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Fortsættes ...

DESCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application No. 61/524,508, filed August 17, 2011.

FIELD OF THE INVENTION

[0002] The present invention relates to recombinant proteins comprising transferrin that is linked to tumstatin or other antiangiogenic protein and methods for producing and using the same. The present invention also relates to expression system that is capable of expressing such recombinant proteins and methods for producing and using the same.

BACKGROUND OF THE INVENTION

[0003] Choroidal neovascularization (CNV) refers to the uncontrolled growth of choroidal vasculature which can lead to severe vision loss in diseases such as pseudoxanthoma elasticum, angioid streaks, histoplasmosis, punctuate inner choroidopathy and wet age related macular degeneration (AMD). Wet AMD occurs when the deposition of drusen (complement components, lipids, and apolipoproteins) causes confined ischemic regions resulting in hypoxia. It is believed that hypoxia leads to an increase in the secretion of vascular endothelial growth factor (VEGF), which activates choroidal endothelial cells to secrete matrix metalloproteinases (MMP). Metalloproteinases degrade the extracellular matrix, thereby allowing for the proliferation of endothelial cells and their migration towards the retina. The effect of MMP eventually results in the development of new blood vessels, or CNV, which can cause retinal detachment and hemorrhage and the formation of sub retinal lesions due to blood and lipid leakage. Once manifested, CNV is a major cause of vision loss in the elderly population of industrialized nations.

[0004] Treatment of CNV is currently limited to a fraction of the patient population and focuses on restraining the detrimental role of VEGF in vascular hyperpermeability and new blood vessel formation. However, VEGF also plays a constructive key role in physiological activities such as wound healing, photoreceptor survival, and maintaining the choroid capillary bed. Currently, Ranibizumab (Lucentis™), Aflibercept (Eylea™) and pegaptanib (Macugen™) are the only two therapeutic agents that have been approved to date to treat CNV. These agents inhibit VEGF. It has been shown that ranibizumab is generally more effective than pegaptanib in treating CNV. Ranibizumab binds to all isoforms of VEGF-A and inhibits VEGF activity including vascular permeability and growth. Other than the two mentioned therapeutic agent, bevacizumab (Avastin™), the parent full length antibody of ranibizumab, is also being explored as an off label treatment for CNV.

[0005] Despite the success of these therapies in treating CNV there are inherent drawbacks in these therapies, including lack of apoptosis in activated endothelial cells, and potential impairment of VEGF related physiological activities such as wound healing. In addition, use of ranibizumab leads to systemic risks including increased rate of thromboembolic events after intravitreal administration in humans. Intravitreal bevacizumab has also been associated with ischemic attack, blood pressure elevation, cerebrovascular accidents, and death. Further, in a clinical trial with patients suffering from CNV, the response rate to ranibizumab was only ~40% in patients with CNV and the gain in number of letters was only 7.2.

[0006] Therefore, there is a need for new and/or more effective therapeutic approach for treating CNV that has reduced side effects and/or better therapeutic efficacy.

SUMMARY OF THE INVENTION

[0007] Some aspects of the invention provide a recombinant protein comprising transferrin that is linked to tumstatin or other similar antiangiogenic protein. In some embodiments, transferrin and tumstatin are directly linked to one another. Yet in other embodiments, transferrin and tumstatin are covalently linked to one another through a linker. Suitable linkers are well known to one skilled in the art.

[0008] Other aspects of the invention provide a plasmid comprising a nucleic acid sequence encoding a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention. Typically, the nucleic acid sequence encoding the recombinant protein is operatively linked to an expression control sequence.

[0009] Still other aspects of the invention provide a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention. In some embodiments, the nucleic acid sequence is operatively linked to an expression control sequence.

[0010] Yet other aspects of the invention provide a recombinant host cell that is transfected with and expresses the recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention.

[0011] Other aspects of the invention provide a method for producing a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention, said method comprising:

transfecting a recombinant host cell with a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention;

culturing the transfected host cell under conditions sufficient to produce the recombinant protein comprising transferrin that is linked to tumstatin; and

recovering the recombinant protein as a substantially purified recombinant protein.

[0012] In some embodiments, the recombinant protein comprises transferrin that is directly linked to tumstatin as defined in the present invention.

[0013] Still other aspects of the invention provide a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention for use in the treatment of a clinical condition associated with choroidal neovascularization (CNV).

[0014] In some embodiments, the clinical condition associated with CNV comprises pseudoxanthoma elasticum, angioid streaks, histoplasmosis, punctuate inner choroidopathy, or wet age related macular degeneration (AMD).

[0015] Compositions of the invention can also be used to treat other diseases such as, but not limited to, cancer; cancer associated neovascularization; corneal angiogenesis; proliferative diabetic retinopathy; neovascular glaucoma; other neovascular and vascular proliferative disorders of the eye; as well as other neovascular and vascular proliferative disorders elsewhere in the body.

[0016] Compositions of the invention can be administered using any of the methods known to one skilled in the art including, but not limited to, intravitreal, intravenous, suprachoroidal, topical, periocular, subcutaneous, intramuscular, subretinal, retrobulbar, intrascleral, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017]

Figure 1 is a graph showing anti-proliferative activity of transferrin-tumstatin, bevacizumab, and tumstatin.

Figure 2 is a graph showing inhibition of endothelial tube formation observed in choroid endothelial cells with different concentrations of tumstatin, transferrin-tumstatin and bevacizumab protein.

Figure 3 is a bar graph showing *in vivo* assessment of CNV lesion size in BN rats.

Figure 4 is a schematic representation transferrin-tumstatin sustained gene expression construct.

DETAILED DESCRIPTION OF THE INVENTION

[0018] Some aspects of the invention provide a recombinant protein comprising transferrin that is linked (i.e., attached) to tumstatin. Transferrin can be linked directly or indirectly (e.g., via a linker) to tumstatin. Other aspects of the invention provide a recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention. Still other aspects of the invention provide recombinant host cells that are transfected with and express the recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a recombinant protein comprising transferrin that is linked to tumstatin as defined in the present invention. Yet other aspects of the invention provide methods for producing and using a recombinant protein comprising transferrin that is linked to tumstatin.

[0019] Tumstatin has been shown to be therapeutically effective in treating CNV. Without being bound by any theory, it is believed that tumstatin has the ability to regress angiogenesis by causing apoptosis, an attribute lacking in current CNV therapies.

[0020] Tumstatin is an endogenous angiogenesis inhibitor which was initially obtained from the C terminus non-collagenous domain (NC1) of Collagen IV present in the basement membrane. It is believed that tumstatin plays a role in inhibiting blood vessel formation by binding to αVβ3 integrins, hindering the proliferation of endothelial cells and also by inducing endothelial cell apoptosis. In the absence of any pathological condition, a balance is maintained between angiogenic (such as VEGF) and antiangiogenic molecules (such as tumstatin). During, hypoxia and ischemia, the balance between angiogenic and antiangiogenic molecules is believed to be perturbed, thereby causing neovascularization. Angiogenesis inhibitors like tumstatin are a part of the body's inherent mechanism to fight neovascularization.

[0021] Tumstatin has many advantages over ranibizumab and bevacizumab and is believed to be therapeutically better than these antibodies. To date, the tumstatin receptor, αVβ3 integrin, is found only on activated endothelial cells and not on normal vessels. Accordingly, it is believed that tumstatin provides a path to target activated endothelial cells, without affecting physiological processes such as wound healing. Tumstatin promotes apoptosis in endothelial cells, and it is believed that apoptosis caused by tumstatin can result in regression of proliferating blood vessels, and thus is advantageous in restoring normal vision. Accordingly, tumstatin can effectively treat neovascularization and prevent vision loss without any significant anti-VEGF therapy related side effects.

[0022] Surprisingly and unexpectedly, the present inventors have discovered that tumstatin's therapeutic efficacy in CNV can be significantly increased by linking tumstatin to another protein. In one particular embodiment, tumstatin was linked to transferrin in order to achieve polarized secretion of the fusion protein from the retinal pigment epithelium (RPE cells). When exposed to the RPE monolayer, the fusion protein was secreted preferably towards the basolateral side, when compared to tumstatin. After transfection with transferrin-tumstatin gene, the transferrin-tumstatin protein formed was secreted more towards the basolateral direction in polarized epithelial cell monolayers, when compared to tumstatin gene product. Basolateral secretion of a recombinant transferrin-tumstatin protein delivered tumstatin in close proximity to the activated and neovascular choroid endothelial cells, thereby enhancing the therapeutic activity of tumstatin. Without being bound by any theory, it is believed that the enhanced basolateral secretion of transferrin-tumstatin is prominent in the eyes of patients suffering from AMD since iron content and transferrin receptor activity is likely elevated in the eyes of these patients. It is believed that the most accessible route for iron elimination is via choroid vasculature. This route also leads to increased secretion of transferrin (and thus the recombinant transferrin-tumstatin protein) towards the choroid.

