METHOD OF PRODUCING RECOMBINANT ADAMTS13 IN CELL CULTURE

CROSS REFERENCES TO APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 61/362,635, filed July 8, 2010~~, the disclosure of which is hereby incorporated herein by reference in its entirety for all purposes~~.

BACKGROUND OF THE INVENTION

[0002] The recombinant expression of therapeutic proteins in cell culture (particularly large-scale cell cultures), including eukaryotic cell culture, and more specifically mammalian cell culture, requires the use of special culture media providing nutrient substances for efficient growth of cells. Cell culture media formulations are often supplemented with a range of additives, including fetal calf serum (FCS), animal derived proteins and/or protein hydrolysates of bovine origin as well as protein hydrolysates derived from plants or yeast. One challenge with such cultures is that the amount of protein and the total and specific activity of the protein produced are often variable across different cell cultures, even when the formulation for the cell culture media is not changed. This variability is especially apparent in the case of large-scale manufacturing processes utilizing cell culture volumes of 10 liters to over 20,000 liters. Cell culture media containing hydrolysates are particularly prone to variability from one cell culture to the next, leading to decreased production of total protein as well as decreased total and specific activity.

[0003] One potential reason for the variability seen across different cell cultures is that contaminants in additives such as hydrolysates vary from one batch to the next. In general, serum or serum-derived substances, such as, e.g., albumin, transferrin or insulin, may comprise unwanted agents that can contaminate the cell cultures and the biological products obtained thereof. Furthermore, human serum derived additives have to be tested for all known viruses,

[0101] In certain aspects, the methods of the invention do not necessarily result in a higher amount of recombinant protein overall, but the recombinant protein (either rVWF or rA13) that is produced shows higher total and specific activity than is seen in proteins produced using standard cell cultures, particularly as compared to proteins produced in cell cultures in which the cell culture medium has not been supplemented with additional copper. In further aspects, the rVWF and rA13 proteins produced in cells cultured in copper supplemented media show consistently increased activity per liter of cell culture as compared to cells cultured in basal cell culture media that has not been supplemented with copper. In still further aspects, the copper supplemented media of the present invention result in increased protein yield, increased number of cells in the culture, and/or increased total activity per liter of culture as compared to media that has not been supplemented with copper.

[0102] Still further advantages of methods and compositions of the invention is that they result a population of proteins containing a high percentage (over 10%) of highly multimerized rVWF.

[0103] Although much of the discussion herein regarding ADAMTS proteins is in terms of ADAMTS 13 (A13), it will be appreciated that because all ADAMTS proteins share a common core domain architecture and common structure-function relationships, the methods and compositions described herein are applicable for production of any ADAMTS proteins, not only rA13.

II. Definitions

[0104] As used herein, "recombinant vWF" includes vWF obtained via recombinant DNA technology. In certain embodiments, vWF proteins of the invention can comprise a construct, for example, prepared as in WO 1986/06096 published on Oct. 23, 1986 and U.S. patent application Ser. No. 07/559,509, filed on Jul. 23, 1990, in the name of Ginsburg et ah~~, which is incorporated herein by reference with respect to the methods of producing recombinant vWF~~. The vWF in the present invention can include all potential forms, including the monomeric and multimeric forms. It should also be understood that the present invention encompasses different forms of vWF to be used in combination. For example, the vWF of the present invention may include different multimers, different derivatives and both biologically active derivatives and derivatives not biologically active.

H96D, R102C, R193W, T196I, H234Q, A250V, R268P, W390C, R398H, Q448E, Q456H, P457L, C508Y, R528G, P618A, R625H, I673F, R692C, A732V, S903L, C908Y, C951 G, G982R, C1024G, A1033T, R1095W, Rl 123C, C1213Y, T1226I, G1239V, R1336W, many of which have been found associated with thrombotic thrombocytopenic purpura (TTP).

ADAMTS 13 proteins also includes polypeptides containing post-translational modifications. For example, ADAMTS 13 has been shown to be modified by N-acetylglucosamine (GlcNAc) at residues 614, 667, and 1354, and it has been predicted that residues 142, 146, 552, 579, 707, 828, and 1235 may also be modified in this fashion.

[0111] Proteolytically active recombinant ADAMTS 13 may be prepared by expression in mammalian cell cultures, as described in Plaimauer et ah, (2002, Blood. 15; 100(10):3626-32) and US 2005/0266528~~, the disclosures of which are herein incorporated by reference in their entireties for all purposes~~. Methods for the expression of recombinant ADAMTS 13 in cell culture are disclosed in Plaimauer B, Scheiflinger F. (Semin Hematol. 2004 Jan;41(l):24-33 and US 201 1/0086413~~, the disclosures of which are herein incorporated by reference in their entireties for all purposes~~.

