TRUNCATED AGGRECANASE MOLECULES

RELATED APPLICATIONS

[001] This application relies on the benefit of priority of U.S. provisional patent application Serial No.: 60/354,592, the entire disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

[002] The present invention relates to the discovery of truncated aggrecanase molecules, processes for producing them, and methods employing these molecules to develop inhibitors to aggrecanase enzymes. The invention further relates to the development of inhibitors of, as well as antibodies to, aggrecanase enzymes. These inhibitors and antibodies may be useful for the treatment of various aggrecanase-associated conditions including osteoarthritis.

BACKGROUND OF THE INVENTION

[003] Aggrecan is a major extracellular component of articular cartilage. It is a proteoglycan responsible for providing cartilage with its mechanical properties of compressibility and elasticity. The loss of aggrecan has been implicated in the degradation of articular cartilage in arthritic diseases such as osteoarthritis.

[004] Osteoarthritis is a debilitating disease which affects at least 30 million Americans (MacLean *et al.*, J Rheumatol 25:2213-8 (1998)). Osteoarthritis can severely reduce quality of life due to degradation of articular cartilage and the resulting chronic pain. An early and important characteristic of the osteoarthritic process is loss of aggrecan from the extracellular matrix (Brandt and Mankin, Pathogenesis of Osteoarthritis, in Textbook of Rheumatology, WB Saunders Company, Philadelphia, PA, at 1355-1373 (1993)). The large, sugar-containing

portion of aggrecan is thereby lost from the extra-cellular matrix, resulting in deficiencies in the biomechanical characteristics of the cartilage.

[005] A proteolytic activity termed "aggrecanase" is believed to be responsible for the cleavage of aggrecan, thereby having a role in cartilage degradation associated with osteoarthritis and inflammatory joint disease. Research has been conducted to identify the enzymes responsible for the degradation of aggrecan in human osteoarthritic cartilage. Aggrecan contains two N-terminal globular domains, G1 and G2, separated by a proteolytically-sensitive interglobular domain, followed by a glycosaminoglycan attachment region and a C-terminal globular domain, G3. At least two enzymatic cleavage sites have been identified within the interglobular domain of aggrecan. One enzymatic cleavage site within the interglobular domain of aggrecan (Asn^{341}-Phe^{342}) has been observed to be cleaved by several known metalloproteases. Flannery *et al.*, J Biol Chem 267:1008-14 (1992); Fosang *et al.*, Biochemical J. 304:347-351 (1994). Cleavage at a second aggrecan cleavage site within aggrecan (Glu^{373}-Ala^{374}) due to IL-1 induced cartilage aggrecan cleavage results in the generation of an aggrecan fragment found in human synovial fluid (Sandy *et al.,* J Clin Invest 69:1512-1516 (1992); Lohmander *et al.*, Arthritis Rheum 36: 1214-1222 (1993); Sandy *et al.*, J Biol Chem 266: 8683-8685 (1991)). Aggrecan cleavage at (Glu^{373}-Ala^{374}) has been attributed to aggrecanase activity (Sandy *et al.*, J Clin Invest 69:1512-1516 (1992). This Glu^{373}-Ala^{374} cleavage site will be referred to as the aggrecanase cleavage site.

[006] Recently, identification of two enzymes, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-11) within the "Disintegrin-like and

Metalloprotease with Thrombospondin type 1 motif" (ADAMTS) family, have been identified which are synthesized by IL-1 stimulated cartilage and cleave aggrecan at the Glu^{373}-Ala^{374} site (Tortorella *et al.*, Science 284:1664-6 (1999); Abbaszade *et al.*, J Biol Chem 274: 23443-23450 (1999)). Aggrecanase-1 is reported to include at least six domains: signal; propeptide; catalytic; disintegrin; TSP type-1 motif and C-terminal. Aggrecanase-2 is also a multidomain protein. It is reported to have a signal sequence; a prodomain; a metalloproteinase domain; a disintegrin domain and a spacer domain between a TSP motif and a TSP sub motif in the C-terminal of the protein. It was generally believed that the TSP domains and the spacer domain are critical for substrate recognition. Specifically, Tortorella *et al* reported that "[T]his region may serve to bind aggrecanase-1 to the glycosaminoglycans of the aggrecan substrate." See Tortorella *et al.*, Science 284:1664-6 (1999).

[007] It is contemplated that there are other, related enzymes in the ADAMTS family which are capable of cleaving aggrecan at the Glu^{373}-Ala^{374} bond and could contribute to aggrecan cleavage in osteoarthritis. It is possible that these enzymes could be synthesized by osteoarthritic human articular cartilage. However, it has been difficult to develop inhibitors and treatment therapies to treat diseases that involve aggrecan cleavage because aggrecanases have been difficult to isolate and purify in large amounts due to poor stability of these molecules and generally low expression levels. Therefore, there is a need to identify novel forms of aggrecanases and further develop ways to isolate and purify aggrecanase proteins in large amounts in order to investigate their role in

disease states and also to develop therapies and compositions to treat diseases involving aggrecan cleavage.

SUMMARY OF THE INVENTION

[008] The present invention is directed to truncated aggrecanase proteins and variants and fragments thereof; nucleotide sequences which encode truncated aggrecanase enzymes of the invention and fragments and variants thereof; and processes for the production of truncated aggrecanases. Truncated aggrecanases of the invention are biologically active and have greater stability and higher expression levels than their full-length counterparts. More specifically, the invention features truncated aggrecanase-1 and aggrecanase-2 enzymes that are more stable and show higher levels of expression than full-length aggrecanase-1 and aggrecanase-2 enzymes, respectively; nucleic acid sequences encoding truncated aggrecanases-1 and 2 of the invention and fragments and variants thereof; and methods of producing truncated aggrecanases 1 and 2, or fragments and variants thereof.

[009] In one embodiment, truncated aggrecanases of the invention comprise aggrecanases that have at least one TSP domain deleted. In another embodiment, truncated aggrecanases of the invention comprise aggrecanases that have at least two TSP domains deleted. Although, TSP domains in aggrecanases have been thought to be important for substrate recognition, and therefore, for the ability of aggrecanase to recognize and subsequently cleave aggrecan, truncated aggrecanase proteins of the invention are biologically-active despite deletion of one or both TSP domains in the proteins.

[010] Truncated aggrecanases of the invention have greater stability and are expressed at higher levels compared with the full-length aggrecanase proteins, thereby facilitating isolation, purification, and use of aggrecanases of the invention in the development of inhibitors and therapies for treatment of diseases. Accordingly, in one embodiment, the invention comprises methods for producing large amounts of purified truncated aggrecanases that may be used for development of inhibitors and treatment therapies.

[011] The invention further includes compositions comprising truncated aggrecanases of the invention and use of such compositions for the development of inhibitors of aggrecanases for treatment of diseases including osteoarthritis. In addition, the invention includes methods for identifying and developing inhibitors of aggrecanase which block the enzyme's activity. The invention also includes antibodies to these enzymes, in one embodiment, for example, antibodies that block aggrecanase activity. These inhibitors and antibodies may be used in various assays and therapies for treatment of conditions characterized by the degradation of articular cartilage. In one embodiment, inhibitors are peptide molecules that bind aggrecanases.

[012] This invention provides amino acid sequences of biologically active truncated aggrecanase molecules that have greater stability compared with the full-length aggrecanase protein.

[013] In one aspect, the invention features biologically active truncated aggrecanase-2 molecules that have at least one TSP domain deleted, such as a protein with an amino acid sequence from amino acid #1 (Met) through amino acid #753 (Glu) of SEQ ID NO: 4; from amino acid # 1 (Met) through amino acid # 752

(Pro) of SEQ ID NO: 6; and from amino acid # 1 (Met) through amino acid # 628 (Phe) of SEQ ID NO: 8, and variants and fragments thereof, including substitution mutants, that exhibit aggrecanase activity.

[014] The invention also features truncated aggrecanase-1 molecules that have at least one TSP domain deleted; for example, an aggrecanase-1 protein with an amino acid sequence from amino acid # 1 (Met) through amino acid # 520 (Ala) of SEQ ID NO: 13; fragments and variants thereof, including substitution mutants, that exhibit aggrecanase activity.

[015] In another embodiment, the invention features biologically-active truncated aggrecanase-2 proteins with at least two TSP domains deleted comprising, for example, a protein with an amino acid sequence from amino acid # 1 through amino acid # 527 (His) of SEQ ID NO: 10 (Figure 10), variants and fragments of thereof, including substitution mutants, that exhibit aggrecanase activity.

[016] Truncated aggrecanases with one or both TSP domains deleted are biologically active and are more stable than the full-length aggrecanase enzymes.

[017] The invention also features nucleic acid molecules that encode truncated aggrecanases of the invention. For example, nucleic acid molecules encoding truncated aggrecanase-2 molecules of the invention include: nucleotide #1 (atg) through nucleotide #2259 (gaa) of SEQ ID NO: 3 (Figure 3), which encodes a polypeptide set forth in SEQ ID NO: 4; nucleotide #1 (atg) through nucleotide #2256 (cct) of SEQ ID NO: 5 (Figure 5), which encodes a polypeptide set forth in SEQ ID NO: 6; nucleotide #1 (atg) through nucleotide #1884 (ttt) of SEQ ID NO: 7 (Figure 7), which encodes the polypeptide set forth in SEQ ID NO:

8; and nucleotide #1 (atg) through nucleotide # 1701 (cat) of SEQ ID NO: 9 (Figure 9), which encodes the polypeptide set forth in SEQ ID NO: 10. Nucleic acid molecules of the invention further include fragments and variants of SEQ ID NOs: 3, 5, 7 and 9 which encode truncated aggrecanase-2 molecules of the invention, nucleotide sequences that hybridize under moderate to stringent conditions with nucleotide sequences of SEQ ID NOs: 3, 5, 7 or 9 and fragments or variants thereof, naturally occurring allelic sequences, and equivalent degenerative codon sequences of aggrecanase-2 nucleic acid sequences disclosed herein.

[018] Nucleic acid molecules encoding truncated aggrecanase-1 molecules of the invention include, for example, nucleotides which encode the polypeptides of SEQ ID NOs: 12 and 13; set forth in Figures 23 and 24 respectively, fragments and variants of nucleic acids that encode truncated aggrecanase-1 molecules of the invention, nucleotide sequences that hybridize under moderate to stringent conditions to nucleic acid sequences that encode truncated aggrecanase-1 molecules of the invention, for example, nucleic acid sequences of Figures 23 and 24, or fragments and variants thereof, naturally occurring allelic sequences and equivalent degenerative codon sequences of aggrecanase-1 encoding nucleic acid sequences. A nucleic acid sequence for full-length aggrecanase-1 molecule is found in Genbank under Accession No.: NM_005099 (Figure 22) Therefore, one skilled in the art can use this sequence to generate a full-length aggrecanase-1 molecule set forth in SEQ ID NO: 11, or use part of the nucleotide sequence to generate a truncated protein, for example, the aggrecanase protein set forth in Figure 12 or 13. For example nucleotide #407

through nucleotide #2132 of the published NM_005099 sequence (Figure 23) (SEQ ID NO: 32) may be used for generating the truncated aggrecanase-1 protein of SEQ ID NO: 12. Similarly, nucleotide #1 through nucleotide #1967 of the published NM_005099 sequence (Figure 24) (SEQ ID NO: 33) may be used for the generation of truncated aggrecanase molecule of Figure 13.

[019] In another aspect, the invention includes aggrecanase molecules that comprise mutations that increase stability and expression levels of truncated aggrecanase molecules compared with their full-length counterparts. Aggrecanases with mutations in their active sites are particularly useful for the synthesis of inhibitors of aggrecanases. Accordingly, In one embodiment, the invention features an aggrecanase-2 molecule with a mutation at amino acid 411 (E411-Q411 mutation) in the active site within the catalytic domain. The amino acid sequence of an aggrecanase-2 molecule with the E411-Q411 mutation is shown in Figure 21 (SEQ ID NO: 30). Mutations that lead to increased stability of the aggrecanase proteins in comparison with their wild-type counterparts can be made in both truncated as well as full-length aggrecanase proteins. It is contemplated that mutations that alter stability of aggrecanase molecules can be made within the catalytic domain of an aggrecanase protein or outside the catalytic domain. Aggrecanase proteins carrying such mutations may be found in nature or may be generated artificially. One skilled in the art can test the affect of a mutation on the stability of an aggrecanase molecule by one of many assays provided. Mutations of the invention include, for example, amino acid substitutions or modifications. Amino acid mutations in aggrecanases of the

invention can be generated by mutagenesis, chemical alteration, or by alteration of DNA sequence used to produce the polypeptide

[020] Aggrecanase-1 molecules can also be generated to include mutations in the catalytic domain in order to increase their stability. For example, Figure 17 features flag-tagged truncated aggrecanase-1 proteins that include an E-to-Q amino acid mutation in the catalytic domain of truncated aggrecanase-1 molecules, the wild-type sequences of which are set forth in SEQ ID NO: 12 and 13, thereby leading to increased stability of aggrecanase-1 proteins compared to full-length wild-type aggrecanase-1 protein, the sequence of which is set forth in SEQ ID NO: 11. These aggrecanases are particularly useful for the development as well as identification of novel inhibitors of aggrecanases.

[021] The invention further features truncated and/or mutant aggrecanase family members and aggrecanase-like proteins with deletions and/or substitution mutations, where a deletion or an amino acid substitution mutation occurs in a region of the protein comparable to that of aggrecanases of the invention.

[022] The invention further includes variants and equivalent degenerative codon sequences of nucleic acid sequences described herein that encode biologically active truncated aggrecanase polypeptides. Additionally, the invention includes nucleic acid molecules that hybridize under moderate to stringent conditions to the nucleic acids of the invention, allelic variants and substitution and deletion mutants of nucleic acids molecules described herein. In one embodiment, truncated aggrecanases and/or aggrecanases carrying at least one amino acid substitution encoded by nucleic acid molecules of the invention are more stable than the corresponding full-length aggrecanase protein and can be

expressed at higher levels than the full-length aggrecanase protein. In one embodiment, mutations are introduced in nucleic acid molecules encoding aggrecanases of the invention that lead to mutations; for example, amino acid substitutions, in the protein encoded by the nucleic acid carrying the mutation. One example of such a mutation is a nucleic acid sequence encoding an aggrecanase-2 protein with an E-to-Q mutation in the active site of molecule. In another embodiment, the invention includes aggrecanase-1 nucleic acid molecules that encode aggrecanase-1 proteins comprising an E-to-Q mutation in the catalytic domain, thereby leading to generation of molecules with greater stability, longer half-lives and increased levels of expression as compared to the full-length aggrecanases 1 and 2.

