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Interactions between collagen IX and biglycan measured by atomic force microscopy

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Abstract

The stability of the lattice-like type II collagen architecture of articular cartilage is paramount to its optimal function. Such stability not only depends on the rigidity of collagen fibrils themselves, but more importantly, on their interconnections. One known interconnection is through type IX and biglycan molecules. However, the mechanical properties of this interaction and its role in the overall stability remain unrevealed. Using atomic force microscopy, this study directly measured the mechanical strength (or the rupture force) of a single bond between collagen IX and biglycan. The results demonstrated that the rupture force of this single bond was 15 pN, which was significantly smaller than those of other known molecule interactions to date. This result suggested that type IX collagen and biglycan interaction may be the weak link in the cartilage collagen architecture, vulnerable to abnormal joint force and associated with disorders such as osteoarthritis.

Keywords: Collagen IX; Biglycan; Atomic force microscopy; Binding force

Optimal function of articular cartilage relies on an architectural network involving many macromolecular interactions within the extracellular matrix (ECM). The key players for these interactions are collagens and proteoglycans that organize into various patterns with differing component concentrations as a function of age and location. The major form of collagen present in articular cartilage is collagen II—a member of the fibrillar collagen family along with types I, III, V, and XI [1]. Other types of collagen involved in the architecture of articular cartilage consist of the fibril-associated collagens with interrupted triple helices (FACIT) and include types IX, XII, and XIV [2]. Collagen II, IX, and XI form a fibril network, or heteropolymer, that has been well described [3].

Specifically, collagen IX—constructed of three genetically distinct chains—distributes across the surface of collagen II [4]. It is a quantitatively minor component (10% in fetal and 1% in adult) of the collagen matrix that consists of four non-collagenous domains, three collagenous domains, and one chondroitin sulfate (glycosaminoglycan, GAG) component per chain [5]. Cremer et al. [6] speculate that collagen IX may serve as a ''spacer'' between individual fibrils or a ''glue'' that binds the collagen II network together, as well as a means for collagen fibrils to interact with PGs.

In addition to collagen, the ECM matrix also contains an array of proteoglycans (PGs) that interact with the collagen network. Biglycan—a member of the small leucine-rich proteoglycan (SLRP) family—is found in the

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pericellular matrix of connective tissue [7]. Composed of a leucine-rich core protein and two chondroitin/dermatan sulfate side chains, biglycan has been observed to interact primarily with collagen II [8].

Given that both collagen IX and biglycan interact with collagen II, it raises the possibility of an interaction between collagen IX and biglycan that would help stabilize the lattice-like architecture of articular cartilage. To the best of our knowledge, such interaction and the accompanying force of interaction has not been demonstrated or quantitated. Measuring the single bond strength between biglycan and collagen IX can offer further clues to the mechanical properties of cartilage [9]. With the development of nanoscale manipulation techniques such as atomic force microscopy (AFM), the mechanical properties between single molecules can be directly assessed [10]. Examples include the biomechanical properties of biomolecules such as DNA and polysaccharides as well as the force elongation of collagen fibrils [11–13]. The aim of this study was to quantify the single bond binding strength between collagen IX and biglycan by directly measuring its rupture force using AFM.

Materials and methods

Experimental facility and procedure. AFM (Novascan Technologies, Ames, IA) was used to directly measure the interaction force between biglycan and collagen IX [14]. Collagen IX was covalently bound to glass coverslips using its core protein to adhere to the coverslip surface (Fig. 1) [15]. The coverslips were sonicated in Sparkleen detergent (Fisher Scientific, Pittsburgh, PA) for 20 min and rinsed thoroughly. They were soaked in 3 M potassium hydroxide for 1 h and then extensively rinsed with deionized water. They were then soaked in 50% H_2SO_4 for 1 h, rinsed in deionized water, and allowed to air-dry in an oven at 60 °C. The coverslips were transferred to a 0.01% v/v solution of 3-aminopropyltriethoxysilane (APS) at room temperature for 30 min. They were washed with deionized water to remove any uncoated APS and allowed to air-dry. Separately, 1 ml of 10 μg/ml collagen IX was placed in PBS (pH 5.5, 150 mM NaCl) and allowed to react with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide for 30 min. The solution was subsequently adjusted to pH 8 using NaOH. Finally, the collagen IX solution was dropped onto the coverslip surface and incubated at room temperature for 30 min. Hydroxylamine was added to stop the reaction, and the coverslips were washed with deionized water.

