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Morphological Changes in Osteoblastic Cells (MC3T3-E1) due to Fluid Shear Stress: Cellular Damage by Prolonged Application of Fluid Shear Stress

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Horikawa, A., Okada, K., Sato, K. and Sato, M. *Morphological Changes in Osteoblastic Cells (MC3T3-E1) due to Fluid Shear Stress: Cellular Damage by Prolonged Application of Fluid Shear Stress.* Tohoku J. Exp. Med., 2000, **191** (3), 127-137 —— We investigated the effect of fluid shear stress on both changes in morphology and in resting level of cytosolic concentrations of Ca^{2+} ($[Ca^{2+}]i$) in MC3T3-E1 cells, osteoblast-like cells derived from the neonatal mouse calvaria. The cells were subjected to fluid shear stress at about 1.0 Pa. Morphological characteristics were compared between stressed and control cells by scanning electron microscopic examination, actin filament evaluation using phalloidin fluorescence and $[Ca^{2+}]i$ measurement using fura-2/AM. The stressed cells changed from a polygonal to a spindle shape after 1-hour exposure to fluid shear stress, whereas control cells remained polygonal, and the stressed cells exhibited an increase in the number of microvilli. The diameter in the long axis of the stressed cells was significantly larger than that of controls, and about half of the stressed cells were oriented nearly along the direction of the fluid shear stress. The arrangement of the actin filaments of the stressed cells changed to parallel, and was positioned along the long axis of the cell process. The resting level of $[Ca^{2+}]i$ in the stressed cells showed a peak at 1 hour after the application of the stress. A more prolonged stress period over 1 hour caused cell shrinkage, an irregularity on the cell surface, and a gradual decrease in the resting level of $[Ca^{2+}]i$, suggesting that prolonged stress time may cause cellular damage. ———— osteoblast-like cell; fluid shear stress; stress; bone; calcium

It is well known that bone is occasionally influenced by several types of mechanical stress and it can adapt to the stress by changing structure and mass. Three types of mechanical stress in the skeletal system have been identified (Goodship et al. 1979; Johnson et al. 1982; Cowin et al. 1991; Lanyon 1993).

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The first is stretching, the second is fluid shear stress, and the third is hydrostatic pressure (Klein-Nulend et al. 1987). Among these, stretching stress has been most widely examined. It has been reported that stretching stress caused changes in morphology (Buckley et al. 1988), membrane phospholipase C (Jones et al. 1991), prostaglandin E2 and cAMP (Somjen et al. 1980), and alkaline phosphatase levels (Neidlinger-Wilke et al. 1994).

In 1990, it was revealed that fluid flow alone could elicit biochemical responses in bone cells (Reich et al. 1990). Particularly, the levels of intracellular cAMP in cultured newborn rat calvarial osteoblasts increased in response to flow in a dose-dependent manner (Reich et al. 1990). Shear stress has also induced increases in $[Ca^{2+}]i$ (Williams et al. 1994; Hung et al. 1995), prostaglandin E2 (Klein-Nulend et al. 1995), and prostaglandin mediated-modulation of transforming growth factor (TGF)-β metabolism in osteoblasts (Klein-Nulend et al. 1996). However, only limited information on morphological changes of osteoblastic cells exposed to fluid shear stress is available in the literature. Therefore, we examined morphological changes in osteoblasts, MC3T3-E1 cells subjected to shear stress, using scanning electron microscopy and actin staining by the immunofluorescent method. Furthermore, we determined a resting level of $[Ca^{2+}]i$ in the stressed and/or non-stressed cells to show the effect of prolonged application of the stress, and it was discussed how fluid shear stress caused morphological changes in the osteoblasts.

Materials and Methods

Cell culture

MC3T3-E1 cells (RCB1126, RIKEN Cell Bank, Tsukuba), which are osteoblast-like cells taken from the neonatal mouse calvaria, were cultured in α-MEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum and 62.5 mg/ml penicillin G and 100 mg/ml streptomycin. The cells were kept in humidified air under 5% CO_2 at 37°C. They were subcultured twice a week using 0.25% trypsin in phosphate-buffered saline (PBS). For the experiments, the cells were seeded at a density of 10 000/cm^2.

