Journal of the Korean Physical Society, Vol. 50, No. 1, January 2007, pp. 313∼319

Direct Measurement of the Force Generated by a Single Macrophage

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(Received 1 September 2006)

During immune responses, macrophages play important roles in phagocytosis and antigen presentation by engulfing pathogenic micro-organisms and cell debris. Since the function of a macrophage highly depends on a series of physical steps including migration, direct contact and strong binding to its target, deployment of cytoplasm and membrane, and intake of the target, here we have investigated the mechanical behavior of a macrophage by manipulating it with a flexible pipette that was used as a force sensor and transducer. We examined the response of a macrophage to mechanical pulling by a positively charged pipette. We observed that the macrophage initially formed strong binding to the pipette and migrated along the direction of pulling for some early period. After that period, the macrophage exerted a huge traction force to pull the pipette back and attempted to retract itself towards its original location. We found that whether it was able to return to the original location depended on the level of applied force. Since the traction force generated by a single macrophage had not been characterized accurately, we measured the force for the first time to our knowledge and found the maximum traction force to be around 80 nN. This quantitative measurement was made possible by a new and convenient method used to calibrate the stiffness of the pipette. Through the study, we acquired a better understanding of the mechanics of and the force generation by a macrophage.

PACS numbers: 87.17.Jj, 87.80.Fe, 87.16.Qp
Keywords: Macrophage, Single cell biophysics, Force generation, Micromanipulation

I. INTRODUCTION

The cell is the basic unit of life. Most remarkable events such as reproduction, homeostasis, metastasis, and movement, which define life, happen in the cell. A single cell that contains all the genetic information can be a life form itself as are prokaryotes and protozoa. Whether a cell is a part of a bigger living organism or an independent life form, its mechanical properties are important for its proper functions because almost all cellular phenomena are cascades of many physical and mechanical processes executed by a multitude of intracellular and extracellular components.

Since an understanding of the mechanical properties of a cell is pivotal for understanding the mechanism of many cellular processes and cell behaviors, a broad range of research works have been conducted [1–8]. First, considering a cell as a lifeless structure, the deformability of various types of cells (red blood cells, leukocytes (white blood cell), myeloid cells, keratocytes, myoblasts, macrophages, *etc.*) has been characterized [2–7]. By these studies, mammalian cells behave as a very soft material with a typical elastic modulus of 0.5 – 50 kPa. However, a yeast cell is 3 – 4 orders of magnitude more rigid with an elastic modulus of about 100 MPa [9]. A modified method suitable to measuring the deformability of non-adherent cells, such as human leukocytes, was developed [5]. In some works, a very large force was applied to a cell in order to break the cell apart and measure the rupture force of the cell [7, 9]. The elas-

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tic properties of those cells were determined by both the cell membrane and the cytoskeleton underneath it. The contribution from each was also estimated in a number of experiments [10,11]. The deformability of a cell provides important knowledge about the shape of the cell and the cell's response to external mechanical stimuli, such as tension and pressure exerted by other neighboring cells. Second, the viscoelastic properties of cells have been characterized by a number of methods [12,13]. The viscoelastic properties of a whole cell are related to the overall mechanical properties of the cell, such as the deformability. The viscoelastic property of the cytoplasm has crucial effects on how intracellular components function inside the cell. It will, for example, affect how enzymes, many building blocks, and metabolites move and diffuse inside the cell. In order to directly measure the viscoelastic properties of a cell, the Brownian motions of a free particle were observed, and in a more active approach, micromanipulation methods using magnetic particles were utilized to manipulate a small particle in the cytoplasm and observe how it behaved [14, 15]. Third, forces are not only mere external stimuli to measure the mechanical properties of a cell, but are switches that induce biologically important phenomena through so-called mechano-transduction. Upon force stimuli, the cell actually modulates the pattern of gene expression [16,17] and modifies its structure, shape, and mechanical properties [2,18–20]. When a stem cell is under pressure, it is known to actually diversify into an epithelial cell. Otherwise, it diversifies into a neuronal cell [21]. Last, mechanical force influences the motility and the migration of a cell by inducing or suppressing growth of the lamellipodium of a cell [20]. This force implies the level of the force involved in movement by pseudopodia. All these experiments provide a mechanical framework with which we can understand the behavior and the function of cells in a more mechanistic point of view.