[0112] As used herein, the term "biologically active derivative", when used in the context of an ADAMTS protein, also embraces polypeptides obtained via recombinant DNA technology. This may include any method known in the art for (i) the production of recombinant DNA by genetic engineering, e.g., via reverse transcription of RNA and/or amplification of DNA, (ii) introducing recombinant DNA into prokaryotic or eukaryotic cells by trans fection, i.e., via electroporation or microinjection, (iii) cultivating said transformed cells, e.g., in a continuous or batch-wise manner, (iv) expressing an ADAMTS protein, e.g., constitutively or upon induction, and (v) isolating said ADAMTS protein, e.g., from the culture medium or by harvesting the transformed cells, in order to (vi) obtain substantially purified recombinant ADAMTS protein, e.g., via ion exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophobic interaction chromatography, and the like. The term "biologically active derivative" includes also chimeric molecules such as e.g. an ADAMTS protein, or functional fragment thereof, in combination with a second polypeptide, e.g., an immunoglobulin Fc domain or an albumin domain, in order to improve the biological/pharmacological properties such as e.g. , half life of the ADAMTS protein in the circulation system of a mammal, particularly a human.

[0118] As used herein, the term "chemically defined medium" or "chemically defined media" refers to a synthetic growth medium in which the identity and concentration of all the components are known. Chemically defined mediums do not contain bacterial, yeast, animal, or plant extracts, although they may or may not include individual plant or animal-derived components (e.g., proteins, polypeptides, etc.). Non-limiting examples of commercially available chemically defined mediums include, various Dulbecco's Modified Eagle's (DME) mediums (Sigma-Aldrich Co; SAFC Biosciences, Inc), Ham's Nutrient Mixture (Sigma-Aldrich Co; SAFC Biosciences, Inc), combinations thereof, and the like. Methods of preparing chemically defined culture mediums are known in the art, for example in U.S. Patent Numbers 6,171 ,825 and 6,936,441 , WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770~~, the disclosures of which are incorporated herein by reference in their entireties for all purposes~~.

[0119] As used herein, the term "oligopeptide-free culture medium" or "oligopeptide-free culture media" refers to a protein-free medium that does not comprise oligopeptides, such as, e.g., oligopeptides derived from a protein hydrolysate. In one embodiment, the medium does not comprise oligopeptides having twenty or more amino acids. In one embodiment of the present invention, the medium does not comprise oligopeptides having fifteen or more amino acids. In another embodiment of the invention, the medium does not comprise oligopeptides having ten or more amino acids. In one embodiment the medium does not comprise oligopeptides having seven or more amino acids. In another embodiment the medium does not comprise oligopeptides having five or more amino acids. In still another embodiment the medium does not comprise oligopeptides having three or more amino acids. According to a further embodiment of the present invention, the medium does not comprise oligopeptides having two or more amino acids. Methods of preparing oligopeptide-free culture medium are known in the art, for example in U.S. Patent Numbers 6,171 ,825 and 6,936,441 , WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770~~, the disclosures of which are incorporated herein by reference in their entireties for all purposes~~.

[0120] As used herein, the term "serum-free culture medium" or "serum-free culture media" refers to a culture medium that is not supplemented with an animal serum. Although oftentimes serum-free mediums are chemically defined mediums, serum- free mediums may be

supplemented with discrete animal or plant proteins or protein fractions. Methods of preparing serum-free culture medium are known in the art, for example in U.S. Patent Numbers 6,171 ,825 and 6,936,441 , WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770~~, the disclosures of which are incorporated herein by reference in their entireties for all purposes~~.

[0121] As used herein, the term "animal protein-free culture medium" or "animal protein-free culture media" refers to a culture medium that is not supplemented with an animal serum, protein, or protein fraction. Although oftentimes animal protein-free culture mediums are chemically defined mediums, animal protein-free culture mediums may contain plant or yeast hydrolysates. Methods of preparing animal protein-free culture medium are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770~~, the disclosures of which are incorporated herein by reference in their entireties for all purposes~~.

[0122] As used herein, the term "basal cell culture medium" or "basal cell culture media" refers to a chemically defined culture medium, an oligopeptide-free culture medium, a serum-free culture medium, or an animal protein-free culture medium that has not been supplemented with a hydrolysate, e.g., a plant or yeast hydro lysate. basal mediums are well known in the art, e.g., DMEM, Ham's F12, DMEM/Ham's F12, Medium 199, McCoy, or RPMI. The basal medium can include a number of ingredients, including amino acids, vitamins, organic and inorganic salts, and sources of carbohydrate. Each ingredient can be present in an amount that supports the cultivation of a cell, such amounts being generally known to a person skilled in the art. The medium can include auxiliary substances, such as buffer substances, e.g., sodium bicarbonate, antioxidants, stabilizers to counteract mechanical stress, or protease inhibitors. If necessary, a non-ionic surfactant such as copolymers and/or mixtures of polyethylene glycols and polypropylene glycols can be added.

