Direct quantification of the rupture force of single hyaluronan/hyaluronan binding protein bonds

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Received 1 December 2003; revised 23 February 2004; accepted 26 February 2004

First published online 9 March 2004

Edited by Michael R. Bubb

Abstract The non-covalent bond between aggrecan and hyaluronan is critical for maintaining the normal structure and function of the extracellular matrix in articular cartilage. The failure of this bond can cause the loss of aggrecan and destruction of the extracellular matrix of articular cartilage. In this study, the rupture force of the single bond between hyaluronan and hyaluronan binding protein – the complex of the hyaluronan binding region of aggrecan and link protein – was directly measured with a nanomechanical testing system as 40 ± 11 pN. The results were compared to a theoretical prediction based on a smart version of the Monte Carlo simulation.

Key words: Rupture force; Hyaluronan; Hyaluronan binding protein; Articular cartilage; Laser tweezers; Monte Carlo simulation

1. Introduction

Proteoglycan aggregate is a major component of the extracellular matrix in articular cartilage, and is responsible for resistance to compression. It consists of a core molecule of hyaluronan (HA) and a number of branches of aggrecan, which bind to HA non-covalently with the help of link protein, a small protein able to bind to both aggrecan and HA [1,2]. Like most HA binding molecules, aggrecan and link protein contain proteoglycan tandem repeats as functional sites to interact with HA [3–6].

The non-covalent bond between aggrecan and HA is critical for maintaining the normal structure of proteoglycan aggregate and contributes to the integrity of the extracellular matrix of articular cartilage. The failure of this bond can cause the detachment of aggrecan from the aggregate and destruction of the integrity of the extracellular matrix of articular cartilage. For example, in link protein-null mice, aggrecan deposition in the cartilage is significantly reduced due to the weaker bond between HA and aggrecan caused by the absence of link protein. As a result, dwarfism and other musculoskeletal abnormalities are reported in these mice [7].

Importantly, to our knowledge the strength of this bond has not yet been determined. The goal of this study is to (1) quantify the bond strength between HA and hyaluronan binding protein (HABP) – the complex of the hyaluronan binding region of aggrecan (G1 domain) and link protein – by directly measuring its rupture force using a nanomechanical testing system and comparing those results to a theoretical prediction based on a smart version of Monte Carlo simulation; and (2) examine whether or not the rupture force of the HA/HABP bond is dependent on the pulling velocity.

2. Materials and methods

2.1. Experimental procedure

Mechanical testing was performed using a single molecule nanomechanical testing system, previously developed in our laboratory [8,9]. In this system, a laser tweezers workstation (Cell Robotics, Albuquerque, NM, USA), which consisted of a laser tweezers 980/1000 module, with a laser wavelength of 980 nm and a continuous wave power diode laser up to 1000 mW, was built in an inverted microscope (Axiovert 135, Carl Zeiss, Thornwood, NY, USA). A piezo-stage with a resolution of 1 nm (P-731.20, Polytec PI, Auburn, MA, USA) and an interferometry system (Micro Development, Zimmerman, MN, USA) were included in order to improve the resolution of the stage movement and molecule displacement measurement to the nanometer level. A two-bead system was adopted to perform the mechanical test on the single molecule nanomechanical testing system. Descriptive details have been provided previously [10]. In brief, a large bead–molecule–small bead linkage was sandwiched between two coverglasses, and was then mounted on the stage. The large bead was immobilized between coverglasses. The small bead was free in the solution except for its linkage to the molecule.

