Boosani et al., 2007).

Statistical Analysis

Statistical differences between control and tumstatin treated LLC tumor groups were calculated using Student's t test. Analysis of variance (ANOVA) was used to determine statistical differences among the groups. A p value <0.001 was considered statistically significant.

Results and Discussion

In the present work, we show evaluation of the purification systems and the methods employed in obtaining the recombinant protein in a biologically active form through cloning, expression and purification of α 3 chain type IV collagen non-collagenous domain (tumstatin) using three different expression systems. The purification profiles and strategies were compared for their efficiencies in yield and biological activity of baculovirus expressed tumstatin *in-vitro* and *in-vivo*.

Purification of tumstatin from Sf-9 cell cultures

The recombinant baculovirus transfer vector pAcHLT-A/ tumstatin (Figure 1A) was co-transfected with linearized Baculogold viral DNA into Sf-9 cells to obtain an infectious complete viral genome. About $2x10^6$ Sf-9 cells were transfected with recombinant virus (MOI-10) and extracts were prepared from 0, 12, 24, 48 and 72 hrs of post-infection as reported earlier (Boosani and Sudhakar, 2006; Sudhakar et al., 1999; Sudhakar et al., 2000). Sf-9 cell extracts were analyzed on a 10% SDS-PAGE gel to monitor the time course expression of tumstatin, where a single band corresponding to 28 kDa was detected whose concentration increased with increase in postviral infection time (Figure 1B, lanes 2 to 6). Where as wild type AcNPV virus infected Sf-9 cells did not show any such expression (data not shown). The over expressed protein cross reacted with anti-tumstatin antibody as observed through Western blotting (data not shown). The coomassie stained gel detailing the purification profile of recombinant tumstatin was shown, wherein about 10 μ g of total protein from each fraction collected during the purification process was loaded



Figure 2: Expression of tumstatin in *E. coli* and mammalian cells. (A) Vector map of pET22b harboring tumstatin: Tumstatin was cloned between NdeI and XhoI sites of bacterial expression vector pET22b. (B) *E. coli* expressed solubile tumstatin protein: Lane 1, shows molecular weight marker; lane 2 shows about 10 μ l of column flow through; lane 3 and 4 show total insoluble and soluble tumstatin protein (total soluble and insoluble tumstatin protein equal volume of PBS and 2 μ l was loaded in each lane). (C) Vector map of plasmid pcBFT harboring tumstatin: Tumstatin was cloned in frame between BamHI and XhoI sites of mammalian expression vector, pcBFT. (D) HEK-293 cell expressed soluble tumstatin: Lane 1 is a molecular weight marker; lane 2 shows cytosolic extracts from HEK-293 cells; lanes 3 and 4 show soluble and insoluble tumstatin fractions similar to B. B and D are coomassie stained 10% SDS-PAGE gel.

in each well of the gel (Figure 1C, lanes 2-3). The Ni-NTA column bound tumstatin protein was eluted, dialyzed and concentrated by 0-80% ammonium sulphate precipitation. About 2 μ l of insoluble and soluble tumstatin protein was diluted in 10 μ l of PBS and analyzed on 10% SDS-PAGE gel to compare their yields (Figure 1C, lanes 4-5).

Purification of tumstatin using bacterial expression system

The tumstatin coding sequence was cloned in frame in pET22b, which resulted in the recombinant plasmid "pET22b/ tumstatin" (Figure 2A). Chemically competent BL21(DE3)AI cells were transformed with "pET22b/tumstatin" and a single colony derived log phase culture was induced with 1mM IPTG and 0.2% L-Arabinose. E. coli cell extracts from different time intervals were analyzed. The time course expression of tumstatin in E. coli was studied to identify the post induction time at which maximum protein induction is observed. The expression of tumstatin protein increased with increase in post induction time (data not shown). After induction, bulk extracts from 1 liter culture were prepared for protein purification using Ni-NTA column as described in the methods section. About 10 ug of total protein from each collected fraction was analyzed on a 10% SDS-PAGE (Figure 2B). About 2 μl of insoluble and soluble tumstatin protein was diluted to 10 µl in PBS and analyzed on a 10% SDS-PAGE gel to compare their yields (Figure 2C, lanes 4-5).