[023] It is expected that other species have DNA sequences that are similar or identical to human aggrecanase enzymes described herein. Accordingly, the invention further includes methods for obtaining other nucleic acid molecules encoding truncated aggrecanases or aggrecanase-like molecules or aggrecanases with amino acid substitutions that alter their biological activity, from humans as well as non-human species. In one embodiment, a method for isolating a nucleic acid sequence encoding an aggrecanase of the invention involves utilizing a nucleic acid sequence disclosed herein or variants or fragments thereof; for example, SEQ ID NO: 1 or a fragment or a variant thereof; SEQ ID NO: 3 or a fragment or a variant thereof; SEQ ID NO: 5 or a fragment or a variant thereof; SEQ ID NO: 7 or a fragment or a variant thereof; SEQ ID NO: 9 or a fragment or a variant thereof, SEQ ID NO: 31 or a fragment or a variant thereof; SEQ ID NO: 32 or a fragment or variant thereof; or SEQ ID NO: 33 or a fragment

or a variant thereof, to design probes for screening libraries for the corresponding gene from other species or coding regions of genes that encode proteins/peptides with aggrecanase activity. Therefore, the invention includes DNA sequences from other species, which are homologous to human aggrecanase sequences, or fragments or variants thereof, and can be obtained using at least one of the DNA sequences provided herein. In addition, the present invention includes DNA sequences that encode fragments or variants of aggrecanases of the invention. The present invention may also include functional fragments of the aggrecanase protein, and DNA sequences encoding such functional fragments, as well as functional fragments of other related proteins. The ability of such a fragment to function is determinable by an assay of the protein in one of many biological assays described for the assay of the aggrecanase protein.

[024] In another aspect, the invention provides methods for producing isolated truncated aggrecanases of the invention. In one embodiment, a human aggrecanase protein of the invention or a variant or fragment thereof may be produced, for example, by culturing a cell transformed with a DNA sequence: from nucleotide #1 through nucleotide #2259, set forth in SEQ ID NO: 3; or from nucleotide #1 through nucleotide #2256, set forth in Figure 5; or from nucleotide #1 through nucleotide #1884, set forth in Figure 7; or from nucleotide #1 through nucleotide #1701, set forth in Figure 9, and recovering and purifying from the culture medium an aggrecanase-2 protein characterized by the amino acid sequence set forth in: Figure 4 from amino acid #1 (Met) through amino acid #753 (Glu); Figure 6 from amino acid #1 (Met) through amino acid # 752 (Pro); Figure 8 from amino acid #1 (Met) through amino acid #628 (Phe); or Figure 10 from amino

acid #1 (Met) through amino acid #567 (His). Similarly, nucleic acid sequences expressing truncated aggrecanase-1 molecules; for example, nucleic acid sequences set forth in Figures 23 and 24 can be used for the production of truncated aggrecanase-1 proteins set forth in Figures 12 and 13, where the aggrecanase-1 proteins are substantially free from other proteinaceous materials with which they are co-produced.

[025] In another embodiment, truncated aggrecanase proteins of the invention may be produced by culturing a cell transformed with a full-length DNA sequence for aggrecanase, and recovering truncated aggrecanase proteins from the culture medium. Accordingly, in one embodiment, a truncated aggrecanase-2 protein including amino acid #1 (Met) to amino acid #753 (Glu), set forth in SEQ ID No: 4, is recovered from the culture medium of a cell transformed with a full-length nucleic acid molecule for aggrecanase-2; for example, nucleic acid molecule set forth in SEQ ID NO: 1. In another embodiment, a truncated aggrecanase-2 molecule including amino acid #1 (Met) to amino acid #752 (Pro), is recovered from culture medium of cells transformed with a nucleic acid molecule for full-length aggrecanase-2. Truncated aggrecanase protein of SEQ ID NO: 4 results from cleavage of the full-length aggrecanase protein, set forth in SEQ ID NO: 2, at E^{753}-G^{754}, yielding a 55kDa protein. The nucleotide and amino acid sequences of the full-length aggrecanase-2 molecule are set forth in SEQ ID NO: 1 and SEQ ID NO: 2 respectively (Accession Nos: NM_007038 and NP_008969) (Figures 1A-1C and 2).

[026] Truncated aggrecanases of the invention that are purified from a culture medium are substantially free from other proteinaceous materials. A

recovered purified aggrecanase protein having at least one TSP domain deleted generally exhibits proteolytic aggrecanase activity by cleaving aggrecan, as disclosed. Therefore, truncated proteins of the invention may be further characterized by the ability to demonstrate aggrecan proteolytic activity in an assay which determines the presence of an aggrecan-degrading molecule. These assays or the development thereof is within the knowledge of one skilled in the art. Such assays may involve contacting an aggrecan substrate with a truncated aggrecanase molecule and monitoring the production of aggrecan fragments (*see*, for example, Hughes *et al.*, Biochem J 305: 799-804 (1995); Mercuri *et al.,* J. Bio Chem. 274:32387-32395 (1999)). For production in mammalian cells, a DNA sequence used for expression of a truncated aggrecanase of the invention further comprises a DNA sequence encoding a suitable propeptide 5' to and linked in frame to the nucleotide sequence encoding the aggrecanase enzyme.

[027] The invention also provides antibodies that bind to isolated aggrecanase proteins of the invention. In one embodiment, such an antibody reduces, inhibits or antagonizes aggrecanase activity. The invention further provides methods for developing and identifying inhibitors of aggrecanase activity comprising the use of a truncated aggrecanase protein with amino acid sequence chosen from, for example, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 13 or a fragment or a variant thereof. In one embodiment, inhibitors of aggrecanase activity prevent cleavage of aggrecan.

[028] Additionally, the invention provides pharmaceutical compositions for inhibiting the proteolytic activity of aggrecanases, wherein the compositions

comprise at least one antibody according to the invention and at least one pharmaceutical carrier. The invention also provides methods for inhibiting aggrecanase activity in a mammal comprising administering to the mammal an effective amount of a pharmaceutical composition according to the invention to inhibit aggrecanase activity.

[029] In another embodiment, the invention includes methods for identifying or developing inhibitors of aggrecanases and the inhibitors produced thereby. In one embodiment, inhibitors of the invention prevent binding of an aggrecanase to an aggrecan molecule. In another embodiment, inhibitors of the invention prevent cleavage of aggrecan by aggrecanase.

[030] The method may entail the identification of inhibitors based on an assay comprising combining at least one aggrecanase protein of this invention with at least one test sample; and determining if the test sample inhibits activity of the aggrecanase protein. The test sample may comprise known or unknown samples, and these samples may be peptides, proteins, chemical compounds (often referred to as small molecules), or antibodies. They may be selected for testing individually, or in batches, such as from a library. The art provides aggrecanase activity assays that could be easily utilized in such a method. Assays for inhibitors may involve contacting a mixture of aggrecan and the inhibitor with an aggrecanase molecule followed by measurement of the aggrecanase inhibition; for instance, by detection and measurement of aggrecan fragments produced by cleavage at an aggrecanase-susceptible site.

[031] The method may also entail the determination of binding sites based on at least one of the amino acid sequence of aggrecanase and the three-

dimensional structure of aggrecanase, and optionally aggrecan. Based on this information, one could developing or identifying a candidate molecule that may inhibit aggrecanase activity based on a structural analysis, such as predicted structural interaction with the binding site. In one embodiment, such a molecule may comprise a peptide, protein, chemical compound, or antibody. Candidate molecules may be later assayed for actual inhibitory activity of the aggrecanase enzyme, as discussed.

[032] Another aspect of the invention therefore provides pharmaceutical compositions containing a therapeutically-effective amount of aggrecanase inhibitors in a pharmaceutically-acceptable vehicle.

[033] Aggrecanase-mediated degradation of aggrecan in cartilage has been implicated in osteoarthritis and other inflammatory diseases. Therefore, these compositions of the invention may be used in the treatment of diseases characterized by the degradation of aggrecan and/or an up regulation of aggrecanase. The compositions may be used in the treatment of these conditions or in the prevention thereof.

[034] The invention further includes methods for treating patients suffering from conditions characterized by a degradation of aggrecan or preventing such conditions. These methods, according to the invention, entail administering to a patient needing such treatment an effective amount of a composition comprising an aggrecanase inhibitor which inhibits the proteolytic activity of aggrecanase enzymes.

[035] The DNA sequences of the present invention are useful, for example, as probes for the detection of mRNA encoding aggrecanase in a given

cell population. Thus, the present invention includes methods of detecting or diagnosing genetic disorders involving aggrecanases, or disorders involving cellular, organ, or tissue disorders in which aggrecanase is irregularly transcribed or expressed. The DNA sequences may also be useful for preparing vectors for gene therapy applications as described below.

[036] A further aspect of the invention includes vectors comprising a DNA sequence as described above in operative association with an expression control sequence therefor. These vectors may be employed in a novel process for producing an aggrecanase protein of the invention in which a cell line transformed with a DNA sequence encoding an aggrecanase protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and an aggrecanase protein is recovered and purified therefrom. This process may employ a number of known cells, both prokaryotic and eukaryotic in origin, as host cells for expression of the polypeptide. The vectors may be used in gene therapy applications. In such use, the vectors may be transfected into the cells of a patient *ex vivo*, and the cells may be reintroduced into a patient. Alternatively, the vectors may be introduced into a patient *in vivo* through targeted transfection.

[037] Additional aspects of the disclosure will be set forth in part in the description, and in part be obvious from the description, or may be learned from practicing the invention. The invention is set forth and particularly pointed out in the claims, and the disclosure should not be construed as limiting the scope of the claims. The following detailed description includes exemplary representations of various embodiments of the invention, which are not restrictive of the invention as

claimed. The accompanying figures constitute a part of this specification and, together with the description, serve to illustrate embodiments and not limit the invention.

DESCRIPTION OF THE DRAWINGS

[038] Figures 1A-1C show the nucleotide sequence of a full-length aggrecanase-2/ADAMTS-5 molecule (SEQ ID NO: 1).

[039] Figure 2 shows the amino acid sequence of a full length aggrecanase-2/ADAMTS-5 molecule (SEQ ID NO: 2) encoded by nucleotide #1 through nucleotide #2915 of SEQ ID NO: 1.

[040] Figure 3 shows the nucleotide sequence of a truncated aggrecanase-2 protein of the invention from nucleotide #1 (atg) through nucleotide 2259 (gaa) (SEQ ID NO: 3).

[041] Figure 4 shows the amino acid sequence of a truncated aggrecanase-2 protein of the invention from amino acid #1 through amino acid #753 (SEQ ID NO: 4), encoded by nucleotide sequence set forth in SEQ ID NO: 3.

[042] Figure 5 shows the nucleotide sequence of a truncated aggrecanase-2/ADAMTS-5 of the invention from nucleotide #1 (atg) through nucleotide #2256 (cct) (SEQ ID NO: 5).

[043] Figure 6 shows the amino acid sequence of a truncated aggrecanase-2 protein of the invention from amino acid #1 through amino acid #752 (SEQ ID NO: 6), encoded by nucleotide sequence set forth in SEQ ID NO: 5.

[044] Figure 7 shows the nucleotide sequence of a truncated aggrecanase-2 molecule of the invention from nucleotide #1 through nucleotide #1884 (SEQ ID NO: 7).

[045] Figure 8 shows the amino acid sequence of a truncated aggrecanase-2 protein of the invention from amino acid #1 through amino acid #628 (SEQ ID NO: 8), encoded by the nucleotide sequence set forth in SEQ ID NO: 7.

[046] Figure 9 shows the nucleotide sequence of a truncated aggrecanase-2 molecule of the invention from nucleotide #1 through nucleotide #1701 (SEQ ID NO: 9).

[047] Figure 10 shows the amino acid sequence of a truncated aggrecanase-2 protein of the invention from amino acid #1 through amino acid #567 (SEQ ID NO: 10), encoded by the nucleotide sequence set forth in SEQ ID NO: 9.

[048] Figure 11 shows the amino acid sequence of a full-length aggrecanase-1 molecule (SEQ ID NO: 11).

[049] Figure 12 shows the amino acid sequence of a truncated aggrecanase-1 molecule including amino acid #1 through amino acid #575 (Pro) (SEQ ID NO: 12).

[050] Figure 13 shows the amino acid sequence of a truncated aggrecanase-1 molecule including amino acid #1 through amino acid #520 (Ala) (SEQ ID NO: 13).

[051] Figure 14 shows the nucleotide sequence of a recombinant truncated aggrecanase-2 protein comprising a peptide linker and a streptavidin tag (SEQ ID NO: 14).

[052] Figure 15 shows the amino acid sequence of a recombinant truncated aggrecanase-2 protein (SEQ ID NO: 15), encoded by nucleotide sequence set forth in SEQ ID NO: 14.

[053] Figure 16 shows aggrecanase activity of conditioned medium from CHO cells expressing wild-type (ADAMTS-5 WT) or active-site mutant (ADAMTS-5 ASM) as detected by the aggrecanase ELISA assay.

[054] Figure 17 shows a schematic representation of flag-tagged truncated aggrecanase-1 molecules with an E-to-Q mutation in the catalytic domain.

[055] Figure 18 shows a schematic representation of streptavidin tagged truncated aggrecanase-2 molecules.

[056] Figure 19 shows a western blot with expression of truncated aggrecanase-2 proteins in comparison with a full-length aggrecanase-2 protein.

[057] Figure 20 shows percent aggrecan cleavage by truncated aggrecanase-2 molecules of the invention in a 3B3 ELISA assay.