In order to form the bead–HA–HABP–bead linkage, the molecules of HA and HABP were each separately coated onto two different sizes of polystyrene beads and then mixed together to allow the formation of the linkage prior to the mechanical testing. Both molecules were obtained from commercial sources. Biotinylated HABP, which includes the hyaluronan binding region of aggrecan and link protein, was purchased from Seikagaku (Tokyo, Japan). HA with a molecule weight $>10^6$ Da was obtained from Acros Organics (Geel, Belgium). Biotinylated HABP was bound to streptavidin-coated polystyrene beads (5.6 μm in diameter, Bangs Laboratories, Fishers, IN, USA) through biotin/streptavidin binding. 5 μl of 0.25 mM biotinylated HABP was combined with 30 μl 0.5 g/ml streptavidin-coated beads in 0.1 M phosphate-buffered saline (pH 7.4) with 1% bovine serum albumin for 1 h at room temperature [11]. In order to reduce the density of HABP on the beads, excessive biotin (Sigma-Aldrich, St. Louis, MO, USA) was also added to impede the binding sites of streptavidin on the beads' surfaces. Afterward, the unbound biotinylated HABP and biotin were removed by centrifugation. In the control group, only biotin was used. HA was bound to another size of amino group-coated polystyrene bead (3.1 μm in diameter, Bangs Laboratories) through amino group/carboxyl group binding. 1 μl of 50 nM HA was reacted with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and *N*-hydroxysuccimide (Pierce Biotechnology, Rockford, IL, USA) in phosphate sodium buffer (pH 5.5) with 0.5 M NaCl. After 15 min, 30 μl 1 g/ml amino group-functioned beads were added and the pH was adjusted to 8. The reaction was quenched by hydroxylamine (Sigma Aldrich) at a final concentration of 10 nM 10 min later [10]. Unbound HA was removed through centrifugation. The two molecule-linked beads were then mixed in an associated buff-

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0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00232-7

er of 0.15 M sodium chloride, 0.01 M MES, 0.005 M EDTA at pH 7.0 and left overnight at 4°C to allow the formation of the binding of HA to HABP [12].

The bond between HA and HABP was ruptured through the beads during the mechanical test. After a large bead–molecule–small bead linkage was identified under microscopy, the laser was turned on to trap the small bead. The large bead was moved away from the small bead with the stage and the molecules were stretched between the beads until the bond between the molecules was broken. A computer program based on the LabVIEW® program (National Instruments, Austin, TX, USA) was created to control the piezo-stage movement and to measure the trapped bead displacement. The deformation of the molecules was measured as the displacement between the beads. The force applied on the molecules was equal to the bead trapping force, which can be calculated as the trapping stiffness times the departure distance of the small bead from the laser beam center [13,14]. The force–displacement curve was recorded and the rupture force was recorded as the highest force the curve reached. A pulling velocity of 0.5 μm/s was adopted in the mechanical test. Additionally, in order to examine the dependence of rupture force of the HA/HABP bond on the pulling velocity, three additional pulling velocities of 0.005 μm/s, 0.05 μm/s and 5 μm/s were also employed. The pulling velocity equals the stage movement velocity, which was controlled by the LabVIEW® program.

2.2. Monte Carlo simulation

Based on the concept that the rupture process of a bond can be modeled as a random event, a smart version of Monte Carlo simulations was made to predict the rupture forces, following the steps previously described by Fritz et al. [15]. The wormlike chain (WLC) model presented by Bustamante et al. [16] was used to fit the rupture force and extension data of the HA/HABP bond. The model was given by

$$F = \frac{k_B t}{p}\left(\frac{1}{4(1-x/L_{\text{contour}})^2} - \frac{1}{4} + \frac{x}{L_{\text{contour}}}\right)$$

where k_B is the Boltzmann constant, T is the temperature in Kelvin, p is the persistence length, and L_{contour} is the contour length.

In the Monte Carlo simulation, the pulling speed v_{pull} is given to be 0.5 μm/s. At each small time interval ($\Delta t = 0.1$ ms), the extension is calculated by $x(t) = v_{\text{pull}} \cdot t$, and the actual force is calculated from the above WLC model. The off-rate is determined from the following equation [15]

$$k_{\text{off}}(F) = k^0_{\text{off}} \cdot e^{F(t)\cdot S_{\text{pot}}/(k_B T)}$$

where k^0_{off} is the off-rate at zero external force, and S_{pot} is the mean width of the binding potential.

The probability of a rupture of the binding at the force level F during the current time interval Δt is given by $P_{\text{rupture}} = k_{\text{off}}(F)\Delta t$. In the Monte Carlo simulation, the occurrence of the bond rupture at this force level is determined by comparing P_{rupture} with a random number P_U within a uniform distribution in [0,1]. When $P_{\text{rupture}} > P_U$, the rupture occurs.

3. Results

The rupture force of a single HA/HABP bond was measured as 40 ± 11 pN (mean ± S.D.) at a pulling velocity of 0.5 μm/s. In total, 75 samples were tested at this pulling velocity. A typical force–displacement curve of the rupture of one single HA/HABP bond is shown in Fig. 1. The persistence length p and the contour length L_{contour} of the molecule were determined as 3.7 nm and 3.1 μm, respectively, using the WLC model [10].

