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Authors: Hayakawa, Kimihide, Hosokawa, Atsushi, Yabusaki, Katsumi, and Obinata, Takashi

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Orientation of Smooth Muscle-Derived A10 Cells in Culture by Cyclic Stretching: Relationship between Stress Fiber Rearrangement and Cell Reorientation

Kimihide Hayakawa[1,2] Atsushi Hosokawa[1], Katsumi Yabusaki[1†] and Takashi Obinata[1,2*]

[1]*Department of Biology, Faculty of Science, and*
[2]*Graduate School of Science and Technology, Chiba University, Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*

ABSTRACT—Mechanical stress causes various responses in cells both *in vivo* and *in vitro*. Realignment of cells and stress fibers is one of the remarkable phenomena that are induced by the stress. However, the mechanism by which their realignment is controlled is largely unknown. In this study, effects of mechanical stretch on the morphology of cultured cells were examined using a cyclic and reciprocal cell stretching apparatus. A10 cells, a cell line derived from rat aortic smooth muscle, were used as a model, since they are spindle-shaped and have remarkable stress fibers aligned along the longitudinal cell axis. Therefore, the orientation of the cell and stress fibers could be easily identified. When the cells were cultured on elastic silicone membranes and subjected to cyclic and reciprocal stretch with an amplitude of 20% at a frequency of 60 cycles per minute, actin stress fibers were aligned obliquely to the direction of stretching with angles of 50 to 70 degrees within about 15 min after the onset of stretching. Then, after 1–3 hr of cyclic stretching, the long axes of a majority of the cells were also reoriented to similar directions to the stress fibers. The stretch-induced cell reorientation was blocked by 1 μM cytochalasin B, but not by colcemid. These results indicate that the orientation of cells and actin filaments are closely related and actin filaments play a critical role in the early step of the cell reorientation.

INTRODUCTION

Many types of cells respond to mechanical stress in a variety of ways both *in vivo* and *in vitro*. Organization pattern of cells, especially orientation of the cells, is known to be affected by the dynamic extracellular environment. Alignment of the endothelial cells and smooth muscle cells which constitute blood vessels is one of the remarkable cases; the former cells are aligned in the direction of the longitudinal vascular axis (White, *et al*., 1983; Wong, *et al*., 1983), while the latter ones are oriented obliquely to the vascular axial direction (Rhodin, 1962). Two types of mechanical stress are responsible for these cellular alignments, namely hydrodynamic shear stress which is given to the endothelium in the direction of blood streaming and periodic stretch in the circumferential direction.

To elucidate the relationship between the mechanical stress and the alignment of the cells in specific direction, various *in vitro* studies have been carried out. Two kinds of mechanical stress have been applied to cultured cells, and their effects on the cells have been examined. One is fluid shear stress that is generated by using a flow chamber; in this case, cultured cells were oriented parallel to the direction of flow (Franke, *et al*., 1984; Levesque and Nerem, 1985). The other is periodic stretching stress; it has been observed that cells and their stress fibers tend to align perpendicularly to the direction of stretching (Dartsch and Betz, 1989; Harris, *et al*., 1992; Iba and Sumpio, 1991; Shirinsky, *et al*., 1989). Endothelial cells were frequently used as a model in these experiments, because they are well aligned in the endothelium of blood vessels *in vivo*. In most cases, the cell orientation has been assessed by the alignment of the actin stress fibers, since they are a major cytoskeletal element which is responsible for the maintenance of cellular shape and is easily detectable by cytochemical methods. However, the temporal relationship between cell orientation and stress fiber alignment under the stress conditions is yet to be investigated. Molecular mechanism(s) of the stress-induced alignment of actin filaments is also poorly understood.

In this study, A10 cells, a cell line derived from rat aortic smooth muscles, were used to examine the response of the cells to the mechanical stress, namely cyclic stretching, with particular attention to rearrangement of cytoskeletal elements and cell orientation. These cells are appropriate for this kind

* Corresponding author: Tel. 043-290-2804;
FAX. 043-290-2807.
E-mail: tobinata@bio.s.chiba-u.ac.jp
† Present address: Biosignal Engineering Division, Kowa Research Institute, Kowa Co., Ltd., 1-25-5 Kannondai, Tsukuba 305, Japan.

of experiments, because they have elongated spindle shapes and actin stress fibers are aligned along the longitudinal cellular axis, so that orientation of the cells and stress fiber alignment can be easily identified. We observed that the orientation of cells and actin filaments are closely related and actin filaments play a critical role in the stress-induced cell reorientation.

MATERIALS AND METHODS

Cell stretching system and cell culture

A mixture of silicone polymer (Silpot 184) (Dow Corning Asia Ltd., Tokyo) and the catalyst was blotted on a glass plate and extended uniformly on the plate with the aid of a centrifugal force. The silicone thin layer was polymerized into membrane sheet at 120°C for 3 hr. The polymerized silicone membrane on the glass plate was cut into small pieces (45 mm×22 mm) and peeled off from the plate. The thickness of the membrane was approximately 0.1 mm. The membrane pieces were washed with acetone, rinsed intensively with water, and stored in ethanol. The silicone membrane was clamped between poly-acetal plates and autoclaved at 120°C for 20 min. Then, the membrane was attached to a stretching apparatus which was driven by a synchronous motor, as shown in Fig. 1. To observe the morphological changes of living cultured cells under a microscope, the bottom of the dishes was made with very thin glass plate (Fig. 1C).