[0023] Based on the foregoing, the present inventors investigated a novel transferrin-tumstatin fusion protein (i.e., a recombinant transferrin-tumstatin protein) as well as tumstatin for their efficacy in inhibiting migration, proliferation, and tube formation of choroid endothelial cells. These activities were compared with those of bevacizumab. Furthermore, the present inventors have determined the ability of the recombinant protein of the present invention to be secreted in a polarized manner in a well established polarized cell model (e.g., Madin-Darby canine kidney, i.e., MDCK, cells).

[0024] As used herein, the term "transferrin" includes therapeutically effective fragments of transferrin. In some embodiments, the term "transferrin" refers to a peptide having at least a peptide fragment of transferrin that can bind integrins. Alternatively, the term "transferrin" typically refers to a peptide having at least 5 amino acids of the full transferrin peptide sequence. Still alternatively, the term "transferrin" refers to a peptide having at least 25%, typically at least 50%, often at least 75%, and still more often at least 90% of the full transferrin peptide sequence, as long as that fragment is capable of selectively binding to integrins. Accordingly, any therapeutically effective fragment of transferrin can be used for targeted uptake and secretion of the recombinant protein towards the neovascular regions (e.g., secretion towards choroid). In some embodiments, the therapeutically effective fragment of transferrin comprises a peptide fragment of transferrin that can bind, typically selectively, integrins.

[0025] Some fragments of transferrin with biological activity are known to one skilled in the art. It should be appreciated that the scope of the invention includes the full-length transferrin and tumstatin proteins as well as fragments of one or both of these proteins as long as such a recombinant protein has a desired biological activity. Whether a particular fragment of transferrin and/or tumstatin has a desired biological activity can be readily determined by one skilled in the art using the *in vitro* and *in vivo* experimentations, such as those disclosed herein. Accordingly, the scope of the invention includes any therapeutic fusion proteins (i.e., recombinant tumstatin-transferrin proteins) for treating neovascular disorders, where tumstatin and/or transferrin can be independently a whole protein or a fragment thereof. It is believed that the recombinant proteins are typically more therapeutically efficient than the parent proteins. Compositions of the invention include recombinant proteins or nucleic acid constructs capable of expressing the recombinant proteins in relevant cells.

Series I Peptides. Fragment of transferrin

[0026] Discussed herein are some of the representative fragments of transferrin that are capable of conferring basolateral secretion and methods for identifying and producing the same.

[0027] Transferrin protein sequence as well as the sequences of various secretory proteins were analyzed and fragments of transferrin suitable for the generation of new fusion proteins capable of basolateral secretion/transport were identified. Analysis of the peptide sequence of various basolaterally secreted proteins such as interleukin 6 (Holtkamp et al., Clin Exp Immunol., 1998, 112(1), 34-43), interleukin 8 (*id.*), and vascular endothelial growth factor A (VEGF-A) (Sonoda et al., AGING, 2010, 2(1), 28-42) led to identification of a surprising similarity in the N terminal amino acids of such proteins. It is believed that proteins including interleukin 6, interleukin 8, and vascular endothelial growth factor A (VEGF-A) are secreted on the basolateral side and have an abundance of leucine amino acid. Furthermore, dileucines (i.e., "LL") are also present near the N terminal of these proteins. Based on these understandings, the present inventors have identified the following representative peptides in transferrin that can be responsible for the basolateral secretion of transferrin-tumstatin: MRLAVGALL (SEQ ID NO:1); MRLAVGALLVC (SEQ ID NO:2); LLVCAVLGLCL (SEQ ID NO:3); GALLVCAVLGLCL (SEQ ID NO:4); LLVCAVLGLCLAV (SEQ ID NO:5); GALLVCAVLGLCLAV (SEQ ID NO:6); and MRLAVGALLVCLLVCAVLGLCLAV (SEQ ID NO:7). Accordingly, in some embodiments any peptide or recombinant peptide comprising these peptides are suitable for effecting basolateral secretion of transferrin-tumstatin. These peptides (i.e., fragments of transferrin disclosed herein), when fused with tumstatin or any other suitable therapeutic protein, result in basolateral secretion of the therapeutic protein (e.g., across retinal pigment epithelium towards the choroid).

Series II Peptides. Peptides of transferrin capably of binding integrins:

[0028] Surprisingly and unexpectedly, the present inventors have also discovered that transferrin protein can interact with αVβ3 integrin receptor, in addition to its expected interaction with transferrin receptor. The recombinant transferrin-tumstatin protein of the invention is believed to interact with the αVβ3 integrin receptor, which is the receptor for tumstatin, and also with the transferrin receptor, due to the presence of transferrin. Using *in silico* docking of transferrin-tumstatin to αVβ3 integrin receptor, the present inventors have identified the amino acids within transferrin protein that interact with the integrin receptor. The following amino acids are some of the representative peptides that are believed to interact with the integrin receptor based on *in silico* docking:

Q(127), N(129), L(131), N(148), I(151), G(152), C(156), L(158), K(163), E(166), K(167), A(168); and

C(246), T(250), R(251), D(259), E(337), I(342), L(345), T(349), E(357), K(359), L(366), E(370), W(377), C(396), I(400), N(402), E(404), A(405), D(406), L(423), V(424), P(425), E(429).

[0029] Based on these interacting amino acids, the following peptides of transferrin were designed as those capable of interacting with integrin receptors: GFQNLNIGCLKEKAVA (SEQ ID NO:8); LLCTRDEILTEKLEWCINEADLVPENY (SEQ ID NO:9); $Transferrin_{127-168}$; $Transferrin_{125-170}$; $Transferrin_{246-259}$; $Transferrin_{244-261}$; $Transferrin_{337-429}$; and $Transferrin_{335-431}$. As used herein, the term $Transferrin_{x-y}$ refers to amino acid sequences of transferrin beginning on amino acid "x" and ending at amino acid "y".

Series III Peptides. Fragment of transferrin capable of binding to transferrin receptor:

[0030] Disclosed below are some of the representative fragments of transferrin that are found to be capable of binding to transferrin receptor.

[0031] It is believed that the following amino acids in transferrin interact with transferrin receptor. $Transferrin_{161-169}$; H(368), R(371), L(372), D(375), E(376), S(378), V(379); PRKPLEKAV (SEQ ID NO:10); $Transferrin1_{59-171}$; $Transferrin_{368-379}$; and $Transferrin_{366-381}$. Accordingly, these fragments of transferrin can also be used in methods of the present invention.

Series IV Peptides. Fragments of tumstatin with anti-angiogenic and/or anti-tumor activity

[0032] The term "tumstatin" includes therapeutically effective fragments of tumstatin. In particular, the term "tumstatin" includes portions or fragments of tumstatin peptide that can increase the delivery of transferrin relative to using transferrin without any additional tumstatin peptide. Accordingly, in some embodiments, the term "tumstatin" refers to a peptide having at least a peptide fragment of tumstatin that can increase the therapeutic effectiveness of transferrin relative to transferrin without such a peptide. Alternatively, the term "tumstatin" refers to a peptide having at least 25%, typically at least 50%, often at least 75%, and more often at least 90% of the full tumstatin peptide sequence. Some fragments of tumstatin with biological activity (e.g., anti-angiogenic activity) are known to one skilled in the art. It should be appreciated that the scope of the invention (as well as the term "tumstatin") includes the full-length tumstatin proteins as well as fragments thereof.

[0033] Tumstatin is an angiogenesis inhibitor that binds to αVβ3 integrin receptor and suppresses tumor growth. Previous deletion mutagenesis studies (see, for example, Eikesdal et al., PNAS, 2008, 105(39), 15040-15045; and Thevenard et al., Int. J. Cancel, 2010, 126, 1055-1066) have identified the following amino acid fragments of tumstatin with antiangiogenic activity: $Tumstatin_{74-98}$; $Tumstatin_{185-203}$; and YSNSG (SEQ ID NO:11).