Among the many types of cells, macrophages play important roles in the removal of pathogens and antigen presentation by engulfing pathogenic micro-organisms and cell debris. Engulfing and ingestion of pathogens and cell debris into a vacuole by a macrophage is called phagocytosis. Some types of macrophages are mobile in order to migrate towards targets. Because the function of a macrophage depends highly on a series of physical steps, including migration, direct contact and strong binding to its target, deployment of the cytoplasm and the membrane, and the intake of the target, and because the activities of a macrophage are diverse and heterogeneous, it is important to study its mechanical behavior at the single-cell level. In our study, we characterized the mechanical properties (the generation of a traction force, the response to an external force, *etc.*) of a single macrophage, which are directly related to migration and phagocytosis of a macrophage. Especially, the traction force by a macrophage has not been characterized precisely, and here we obtained the quantitative values for the force. In order to obtain quantitative results for force generation, we calibrated our force sensor, a flexible tapered pipette by using an atomic force microscope (AFM) cantilever with a known spring constant. With a calibrated pipette, we measured the traction force generated by a macrophage precisely. Through the study, we acquired a better understanding of the mechanics of and the force generation by a macrophage.

II. MATERIALS AND METHODS

1. Preparation of Macrophage Samples

A macrophage cell line (RAW 264.7) was chosen as a model system to test the mechanical properties of a macrophage and its behavior under an external force. A macrophage stock stored in liquid nitrogen was transferred to a Dulbecco's Modified Eagle Medium (Gibco) kept at 37 °C, which contained 10 % FBS (fetal bovine serum) and 1× antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin). 5 % CO_2 was introduced into the incubator to maintain a proper pH in the medium. After one day, the cells were washed with phosphate-buffered saline and counted by using a hemato-cytometer. 2×10^4 cells were then transferred to a 65-mm dish and incubated one more day under the same conditions. The sample was then ready to be used.

2. Preparation of the Pipette Force Transducer

In order to exert a mechanical force on a macrophage and measure the force generated by it with a flexible pipette, a thin-walled hollow pipette (Sutter, BF150-117-10) was pulled with a Sutter P-97 pipette puller. The flexibility of the tapered pipette was adjusted to the desired range by varying the pulling conditions. The tip of the pipette was melted to form a small blob with a diameter around $6 \sim 10$ μm. This round blob was the target object on which the macrophage exerted a force.

Because the cell membrane takes a negative charge, the interaction between the pipette and a macrophage is facilitated when the pipette is positively charged. Therefore, we coated the pipette with a polymer material bearing positive charge, APTES (3-aminopropyltriethoxysilane). The detailed protocol of coating APTES on a glass pipette is shown elsewhere [22]. Here, we describe the essential steps. Because the cleanness of the pipette surface is critical for the quality of the coating, the pipette surface was treated with ozone plasma three times. Then, the pipette was immersed in 1 % APTES solution in ethanol and incubated for 5 hours to crosslink APTES to the glass. After having been rinsed with deionized water, the pipette was baked in an oven at 110 °C for two hours. After sonication in toluene for 5 minutes, it was further rinsed with

deionized water 20 times and was ready to be used for our experiments.

3. Setup for Force Calibration and Measurements

The experiments were conducted with a phase-contrast inverted microscope (Olympus, IX71) to visualize clearly the shapes and the boundaries of the cells. In order to manipulate a cell by using a flexible pipette, we mounted it on an XYZ manipulator (Narishige, MHW-3), whose minimum graduation in position was 0.2 μm. Images were taken with a back-illuminated electron multiplying CCD (EMCCD: Andor, IXON DV887-BV). For recording slow processes, we used a repetition rate of 1 Hz and a typical exposure time of 50 msec. The images recorded were then analyzed by using a C program (written by B. J.) to determine the position of the pipette (and the cell).