Apparatus for applying fluid shear stress

We used a rotating disk apparatus that was described previously (Ando et al. 1987). A flat disk of stainless steel was held on the surface of the tissue culture medium and was rotated by a DC motor. The fluid shear stress (t) induced by the device is given by $t=\mu\omega r/d$ (μ, the medium viscosity; ω, the angular velocity of rotation; r, the distance from the cell to the center of the culture dish; d, the depth of the medium). About 1.0 Pa fluid shear stress was used in this experiment ($\mu=0.7$ mPa.s, $\omega=2\pi$, $r=6$ cm, and $d=0.2$ mm), since Tsay and Weinbaum (1991) showed that 0.8–3.0 Pa is the biological magnitude range for bone cells in their model of the flow system through cross-linked fiber filled channels.

Scanning electron microscopy

Coverslips (NUNC, Roskilde, Denmark), 3×3 cm in size, were used for observation under a scanning electron microscope (SEM). In order to survey the morphological changes in MC3T3-E1 cells, the application of fluid shear stress was started 24 hours after the seeding of the cells (non-confluent). The cells were divided as follows into 3 groups according to the duration of fluid shear stress: The 1-hour group, the 6-hour group and the 12-hour group, and cells not subjected to the stress were used as controls. The cells were fixed in 3% glutaraldehyde in PBS for 4 hours at room temperature immediately after the exposure to the stress was finished. After postfixation by OsO_4, the cells were rinsed with phosphate buffer for 24 hours and dehydrated sequentially with 70%, 90% and 100% ethanol every 1 hour, and freeze-dried using *t*-butyl alcohol. Finally, coverslips were mounted onto a stage and coated with Au ion, and then examined under the SEM (JSM-T200, JEOL, Tokyo). The diameter in the long axis and the area of the cytoplasm of the cells were measured and analyzed by the public domain NIH Image program (developed at the U.S. National Institute of Health) in 30 randomly selected cells in each group. The cells subjected to the fluid shear stress for 1 hour were classified into 3 subgroups (α, β, γ) on the basis of the direction of the long axis of the cells, as shown in Fig. 1. Cells in subgroup α were positioned nearly along the direction of the fluid shear stress. The subgroups β and γ included cells that were not positioned along the direction of the fluid shear stress.

Staining of actin filament for immunofluorescence

The cells were cultured on glass coverslips (S-0317, Matsunami, Osaka), and the application of fluid shear stress was started 48 hours after the seeding of the cells. Staining of actin filament for immunofluorescence was performed in these sub-confluent MC3T3-E1 cells after 1 hour of stress, and cells not exposed to the stress were used as controls. After rinsing, the cells on the coverslips were fixed in 3% paraformaldehyde and 2% sucrose in 0.1 mol phosphate buffer for 30 minutes. After rinsing, the glass coverslips were preincubated in 0.1% Triton X-100 in 10 mM PBS solution for 10 minutes, and incubated with FITC-conjugated phalloidin (1×10^{-6} mol/liter, Sigma, St. Louis, MO, USA) for 30 minutes at room temperature. After incubation, the coverslips were enclosed using 1 g of glycerin-TBS (D-2522, Sigma) for observation under a fluorescence microscope.

Measurement of $[Ca^{2+}]i$

Measurement of $[Ca^{2+}]i$ was performed by microfluorometry using Fura-2/AM (Dojindo, Kumamoto). MC3T3-E1 cells were seeded on collagen-coated glass coverslips (Type I, Koken, Tokyo). The cells were exposed to fluid shear stress 24 hours after seeding (non-confluent), and the cells were divided into 4 groups