[0034] The present inventors have also designed novel peptides based on the above peptide sequence by *in silico* protein modeling. In one particular embodiment, D (aspartic acid) was replaced with H (histidine) at two points as shown below:

TMPFLFCNVN**H**VCNFASRN**H**YSYWL (SEQ ID NO:12)

[0035] It is believed that replacing H with D results in a net favorable electrostatic interaction between the positive charge of H and the other protein charges. In some other embodiments, the hydrophobic residues alanine (A) and leucine (L) were replaced with arginine (R), a hydrophilic amino acid. Some of these modified peptides include: TMPFLFCNVNDVCNF**R**SRNDYSYWL (SEQ ID NO:13); TMPFLFCNVNDVCNFASRNDYSYW**R**(SEQ ID NO:14); CNYYSNSYSFWL**R**SLNPER (SEQ ID NO:15); and CNYYSNSYSFWLAS**R**NPER (SEQ ID NO:16).

[0036] Arginine contains a guanidinium group, which is responsible for its polarity. It is believed that replacing hydrophobic residues with hydrophilic arginine helps in improving the stability of the protein by virtue of additional hydrogen bonds formed with the solvent. Furthermore, it is believed that arginine, whose guanidinium group is positively charged at pH 7.4, will introduce a charge on the surface and provide an avenue for additional interactions on the protein surface. Any of the above natural sequences or modified fragments can be used in creating fusion or recombinant proteins of the invention. Accordingly, it should be appreciated that the terms "transferrin" and "tumstatin" includes those modifications where one or more of the amino acid residues are replaced with a non-wild type amino acids including homolog amino acids. One skilled in the art can readily determine a suitable amino acid substitution having read the present disclosure.

[0037] As discussed above, in addition to the full-length recombinant proteins of transferrin and tumstatin, the scope of the invention includes recombinant proteins in which one or both of the full-length proteins of transferrin and/or tumstatin are replaced with any of the corresponding partial peptides disclosed above.

[0038] Accordingly, the scope of the invention includes recombinant proteins where any combination of a peptide/protein from peptides disclosed in Series I or III Peptides above or transferrin itself with the peptide in Series II or IV Peptides above or tumstatin itself (e.g., a

Series I Peptide fused with any therapeutic protein or peptide such as tumstatin or a peptide of Series II or IV Peptide; and Series II Peptide fused with Series I Peptide, etc.). Additionally, a Series I Peptide can be combined with any new therapeutic macromolecule for targeted uptake/transport/secretion.

[0039] . In the Examples, procedures that are constructively reduced to practice are described in the present tense, and procedures that have been carried out in the laboratory are set forth in the past tense.

EXAMPLES

Materials and Methods

EXAMPLE 1

[0040] *Materials:* Transwell® filters (0.4μm pore size) were purchased from Corning Inc.,NY. Bovine serum albumin was purchased from Sigma Aldrich (MO). BD Matrigel Matrix Growth Factor Reduced was purchased from BD Biosciences (CA). DNA ladder, Lipofectamine® 2000 reagent and DAPI stain was purchased from Invitrogen Corporation (CA). The restriction enzymes used were purchased from New England Biolabs (MA). The QuikChange® Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (CA). Transferrin gene, choroid endothelial cells (RF/6A), and the RF/6A cell media were purchased from American Type Culture Collection, VA. QIAGEN® plasmid Giga kit was purchased from QIAGEN Inc. (CA). TALON® metal affinity resin (Catalogue # 635502) was purchased from Clonetech Laboratories, Inc. (CA). BCA® Protein Assay Kit, used to estimate protein content, was purchased from Pierce Biotechnology, Inc. (IL) (Catalogue # 23225). Readymade gels, 10% Ready Gel Tris-HCl® from Bio-Rad laboratories, Inc. (CA) and EZ Run® prestained protein ladder from Fisher Scientific (PA) were used during SDS PAGE gel electrophoresis. A BCA ® protein assay kit was purchased from Thermo Fisher Scientific (IL).

[0041] *Construction of plasmids:* Four different constructs were prepared for the study, containing the following cDNAs, namely, (a) Tumstatin; (b) Tumstatin-EGFP; (c) Transferrin-tumstatin (see Figure 4 which schematically illustrates transferrin-tumstatin sustained gene expression construct. A similar construct with transferrin is useful for long-term expression of tumstatin); and (d) Transferrin-tumstatin-EGFP. All primers were purchased from Integrated DNA Technologies Inc. (CA) for use in this experiment. Tumstatin cDNA was PCR amplified using forward primer (5'-CGATGGATCCGCAACCTGGACAACGAGAGGCTT-3') (SEQ ID NO:17) and reverse primer (5'-CGATCTCGAGAGTGTCTTTTCTTCATGCACACC-3') (SEQ ID NO:18) and ligated into vector PSecTag2B as a *BamH1* and *Xho1* fragment. EGFP was cloned into PSecTag2B vector as a *Hind III* and *BamH1* fragment using the pEGFP vector (Clonetech

Laboratories, CA) as a template and using forward primer (5'-ATCGATAAGCTTTGTGAGCAAGGGCGAGGAGC-3') (SEQ ID NO:19) and reverse primer (5'-ATCGATGGATCCCTTGTACAGCTCGTCCATGC-3') (SEQ ID NO:20).

[0042] The IgK secretory sequence was swapped for transferrin by first digesting our construct with Nhe1 plus Sfi1 to remove the IgK sequence. Transferrin was amplified using the forward primer (5'-AGTCGCTAGCATGAGGCTCGCCGTG GGAGCCC-3') (SEQ ID NO:21) and reverse primers (5'- AGTCGCGGCCGGCTGG GCCAGGTCTACGGAAAGTGCAGGCT-3') (SEQ ID NO:22) containing and restriction sites, Nhe1 and Sfi1 sites, respectively, and cloned into the linearized vector. Transferrin was incorporated into the PsecTag2B containing Tumstatin and pEGFP. The Igk gene portion of PSecTag2B was removed during this process. The same forward and reverse primers were used to incorporate Transferrin into a PsecTag2B vector containing only Tumstatin to make transferrin-tumstatin plasmid.

[0043] All plasmids were grown using the dH5α strain of E.Coli bacteria and amplified using QIAGEN® plasmid Giga kit. A1% agarose gel was prepared in TAE buffer and used to study the cDNA constructs. Pictures were taken using the GelDoc XR® imaging system (Bio-Rad laboratories, Inc.CA).

[0044] _Production and purification of protein:_ The PSecTag2B vector used to construct plasmids, as mentioned above, has a six histidine tag that can aid in purification of the protein secreted in the media. The plasmid was transfected in ARPE (human retinal pigment epithelium) cells to express the fusion proteins created. ARPE cells were grown until 80% confluency was attained. Transient transfection of the ARPE cells was performed using the Lipofectamine® 2000 reagent. TALON® metal affinity resin was used to purify the histidine tagged protein. BCA® Protein Assay Kit was used to estimate the elute's protein content. A protein estimation standard curve was made using Bovine serum Albumin (Sigma Aldrich, MO).

[0045] _Confocal microscopy of ARPE cells:_ The tumstatin-EGFP plasmid was studied in APRE cells to see the functioning of EGFP gene in the cells. ARPE cells were grown until 80% confluency was attained. Transient transfection of the ARPE cells was performed using Lipofectamine® 2000 reagent. DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) staining was done for the cell nuclei. ARPE cells with no transfection with tumstatin -EGFP plasmid and only DAPI staining were used as control.

[0046] To study the internalization of transferrin-tumstatin-EGFP and tumstatin-EGFP, protein choroidal endothelial cells were exposed to transferrin-tumstatin-EGFP and tumstatin-EGFP protein for 24 hours. After 24 hours the cells were washed with cold PBS pH 7.4 followed by washing with cold acidic buffer (pH 5.0) and fixed using 4% paraformaldehyde and stained with DAPI. Cells were observed under a Nikon C1 si® confocal microscope.

[0047] _Polarized secretion of transferrin-tumstatin-EGFP fusion protein:_ The MDCK cell line was chosen for the fusion protein polarized secretion study as it is a polarized system that is well understood. MDCK cells have routes that deliver membrane proteins to the apical or basolateral surface.

[0048] MDCK cells were plated on Transwell filters and Trans epithelial electrical resistance (TEER) was measured using an EVOM® resistance meter (World Percision Instruments, CA). When TEER was above 300 Ω, the cells were transfected with tumstatin-EGFP or transferrin-tumstatin-EGFP plasmid as described above. After 24 hours of transfection, the MDCK cells were fixed and stained using DAPI nuclei stain. The media was collected from both the basolateral and the apical side separately and fusion proteins were quantified using a BCA® protein assay kit.