III. Cell Culture Media and Cell Culture Supernatant

[0123] One aspect of the present invention relates to cell culture media for producing rVWF and/or rA13 that have increased activity as compared to rVWF and rA13 produced with basal cell culture media. In one aspect, the present invention relates to cell culture media for producing rVWF and/or rA13 in which basal cell culture media is supplemented with one or

	About 10 µg/L	Var. 25	Var. 65	Var. 105	Var. 145	Var. 185	Var. 225	Var. 265	Var. 305	Var. 345	Var. 385	Var. 425
	1-20 µg/L	Var. 26	Var. 66	Var. 106	Var. 146	Var. 186	Var. 226	Var. 266	Var. 306	Var. 346	Var. 386	Var. 426
	2-20 µg/L	Var. 27	Var. 67	Var. 107	Var. 147	Var. 187	Var. 227	Var. 267	Var. 307	Var. 347	Var. 387	Var. 427
	1-10 µg/L	Var. 28	Var. 68	Var. 108	Var. 148	Var. 188	Var. 228	Var. 268	Var. 308	Var. 348	Var. 388	Var. 428
	2-10 µg/L	Var. 29	Var. 69	Var. 109	Var. 149	Var. 189	Var. 229	Var. 269	Var. 309	Var. 349	Var. 389	Var. 429
	1-6 µg/L	Var. 30	Var. 70	Var. 110	Var. 150	Var. 190	Var. 230	Var. 270	Var. 310	Var. 350	Var. 390	Var. 430
	2-6 µg/L	Var. 31	Var. 71	Var. 111	Var. 151	Var. 191	Var. 231	Var. 271	Var. 311	Var. 351	Var. 391	Var. 431
	3-6 µg/L	Var. 32	Var. 72	Var. 112	Var. 152	Var. 192	Var. 232	Var. 272	Var. 312	Var. 352	Var. 392	Var. 432
	4-6 µg/L	Var. 33	Var. 73	Var. 113	Var. 153	Var. 193	Var. 233	Var. 273	Var. 313	Var. 353	Var. 393	Var. 433
	1-5 µg/L	Var. 34	Var. 74	Var. 114	Var. 154	Var. 194	Var. 234	Var. 274	Var. 314	Var. 354	Var. 394	Var. 434
	2-5 µg/L	Var. 35	Var. 75	Var. 115	Var. 155	Var. 195	Var. 235	Var. 275	Var. 315	Var. 355	Var. 395	Var. 435
	3-5 µg/L	Var. 36	Var. 76	Var. 116	Var. 156	Var. 196	Var. 236	Var. 276	Var. 316	Var. 356	Var. 396	Var. 436
	4-5 µg/L	Var. 37	Var. 77	Var. 117	Var. 157	Var. 197	Var. 237	Var. 277	Var. 317	Var. 357	Var. 397	Var. 437
	1-4 µg/L	Var. 38	Var. 78	Var. 118	Var. 158	Var. 198	Var. 238	Var. 278	Var. 318	Var. 358	Var. 398	Var. 438
	2-4 µg/L	Var. 39	Var. 79	Var. 119	Var. 159	Var. 199	Var. 239	Var. 279	Var. 319	Var. 359	Var. 399	Var. 439
	3-4 µg/L	Var. 40	Var. 80	Var. 120	Var. 160	Var. 200	Var. 240	Var. 280	Var. 320	Var. 360	Var. 400	Var. 440

*ND = not defined
*NMT = no more than
*AL = at least

[0132] In some embodiments, the copper supplemented media of the present invention are produced by supplementing basal media that is animal protein-free and/or chemically defined. Methods of preparing animal protein-free and chemically defined culture mediums are known in the art, for example in U.S. Patent Numbers 6,171,825 and 6,936,441 , WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770, ~~the disclosures of which are incorporated herein by reference in their entireties for all purposes~~. In one embodiment, the basal culture medium used in the methods described herein is animal protein-free or oligopeptide-free medium. In certain embodiments, the culture medium may be chemically defined. In certain embodiments, the culture media may contain at least one polyamine at a concentration of about 0.5 mg/L to about 10 mg/L.

[0133] In further embodiments and in addition to any of the description provided above, culture media of the invention are provided in which a basal medium is supplemented with copper and at least one of calcium, zinc, and/or vitamin B3. In certain embodiments, the medium may be an animal protein-free, oligopeptide-free, or chemically defined medium. In certain embodiments, the animal protein-free or oligopeptide free medium is prepared as taught in U.S. Patent Numbers 6,171,825 and 6,936,441, WO 2007/077217, and U.S. Patent Application Publication Numbers 2008/0009040 and 2007/0212770~~, the disclosures of which are incorporated herein by reference in their entireties for all purposes, both of which are incorporated herein by reference in their entireties for all purposes,~~ and supplemented with additional copper and optionally one or more of calcium, zinc, and vitamin B3. In a specific embodiment, the chemically defined culture medium may be similar to a Dulbecco's Modified Eagle's Media / Ham's F 12 1 : 1 mixture (DMEM/Ham's F12), which has been supplemented with additional copper and optionally calcium, zinc, and/or vitamin B3, in order to increase the specific activity of rVWF or rA13 expressed in a cell cultured in the medium. In yet other embodiments, the culture medium is animal component free. In another embodiment, the culture medium contains protein, e.g., animal protein from serum such as fetal calf serum. In another embodiment, the culture has recombinant proteins exogenously added. In another embodiment, the proteins are from a certified pathogen free animal.