[058] Figure 21 shows the amino acid sequence for a full-length ADAMTS-5 protein with an E-to-Q mutation at amino acid position 411 (SEQ IS NO: 30).

[059] Figures 22A and 22B show a nucleic acid sequence encoding a full-length aggrecanase-1 protein (SEQ ID NO: 31).

[060] Figure 23 shows a nucleic acid sequence encoding a truncated aggrecanase-1 molecule; for example, the protein set forth in Figure 12 (SEQ ID NO: 32).

[061] Figure 24 shows a nucleic acid sequence encoding a truncated aggrecanase-1 molecule; for example, the protein set forth in Figure 13 (SEQ ID NO: 33).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[062] In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[063] The term "**aggrecanase**" refers to a family of polypeptides that are capable of cleaving the aggrecan protein. Generally, these are proteins that cleave aggrecan at the Glu^{373}-Ala^{374} aggrecanase cleavage site. Aggrecanases of the present invention encompass but are not limited to the sequences of SEQ ID NO: 11 (aggrecanase-1) and SEQ ID NO: 2 (aggrecanase-2). The term "aggrecanase" includes naturally-occurring variants SEQ ID NOs: 11 and 2, as well as fragments of the sequences encoded by SEQ ID NOs: 11 and 2 that are active in at least one of the assays provided. For example, included in this definition are amino acid sequences substantially similar or substantially identical to the amino acid of SEQ ID NOs: 11 or 2 or fragments thereof; or an amino acid sequence at least about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical to the amino acid sequence of SEQ ID NO: 11 or 2, or a fragment thereof.

[064] The term aggrecanase further includes the proteins encoded by the nucleic acid sequence of SEQ ID NO: 31 and 1 (aggrecanase-1 and 2

respectively) disclosed, fragments and variants thereof. In one embodiment, the nucleic acids of the present invention will possess a sequence which is either derived from, or is a variant of a natural aggrecanase encoding gene, or a fragment thereof.

[065] The term "**antibody**" refers to an immunoglobulin, or a fragment thereof, and encompasses any polypeptide comprising an antigen-binding site. The term includes but is not limited to polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies. It also includes, unless otherwise stated, antibody fragments such as Fab, $F(ab')_2$, Fv, scFv, Fd, dAb, and other antibody fragments which retain the antigen binding function.

[066] The term "**biological activity**" refers to at least one cellular process interrupted or initiated by an aggrecanase enzyme binding to aggrecan. Generally, biological activity refers to proteolytic cleavage of aggrecan by aggrecanase. Aggrecanase activities include, but are not limited to, binding of aggrecanase to aggrecan and cleavage of aggrecan by aggrecanase. Activity can also include a biological response resulting from the binding to or cleavage of aggrecan by aggrecanases of the invention.

[067] The term "**deletion**" as used herein is the removal of at least one amino acid from the full-length amino acid sequence of an aggrecanase. The term deletion also refers to removal of nucleotides from a nucleic acid sequence encoding an aggrecanase, thereby resulting in a nucleic acid that encodes a truncated protein.

[068] A deletion in a nucleic acid molecule encoding an aggrecanase or an aggrecanase protein can be made anywhere in the nucleic acid molecule or the protein as desirable. For example, a deletion may occur anywhere within the protein; for example, N-terminus, C-terminus or any other part of an aggrecanase protein, and can include removal of at least one amino acid; for example, from about 1 to about 5 amino acids, from about 5 to about 10 amino acids, from about 10 to about 20 amino acids, from about 20 to about 30 amino acids, from about 30 to about 50 amino acids, from about 50 to about 100 amino acids, from about 100 to about 150 amino acids, from about 150 to about 200 amino acids, from about 200 to about 250 amino acids, from about 250 to about 300 amino acids, from about 300 to about 350 amino acids, from about 350 to about 400 amino acids, from about 400 to about 450 amino acids, from about 450 to about 500 amino acids, or greater than 500 amino acids.

[069] Deletions can also be made in nucleic acid molecules that encode aggrecanases of the invention; for example, deletions can be made in the region of a nucleic acid that encodes for a TSP domain of an aggrecanase. Such deletions typically encompass the 3' region of a nucleic acid molecule encoding an aggrecanase of the invention. However, it is contemplated that deletions can be made anywhere in a nucleic acid expressing an aggrecanase molecule. One skilled in the art can test truncated aggrecanases of the invention for activity in one of many assays disclosed.

[070] An amino acid deletion according to the invention comprises the removal of at least one amino acid from the N-terminus of an aggrecanase protein. In another embodiment, a deletion comprises removal of at least one

amino acid from the C-terminus of an aggrecanase protein. In yet another embodiment, a deletion comprises removal of amino acids from a region lying between N-terminal end and C-terminal end of an aggrecanase molecule. In one embodiment, such a deletion involves removal of an entire domain of an aggrecanase of the invention. For example, aggrecanases of the invention comprising a deletion include aggrecanase-1 molecules that have one TSP domain deleted or aggrecanase-1 molecules that have two TSP domains deleted, or aggrecanase-2 molecules that have one TSP domain deleted, or aggrecanase-2 molecules that have two TSP domains deleted. Aggrecanases according to the invention may comprise deletion of all TSP domains within an aggrecanase protein. It is contemplated that other domains within aggrecanase molecules may also be deleted to generate biologically-active truncated aggrecanases that are more stable than the full-length counterpart.

[071] The term **"fragment"** as used herein, refers to a portion of an aggrecanase protein of the invention, for example, a portion of amino acid sequences set forth in SEQ ID NO: 2, 4, 6, 8, 10, 11, 12, and 13. In one embodiment, a fragment of a protein refers to an amino acid sequence that has aggrecanase activity in one of many assays provided. The term "fragment" also includes nucleotide sequences that are long enough to encode peptides that exhibit aggrecanase activity. However, fragments of a nucleotide sequence may or may not encode protein fragments that retain aggrecanase biological activity. Protein and nucleic acid fragments of the invention include portions of other nucleic acid molecules or proteins that are substantially identical to at least one portion of the nucleotide sequences set forth in SEQ ID NOs: 1, 3, 5, 7, 9, 31, 32,

or 33, or at least one portion of amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, 8, 10, 11, 12, or 13, respectively. Fragments of nucleic acid sequences may range, for example, from at least about 20 nucleotides, from at least about 50 nucleotides, from at least about 100 nucleotides, from at least about 150 nucleotides, from at least about 200 nucleotides, from at least about 250 nucleotides, from at least about 300 nucleotides, from at least about 400 nucleotides, from at least about 500 nucleotides, from at least about 600 nucleotides, from at least about 700 nucleotides, from at least about 800 nucleotides, up to the entire length of the nucleic acid sequence set forth in SEQ ID NO: 1. In one embodiment, nucleic acid fragments encode peptides that have aggrecanase activity. Protein fragments may range, for example, from at least about 5 amino acids, from at least about 10 amino acids, from at least about 20 amino acids, from about 30 amino acids, from about 40 amino acids, from about 50 amino acids, from at least about 100 amino acids, from at least about 150 amino acids, from at least about 200 amino acids, from at least about 250 amino acids, from at least about 300 amino acids, from at least about 350 amino acids, from at least about 400 amino acids, up to the entire length of the amino acid sequence set forth in SEQ ID NO: 2. Fragments of nucleic acids of the invention can arise from 3' portions, 5' portions or any other part of a nucleic acid sequence. Similarly, protein fragments can arise from N-terminus portion, C-terminus portion or any other part of a protein. In one embodiment, fragments of proteins retain aggrecanase activity. In another embodiment, protein fragments of the invention arise from a portion of a protein that has aggrecanase activity.

[072] The term "**effective amount**" refers to a dosage or amount of a composition at least one aggrecanase inhibitor or antibody of the invention that is sufficient to treat a patient.

[073] The term "**inhibit**" or "**inhibition**" of aggrecanase or aggrecanase activity refers to a reduction, inhibition of otherwise diminution of at least one activity of aggrecanase due to binding of an inhibitor to the aggrecanase or aggrecan. The reduction, inhibition, or diminution of binding can be measured by one of many assays provided. Inhibition of aggrecanase activity does not necessarily indicate a complete negation of aggrecanase activity. A reduction in activity can be, for example, at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more. In one embodiment, inhibition is measured by a reduction in the detection of cleavage products of aggrecan. Inhibitors of the present invention include, but are not limited to, antibodies, proteins, peptides, and chemical compounds (often referred to as small molecules).

[074] The term "**isolated**" describes a nucleic acid molecule or polypeptide molecule that is substantially free of its natural environment. For instance, an isolated protein is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which it is derived. The term "isolated" also refers to an aggrecanase protein according to the invention which is free from association with other proteases and retains aggrecanase proteolytic activity. In addition, the term "isolated" refers to nucleic acid molecules that encode aggrecanases of the invention and are free from other cellular material and contaminants.

[075] The term "**neoepitope antibody**" refers to an antibody that specifically recognizes a new N- or C-terminal amino acid sequence generated by proteolytic cleavage but which does not bind to such an epitope on the intact (uncleaved) substrate.

[076] The term "**operative association**" with an expression control sequence generally refers to the presence of a specific nucleotide sequence or sequences that control or affect transcription rate or efficiency of a nucleotide molecule linked to the sequence. For example, a promoter sequence that is located proximally to the 5' end of an aggrecanase coding nucleotide sequence may be in operative association with the aggrecanase encoding nucleotide sequence. Expression control sequences include, but are not limited to, for example, promoters, enhancers, and other expression control sequences, or any combination of such elements, either 5' or 3' to an aggrecanase encoding nucleotide sequence in order to control its expression. Not all of these elements are required, however. A skilled artisan can select the appropriate expression control sequences, for example, depending on desired expression levels for the aggrecanases of the invention.

[077] The term "**specific binding**" of an antibody means that the antibody binds to at least one aggrecanase molecule of the present invention and the antibody will not show any significant binding to molecules other than at least one novel aggrecanase molecule. The term is also applicable where, e.g., an antigen binding domain of an antibody is specific for a particular epitope, which is represented on a number of antigens, and the specific binding member (the antibody) carrying the antigen binding domain will be able to bind to the various

antigens carrying the epitope. Therefore, it is contemplated that an antibody of the invention will bind to an epitope on multiple novel aggrecanase proteins. Typically, the binding is considered specific when the affinity constant K_a is higher than 10^8 M^{-1}. An antibody is said to "specifically bind" to an antigen if, under appropriately selected conditions, such binding is not substantially inhibited, while at the same time non-specific binding is inhibited. The conditions are usually defined in terms of concentration of antibodies, ionic strength of the solution, temperature, time allowed for binding, concentration of additional molecules associated with the binding reaction (e.g., serum albumin, milk casein), etc. Such conditions are well known in the art, and a skilled artisan using routine techniques can select appropriate conditions.

[078] The term "**stability**" as used herein, generally refers to a decrease in the rate of degradation of a protein, thereby increasing its half-life, solubility and/or expression levels. Several factors affect protein stability *in vitro* and *in vivo*, for example, pH, salt concentration, temperature, protein degradation, for example by proteases, metal ions, autocatalysis of proteins, hydrophobicity etc. In one embodiment, the invention includes truncated aggrecanases that are more stable than their full-length counterparts. In another embodiment, the invention includes aggrecanase active-site mutants that are more stable than their wild-type counterparts. Conditions that make a protein more stable generally include conditions that keep the protein in a folded conformation for longer than normal, thereby preserving its biological activity for a longer period of time. An increase in stability of a protein generally increases its half-life and expression levels, thereby

making it possible to purify the protein in large amounts for therapeutic purposes and for development of inhibitors.

[079] The term **"highly stringent"** or **"high stringency"** describes conditions for hybridization and washing used for determining nucleic acid-nucleic acid interactions. Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. The stringency conditions are dependent on the length of the nucleic acid and the base composition of the nucleic acid and can be determined by techniques well known in the art. Generally, stringency can be altered or controlled by, for example, manipulating temperature and salt concentration during hybridization and washing. For example, a combination of high temperature and low salt concentration increases stringency. Such conditions are known to those skilled in the art and can be found in, for example, "Current Protocols in Molecular Biology," John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Both aqueous and nonaqueous conditions as described in the art can be used. One example of highly stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 50°C. A second example of highly stringent hybridization conditions is hybridization in 6X SSC at about 45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 55°C. Another example of highly stringent hybridization conditions is hybridization in 6X SSC at about 45°C,

followed by at least one wash in 0.2X SSC, 0.1% SDS at 60°C. A further example of highly stringent hybridization conditions is hybridization in 6X SSC at about 45°C, followed by at least one wash in 0.2X SSC, 0.1% SDS at 65°C. Highly stringent conditions include hybridization in 0.5M sodium phosphate, 7% SDS at 65°C, followed by at least one wash at 0.2X SSC, 1% SDS at 65°C.

[080] The phrase **"moderately stringent"** or **"moderate stringency"** hybridization refers to conditions that permit a nucleic acid to bind a complementary nucleic acid that has at least about 60%, at least about 75%, or at least about 85%, identity to the nucleic acid; with greater than about 90% identity to the nucleic acid especially preferred. Moderately stringent conditions comprise but are not limited to, for example, hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C., followed by washing in 0.2 X SSPE, 0.2% SDS, at 65°C. (see, e.g., Sambrook et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989).

[081] The phrase **"substantially identical"** or **"substantially similar"** means that the relevant amino acid or nucleotide sequence will be identical to or have insubstantial differences (through conserved amino acid substitutions) in comparison to the sequences which are disclosed. Nucleotide and polypeptides of the invention include, for example, those that are at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical in sequence to nucleic acid molecules and polypeptides disclosed.

[082] For polypeptides, at least 20, 30, 50, 100, or more amino acids will be compared between the original polypeptide and the variant polypeptide that is substantially identical to the original. For nucleic acids, at least 50, 100, 150, 300 or more nucleotides will be compared between the original nucleic acid and the variant nucleic acid that is substantially identical to the original. Thus, a variant could be substantially identical in a region or regions, but divergent in others, while still meeting the definition of "substantially identical." Percent identity between two sequences is determined by standard alignment algorithms such as, for example, Basic Local Alignment Tool (BLAST) described in Altschul *et al.*, J. Mol. Biol., 215:403-410 (1990), the algorithm of Needleman *et al.*, J. Mol. Biol., 48:444-453 (1970), or the algorithm of Meyers *et al.*, Comput. Appl. Biosci., 4:11-17 (1988).