[0049] *Cell proliferation assay:* Choroid endothelial cells (RF/6A) were used to study the effect of tumstatin protein and transferrin-tumstatin protein on the proliferation of cells under the effect of VEGF 165. An MTT assay was used to evaluate cell proliferation.

[0050] The choroid endothelial cells (RF/6A, passage# 9) were plated in a 96-well plate at a seeding density of about 20,000 cells/well and allowed to adhere to the well for 24 hours. After 24 hours, cells were incubated with solutions of VEGF 165 (R&D systems, MN) in a concentration of 50 ng/ml. The proliferation of RF/6A cells was induced by using 50 ng/mL of VEGF 165. Out of the 96 wells, 3 wells were kept as controls, containing only RF/6A cells and 50 ng/mL of VEGF 165. The remaining wells contained bevacizumab, tumstatin, or transferrin-tumstatin in varied concentrations.

[0051] These concentrations were utilized for bevacizumab: 1.5 - 500 nM. The media was aspirated out and 200 µl of fresh serum free media was added to each well at the end of 24 hours. The MTT reagent (Sigma Aldrich, MO), i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide), (20 µl of 5 mg/ml MTT dissolved in PBS pH 7.4) was added to each well and incubated at 37°C for 3 h. The medium was aspirated out and the formazan crystals formed were dissolved in 200 µl of DMSO. After adding DMSO, the crystals dissolved and the color absorbance was measured at 570 nm using a microplate reader. In the next experiment, tumstatin or transferrin-tumstatin was used instead of bevacizumab in the following concentrations: 7.8-1000 nM. The same methods, as described above, were followed. Transferrin-tumstatin protein was used in the concentrations of 1.5- 500 nM (n=3 for each concentration).

[0052] *Tube formation assay:* Matrigel® was thawed at 4°C overnight. A 48 well plate was prepared for the tube formation assay by spreading 75µl of the thawed matrigel in the bottom of each of the wells. The plate was kept at 37°C for 30 minutes to polymerize the matrigel. Choroidal endothelial cells were transferred to the 48 well plate containing the matrigel. Each well contained $6x10^3$ cells. Three wells were kept as control with no tumstatin incorporated. The additional 45 wells contained the tumstatin protein in the concentrations described above for the proliferation assay. The plate was kept at 37°C for 18 hours. Tube formation was analyzed using a light microscope. The same methods were used to conduct the transferrin-tumstatin and bevacizumab protein experiments. Concentrations ranging from 0.15 to 500nM (n=3 for each concentration) were utilized.

[0053] *Cell migration assay: In vitro* cell migration assays were performed using a Matrigel invasion chamber (8-μm pore size, Becton Dickinson, MA). A suspension of 5 × 10^5 cells in 0.5 ml of serum free media were added to the Matrigel chamber. Wells were filled with 1ml of 10ng/ml VEGF solution. The chambers were incubated at 37°C for 24 hours in a 95% air/5% CO_2 incubator. The cells on the lower surface of the membrane were stained with Haematoxylin and Eosin stain. The invading cells were photographed under a Nikon microscope at 40x magnification and counted in five fields of three membranes for each concentration. The same methods were used to conduct the transferrin-tumstatin and bevacizumab protein experiments. Concentrations ranging from 0.15 to 500nM (n=3 for each concentration) were utilized.

[0054] *Molecular docking:* An Accelery's discovery visualizer v2.5.1.9167 (Accelry's, Inc.CA) was used to study *in silico* docking of tumstatin and transferrin-tumstatin to the αVβ3 integrin receptor. The crystal structure of the non-collagenous domain of collagen IV (PDB # 1LI1) was used as a reference to develop a homologous model for tumstatin. The crystal structure of iron-free human serum transferrin (PDB # 2HAV) was used as a reference to develop a homologous model for transferrin. The proteins were prepared and energy minimization performed. Transferrin-tumstatin fusion proteins were created by fusing tumstatin to the C terminus of transferrin with a peptide bond. For the tumstatin with αVβ3 integrin docking studies, a homology model of tumstatin was used as a ligand and docked onto the crystal structure of the extracellular domain of αVβ3 integrin (PDB # 1JV2). For the transferrin-tumstatin with αVβ3 integrin docking studies, the fusion protein was docked to the αVβ3 integrin receptor (PDB # 1JV2).

[0055] *Apoptosis assay:* Apoptosis was studied using the DeadEnd colorimetric TUNEL (TdT-mediated dUTP nick end labeling) system (Promega Corporation, WI). Choroid endothelial cells (1 x 10^5 cells / well) were plated on cover slips in a 12 well plate and allowed to adhere for 24 hours. After 24 hours, the cells were exposed to different concentrations of bevacizumab (1, 10 and 100 nM), tumstatin (100, 250 and 500 nM), and transferrin-tumstatin (1,10 and 100 nM) protein. After 24 hours of protein exposure the cells were then washed, fixed with 4% paraformaldehyde, and permeabilized using 0.2% Triton® X-100 solution in phosphate buffer pH 7.4. The cells were further stained as per the standard protocol provided along with the DeadEnd™ colorimetric TUNEL assay system. The cells were studied under a light microscope at 40x magnification.

[0056] *Induction of CNV in Brown Norway rats and choroidal flatmounts :* Adult male brown norway rats (150-180g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA). Rats were anesthetized using an intraperitoneal injection of 40-80mg/ml ketamine and 10-12 mg/kg xylazine mixture. Induction of laser burns was performed as follows. Pupils were dilated by topical administration of 1% tropicamide solution. The fundus was visualized after placing a coverslip on the eye and insillation of 2.5% hypromellose solution. Eight laser spots (100 mm, 150 mW, 100 ms) concentric with the optic nerve were placed in the right eye of each rat using a 532 nm diode laser (Oculight Glx; Iridex Inc., Mountain View, CA, USA) and a slit lamp (Zeiss slit lamp 30SL; Carl Zeiss Meditec Inc., Dublin, CA, USA). Left eye was used as a control for each animal. The Bruch's membrane breakage was confirmed by the end point "bubble formation". Rats showing intraocular hemorrhage on laser administration were excluded from the study. CNV lesions were allowed to develop for 14 days after induction of laser burns. At the end of 14 days, rats were administered one of the following treatments intravitreally, (a) PBS pH 7.4, (b) bevacizumab, (c) tumstatin protein, and (d) transferrin-tumstatin protein. Rats were euthanized at the end of 14 days of treatment and eyes enucleated.

[0057] For choroidal flatmount, the rats anesthetized using an intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine mixture. Rats were infused with 10 ml of PBS (pH 7.4) followed by infusion with 10 ml 4% paraformaldehyde. Finally, 4 ml of 50 mg/ml fluorescein isothiocyanate (FITC)-dextran solution ($2x10^6$ Da) was infused. The eyes were then enucleated and flatmounts prepared. The flatmounts were imaged with a Nikon EZ-Cl confocal microscope using 488 and 568 nm excitation wavelengths. CNV areas were obtained using ImageJ software.

Results

[0058] *Construction of plasmids:* Picture of 1% agarose gel were taken (not shown) of tumstatin, tumstatin-EGFP, transferrin-tumstatin-EGFP, and transferrin-tumstatin plasmid, which were 5813, 6521, 8550 and 7842 base pairs, respectively. A 1Kb DNA ladder was run as a size marker. The SDS gel electrophoresis image of tumstatin (28 kDa) and transferrin-tumstatin-EGFP (135 kDa) protein were also taken (not shown). An electropherogram was also taken for base pair sequencing result for tumstatin after its insertion in PSecTag2B. Sequencing was done using capillary electrophoresis (Applied Biosystems, CA). Expression of tumstatin-EGFP gene was studied in ARPE cells and images were taken (not shown). Confocal microscopy pictures (100X) of ARPE cells expressing the tumstatin-EGFP protein were also taken (not shown). DAPI staining was performed to stain cell nuclei. ARPE cells with no tumstatin-EGFP transfection were used as controls to cancel out any background fluorescence.

[0059] Internalization of transferrin-tumstatin-EGFP protein was determined. Intense EGFP signal was observed in RF/6A cells upon exposure to transferrin-tumstatin-EGFP protein. Any protein on cell surface was expected to be removed by acidic buffer washes. This was due to the pKa of the lipid phospholipids that constituted the cell membrane. The lowest pKa of the phosphate groups is ~2, which indicates that the phosphate groups will either be in an uncharged state of H_3PO_4 or have a single negative charge ($H_2PO_4^-$) at pH 2. At pH higher than ~2, the probability of having two negative charges (HPO_4^{-2}) increases. Therefore the EGFP signal was supposed to be due to the internalized protein only. Exposure of RF/6A cells to the tumstatin-EGFP protein resulted in no observable internalization.