[0134] In certain embodiments, the culture media contains at least one polyamine at a concentration of at or about between 0.5 mg/L and 30 mg/L. In another embodiment, the culture medium contains at least one polyamine at or about between 0.5 mg/L and 10 mg/L. In one embodiment, the culture medium contains at least one polyamine at or about between 2 mg/L and 8 mg/L. In certain embodiments the polyamine is from the group of ornithine, putrescine, spermine or spermidine, or the like. In a preferred embodiment, the polyamine is putrescine. In a specific embodiment, the culture medium contains at or about between 2 mg/L and 8 mg/L putrescine.

[0135] In one embodiment, the culture media contains at least one polyamine at a concentration of at or about between 0.5 mg/L and 30 mg/L and a copper and ammonium combination according to any one of variations 1 to 440, as set forth in Table 1. In another embodiment, the culture medium contains at least one polyamine at or about between 0.5 mg/L and 10 mg/L and a

and temperature. In certain embodiments, the rVWF or rA13 is recovered after harvesting the supernatant of a continuous culture. An exemplary method for continuous cell cultivation is described in WO/201 1/012725 (Grillberger et al.), the content of which is hereby incorporated by reference in its entirety for all purposes.

[0164] In a perfusion culture, the extracted medium is depleted of cells, which are retained in the culture vessel, for example, by filtration or by centrifugal methods that lead to the reintroduction of the cells into the culture. However, typically membranes used for filtration do not retain 100% of cells, and so a proportion is removed when the medium is extracted. It may not be crucial to operate perfusion cultures at very high growth rates, as the majority of the cells are retained in the culture vessel. In certain embodiments, the rVWF or rA13 is recovered after harvesting the supernatant of a perfusion culture.

[0165] Stirred-tank reactor system can be used for batch and continuous cell cultures operated under suspension or adherent modes. Generally, the stirred-tank reactor system can be operated as any conventional stirred-tank reactor with any type of agitator such as a Rushton, hydrofoil, pitched blade, or marine.

[0166] In certain embodiments, the cell-culture methods of the invention may comprise the use of a microcarrier. In some embodiments, the cell-cultures of the embodiments can be performed in large bioreactors under conditions suitable for providing high volume-specific culture surface areas to achieve high cell densities and protein expression. One means for providing such growth conditions is to use microcarriers for cell-culture in stirred tank bioreactors. The concept of cell-growth on microcarriers was first described by van Wezel (van Wezel, A.L., Nature 216:64-5 (1967)) and allows for cell attachment on the surface of small solid particles suspended in the growth medium. These methods provide for high surface-to-volume ratios and thus allow for efficient nutrient utilization. Furthermore, for expression of secreted proteins in eukaryotic cell lines, the increased surface-to-volume ratio allows for higher levels of secretion and thus higher protein yields in the supernatant of the culture. Finally, these methods allow for the easy scale-up of eukaryotic expression cultures.

[0167] The cells expressing vWF and/or rA13 can be bound to a spherical or a porous microcarrier during cell culture growth. The microcarrier can be a microcarrier selected from the group of microcarriers based on dextran, collagen, plastic, gelatine and cellulose and others as

is at least 2.4 μg/L, 3 μg/L, 4 μg/L, 5 μg/L, 6 μg/L, 7 μg/L, 8 μg/L, 9 μg/L, 10 μg/L, 15 μg/L, 20 μg/L, or higher. In other embodiments, the final copper concentration of the supplemented basal culture media is between 2-20 μg/L, 2-10 μg/L, 3-8 μg/L, or 4-6 μg/L. In a preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 50 mU^g rVWF. In a more preferred embodiment, the recovered supernatant has a rVWF specific ristocetin cofactor activity of at least 70 mU^g rVWF.

[0200] Recombinant vWF can be produced by expression in a suitable eukaryotic host system. Examples of eukaryotic cells include, without limitation, mammalian cells, such as CHO, COS, HEK 293, BHK, SK-Hep, and HepG2; insect cells, e.g., SF9 cells, SF21 cells, S2 cells, and High Five cells; and yeast cells, e.g., Saccharomyces or Schizosaccharomyces cells. In one embodiment, the vWF can be expressed in yeast cells, insect cells, avian cells, mammalian cells, and the like. For example, in a human cell line, a hamster cell line, or a murine cell line. In one particular embodiment, the cell line is a CHO, BHK, or HEK cell line. Typically, mammalian cells, e.g., CHO cells from a continuous cell line, can be used to express the vWF of the present invention.

