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ARTICLES

Fluid Flow-Induced Tyrosine Phosphorylation and Participation of Growth Factor Signaling Pathway in Osteoblast-Like Cells

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Abstract To investigate how mechanical loading stimulates bone cells, we subjected murine osteoblast-like cells, MC3T3E1, to fluid flow generated by shaking culture dishes. Since we had previously found that egr-1 mRNA is up-regulated by the flow, and that the regulation involves tyrosine kinase, we examined which proteins are tyrosine-phosphorylated by flow. Western blotting and immunoprecipitation of cell lysates showed tyrosine phosphorylation enhancement of many proteins, including ERK2 and Shc, and activation of ERK1/2. Although these responses did not occur in serum-free media, addition of EGF or bFGF recovered the responses. AG1478, an inhibitor of EGF receptor kinase activity, abolished tyrosine phosphorylation enhancement, ERK1/2 activation, and egr-1 mRNA accumulation induced by the flow of EGF-containing serum-free media. These results suggest that growth factor signaling pathways are involved in these responses. Repetition of fluid flow induced repeatedly up-regulation of egr-1 mRNA. Such events may also occur in bone under mechanical loading. J. Cell. Biochem. 76:529–538, 2000.

Key words: mechanical stress; EGF; bFGF; egr-1; Shc; ERK

The architecture and homeostasis of bone are maintained by mechanical loading, but the mechanism is unclear. Dodds et al. [1993] showed that the activity of glucose 6-phosphate dehydrogenase (G6PD) increased in osteoblasts and osteocytes immediately after forced mechanical loading of rat fibulae for 5 min. Lean et al. [1996] and Inaoka et al. [1995] found that c-fos mRNA increased on the bone surface and in osteocytes within 30 min of mechanical loading of rat tail vertebrae for 5 min. These immediate responses following mechanical loading suggest that bone cells respond directly to mechanical force.

Regarding culture systems, there are many studies concerning how bone cells respond to mechanical stimuli. Increases in prostaglandin release have been found by many researchers [Brighton et al., 1991; Klein-Nulend et al., 1995a,b, 1997; Ozawa et al., 1990; Reich and Frangos, 1991]. Increases in cAMP levels [Brighton et al., 1991; Carvalho et al., 1994; Reich et al., 1990; Sandy et al., 1989], NO release [Johnson et al., 1996; Klein-Nulend et al., 1995b; Pitsillides et al., 1995], or IP3 levels [Carvalho et al., 1994; Reich and Frangos, 1991; Sandy et al., 1989], change in membrane potential [Duncan et al., 1996; Salter et al., 1997], and localization of PKC, cytoskeletal proteins, or focal contact proteins [Carvalho et al., 1994] have also been reported by various researchers. These results were obtained using various stimulation methods, such as dish deformation, hydrostatic pressure, osmotic pressure, or fluid flow. Although differences in results between these distinct methods are not found, whether the same trigger is set off by different stimuli is unclear. Some discrepancies between results from in vivo, organ culture, and cell culture methods suggest the important role of the stimulation method.

In bone, a calcified tissue speculated to deform less than soft tissues under mechanical force, stress-induced flow through canaliculi is

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generated by cyclic mechanical loading [Dillaman et al., 1991; Piekaski and Munro, 1977]. Turner et al. [1994] inferred that bone formation by mechanical loading depends on the stress-induced fluid flow, based on their observation that both the bone formation rate and the stress-generated potential increase in proportion to the increase in loading frequency. Owan et al. [1997] showed that osteoblasts are more responsive to fluid forces than to mechanical strain in cell culture systems.

On the basis of these studies, we stimulated clonal murine osteoblast-like cells, MC3T3E1 [Sudo et al., 1983], with a flow of culture media. We had previously found that fluid flow induces an increase in egr-1 mRNA level, the induction being mediated by tyrosine kinase(s) and factor(s) in serum [Ogata, 1997]. To examine the signal transduction of fluid flow, we studied proteins which are tyrosine-phosphorylated and factors in serum which participate in the induction. In this paper, we show tyrosine phosphorylation enhancement of many proteins, including ERK2 and Shc, and activation of ERK1/2, in serum-containing media. Furthermore, we show that EGF and bFGF are candidates for the requisite factors in serum and that these growth factor signaling pathways are involved in the fluid flow-induced response.