A smart version of Monte Carlo simulation was found to agree well with the experimental data. From such a fit an off-rate $K^0_{\text{off}} = 0.0044\ \text{s}^{-1}$ at zero external force and a mean width of the HA/HABP bond potential $S_{\text{pot}} = 4.1$ nm were determined. Six hundred samples have been generated in the Monte Carlo simulation. The probability distribution of the rupture force is shown and compared with experimental data in Fig. 2. The simulated rupture forces have a mean value 40 pN with a standard deviation 11 pN.

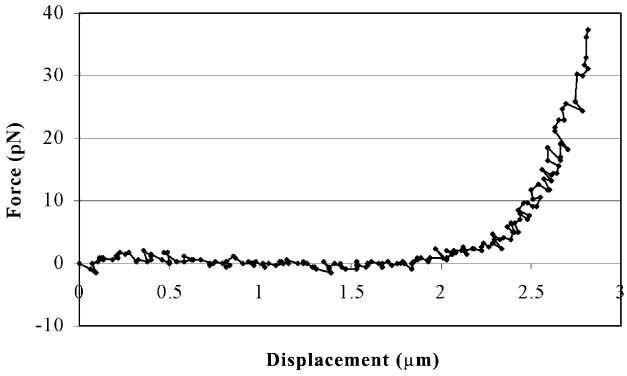

Fig. 1. A typical force–displacement curve of HA elongation. The maximal force represents the rupture force of the bond between HA and HABP. The pulling velocity was 0.5 μm/s.

In order to identify the dependence of rupture force of the HA/HABP bond on the pulling velocity, the rupture force of the HA/HABP bond was tested at the different pulling velocities of 0.005 μm/s, 0.05 μm/s and 5 μm/s. The rupture force of HA/HABP was detected as 11 ± 4.0 pN under the pulling velocity of 0.005 μm/s, 28 ± 11 pN under the pulling velocity of 0.05 μm/s, and 59 ± 11 pN under the pulling velocity of 5 μm/s. The rupture force of HA/HABP increased significantly with a faster pulling velocity ($P < 0.001$) and showed a logarithmic dependence on pulling velocity (Fig. 3).

4. Discussion

In this study, we directly measured the rupture force of the single bond between HA and HABP, the critical intermolecular interaction that ensures the integrity in the proteoglycan aggregate in the extracellular matrix of articular cartilage. Knowledge concerning the strength of this bond may help us to understand the extracellular matrix molecular interactions in articular cartilage under mechanical environment, and may potentially help us to understand the mechanism of some articular cartilage degenerative diseases, such as osteoarthritis. As this is the first investigation, to our knowledge, to directly quantify the strength of the single HA/HABP bond, some technical issues must be addressed to ensure the accuracy of the results.

During this experiment, single HA/HABP bonds were achieved by adopting some specific measures described previously [10,17,18]. First, the molecules were dissolved and diluted at low concentration in order to maximize the presence of single molecules in the solution. Second, biotin was added to impede the binding of biotinylated HABP to the streptavidin-coated beads to help reduce the density of the HABP on the beads. Third, the persistence length of the molecule of each sample was measured using the WLC model in order to ensure the HA between the two beads was a single molecule. The persistence length of the molecules between the beads was found to be 3.7 ± 1.4 nm, which was similar to that of single HA reported previously by Fujii et al. (4.5 ± 1.2 nm) [10]. These measures ensured that the HA/HABP bonds tested in this experiment were single ones.