according to the duration of the stress: the 5-minute group, the 1-hour group, the 6-hour group and the 12-hour group. Unstressed cells were used as controls. The number of cells tested in each group was 20. After the exposure to the stress, the coverslips with cells were washed with HEPES-buffered saline (HBS; 135.5 mM NaCl, 5.9 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ $6H_2O$, 10 mM glucose and 12 mM HEPES-NaOH, pH 7.4) and loaded with 5 mM Fura-2/AM for 2 hours at room temperature (20–27°C). After washing with HBS, the coverslips were mounted in a chamber placed on an inverted fluorescence microscope (Nikon TMD, Tokyo). Fluorescence was generated by double beam excitation (340 and 360 nm), and emission was monitored with a cut-off below 420 nm, as described by Sato et al. (1991). The cells loaded with Fura-2/AM were excited at 360 nm both at the start and at the end of the measurements, and they were excited at 340 nm during the measurements. The fluorescent images of the cells were displayed and analyzed with a video-frame memory system (C1901 MARKII; Mitsubishi Kasei, Tokyo) equipped with a computerized image-analysis unit. The value of $[Ca^{2+}]i$ was obtained from the ratio of fluorescence intensity at 340 nm to that at 360 nm by referring the value to a calibration curve obtained in vitro (Sato et al. 1991). All measurements were performed at room temperature (22–27°C).

Statistical analysis

Statistical analysis was performed by the Student's *t*-test. Differences between the groups were considered significant when the p value of comparison was 0.05 or smaller.

Results

Effect of fluid shear stress on morphological changes in MC3T3-E1 cells

Before application of the fluid shear stress, MC3T3-E1 cells were flat and polygonal in appearance. After 1 hour of continuous application of the fluid shear stress, they exhibited a spindle shape. The nucleus also changed to a spindle shape, and the cell surface of the stressed cells exhibited an increase in the number of microvilli. The cell borders were not obvious in control cells but so distinct in stressed cells (Fig. 1). After exposure to the stress for 6 hours, the cells showed a marked shrinkage and an increased irregularity of the cell surface. These changes were most marked in the 12-hour group (Fig. 2).

The diameter in the long axis of MC3T3-E1 cells exposed to fluid shear stress for 1 hour was larger than that of control cells (71.7 ± 20.7 mm vs. 48.6 ± 9.3 mm, mean $\pm$ S.D., $p < 0.001$), suggesting that shear stress induced a formation of long cell processes. After exposure to the stress for 6 hours and 12 hours, the diameter in the long axis of the stressed cells was larger than that of the control cells (6-hour group, 61.2 ± 13.1 mm vs. 46.8 ± 12.1 mm, $p = 0.041$; 12-hour group, 86.2 ± 16.7 mm vs. 64.0 ± 12.5 mm, $p < 0.001$, Fig. 3A).

After exposure to the stress for 1 hour, the area of the cytoplasm of the cells

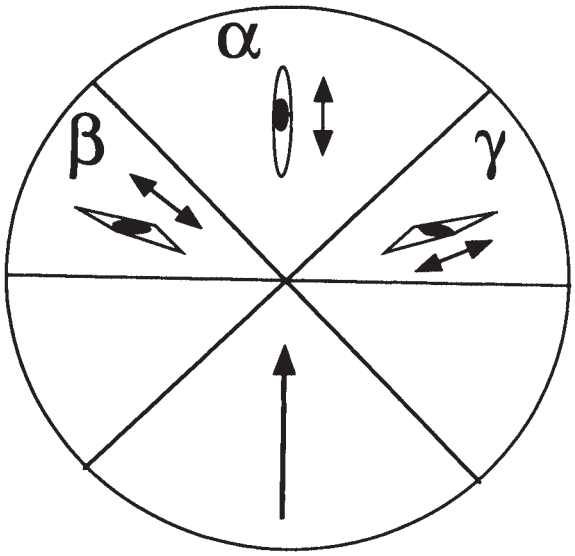

Fig. 1. Schematic illustration of subgroups of cells according to the direction of the long axis. MC3T3-E1 cells were divided into 3 subgroups (α, β, γ) according to the direction of the long axis of the cells. Forty-seven percent of cells were in subgroup α. ←, direction of fluid shear stress; ↔, direction of the long axis of the cell; ⬭, MC3T3-E1 cell.