4. Force Calibration

In order to measure the force quantitatively, we calibrated the spring constant of the pipette. The spring constant of the flexible pipette is defined as the ratio of the force exerted to the tip of the pipette to its displacement. The displacement of the tip is defined as the distance by which the tip is bent from its relaxed position. For calibration, a soft cantilever (BS-SiNi, BudgetSensors, Bulgaria; gift from PucoTech, Inc, Korea), whose spring constant was known to be 0.06 N/m, was used as a reference. By pushing the pipette against a fixed cantilever or pulling the former from the latter when they were stuck, the spring constant of the pipette could be estimated based on that of the cantilever. For details, see below in Results.

5. Measurement of the Force by the Macrophage

Although the characters of individual macrophages may differ markedly, the shape and the size of a macrophage can still be useful criteria to characterize its condition and by which to sort it. To reduce the heterogeneity of the macrophages studied in our experiments, we selected cells that met the following criteria: First, cells had to be activated; in other words, capable of conducting their functions in immune responses. Activated macrophages (Figs. 1 (b) [ii,iii]) look significantly different from their inactivated and round counterparts (Fig. 1 (b) [i]). Second, among activated cells, ones with elongated, dipolar shapes (Fig. 1 (b) [ii]: a typical aspect

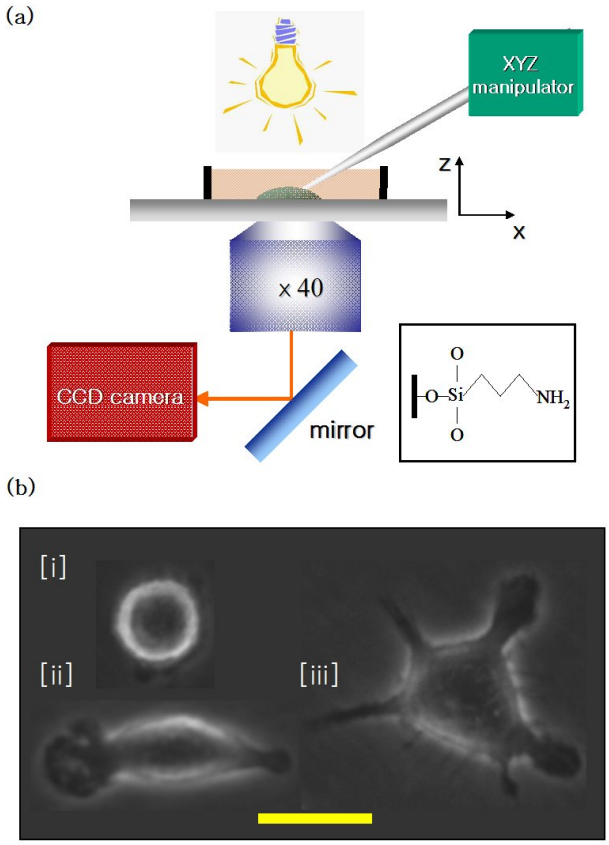

Fig. 1. (a) Schematic diagram of the experimental setup. A Petri dish holding the sample (pink) is placed over an inverted optical microscope. A tapered pipette mounted on a XYZ micromanipulation stage is used to manipulate a macrophage (green crescent) in 3D. Light from the lamp illuminates the sample dish, and images are collected by a CCD camera. The inset shows the chemical structure of the APTES and its attachment to the pipette surface. (b) Typical shapes and sizes of macrophages in different categories: [i] inactivated, [ii] activated, dipolar, and [iii] activated, star-shaped (Scale bar: 20 μm).

ratio of the cell body is 2 : 1), which are the majority, were selected rather than expanded star-shaped ones (Fig. 1 (b) [iii]). Last, the horizontal and the vertical dimensions of the cell bodies used for our studies were approximately 36 μm and 18 μm, respectively, within 30 %. The size of a cell was used as an indicator of the age and the maturity of a cell.