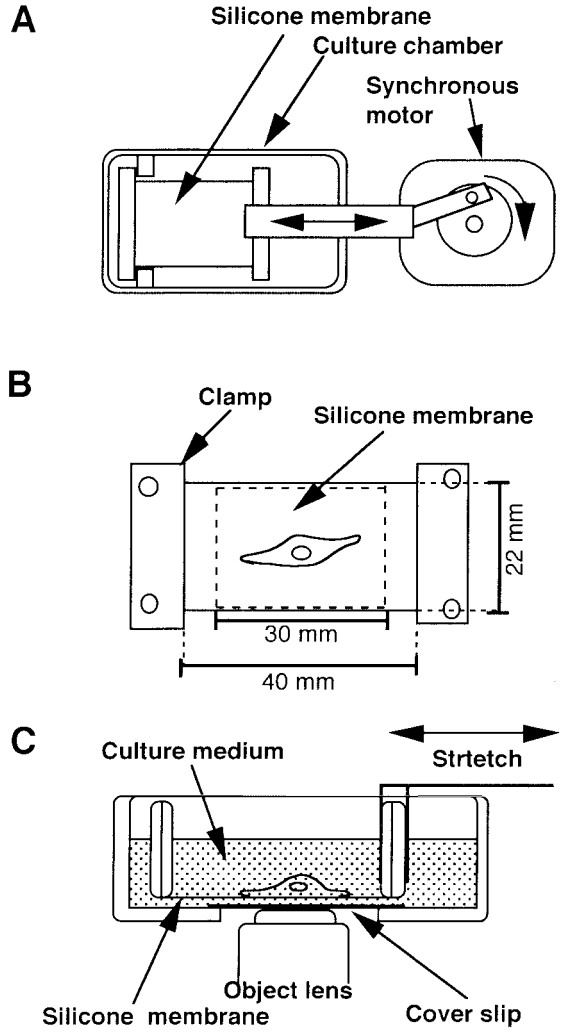

Fig. 1. Schematic representation of the cell-stretching apparatus used in this study. Cells were cultured on silicone membrane as shown in (B), and then, the membrane was attached to the stretching apparatus driven by a synchronous motor as shown in (A). For taking micrographs during the course of stretching, the culture dish, the bottom of which was made with thin glass plate, was used (C). Because the working distance of the lens was short, maximum thickness of the glass plate was 0.17 mm. Cells were plated on a collagen-coated membrane and then, the membrane was stretched at the frequency of 60 cycles per min with an amplitude of 20% stretch. The area framed by dotted line was used for microscopic observations.

A10 cells, a cell line derived from rat vascular smooth muscle (Kimes and Brandt, 1976), were maintained on plastic dishes in the Dulbecco's-modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 95% air with 5% CO_2. For stretching, the cells were removed from the culture dish with 0.25% trypsin and plated on a silicone membrane coated with 0.05% porcine Type I collagen (Koken, Tokyo) in DMEM containing 10% FBS for 24 hr before stretching. The culture medium was replaced with a stretching culture medium (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 20 mM Hepes, pH 7.4) before stretching. The membrane was stretched reciprocally up to 1.2 times of the original length at a frequency of 60 cycles per minute (1 Hz).

Immunofluorescence microscopy

A10 cells on the silicone membrane were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) (0.15 M NaCl and 10 mM phosphate buffer, pH 7.0) containing 2 mM EDTA and 5 mM $MgCl_2$ for 10 min at room temperature, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. The specimens were washed with PBS containing 10 mM glycine, and treated with 2% bovine serum albumin (BSA) in PBS containing 0.05% NaN_3 for 20 min. They were then exposed to anti-tubulin antibody (TUB 2.1) (ICN Pharmaceuticals, Inc., USA) for 90 min at room temperature, followed by treatment with a mixture of fluorescein (FITC)-labeled goat anti-mouse IgG (GAM) (Tago: Burlingame, California, USA) and rhodamine (RITC)-labeled phalloidin (Wako: Tokyo, Japan) for 60 min. Washing with PBS was carried out after the respective antibody treatments. After the final wash, specimens were mounted in a solution containing 10 mg/ml *p*-phenylendiamine, 90% glycerol and PBS, pH 8.0, and examined under a ZEISS Axioskop epifluorescence microscope.

Other procedures

Cytochalasin B was purchased from Research Biochemicals International (Natick, MA, USA), and dissolved in dimethyl sulfoxide (DMSO) at 1 mM and stored at –20°C. Colcemid (Sigma, St. Louis, USA) was dissolved in DMSO at 5 mg/ml and stored at –20°C. Each reagent was diluted into appropriate concentrations for respective experiments with the stretching culture medium just before use.