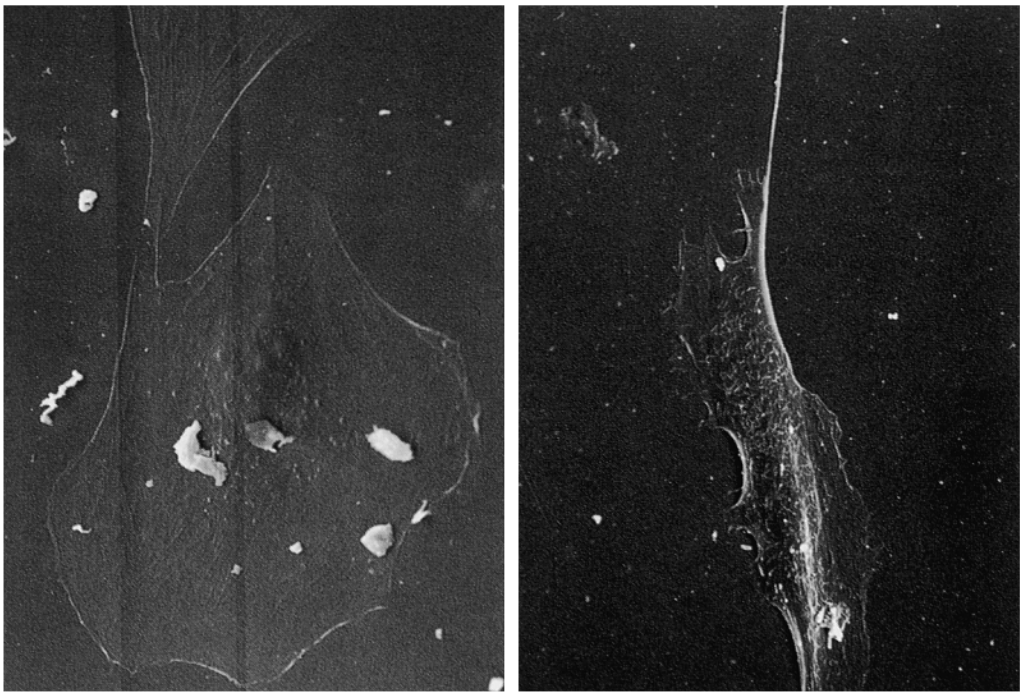

Fig. 2. Morphological features of non-confluent cells exposed to fluid shear stress. Control cells show a flat and polygonal shape (left, ×800). Cells after the application of the stress for 1 hour show a spindle shape. The diameter in the long axis of MC3T3-E1 cells that were subjected to fluid shear stress was larger than that of the control cells. The cell surface of the stressed cells exhibited an increase in the number of microvilli (right, ×800).