***Polarized secretion of transferrin-tumstatin-EGFP fusion protein*:**

[0060] MDCK cells were used to study the basolateral secretion of the transferrin-tumstatin-EGFP protein. The images taken of this experiment showed that the transferrin-tumstatin-EGFP protein was secreted on the basolateral side of the cells. When the basolateral side was examined under a confocal microscope, the cell boundaries were not visible. This may be the result of the porous nature of the transwell filters that does not allow light to be reflected back to the microscope and can also hinder light path to the cells. This can be a possible reason for lack of visibility of cells from the filter's basolateral side. Although cells were not visible from the basolateral side, DAPI staining and EGFP signal from the secreted protein can clearly be observed on the basolateral side. Cells can clearly be observed when filter's apical side was studied under a confocal microscope. Protein secretion was observed on the basolateral surface of the cells. Such occurrence can be explained in at least two ways. The protein may be bound to the receptors on the cell surface, or the protein may be embedded in the filter (for the basolaterally secreted protein). A quantification of the protein secreted in the basolateral and apical medium further confirmed the secretory pathway of the transferrin-tumstatin-EGFP protein.

[0061] Transferrin-tumstatin-EGFP protein collected from the apical and basolateral media was purified. The protein content in the apical and basolateral media was determined. The apical media contained 23.17% and the basolateral side contained 76.83% of the transferrin-tumstatin-EGFP protein. The total protein secreted in both media was considered to be 100%. Tumstatin-EGFP protein (control) was purified from the apical and basolateral media collected after the completion of polarized MDCK cell study. The apical media contained 68.84% and the basolateral side contained 31.16% of the tumstatin-EGFP protein. These data show that having a transferrin-tumstatin fusion protein can significantly increase the basolateral secretion of the fusion protein. In the absence of transferrin, tumstatin was secreted more on the apical side (68.84%) in comparison to only 23.17% being secreted apically in the presence of transferrin in the fusion protein.

[0062] *Cell proliferation assay*: Cell proliferation induced by VEGF 165 was inhibited by the presence of tumstatin and transferrin-tumstatin protein. Figure 1 shows the cell proliferation under the effect of tumstatin, transferrin-tumstatin and bevacizumab proteins. MTT assay was used to judge the anti-proliferative activity of tumstatin (●), transferrin-tumstatin (Δ) and bevacizumab (■) on choroidal endothelial cell proliferation stimulated by VEGF (50 ng/ml). Absorbance after treatment with MTT reagent was measured using UV-Vis spectrophotometer. Data is expressed as mean ± S.D. for n = 3. As the graph shows, transferrin-tumstatin protein was highly efficacious in reducing cell proliferation when compared to tumstatin alone. The IC_{50} of the transferrin-tumstatin protein was found to be 5.97 nM. It was found to be 185.7 nM for the tumstatin protein alone. Bevacizumab was also tested for inhibition of cell proliferation and its efficacy was found to be at an IC_{50} of 7.78 nM.

[0063] Transferrin-tumstatin protein was more efficient at inhibiting cell proliferation than Bevacizumab. Transferrin-tumstatin was also able to inhibit a greater number of cells, above the IC_{50} of the protein, in comparison to bevacizumab. At a concentration of 15.6 nM transferrin-tumstatin inhibition was nearly 84% whereas bevacizumab inhibition is ~63%.

[0064] *Tube formation assay*: Representative images (not shown) of tube formation were taken under different concentrations of tumstatin, transferrin-tumstatin and bevacizumab. Tube formation was seen to decrease with increase in concentration of tumstatin and transferrin-tumstatin protein. Both tumstatin and transferrin-tumstatin protein were able to inhibit tube formation, whereas no noticeable inhibition of tube formation was observed under the effect of bevacizumab. In the tube formation assay performed using transferrin-tumstatin fusion protein, a substantially complete prevention of formation of tubes was observed at 125 nM concentration. Figure 2 shows a sigmoidal fit graph of all the three proteins tested. Tube formation was evaluated after 18 hours. Data is expressed as mean ± S.D. for n = 3.

[0065] *Invasion assay*: Invasion assay mimics the basement membrane *in vivo* and cells tend to migrate towards a chemoattractant such as VEGF. Images showed the decrease in number of invading cells under the effect of tumstatin and transferrin-tumstatin protein. Significant decrease in the number of cells invading the Matrigel invasion chamber membrane was observed as the concentration of tumstatin protein was increased in the media. Images (not shown) of cells invading membrane under the effect of tumstatin and transferrin-tumstatin were also taken. VEGF (10ng/ml) was used as the chemoattractant and it showed considerable invasion of the membrane in the absence of either tumstatin or transferrin-tumstatin protein. However, the effect of VEGF in inducing membrane invasion was reduced in the presence of either of the two proteins, even though VEGF was present. This indicates to the efficacy of tumstatin and transferrin-tumstatin protein in reducing cell migration and invasion even in the presence of stimulants such as VEGF.

[0066] The invasion was reduced significantly by transferrin-tumstatin protein when compared to only tumstatin protein. The effect of transferrin-tumstatin protein was significantly different than tumstatin at the concentrations of 15 and 125nM ($P< 0.05$). However, the difference was not substantially significantly at higher concentrations of 250 and 500nM. This indicates that the invasive tendency was saturated (the cells are no more invasive and attracted towards VEGF) and further invasion was not affected at higher concentrations. This effect is different than that observed in cell proliferation and tube formation studied using the two proteins. Bevacizumab exhibited lower IC_{50} values of 8.0 nM in comparison to 12.14 nM for transferrin-tumstatin and indicates an important role of VEGF in cell invasion.

[0067] *Molecular docking*: Molecular models were made *in silico* for tumstatin and transferrin-tumstatin docked to the $\alpha V\beta 3$ integrin receptor. The energy values for the two different docked proteins indicated that the docking of transferrin-tumstatin (interaction energy of -130.43 kcal/mol) is more energetically favorable than the docking of tumstatin (interaction energy of -113.28 kcal/mol) alone.

[0068] _Apoptosis assay:_ Representative images (not shown) were taken of cells exhibiting apoptosis under the effect of tumstatin, transferrin-tumstatin and bevacizumab. Tumstatin and transferrin-tumstatin protein induced apoptosis in choroid endothelial cells at all three concentrations tested (100,250 and 500 nM and 1, 10 and 100 nM, respectively). No significant apoptosis was induced by bevacizumab (1, 10 and 100 nM concentrations) and was similar to what was observed in control PBS.

Induction of CNV in Brown Norway rats and choroidal flatmounts:

[0069] Transferrin-tumstatin was found to be more efficacious in reducing laser induced CNV lesion area as compared to bevacizumab and tumstatin (Figure 3). CNV lesion size was quantified at the end of 14 days of treatment in choroidal flatmounts. A quantitative comparison of the CNV lesion size is shown in Figure 3 (* indicates $p<0.05$ when compared to tumstatin treatment. † indicates $p<0.05$ when compared to bevacizumab. Data is expressed as mean ± S.D. for n=42-48 lesions in each group). Transferrin-tumstatin treated rats had a significantly lower lesion size in comparison to bevacizumab and tumstatin treated rats.

Discussion

[0070] Some aspects of the invention provide a recombinant protein comprising transferrin that is linked to tumstatin. The recombinant proteins (in particular transferrin-tumstatin recombinant (e.g., fusion) protein) of the invention have enhanced binding to αVβ3 integrin and inhibited endothelial cell proliferation, migration, and tube formation with 21, 25 and 31-fold higher activity, respectively, relative to tumstatin alone. Further, transferrin-tumstatin was shown to be more effective at inhibiting choroidal endothelial cell proliferation and tube formation compared to bevacizumab. Moreover, transferrin-tumstatin recombinant protein showed a superior efficacy for inhibiting CNV *in vivo* compared to transferrin alone. Other aspects of the invention provide a plasmid expression system that is capable of producing a recombinant protein comprising transferrin that is linked to tumstatin (i.e., transferrin-tumstatin fusion protein) as defined in the present invention. In some embodiments, the plasmid expression system of the invention allows preferential secretion of the protein towards the basolateral side of confluent cell monolayers.