After macrophages had been spread on the dish at an optimal areal density (several cells per each viewing field of 128 μm × 128 μm), the sample dish was scanned to find the ones suitable for the study based on the criteria described above. The macrophages to be studied had to be, at least initially, well separated from others and judged to be healthy by their shape and size. Once a macrophage was selected, it was approached by a flexible pipette and touched by the pipette's blob. After the macrophage and the pipette became attached to each

other, the pipette was retracted from the macrophage. It was obvious from video images that only one cell was bound to the pipette. Under phase contrast microscopy, the boundary of the macrophage that wrapped the tip of the pipette was visible. In addition, the pipette approached the Petri dish at a large angle (∼30°) so that no other parts of the pipette except the tip or blob would be within reach by cells on the dish during the experiments. The response of the macrophage was monitored and the positions of both were recorded throughout the experiments. The recording was interrupted every 1000 sec and 3000 sec prior to and during the pipette retraction, respectively. At the short-interval interruptions, the macrophage was checked for its binding and phagocytic inclination to the target. At the long-interval ones, the video images were saved due to memory limit. Images were then analyzed after collecting all the data. Because the blob of the pipette, where the pipette contacted the macrophage, appeared conspicuously bright in image, it was conveniently tracked to obtain the positional information on them. The details on how to analyze the data are also described in Results.

III. RESULTS

1. Calibration of the Spring Constant of the Pipettes

In order to determine the force generated by a macrophage quantitatively, we calibrated a flexible tapered pipette by using an AFM cantilever with a known spring constant. The cantilever was laid to the side on a 35-mm Petri dish and fixed firmly to the dish with epoxy (as shown in Fig. 2 (a)). The tapered pipette to be calibrated approached at the same height as that of the cantilever so that the blob of the pipette met and contacted the tip of the cantilever as shown in Figs. 2 (a)-[I-III]. The head of the pipette was pushed against the tip of the cantilever, and both the pipette and the cantilever were bent with large radii of curvature. The ratio of their horizontal displacements yields that of their spring constants. That is

$$k_{pipette} = k_{cantilever} \times d_c/d_p = k_{cantilever} \times r_c/r_p,$$

where d_c and d_p are the displacements of the cantilever and the pipette, respectively and r_c and r_p the slopes of the time-displacement curves for the cantilever and the pipette, respectively (Fig. 2 (b)). At constant rate of displacement, the ratio of the slopes is the same as that of the displacements.

With the spring constant of the cantilever known, that of the pipette can be determined. The value of the spring constant may differ from pipette to pipette because the pulling process by the puller is not completely controllable. Therefore, in our experiments, we calibrated every pipette after measurement, the reason being that the surface charge of the pipette may be altered by touching the cantilever. The calibration was conducted either by pushing them against each other or by pulling them apart if they stuck to each other because of electrostatic interaction. Fig. 2 (b) shows the displacements of the pipette and the cantilever as a function of time. The slope of the cantilever displacement is 0.065 μm/sec and that of the pipette displacement 0.88 μm/sec. The data indicate that the spring constant of the pipette is 4.5 nN/μm. In order to check how variable the spring constant of the pipette pulled in an identical manner was, we calculated the mean and the error of the spring constants of 3 different pipettes. From the result, the spring constant of pipette was 5.2 ± 0.6 nN/μm.