[0201] In certain embodiments, the nucleic acid sequence comprising a sequence coding for vWF can be a vector. The vector can be delivered by a virus or can be a plasmid. The nucleic acid sequence coding for the protein can be a specific gene or a biologically functional part thereof. In one embodiment, the protein is at least a biologically active part of vWF.

[0202] A wide variety of vectors can be used for the expression of the vWF and can be selected from eukaryotic expression vectors. Examples of vectors for eukaryotic expression include: (i) for expression in yeast, vectors such as pAO, pPIC, pYES, pMET, using promoters such as AOX1 , GAP, GAL1 , AUG1 , etc; (ii) for expression in insect cells, vectors such as pMT, pAc5, pIB, pMIB, pBAC, etc., using promoters such as PH, plO, MT, Ac5, OpIE2, gp64, polh, etc., and (iii) for expression in mammalian cells, vectors such as pSVL, pCMV, pRc/RSV, pcDNA3, pBPV, etc., and vectors derived from viral systems such as vaccinia virus, adeno-associated viruses, herpes viruses, retroviruses, etc., using promoters such as CMV, SV40, EF-1 , UbC, RSV, ADV, BPV, and β-actin. An exemplary vector for expressing rVWF is described by Kaufman et al. (Mol Cell Biol. 1989 Mar;9(3): 1233-42), ~~the content of which is hereby incorporated by reference in its entirety for all purposes~~.

the DUKX-B 1 1 subclone (CHO, Uriaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse Sertoli cells (TM4, Mather, Biol. Reprod, 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N. Y. Acad. Sci, 383:44-68 (1982)); MRC 5 cells; FS4 cells; and the human hepatoma line (Hep G2). Preferably, the cell line is a rodent cell line, especially a hamster cell line such as CHO or BHK.

[0223] A wide variety of vectors can be used for the expression of an ADAMTS protein (e.g., ADAMTS 13) and can be selected from eukaryotic and prokaryotic expression vectors. In certain embodiments, a plasmid vector is contemplated for use in expressing an ADAMTS protein (e.g., ADAMTS 13). In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector can carry a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. The plasmid will comprise a nucleotide sequence encoding an ADAMTS protein (e.g., ADAMTS 13) operable linked to one or more control sequences, for example, a promoter.

[0224] A preferred method of preparing stable CHO cell clones expressing a recombinant ADAMTS protein is as follows. A DHFR deficient CHO cell line DUKX-B1 1 is transfected with a DHFR expression vector to allow for expression of the relevant recombinant protein. An exemplary method is described by Plaimauer et al. (Blood. 2002 Nov 15; 100(10):3626-32. Epub 2002 Jul 12)~~, the content of which is hereby incorporated by reference in its entirety for all purposes~~. Selection is carried out by growth in Hypoxanthine/Thymidine (HT) free media and amplification of the relevant region coding for expression of the recombinant ADAMTS protein and DHFR gene is achieved by propagation of the cells in increasing concentrations of methotrexate. Where appropriate, CHO cell lines may be adapted for growth in serum and/or protein free medium, essentially as described in US 6,100,061 (Reiter et al., Immuno Aktiengesellschaft) .

[0225] In another preferred embodiment, stable HEK293 cells are prepared by transfecting with a construct containing a hygromycin selectable marker and selecting transformants by antibiotic resistance.

[0226] The ability of certain viruses to infect cells or enter cells via receptor-mediated endocytosis, and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign nucleic acids into cells (e.g., mammalian cells). Accordingly, in certain embodiments, a viral vector is used to introduce a nucleotide sequence encoding an ADAMTS protein (e.g. , ADAMTS 13) into a host cell for expression. The viral vector will comprise a nucleotide sequence encoding an ADAMTS protein (e.g., ADAMTS 13) operable linked to one or more control sequences, for example, a promoter. Alternatively, the viral vector may not contain a control sequence and will instead rely on a control sequence within the host cell to drive expression of the ADAMTS protein. Non-limiting examples of virus vectors that may be used to deliver a nucleic acid include Adenoviral vectors, AAV vectors, and Retroviral vectors.

[0227] In one embodiment, an Adenovirus expression vector include those constructs containing adenovirus sequences sufficient to support packaging of the construct and to ultimately express an ADAMTS construct that has been cloned therein. Adenoviral vectors allow for the introduction of foreign sequences up to 7 kb (Grunhaus et al., Seminar in Virology, 200(2):535-546, 1992)).