For the biglycan-modified AFM tip, a gold cantilever tip was submerged in a piranha solution (H_2SO_4:30% H_2O_2 = 3:1, v/v) for 5 min and then washed three times with absolute ethanol (Fig. 1) [15]. One clean tip was tested against a clean coverslip for the control event. Five millimolar of 2-mercaptoethanol in ethanol was added to react with the tip for 24 h at room temperature. The tip was then washed three times with absolute ethanol followed by 1 rinse with toluene. After rinsing, 2% of 3-mercaptopropyltrimethoxysilane (MTS) in toluene was added to react with the tip for 2 h under an argon atmosphere at room temperature. The tip was then rinsed with toluene and allowed to air-dry. The tip was placed in 2 mM *N*-γ-maleimidobutyryloxy succinimide ester (GMBS) in absolute ethanol for 1 h at room temperature. The tip was rewashed with 95% ethanol three times and then deionized water three times. Finally, 1 ml of 10 μg/ml biglycan in phosphate-buffered saline (PBS: 20 mM Na_2HPO_4, 150 mM NaCl, and Milli-Q water, pH 7.0) was added to react to the tip for 30 min at room temperature.

Binding force measurement. The interaction between collagen IX and biglycan was measured by the AFM tip approaching the coverslip perpendicularly [16,17]. As the tip approached the surface and remained there for 10 s, a bond between collagen IX and biglycan formed. When the tip withdrew from the surface, the force generated by the bending of the tip stretched the molecules and subsequently ruptured the bond. A force distance curve of the approach and retract events were generated (Fig. 2). The instantaneous event of bond rupture created a sudden jump in the force distance curve. The value of these sudden jumps represents the rupture event of the binding between the collagen IX and the biglycan. To obtain the force–displacement curve [18], the surface interaction force can be detected continuously.

We tested the interaction force at 4 random locations with 40–50 tests collected at each location. A soft cantilever (0.03 N/m) (MakroMasch, Portland, Oregon) was used in this experiment, and the approach and retract rate of the tip to the surface was kept at a constant speed of 0.05 μm/s [19]. The tip indentation forces were kept relatively constant at 1 nN. The binding force between collagen IX and biglycan was computed for all sudden increases in the force–distance curve for each of the tests.

Results

The histogram of all the rupture forces is shown in Fig. 3. The distribution of single force measurements ranged from 8 to 51 pN with the majority of the tests falling between 12 and 18 pN. Maximal peak force to separate the binding force between collagen IX and biglycan was around 15 pN. For tips and surfaces without biglycan or collagen IX bond, no obvious rupture events were

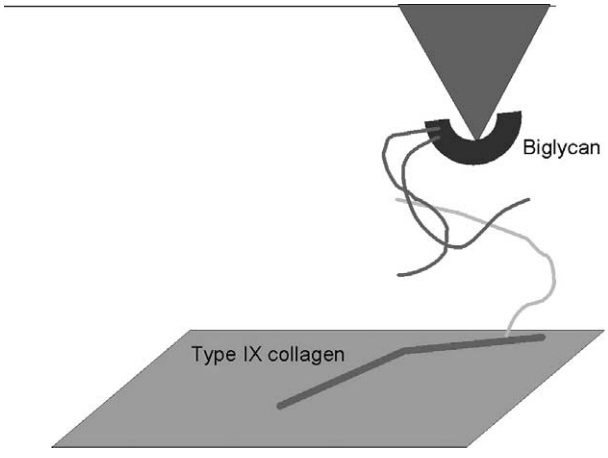

Fig. 1. Collagen IX was covalently immobilized to a coverslip surface, and biglycan was immobilized over AFM probe.

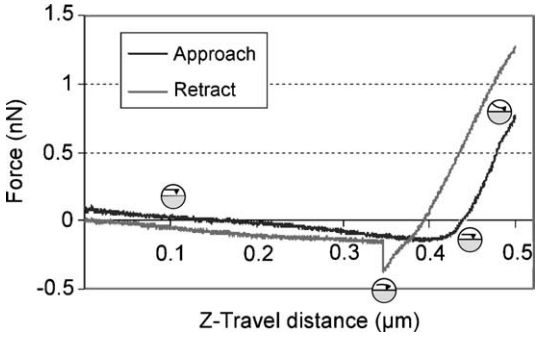

Fig. 2. Typical force versus *Z* distance curve generated by the AFM. The measurements corresponding to different states of cantilever during approach and retraction are noted.