MATERIALS AND METHODS

Materials

Epidermal growth factor (EGF) (recombinant human) and basic-fibroblast growth factor (bFGF) (recombinant human) were obtained from R & D System, Inc. (Minneapolis, MN). Tyrphostin AG1478 was obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP) conjugated anti-phosphotyrosine antibodies (PY20), anti-ERK1 antibodies (MK12), anti-Shc affinity purified polyclonal antibodies, and anti-Shc monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-active form ERK1/2 affinity-purified polyclonal antibody (anti-ACTIVE MAPK pAb) was obtained from Promega Corporation (Madison, WI). HRP-conjugated goat anti-rabbit IgG(H+L) was obtained from Kirkegaard & Perry Lab. Inc. (Gaithersburg, MD). HRP-conjugated goat anti-mouse IgG was obtained from Chemicon International Inc. (Temecula, CA). Protein G Plus/Protein A Agarose suspension was purchased from CALBIOCHEM-NOVABIOCHEM Co. (Cambridge, MA).

The cDNA for mouse egr-1 was provided by Dr. D. Nathans [Christy et al., 1988]. The cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was provided by Dr. Sevgi Rodan (Merck Research Laboratory, West Point, PA).

Cell Cultures

Murine osteoblast-like cells, MC3T3E1 [Sudo et al., 1983], were grown in α-MEM containing 5% fetal bovine serum (FBS) in 100-mm (for immunoprecipitation) or 60-mm (for Western blotting and Northern blotting) diameter polystyrene tissue culture dishes, without any additional treatment of the dishes, in a culture chamber at 37°C under 5% CO_2 in air. The media of cells in the sub-confluent were replaced and cells were cultured for another 16 h, followed by fluid flow experiments. Only in the experiment for Figure 5, culture flasks were used as described in the legend.

Fluid Flow Experiments

The flow of the culture media was generated by shaking the culture dishes on a shaker. Five hours before beginning the fluid flow, the media were replaced by the media indicated in the figure for each experiment, except the experiments shown in Figures 9 and 12. The fluid flow-loaded cells were then put on a shaker on the lower shelf in a culture chamber; non-loaded control cells were put on the upper shelf of the same chamber. The chamber was not disturbed for 5 h. The shaker was shaken horizontally with an amplitude of 3 cm at a rate of 60 shakes/min for 1 or 5 min, as described in each figure legend. In the experiment shown in Figure 9, the interval from media replacement to shaking differed as described in the figure legend. In the experiment shown in Figure 12, 16 h before the first shaking, the media were replaced. Shaking was performed nine times as described in the figure legend.

Cell Lysis for Protein Analysis

The dishes were shaken for 1 or 5 min. Immediately before shaking and 0.1, 2, 3, and 10 min after shaking, the dishes were put on ice and quickly washed three times with ice-cold PBS. Ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH7.4, 2 mM EDTA, 0.5% NP40, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 mM phenylmethylsulfunyl fluoride

(PMSF)) was then added and the dishes were incubated for 20 min on ice. The cell lysates were scraped, passed through a 26G needle, and transferred to test tubes, followed by centrifugation for 20 min at 15,000*g*. The supernatants were used for Western blotting or immunoprecipitation. These procedures were always performed at the same time for both the shaken cells and non-shaken control cells.

Western Blotting

To the supernatants containing 20 µg total protein, an equal volume of 2× sample buffer (0.125 M Tris.HCl pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 0.001% bromophenol blue) was added and the proteins were fractionated using 10% or 8% SDS-polyacrylamide (29:1 acrylamide/bisacrylamide) gel electrophoresis (SDS-PAGE) and electrotransferred onto PVDF membranes (Immobilon, MILLIPORE, Bedford, MA). The membranes were incubated in a blocking buffer consisting of wash buffer (see below) containing 1% BSA for blotting with PY20, or 5% non-fat dry milk for blotting with other antibodies. This was followed by incubation in a primary antibody solution, where HRP-PY20 (0.1 µg/ml) and anti-ACTIVE MAPK pAb (25 ng/ml) were diluted in a blocking buffer with 1% BSA, and anti-ERK1 (MK12, 50 ng/ml) and anti-Shc monoclonal antibodies (1 µg/ml) were diluted in a blocking buffer containing 5% non-fat dry milk. The membranes were washed five times, for 5 min each time, with wash buffer (10 mM Tris.HCl pH 7.6, 100mM NaCl, 0.1% Tween 20), and incubated with HRP-conjugated anti-rabbit IgG (50 ng/ml) diluted in a blocking buffer with 1% BSA for anti-ACTIVE MAPK pAb blots, and with HRP-conjugated anti-mouse IgG (1:5000) diluted in a blocking buffer with 5% non-fat dry milk for MK12 and anti-Shc monoclonal antibodies blots. This was followed by washing five times with wash buffer. The target proteins were detected using ECL Western blotting detection reagent (Ammersham Pharmacia Biotech, Buckinghamshire, UK).