RESULTS

When the silicone membrane, the substratum for the cells, was stretched in the stretching system that we devised, living A10 cells on the membrane were obviously stretched, as shown in Fig. 2. The magnitude of the cell stretching was indistinguishable from that of the membrane stretching. Stretching of the membrane at 20% amplitude caused 20% (±1 %) increase in the cell length along the direction of stretching. The ratio of compression of the membrane as well as compression of the cells perpendicular to the direction of stretching was about 8%. The ratio of stretching and com-

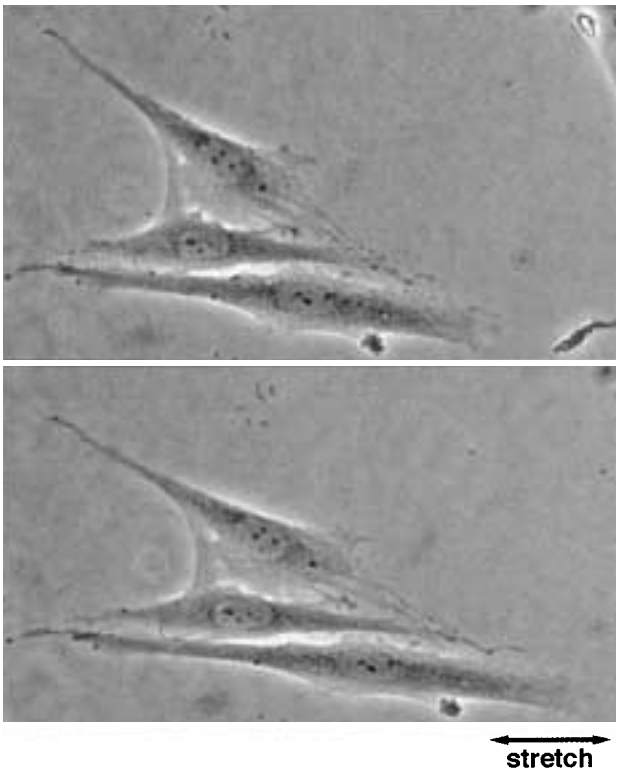

Fig. 2. Micrographs of the cells on the membrane before stretch (top) and the same cells on the stretched membrane (bottom) were taken with phase-contrast optics. Obviously, the cells were stretched during the membrane stretching.

pression was almost uniform over the membrane area used for observations (see Fig. 1B); we measured the magnitude of stretching and compression at different regions of the membrane and found that the variation among the regions was ±1%.

When the cells were cultured on the membrane before stretching, A10 cells were oriented randomly on the silicone membrane (Fig. 3B). 60 min after the onset of cyclic stretching at a frequency of 60 cycles per min and at 20 % amplitude, considerable numbers of A10 cells started to align obliquely to the direction of stretching with angles of 50 to 80 degrees (Fig. 3C), and 3 hr after stretching, more than 80 % of the cells were aligned at 50 to 80 degrees to the direction of stretching (Fig. 3D). The cells were scarcely detached from the substratum during cyclic stretching. Static stretching of the membrane caused neither realignment of the cells (Fig. 3E) nor rearrangement of the stress fibers (data not shown). These results indicate that cyclic stretching is necessary for the rapid reorientation of the cells and the rearrangement of the stress fibers.

In the A10 cells, stress fibers were clearly detectable; they were aligned along the long axis of the cells as shown in Fig. 4A. The stretch-induced reorientation of the cells was accompanied by drastic rearrangement of the stress fibers. When cyclic stretching was applied to the cells for 30 min, the cells were not reoriented obliquely to the direction of stretch-