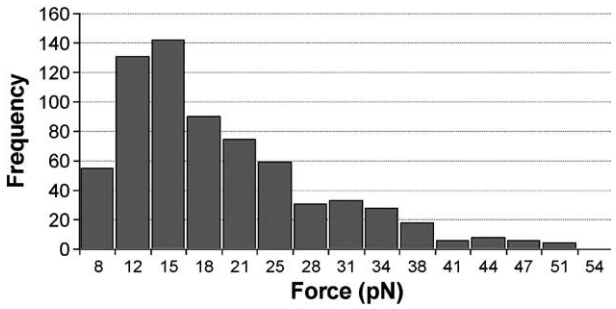

Fig. 3. Histogram of the adherence force between a biglycan-coated AFM tip and collagen-coated coverslip surface.

recorded. Multiple ruptures for each single test were recorded in most of the tests. Two peaks over 31 and 44 pN showed signs of peak frequency from the 180 tests and 650 total rupture forces. The peaks at 31 and 44 pN from force histogram represent double and triple bond ruptures between collagen IX and biglycan.

Discussion

In this study, we directly measured the rupture force between type IX collagen and biglycan, a newly described potential molecular interaction on the surface of type II collagen. The force–distance curve generated from the AFM data displayed a sharp adhesion point in the cantilever's retract phase (Fig. 2). This observation indicates that the force measured represents the detachment force of the bond between collagen IX and biglycan [18]. To confirm that the collagen IX–biglycan interaction existed under full discontinuities in the force curves, a clean tip and coverslip without modified biglycan and collagen IX were tested for comparison. There was no apparent force discontinuity in the retracting curve for the tip and surface without modified biglycan and collagen IX.

This study demonstrated that the single binding force between the collagen IX and biglycan was ~15 pN, which is significantly lower than those of other known molecule interactions to date. For example, the rupture force between collagen I and decorin was found to range from 32 pN between GAG and collagen to 55 pN between protein core and collagen [20]. This result suggested that the collagen IX and biglycan interaction may be the weak link in cartilage collagen architecture, making cartilage vulnerable to abnormal joint force and therefore associated with disorders such as osteoarthritis.

One critical part of the experiment was to ensure most samples to be single bonds. The apical radius of the AFM tip used in this study was less than 20 nm, and the tip was cone-shaped. The size of the core protein of biglycan was about 6 nm, and the length of GAG chain was about 100 nm [16]. When the tip reached the surface and remained for 10 s, the opportunity existed for multiple molecules to be involved in interactions. A distribution of rupture sequences was created because molecules attached at different locations on the tip and with different lengths of GAG chain. The majority of molecules were broken via single bonds; however, double and triple bonding with two to three molecules broken nearly simultaneously was occasionally recorded. With the maximal peak among the distribution of rupture force being at 15 pN, additional peaks were noted at around 31 and 44 pN which are approximately 2 and 3 times 15 pN.

The experiment was carefully designed such that the bond between the AFM tip and biglycan, and between the coverslip and collagen IX was much stronger than the collagen IX and biglycan interaction. The bond between the AFM tip's surface was modified through MTS and GMBS to establish the NHS ester group activated functional legend. The NHS ester group activated tip binds to the amino group of the core protein of biglycan. The chemical bond between the tip and biglycan was a covalent bond. The bond strength of a covalent bond is greater than 1 nN. The coverslip was modified with APS in order to bind the GAG chain of collagen IX. To activate its carboxyl group, collagen IX was modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide. Hydroxylamine was added to the collagen IX coverslip solution to quench the unbound carboxyl on the molecule. The bond formed between the coverslip and collagen IX was also a covalent bond; consequently, the interaction between collagen IX and biglycan was the weakest bond. Therefore, the rupture force recorded should represent the interaction between collagen IX and biglycan.

Collagens play a major role in the mechanical properties of articular cartilage as they resist compressive forces. The predominant collagen in hyaline cartilage tissue is collagen II in the form of a heteropolymer with collagen IX, XI, and matrix proteoglycans [4]. Collagen IX contains 3 collagenous domains and 4 non-collagenous (NC) domains with an important domain being the COL2 domain. At this domain, collagen IX crosslinks with collagen II [3]. The NC4 domain protects the surface of the fibril and serves as a point of interaction with proteoglycan [2]. Interactions between collagens and proteoglycans are required in the maintenance of cartilage. Collagen IX on the surface of collagen fibrils may also play a role in cartilage homeostasis by binding growth factors, while the interactions with collagen II may strongly influence the shear properties of cartilage. It has been noted that reduced function of collagen IX would contribute to degeneration of articular cartilage. Loss of collagen IX, resulting in loss of stabilization in the fibril network, is accompanied by swelling of the cartilage and the early onset of osteoarthritis [21].