Immunoprecipitation

The total protein 500 µg of the cell lysate supernatants was transferred to 1.5 ml microtest-tubes and adjusted to 1,000 µl with a lysis buffer. Anti-Shc polyclonal antibodies or normal rabbit IgG (2.5 µg) were added and incubation was done overnight at 4°C. Next, 15 µl Protein G Plus/Protein A Agarose suspension was added to each tube and incubated, with agitation, for 6 h at 4°C. The precipitates were washed five times with a lysis buffer. A 70 µl 2× sample buffer was added to 40 µl precipitates and boiled for 5 min at 100°C, followed by centrifugation. Seventy µl of the supernatants were used in SDS-PAGE and Western blotting.

Stripping of Blots and Re-Immunoblotting

Stripping of blots was performed by incubating the membranes for 30 min at 65°C in a solution containing 62 mM Tris-HCl pH 6.8, 2% SDS and 100 mM 2-mercapthoethanol. The stripped membranes were washed with wash buffer, followed by blocking, and re-immunoblotting.

RNA Extraction and Northern Blotting

The dishes were shaken for 1 min. Immediately before shaking or 30 min after shaking, the cells were denatured with 4M guanidinium isothiocyanate containing 0.7% 2-mercaptoethanol. Total RNA was extracted using the acid-guanidinium thiocyanate-phenol-chroloform method. Ten (^{32}P-labeling) or 20 (alkaline phosphatase [AP] labeling) µg of total RNA was fractionated by electrophoresis in 1% agarose/formaldehyde gels and transferred onto nylon filters. For ^{32}P-labeling, Gene Screen (Biotechnology System, Boston, MA) was used and fixed by UV light. For AP labeling, Hybond N$^+$ (Amersham Pharmacia Biotech) was used. This was fixed by soaking in 0.05N-NaOH for 5 min followed by rinsing in 2 × SSC for 60 sec and by baking in an oven at 80°C for 2 h. Pre-hybridization, hybridization, wash, and detection were performed according to the usual method for ^{32}P-labeling and the Alk Phos Direct kit (Amersham Pharmacia Biotech) protocol for AP labeling, except that a hybridization temperature of 62°C was used for AP labeling.

RESULTS

Media Flow Enhances Tyrosine Phosphorylation of Many Proteins, Including Shc and ERK1/2

The culture dishes of the clonal murine osteoblast-like cells, MC3T3E1, were shaken on a shaker as described in Materials and Methods. Figure 1 shows the effect of shaking on tyrosine phosphorylation in MC3T3E1 cells cultured in media containing 10% fetal bovine serum (FBS). The tyrosine phosphorylation of proteins of mo-