[0071] Experiments showed that recombinant protein of transferrin-tumstatin inhibited endothelial cell proliferation (IC_{50} of 5.97 nM) with 31-fold and 1.3-fold greater potency than tumstatin (IC_{50} of 185.7 nM) and bevacizumab (IC_{50} of 7.78 nM), respectively. Without being bound by any theory, it is believed that the 31-fold greater efficacy of transferrin-tumstatin over tumstatin and 1.3-fold better activity over bevacizumab is the result of two possible mechanisms. First, it is believed that transferrin improved the binding efficiency of tumstatin to αVβ3 integrin receptor. Using *in silico* modeling, the present inventors have determined that transferrin has binding interactions with αVβ3 integrin. *In silico* modeling also showed that the fusion protein exhibited better interaction energy than tumstatin alone (-130.43 kcal/mol vs -113.28 kcal/mol). The second possible mechanism of improved activity of recombinant protein transferrin-tumstatin is believed to be internalization of the fusion protein. It was observed that transferrin-tumstatin is internalized by choroidal endothelial cells. This internalization was not evident for tumstatin.

[0072] It is possible that transferrin-tumstatin may also be acting via a pathway that is activated only after internalization. Other endogenous antiangiogenic proteins such as endostatin and angiostatin have been shown to form complexes with RGD containing proteins such as fibronectin and vitronectin. Complex formation is essential for activity of these antiangiogenic proteins and these proteins were found to be inactive in mice lacking either fibronectin or vitronectin. Fibronectin is secreted by endothelial cells in a significant amount (~3.0μg/10^5 cells/ day), and once internalized, accumulation of RGD containing proteins such as fibronectin in the cytoplasm of the cell induces apoptosis. Thus, it is possible that fusion of transferrin to tumstatin increases the cytoplasmic content of RGD containing proteins such as fibronectin, thereby causing apoptosis of cells. This possibility is not significant for tumstatin as it did not show any signs of internalization in experiments. For tumstatin, its binding to $\alpha V\beta 3$ integrin is believed to be the principal mechanism of action.

[0073] Again without being bound by any theory, it is believed that recombinant protein of transferrin-tumstatin inhibits cell proliferation by inhibiting protein synthesis and inducing apoptosis, similar to tumstatin alone. The present inventors have observed greater anti-apoptotic activity for recombinant transferrin-tumstatin protein than tumstatin alone. Bevacizumab did not exert any observable apoptotic activity in choroid endothelial cells. Lack of apoptotic activity has been previously reported for bevacizumab in corneal endothelial cells, retinal ganglion cells, and retina-RPE-choroid cultures.

[0074] Experiments showed that recombinant protein of transferrin-tumstatin inhibited tube formation in choroidal endothelial cells with 25 fold more efficacy than tumstatin alone. It is believed that internalization of recombinant protein of transfferin-tumstatin leads to complex of transferrin-tumstatin and fibronectin that can bind to other activated endothelial cells more strongly and prevent tube formation. Bevacizumab did not show any noticeable inhibition of tube formation in choroidal endothelial cells. It is generally believed that tube formation occurs under the effect of collagen IV, VIII, XV, XVIII, and laminin 8 and 10, which are present in the basement membrane. These components tend to promote stability, adhesion, and migration of endothelial cells. Growth factors such as VEGF are also present in the basement membrane matrix. However, VEGF does not appear to play an important role in tube formation, as tube formation occurs with high efficiency even in the presence of bevacizumab, which is known to act by neutralizing VEGF in the extracellular matrix.

[0075] It is believed that during angiogenesis, after degradation of basement membrane, endothelial cells migrate onto a provisional basement membrane-like matrix. VEGF is known to play an important role in the migration of endothelial cells, and the present inventors have studied this process using a Boyden chamber cell migration assay. One of the purposes of this assay was to assess the impact of VEGF on invasion of basement membrane by endothelial cells that are further going to form blood vessels. Cell migration under the effect of 10 ng/ml of VEGF was significantly lower when recombinant protein transferrin-tumstatin was used than when tumstatin was used alone. It has been shown that tumstatin inhibits focal adhesion kinase (FAK) phosphorylation. Lack of FAK activation eventually leads to inhibition of cell migration. It is believed that recombinant protein transferrin-tumstatin exerts similar effects, although to a greater extent.

[0076] Another major advantage of fusing transferrin to tumstatin is the preferential basolateral secretion of the fusion protein. Nearly 75% of recombinant protein transferrin-tumstatin was secreted towards the basolateral side of the cell in comparison to only ~35% of tumstatin protein alone. This provides recombinant protein transferrin-tumstatin with the capability to target the proliferating choroid endothelial cells following intravitreal drug delivery. After intravitreal delivery of therapeutic agents, RPE cells act as a barrier to further movement of therapeutic agents due to the tight junction nature of these cells. Transferrin-tumstatin is advantageous over tumstatin or bevacizumab by virtue of its basolaterally secreting nature. Intravitreally administered transferrin-tumstatin places tumstatin towards the choroid endothelial cells and in close vicinity to the site of disease.

[0077] *In vivo* studies indicated the superior efficacy of transferrin-tumstatin in inhibiting choroidal neovascularization. Lesion size in BN rats eyes treated with transferrin-tumstatin was significantly lower compared to rats treated with bevacizumab or tumstatin.

[0078] These results indicate that transferrin-tumstatin is a better therapeutic agent than bevacizumab because among other things (a) transferrin-tumstatin is a better inhibitor of cell proliferation than bevacizumab, (b) transferrin-tumstatin induces cell apoptosis in choroid endothelial cells, which is absent with bevacizumab, (c) transferrin-tumstatin inhibits tube formation, whereas bevacizumab is ineffective in inhibiting tube formation. In comparison to tumstatin alone, transferrin-tumstatin exerts ≥ 20-fold potency in all the in vitro assays used to assess neovascularization. More significantly, transferrin-tumstatin was more efficacious *in vivo* in reducing laser induced CNV compared to bevacizumab and tumstatin.

EXAMPLE 2

[0079] *Production and purification Tf-T protein*: The PSecTag2B vector used to construct plasmids has a six histidine tag that helps in purification of the protein secreted in the media. ARPE cells (passage # 24) were grown in a 12 well plate till 80 % confluency. Amplified Tf-T (transferrin-tumstatin) plasmid (cloned in PSecTag2B vector) was diluted in DMEM/F12 media and mixed with lipofectamine reagent. The plasmid and lipofectamine complex was added to each well containing cells and media. The complex was mixed gently by rocking the wells back and forth. The cells were incubated at 37°C in a 5% CO_2 incubator for 24 hours. At the end of 24 hours, media was collected and purified. TALON® metal affinity resin was used to purify the fusion protein. The media collected from the cells was mixed with the resin and incubated for

20 minutes at room temperature with mild agitation. Protein bound to the resin was eluted using an aqueous solution of 50 mM sodium phosphate, 300 mM sodium chloride, and 150 mM imidazole at pH 7.0. Later, imidazole was removed from the protein solution by dialyzing (using a 2000 MWCO dialysis bag) against a similar buffer without imidazole. BCA® Protein Assay Kit was used to estimate the protein content. Three separate batches were purified as described and assessed for reproducibility using SDS gel electrophoresis (PAGE), fluorescence spectroscopy, and circular dichroism.

[0080] For gel electrophoresis, 5 µg of protein was mixed with 4x loading dye and boiled for 5 minutes. Samples were run on 4-20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). Circular dichroism (CD) in the far-UV spectral region (190-250 nm) was used to study Tf-T and tumstatin secondary structure. CD spectra were obtained on an AVIV model 62 DS spectropolarimeter (AVIV Biomedical, Inc., NJ). Protein solution was transferred into a 1 mm path length quartz cell and placed in a thermostatic cell holder. Data were collected at 0.25 nm intervals utilizing a 2 nm bandwidth. A 100nM solution of tumstatin and Tf-T was prepared in 5mM phosphate buffer pH 7.4. Fluorescence spectroscopy was performed by exciting the protein at 280 nm. Emission spectrum was collected from 300-400 nm wavelengths. The experiment was performed in a Spectramax M5 microplate reader (Molecular Devices, LLC).

[0081] *Characterization of tumstatin and Tf-T protein:* Characterization of Tf-T was performed using SDS gel electrophoresis (PAGE), size exclusion chromatography (SEC), circular dichroism, and dynamic light scattering. For gel electrophoresis, 5 µg of protein was mixed with 4x loading dye and boiled for 5 minutes. Samples were run on 4-20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). SEC was performed using an Agilent SEC-3 column (I.D. 7.8 mm and length 300mm). The flow rate for the injected samples was 2ml/minute. The sample volume injected was 25µl. Mobile phase used for SEC was phosphate buffer saline (PBS) pH 7.4.