Purification of tumstatin using mammalian expression system

The coding sequence for tumstatin was also cloned in eu-

karyotic expression vector pcBFT (Figure 2C). Human embryonic kidney cells (HEK-293) were transfected with recombinant plasmid pcBFT/tumstatin and selected using G-418 sulphate. About 2 liters of HEK-293 culture medium supernatant from cultured confluent cells containing the secreted tumstatin protein was pooled, concentrated and subjected to antitumstatin antibody based affinity chromatography. Different fractions obtained during purification process were analyzed by SDS-PAGE and coomassie staining (Figure 2D). The cytosolic extracts, soluble and insoluble protein obtained after 0-80% Ammonium sulphate precipitation were analyzed on a 10% SDS-PAGE gel (Figure 2F, lanes 2-4).

Comparison of the three expression systems

The soluble and insoluble tumstatin protein from three different expression systems (Sf-9 insect cells, E. coli and HEK-293 cells) after dialysis was quantified and compared (Table 2). Expression of bulk amounts of tumstatin using bacterial system is less laborious and requires shorter time compared to Sf-9 insect cells and HEK-293 cell systems. The advantage of using bacterial system is that one can express bulk amounts of recombinant tumsatin protein in a shorter period of time. Although bacterial expression results in high yields of recombinant tumstatin protein, most of it is insoluble after dialysis. Refolding of the insoluble protein is laborious, and may also lead to improper folding of the proteins. Added to this the small fraction of soluble tumstatin protein obtained contains endotoxins, making the protein inapplicable for in-vitro studies. Hence expression of this extracellular matrix type IV collagen derived tumstatin protein in E. coli does not appear to be an ideal system for bulk production in its biologically active form. Further, the down stream processes involved in removal of endotoxins and protein refolding will greatly decrease the net yield.

HEK-293 cell expression system is too laborious, time consuming and expensive, besides bulk production of recombinant protein requires longer propagation of transfected HEK-293 cells. Most of the protein is soluble which eliminates the need for protein refolding, and secretion of the protein into the medium supernatant is an added advantage. However, a large amount of culture media needs to be pooled for purification and the yield obtained is very less which is not sufficient to carryout in-vivo studies. For bulk production in HEK-293 cells, very large volumes of medium supernatant needs to be pooled and stored before purification, and this requires prolonged freezing and thawing affecting the yield of the tumstatin protein. In addition secreted tumstatin proteins may have short half-life and since the culture conditions were maintained at 37°C, the over expressed tumstatin proteins are liable for degradation by proteases.

For purification of recombinant tumstatin protein using baculovirus expression system, a significant amout of time is required to develop the infectious virus, this is the only time consuming step involved but once the recombinant virus is made with high titer, the purification procedures are similar to bacterial system. About 95% of the recombinant protein expressed in *Sf-9* cells was soluble, while the protein expressed in HEK-293 cells and *E. coli* systems yielded 98% and 5% of total soluble protein respectively (Table 2). Several factors

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Expression host	E. coli	HEK-293 cells	Sf-9 cells			
Culture size	1 Liter	150mm x 20mm (2 liter medium supernatant)	5 plates (150mm x 20mm) $\sim 3x10^7$ cells/plate			
Total protein	~ 700 mg	~ 100 mg	~ 350 mg			
Column matrix	Ni-NTA agarose	Affinity chromatography	Ni-NTA agarose			
Column volume	15 ml	10 ml	10 ml			
Total column bound protein	~ 82 mg	~60 mg	~ 150 mg			
Total column eluted protein	~ 27 mg	~11 mg	~ 62 mg			
Insoluble protein	~23.6 mg	~0.55 mg	~1.35 mg			
Soluble protein	~1.415 mg	~5.516 mg	~24.753 mg			
% yield of soluble protein	~5.65 %	~90.9 %	~94.8 %			
Endotoxin	+	-	-			

Table 2: Comparing different expression systems for tumstatin expression and purification.





(A) Protein synthesis assay: Inhibition of protein synthesis by tumstatin in endothelial cells: Uptake of radioactive methionine in serum starved HUVECs after 24 hrs incubation with media containing 10% FCS and various concentrations of tumstatin. Methionine incorporation in cell lysates was measured using liquid scintillation counter. The data shown is from three independent experiments. (B) Cell proliferation assay: The graph summarizes results from three independent experiments on the relative [³H]-thymidine incorporation in HUVECs upon treatment with various concentrations tumstatin for 48 hr. (C) Cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT): The graph shows decrease in cell viability in HUVECs with increase in tumstatin concentration. A-C, NC represents negative control (cells grown in 0.1% FCS medium) and PC represents positive control (cells grown in 10% FCS medium).

such as high viral titer, higher *Sf-9* cell viability, temperature etc. needs to be optimized which will further enhance the net yield of the expressed tumstatin protein in baculovirus expression system. Our results indicate that bacculovirus expression system produces tumstatin protein without affecting its native properties, and in soluble quantities much higher than *E. coli* and HEK-293 systems. This baculovirus expressed purified soluble tumstatin was used in functional studies described below. The biologically activity and the quantity of the soluble tumstatin protein obtained using bacculovirus system was significantly higher compared to *E. coli* or HEK-293 systems. Micromolar quantities of protein purified using baculovirus was effective in some of the angioinhibitory studies tested here.