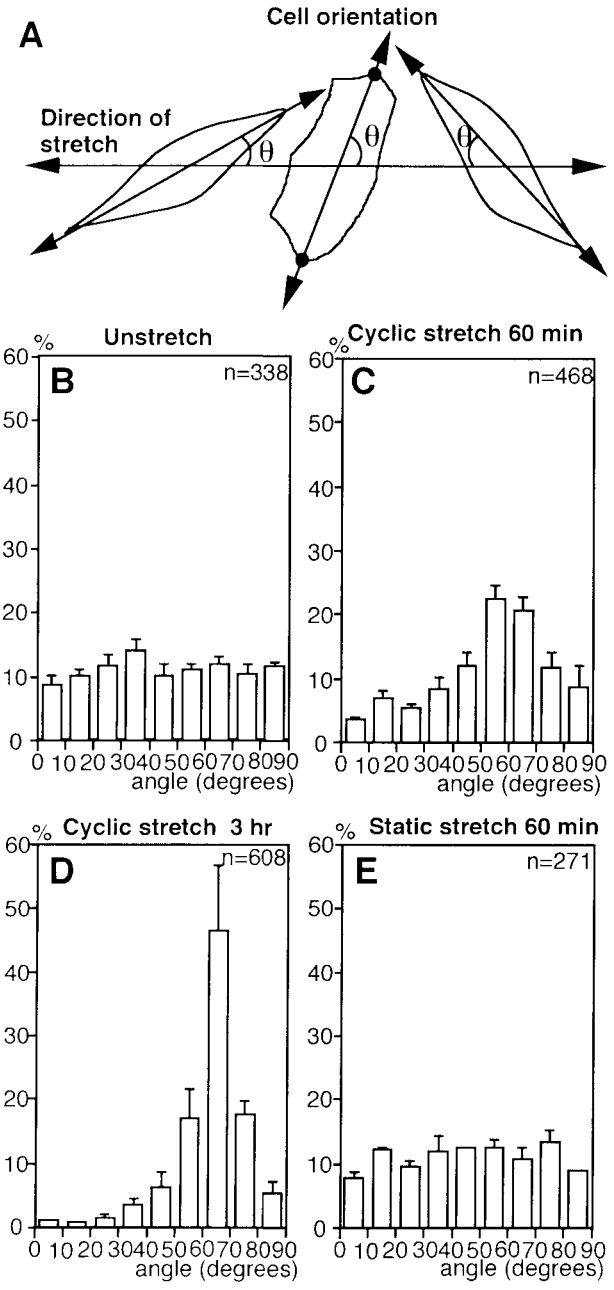

Fig. 3. Stretch-induced orientation of cultured A10 cells. The cells were cultured on the membrane and stretched at 20% amplitude and at the frequency of 60 cycles per min. The acute angles (θ) between the long axis of the cells and the direction of stretching were measured as shown in (A). In the case of the cells with deformed shape, the points of cell periphery which gave longest distance were selected and the line drawn between the two points was designated as the direction of the long axis of the cells. The populations of the cells oriented at each angle range as shown in the abscissa were counted. The ordinate shows the percentage of cells in each angle range; the values are the average (± SE) of the data from four independent experiments. Total numbers of cells counted (n) are given in the respective figure panels. Before stretching (B), the cells were oriented randomly. However, after stretching for 60 min (C), a majority of the cells became oriented at angles of about 50 to 80 degrees to the direction of stretching. 3 hr after cyclic stretching (D), more than 80 % of the cells oriented at angles of about 50 to 80 degrees. Non-cyclic (static) stretch caused no significant orientation of cells (E).

