Mechanical response of single myoblasts to various stretching patterns visualized by scanning probe microscopy*

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Summary. The mechanical memory effect of single cells was reported in our recent study. In order to clarify this effect, various sequential stimuli of uniaxial deformation were applied to cells by deformable culture dishes and a deformation device, and the local stiffness distribution of single C2C12 myoblasts was visualized by scanning probe microscopy. Following a single step stretching, cellular stiffness first increased steeply and then gradually decreased for two hours. By a single step stretching 30 min after a long pulse-like deformation with a pulse duration of 30 min, the cells responded in the same way. On the other hand, they did not respond to a single step stretching 30 min after a short pulse-like deformation with a pulse duration of 0.5 min. These results indicated that cellular mechanical response to external deformation is affected strongly by a preceding deformation and that the duration time of the preceding deformation is an important factor in the change in mechanical response. We consider that the change in mechanical response contributes to a regulatory mechanism of cellular contractile force.

Introduction

Cells or tissues in humans are always exposed to mechanical stimuli that strongly affect their formation or function. Repetitive loading causes the hypertrophy of muscle cells, weight bearing induces bone mineralization and cyclic compression promotes a matrix synthesis in cartilage (Smith et al., 2000; Powel et al., 2002; Banes et al., 2003; Turner, 2006). Single cells are not only exposed passively to external stimuli but also respond actively through cellular contractile forces which are generated by the stress fiber network consisting of filamentous actin and myosin beneath the cellular membrane. The intracellular contractile force can generate an intercellular force contacting cells in tissues through cell-cell adhesion or through their surrounding extracellular matrix. This is believed to be an important factor for the macroscopic morphogenesis of cells in tissues like cartilage or alveolus.

The mechanical response of cells has been investigated intensively. For example, fibroblasts embedded in collagen gel modified the degree of tension on the gel in response to external oscillating forces (Brown et al., 1998). Increased external loading was followed by a diminution of cell-mediated contraction whereas a reduction in the external force induced cellular contraction. This response apparently results from the

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interaction between cells and collagen gel. Furthermore, the authors measured the mechanical response of single cells by scanning probe microscopy (SPM). The stiffness of single fibroblasts increases/decreases rapidly and decreases/increases gradually when the cells are stretched/contracted by a single-step deformation. These results indicate that so-called "tensional homeostasis" exists at the single cell level (Mizutani et al., 2004). Moreover, this mechanical response originates in the diphosphorylation of myosin regulatory light chain of myosin II combined with F-actin, which increases cellular tension acting on stress fibers (Mizutani et al., 2006).

SPM is unique positioned to visualize not only the surface topography but also the spatial distribution of the local viscoelasticity of living cells in a culture medium (Radmacher et al., 1996; Haga et al., 2000). Cellular stiffness of fibroblasts measured by SPM mainly reflects the intracellular tension of stress fibers (Nagayama et al., 2004). Moreover, SPM is a powerful tool to investigate cellular mechanical responses by combination with various kinds of techniques such as fluorescence microscopy using green fluorescent protein reporters, micromanipulators, and uniaxial stretching devices. Recently, the tensional memory effect of tensional responses of single cells has been observed by SPM: the cellular mechanical response of single fibroblasts to single step stretching was weakened after a series of multi-step deformations (Tamura et al., 2007).

Thus, the present study was designed to elucidate the mechanism of the tensional memory effect by SPM. We have examined how intracellular tension responds to various patterns of external mechanical stimuli like multi-step deformation by the combined use of SPM and a computer controlled stretching device. The stretching device can control the deformation of an elastic culture dish and so generate various deformations of cells.

Materials and Methods

Experimental setup

A commercial SPM was used for measuring the topography and local stiffness of living cells (BioScope NanoScope IIIa; Veeco Instruments, Woodbury, NY, USA). This was combined with an inverse optical microscope (Eclipse TE2000-U; Nikon, Tokyo) on a vibration isolator. Since the SPM is a top-loading type, a relatively large space can be available for setting a cell deformation device, as described below. This instrument was located in a thermally insulating box, together with a control unit for the atmospheric temperature. This system can control sample temperature at 36 ± 1°C. The cell deformation device was located on the x-y stage of the optical microscope.

