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Implications of heterogeneous bead behavior on cell mechanical properties measured with magnetic twisting cytometry

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Abstract

Magnetic twisting cytometry (MTC) measures cellular mechanical properties, such as cell stiffness and viscosity, by applying mechanical stress to specific cell surface receptors via ligand-coated ferromagnetic beads. MTC measures simultaneously the rotation of approximately 50,000 beads attached to 20,000 - 40,000 cells. Here we show direct evidence of heterogeneous bead behavior and examine its consequences in the interpretation of cell mechanical properties.

Keywords; Cell mechanics; Cytoskeleton; Cell stiffness

1. Introduction

Cells can respond to environmental stimuli by changing their mechanical properties. For example, muscle cells contract or relax, endothelial cells can become stiff or flexible, and neutrophils can change shape. The accurate measurement of the cell's mechanical state is thus paramount in understanding mechanotransduction and cytoskeletal structure and function.

Numerous techniques are currently used to probe the mechanical properties of single cells: micropipette aspiration [1,2] and cell poking [3,4] can be used to measure whole cell deformability, and laser tweezers [5] and atomic force microscopy [6] can be used to measure local distortion properties. A technique developed in our institution is magnetic twisting cytometry (MTC). MTC measures the mechanics of approximately 20,000–40,000 cells simultaneously, and probes mechanical properties at specific receptor sites. Briefly, MTC works by twisting ferromagnetic beads (4.5 μm diameter) in a magnetic field. These beads are coated with a ligand specific for the receptor site of interest. A magnetic torque is applied to the beads, and the bead rotation is measured with magnetic sensors. MTC has been successfully applied to show that external mechanical forces are transmitted across the cell surface and through the cytoskeleton via transmembrane cell adhesion molecules such as integrins [7–9], E-selectins [10], E-cadherins [11], and the urokinase receptors [12].

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MTC has also been used to quantitate cell mechanical properties [7,11,13]. It is inviting to employ cell mechanical properties obtained with MTC to model cytoskeletal structure, to calculate the transit time of neutrophils through capillaries, or to predict the deformation of endothelial cells under fluid shear stress. Such models and calculations, however, require reliable and accurate measurements of cell mechanical properties and a basic understanding of the meaning of these measurements.

The aim of this report is to investigate how details of bead attachment influence the stress experienced by the cells during magnetic twisting, and how bead attachment affects the measurement of cell mechanical properties. We used scanning electron microscopy (SEM) to study the attachment between beads and cultured cells and found that the nature of bead attachment is non-uniform. Based on these SEM findings we hypothesized that bead movement during magnetic twisting is heterogeneous. To test this hypothesis we applied repeated bead magnetizations during uninterrupted twisting and measured the decay of the remanent magnetic signal of the beads. This protocol demonstrated functional evidence of heterogeneous bead rotation. Consequently, heterogeneous bead behavior can confound estimates of cell mechanical properties.

2. Methods

2.1. *Cells*

Cultured bovine capillary endothelial cells (BCE) and human airway smooth muscle cells (HASM) were serum deprived for 2 days, trypsinized, and plated in defined medium on 96-well plates as described in Refs. [8,14].

2.2. *Magnetic twisting cytometry*

Detailed descriptions of the magnetic twisting cytometry technique have been published elsewhere [8,15]. In brief, ferromagnetic (Fe_3O_4) microbeads (4.5 μm diameter, specific magnetic saturation moment = 5 Am^2/kg) [16] were coated with a synthetic RGD peptide (Peptite 2000, Telios) at a concentration of 50 μg/ml. The RGD peptide is a specific ligand for integrin receptors [17]. The RGD-coated beads were dispersed in serum-free medium and added to each well at 20 μg per well (1-2 beads per cell) for 20 min. Unbound beads were washed away prior to MTC measurements.

During measurement, each cell well was placed into the magnetic twisting cytometer and kept at 37°C. A 100 μs 1000 G homogeneous magnetic pulse was then applied to magnetize the beads in the horizontal direction. A fluxgate magnetometer (Foerster, Reutlingen, Germany) was used to measure the remanent magnetic field of the beads in the horizontal direction (B_r). Values for B_r were typically on the order of 1 nT. To improve signal-to-noise ratio, the cell well was rotated around the vertical axis at 6.5 Hz, and B_r was determined by lock-in amplification. Also, the entire apparatus was shielded from external magnetic fields by four mu-metal cylinders that were closed on both ends (Amuneal, Philadelphia, USA).

A magnetic "twisting" field (< 100 G), H, is applied in the vertical direction to twist the beads upward. Cell mechanical properties can be derived from twisting torque and angular bead rotation.