Functional characterization of the baculovirus expressed recombinant human tumstatin protein

Inhibition of protein synthesis in HUVECs was tested using baculovirus expressed recombinant tumstatin protein. Protein synthesis in HUVECs was inhibited in a dose dependent manner, where a concentration of 1.0 μ M tumstatin showed about 50% inhibition of protein synthesis (Figure 3A). Similarly, we also tested and observe dose dependent inhibition of HUVECs

proliferation upon treatment with tumstatin (Figure 3B). Also using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay was carried using different doses of tumstatin on endothelial cells and observed dose dependent inhibition of cell viability (Figure 3C).

Migration of endothelial cells has been shown to have an important early role in neovascularization (Kung et al., 2000; Sudhakar et al., 2005). Thus, we tested baculovirus expressed recombinant tumstatin for endothelial cell migration. Tumstatin showed a weaker inhibition of HUVECs migration across fibronectin coated membrane towards bFGF in a Boyden chamber compared to arresten (Figure 4A). The numbers of migrated endothelial cells towards underside of the Boyden chamber in three independent experiments were scored and assessed (Figure 4B). Interestingly arresten showed about 82% of migration inhibitory effect on HUVECs where as tumstatin showed about 25% of migration inhibition (Figure 4B). These results indicate that baculovirus expressed arresten is a more potent inhibitor of endothelial cell migration compared to tumstatin expressed in the same system.

Tube formation assay, another characteristic angioinhibitory activity of tumstatin was also tested. Addition of 1.0 μM re-



Figure 4: Migration and Tube formation assays. (A) Migration assay: Photographs of HUVEC cells from the underside of Boyden chamber membrane. Number of cells migrated in ICM (incomplete medium) used as negative control. Number of migrated cells in ICM with 25 ng/ml bFGF or with bFGF and 1 μ M tumstatin or 1 μ M α 1(IV)NC1 were viewed using Olympus CK2 light microscope and representative fields (200x magnification) are shown. (B) Migration assessment assay: The graph displays average number of cells migrated in three independent experiments. (C) Tube formation assay: Tube formation was evaluated after 24 hrs on MatrigelTM matrix with and without 1 μ M tumstatin or with 1 μ M α 1(IV)NC1 proteins. Tube formation was visualized in Olympus CK2 light microscope and representative fields at 100x magnification are shown. (D) Tube formation assessment: The graph displays average number of tubes in three independent experiments. In A and C, arrows indicate migrated endothelial cells and tube formation respectively.

combinant tumstatin protein to HUVECs culture media significantly inhibited tube formation on matrigel matrix (Figure 4C, middle panel). Previously we showed similar inhibition using arresten used as positive control here (Figure 4C, right) (Boosani and Sudhakar, 2006; Sudhakar et al., 2005). No significant effect of tumstatin on HUVECs proliferation was observed in cells that were not stimulated with FCS or bFGF indicating that tumstatin has no cytotoxic effects (data not shown).

Signal transduction cascades induced by baculovirus expressed tumstatin

In endothelial cells, ligand upon binding to integrins induces phosphorylation of focal adhesion kinase (FAK), which serves as a platform for different downstream signals (Sudhakar et al., 2003; Zachary and Rozengurt, 1992). Recombinant baculovirus expressed purified tumstatin inhibited phosphorylation of FAK in endothelial cells cultured on fibronectin, confirming our previous observations that, this protein was working properly (Figure 5A) (Boosani et al., 2007; Maeshima et al., 2002; Sudhakar et al., 2003). Downstream to FAK, protein kinase B (PKB/Akt), phosphatidyl-3-kinase (PI3 kinase) plays an important role in mediating pathways involved in endothelial cell survival (Boosani et al., 2007; Hemmings, 1997; Maeshima et al., 2002; Shiojima and Walsh, 2002; Sudhakar et al., 2003). Akt is also known to regulate protein synthesis by phosphorylating eukaryotic initiation factor 4E-binding protein (4E-BP1) via mTOR kinase (Downward, 1998; Maeshima et al., 2002; Miron et al., 2001; Sudhakar et al., 2003). Recombinant baculovirus expressed tumstatin inhibited sustained phosphorylation of Akt (Figure 5B), and activation of PI3 kinase in HUVECs cultured on fibronectin (data not shown). Downstream to Akt, mTOR directly phosphorylates eukaryotic initiation factor 4E(eIF4E)-binding protein (4E-BP1), and the unphosphorylated 4E-BP1 interacts with eIF4E inhibiting Capdependent protein synthesis (Boosani et al., 2007; Kumar et al., 2000; Maeshima et al., 2002; Pause et al., 1994; Sudhakar et al., 2003). Our studies using baculovirus expressed tumstatin confirmed the suppression mTOR activity and inhibition of 4E-BP1 phosphorylation in HUVECs (Figure 5C).