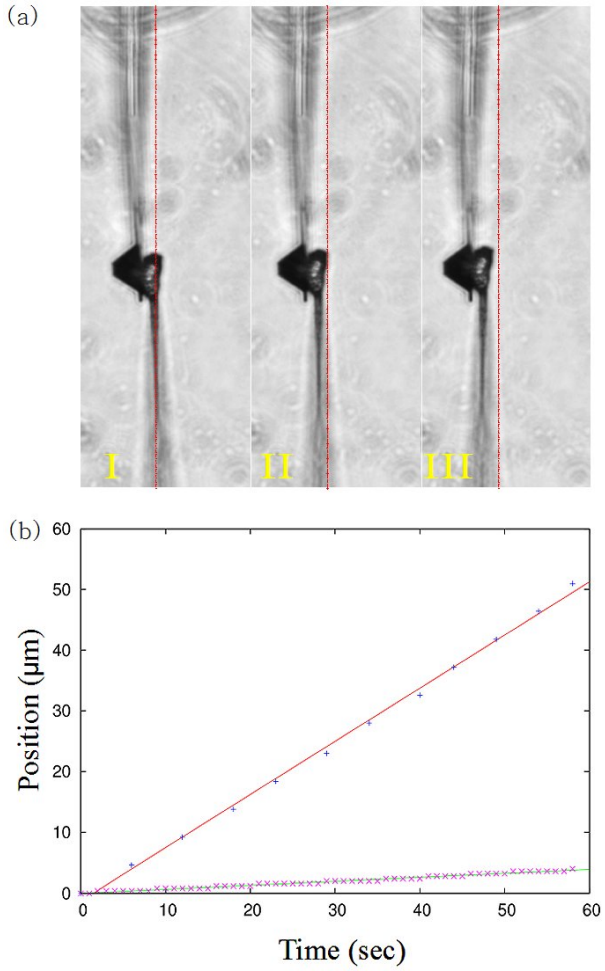

Fig. 2. (a) Three sequential images (I, II, and III) during calibration. Shown as a rod with a solid trapezoid and a rod with an oval blob in each frame are the cantilever (upper left) and the pipette (lower right), respectively. The pipette pushes the cantilever to the left. The dotted lines indicate the initial location of the pipette. (b) The pipette and the cantilever push each other at constant rate. The data are shown in time vs. displacements. The ratio of the slopes of the two lines yields that of the spring constants of the two force sensors.