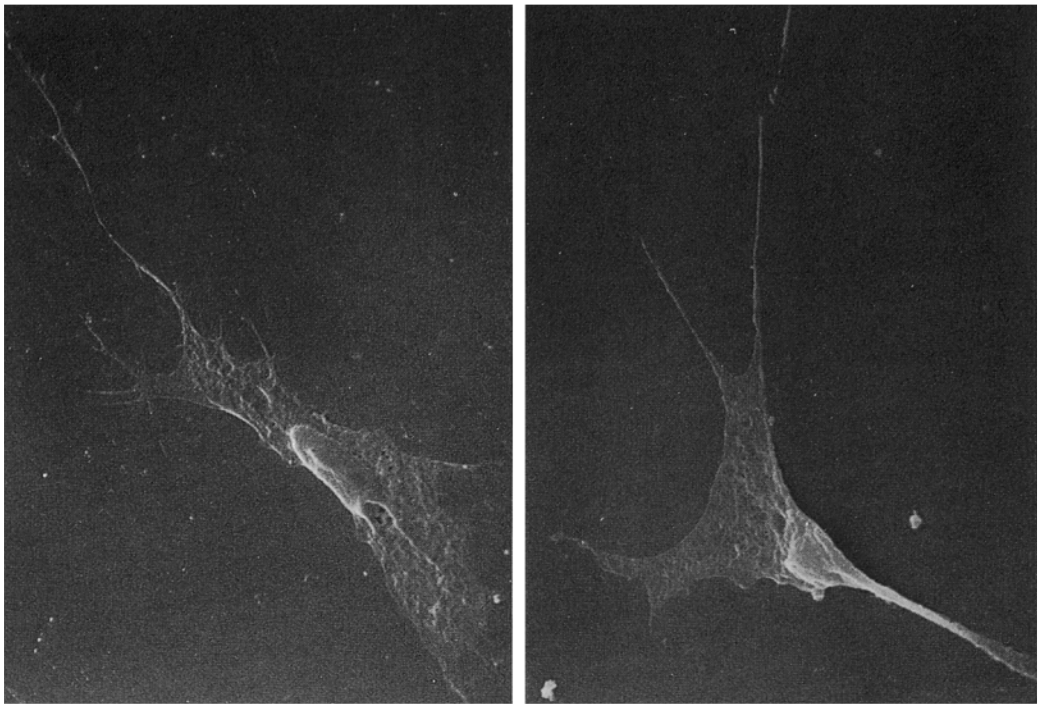

Fig. 3. Scanning electron microscopic photograph of the cells exposed to the stress for 6 hours shows the marked shrinkage of the cell and increased irregularity of the cell surface (left, ×800), and these changes were marked in the cells exposed to the stress for 12 hours (right, ×800).

was not significantly different between stressed cells and controls (2095.17±887.0 mm^2 vs. 2239.9±735.4 mm^2, $p=0.5775$). But, after exposure for 6 hours and 12 hours, the area of the cytoplasm of the stressed cells was smaller than that of the control cells (6-hour group, 1020.5±800.7 mm^2 vs. 1478.4±515.1 mm^2, $p=0.0379$; 12-hour group, 403.4±127.5 mm^2 vs. 1716.8±690.5 mm^2, $p<0.001$, Fig. 3B).

With regard to cell direction 1 hour after the stress, 47% of the cells were positioned nearly along the direction of the fluid shear stress, and classified in subgroup α. Fifty-three percent of stressed cells were not positioned along the direction of the fluid shear stress; 28% of the cells were classified in subgroup β, and 25% in subgroup γ.

Effect of fluid shear stress on changes in actin filaments of MC3T3-E1 cells

In the control cells, thin radial actin filaments were observed. In the cells subjected to the stress for 1 hour, the radial arrangement of the actin filaments had changed to parallel, and was positioned along the direction of the long axis of the cytoplasmic process (Fig. 5).

Effect of fluid shear stress on the resting level of $[Ca^{2+}]i$ *in MC3T3-E1 cells*

There was a significant difference in the resting level of $[Ca^{2+}]$i between the stressed and the control cells in the 5-minute group. The averaged resting level of $[Ca^{2+}]$i in stressed cells was 97.3±10.8 nM, and that in control cells was 77.8±