Figure 5: Regulation of tumstatin on the FAK/mTOR/4EBP1 Capdependent translation signaling.

(A) FAK phosphorylation: Immunoblots of phosphorylated FAK (upper blot) and total FAK (lower blot) showing inhibition of sustained phosphorylation by tumstatin. (B) Akt phosphorylation: Immunoblots of phosphorylated Akt (upper blot) and total Akt (lower blot) showing inhibition of sustained phosphorylation by tumstatin. (C) mTOR and 4E-BP1 phosphorylation: Autoradiograph of the auto-phosphorylated mTOR (upper blot) and phosphorylated 4E-BP1 (lower blot) from (HA)-mTOR transfected HUVECs. A-C. (P) and FN represents phosphorylation and fibronectin. A-C. Relative densities of phosphorylated FAK, Akt and mTOR was measured using NIH image software (top graph).

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Figure 6: In-vivo tumor angiogenesis in C57BL/6 mice.

(A) The graph showing the growth of C57BL/6 mice LLC tumors with and without tumstatin injections. The results are shown as mean ± SEM. p < 0.001 compared with tumor mice without tumstatin injection as control group. (B) Frozen sections (4 µm) from control and tumstatin treated tumors were stained with anti-CD31 antibody and the number of CD31 positive blood vessels were counted, blood vessel quantification results were shown as the mean \pm SE. *p < 0.005; compared to mice with and without treatment. (C) The circulating blood VEGFR2 positive endothelial cells in the tumor bearing control and tumstatin treated mice were quantification, results were shown as the mean \pm SE. *p < 0.005; compared to mice with and without treatment. In panel B and C tumor CD-31 positive blood vessels and circulating blood endothelial cells were shown (arrow) in 10 fields at 200x magnification. Scale bar: 50 µm.

Regulation of tumor angiogenesis by tumstatin in C57BL/6 mice

Earlier researchers demonstrated that tumstatin halts the rate of tumor growth and angiogenesis in-vivo (Boosani et al., 2007; Miyoshi et al., 2006; Pedchenko et al., 2004). Here we have examined the growth of LLC tumors and tumor angiogenesis inhibition upon baculovirus expressed tumstatin treatment in tumor bearing C57BL/6 mice. Similarly as explained in our earlier studies, in this study LLC tumors were allowed to reach about 150 mm³ in size, and then 30 µg/mouse/day tumstatin was administered intravenously (Figure 6A). The untreated control C57BL/6 mouse tumor group demonstrated an increased rate of tumor growth, numbers of CD31 positive blood

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vessels, where as the tumstatin treated experimental tumor mice demonstrated a significant inhibition of tumor growth and numbers of CD31 positive blood vessels (Figure 6B, tumstatin treated). To support inhibition of tumor growth and tumor angiogenesis, we have also measured circulating VEGFR2 positive endothelial cells from control and tumstatin treated tumor bearing mice blood, and the results conform significant inhibition of circulating endothelial cells upon tumstatin treatment. (Figure 6C, tumstatin treated). These results demonstrate that baculovirus expressed tumstatin is biologically active and regulating its angioinhibitory and antitumor activities in-vitro and in-vivo.

In summary, baculovirus expressed recombinant tumstatin protein showed its characteristic angioinhibitory and antitumor properties such as inhibition of FAK activation, inhibition of mTOR, 4E-BP1 and Cap dependent protein synthesis, inhibition of proliferation, tube formation and tumor growth. Our results suggest that baculovirus expression is an effective system to produce bulk quantities of tumstatin, and more importantly retaining the biological activity of the expressed protein. Since several endogenous angiogenesis inhibitors derived from type IV collagen non-collagenous domains are being investigated in cancer therapy and also in choriodial neovascularization related eye diseases, there is a need for a suitable expression system that produces recombinant tumstatin protein in high quality and yield. The comparitive expression of tumstatin in three different expression systems conformed that the baculovirus expression system is more usefull expression system for bulk production of soluble biologically active tumstatin for its preclinical and clinical use.

Disclosure of potential conflicts of intrest

None

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