[0228] In another embodiment, an adeno-associated virus (AAV) can be used to introduce a nucleotide sequence encoding an ADAMTS protein (e.g. , ADAMTS 13) into a host cell for expression. AAV systems have been described previously and are generally well known in the art (Kelleher and Vos, Biotechniques , 17(6): 1 1 10-7, 1994; Cotten et al, Proc Natl Acad Sci USA, 89(13):6094-6098, 1992; Curiel, Nat Immun, 13(2-3): 141-64, 1994; Muzyczka, Curr Top Microbiol Immunol, 158:97-129, 1992). Details concerning the generation and use of rAAV vectors are described, for example, in U.S. Patent Nos. 5,139,941 and 4,797,368, ~~each incorporated herein by reference in their entireties for all purposes~~.

[0229] In one embodiment, a retroviral expression vector can be used to introduce a nucleotide sequence encoding an ADAMTS protein (e.g., ADAMTS 13) into a host cell for expression. These systems have been described previously and are generally well known in the art (Mann et

al, Cell, 33: 153-159, 1983; Nicolas and Rubinstein, In: Vectors: A survey of molecular cloning vectors and their uses, Rodriguez and Denhardt, eds., Stoneham: Butterworth, pp. 494-513, 1988; Temin, In: Gene Transfer, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986). In a specific embodiment, the retroviral vector is a lentiviral vector (see, for example, Naldini et al, Science, 272(5259):263-267, 1996; Zufferey et ah, Nat Biotechnol, 15(9): 871-875, 1997; Blomer et al, J Virol, 71(9):6641-6649, 1997; U.S. Pat. Nos. 6,013,516 and 5,994,136).

[0230] Non-limiting examples of vectors for prokaryotic expression include plasmids such as pRSET, pET, pBAD, etc., wherein the promoters used in prokaryotic expression vectors include lac, trc, trp, recA, araBAD, etc. Examples of vectors for eukaryotic expression include: (i) for expression in yeast, vectors such as pAO, pPIC, pYES, pMET, using promoters such as AOX1 , GAP, GAL1 , AUG1 , etc; (ii) for expression in insect cells, vectors such as pMT, pAc5, pIB, pMIB, pBAC, etc., using promoters such as PH, plO, MT, Ac5, OpIE2, gp64, polh, etc., and (iii) for expression in mammalian cells, vectors such as pSVL, pCMV, pRc/RSV, pcDNA3, pBPV, etc., and vectors derived form viral systems such as vaccinia virus, adeno-associated viruses, herpes viruses, retroviruses, etc., using promoters such as CMV, SV40, EF-1, UbC, RSV, ADV, BPV, and β-actin. An exemplary vector for expressing rA13 is described by Plaimauer et al. (Blood. 2002 Nov 15;100(10):3626-32. Epub 2002 Jul 12)~~, the content of which is hereby incorporated by reference in its entirety for all purposes~~.

[0231] In certain embodiments, the cell-culture methods of the invention may comprise the use of a microcarrier. The present invention provides, among other aspect, methods of large-scale ADAMTS protein expression. In some embodiments, the cell-cultures of the embodiments can be performed in large bioreactors under conditions suitable for providing high volume-specific culture surface areas to achieve high cell densities and protein expression. One means for providing such growth conditions is to use microcarriers for cell-culture in stirred tank bioreactors. In another embodiment, these growth requirements are met via the use of a suspension cell culture.

V. Specific embodiments

A. Recombinant Von Willebrand Factor (rVWF)

The optimal pharmaceutical formulation is determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712~~, the disclosure of which is hereby incorporated by reference~~. Such formulations influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose is calculated according to body weight, body surface area or organ size. Appropriate dosages may be ascertained through use of established assays for determining blood level dosages in conjunction with appropriate dose-response data. The final dosage regimen is determined by the attending physician, considering various factors which modify the action of drugs, e.g. the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. By way of example, a typical dose of a recombinant vWF of the present invention is approximately 50 U/kg, equal to 500 g/kg. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

VII. Methods of treatment

[0301] The present invention further contemplates methods of treating a patient in need of rVWF or rA13 produced according to the methods described herein. Such methods of treatment can include administration of pharmaceutical formulations comprising the recombinant rA13 or the high molecular weight, recombinant vWF of the present invention.

[0302] In another aspect, the present invention provides methods for therapeutic or prophylactic treatments comprising the administration of an rVWF or rA13 composition provided herein. Generally, for therapeutic applications, formulations are administered to a subject with a disease or condition associated with ADAMTS13 or VWF dysfunction or otherwise in need thereof, in a "therapeutically effective dose." Formulations and amounts effective for these uses will depend upon the severity of the disease or condition and the general state of the patient's health. Single or multiple administrations of the formulations may be administered depending on the dosage and frequency as required and tolerated by the patient.