It was critical to ensure that the bond broken during the

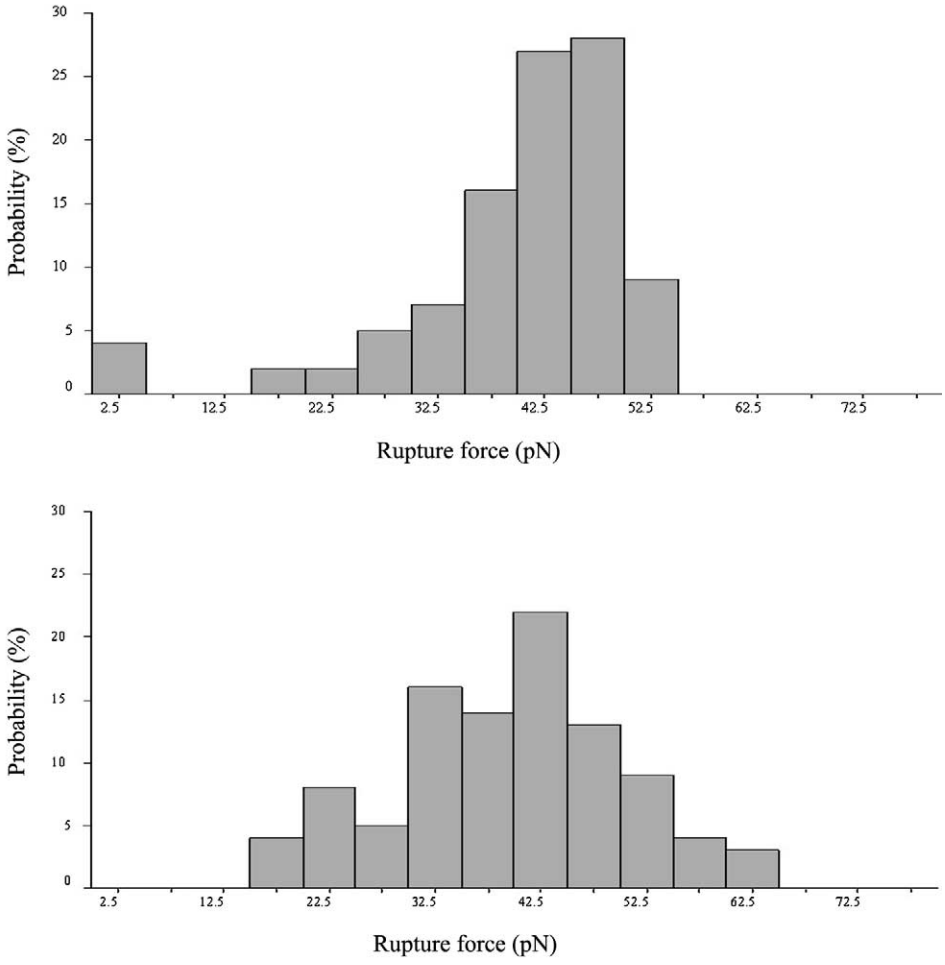

Fig. 2. Force-dependent bond rupture probabilities for the single bond between HA/HABP predicted by Monte Carlo simulation (top panel) was compared to that measured in the experiment (bottom panel).

experiment was the bond between HA and HABP. In order to achieve this, we linked the molecules of HA and HABP to the beads with stronger bonds. HA was attached to the bead with a covalent bond, whose rupture force was at least 1400 pN [19]. Biotin was linked to HABP also through a covalent bond according to the manufacturer's protocol. The linkage between HABP and the bead was the bond between biotin and streptavidin, one of the strongest known non-covalent

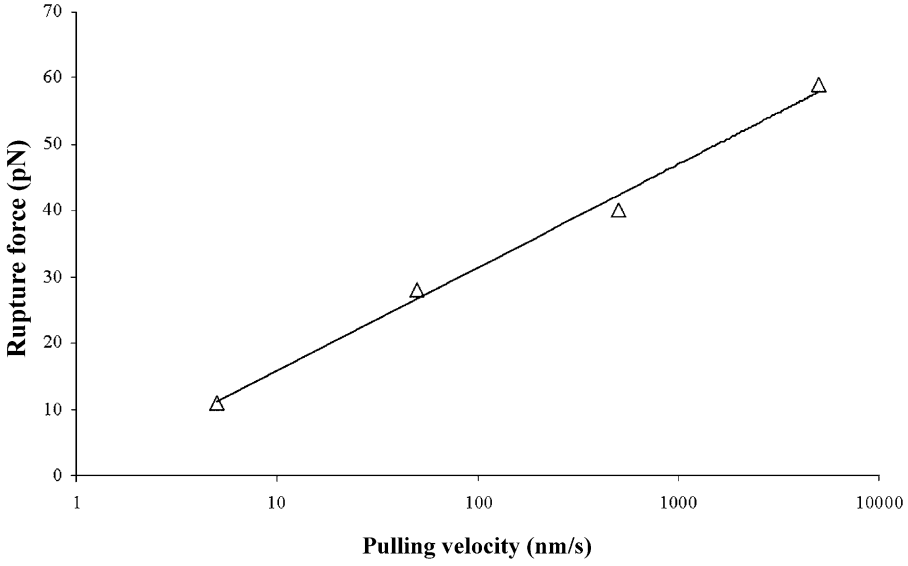

Fig. 3. The rupture force of a single bond between HA/HABP showed a logarithmic dependence on pulling velocity.

bonds. The rupture force of a single bond between biotin and streptavidin was reported as 257 pN [20], which is much higher than the rupture force we measured in this experiment. Therefore, it is reasonable to conclude that the bond ruptured during the experiment was the non-covalent bond between HA and HABP.