Collagen IX may serve as a site of interaction between adjacent collagen fibrils or other interspersed proteoglycans, such as biglycan which is abundant in fibrocartilage and helps to stabilize the matrix. Biglycan contains an N-terminal domain and two chondroitin/dermatan sulfate side chains, as opposed to decorin which has only one GAG side chain. Biglycan binds to other macromolecules and aids in the formation of the network in the pericellular matrix [5]. Within this pericellular area, biglycan is found

to interact primarily with collagen II. Small interstitial proteoglycans such as biglycan interact with collagen fibrils via GAG side chains. This interaction plays an important role through influencing chondrocyte proliferation and regulating collagen fibrillogenesis. It also serves to stabilize the matrix and ultimately maintain the integrity of connective tissue [8]. In a canine model, the mRNA levels in osteoarthritic cartilage were found to increase 3.9 times, potentially producing an alteration in cartilage formation [22]. Alternatively, in biglycan-deficient mice, impaired gait and premature osteoarthritis progressively developed [23].

Some technical issues should be addressed to understand the use of AFM as a means to measure the rupture force between collagen IX and biglycan. In previous applications, AFM has been used to assess biological systems below the ultrastructural level, such as with DNA and polysaccharides [11,13,24]. AFM can detect binding forces ranging from 10 pN to 10 μN. Depending on the characteristics of the interaction, some tests can be repeated up to 50 times. Therefore, AFM is well suited for the study of collagen fibril interaction forces. However, it is imperative that experimental techniques be carefully executed to achieve accurate results. The background vibrating and acoustic noise should be well controlled to diminish the interference from the environment. In our experiments, the force–displacement curve was recorded at least 30 min after the system was under the solution to let the system reach thermal equilibrium. The standard deviation of the unbending area of the force–displacement curve is less than 3 pN in our system. Finally, to calculate the interaction force between isolated single molecules, the molecular density distributed on the tip and coverslip should be sufficiently low to allow minimal molecular interaction. In our study, painstaking preparation of partially immobilized molecules on the biglycan tip samples and the collagen IX coverslips was done to produce a minimal number of bonding events [17].

On the molecular level, the determination of collagen fibril biomechanical properties can be used to identify monomer interactions involving specific molecular components [14]. Knowledge of the force required to rupture the collagen IX-biglycan bond provides greater insight into extracellular matrix interactions within articular cartilage when subjected to the mechanical environment. A determination of the extracellular matrix interactions is critical to understanding the mechanism of articular cartilage function. It can be presumed that the collagen IX–biglycan interaction contributes to the stabilization of the matrix surrounding collagen II leading to increased resistance of cartilage under mechanical force. This intermolecular interaction may also help lead to a greater understanding into the mechanism of dysfunction of articular cartilage such as in rheumatoid arthritis and osteoarthritis [25,26]. To the best of our knowledge, this study is the first investigation to directly measure the force of single collagen IX and biglycan bonds.

In summary, an intermolecular interaction force between collagen IX and biglycan was measured to be about 15 pN. This data set offers further insights into the biomechanical properties of cartilage, suggesting that this bond might be the weak link in cartilage collagen architecture. This information may lead to further understanding of articular cartilage dysfunction.