0.66 µg/L Cu^{2+}	1.29	1049	821	1862
2 µg/L Cu^{2+}	2.17	2470	1146	1749

[0323] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. ~~All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.~~

WHAT IS CLAIMED IS:

1. A method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of:

(a) providing a basal cell culture media;

(b) supplementing the basal cell culture media with copper to provide a final copper concentration of from 1.0 μg/L to 20.0 μg/L;

(c) providing one or more cells comprising a nucleic acid encoding a rA13 protein;

(d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant, wherein the NH^{4+} content of the cell culture supernatant is maintained at a concentration below 10 mM; and

(e) recovering at least a portion of the culture supernatant,

wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

2. The method of claim 1, wherein the basal cell culture media is an animal protein free culture media.

3. The method of claim 1 or 2, wherein the basal cell culture media is a protein free culture media.

4. The method according to any one of claims 1 to 3, wherein the basal cell culture media is a chemically defined culture media.

5. The method according to any one of claims 1 to 4, wherein the final copper concentration of the supplemented basal cell culture media is at least 2 μg/L copper.

6. The method according to any one of claims 1 to 4 wherein the final copper concentration of the supplemented basal cell culture media is at least 4 μg/L copper.

7. The method of claim 1, wherein the final copper concentration of the supplemented basal cell culture media is between 1 µg/L and 6 µg/L copper.

8. The method of claim 1, wherein the final copper concentration of the supplemented basal cell culture media is between 2 µg/L and 4 µg/L copper.

9. The method according to any one of claims 1 to 8, wherein copper supplementing the basal cell culture media is provided as a copper salt, a copper chelate, or a combination thereof.

10. The method of claim 9, wherein the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

11. The method according to any one of claims 1 to 10, wherein the one or more cells are mammalian cells.

12. The method of claim 11, wherein the mammalian cells are CHO cells.

13. The method according to any one of claims 1 to 12, wherein culturing the one or more cells comprises batch cultivation of the cells.

14. The method according to any one of claims 1 to 12, wherein culturing the one or more cells comprises continuous cultivation of the cells.

15. The method of claim 14, wherein the continuous cultivation of cells is performed in chemostatic mode.

16. The method of claim 14, wherein the continuous cultivation of cells is performed in perfusion mode.

17. The method according to any one of claims 1 to 16, wherein the one or more cells is cultured in at least 100 L of the supplemented basal cell culture media.

18. The method according to any one of claims 1 to 17, wherein the cell density is maintained at less than $4.0x10^6$ cells per mL during the step of culturing the one or more cells.

19. The method of claim 18, wherein the cell density is maintained at less than $3.5x10^6$ cells per mL during the step of culturing the one or more cells.

20. The method of claim 18, wherein the cell density is maintained at less than $3.0x10^6$ cells per mL during the step of culturing the one or more cells.

21. The method of claim 18, wherein the cell density is maintained at between $3.0x10^6$ cells per mL and $4.0x10^6$ cells per mL during the step of culturing the one or more cells.

22. The method according to any one of claims 1 to 21, wherein the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from the portion of culture supernatant.

23. The method according to any one of claims 1 to 22, wherein at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

24. The method of claim 23, wherein at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

25. The method according to any one of claims 1 to 24, wherein the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 800 mU/μg.

26. The method according to any one of claims 1 to 24, wherein the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 1200 mU/μg.

27. The method according to any one of claims 1 to 24, wherein the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 1600 mU/μg.

28. The method according to any one of claims 1 to 27, wherein the NH_4^+ content of the cell culture supernatant is maintained at a concentration below 5 mM.

29. The method according to any one of claims 1 to 28, wherein the method further comprises a rA13 enrichment step.

30. A cell culture supernatant comprising recombinant ADAMTS13 (rA13), wherein the supernatant is prepared by a method according to any one of the preceding claims.

31. The cell culture supernatant comprising recombinant ADAMTS13 (rA13) of claim 30, wherein the supernatant contains at least 6 U FRETS-VWF73 activity per mL.

32. The cell culture supernatant of claim 32, wherein the supernatant contains at least 8 U FRETS-VWF73 activity per mL.

33. The cell culture supernatant of claim 32, wherein the supernatant contains at least 10 U FRETS-VWF73 activity per mL.

34. The cell culture supernatant comprising recombinant ADAMTS13 (rA13) of claim 30, wherein the supernatant contains at least 3 µg rA13 per mL.

35. The cell culture supernatant of claim 34, wherein the supernatant contains at least 4 µg rA13 per mL.

36. The cell culture supernatant of claim 34, wherein the supernatant contains at least 5 µg rA13 per mL.

37. A recombinant ADAMTS13 (rA13) composition prepared by a method according to any one of claims 1 to 29.

38. The composition of claim 37, wherein the composition is formulated for pharmaceutical administration.

39. The composition of claim 37 or 38, wherein the composition is formulated for intravenous administration.

WHAT IS CLAIMED IS:

1. A method for producing a recombinant ADAMTS13 (rA13) composition, the method comprising the steps of:

(a) providing a basal cell culture media;

(b) supplementing the basal cell culture media with copper to provide a final copper concentration of from 1.0 μg/L to 6.0 μg/L;

(c) providing one or more cells comprising a nucleic acid encoding a rA13 protein;

(d) culturing the one or more cells in the copper supplemented cell culture media such that rA13 is expressed and excreted from the cells into a culture supernatant, wherein the NH^{4+} content of the cell culture supernatant is maintained at a concentration below 10 mM; and

(e) recovering at least a portion of the culture supernatant,

wherein at least 1500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

2. The method of claim 1, wherein the basal cell culture media is an animal protein free culture media.

3. The method of claim 1 or 2, wherein the basal cell culture media is a protein free culture media.

4. The method according to any one of claims 1 to 3, wherein the basal cell culture media is a chemically defined culture media.