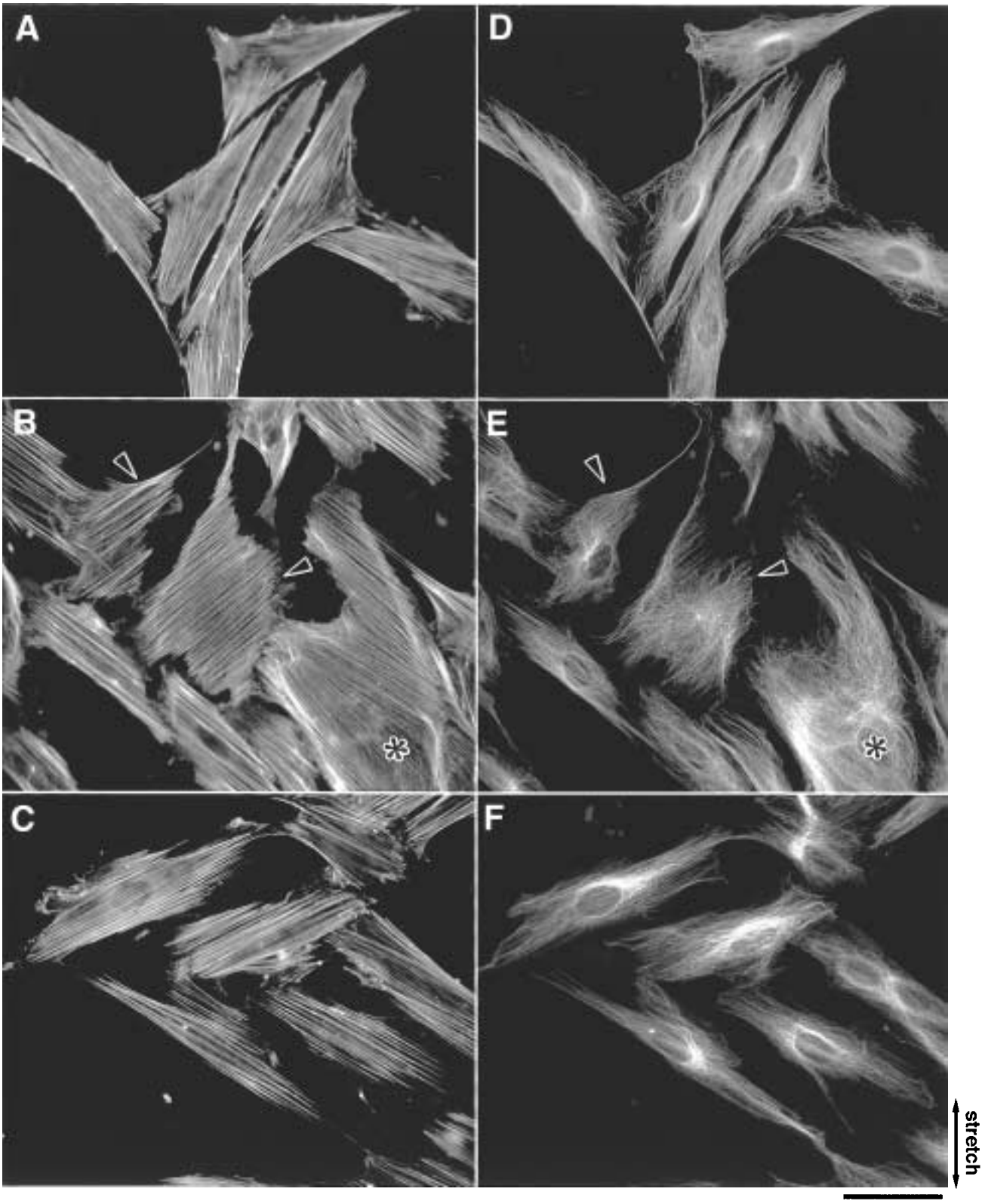

Fig. 4. Fluorescence micrographs showing reorientation of cells, stress fibers and microtubules during cyclic stretching. A10 cells were cultured on the membrane and stretched at 20% amplitude and at the frequency of 60 cycles per min. Before stretch (A, D), 30 min after stretching (B, E), or 3 hr after stretching (C, F), the cells were dually stained with rhodamine-phalloidin (A, B, C) and anti-tubulin antibody (D, E, F). In the intermediate stage, 30 min after stretching, the cells were not reoriented yet, but the stress fibers were already aligned at angles of 50 to 70 degrees to the direction of stretching (B). In 3 hr, the cells completed reorientation and the stress fibers in the cytoplasm were present in the direction of the long axis of the cells (C). In contrast, ordered alignment of the microtubules was scarcely observed throughout the experiment, except that some microtubules in the cell periphery existed along the longitudinal cell axis. Bar: 20 μm.

ing, although cell morphology was altered. Interestingly, however, the actin stress fibers were already aligned at the angles of around 50–70 degrees to the direction of stretching at this time point (Fig. 4B), as at 3 hr after stretching (Fig. 4C). In a small number of cells, orientation angles of stress fibers were somewhat varied in a single cell (marked by asterisks). Cells with polygonal or asymmetric shapes were occasionally observed (marked by arrowheads); some cells were elongated at one side and spread at the other region on the substrate. In the cells that were stretched repetitively for 3 hr, a majority of the cells were aligned at 50 to 80 degrees to the direction of stretching as mentioned above and they showed elongated spindle shape as the control unstretched cells. Therefore, at this moment, stress fibers were present along the long axis of the cells (Fig. 4C).

Microtubules were also clearly visible in the A10 cells (Fig. 4D); in the control unstretched cells, they were mostly aligned roughly along the longitudinal cell axis, but they showed a wavy pattern and were not aligned as clearly as stress fibers. As judged by the immuno-staining patterns, localization of microtubules was not changed significantly during stretching (Fig. 4E, F), even when the stretch-dependent alignment of the stress fibers was initiated. Only the microtubules in the limited area, especially in the cell periphery region, were partly aligned in the same direction as the stress fibers.

To clarify the relationship between stress fiber rearrangement and cell reorientation during cyclic stretching, time courses of stretch-induced realignment of stress fibers and cell orientation were examined. The results are summarized in Fig. 5. As early as 5 min after the onset of the cyclic stretching, rearrangement of the stress fibers was initiated. However, the orientation angles of stress fibers were not determined in considerable numbers of cells, because stress fibers were significantly disrupted and the fragmented fibers were oriented in various directions. 15 min after stretching, stress fibers in most of the cells were already aligned obliquely to the direction of stretching with angles of 50 to 70 degrees (Fig. 5C). In contrast, reorientation of cells started 15 min after stretching, proceeded gradually and settled at 3 hr after stretching. Thus, cell reorientation was significantly delayed from rearrangement of the stress fibers (Fig. 5C, D, E). 3 hr after stretching, good correlation between stress fiber alignment and cell orientation was observed (Fig. 5F). These results clearly indicate that rearrangement of stress fibers preceded cell orientation. Stress fibers seemed to play an important role in cell orientation, but the stress fiber arrangement did not immediately lead to cell reorientation. The results suggest that the cell reorientation does not simply depend on the arrangement of actin filaments and may be controlled by some additional mechanism(s) including alteration of cell adhesion to substrate.