2.3. *Determination of cell mechanical properties*

During twisting, the magnitude of the specific torque T_{mag} (that is torque per unit bead volume) applied to a bead by the magnetic field is

$$T_{mag} = cH \cos \alpha, \quad (1)$$

where $\pi/2 - \alpha$ is the angle of the bead's magnetic moment relative to the twisting field (this choice of a means that for an initial angle of $\alpha = 0$, the final angle a also represents the angular rotation, or strain). c is the "bead constant", expressed as torque per unit bead volume per Gauss. c is determined, as described in Ref. [15], by placing beads in a fluid of known viscosity, and measuring the angular velocity while twisting. The bead's specific magnetic saturation moment of 5 Am^2/kg thus results in a c of 4.1 $dyn/cm^2/G$.

In principle, when a bead is placed in an infinite elastic material and rotated, the material exerts

a torque T_{material} on the bead, of magnitude

$$T_{material} = G\alpha, \tag{2}$$

where G is the shear modulus. At dynamic equilibrium, the net torque on the bead is zero. In our case the magnetic and material torque vectors point in opposite directions. It follows that their magnitudes are equal: $T_{\text{mag}} = T_{\text{material}}$.

In practice, however, there are at least two confounding factors. First, T_{mag} can substantially deviate from Eq. (1) if beads are not far away from each other, but are clustered, since then their magnetic fields interact. Second, if the material is finite, inhomogeneous, anisotropic, nonlinear, or not purely elastic, then the relationship between T_{material} and a is no longer given by the simple Eq. (2). In particular, using Eq. (2) together with measurements of T_{material} and a to estimate the shear modulus G will be in error.

For a single bead or a homogeneous bead population, α can be calculated as

$$\alpha = \cos^{-1}\frac{B_r(t)}{B_0}, \tag{3}$$

where B_0 is the bead's remanent magnetic field in the horizontal direction immediately after magnetization, and $B_r(t)$ is the bead's remanent magnetic field in the horizontal direction during twist (Fig. 1). If beads behave heterogeneously (either due to heterogeneous bead properties or heterogeneous material properties), α is distributed among the beads. In that case, Eq. (3) overestimates the mean angular rotation because

$$\cos^{-1}\frac{\bar{B}_r(t)}{\bar{B}_0} \geqslant \bar{\alpha}. \tag{4}$$

To show functional evidence of heterogeneous bead behavior, we measured B_r during continuous twisting with repetitive bead magnetization. B_0 is determined during the first 20 s after the first magnetization.

After remagnetization we would expect the remanent field to reset to B_0 and continue with the same or slightly higher slope vs. time it had immediately before remagnetization, but only if all beads behaved homogeneously. However, if there were two (or more) populations of beads, then we would expect the remanent field first to reset to B_0, then to drop rapidly, followed by a more gradual decay. The rapid signal drop would correspond to loosely bound beads quickly rotating to 90°, and the subsequent gradual decay in signal would correspond to tightly bound beads rotating more slowly because their rotation is impeded by cellular attachments.

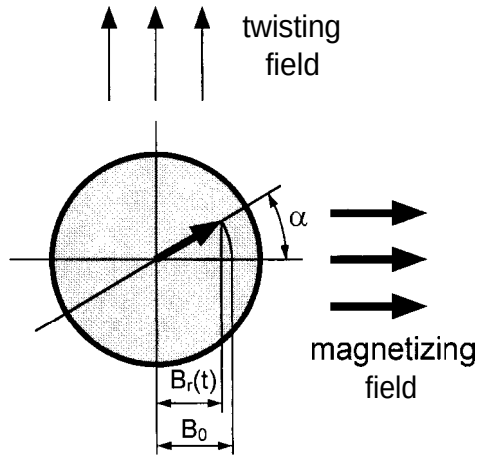

Fig. 1. Schematic diagram of a bead, magnetized in the horizontal direction, and twisted in the vertical direction, showing the total remanent field of the bead (B_0), the bead's remanent field in the horizontal direction ($B_r(t)$), and the angular rotation α.

2.4. *Determination of bead attachment*

We used scanning electron microscopy (SEM) (Amray 1000A, USA) equipped with X-ray spectroscopy (EDX) (Kevex μX7000, USA) to evaluate the distribution of contact area between beads and cultured cells. The iron content of each bead was assessed by EDX to exclude round, non-magnetic particles from our analysis. After cell mechanical properties were measured with MTC (between 30 and 60 min after adding the beads), cells were fixed in the cell wells with glutaraldehyde, dehydrated with alcohol, and critical point dried. The bottom of the cell wells were cut off with a hot tungsten wire, and cells were sputter coated with gold-palladium.