[0082] CD in the far-UV spectral region was used to study Tf-T and tumstatin secondary structure. CD spectra were obtained on an AVIV model 62 DS spectropolarimeter (AVIV Biomedical, Inc., NJ). Protein solution was transferred into a 1 mm pathlength quartz cell and placed in a thermostatic cell holder. Data were collected at 0.25 nm intervals utilizing a 2 nm bandwidth. Malvern Nanosizer was used to evaluate the particle size of a 1mg/ml solution of tumstatin and Tf-T.

[0083] *Secretion of tumstatin and Tf-T fusion protein:* For protein secretion study, RPE cell monolayer was chosen to evaluate the secretion pattern of Tf-T. Initially cells were characterized for their electrical resistance, tight junction formation, and nuclear staining pattern. RPE cells were plated on Transwell filters and transepithelial electrical resistance (TEER) was measured using an EVOM® resistance meter (World Precision Instruments, CA). When TEER was >200 $\Omega.cm^2$, tight junction formation was also confirmed by ZO-1 staining. Filters with cells having a TEER > 200 $\Omega.cm^2$ were fixed for 30 minutes at room temperature by addition of 10% formalin (0.5 ml on apical and 1.5 ml on basolateral side). Cells were further permeabilized by adding 0.1% triton X 100 containing 5% goat serum (0.5 ml on apical side) for 1 hour at room temperature. Primary ZO1 antibody (0.5 ml on apical side), diluted to 1: 100 dilutions, was added to the cells and incubated for 1 hour at room temperature. Secondary FITC labeled antibody (0.5 ml on apical side), diluted to 1: 100 dilutions, was added to cells and incubated for 1 hour at room temperature. Cells were incubated with 3 µg/ml DAPI (4',6-diamidino-2-phenylindole) for 5 minutes to stain the nuclei. Filters were cut out with a sharp blade and transferred to a glass slide, fixed by adding SuperMount mounting media (BioGenex, CA) and visualized under a confocal microscope.

[0084] To evaluate the secretion pattern, cells were incubated with 200µg tumstatin or Tf-T protein on the apical side. After 24 hours of transfection, the media was collected from both the basolateral and the apical side separately and purified using TALON metal affinity resin. Tumstatin and Tf-T proteins were quantified using a BCA® protein assay kit.

[0085] *Stability of Tf-T protein:* Tf-T protein was assessed for its stability in (a) pH 4.0 and 8.0 buffers, (b) tris(hydroxymethyl)aminomethane (TRIS) and phosphate citrate buffer at pH 7.0, and (c) ionic strengths ranging from 10 mM to 250 mM sodium chloride at pH 7.0. The protein solutions were exposed to the mentioned conditions for 48 hours and stability assessed using circular dichroism and fluorescence spectroscopy. SDS PAGE was used in addition of circular dichroism and fluorescence spectroscopy to assess pH stability.

Early time point efficacy studies in CNV* induced *Brown Norway rats:

[0086] Adult male Brown Norway (BN) rats (150-180 gm) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA). Rats were anesthetized using an intraperitoneal injection of 40-80 mg/ml ketamine and 10-12 mg/kg xylazine mixture. Induction of laser burns was performed as described in Example 1. CNV lesions were allowed to develop for 7 days after induction of laser burns. At the end of 7 days, rats were administered one of the following treatments intravitreally, (a) PBS pH 7.4, (b) bevacizumab, or (c) Tf-T protein. The development of CNV lesions before and after treatment was monitored using fluorescein angiography. For fluorescein angiography, rats were anaesthetized, pupils were dilated by topical administration of 1% tropicamide solution, and 200 µl of a 1% sodium fluorescein was administered to rats through the tail vein. Leakage from the lesions was immediately monitored using Genesis Df fundus camera (Kowa Optimed Inc, CA). CNV areas were obtained using ImageJ software.

Results

[0087] *Production and purification of Tf-T protein*: Single bands of 100Kda Tf-T protein were attained in the three batches of Tf-T prepared and tested. Tf-T produced by the described method had similar fluorescence and circular dichroism spectra.

[0088] *Characterization of tumstatin and Tf-T protein:* A single band at 100 kDa and 28 kDa in SDS PAGE and a single peak in SEC showed the purity of Tf-T and tumstatin. Particle size for Tf-T was 8.0 nm with a polydispersity index (PDI) of 0.414. Particle size of tumstatin was 3.9 nm with a PDI of 0.359. CD scan of Tf-T circular dichroism spectra indicated that Tf-T has a majority of β sheets and turns (~60 %) along with the presence of α-helices too (~32 %). Tumstatin's structure is also rich in β sheets and turns (~50 %). However, tumstatin has less α-helices (~16 %) and has more of a random coiled structure (~40 %). Therefore, the circular dichroism spectra are different for both the proteins.

[0089] *Secretion of Tf-T fusion protein:* RPE cells formed tight junctions when grown on a Transwell filter for 4 weeks. Tight junction formation was also confirmed by the staining of ZO-1 protein. The RPE cells showed a clear staining pattern for ZO-1 and outlined the uniform polygonal shape of the RPE cells within the monolayer. Following apical exposure of the proteins, RPE cells secreted 126.8 µg/ml of Tf-T protein to the basolateral side. On the contrary, only 12.3 µg/ml tumstatin was secreted towards the basolateral side.

[0090] *Stability of Tf-T protein:* Tf-T was found to be most stable at pH 7.0. SDS PAGE showed that the band intensity decreases at pH 4-6 in comparison to the control Tf-T, indicating degradation of the protein. Furthermore, fluorescence and circular dichroism indicated a decrease in signal at all pH values except pH 7.0. As the protein was found to be most stable at pH 7.0, further stability studies (ionic strength and buffer) were performed at pH 7.0.

[0091] The fluorescence and circular dichroism spectra of Tf-T exposed to NaCl 10mM to 250mM in phosphate citrate and TRIS buffer were obtained. Tf-T showed no effect of ionic strength changes in the presence of phosphate citrate buffer. However, Tf-T signal was reduced in the presence of NaCl 250mM and TRIS buffer. Thus, phosphate citrate buffer is better suited for the protein.

Early time point efficacy studies in CNV* induced *Brown Norway rats:

[0092] When treatment was initiated at an earlier time point (7 days after CNV induction) Tf-T treated rats had a lower lesion size compared to bevacizumab treated rats. This indicates that Tf-T is efficacious as a preventive treatment for choroidal neovascularization.

SEQUENCE LISTING

[0093]

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<120> TRANSFERRIN-TUMSTATIN FUSION PROTEIN AND METHODS FOR PRODUCING AND USING THE SAME

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<210> 7
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Val Leu Gly Leu Cys Leu Ala Val
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Ser Arg Asn His Tyr Ser Tyr Trp Leu
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Ser Arg Asn Asp Tyr Ser Tyr Trp Leu
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Ser Arg Asn Asp Tyr Ser Tyr Trp Arg
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<400> 22
agtcgcggcc ggctgggcca ggtctacgga aagtgcaggc t 41

REFERENCES CITED IN THE DESCRIPTION

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- US61524508B [0093]

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- **SONODA et al.**AGING, 2010, vol. 2, 128-42 [0027]
- **EIKESDAL et al.**PNAS, 2008, vol. 105, 3915040-15045 [0033]
- **THEVENARD et al.**Int. J. Cancel, 2010, vol. 126, 1055-1066 [0033]

Patentkrav

1. Rekombinant protein omfattende transferrin, der er forbundet til tumstatin.

2. Det rekombinante protein ifølge krav 1, hvor transferrin og tumstatin er direkte forbundet til hinanden.

3. Det rekombinante protein ifølge krav 1, hvor transferrin og tumstatin er kovalent forbundet til hinanden via en linker.

4. Plasmid omfattende en nukleinsyresekvens, der koder for et rekombinant protein omfattende transferrin, der er forbundet til tumstatin som ifølge krav 1.

5. Plasmidet ifølge krav 4, hvor nukleinsyresekvensen, der koder for det rekombinante protein er funktionelt forbundet til en ekspressionskontrol-sekvens.

6. Rekombinant nukleinsyremolekyle omfattende en nukleinsyresekvens, der koder for et rekombinant protein omfattende transferrin, der er forbundet til tumstatin som ifølge krav 1.