Uniaxial deformation device

We have developed a uniaxial deformation device (UDD) for stretching or contracting living cells along the x axis in culture conditions using elastic cell culture dishes (Tamura et al., 2007). This can precisely control the parameters of applied deformation patterns to cells such as the amount of deformation or duration. UDD consists of two motor-drive uniaxial slide stages (TAM-401SOMEB-MDC(15); Sigma Koki, Tokyo), a motor controller (OMEC-2BG; Sigma Koki), and steel clamps for the dishes (Fig. 1). Elastic culture dishes were made of semi-transparent silicone rubber (SH 9555; Dow Corning, Midland, MI, USA) and were a rectangular solid-shape with a depression as cell culture chamber. The outer overall sizes were 60×40×7 mm³, and the inner chamber sizes were 30×30×5 mm³. We chose 2 mm for the thickness of the chamber bottom. This thickness was suitable both for reduction of the vibration noise on the SPM measurement and for the optical microscopy observation at high magnifications. For fluorescence microscopy experiments, we used other elastic culture dishes without a chamber bottom. The outer overall sizes were 60×40×5 mm³, and the inner chamber sizes were 30×30×5 mm³. We attached a <100 μm-thick membrane made of transparent silicone rubber (SYLGARD 184; Dow Corning) as a chamber bottom to the dishes.

The elastic culture dishes were fixed on two uniaxial slide stages by steel clamps, as shown in Figure 1. We performed the following procedure before the cell culture: The dishes were clamped on slide stages, stretched at a strain of 0.3, and kept overnight. The dishes were then contracted to a strain of 0.15, attached with steel props to maintain the strain, and removed from slide stages. After being sterilized by ultraviolet light for several hours, they were coated with fibronectin (F. Hoffmann-La Roche,
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The measurement consisted of the following three steps: First, the loading force acting on the cantilever was measured with an indentation of the cantilever into the surface of a sample. This measurement is generally called the force curve measurement. Second, the data was fitted to the following equation derived from the Hertzian contact model (Hertz, 1881):

$$z - z_0 = \frac{F}{k} + \frac{\pi F (1 - \nu^2)}{2E \tan \alpha},$$

where $z$ is the $z$-position of the piezo scanner, $F$ is the loading force, $\nu$ is the Poisson's ratio of the sample, and $\alpha$ is the half-opening angle of the tip. The height of the sample $z_0$ and Young's modulus $E$ were acquired as fit parameters when the force curve data was fitted to the Hertzian contact model using the Levenberg-Marquardt method (Marquardt, 1963; Levenberg, 1994). For simplification, we assumed $\nu$ as 0.5. Third, the above two processes were adapted for each pixel point in a scan area. We measured the force curves for 32 pixels × 32 lines (1024 pixels) or 64 pixels × 64 lines (4096 pixels) in one frame. To complete the scan of one frame, the 1024 pixels-measurement and the 4096 pixels-measurement took ~8 min and ~32 min, respectively. Prior to the force curve analysis, the measured force curves were passed through a 300 Hz low-pass filter (3611 Multifunction Filter, NF, Japan) to reduce the noise on the force curves.

Basel, Switzerland) to support the cell adhesion. Then the cells were cultured in the dish. The present preparation can apply stretching at a strain within ±0.1.

**Topography and stiffness visualization by SPM**

The SPM was equipped with a piezo scanner with a maximal scan range of 100 µm (x-y axis) and 10 µm (z axis) to the control position of a cantilever. We used a silicon-nitride cantilever with a spring constant of 0.01 N/m for living cell measurements. The tip of the cantilever was pyramidal in shape, with an opening angle of 35° (MLCT-AUNM, Veeco Instruments). The spatial distribution of cellular stiffness and topography were simultaneously measured by the force volume mode (Nagayama et al., 2001).