[083] The term **"treating"** or **"treatment"** refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment may include individuals already having a particular medical disorder as well as those who may ultimately acquire the disorder (i.e., those needing preventative measures). Treatment may regulate aggrecanase activity or the level of aggrecanase to prevent or ameliorate clinical symptoms of at least one disease. The inhibitors and/or antibodies may function by, for example, preventing the interaction or binding of aggrecanase to aggrecan, or by reducing or inhibiting aggrecanase activity.

[084] The term **"truncated"** as used herein, refers to nucleotides encoding aggrecanases of the invention that are missing at least one nucleotide, or aggrecanases of the invention that are missing at least one amino acid, which are

found in a full-length aggrecanase nucleic acid molecule or amino acid sequence, respectively. Truncated proteins of the invention are generally shorter in length compared with their full-length counterparts, usually due to a deletion of at least one nucleotide in a nucleic acid sequence encoding a truncated protein or in at least one amino acid of the corresponding full-length protein.

[085] In one embodiment, for example, truncated aggrecanases of the invention include truncated aggrecanase-1 proteins comprising an amino acid sequence set forth in SEQ ID NO: 12 comprising deletion of amino acid #576 through the C-terminal end of the full-length aggrecanase-1 protein, set forth in SEQ ID NO: 11; and SEQ ID NO: 13 comprising deletion of amino acid # 521 through the C-terminal end of the full-length aggrecanase-1 protein, set forth in SEQ ID NO: 11. In another embodiment, truncated aggrecanases of the invention comprise aggrecanase-2 truncated proteins; for example, as set forth in SEQ ID NO: 4 comprising deletion of amino acid #754 through the C-terminal end of the full-length aggrecanase-2 protein, set forth in SEQ ID NO: 2; SEQ ID NO: 6 comprising deletion of amino acid #753 through the C-terminal end of the full-length aggrecanase-2 protein, set forth in SEQ ID NO: 2; SEQ ID NO: 8 comprising deletion of amino acid #629 through the C-terminal end of the full-length aggrecanase-2 protein, set forth in SEQ ID NO: 2; and SEQ ID NO: 10 comprising deletion of amino acid #568 through the C-terminal end of the full-length aggrecanase-2 protein, set forth in SEQ ID NO: 2. Such a deletion can be made either in a nucleic acid that encodes an aggrecanase or be made in the protein itself subsequent to the formation of the protein. Accordingly, the term "truncated" as used herein refers to nucleic acids that encode aggrecanases that

are truncated as well as truncated aggrecanases themselves. Nucleic acids encoding for truncated proteins of the invention are depicted in SEQ ID NOs: 3, 5, 7, 9, 11, 32 and 33. Truncated proteins of the invention can be expressed as fusion proteins.

[086] Truncated aggrecanases of the invention have greater stability than the corresponding full-length aggrecanase molecule. Truncated aggrecanases of the invention can also be expressed at higher levels both *in vivo* and *in vitro* than the corresponding full-length aggrecanase proteins. Truncated aggrecanases of the invention are biologically active and may include other alterations, such as, amino acid substitutions, modifications, or deletions in other parts of the protein.

[087] The term "**variant**" refers to nucleotide and amino acid sequences that are substantially identical or similar to the nucleotide and amino acid sequences provided, respectively. Variants can be naturally occurring; for example, naturally occurring human and non-human nucleotide sequences that encode aggrecanase or aggrecanase-like proteins, or be generated artificially. Examples of variants are aggrecanases resulting from alternative splicing of the aggrecanase mRNA, including both 3' and 5' spliced variants of the aggrecanases of the invention, point mutations and other mutations, or proteolytic cleavage of the aggrecanase protein. Variants of aggrecanases of the invention include nucleic acid molecules or fragments thereof and amino acid sequences and fragments thereof, that are substantially identical or similar to other nucleic acids (or their complementary strands when they are optimally aligned (with appropriate insertions or deletions)) or amino acid sequences respectively. In one embodiment, there is at least about 50% identity, at least about 55% identity, at

least about 60% identity, at least about 65% identity, at least about 70% identity, at least about 75% identity, at least about 80% identity, at least about 85% identity, at least at least about 90%, at least about 92% identity, at least about 93% identity, at least about 94% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, or at least about 99% identity between a nucleic acid molecule or protein of the invention and another nucleic acid molecule or protein respectively, when optimally aligned. Variants, as defined, include both naturally occurring nucleic acid sequences as well as equivalent degenerative codon sequences of the aggrecanases of the invention. Additionally, variants include proteins or polypeptides that exhibit aggrecanase activity, as defined.

[088] To assist in the identification of the sequences listed in the specification and figures, the following table (Table 1) is provided, which lists the SEQ ID NOs, the figure location, and a brief description of each sequence.

Table 1

a.a. = amino acid

SEQUENCES	FIGURES	DESCRIPTION
SEQ ID NO: 1	Figures 1A-1C	full-length nucleotide sequence of ADAMTS-5 (aggrecanase-2)
SEQ ID NO: 2	Figure 2	full-length a.a. sequence of ADAMTS-5 encoded by SEQ ID NO: 1
SEQ ID NO: 3	Figure 3	a nucleotide sequence from nucleotide #123 to nucleotide #2382 of SEQ ID NO: 1
SEQ ID NO: 4	Figure 4	a.a. sequence of a truncated aggrecanase-2 molecule encoded by SEQ ID NO: 3
SEQ ID NO: 5	Figure 5	a nucleotide sequence from nucleotide #123 to nucleotide # 2379 of SEQ ID NO: 1
SEQ ID NO: 6	Figure 6	a.a. sequence of a truncated aggrecanase-2 molecule encoded by SEQ ID NO: 5
SEQ ID NO: 7	Figure 7	a nucleotide sequence from nucleotide #123 to nucleotide #2007 of SEQ ID NO: 1
SEQ ID NO: 8	Figure 8	a.a. sequence of a truncated aggrecanase-2 molecule encoded by SEQ ID NO: 7
SEQ ID NO: 9	Figure 9	a nucleotide sequence from nucleotide #123 to nucleotide #1824 of SEQ ID NO: 1
SEQ ID NO: 10	Figure 10	a.a. sequence of a truncated aggrecanase-2 molecule encoded by SEQ ID NO: 9

SEQUENCES	FIGURES	DESCRIPTION
SEQ ID NO: 11	Figure 11	a.a. sequence of full-length aggrecanase-1 protein encoded by SEQ ID NO: 31
SEQ ID NO: 12	Figure 12	a.a. sequence of truncated aggrecanase-1 protein including a.a #1 Met) through a.a #575 (Pro) encoded by SEQ ID NO: 32
SEQ ID NO: 13	Figure 13	a.a. sequence of truncated aggrecanase-1 protein including a.a. #1 (Met) through a.a. #520 (Ala) encoded by SEQ ID NO: 33
SEQ ID NO: 14	Figure 14	a nucleotide sequence of a recombinant truncated aggrecanase-2 protein with a peptide linker and a streptavidin tag
SEQ ID NO: 15	Figure 15	a.a. sequence of a recombinant aggrecanase-2 protein encoded by SEQ ID NO: 14.
SEQ ID NO: 16		Primer
SEQ ID NO: 17		Primer
SEQ ID NO: 18		Primer
SEQ ID NO: 19		Primer
SEQ ID NO: 20		oligonucleotide
SEQ ID NO: 21		oligonucleotide
SEQ ID NO: 22		peptide linker
SEQ ID NO: 23	Figure 18	streptavidin -tag
SEQ ID NO: 24		oligonucleotide
SEQ ID NO: 25		oligonucleotide
SEQ ID NO: 26		catalytic motif
SEQ ID NO: 27		Nucleotide insert
SEQ ID NO: 28		insert containing Xho1 site
SEQ ID NO: 29		68 bp adapter
SEQ ID NO: 30	Figure 21	aggrecanase-2 protein with an E-to-Q mutation at a.a. position 411

SEQUENCES	FIGURES	DESCRIPTION
SEQ ID NO: 31	Figures 22A and 22B	full-length nucleotide sequence of aggrecanase-1
SEQ ID NO: 32	Figure 23	Nucleotide sequence of a truncated aggrecanase-1
SEQ ID NO: 33	Figure 24	Nucleotide sequence of a truncated aggrecanase-1

II. Novel Aggrecanase Molecules

[089] In one embodiment, the nucleotide sequence of a truncated aggrecanase-2 of the invention is set forth in SEQ ID NO: 3, wherein the nucleic acid sequence includes nucleotide # 123 (atg) through nucleotide # 2382 (gaa) of the full-length nucleic acid sequence of aggrecanase-2, set forth in SEQ ID NO: 1. In another embodiment the nucleotide sequence of a truncated aggrecanase-2 is set forth in Figure 5 from nucleotide #1 again beginning with the "A" of the (atg) through nucleotide #2256 (cct). In another embodiment the nucleotide sequence of a truncated aggrecanase-2 of the invention comprises nucleotide #1 (atg) through #1884 (ttt) set forth in Figure 7, which comprises deletion of nucleotide sequence encoding a TSP domain. In yet another embodiment, the nucleic acid sequence of a truncated aggrecanase-2 protein of the invention comprises nucleotide #1 (atg) through nucleotide #1701 (cat) which encodes an aggrecanase-2 protein having both TSP domains deleted. The invention further includes naturally-occurring nucleic acid sequences and fragments and variants thereof, as well as those that are artificially generated and equivalent degenerative codon sequences of the sequence described above as set forth in Figures 3, 5, 7, and 9, as well as fragments thereof which encode polypeptides that exhibit aggrecanase activity.

[090] In another embodiment, the nucleotide sequences of the invention include nucleic acid sequences that encode truncated aggrecanase-1 molecules. For example, a truncated aggrecanase-1 protein, set forth in Figure 12, can be encoded by the nucleic acid sequence set forth in Figure 23 (SEQ ID NO: 32). Yet another truncated aggrecanase-1 protein of the invention is set forth in Figure 13, which includes deletion of a TSP domain. A nucleic acid sequence that encodes such a truncated aggrecanase-1 protein is set forth in Figure 24 (SEQ ID NO: 33).

[091] The amino acid sequence of an isolated truncated aggrecanase-2 molecule is set forth in Figure 4 from amino acid #1 (Met) through #753 (Glu). The invention further features amino acid sequences encoded by nucleotide sequences of Figures 5, 7, and 9, which encode truncated aggrecanase-2 proteins, amino acid sequence of which are set forth in: Figure 6 including amino acid #1 (Met) through #752 (Pro); Figure 8 including amino acid #1 (Met) through amino acid #628 (Phe); and Figure 10 including amino acid #1 (Met) through amino acid #567 (His). The invention further includes amino acid sequences for truncated aggrecanase-1 molecules. The amino acid sequence for a truncated aggrecanase-1 molecule is set forth in Figure 23, which may be encoded by the nucleotide sequence of Figure 12. Yet another truncated aggrecanase-1 molecule of the invention is featured in Figure 13, which may be encoded by the nucleic acid sequence of Figure 24. The invention further includes fragments and variants of the amino acid sequences of polypeptides that exhibit aggrecanase activity.

[092] A human aggrecanase protein of the invention or a fragment thereof may be produced by culturing a cell transformed with a DNA sequence of Figure 3 from nucleotide #1 (atg) through #2259 (gaa) and recovering and purifying from the culture medium a protein characterized by the amino acid sequence set forth in Figure 4 from amino acid #1 (Met) through #753 (Glu) substantially free from other proteinaceous materials with which it is co-produced.

[093] In another embodiment, the aggrecanase protein of the invention may be produced by culturing a cell transformed with the DNA sequence of Figure 5 from nucleotide # 1 (atg) through nucleotide #2256 (cct) and recovering and purifying the aggrecanase protein comprising an amino acid sequence of Figure 6 from amino acid #1 (Met) through amino acid #752 (Pro). In another embodiment, the aggrecanase protein of the invention may be produced by culturing a cell transformed with the DNA sequence of Figure 5 from nucleotide #1 (atg) to nucleotide #2256 (cct) with an additional GAA following CCT and recovering and purifying the aggrecanase protein comprising an amino acid sequence of Figure 4 from amino acid #1 (Met) through amino acid #753 (Glu). In yet another embodiment aggrecanase proteins of the present invention may be produced by culturing a cell transformed with the DNA sequence of Figures 1A-1C (SEQ ID NO: 1) and recovering and purifying a truncated aggrecanase protein from the culture medium comprising amino acid #1 through amino acid #752 (SEQ ID NO: 6), or amino acid # 1 through amino acid #753 (SEQ ID NO: 4), produced due to cleavage of the full-length aggrecanase-2 protein set forth in SEQ ID NO: 2.

[094] In a further embodiment, a protein recovered from a cell culture medium includes amino acids #1 through #628 (SEQ ID NO: 8); amino acids #1

though 567 (SEQ ID NO: 10); amino acids # 1 through 575 (SEQ ID NO: 12) or amino acids #1 through 520 (SEQ ID NO: 13). Purified expressed proteins are substantially free from other proteinaceous materials with which they are co-produced, as well as from other contaminants. A recovered purified protein is contemplated to exhibit proteolytic aggrecanase activity by cleaving aggrecan. Thus, proteins of the invention may be further characterized by their ability to demonstrate aggrecan proteolytic activity in an assay which determines the presence of an aggrecan-degrading molecule. These assays or the development thereof is within the knowledge of one skilled in the art. Such assays may involve contacting an aggrecan substrate with the aggrecanase molecule and monitoring the production of aggrecan fragments (*see*, for example, Hughes *et al.*, Biochem J 305: 799-804(1995); Mercuri *et al,* J. Bio Chem. 274:32387-32395 (1999)).

[095] For production in mammalian cells, the DNA sequence further comprises a DNA sequence encoding a suitable propeptide 5' to and linked in frame to the nucleotide sequence encoding an aggrecanase enzyme. Aggrecanase proteins of the invention recovered from a culture medium are purified by isolating them from other proteinaceous materials with which they are co-produced and from other contaminants present. The isolated and purified proteins may be characterized by the ability to cleave aggrecan substrate.