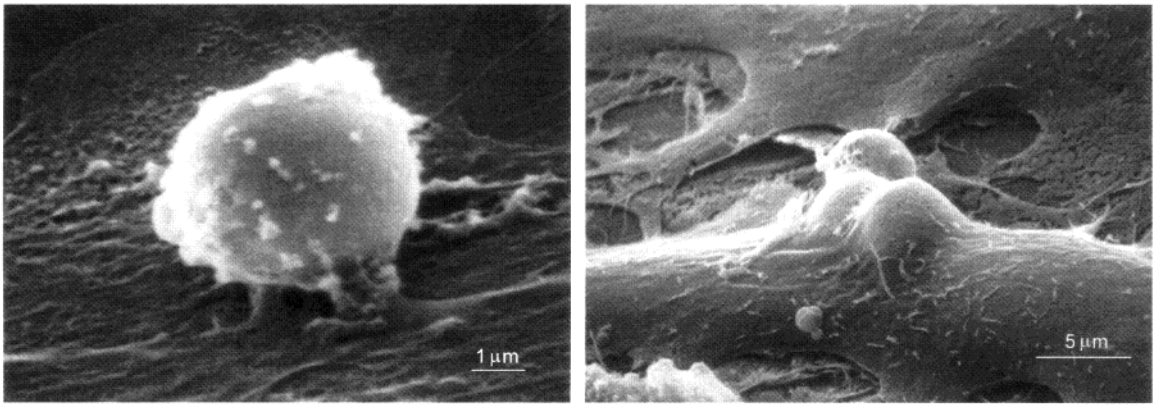

Fig. 2. SEM image of RGD-coated Fe_3O_4 beads on HASM cells. Left: non-internalized bead, right: beads embedded in cells.

3. Results

3.1. Scanning EM

The scanning EM of HASM- and BCE-cells revealed internalized and non-internalized beads (Fig. 2). Typically, 50% of the beads attached to BCE cells and 20% of the beads attached to HASM cells are internalized. In addition, in BCE cells we found 15% of the beads attached to the cell membrane not via specific receptor–ligand binding but indirectly via an extracellular matrix protein network. We found no such indirect bead attachment in HASM cells.

3.2. MTC

During twisting, the apparent rotation of the beads on BCE cells was at first rapid then more gradual (Fig. 3 left). We then remagnetized the beads perpendicular to the continuously applied twisting field. This reset the ratio $B_r(t)/B_0$ to 1 and restored the torque acting on the beads to its initial value. The time courses of B_r after each remagnetization remained unchanged: a rapid drop of about 20% of B_0, which then proceeded more gradually (Fig. 3 right).

We obtained different results in HASM cells with a similar protocol of repeated bead magnetization

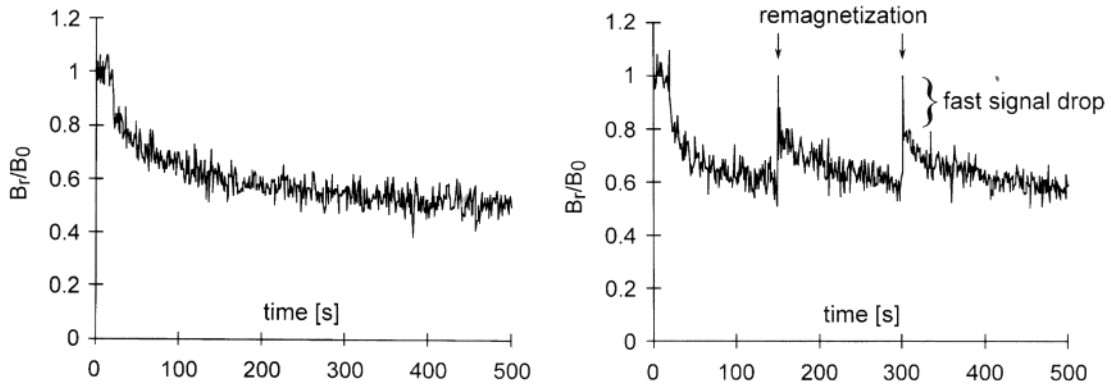

Fig. 3. BCE cells during continuous 20 G twist with initial magnetization (left) and repetitive magnetization (right). (Signal during magnetization cannot be measured and is set to 1.)

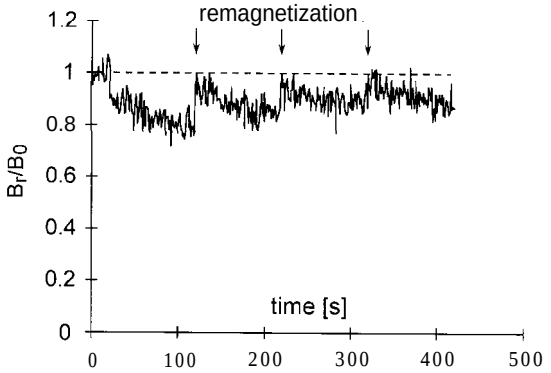

Fig. 4. HASM cells during continuous 25 G twist with repetitive magnetization.