To clarify roles of cytoskeleton in stretch-induced morphological change of A10 cells, cells were stretched in the presence or absence of chemical agents that disrupt cytoskeletal elements. When A10 cells were treated with 1 μM cytochalasin B for 30 min and then subjected to cyclic

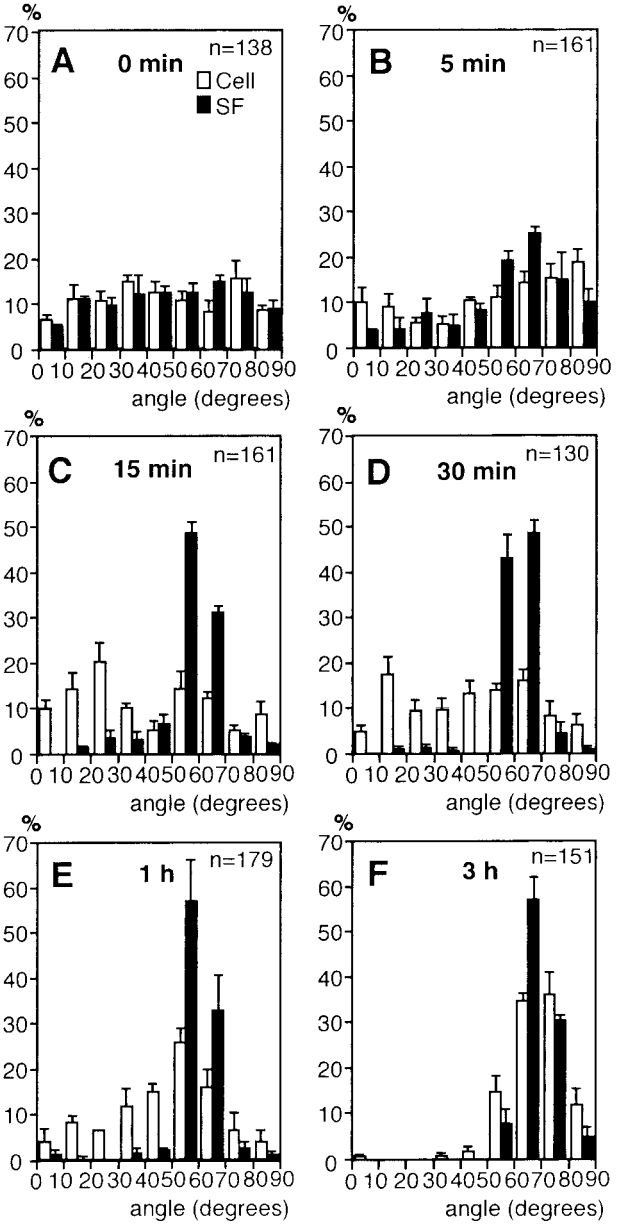

Fig. 5. Time course of stretch-induced realignment of stress fibers and cell orientation. A10 cells cultured on the membrane were stretched at 20 % amplitude and at the frequency of 60 cycles per min. Orientation angles of the cells and the stress fibers in the cells were measured before stretch (A), 5 min (B), 15 min (C), 30 min (D), 1 hr (E), and 3 hr (F) after stretching as shown in Fig. 3. Orientation angles of the stress fibers were easily determined before stretch and at 1 to 3 hr after stretching, because all the stress fibers were aligned in nearly the same directions within a single cell. At the intermediate stages of cell reorientation during stretching, the stress fibers in a single cell were not always aligned in the same directions. In such cases, the stress fibers located in the center of the cells were selected as the representatives. The populations of the cells oriented (open bar) and the populations of the stress fibers oriented (closed bar) were counted at each angle range as given in the abscissa. The populations of the stress fibers mean that the populations of the cells having stress fibers oriented in the respective angle range. The ordinate shows the percentage of the cells in each angle range; the values are the average (± SE) of the data from three independent experiments. Total numbers of cells examined (n) are shown in each figure.