[096] Aggrecanase proteins provided herein also include proteins encoded by the sequences similar to those of Figure 3 from nucleotide #1 (atg) through # 2259 (gaa); Figure 5 from nucleotide #1 (atg) through nucleotide #2256 (cct); Figure 7 from nucleotide #1 (atg) through nucleotide #1884 (ttt); Figure 9 from nucleotide #1 (atg) through nucleotide #1701 (cat); Figure 23 from nucleotide #

1(atg) through nucleotide #1725 (cca); and Figure 24 from nucleotide #1 (atg) through nucleotide #1560 (gct), but into which modifications or deletions are naturally provided (e.g., allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Figure 4 from amino acid #1 (Met) through amino acid # 753 (Glu); Figure 6 from amino acid #1 (Met) through #752 (Pro); Figure 8 from amino acid #1 (Met) through amino acid # 628 (Phe); Figure 10 from amino acid #1 (Met) through amino acid # 567 (His); Figure 12 from amino acid # 1 (Met) through amino acid # 575 (Pro); or Figure 13 from amino acid #1 (Met) through amino acid # 520 (Ala). These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with aggrecanase molecules, may possess biological properties in common therewith.

[097] It is known, for example, that numerous conservative amino acid substitutions are possible without significantly modifying the structure and conformation of a protein, thus maintaining the biological properties as well. For example, it is recognized that conservative amino acid substitutions may be made among amino acids with basic side chains, such as lysine (Lys or K), arginine (Arg or R) and histidine (His or H); amino acids with acidic side chains, such as aspartic acid (Asp or D) and glutamic acid (Glu or E); amino acids with uncharged polar side chains, such as asparagine (Asn or N), glutamine (Gln or Q), serine (Ser or S), threonine (Thr or T), and tyrosine (Tyr or Y); and amino acids with nonpolar side chains, such as alanine (Ala or A), glycine (Gly or G), valine (Val or

V), leucine (Leu or L), isoleucine (Ile or I), proline (Pro or P), phenylalanine (Phe or F), methionine (Met or M), tryptophan (Trp or W) and cysteine (Cys or C). Thus, these modifications and deletions of the native aggrecanase may be employed as biologically-active substitutes for naturally-occurring aggrecanase and in the development of inhibitors other polypeptides in therapeutic processes. It can be readily determined whether a given variant of aggrecanase maintains the biological activity of aggrecanase by subjecting both aggrecanase and the variant of aggrecanase, as well as inhibitors thereof, to the assays described in the examples.

[098] The invention also includes aggrecanase molecules comprising amino acid substitutions that increase the stability of the aggrecanase molecules. For example, amino acid sequence of an aggrecanase-2 molecule with an E-to-Q mutation at position 411 of the protein is provided in SEQ ID NO: 30. Figure 17 further provides schematic representations of amino acid mutations E-to-Q within the catalytic domains of truncated aggrecanase-1 molecules of Figures 12 and 13. Although the E-to-Q mutation is in the active site of the molecule and serves to prevent degradation of aggrecanases of the invention, and to thereby increase stability and half-life of aggrecanases, it is contemplated that amino acid mutations can be made in other regions of the protein that increase stability of aggrecanase molecules.

[099] In one embodiment, active site mutations are introduced into aggrecanases to intentionally block the catalytic activity of the enzyme. This is especially useful for the purposes of crystallization and structural determination of aggrecanases and subsequently to identify and develop inhibitors of

aggrecanases. Increased stability of truncated or active-site mutant aggrecanases of the invention makes it possible to purify and isolate large amounts of aggrecanase molecules for subsequent use in the development of inhibitors for treatment of diseases. The E-to-Q mutation makes the aggrecanases biologically inactive, thereby enabling purification of inactive protein in large amounts for use in gene therapy in patients and also for development of inhibitors.

[0100] Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important amino acid residues of the proteins or polypeptides of the invention or to increase or decrease the activity of the aggrecanases of the invention described. Exemplary amino acid substitutions are set forth in Table 2.

Table 2: Amino Acid Substitutions

Original Residues	Exemplary Substitutions	More Conservative Substitutions
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser, Ala	Ser
Gln (Q)	Asn	Asn
Gly (G)	Pro, Ala	Ala
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, 1, 4 Diamino-butyric Acid, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala, Tyr	Leu
Pro (P)	Ala	Gly
Ser (S)	Thr, Ala, Cys	Thr

Thr (T)	Ser	Ser
Trp (W)	Tyr, Phe	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

[0101] In certain embodiments, conservative amino acid substitutions also encompass non-naturally-occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems.

[0102] Other specific mutations of the sequences of aggrecanase proteins described herein involve modifications of glycosylation sites. These modifications may involve O-linked or N-linked glycosylation sites. For instance, the absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at asparagine-linked glycosylation recognition sites. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Additionally, bacterial expression of aggrecanase-related protein will also result in production of a non-glycosylated protein, even if the glycosylation sites are left unmodified.

III. Aggrecanase Nucleotide Sequences

[0103] Nucleic acids within the scope of the invention include isolated DNA and RNA sequences that hybridize to the native aggrecanase DNA sequences

disclosed under conditions of moderate to high stringency. Conditions of high stringency generally refer to hybridization and washing conditions that employ higher temperatures and lower salt concentrations. Additionally, inclusion of formamide also increases stringency. For example, hybridization conditions at 60-65°C in the absence of formamide or at 42°C with 50% formamide, are both high stringency conditions.

[0104] Further included in the present invention are DNA sequences which hybridize under high to moderate stringent conditions with the DNA sequence of SEQ ID NOs: 3, 5, 7, 9, 32 or 33 and encode a truncated aggrecanase protein having the ability to cleave aggrecan. In one embodiment, DNA sequences include those which hybridize under high stringent conditions (*see* Maniatis *et al., Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, at 387-389 (1982)). Such stringent conditions comprise; for example, 0.1X SSC, 0.1% SDS, at 65°C. DNA sequences identified by hybridization include, for example, DNA sequences that encode a protein which is at least about 80% identical, at least about 90% identical, or at least about 95% identical to the sequence of the proteins of the invention; for example, amino acid sequences set forth in SEQ ID NOs: 4, 6, 8, 10, 12 and 13. DNAs that are equivalents to the DNAs of SEQ ID NOs: 3, 5, 7, 9, 32, or 33 will also hybridize under moderately-stringent conditions to the DNA sequence encoding the peptide sequence of SEQ ID NO: 4, 6, 8, 10, 12 or 13, respectively. It is understood, however, that under certain conditions, nucleic acid molecules that encode truncated proteins will hybridize to nucleic acid molecules encoding full-length proteins.

[0105] Conditions of moderate stringency are known in the art and are defined by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Vol. 1, Cold Spring Harbor Press (2nd ed. 1989). In one embodiment, for example, conditions of moderate stringency include use of a prewashing solution of 5X SSC/0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C-60°C temperature and washing overnight in 5X SSC overnight at about 55°C. The skilled artisan will recognize that the conditions may be adjusted as necessary according to factors such as the length and composition of the nucleic acid sequences.

[0106] Still a further aspect of the invention are DNA sequences encoding truncated aggrecanases having aggrecanase proteolytic activity or other yet undisclosed or undiscovered activities of aggrecanases.

[0107] In yet another embodiment, nucleic acids of the invention include nucleic acid molecules that encode aggrecanases comprising mutations that lead to increase in stability of such molecules.

[0108] Finally, allelic or other variations of the sequences of the invention; for example, SEQ ID NOs: 3, 5, 7, 9, 32 and 33, encoding amino acid sequences set forth in SEQ ID NOs: 4, 6, 8, 10, 12 and 13 respectively, or peptide sequence variants of aggrecanases of the invention that have aggrecanase activity, are also included in the present invention. Additionally, the present invention includes fragments of the DNA sequences of the invention and variants of such sequences that encode a protein with aggrecanase activity.

[0109] Similarly, DNA sequences which code for aggrecanase proteins but which differ in codon sequence due to the degeneracies of the genetic code or

allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change), also encode novel aggrecanases described herein. Variations in the DNA sequences of Figures 1, 3, 5, 7, 9, 31, 32 and 33, which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life, or production of the polypeptides encoded, are also encompassed in the invention.

[0110] Another aspect of the present invention includes vectors for use in a method of expression of these novel aggrecanase polypeptides. Preferably, vectors of the invention contain a I DNA sequence described above which encodes a truncated aggrecanase or an active site mutant aggrecanase of the invention. Additionally, vectors contain appropriate expression control sequences permitting expression of aggrecanase protein sequences of the invention. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention. Additionally, the sequence of Figure 3 from nucleotide #1 (atg) through # 2259 (gaa), or Figure 5 from nucleotide #1 (atg) through #2256 (cct), or Figure 7 from nucleotide # 1 (atg) through nucleotide # 1884 (ttt), or Figure 9 from nucleotide #1 (atg) through nucleotide # 1701 (cat), or Figure 23 from nucleotide #1 (atg) through nucleotide # (cca), or Figure 24 from nucleotide #1 (atg) through nucleotide # (gct), or other sequences encoding aggrecanase proteins could be manipulated to express composite aggrecanase molecules. Thus, the present invention includes chimeric DNA molecules encoding an aggrecanase protein comprising a fragment from nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 9, 32, and 33 linked in correct reading

frame to a DNA sequence encoding another aggrecanase polypeptide. A DNA molecule as set forth in SEQ ID NOs: 3, 5, 7, 9, 32, or 33, or a variant or fragment thereof, may be linked either 5' or 3' to a DNA molecule encoding another aggrecanase.

IV. Production of Aggrecanase Proteins

[0111] Another aspect of the present invention provides methods for producing aggrecanase proteins. In one embodiment, a method of the present invention involves culturing a suitable cell line, which has been transformed with a DNA sequence encoding a aggrecanase protein of the invention under the control of known regulatory sequences. The transformed host cells are cultured and the aggrecanase proteins are recovered and purified from the culture medium. The purified proteins are substantially free from other proteins with which they are co-produced, as well as from other contaminants.

[0112] Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening, product production, and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), Kaufman *et al.,* Mol. Cell. Biol., 5(7):1750-1759 (1985), or Howley *et al.*, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. The mammalian cell line CV-1 may also be suitable.

[0113] Bacterial cells may also be suitable hosts. For example, the various strains of *E. coli* (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, Pseudomonas, other bacilli and the

like may also be employed in this method. For expression of aggrecanase proteins of the invention in bacterial cells, DNA encoding the propeptide of an aggrecanase is generally not necessary.

[0114] Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller *et al.*, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

[0115] Various vectors disclosed may be employed in the method of transforming cell lines and usually contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Regulatory sequences for such vectors are known to those skilled in the art and may be selected, depending upon the host cells. Such selection is routine and does not form part of the present invention.

[0116] One skilled in the art can construct mammalian vectors by employing a nucleic acid sequence disclosed; for example, SEQ ID NOs: 1, 3, 5, 7, 9, 31, 32, and 33.

[0117] Truncated aggrecanase proteins of the invention can also be expressed as fusion proteins including the protein sequence; for example, the sequence set forth in SEQ ID NO: 4, 6, 8, 10, 12 or 13, or a fragment or a variant thereof, and, for example, a tag; i.e., a second protein or at least one amino acid, from about 2 to 50 amino acids, or from about 50 to about 100 amino acids, which are added to the amino terminus of, the carboxy terminus of, or any point within

the amino acid sequence of an aggrecanase protein, or a fragment or variant thereof. Typically, such amino acid tags are made to stabilize the resulting fusion protein or to simplify purification of an expressed recombinant form of the corresponding aggrecanase protein or a fragment or a variant of such protein, including, for example, a truncated form of an aggrecanase protein of the invention. Such tags are known in the art. Representative examples of such tags include sequences which encode a series of histidine residues, the epitope tag FLAG, the Herpes simplex glycoprotein D, beta-galactosidase, maltose binding protein, streptavidin tag or glutathione S-transferase. In one embodiment, a nucleic acid sequence encoding a tag is linked in frame to a nucleic acid sequence encoding an aggrecanase of the invention for subsequent production of a recombinant or fusion aggrecanase protein including the tag. Such recombinant or fusion aggrecanase proteins include, for example, truncated aggrecanase-1 enzymes with a C-terminal FLAG tag, as shown in Figure 17, and truncated aggrecanase-2 enzymes with a C-terminal Streptavidin tag, as shown in Figure 18. A nucleic acid sequence encoding a truncated aggrecanase-2 protein comprising a streptavidin tag is set forth in Figure 14 (SEQ ID NO: 14), which encodes a truncated aggrecanase-2 fusion protein set forth in Figure 15.

[0118] Similarly, aggrecanases that contain amino acid mutations which lead to increased stability, expression levels, and/or half-lives can also be produced as fusion proteins using, for example, the amino acid sequence set forth in SEQ ID NO: 30, or a fragment or variant thereof, and, for example, a tag as discussed above. Examples of aggrecanase-1 and -2 fusion proteins of the

invention, including aggrecanases with amino acid mutations in their catalytic domain, are set forth in Figures 14, 15, 17 and 18.

V. Generation of Antibodies

[0119] Isolated proteins of the present inventions may be used to generate antibodies, either monoclonal or polyclonal, to aggrecanase and/or other aggrecanase-related proteins, using methods of antibody production that are generally known in the art. Thus, the present invention also includes antibodies to aggrecanase or other related proteins. The antibodies include both antibodies that block aggrecanase activity and antibodies that do not. The antibodies may be useful for detection and/or purification of aggrecanase or related proteins, or for inhibiting or preventing the effects of aggrecanase. Aggrecanases of the invention or portions thereof may be utilized to prepare antibodies that specifically bind to aggrecanase.

[0120] Antibodies can be made, for example, via traditional hybridoma techniques (Kohler and Milstein, Nature 256:495-499 (1975)), recombinant DNA methods (for example, U.S. Pat. No. 4,816,567), or phage display techniques using antibody libraries (Clackson *et al.*, Nature 352: 624-628 (1991); Marks *et al.*, J. Mol. Biol. 222:581-597 (1991)). For various antibody production techniques, see Antibodies: A Laboratory Manual, eds. Harlow *et al.*, Cold Spring Harbor Laboratory (1988).