5. The method according to any one of claims 1 to 4, wherein the final copper concentration of the supplemented basal cell culture media is at least 2 μg/L copper.

6. The method according to any one of claims 1 to 4 wherein the final copper concentration of the supplemented basal cell culture media is at least 4 μg/L copper.

7. The method of claim 1, wherein the final copper concentration of the supplemented basal cell culture media is between 2 μg/L and 4 μg/L copper.

8. The method according to any one of claims 1 to 7, wherein copper supplementing the basal cell culture media is provided as a copper salt, a copper chelate, or a combination thereof.

9. The method of claim 8, wherein the copper salt is selected from the group consisting of copper sulfate, copper acetate, copper carbonate, copper chloride, copper hydroxide, copper nitrate, and copper oxide.

10. The method according to any one of claims 1 to 9, wherein the one or more cells are mammalian cells.

11. The method of claim 10, wherein the mammalian cells are CHO cells.

12. The method according to any one of claims 1 to 11, wherein culturing the one or more cells comprises batch cultivation of the cells.

13. The method according to any one of claims 1 to 11, wherein culturing the one or more cells comprises continuous cultivation of the cells.

14. The method of claim 13, wherein the continuous cultivation of cells is performed in chemostatic mode.

15. The method of claim 13, wherein the continuous cultivation of cells is performed in perfusion mode.

16. The method according to any one of claims 1 to 15, wherein the one or more cells is cultured in at least 100 L of the supplemented basal cell culture media.

17. The method according to any one of claims 1 to 16, wherein the cell density is maintained at less than $4.0x10^6$ cells per mL during the step of culturing the one or more cells.

18. The method of claim 17, wherein the cell density is maintained at less than $3.5x10^6$ cells per mL during the step of culturing the one or more cells.

19. The method of claim 17, wherein the cell density is maintained at less than $3.0x10^6$ cells per mL during the step of culturing the one or more cells.

20. The method of claim 17, wherein the cell density is maintained at between $3.0x10^6$ cells per mL and $4.0x10^6$ cells per mL during the step of culturing the one or more cells.

21. The method according to any one of claims 1 to 20, wherein the step of recovering at least a portion of the culture supernatant comprises filtration or centrifugation to remove cells from the portion of culture supernatant.

22. The method according to any one of claims 1 to 21, wherein at least 2000 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

23. The method of claim 22, wherein at least 2500 Units FRETS-VWF73 activity per liter supplemented basal cell culture media per day is present in the recovered culture supernatant.

24. The method according to any one of claims 1 to 23, wherein the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 800 mU/μg.

25. The method according to any one of claims 1 to 23, wherein the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 1200 mU/μg.

26. The method according to any one of claims 1 to 23, wherein the recovered supernatant has a rA13 specific FRETS-VWF73 activity of at least 1600 mU/μg.

27. The method according to any one of claims 1 to 26, wherein the NH_4^+ content of the cell culture supernatant is maintained at a concentration below 5 mM.

28. The method according to any one of claims 1 to 27, wherein the method further comprises a rA13 enrichment step.

29. A cell culture supernatant comprising recombinant ADAMTS13 (rA13), wherein the supernatant is prepared by a method according to any one of the preceding claims.

30. The cell culture supernatant comprising recombinant ADAMTS13 (rA13) of claim 29, wherein the supernatant contains at least 6 U FRETS-VWF73 activity per mL.

31. The cell culture supernatant of claim 30, wherein the supernatant contains at least 8 U FRETS-VWF73 activity per mL.

32. The cell culture supernatant of claim 30, wherein the supernatant contains at least 10 U FRETS-VWF73 activity per mL.

33. The cell culture supernatant comprising recombinant ADAMTS13 (rA13) of claim 29, wherein the supernatant contains at least 3 μg rA13 per mL.

34. The cell culture supernatant of claim 33, wherein the supernatant contains at least 4 μg rA13 per mL.

35. The cell culture supernatant of claim 33, wherein the supernatant contains at least 5 μg rA13 per mL.

36. A recombinant ADAMTS13 (rA13) composition prepared by a method according to any one of claims 1 to 28.

37. The composition of claim 36, wherein the composition is formulated for pharmaceutical administration.

38. The composition of claim 36 or 37, wherein the composition is formulated for intravenous administration.