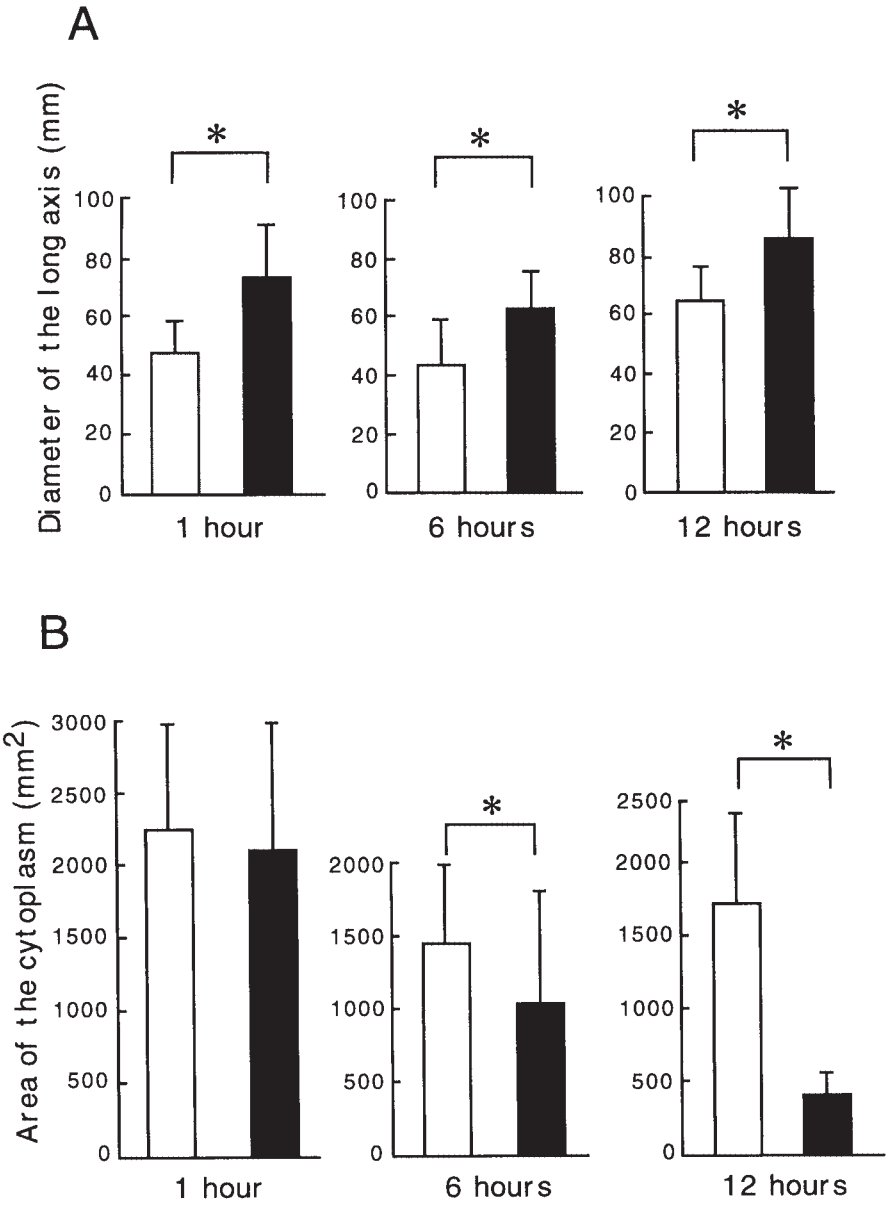

Fig. 4. Morphometrical data of non-confluent cells exposed to fluid shear stress. A: Changes in the length of cell processes after the stress. The diameters in the long axis of MC3T3-E1 cells that were subjected to the fluid shear stress for 1 hour (left), 6 hours (middle), and 12 hours (right) were significantly larger than those of the control cells (white bar, control; black bar, stressed group). B: Changes in the area of the cytoplasm after the stress. After 6 (middle) and 12 hours (right) of the fluid shear stress, the area of the cytoplasm of control cells was larger than that of stressed cells (white bar, control; black bar, stressed group).

10.8 nM ($p<0.001$). In the 1-hour and 6-hour groups, the resting level of $[Ca^{2+}]i$ in the stressed cells was still significantly higher than that in the control cells (1-hour group, 125.5 ± 15 nM vs. 77.5 ± 15 nM, $p<0.001$; 6-hour group, 93 ± 15 nM vs. 51.0 ± 3.16 nM, $p<0.001$). However, the resting level of $[Ca^{2+}]i$ in stressed cells was lower than that in the control cells in the 12-hour group (50.0 ± 4.79 nM vs. 75.0 ± 8.81 nM, $p<0.001$, Fig. 6).