[0121] Antibodies of the invention may be used in the treatment of the diseases described below. Antibodies can also be used in the assays and methods of detection described.

VI. Development of Inhibitors

[0122] Various conditions such as osteoarthritis are known to be characterized by degradation of aggrecan. Therefore, truncated aggrecanases of the invention and aggrecanases with mutations that lead to increased stability and expression levels of aggrecanases, make it possible to generate aggrecanase molecules in large amounts in order to develop inhibitors to aggrecanases.

[0123] The invention therefore provides compositions comprising an aggrecanase inhibitor. Inhibitors may be developed using an aggrecanase molecule of the invention in screening assays involving a mixture of aggrecan substrate with an inhibitor of aggrecanase activity followed by exposure to aggrecan. Inhibitors can be screened using high throughput processes, such as by screening a library of inhibitors. Inhibitors can also be made using three-dimensional structural analysis and/or computer aided drug design. The method may entail determination of binding sites for inhibitors based on the three dimensional structure of aggrecanase and aggrecan and developing molecules reactive with a binding site on aggrecanase or aggrecan. Candidate molecules are assayed for inhibitory activity. Additional standard methods for developing inhibitors of aggrecanase molecules are known to those skilled in the art. An assay for identification and development of aggrecanase inhibitors involves, for example, contacting a mixture of aggrecan and an inhibitor with an aggrecanase molecule followed by measurement of the degree of aggrecanase inhibition; for instance, by detection and measurement of aggrecan fragments produced by cleavage at an aggrecanase susceptible site. Inhibitors may be proteins, peptides, antibodies, or chemical compounds. In one embodiment, inhibitors are

peptide molecules that bind an active site on aggrecanase molecules. For example, active site mutants of aggrecanase-1 and aggrecanase-2 molecules can be used for the development of peptide inhibitors. Aggrecanase-1 molecules that comprise an E-to-Q amino acid change within the catalytic domain are set forth in Figure 17. Similarly, amino acid sequence of an aggrecanase-2 molecule with an E to Q change at position 411 within the catalytic domain is set forth in Figure 21 (SEQ ID NO: 30).

VII. Disease Treatment and Diagnosis

[0124] Inhibitors of aggrecanase activity may be used in the treatment of diseases described below. Inhibitors can also be used in the assays and methods of detection described. Various diseases that are contemplated as being treatable by using inhibitors of aggrecanases of the invention include, but are not limited to, osteoarthritis, cancer, inflammatory joint disease, rheumatoid arthritis, septic arthritis, periodontal diseases, corneal ulceration, proteinuria, coronary thrombosis from atherosclerotic plaque rupture, aneurysmal aortic disease, inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, brain and hematopoietic malignancies, osteoporesis, Parkinson's disease, migraine, depression, peripheral neuropathy, Huntington's disease, multiple sclerosis, ocular angiogenesis, macular degeneration, aortic aneurysm, myocardial infarction, autoimmune disorders, degenerative cartilage loss following traumatic joint injury, head trauma, dystrophobic epidermolysis bullosa, spinal cord injury, acute and chronic neurodegenerative diseases, osteopenias, tempero mandibular joint disease, demyelating diseases of the nervous system, organ

transplant toxicity and rejection, cachexia, allergy, tissue ulcerations, restenosis, and other diseases characterized by altered aggrecanase activity or altered aggrecanase level.

[0125] Inhibitors and antibodies of the invention that inhibit activity of aggrecanases and/or compounds that lower expression of aggrecanases may be used in the treatment of any disease in a mammal that involves degradation of the extracellular matrix. An effective amount of at least one of aggrecanase antibodies or inhibitors, in a pharmaceutically acceptable vehicle can be used for treatment of diseases, such as osteoarthritis, or other diseases disclosed which are characterized by degradation of matrix proteins, such as aggrecan, by aggrecanases and aggrecanase-related proteins.

VIII. Administration

[0126] Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of an aggrecanase inhibitor or antibody. Therefore, these compositions of the invention may be used in the treatment of diseases characterized by the degradation of aggrecan by an aggrecanase enzyme or a protein with aggrecanase-like activity.

[0127] The invention includes methods for treating patients suffering from conditions characterized by a degradation of aggrecan. These methods, according to the invention, entail administering to a patient needing such treatment, an effective amount of a composition comprising an aggrecanase inhibitor which inhibits the proteolytic activity. It is contemplated that inhibitors of the invention may function either by inhibiting aggrecanase activity or simply by regulating levels of aggrecanases in a disease state.

[0128] Inhibitors of the present invention are useful to diagnose or treat various medical disorders in humans or animals. In one embodiment, the antibodies of the invention can be used to inhibit or reduce at least one activity associated with an aggrecanase protein, relative to an aggrecanase protein not bound by the same antibody. In one embodiment, inhibitors of the invention can inhibit or reduce at least one of the activities of an aggrecanase molecule relative to the aggrecanase that is not bound by an antibody. In certain embodiments, an activity of an aggrecanase, when bound by at least one of the presently disclosed antibodies, is inhibited at least 50%, may be inhibited at least 60, 62, 64, 66, 68, 70, 72, 72, 76, 78, 80, 82, 84, 86, or 88%, may be inhibited at least 90, 91, 92, 93, or 94%, or may be inhibited at least 95% to 100% relative to the aggrecanase protein that is not bound by at least one of the presently disclosed antibodies.

[0129] Generally, compositions of the present are administered to a patient so that antibodies or their binding fragments are administered at a dose ranging from about 1 μg/kg to about 20 mg/kg, about 1 μg/kg to about 10 mg/kg, about 1 μg/kg to about 1 mg/kg, about 10 μg/kg to about 1 mg/kg, about 10 μg/kg to about 100 μg/kg, about 100 μg to about 1 mg/kg, or about 500 μg/kg to about 1 mg/kg. Antibodies are administered as a bolus dose, to maximize the interval of time that the antibodies can circulate in the patient's body following their administration to the patient. Continuous infusion may also be used after an initial bolus dose

[0130] In another embodiment, the invention is directed to administration of inhibitors of aggrecanases, such as proteins, peptides, antibodies, and chemical compounds. The effective amount of an inhibitor is a dosage which is useful for reducing activity of aggrecanases to achieve a desired biological outcome.

Generally, appropriate therapeutic dosages for administering an inhibitor may range, for example, from about 5 mg to about 100 mg, from about 15 mg to about 85 mg, from about 30 mg to about 70 mg, or from about 40 mg to about 60 mg. Inhibitors can be administered in one dose, or at intervals such as once daily, once weekly, or once monthly. Dosage schedules for administration of an aggrecanase inhibitor can be adjusted based on, for example, the affinity of the inhibitor for its aggrecanase target, the half-life of the inhibitor, and the severity of the patient's condition. Generally, inhibitors are administered as a bolus dose, to maximize their circulating levels. Continuous infusions may also be used after the bolus dose.

[0131] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell culture or experimental animal models, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Inhibitors, which exhibit large therapeutic indices, are generally preferred.

[0132] The data obtained from cell culture assays and animal studies can be used in formulating a range of dosages for use in humans. The dosage of such compounds may lie within a range of circulating concentrations that exhibit an ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any inhibitor used according to the present invention, a therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in

animal models to achieve a circulating plasma concentration range that exhibits an IC_{50} (*i.e.*, the concentration of the test antibody which achieves a half-maximal inhibition of symptoms) as determined by cell culture assays. Levels in plasma may be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by suitable bioassays. Examples of suitable bioassays include DNA replication assays, transcription-based assays, GDF protein/receptor binding assays, creatine kinase assays, assays based on the differentiation of pre-adipocytes, assays based on glucose uptake in adipocytes, and immunological assays.

[0133] Therapeutic methods of the invention include administering an aggrecanase inhibitor composition topically, systemically, or locally as an implant or a device. The dosage regimen for the administration of composition will be determined by the attending physician based on various factors which modify the action of the aggrecanase protein, the site of pathology, the severity of disease, the patient's age, sex, and diet, the severity of any inflammation, time of administration and other clinical factors. Generally, systemic or injectable administration will be initiated at a dose which is minimally effective, and the dose will be increased over a preselected time course until a positive effect is observed. Subsequently, incremental increases in dosage will be made limiting to levels that produce a corresponding increase in effect, while taking into account any adverse affects that may appear. The addition of other known factors, to a final composition, may also affect the dosage.

[0134] Progress can be monitored by periodic assessment of disease progression. The progress can be monitored, for example, by X-rays, MRI or other imaging modalities, synovial fluid analysis, and/or clinical examination.

IX. Assays and Methods of Detection

[0135] The inhibitors and antibodies of the invention can be used in assays and methods of detection to determine the presence or absence of, or quantify aggrecanase in a sample. The inhibitors and antibodies of the present invention may be used to detect aggrecanase proteins, *in vivo* or *in vitro*. By correlating the presence or level of these proteins with a disease, one of skill in the art can diagnose the associated disease or determine its severity. Diseases that may be diagnosed by the presently disclosed inhibitors and antibodies are set forth above.

[0136] Detection methods for use with antibodies are well known in the art and include ELISA, radioimmunoassay, immunoblot, western blot, immunofluorescence, immuno-precipitation, and other comparable techniques. The antibodies may further be provided in a diagnostic kit that incorporates at least one of these techniques to detect a protein (e.g., an aggrecanase protein). Such a kit may contain other components, packaging, instructions, or other material to aid the detection of an aggrecanase protein, and instructions regarding use of the kit. When protein inhibitors, for example, peptide inhibitors, are used in such diagnostic assays, protein-protein interaction assays can be employed.

[0137] Where inhibitors are intended for diagnostic purposes, it may be desirable to modify them; for example, with a ligand group (such as biotin) or a detectable marker group (such as a fluorescent group, a radioisotope or an enzyme). If desired, the antibodies (whether polyclonal or monoclonal) may be

labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms, electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase can be detected by its ability to convert tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. Other suitable binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art.

[0138] The following examples illustrate practice of the present invention in isolating and characterizing human aggrecanase and other aggrecanase-related proteins, obtaining the human proteins and expressing the proteins via recombinant techniques.

EXAMPLES

Example 1: Cloning of ADAMTS-5 (Aggrecanase-2)

[0139] PCR primers were designed to the published sequence for human ADAMTS-5 (GenBank Accession # AF142099). The full-length coding sequence for ADAMTS-5 was amplified from a human uterus cDNA library (Genetics Institute/Wyeth Research) using the Advantage-GC PCR kit (Clontech).

[0140] ADAMTS-5 was isolated using PCR. Tissue expression pattern of aggrecanase-5 was determined by PCR amplification of 7 different oligo dT-primed human cDNA libraries including placenta, brain, muscle, lung, heart, uterus and spinal cord. PCR primers that amplified 5' and 3' regions of the ADAMTS-5 cDNA were used. Primer sequences for amplifying the 5' portion of

ADAMTS-5 were as follows: 5' primer : 5'GACTGACTGAATTCATACCCATAAAGTCCCAGTCGCGCA (SEQ ID NO: 16), which incorporated an 8 bp tail (GACTGACT), and an EcoR1 site (GAATTC) upstream of the start codon (ATG) of the ADAMTS-5 sequence and 3' primer: 5' CAGGGCTTAGATGCATCAATGCTGG (SEQ ID NO: 17). Primer sequences for amplifying 3' portion of ADAMTS-5 were as follows: 5' primer: 5' TACCAGCATTG ATGCATCTAAGCCCT (SEQ ID NO: 18) and 3' primer 5' AAATGGGCGCGGC CGCTGCATCGGTGCTGAATCCTCCAGTTATCT (SEQ ID NO: 19), which incorporated an 8 bp tail (AAATGGGC) and a Not1 site (GCGGCCGC) downstream of the stop codon (TAG) for ADAMTS-5. PCR products of the appropriate size, 5' amplification product of 1404 bp and 3' amplification product of 1519 bp were found using a uterus cDNA library as substrate. The Advantage-GC PCR Kit from Clontech was used for the PCR reactions. Reaction conditions were those recommended by the manufacturer; with the following exceptions: the amount of GC Melt used was 10 µl per 50 µl reaction; the amount of Not1 linearized library used was 0.2 ng/µl reaction; and the amount of each oligo used was 2 pmol/ul reaction. Cycling conditions were as follows: 95°C for 1 min, one cycle; followed by 30 cycles consisting of 95°C for 15 sec/68°C for 2 min. The 2 overlapping PCR products resulting from the amplifications were digested with EcoR1 and Nsi1 (5' product) or Nsi1 and Not1 (3' product) and ligated into the CHO expression vector pHTop_new, digested with EcoR1 and Not1 using standard ligation enzyme, buffers and conditions. Ligated products were used to transform ElectroMAX DH10B cells from Life Technologies (Carlsbad, CA). Cloned PCR fragments of ADAMTS-5 were sequenced to verify sequence.

[0141] Nucleotide sequence for full-length ADAMTS-5 protein was the consensus sequence derived from the PCR products. Two silent changes were reflected in this sequence as compared to the published sequence for ADAMTS-5. These changes were a G to an A at nucleotide # 711 and an A to a G at nucleotide # 2046 (numbering starts at 1 for the A of the ATG start codon for ADAMTS-5). The full-length cDNA, including an open reading frame (ORF) for ADAMTS-5 ORF and 5' and 3' untranslated regions (UTRs), was subcloned into the mammalian expression vector pED6-dpc2.