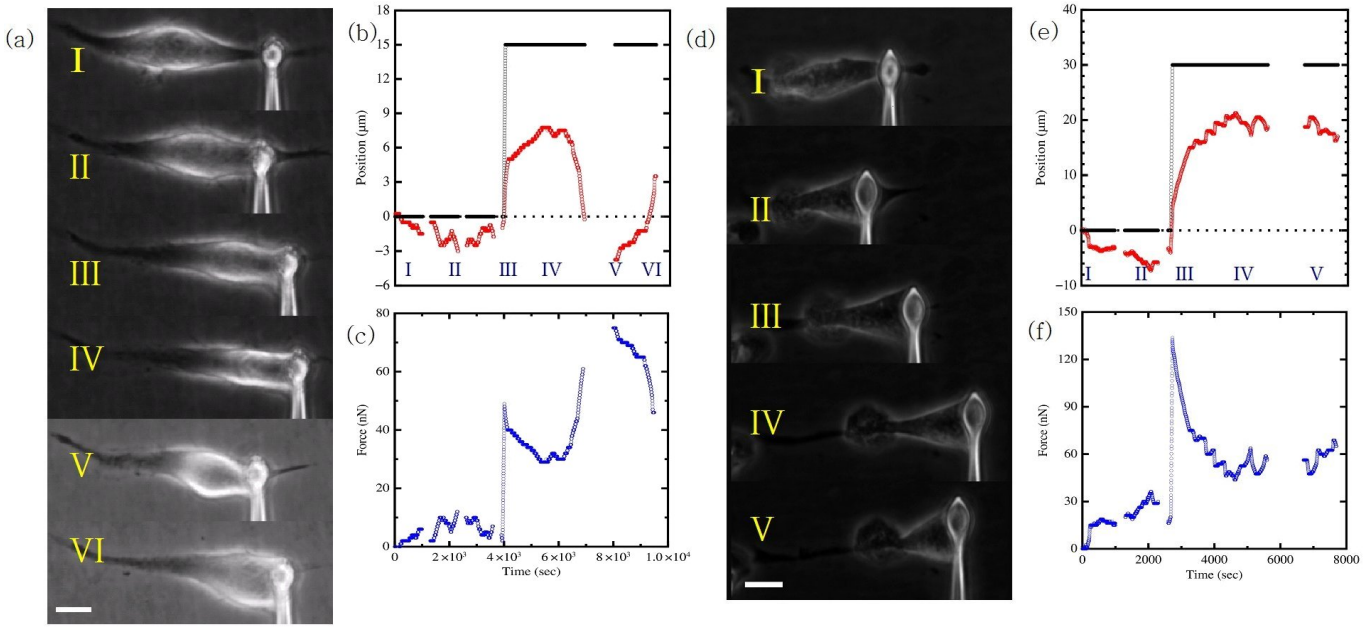

Fig. 3. (a) Six sequential images (I to VI) showing the positions of the pipette and the macrophage. Each image corresponds to the time point labeled with the same Roman letters in Fig. 3 (b). The long bulge-shaped body aligned horizontally in each image is a macrophage and the narrow match-shaped rod aligned vertically is a pipette. (b) "Position" of the pipette stem by translation (black) and that of the blob (*i.e.*, the contact point of the macrophage (red)): [I] initial touch between the macrophage and the pipette; [II] as the macrophage approached and then embraced the pipette, the pipette was pulled toward the macrophage; [III] as soon as the pipette was wrapped, it was translated by 15 μm and the macrophage was dragged quickly, but the actual movement was less than the total translation; [IV] the macrophage slowly dragged along the pulling; [V] the macrophage exerted a huge traction force and moved to the left; [VI] after the impulsive force, the macrophage moved back to the right. (c) The force exerted to the macrophage. (d) Five sequential images (I to V) showing the positions of the pipette and the macrophage. Each image corresponds to the time point labeled with the same Roman letters in Fig. 3 (e). (e) Same convention as (b): [I] same as (b)-[I], [II] same as (b)-[II], [III] same as (b)-[III] except that the total translation is 30 μm, [IV] in the force-balanced equilibrium. (f) Same as (c).

2. The Behavior of a Macrophage under a Pulling Force

When a positively charged pipette was in contact with a macrophage, the macrophage always grabbed the pipette firmly and established a strong attachment to it. As indicated in Figs. 3 (b) and (e) by negative values of the position of the blob prior to pulling, the macrophage tended to attract the pipette right after binding to it. The macrophage nearly encompassed the blob, as revealed in images in Fig. 3 (a) and (d). This suggests that the macrophage sensed the positively charged blob as a target for phagocytosis and aimed at engulfing it. The round blob, however, failed to be fully phagocytosed because it was connected to the pipette via the rather thick stem. In order to examine how a macrophage responded to an external force, we instantly exerted a pulling force to the macrophage by translating the pipette horizontally and away from it by either 15 μm or 30 μm. At the moment of the translation, the pipette remained attached to the macrophage with the head of the pipette being bent. From Hooke's law, the total initial force is $k_{pipette}\ d_p$ (in general, d_p = the translation distance of the pipette (black in Fig. 3 (b)) - the position of the macrophage (red in Fig. 3 (b))). Depending on the initial translation distance, the response by a macrophage was somewhat different.

When the initial translation distance by the pipette was 15 μm, the macrophage moved quickly by about 5 μm along the direction of pulling, but its movement markedly slowed down as the applied force decreased as a result of the reduced displacement of the pipette. The forces by the pipette and the macrophage were then balanced briefly and during such a stable period, the macrophage only took a few sporadic backward or forward steps. After the stable period, the macrophage retracted itself in the direction opposite to the pulling and generated a large amount of traction force. As shown in Fig. 3 (b), the retraction happened rapidly. Consequently, the pipette was bent by more than the total translation distance, 15 μm. The maximum force exerted by the macrophage was then bigger than the initial pulling force by the pipette. We repeated the same measurements on five independent cells and observed similar results.

When the initial translation distance by the pipette

was 30 μm, the macrophage moved quickly by 8 – 9 μm and then more slowly by 10 μm along the pulling direction. After that, it moved slowly in steps as the applied force decreased. Again, the forces by the pipette and the macrophage were balanced, but it remained there for a more extended period, showing some backward or forward steps. Since the initial pulling force here was nearly twice as much as the previous case, the macrophage was never able to move significantly backward from the equilibrium point, and a prolonged equilibrium was established after the macrophage walked by 20 μm from the initial position. We repeated the same measurements on four independent cells and observed similar results. Again, we estimated the maximum force exerted by the macrophage from this tug-of-war state.

3. Measurement of the Traction Force Generated by a Macrophage

Shown in Fig. 3 (c) is the force exerted to the macrophage when the initial translation distance of the pipette was 15 μm. This result was obtained simply by multiplying the displacement of the pipette, d_p, by the spring constant of the pipette, $k_{pipette}$. The forward movement of the macrophage slowed down significantly when the applied force was reduced to 40 nN, which implies that this level of force was readily resistible by a macrophage. Interestingly and remarkably, the macrophage boosted its traction force up to ∼80 nN after the stable period had spanned from 5000 to 6500 sec. However, the maximum force observed around 8000 sec could not be sustained long, and the force dropped gradually towards the equilibrium value, indicating the impulsive nature of force generation.