A smart version of Monte Carlo simulation was used to predict the rupture force of HA/HABP at the pulling velocity of 0.5 μm/s and was compared to the experimental data. A mean width of the HA/HABP bond of 4.1 nm was predicted by Monte Carlo simulation. This value is higher than published data of mean widths of other bonds [15,21]. Since the HABP includes two components – hyaluronan binding region of aggrecan and the link protein, both of which can bind to HA independently – the bond between HA/HABP may include two bonds – the bond of hyaluronan binding region of aggrecan to HA and the bond of link protein to HA. Therefore, the mean width of HA/HABP may include those of both bonds, and probably some distance between. The mean value and standard deviation of predicted data showed good agreement with the experimental data. However, there was some difference between the distributions of predicted rupture force and experimental data. There was no clear cut-off at $\sim$50 pN in the latter compared to predicted results. One explanation is that the experimental data included few multiple bonds. Although various measures were adopted to achieve single molecule binding, a small number of multiple bonds may still have formed. Those multiple bonds could be ruptured simultaneously, showing single peaks on the force–displacement curve with higher rupture force than that of single bonds [22].

Another important measure of the bond between HA and aggrecan, the dissociation constant (K_D), had been studied previously. For example, Watanabe et al. measured the K_D of the binding between HA and aggrecan as 2.26×10^{-7} M, and the K_D of the HA/link protein bond as 0.89×10^{-7} M [6]. The K_D is a measure of the equilibrium of the binding and dissociation of molecules due to spontaneous binding and dissociation. This is an important concept in the study of biological binding phenomena. The rupture force, as measured in this report, is a quite distinct measurement, which is not directly derivable from the K_D. It should be noted that the K_D itself does not reflect the rate of association and dissociation, but rather the equilibrium between these processes.

In this experiment, we identified that the rupture force of the HA/HABP bond increased significantly with the pulling velocity. The dependence of rupture force on pulling velocity had been reported in other non-covalent bonds previously, such as the bond between P-selectin/P-selectin glycoprotein ligand-1 [15] and that between biotin/avidin [23]. Previous literature in macromolecular biomechanics suggests that for non-covalent bonds in general, there are three regimes in the pulling velocity [24]. In the very slow loading regime, the intrinsic dissociation rate of the bond is much faster than the rate at which the force reaches any appreciable level, and the rupture force tends to zero. At the opposite extreme, the fast loading regime, the applied load reaches the maximum rupture force much faster than the bond dissociation rate. Therefore, the measured rupture force is not expected to further increase with the loading rate. In the intermediate regime, when the loading rate is comparable to the force-activated dissociation rate, the rupture force depends strongly on the loading rate. Our data suggest that for the bond between HA and HABP, at least, the pulling velocities of 0.05 μm/s and 0.5 μm/s fall within the intermediate regime. Previous studies of the rupture of non-covalent bonds [15,21,25] showed that the rupture force of the bond between P-selectin/P-selectin glycoprotein ligand-1, the bond of titin folding and that between integrin $\alpha_5\beta_1$/fibronectin were logarithmically dependent on pulling velocity. The rupture force of the bond between HA/HABP measured in this experiment was also in conformity with a logarithmic dependence on pulling velocity. However, since the energy landmarks of the unbinding of the streptavidin/biotin bond was characterized by the rupture forces of that bond at a wide range of pulling velocities over 7 orders of magnitudes [24], our results at four pulling velocities over three orders of magnitudes may not be enough to characterize the energy landmarks of the bond between HA/HABP. More testing at a wider range of pulling velocities, as a part of a larger investigation, may need to be done in the future.

In summary, we measured the rupture force of the single bond between HA/HABP – the critical intermolecular interaction that ensures the integrity in the proteoglycan aggregate in the extracellular matrix of articular cartilage – as 40 ± 11 pN at the pulling velocity of 0.5 μm/s using a nanomechanical testing system. The results showed a good fit to a theoretical prediction based on a smart version of the Monte Carlo simulation.

Acknowledgements: This study was supported by a grant from the Arthritis Foundation.

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