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References

[1] V. Ottani, D. Martini, M. Franchi, A. Ruggeri, M. Raspanti, Hierarchical structures in fibrillar collagens, Micron 33 (2002) 587–596.
[2] M. van der Rest, E. Aubert-Foucher, B. Dublet, D. Eichenberger, B. Font, D. Goldschmidt, Structure and function of the fibril-associated collagens, Biochem. Soc. Trans. 19 (1991) 820–824.
[3] D.R. Eyre, J.J. Wu, R.J. Fernandes, T.A. Pietka, M.A. Weis, Recent developments in cartilage research: matrix biology of the collagen II/IX/XI heterofibril network, Biochem. Soc. Trans. 30 (2002) 893–899.
[4] D.R. Eyre, Collagens and cartilage matrix homeostasis, Clin. Orthop. Relat. Res. (2004) S118–S122.
[5] P.J. Roughley, E.R. Lee, Cartilage proteoglycans: structure and potential functions, Microsc. Res. Tech. 28 (1994) 385–397.
[6] M.A. Cremer, E.F. Rosloniec, A.H. Kang, The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease, J. Mol. Med. 76 (1998) 275–288.
[7] P. Bianco, L.W. Fisher, M.F. Young, J.D. Termine, P.G. Robey, Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues, J. Histochem. Cytochem. 38 (1990) 1549–1563.
[8] D.H. Vynios, N. Papageorgakopoulou, H. Sazakli, C.P. Tsiganos, The interactions of cartilage proteoglycans with collagens are determined by their structures, Biochimie 83 (2001) 899–906.
[9] A.S. Wolberg, D.W. Stafford, D.A. Erie, Human factor IX binds to specific sites on the collagenous domain of collagen IV, J. Biol. Chem. 272 (1997) 16717–16720.
[10] D. Leckband, Measuring the forces that control protein interactions, Annu. Rev. Biophys. Biomol. Struct. 29 (2000) 1–26.
[11] H. Clausen-Schaumann, M. Rief, C. Tolksdorf, H.E. Gaub, Mechanical stability of single DNA molecules, Biophys. J. 78 (2000) 1997–2007.
[12] J.S. Graham, A.N. Vomund, C.L. Phillips, M. Grandbois, Structural changes in human type I collagen fibrils investigated by force spectroscopy, Exp. Cell Res. 299 (2004) 335–342.
[13] M.F. Reif, F. Oesterhelt, B. Heymann, H.E. Gaub, Single molecule force spectroscopy on polysaccharides by atomic force microscopy, Science 275 (1997) 1295–1297.
[14] Y.S. Lo, N.D. Huefner, W.S. Chan, F. Stevens, J.M. Harris, T.P. Beebe, Specific interactions between biotin and avidin studied by atomic force microscopy using the Poisson statistical analysis method, Langmuir 15 (1999) 1373–1382.
[15] J. Zlatanova, S.M. Lindsay, S.H. Leuba, Single molecule force spectroscopy in biology using the atomic force microscope, Prog. Biophys. Mol. Biol. 74 (2000) 37–61.
[16] J.K. Stuart, V. Hlady, Feasibility of measuring antigen–antibody interaction forces using a scanning force microscope, Colloids Surf. B Biointerfaces 15 (1999) 37–55.

[17] M. Grandbois, M. Beyer, M. Rief, H. Clausen-Schaumann, H.E. Gaub, How strong is a covalent bond? Science 283 (1999) 1727–1730.
[18] S. Kidoaki, T. Matsuda, Mechanistic aspects of protein/material interactions probed by atomic force microscopy, Colloids Surf. B Biointerfaces 23 (2002) 153–163.
[19] J.M. Williams, T.J. Han, T.P. Beebe, Determination of single-bond forces from contact force variances in atomic force microscopy, Langmuir 12 (1996) 1291–1295.
[20] M.L. Yeh, X. Liu, Z.P. Luo, Quantification of the binding strength of type I collagen and decorin using optical tweezers, in: Trans. of the 51st Annual Meeting, Orthopaedic Research Society 30 (2005) 922.
[21] J.J. Wu, P.E. Woods, D.R. Eyre, Identification of cross-linking sites in bovine cartilage type IX collagen reveals an antiparallel type II–type IX molecular relationship and type IX to type IX bonding, J. Biol. Chem. 267 (1992) 23007–23014.
[22] G.S. Dourado, M.E. Adams, J.R. Matyas, D. Huang, Expression of biglycan, decorin and fibromodulin in the hypertrophic phase of experimental osteoarthritis, Osteoarthritis Cartilage 4 (1996) 187–196.
[23] L. Ameye, D. Aria, K. Jepsen, A. Oldberg, T. Xu, M.F. Young, Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis, FASEB J. 16 (2002) 673–680.
[24] O.H. Willemsen, M.M. Snel, A. Cambi, J. Greve, B.G. De Grooth, C.G. Figdor, Biomolecular interactions measured by atomic force microscopy, Biophys. J. 79 (2000) 3267–3281.
[25] T. Xu, P. Bianco, L.W. Fisher, G. Longenecker, E. Smith, S. Goldstein, J. Bonadio, A. Boskey, A.M. Heegaard, B. Sommer, K. Satomura, P. Dominguez, C. Zhao, A.B. Kulkarni, P.G. Robey, M.F. Young, Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice, Nat. Genet. 20 (1998) 78–82.
[26] T. Kojima, F. Mwale, T. Yasuda, C. Girard, A.R. Poole, S. Laverty, Early degradation of type IX and type II collagen with the onset of experimental inflammatory arthritis, Arthritis Rheum. 44 (2001) 120–127.