(Fig. 4). Here, however, a fast signal drop was only apparent with the first twist. Subsequent bead remagnetization restored the B_r signal to 95% of its initial value.

4. Discussion

Scanning EMs have revealed considerable inhomogeneity of bead attachment. We found a coexistence of internalized and non-internalized beads. Moreover, in BCE cell cultures we found that about 15% of the beads are only indirectly attached to the cells. We hypothesized that beads with small contact area or indirect attachment experience less elastic recoil when twisted than internalized beads or beads with larger contact area. We tested this hypothesis by applying a constant twisting field over several minutes during which we repeatedly remagnetized the beads. If beads behaved homogeneously, we would expect no rapid drop in the remanent signal following remagnetization. In BCE cells, however, we measured a fast signal drop of 20% of B_0 even after several bead remagnetizations (Fig. 3 right). We believe that the fast signal drop is due to rapid bead rotation that cannot be explained by a single bead population. Instead, by assuming a distribution of two bead populations, weakly and strongly bound beads, 20% of the signal can be attributed to weakly bound beads that are virtually free to rotate. In HASM cells, by contrast, we measured a fast signal drop after remagnetization of only 5% of B_0. This result is in agreement with our findings with scanning EM; no indirectly attached beads were found in HASM cells.

Heterogeneous bead behavior has profound consequences to the interpretation of cell stiffness. Beads cannot rotate further than 90" unless they are remagnetized, because at this angle the magnetic torque has fallen to zero. At low twisting fields, weakly bound or free beads may rotate maximally, whereas more strongly bound beads can still rotate further when subjected to higher twisting fields. Thus, the cells appear to be stiffer when subjected to progressively higher twisting fields. Such a "stiffening response" has been reported and interpreted as evidence for underlying "tensegrity architecture" of the cytoskeleton [7,8]. However, here we propose an alternate explanation for the stiffening response observed in MTC. The higher inhomogeneity of bead attachment in BCE cells compared to HASM cells suggest a more pronounced stiffening response in BCE cells. This is indeed the case: We found that the stiffness of BCE cells increased by 130% when the twisting field is increased from 10 to 30 G, whereas the stiffness of HASM cell increased by only 40%. We thus conclude that part of the stiffening response may be due to bead inhomogeneity.

Bead inhomogeneity also compromises our method to compute the mean angular bead rotation using Eq. (3). The $\cos^{-1}$ function puts higher weight on weakly bound beads with a larger rotation, thus overestimating the mean angular bead rotation and underestimating the mean shear modulus or cell stiffness as calculated with Eq. (2). Consequently, values for cell stiffness in the order of $10–100\,\mathrm{dyn/cm^2}$ as previously reported with MTC would be higher if free or weakly bound beads were excluded.

To illustrate the influence of bead inhomogeneity on stiffness values and stiffening response, we modeled two bead populations – unbound beads that can rotate freely, and beads bound to cells that have a constant shear modulus of $200\,\mathrm{dyn/cm^2}$. Fig. 5 shows the apparent stiffness as calculated with Eq. (2) that we would obtain with MTC measurements. The fraction of unbound beads was changed between 1% and 20%. Fig. 5

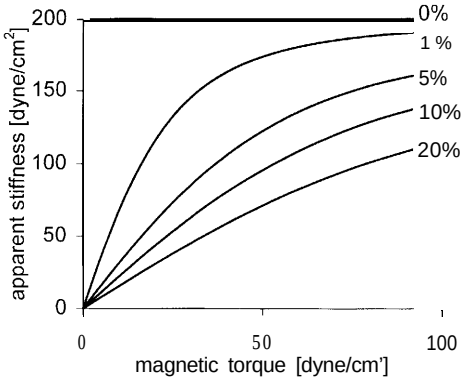

Fig. 5. Apparent stiffness vs. stress as measured with MTC at different fractions of unbound beads. Cell stiffness is assumed to be 200 dyn/cm'

demonstrates that a fraction of unbound beads as low as 1% can severely compromise cell stiffness measurements at low magnetic torque, but with increasing torque apparent stiffness approximates cell stiffness.

We conclude that the apparent shear modulus obtained by MTC may underestimate the absolute shear modulus, but these underestimates are minimized when the magnetic torque is greater. Also, measurements of relative changes in shear modulus in a given preparation are likely to be accurate.

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