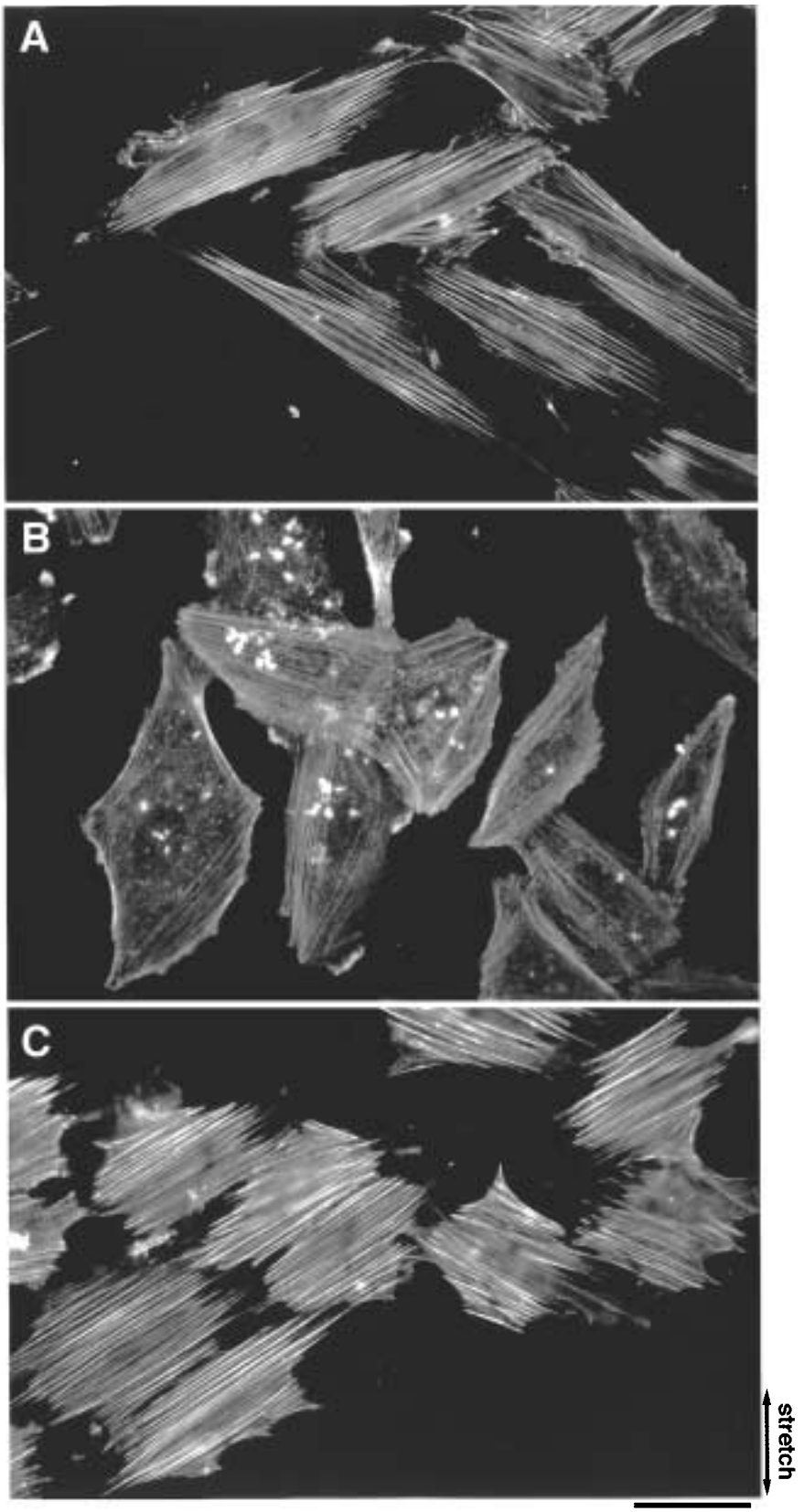

Fig. 6. Roles of actin filaments and microtubules in stretch-induced reorientation of cultured A10 cells. A10 cells were pre-cultured for 30 min in the presence of cytochalasin B (B), colcemid (C) or in the absence of these agents (A), and then, the membranes were stretched for 3 hr, as in Fig. 3. The cells were stained with rhodamine-phalloidin. In the presence of cytochalasin, the stress fibers were significantly disrupted and stretch-induced orientation of the cells was inhibited almost completely. In contrast, colcemid-treated cells responded to stretching and the stress fibers were obliquely aligned to the direction of stretching by cyclic stretching for 3 hr. Bar: 20 μm.

stretching in the presence of cytochalasin B, stress fibers were considerably diminished and stretch-induced cell realignment was significantly blocked (Fig. 6B). The residual stress fibers seemed to be aligned in varied angles to the direction of stretching. Experiments at higher concentrations of cytochalasin which completely disrupt stress fibers were difficult, because the cells were easily detached from the silicone membrane during stretching under these conditions. In contrast, pre-incubation of the cells with colcemid at 7.5 μg/ml for 30 min led to disassembly of microtubules (data not shown). When the colcemid-treated cells were cyclically stretched in the presence of colcemid at the same concentration, rearrangement of stress fibers as well as cell reorientation was induced (Fig. 6C). However, elongated and spindle-shaped morphology of A10 cells as seen in the control culture (Fig. 6A) became less clear in the presence of colcemid. Microtubules may be partly involved in maintaining the spindle-shaped cell morphology. These results indicate that stress fibers play a critical role in stretch-induced cell reorientation, but microtubules are not essential for receiving signals of mechanical stimulation and for rearrangement of actin cytoskeleton and cell reorientation.

DISCUSSION

In this study, the response of cultured cells to repetitive stretching stress was examined with special attention to the changes in cell orientation and stress fiber arrangement using our original cell stretching apparatus. With this apparatus, morphological changes of the cultured cells during stretching could be traced by time lapse microscopy. Dynamics of actin in the process of realignment of stress fibers could be visualized at real time by introducing fluorescence-labeled actin into the cells or by expressing of GFP-tagged actin in the cells by cDNA transfection methodology. Such kinds of studies are now in progress.

A10 cells were used as a cell model in this study, since they have an elongated spindle shape and remarkable stress fibers that are clearly aligned along the longitudinal cell axis and, therefore, changes in orientation of the cell and stress fibers could be easily identified. Previous investigators have examined stretch-induced realignment of cells and/or stress fibers mostly in endothelial cells (Dartsch and Betz, 1989; Iba and Sumpio, 1991; Shirinsky, *et al*., 1989; Sokabe, *et al*., 1997; Takemasa, *et al*., 1998). We have examined a myoblast cell line (C2 cells) and fibroblasts of primary cultures as well. In both of these cells, stress fibers are visible, but the polarity of cell shape is not so clear as A10 cells. Using A10 cells is advantageous for examining stretch-induced morphological changes of cells, particularly for precisely determining their orientation angles to the direction of stretching. As another excellent cell model with bipolar shapes, human melanocytes have been reported (Wang *et al*., 1995).