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First of all, we confirmed that the previously obtained properties of cellular responses to external deformation (Tamura et al., 2007) are reproduced by the present measuring system. Figure 2 shows a typical time-lapse sequence of stiffness distributions of living myoblasts by SPM when the cells were stretched in a single step with the strain of 0.07. Many stiff fibers are visible clearly on the cell both before and after the stretching. These fibers correspond to stress fibers beneath the cell membrane from immunofluorescence experiments as described previously (Nagayama et al., 2004). There was no obvious change either in its stress fiber network or in cellular morphology by the present strain of stretching, except for small elongation induced by the strain of 0.07. The cellular stiffness became larger just after stretching and then turned to be smaller for the following two hours. To evaluate time dependence of the stiffness change, the local stiffness was averaged over the 9 × 9 pixels area on a central part of the cells. The averaged values were stable before stretching, increased steeply after the stretching and then decreased gradually for the following two hours. Since the cellular stiffness measured by SPM attributes mainly to the cellular contraction force along stress fibers, the cellular stiffness responses observed in the present study are considered to the result from the responses of the contraction force to external deformations. Then this result indicates that the cells show quick tensional

Sample preparation
C2C12 mouse myoblasts (NIH-3T3; RIKEN Cell Bank, Tsukuba) were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Invitrogen) and 1% antibiotics (Sigma-Aldrich, St. Louis, MO, USA). Trypsinised cells were spouted to the elastic culture dish. After culturing over a day, the medium changed into HEPES-buffered medium (pH 7.2) and the dish was fixed on UDD.

Fluorescence microscopy
Cells were rinsed with phosphate-buffered saline (PBS), followed by fixation with 4% formaldehyde/PBS for 10 min. They were then washed twice with PBS and treated with 0.5% bovine serum albumin in PBS to block nonspecific staining. The cells were stained with fluorescence-labeled phalloidin (MFP-488 phalloidin; Mo Bi Tec, Göttingen, Germany) for 3 h. The cells were subsequently rinsed three times with PBS. To avoid loss of fluorescence intensity, the cells were covered with 1,4-diazobicyclo[2.2.2]octane (DABCO; Wako Pure Chemicals, Osaka) diluted in glycerol. After staining, we observed the stained cells by using confocal laser scanning microscopy (C1 confocal imaging system; Nikon).

Results
First of all, we confirmed that the previously obtained properties of cellular responses to external deformation (Tamura et al., 2007) are reproduced by the present measuring system. Figure 2 shows a typical time-lapse sequence of stiffness distributions of living myoblasts by SPM when the cells were stretched in a single step with the strain of 0.07. Many stiff fibers are visible clearly on the cell both before and after the stretching. These fibers correspond to stress fibers beneath the cell membrane from immunofluorescence experiments as described previously (Nagayama et al., 2004). There was no obvious change either in its stress fiber network or in cellular morphology by the present strain of stretching, except for small elongation induced by the strain of 0.07. The cellular stiffness became larger just after stretching and then turned to be smaller for the following two hours. To evaluate time dependence of the stiffness change, the local stiffness was averaged over the 9 × 9 pixels area on a central part of the cells. The averaged values were stable before stretching, increased steeply after the stretching and then decreased gradually for the following two hours. Since the cellular stiffness measured by SPM attributes mainly to the cellular contraction force along stress fibers, the cellular stiffness responses observed in the present study are considered to the result from the responses of the contraction force to external deformations. Then this result indicates that the cells show quick tensional
response to quick deformations like elastic materials and tensional homeostasis. Next, we applied a single step stretching for 30 min, short pulse-like deformations repeated 30 times with the pulse duration of 0.5 min and the pulse period of 1 min, and then a single step stretching 50 min after the repeated pulse-like deformations (Fig. 3). The cells did not respond to the second single step stretching after the repeated deformations, although the cells did to the first single step stretching as mentioned above. These results are in good agreement with the results reported previously (Tamura et al., 2007), and also indicate that the present experimental set up can reproduce the reported mechanical response of single cells.

We firstly examined the influence of the first single step stretching on the cellular response to the second one. For this purpose, the first single step stretching for 30 min were applied to cells, followed by the second single step stretching successively after an interval of 30 min. Figure 4 shows a time-lapse sequence of stiffness distributions. The cell did not move nor change its shape largely. The cellular stiffness became larger just after both the first and the second stretching and then became smaller for several minutes. Time dependence of the averaged cellular stiffness shows that the cells responded markedly to the second stretching as well as to the first stretching. This result indicates that a long pulse-like deformation with a pulse duration of 30 min does not have any obvious influence on cellular responses to the following stretching. It also indicates that an interval of deformation stimuli of 30 min is enough long to allow the independence of the cellular response from sequential
Fig. 5. Time-lapse cellular stiffness images measured by SPM when a single step stretching was applied 30 min after a short pulse-like deformation (A). Time dependence of the averaged cellular stiffness with a schematic drawing of the deformation pattern of cells (B).