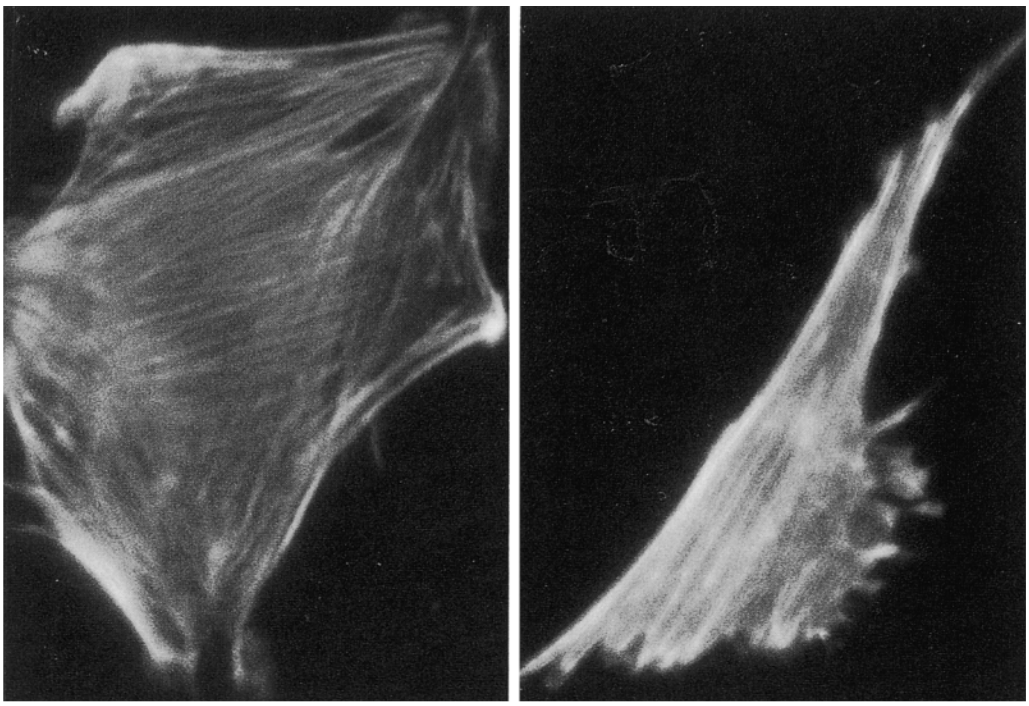

Fig. 5. Fluorescent microphotograph with phalloidin-FITC stain (original magnification, ×400). Control cells show radial actin filaments (left). Cells exposed to the stress for 1 hour show actin filaments parallel to the direction of the long axis of the cell processes.

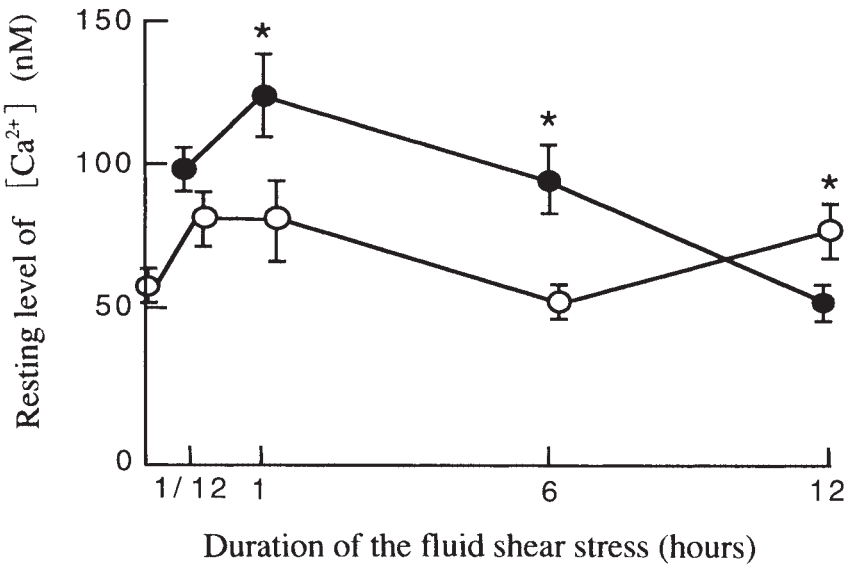

Fig. 6. Resting level of $[Ca^{2+}]i$ of the stressed and control cells. The $[Ca^{2+}]i$ of stressed MC3T3-E1 cells showed a peak after application of the stress for 1 hour and gradually decreased over time ($n=20$, $p<0.001$. open circle, control, closed circle, stressed cell).