7. Det rekombinante nukleinsyremolekyle ifølge krav 6, hvor nukleinsyresekvensen er funktionelt forbundet til en ekspressionskontrol-sekvens.

8. Rekombinant værtscelle, der er transfekteret med og udtrykker det rekombinante nukleinsyremolekyle omfattende en nukleinsyresekvens, der koder for et rekombinant protein omfattende transferrin, der er forbundet til tumstatin som ifølge krav 6.

9. Fremgangsmåde til fremstilling af et rekombinant protein omfattende transferrin, der er forbundet til tumstatin som ifølge krav 1, hvilken fremgangsmåde omfatter:

at transfektere en rekombinant værtscelle med et rekombinant nukleinsyremolekyle omfattende en nukleinsyresekvens, der koder for et rekombinant protein omfattende transferrin, der er forbundet til tumstatin som ifølge krav 6;

at dyrke den transfekterede værtscelle under forhold der er tilstrækkelige til at fremstille det rekombinante protein omfattende transferrin, der er forbundet til tumstatin; og

at genvinde det rekombinante protein som et i alt væsentligt oprenset rekombinant protein.

10. Fremgangsmåden ifølge krav 9, hvor det rekombinante protein omfatter transferrin, der er direkte forbundet til tumstatin som ifølge krav 2.

11. Rekombinant protein omfattende transferrin, der er forbundet til tumstatin som defineret i krav 1 til anvendelse i behandlingen af en klinisk tilstand associeret med choroidal neovaskularisering (CNV).

12. Det rekombinante protein som defineret i krav 1 til anvendelsen som defineret i krav 11, hvor den kliniske tilstand associeret med CNV omfatter pseudoxanthoma elasticum, angioide striber, histoplasmose, punktformig indre choroidpati, eller våd, aldersbetinget makulær degeneration.

DRAWINGS

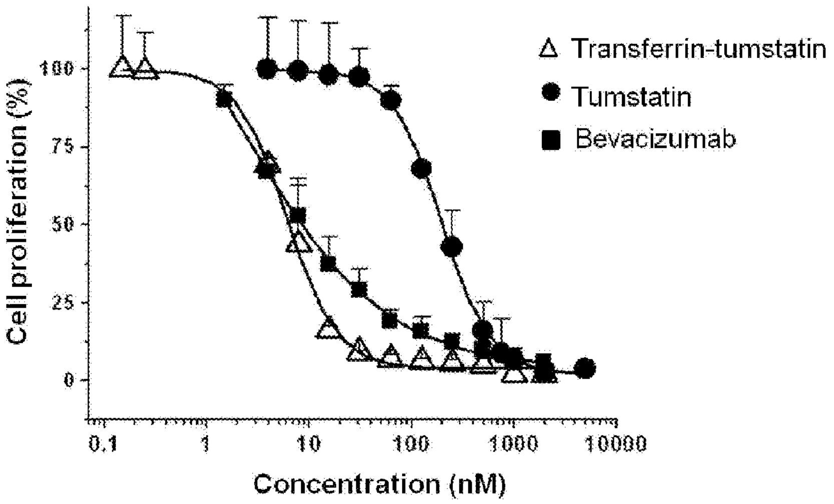
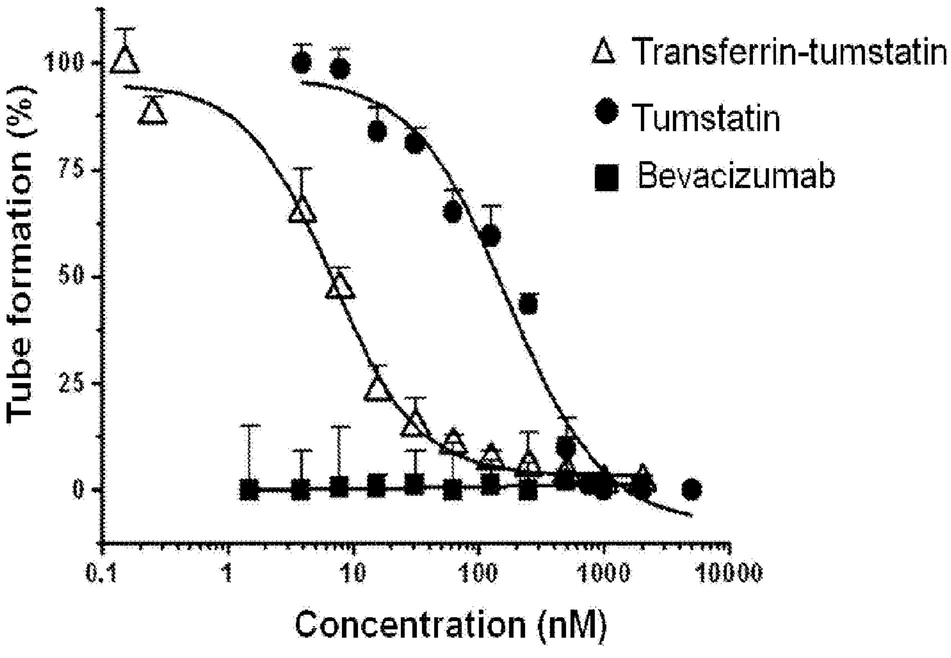


FIGURE 1



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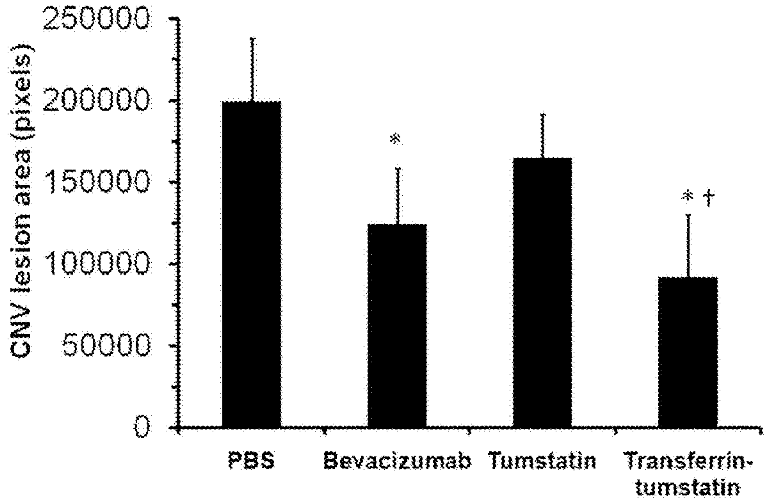

FIGURE 3

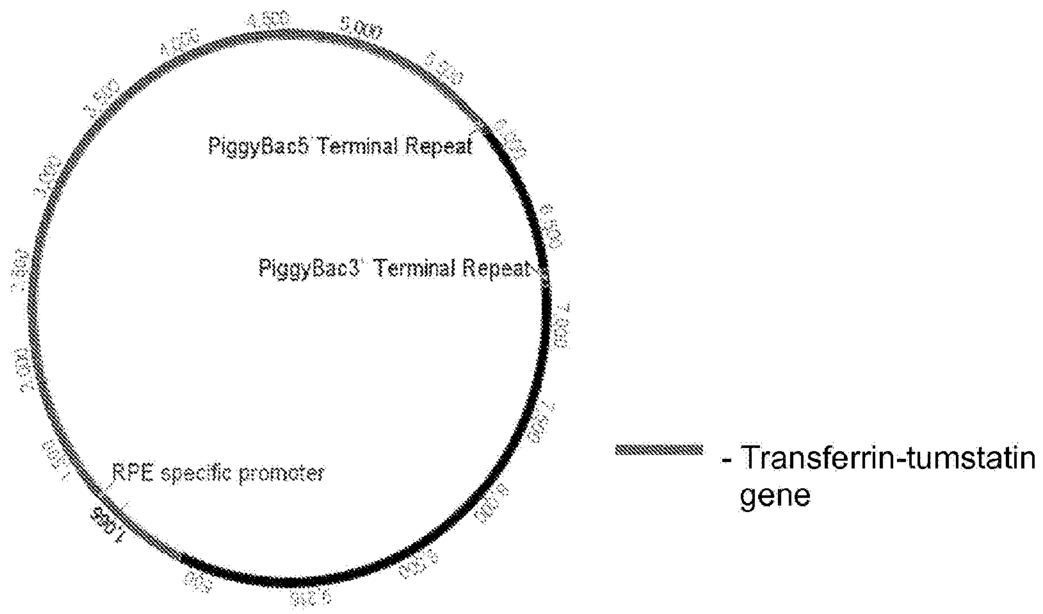

FIGURE 4