[0142] A cDNA expressing the full-length human aggrecanase-2 (hAgg-2)/ADAMTS-5 protein was cloned into the expression plasmid pHTop. This plasmid was derived from pED (Kaufman, *et al.*, Nucleic Acids Res. 19:4485-4490 (1991) by removing the majority of the adenomajor late promoter and inserting six repeats of the tet operator (Gossen and Bujard, Proc. Natl. Acad. Sci. U.S.A. 89:5547-5551 (1992)). A CHO cell line stably expressing hAgg-2 was obtained by transfecting pHTop/hAgg-2 into CHO/A2 cells and selecting clones in .05 μM methotrexate. Clones were screened for Aggrecanase-2 expression by western analysis of conditioned media using a polyclonal antibody specific for Aggrecanase-2. The CHO/A2 cell line was derived from CHO DUKX B11 cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. U.S.A. 77:4216-4220 (1980)) by stably integrating a transcriptional activator, a fusion between the tet repressor and the herpes virus VP16 transcription activation domain (Gossen and Bujard, Proc. Natl. Acad. Sci. U.S.A. 89:5547-5551 (1992)).

[001] A truncated ADAMTS-5 protein including a C-terminal protein purification tag was constructed using 3 DNA fragments from the full-length

ADAMTS-5 construct described above and a synthetic DNA duplex. ADAMTS-5 was truncated at amino acid residue 752 (Pro) of the full-length aggrecanase protein. Proline at 752 in the full length protein is N-terminal of a cleavage site in the full-length protein. The 3 DNA fragments consisted of a 5777 bp SgrA1/Not1 fragment containing the pHTop_new vector backbone and a 5' portion of ADAMTS-5, a 1756 bp SgrA1/BspH1 fragment containing ADAMTS-5, and a 304 bp BspH1/BsrG1 fragment containing ADAMTS-5. The 4 oligonucleotide fragments used to build the 110 bp synthetic duplex were as follows: oligonucleotide fragment 1: 5'GTACAAAGATTGTTGGAACCTTTAATAAGAAAA GTAAGGGTTACACTGACGTGGTGAGGATTC (SEQ ID NO: 20); oligonucleotide fragment 2: 5' CTGGATCCGGATCTGCTTGGAGCCACCCGCAGTTCGAAA AATAAGGC (SEQ ID NO: 21) which encodes a GSGSA (SEQ ID NO: 22) peptide linker followed by a WSHPQFEK (SEQ ID NO: 23) Strep-tag II protein purification tag from IBA fused C-terminus to amino acid 752 of the truncated ADAMTS-5 protein, oligonucleotide fragment 3: 5' GGCCGCCTTATTTTTCGAACTGCGGGTGGCTC CAAGCAGATCCGGATCCAGGAATCCTCAC (SEQ ID NO: 24) and oligonucleotide fragment 4: 5' CACGTCAGTGTAACCCTTACTTTTCTTATTAAAGGTTCCAACA ATCTTT (SEQ ID NO: 25). Each oligonucleotide fragment was diluted to a final concentration of 10 pmol/ul with sterile water. Equal volumes (10 µl each) of oligonucleotide fragments 1 and 4 were mixed. Equal volumes (10 µl each) of oligonucleotide fragments 2 and 3 were mixed. Mixtures were heated to 95°C for 5 minutes and then allowed to cool to room temperature. 1 µl of each mixture was

used in the final ligation with the 3 ADAMTS-5 DNA fragments described above. Standard ligation enzyme, buffers and conditions were used. Ligated products were used to transform ElectroMAX DH10B cells from Life Technologies (Carlsbad, CA). The cloned synthetic oligonucleotides were sequenced to determine fidelity and verify sequence. Figure 18 is a schematic representation of Streptavidin-tagged aggrecanase-2 proteins of the invention.

Example 2: Expression of ADAMTS-5-wild type(WT) and Active Site Mutant (ASM)

[0143] CHO/A2 cells were transfected with ADAMTS-5-WT or ASM expression vectors using Lipofectin (Life Technologies, Inc.). The cells were plated in the presence of .02, .05, and 0.1 mM Methotrexate (MTX) selection and incubated at 37°C, 5% CO_2 for 2 weeks. Colonies were picked and expanded into cell lines while cultured in selection medium.

[0144] In another experiment, CHO cells were transfected with active site mutants for aggrecanase-1, as depicted in Figure 17.

Example 3: Subcloning of ADAMTS-5 Active Site Mutant

[0145] The E411-Q411 point mutation in ADAMTS-5 was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenesis was performed on pHTop/Agg-2 using the QuickChange Site Directed Mutation Kit (Stratagene, catalog # 200518). The single base pair mutation, 2597-G to 2597-C resulted in a single amino acid change at position 411 (E-to-Q) in the catalytic domain of aggrecanase-2 protein (SEQ ID NO: 30). This mutation was shown to inactivate the catalytic activity of mini-stromelysin-1

(Steele *et al.*, Protein Engineering 13:397-405(2000)). A CHO cell line stably expressing hAgg-2(E411Q) active site mutant was obtained by transfecting pHTop/hAgg-2(E411Q) into CHO/A2 cells and selecting clones in .05 μM methotrexate. Clones were screened for Aggrecanase-2 expression by western analysis of conditioned media using a polyclonal antibody specific for Aggrecanase-2.

Additionally, E-to-Q mutation in the active site of aggrecanase-1 molecules of the invention is generated. Figure 17 is a schematic representation of recombinant truncated aggrecanase-1 molecules of the invention that include an E-to-Q mutation in the catalytic domain. Nucleic acid sequences encoding these aggrecanase-1 molecules are cloned into an appropriate vector; for example, pHTop disclosed, and subsequently transfected into an appropriate cell line; for example, CHO cells. Stable transfectants for aggrecanases of the invention can be selected as described above. Expression levels of active site aggrecanase mutants can be detected using an antibody specific for the aggrecanase being expressed.

Example 4: Western Blotting

[0146] Conditioned medium from CHO cells expressing ADAMTS-5 WT or ASM were loaded on a 12% SDS-PAGE gel under reducing conditions. The samples were then transferred to a nitrocellulose membrane. ADAMTS-5 protein was detected by a polyclonal antibody against ADAMTS-5, followed by goat-anti-rabbit IgG-HRP and a chemiluminescent substrate (Pierce, Milwaukee, WI).

[0147] In another experiment, conditioned medium from CHO cells expressing truncated aggrecanase-2 proteins was loaded onto a 12 % SDS-PAGE. The results of such an experiment are shown in Figure 19. Briefly, CHO cells were transfected with a nucleic acid encoding either truncated aggrecanase 2 from amino acid #1 through amino acid #567, or truncated aggrecanase-2 from amino acid # 1 through amino acid #628, or a nucleic acid expressing a full-length aggrecanase-2 molecule. Stable CHO cell lines were developed expressing either aggrecanase-2 from amino acid #1 through 567, or from amino acid #1 through 628, or full-length aggrecanase-2 molecule. Cells were subsequently harvested for proteins and expression levels for the various aggrecanase-2 molecules was determined by Western blot analysis using an antibody specific to aggrecanase-2. As demonstrated by the experiment set forth in Figure 19, truncated aggrecanase proteins are expressed at higher levels, due to higher stability, as compared with the full-length aggrecanase-2 protein.

Example 5: Microcapillary HPLC-Mass Spectrometry

[0148] Recombinant ADAMTS-5-WT or ASM proteins were purified by HP-HPLC and further analyzed by 1D-SDS-polyacrylamide gel electrophoresis. Proteins were visualized by Coommassie blue staining, and protein bands of interest were excised manually, then reduced, alkylated and digested with trypsin or endopeptidase Lys-C (Promega, Madison, WI) *in situ* using an automated in-gel digestion robot. After digestion, the peptide extracts were concentrated and separated by microelectrospray reversed-phase HPLC. Peptide analyses were done on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA). Automated analysis of MS/MS data was performed using the SEQUEST computer algorithm incorporated into the Finnigan Bioworks data analysis package (ThermoQuest, San Jose, CA) using the database of proteins derived from the complete genome.

Example 6: Biological Activity of Expressed Aggrecanase

[0149] To measure the biological activity of the expressed aggrecanase proteins; for example, truncated aggrecanases of the invention disclosed, the proteins are recovered from the cell culture and purified by isolating the aggrecanase-related proteins from other proteinaceous materials with which they are co-produced as well as from other contaminants. Purification is carried out using standard techniques known to those skilled in the art. The isolated protein may be assayed in accordance with the following assays:

[0150] Assays specifically to determine if the protein is an enzyme capable of cleaving aggrecan at the aggrecanase cleavage site:

[0151] Fluorescent peptide assay: Expressed protein is incubated with a synthetic peptide which encompasses amino acids at the aggrecanase cleavage site of aggrecan. Either the N-terminus or the C-terminus of the synthetic peptide is labeled with a flourophore and the other terminus includes a quencher. Cleavage of the peptide separates the flourophore and quencher and elicits flourescence. From this assay it is determined that the expressed aggrecanase protein can cleave aggrecan at the aggrecanase site, and relative fluorescence is a determination the relative activity of the expressed protein.

[0152] Neoepitope western: Expressed aggrecanase protein is incubated with intact aggrecan. After several biochemical manipulations of the resulting sample (dialysis, chondroitinase treatment, lyophilization and reconstitution) the sample is run on an SDS PAGE gel. The gel is incubated with an antibody that is specific to a site on aggrecan which is only exposed after aggrecanase cleavage. The gel is transferred onto nitrocellulose paper and developed using a secondary antibody (called a western assay) which subsequently results in a banding pattern indicative of products with a molecular weight consistent with aggrecanase generated cleavage products of aggrecan. This assay results in the finding that the expressed aggrecanase protein cleaved native aggrecan at the aggrecanase cleavage site, and also gives the molecular weight of the cleavage products. Relative density of the bands can give an indication of relative aggrecanase activity.

[0153] Assay to determine if an expressed protein can cleave aggrecan anywhere in the protein (not specific to the aggrecanase site).

[0154] Aggrecan ELISA: Expressed protein is incubated with intact aggrecan which had been previously adhered to plastic wells. The wells are washed and then incubated with an antibody that detects aggrecan. The wells are developed with a secondary antibody. If the original amount of aggrecan remains in the wells, the antibody staining is dense. Whereas, if aggrecan was digested by aggrecanase activity of the expressed aggrecanase protein, the aggrecan comes off the plate and the subsequent staining of the aggrecan-coated wells by the antibody is reduced. This assay tells whether an expressed protein is capable of cleaving aggrecan (anywhere in the protein, not only at the aggrecanase site) and can further determine relative aggrecan cleavage.

[0155] The results of one such experiment are shown in Figure 20, where aggrecan cleavage was detected after incubation of aggrecan with truncated aggrecanase-2 molecules using a 3B3 ELISA assay. The results of such an experiment, shown in Figure 20 include % aggrecan cleavage subsequent to incubation with truncated aggrecanases; for example, aggrecanase-2 from amino acid #1 through 567 and aggrecanase-2 from amino acid #1 through 628. As shown in Figure 20, truncated aggrecanases of the invention having one or both TSP domains deleted are biologically active.

[0156] Protein analysis of isolated proteins is conducted using standard techniques such as SDS-PAGE acrylamide (Laemmli, Nature 227:680 (1970)) stained with silver (Oakley, *et al.*, Anal Biochem. 105:361 (1980)) and by immunoblot (Towbin, et al., Proc. Natl. Acad. Sci. USA 76:4350 (1979)). Using the above described assays, expressed aggrecanase-related proteins are

evaluated for their activity and useful aggrecanase-related molecules are identified.

[0157] Activity Assay: Mirotiter plates (Costar) were coated with hyaluronic acid (ICN), followed by chondroitinase (Seikagaku Chemicals)-treated bovine aggrecan. Conditioned medium from CHO cells expressing WT ADAMTS-5 or ADAMTS-5 ASM was added to the aggrecan-coated plates. Aggrecan cleaved at the E^{373}-A^{374} within the interglobular domain was washed away. The remaining uncleaved aggrecan was detected with the 3B3 antibody (ICN), followed by anti-IgM-HRP secondary antibody (Southern Biotechnology). Final color development was with 3,3", 5,5" tetramethylbenzidine (TMB, BioFx Laboratories).

[0158] ADAMTS-5 is synthesized in the inactive pro-form (~90 kDa) and can be processed by furin to yield the mature species of ~70 kDa. Conditioned medium from CHO lines transfected with ADAMTS-5 expressed a small amount of active, mature protein. The predominant species expressed was a protein of ~ 55 kDa, representing a cleavage product of the mature protein.

[0159] Conditioned medium from the CHO stable lines transfected with ADAMTS-5 showed that the enzyme was cleaved to yield a species of ~55 kDa. Mass spectrometry and N-terminal sequencing of the clipped form revealed that the cleavage occurred between E^{753}-G^{754} residues in the spacer domain. In the presence of EDTA or a non-specific hydroxamate metalloprotease inhibitor, the full length mature, ~70 kDa protein, was preserved. This cleavage is autocatalytic and not due to an enzyme present in the CHO conditioned medium. Site-specific mutagenesis was performed to mutate the E^{411}-Q^{411} within the catalytic HELGH motif (SEQ ID NO: 26) of ADAMTS-5. Stable CHO lines were made with this

ADAMTS-5 active site-mutant (ADAMTS-5 ASM). Conditioned medium from CHO stable lines transfected with ADAMTS-5 ASM lacked aggrecanase activity as shown by ELISA (Figure 16). The full-length ~70 kDa protein rather than the clipped 55 kDa form, was predominant in a western blot of conditioned media from CHO cells expressing ADAMTS-5 ASM.

[0160] The above described examples demonstrate that recombinant ADAMTS-5 was susceptible to proteolytic cleavage at residue E^{753}-G^{754} in the spacer domain. This cleavage reduced the size of the mature protein to ~55 kDa and was inhibited by EDTA and a non-specific hydroxamate metalloprotease inhibitor. A point mutation in the catalytic domain of ADAMTS-5 inactivated the enzymatic activity of the protein and protected the full-length protein from cleavage. It is therefore contemplated that the proteolytic processing was autocatalytic and not due to a protease present in the conditioned medium of the CHO cells.

Example 7: Expression of Aggrecanase

[0161] In order to produce murine, human or other mammalian aggrecanase-related proteins, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts including insect host cell culture systems by conventional genetic engineering techniques. Expression system for biologically active recombinant human aggrecanase is contemplated to be stably transformed mammalian cells, insect, yeast or bacterial cells.