Discussion

The present study showed that the application of fluid shear stress to MC3T3-E1 cells induced several morphological changes such as the spindle shape

with cell shrinkage, formation of long cell processes, and formation of microvilli on the cell surface. The stress also made about half of the cells to be positioned along the stress-applied direction, and actin filaments along the long axis of cell processes. In addition, the stress increased the resting level of $[Ca^{2+}]i$ in the cells.

The most characteristic morphological change was the spindle-like change with long cell processes. Palumbo et al. (1990) and Mikuni-Takagaki et al. (1995) suggested that partial elongation of the processes of osteoblasts is a sign of differentiation into osteocytes. However, morphological changes in the cell processes in the current study were observed in only 1 hour application of the fluid shear stress. Because 1 hour of stress is too short to induce cell differentiation (Mikuni-Takagaki et al. 1995), it is unlikely that differentiation from osteoblast to osteocyte could be induced by the fluid shear stress.

The present results obviously showed that continuous application of stress for 1 hour increased the resting level of $[Ca^{2+}]i$. In osteoblasts, fluid shear stress rapidly increased $[Ca^{2+}]i$ (Williams et al. 1994; Hung et al. 1995). In addition, it has been often observed that after exposure of neurons to Ca^{2+} channel agonists for a long time, $[Ca^{2+}]i$ was maintained at an increased level for a long time even after removal of agonists (our observations). Considering these facts, the present findings of the stress-induced increase in the resting level of $[Ca^{2+}]i$ might result from the induction of $[Ca^{2+}]i$ increase by the stress. Since the rise in $[Ca^{2+}]i$ has been thought to play the role of an intracellular messenger in the mechanical stress transduction mechanism (Hung et al. 1995), we speculated that osteoblastic MC3T3-E1 cells were mechanosensitive.

The present study firstly demonstrated that the stress induced an increase in the number of microvilli in osteoblastic cells, and prolonged fluid shear stress induced irregularity on cell surface with cell shrinkage. Since these morphological changes were also observed in endothelial cells under several cell-damaged conditions (Lossinsky et al. 1995), they might be features of cell damage of osteoblasts. Interestingly, the resting level of $[Ca^{2+}]i$ in the stressed cells showed a peak value at 1-hour application of stress and then gradually decreased with a prolongation of the application time. These data indicate that the intracellular response to shear stress increased with a short application time of stress but decreased with a longer application time. Irregularity on cell surface and cell shrinkage, namely cell damage, might be associated with the prolonged increase in the resting level of $[Ca^{2+}]i$ shown in this study.

Buckley et al. (1988) showed calvarial osteoblast-like cells positioned 90 degrees to the applied stretching stress. In endothelial cells, Remuzzi et al. (1984) revealed that the long axis of cell processes in almost all endothelial cells was positioned parallel to the direction of the fluid shear stress. However, only half of MC3T3-E1 cells had cell processes positioned parallel to the stress direction, and the arrangement of actin filaments after the application of the stress was parallel

to the long axis of the cell processes. This result suggests that the directional response of osteoblastic cells to fluid shear stress is somewhat different from that of endothelial cells, and furthermore there is a difference in directional response between shear stress and stretch stress in osteoblastic cells. The stress induced an increase of messenger RNA expression of TGF β1 in osteoblast-like cells (Sakai et al. 1998), and the increase was inhitied by both the cation channel blocker and an Ca^{2+} channel blocker. It could be assumed that an increase in $[Ca^{2+}]i$ in response to shear stress leads to the induction of TGF β1, and as a result, may promote actin re-arrangement, causing morphological changes. However, a relationship between these morphological and $[Ca^{2+}]i$ changes, and function of osteoblasts was not examined in the current study, since our observations were performed within 12 hours after the start of the stress. This relationship should be further investigated under a longer observations.

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