Shown in Fig. 3 (f) is the force exerted to the macrophage when the initial translation distance was 30 μm. The forward movement of the macrophage slowed down significantly when the applied force was reduced to about 80 nN, which implies that this level of force was resistible by the macrophage. As mentioned before, due to higher level of the applied force, the macrophage remained in prolonged equilibrium. The maximum force exerted by the macrophage from the tug-of-war was ∼60 nN.

IV. DISCUSSIONS AND CONCLUSIONS

In this study, we investigated the responses of a macrophage to an external force and directly measured the traction force generated by the macrophage. In order to measure quantitatively the force generated by the macrophage, we introduced an easy one-step calibration method that utilized an AFM cantilever with a known spring constant to determine the stiffness of the pipette. A strong attachment between a macrophage and a "target" pipette was established because the pipette coated with the positively charged APTES interacted strongly with the macrophage via the electrostatic attraction. Presumably due to such an attractive interaction, the macrophage in contact with the pipette spontaneously pulled it to engulf it. When the macrophage was pulled with two different levels of force, 50 nN and 130 nN, it initially followed the pipette until the pulling force dropped to 40 nN and 80 nN, respectively. In the case with the lower pulling force, it was able to retract all the way to the original location by exerting forces up to 80 nN after a short stable period. In the case with the higher pulling force, however, it just remained in force-balanced equilibrium by generating about 60 nN of force steadily. The relation between the initial pulling force and the traction force needs to be investigated more thoroughly in the future.

From these measurements, we learned that the macrophage could exert forces as high as ∼80 nN spontaneously. Cells with weaker anchorage or smaller size might generate less forces (50 ∼ 70 nN), but even in such cases, their responses would still be very similar in a qualitative manner. Because the substrate on which the macrophage was anchored was firm and rigid and it maintained a rear anchoring position even when it moved back and forth, the force was entirely generated by the macrophage and was not limited by the softness of the substrate [23].

In addition, we learned how a cell responded to an external force. As seen here, a cell may be stretched and expanded along the direction of pulling when a strong force is suddenly applied to it. Whether a cell was pulled at 50 nN or 130 nN, the initial responses by the cell were similar. If a cell was pulled by surprise, it could not effectively resist and just was dragged along almost helplessly. However, it entered a stable tug-of-war period soon once the applied force dropped to 40 – 50 nN. This level of force seemed to be resistible from the cell's own mechanical capability. As for the 15-mm translation, the stable period might be an important preparation stage for generating a huge traction force. During this period, the macrophage might synthesize and deploy the extra cytoskeleton elements (*i.e.*, actins and microtubules) and motor proteins (*i.e.*, myosins and dyneins) necessary to alter the structure and to create force. It would be interesting in the future to see what happens inside the cell while a cell stimulated mechanically reveals the aforementioned responses and creates a huge traction force.

There are several things worth mentioning. Although the precisions of our method for the force and the position measurements may be limited by the resolution of optical microscopy because measurements of the two quantities are mainly based on an analysis of optical images, our experimental geometry and technique are advantageous over more common AFM-based techniques in the following aspects. First, it is easy and cheap to implement such a setup in most biology and physics labs.

All that are needed are basically an optical microscope, a XYZ micromanipulator, and a CCD camera. Second, with the technique, we could exert a force to cells horizontally while visualizing them, and we could study how the lateral force affected the cell's behavior. Third, we could not only measure the elastic or viscoelastic properties of the cell but also monitor its migration or motility. Utilizing these advantages, we could directly relate an external mechanical stimulus to the cell's movement.

In summary, we showed how to study the mechanical properties of a cell and to determine the physical quantities involved in the mechanics of the cell, such as the traction force. It would be very interesting to see the method shown here widely used to investigate the physical aspects of cell and to connect the physical properties of a cell to biological pathways inside the cell.

ACKNOWLEDGMENTS

This work was supported by grant No. R01-2005-000-10477-0 from the Basic Research Program of the Korea Science and Engineering Foundation, by a Korea Research Foundation grant funded by Korea Government (MOEHRD, Basic Research Promotion Fund, KRF-2005-070-C00054) and by the Seoul R&DB Program. This work was also partially supported by the Ministry of Health (D. J. A.).

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