[0162] One skilled in the art can construct mammalian expression vectors by employing the nucleotide sequence of Figure 3 from nucleotide #1 (atg) through # 2259 (gaa); nucleotide sequence of Figure 5 from nucleotide #1 (atg) through nucleotide #2256 (cct); nucleic acid sequence of Figure 7 from nucleotide #1 (atg) through nucleotide #1884 (ttt); or nucleic acid sequence of Figure 9 from nucleotide #1 (atg) through nucleotide #1701 (cat) or other DNA sequences encoding aggrecanases or aggrecanase-like proteins or other modified sequences and known vectors, such as pCD (Okayama *et al.*, Mol. Cell Biol., 2:161-170 (1982)), pJL3, pJL4 (Gough *et al.*, EMBO J., 4:645-653 (1985)) and pMT2 CXM.

[0163] The mammalian expression vector pMT2 CXM is a derivative of p91023(b) (Wong *et al.*, Science 228:810-815, 1985) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 CXM have been described (Kaufman, R.J., 1985, Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in *E. coli*.

[0164] Plasmid pMT2 CXM is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which

can be ligated and used to transform *E. coli* HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2 CXM is then constructed using loopout/in mutagenesis (Morinaga, *et al.*, Biotechnology 84: 636 (1984)). This removes bases 1075 to 1145 relative to the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence:

5' PO-CATGGGCAGCTCGAG 3' (SEQ ID NO:27) at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I. A derivative of pMT2CXM, termed pMT23, contains recognition sites for the restriction endonucleases PstI, EcoRI, SalI and XhoI. Plasmid pMT2 CXM and pMT23 DNA may be prepared by conventional methods.

[0165] pEMC2β1 derived from pMT21 may also be suitable in practice of the invention. pMT21 is derived from pMT2 which is derived from pMT2-VWF. As described above EcoRI digestion excises the cDNA insert present in pMT-VWF, yielding pMT2 in linear form which can be ligated and used to transform *E. Coli* HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods.

[0166] pMT21 is derived from pMT2 through the following two modifications. First, 76 bp of the 5' untranslated region of the DHFR cDNA including a stretch of 19 G residues from G/C tailing for cDNA cloning is deleted. In this process, a XhoI site is inserted to obtain the following sequence immediately upstream from DHFR: 5' -

CTGCAGGCGAGCCTGAATTCCTCGAGCCATCATG (SEQ ID NO: 28)

PstI EcoRI XhoI

[0167] Second, a unique ClaI site is introduced by digestion with EcoRV and XbaI, treatment with Klenow fragment of DNA polymerase I, and ligation to a ClaI linker (CATCGATG). This deletes a 250 bp segment from the adenovirus associated RNA (VAI) region but does not interfere with VAI RNA gene expression or function. pMT21 is digested with EcoRI and XhoI, and used to derive the vector pEMC2B1.

[0168] A portion of the EMCV leader is obtained from pMT2-ECAT1 (S.K. Jung, *et al*, J. Virol 63:1651-1660 (1989)) by digestion with EcoRI and PstI, resulting in a 2752 bp fragment. This fragment is digested with TaqI yielding an EcoRI-TaqI fragment of 508 bp which is purified by electrophoresis on low melting agarose gel. A 68 bp adapter and its complementary strand are synthesized with a 5' TaqI protruding end and a 3' XhoI protruding end which has the following sequence:

5'

CGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTT

T

TaqI

GAAAAACACGATTGC 3' (SEQ ID NO: 29)

XhoI

[0169] This sequence matches the EMC virus leader sequence from nucleotide 763 to 827. It also changes the ATG at position 10 within the EMC virus leader to an ATT and is followed by a XhoI site. A three way ligation of the pMT21 EcoRI-16hoI fragment, the EMC virus EcoRI-TaqI fragment, and the 68 bp oligonucleotide adapter TaqI-16hoI adapter resulting in the vector pEMC2β1.

[0170] This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the majority of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, DHFR and β-lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

[0171] The construction of vectors may involve modification of the aggrecanase-related DNA sequences. For instance, a cDNA encoding an aggrecanase can be modified by removing the non-coding nucleotides on the 5' and 3' ends of the coding region. The deleted non-coding nucleotides may or may not be replaced by other sequences known to be beneficial for expression. These vectors are transformed into appropriate host cells for expression of aggrecanase or aggrecanase-like proteins. Additionally, the sequence of Figure 3 from nucleotide #1 (atg) through nucleotide #2259 (gaa), or Figure 5 from nucleotide #1 (atg) through nucleotide #2256 (cct), or Figure 7 from nucleotide # 1 (atg) through nucleotide #1884 (ttt); or Figure 9 from nucleotide #1 (atg) through nucleotide #1701 (cat), or fragments of variants thereof or other sequences encoding aggrecanases of the invention or proteins that have aggrecanase activity can be manipulated to express an aggrecanase or aggrecanase-like protein by deleting aggrecanase encoding propeptide sequences and replacing them with sequences encoding the complete propeptides of other aggrecanase proteins.

[0172] One skilled in the art can manipulate the sequences of Figure 3 from nucleotide #1 (atg) through nucleotide #2259 (gaa); or Figure 5 from nucleotide #1 (atg) through nucleotide #2256 (cct); or Figure 7 from nucleotide #1 (atg) through

nucleotide #1884 (ttt); or Figure 9 from nucleotide #1 (atg) through nucleotide #1701 (cat); or nucleotide sequences encoding aggrecanases molecules of Figures 12 and 13 set forth in Figures 23 and 24, or fragments and variants thereof, by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression of truncated aggrecanase molecules. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). A modified aggrecanase encoding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi *et al.*, Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells to express an aggrecanase protein of the invention. For a strategy for producing extracellular expression of aggrecanase-related proteins in bacterial cells, see, e.g. European patent application EPA 177,343.

[0173] Similar manipulations can be performed for construction of an insect vector (*see, e.g.*, procedures described in published European patent application 155,476) for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. (*See, e.g.*, procedures described in published PCT application WO86/00639 and European patent application EPA 123,289).

[0174] A method for producing high levels of a aggrecanase-related protein of the invention in mammalian, bacterial, yeast or insect host cell systems may

involve the construction of cells containing multiple copies of the heterologous aggrecanase-related gene. The heterologous gene is linked to an amplifiable marker; e.g., the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types.

[0175] For example, a plasmid containing a DNA sequence for an aggrecanase or aggrecanase-like protein of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 (Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)) can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by various methods including calcium phosphate coprecipitation and transfection, electroporation or protoplast fusion. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (e.g. sequential steps in 0.02, 0.2, 1.0 and 5 μM MTX) as described in Kaufman *et al.*, Mol Cell Biol., 5:1750 (1983). Transformants are cloned, and biologically active aggrecanase expression is monitored by at least one of the assays described above. Aggrecanase protein expression should increase with increasing levels of MTX resistance. Aggrecanase polypeptides are characterized using standard techniques known in the art such as pulse labeling with ^{35}S methionine or cysteine and polyacrylamide gel electrophoresis. Similar procedures can be followed to produce other aggrecanases or aggrecanase-like proteins.

[0176] As one example an aggrecanase nucleotide sequence of the present invention is cloned into the expression vector pED6 (Kaufman *et al.*, Nucleic Acid Res. 19:44885-4490 (1991)). COS and CHO DUKX B11 cells are transiently transfected with an aggrecanase sequence of the invention (+/- co-transfection of PACE on a separate pED6 plasmid) by lipofection (LF2000, Invitrogen). Duplicate transfections are performed for each molecule of interest: (a) one transfection set for harvesting conditioned media for activity assay and (b) one transfection set for 35-S-methionine/cysteine metabolic labeling.

[0177] On day one media is changed to DME(COS) or alpha (CHO) media + 1% heat-inactivated fetal calf serum +/- 100 µg/ml heparin on wells of set (a) to be harvested for activity assay. After 48h (day 4), conditioned media is harvested for activity assay.

[0178] On day 3, the duplicate wells of set (b) were changed to MEM (methionine-free/cysteine free) media + 1% heat-inactivated fetal calf serum +100µg/ml heparin + 100µCi/ml 35S-methionine/ cysteine (Redivue Pro mix, Amersham). Following 6h incubation at 37°C, conditioned media is harvested and run on SDS-PAGE gels under reducing conditions. Proteins are visualized by autoradiography.

Example 8: Biological Activity of Expressed Aggrecanase

[0179] To measure the biological activity of the expressed aggrecanase-related proteins obtained in the examples above, the proteins are recovered from the cell culture and purified by isolating the aggrecanase-related proteins from other proteinaceous materials with which they are co-produced as well as from

other contaminants. The purified protein may be assayed in accordance with assays described above. Purification is carried out using standard techniques known to those skilled in the art.

[0180] Protein analysis is conducted using standard techniques such as SDS-PAGE acrylamide (Laemmli, Nature 227:680 (1970)) stained with silver (Oakley, *et al.* Anal. Biochem. 105:361 (1980)) and by immunoblot (Towbin, *et al.* Proc. Natl. Acad. Sci. USA 76:4350 (1979)).

[0181] The foregoing descriptions detail presently preferred embodiments of the present invention Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

We claim:

1. A biologically-active aggrecanase protein, wherein the aggrecanase protein comprises a deletion of all or a portion of at least one TSP domain.

2. An aggrecanase protein according to claim 1, wherein the protein comprises a deletion of all or a portion of one TSP domain.

3. An aggrecanase protein according to claim 2, wherein the protein comprises an amino acid sequence chosen from SEQ ID NOs: 4, 6, 8, and 13, or a fragment or variant.

4. An aggrecanase protein according to claim 1, wherein the protein comprises a deletion of all or a portion of two TSP domains.

5. An aggrecanase protein according to claim 4, wherein the protein comprises an amino acid sequence chosen from SEQ ID NO. 10, or a fragment or variant.

6. An antibody that binds to an aggrecanase protein of any one of claims 1-5.

7. An antibody of claim 6, wherein the antibody is chosen from at least one of polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies.

8. An antibody of claim 6, wherein the antibody specifically binds to the aggrecanase protein.

9. An antibody of claim 6, wherein the antibody is chosen from a full length antibody, Fab, $F(ab')_2$, Fv, scFv, Fd, and dAb.

10. An isolated DNA molecule comprising a nucleotide sequence encoding the aggrecancase protein of claim 1.

11. An isolated DNA molecule of claim 10, wherein the DNA molecule comprises a nucleotide sequence chosen from:

a) the sequence of SEQ ID NO: 3 from nucleotide #1 (atg) through #2259 (gaa);

b) the sequence of SEQ ID NO: 5 from nucleotide #1 (atg) through #2256 (cct);

c) the sequence of SEQ ID NO: 7 from nucleotide #1 (atg) through #1884 (ttt);

d) the sequence of SEQ ID NO: 9 from nucleotide #1 (atg) through #1701 (cat);

e) the sequence of SEQ ID NO: 32 from nucleotide #1 (atg) through # (cca);

f) the sequence set forth in SEQ ID NO: 33 from nucleotide #1 (atg) through # (gct);

g) sequences that hybridize under stringent conditions with SEQ ID NOs: 3, 5, 7, 9, 32, and 33; and

h) fragments and variants of (a) – (g).

12. A vector comprising a DNA molecule of claim 11 in operative association with an expression control sequence.

13. A host cell transformed with a DNA sequence of claim 11.

14. A method for producing a purified truncated aggrecanase protein, said method comprising:

(a) culturing a host cell transformed with a DNA molecule according to claim 10; and

(b) recovering and purifying said aggrecanase protein encoded by the DNA molecule from the culture medium.

15. A method for producing a purified truncated aggrecanase protein, said method comprising:

(a) culturing a host cell transformed with a DNA molecule of at least one of SEQ ID NOs: 3, 5, 7, 9, 32, 33, or a fragment or variant; and

(b) recovering and purifying said aggrecanase protein from the culture medium with amino acid sequence of at least one of SEQ ID NOs: 4, 6, 8, 10, 12, and 13, respectively, or fragment or variant.

16. An antibody that binds to a purified truncated aggrecanase protein of claim 14 or 15.

17. An antibody of claim 16, wherein the antibody is chosen from at least one of a polyclonal, monoclonal, monospecific, polyspecific, non-specific,

humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies.

18. An antibody of claim 16, wherein the antibody specifically binds to the aggrecanase protein.

19. An antibody of claim 16, wherein the antibody is chosen from a full length antibody, Fab, $F(ab')_2$, Fv, scFv, Fd, and dAb.

20. A method of developing inhibitors of aggrecanase comprising:

(a) combining at least one aggrecanase protein set forth in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, or a fragment or variant, with at least one test sample; and

(b) determining if the test sample inhibits activity of the aggrecanase protein of (a).

21. The method of claim 20, wherein the test sample comprises at least one of a peptide, protein, chemical compound, or antibody.

22. A method of developing inhibitors of aggrecanase comprising:

(a) utilizing at least one of:

(i) an amino acid sequence of at least one aggrecanase protein set forth in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, or a fragment or a variant;

(ii) a three-dimensional structure of at least one aggrecanase protein set forth in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, or a fragment or a variant;

(b) identifying candidates that may inhibit aggrecanase activity based on a structural analysis.

23. The method of claim 22 further comprising:

(c) combining at least one aggrecanase protein set forth in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, or a fragment or a variant, with at least one candidate in (b);

(d) determining if the candidate inhibits aggrecanase activity.

24. The method of claim 22, wherein the candidate comprises at least one of a protein, peptide, chemical compound, or antibody.

25. A method of inhibiting aggrecan cleavage in a mammal, the method comprising administering to said mammal an effective amount of a composition comprising an inhibitor of an aggrecanase according to claim 1.

26. The method of claim 25, wherein the inhibitor comprises at least one of a protein, peptide, chemical compound, or antibody.

27. An aggrecanase protein, wherein the protein comprises a mutation that increases stability of the aggrecanase protein relative to the native aggrecanase protein.

28. The aggrecanase protein of claim 27, wherein the mutation comprises an amino acid substitution.

29. The aggrecanase protein of claim 28, wherein the amino acid substitution is an E-to-Q change in active site of the aggrecanase protein.

30. The aggrecanase protein of claim 29, comprising the amino acid sequence set forth in SEQ ID NO: 30.