When A10 cells on silicone membranes were stretched cyclically in our experimental apparatus, they started to reorient within 1 hr, and after 3 hr of stretching, a majority of the cells aligned obliquely to the direction of stretching at 60 to 70 degrees. The stress fibers were also aligned in the same direction with oblique angles as the cells. Many previous reports described that the stress fibers as well as cells were oriented perpendicularly to the direction of stretching under stretching stress (Iba and Sumpio, 1991; Shirinsky, *et al*., 1989; Sokabe, *et al*., 1997). Takemasa *et al*. (1997), however, demonstrated that endothelial cells were aligned obliquely to the direction of stretching and the orientation angles of the cells depended on stretching amplitude by detailed analysis using their own stretching apparatus. Our observations are consistent with those by Takemasa *et al*. (1998); In the case of 20% stretch of the membrane, the orientation angles of stress fibers in their report were in the similar range to those in our study.

Since stress fibers are most remarkably aligned in cyclically stretched cells among various cytoskeletal elements, they have been regarded as being necessary for the elongated shape of the stretched cells (Iba and Sumpio, 1991). In our observations, the stress fibers started to realign obliquely to the direction of stretching soon after the onset of stretching, even 5 min after stretching, and the realignment was almost completed within 15 min, much earlier than reorientation of the cells. In contrast, the distribution pattern of the microtubules was not markedly altered. The stress fiber rearrangement appears the earliest response among the morphologically detectable responses of the cells in cyclic stretching. Since disassembly of actin filaments by cytochalasin B inhibited reorientation of the cells, it is concluded that actin cytoskeleton, but not microtubules, play a critical role in the stretch-induced cell reorientation. Actin cytoskeleton may function as the machinery that is responsive to the mechanical stress. How actin filaments are rearranged so quickly in response to mechanical stress is a matter of interest. Takemasa *et al* (1998) reported that length of stress fibers changes during cyclic stretching because of membrane deforming, and the stress fibers become aligned at a particular angle to minimize their length alteration in cyclic deforming field. Stress fibers rearranged at this angle may be suitable for avoiding compressing stress and could be maintained during stretching. Actin-binding proteins such as cofilin, ADF, profilin and gelsolin (for review see Pollard and Cooper, 1986; Bamburg, 1999) may be involved in rearrangement of actin filaments. Which actin-binding proteins are particularly important in stretch-induced actin dynamics and how they function during this process remain for further investigations.

The analysis of the time courses of the stress fiber realignment and cell reorientation indicated that stress fiber rearrangement preceded cell reorientation. Considerable time lag between the reorientation of the cells and stress fiber realignment was observed. At the intermediate phase during the cell reorientation, cells showed strange morphology; for example, polygonal and asymmetric shapes were occasionally observed. It appeared that cell reorientation was difficult to proceed in the beginning, because the cells remained attached to the substrate during early phase of stretching although realignment of the stress fibers had been already

completed. Thus, it is suggested that the cell reorientation does not simply depend on the arrangement of actin filaments and may be controlled by some additional mechanism(s) including modulation of cell-substrate adhesion.

Recently, much information has been accumulated as to the cytoplasmic response which might be involved in stretch-induced rearrangement of cell and stress fibers. It has been reported that stretch-activated (SA) ion channels plays an important role in stretch-induced orientation of endothelial cells (Sokabe, *et al*., 1997). Lee *et al*. (1999) demonstrated that the calcium transients mediated by SA channels are involved in the regulation of cell movement, especially, in the retraction and detachment of the rear ends of locomoting cells. According to Naruse *et al*. (1998), the increased Ca^{2+} ions through SA channels lead to activation of calcineurin, a calcium-calmodulin dependent protein phosphatase, which then activates $pp60^{c\text{-}src}$, a non-receptor type of tyrosine kinase. The activated $pp60^{c\text{-}src}$ is necessary for stretch-induced cell orientation (Sai, *et al*., 1999). On the other hand, Lawson and Maxfield (1995) have revealed that calcineurin is involved in integrin recycling in the case of chemotactic movement of neutrophils. Retta *et al*. (1996) demonstrated that phenylarsine oxide, an inhibitor of tyrosine phosphatase, inhibits loss of focal adhesion which are induced by serum starvation and by cytochalasin D treatment. In our preliminary investigation, phenylarsine oxide inhibited stretch-dependent reorientation of A10 cells but not rearrangement of the stress fibers. It is likely that reinforcement of cell adhesion to substratum by phenylarsine oxide led to inhibition of the cell reorientation. On the basis of our observations and other recent information, it is conceivable that stretch-induced cell reorientation is caused by a combination of rearrangement of the stress fibers and alteration of the focal adhesion sites which may be regulated by calcineurin-dependent pathway in a Ca^{2+}-dependent manner.

ACKNOWLEDGMENTS

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