Secondly, we examined the influence of the duration time of short pulse-like deformations on cellular responses to successive stimuli. We applied the following series of deformations: the first single step stretching for 30 min, a short pulse-like deformation with a pulse duration of 0.5 min, and then a second single step stretching 30 min after a short pulse-like deformation. Figure 5 shows time-lapse cellular stiffness images, when the above series of the deformations was applied to a cell. The cell examined neither moved nor changed its direction by the deformations. The cell became much stiffer just after the first stretching and then turned softer for 50 min. We could not measure a stiffness image in the short pulse-like deformation because of the measuring limit of the SPM observation. The cellular stiffness did not change after the second single step stretching. The time dependence of the cellular stiffness clearly showed the suppression of cellular responses after the short pulse-like deformation. This result indicates that a combination of the short and long pulse-like deformations completely suppresses cellular responses to external stretching.

In order to examine the influence of mechanical stimuli to stress fiber networks—which generate contractile force—we further observed stress fiber networks of cells by fluorescence microscopy when a short pulse-like deformation with a pulse duration of 0.5 min following a long pulse-like deformation with a pulse duration of 30 min or only a long pulse-like deformation was applied (Fig. 6). Fluorescence images clearly showed that stress fibers remained in cells after applications of both long and short pulse-like deformations. This indicates that the suppression of a mechanical response to a short pulse-like deformation does not result from a disassembly of stress fiber networks in cells.
Discussion

The response to the cellular stiffness observed in the present study is considered to result from the response to the contraction force of the external deformation since the cellular stiffness measured by SPM is mainly attributed to the cellular contraction force along stress fibers (Nagayama et al., 2004). Since the stress fiber networks were not broken by the present deformations, we consider that the observed changes in cellular stiffness arise mainly from the response to the contraction force along stress fibers in cells from external stimuli.

In the present study, we also showed that the successive short pulse-like deformation or a combination of short and long pulse-like deformations suppresses the cellular response to the successive stimuli, suggesting that the mechanical cellular response depends strongly on the patterns of previously applied deformations. This implies that the cells can memorize their experiences of the applied mechanical stimuli. We further showed that a long pulse-like deformation with a pulse duration of 30 min does not affect cellular response. This finding indicates that the duration time of deformations is one of the critical factors for the expression of a memory effect of cellular response. This finding suggests that the memory effect is independent from the magnitude of the contraction force before stretching. Therefore, we consider that the memory effect seems to be related to an upstream regulatory mechanism of the contraction force.

The contraction force is generated by the interaction between actin filaments and myosin II. The phosphorylation level of myosin II regulatory light chain (MRLC) controls the contraction force. Two phosphorylatable sites of MRLC are responsible for the motor activity of myosin II. MRLC can be in either an un-, mono- or diphosphorylated state. In our previous study (Mizutani et al., 2006), we showed that the diphosphorylation of MRLC is responsible especially for increasing the cellular contraction force along stress fibers when the cells are stretched externally, and suggested that the mechanical response is attributed to such a specific property of diphosphorylation state of MRLC. Therefore, a regulatory mechanism of the diphosphorylation state of MRLC, i.e. kinases or phosphatases of MRLC, may exhibit the memory effect, depending strongly on the duration time of external deformation.

In conclusion, the present study revealed that cultivated myoblasts have a mechanical memory effect in response to the external deformation of the cell, and the duration time of deformations is one of the critical factors for expression of the memory effect. We thus consider that cells in muscle tissues express the mechanical memory effect because the cells are always exposed to short
mechanical stimuli induced by the contraction and/or relaxing of neighboring cells or whole muscle tissues. Such stimuli clearly affect the properties of muscle tissues (Powell et al., 2002). This memory effect derived from myoblasts could also be related to myogenesis and the maintenance of muscle tissues.

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