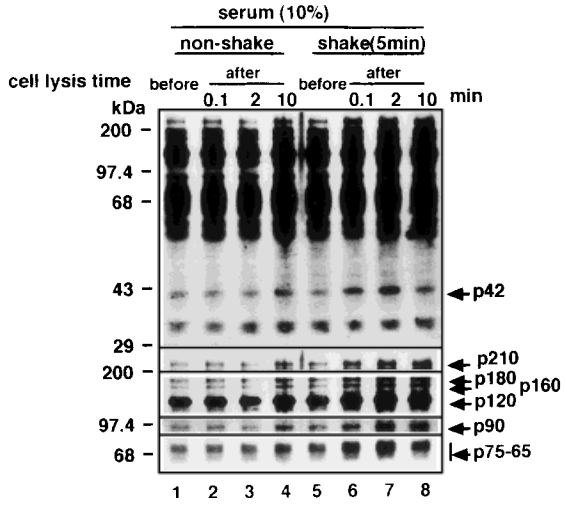

Fig. 1. Western blotting with anti-phosphotyrosine antibodies. MC3T3E1 cells were cultured in 10% FBS-containing αMEM for 5 h on a shaker for loaded cells **(lanes 5–8)**, or on an upper shelf for non-loaded control cells **(lanes 1–4)**, in the same chamber. Immediately before shaking, a dish in each group was lysed (lanes 1 and 5). After that, the dishes on the shaker were shaken for 5 min and a dish in each group was lysed successively 0.1, 2, and 10 min after shaking. The lysates were fractionated with 8% gel-SDS-PAGE and electroblotted onto a membrane, followed by immunoblotting with anti-phosphotyrosine antibodies. The top picture shows the whole image of the Western blots. The lower pictures show the images from different exposure times using the same filter to display the change in the tyrosine phosphorylation level from 210 to 65 kilodalton proteins. Molecular weights of the proteins in which tyrosine phosphorylation was enhanced after shaking are indicated with arrows at the right side of the picture.

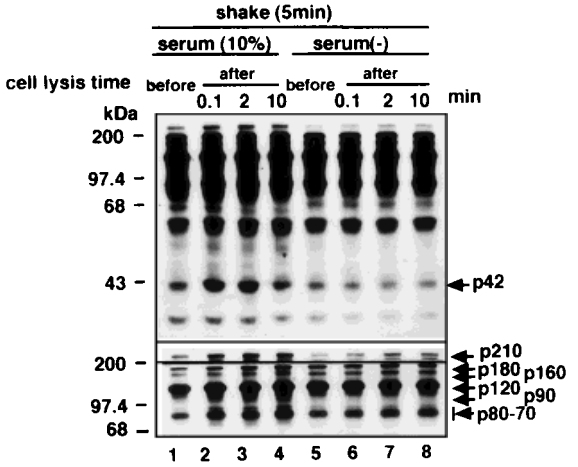

Fig. 2. Western blotting with anti-phosphotyrosine antibodies. MC3T3E1 cells were cultured in αMEM containing 10% FBS **(lanes 1–4)**, or non-containing FBS **(lanes 5–8)**, for 5 h on a shaker. A dish in each group was lysed immediately before shaking (lanes 1 and 5). After that, the remaining dishes were shaken for 5 min and a dish in each group was lysed successively 0.1, 2, and 10 min after shaking. The lysates were treated as described in Figure 1.

lecular weight of 210, 180, 160, 120, 90, 75–65, and 42 kilodaltons was enhanced 0.1 (lane 6), 2 (lane 7), and 10 (lane 8) min after shaking for 5 min, compared with that just before shaking (lane 5), while in non-shaken control cells (lanes 1–4) variation in tyrosine phosphorylation levels was hardly observed. A slight enhancement of tyrosine phosphorylation in lane 4 may be due to the effect of temperature or other changes.

Figure 2 shows the effect of FBS on tyrosine phosphorylation enhancement by shaking. In the cells cultured in 10% FBS-containing media (lanes 1–4), up-regulation of tyrosine phosphorylation after shaking was observed in proteins of the same molecular weight as those in Figure 1. In the cells cultured in non-FBS-containing media (lanes 5–8), where the tyrosine phosphorylation level as a whole was suppressed compared with the left 4 lanes, a difference in tyrosine phosphorylation levels before and after shaking was not observed. The bands of 80–65 kilodaltons showed a slight difference in the separation between experiments, although tyrosine phosphorylation was always increased after shaking.

The tyrosine phosphorylation enhancement after shaking of the 42-kilodalton protein was especially clear in both Figures 1 and 2. Since the molecular weight was close to that of ERK2 in MAP kinases, which mediate the signal transduction of various extracellular stimulations to nuclear events and are activated in endothelial cells subjected to shear stresses [Davies, 1995], Western blotting was performed with anti-active form ERK1/2 antibodies. The results showed that activation of ERK1/2 was induced in the cells cultured in 10% FBS-containing media after shaking (upper panel in Fig. 3), and if the media did not contain FBS, shaking did not induce ERK1/2 activation (lower panel in Fig. 3).

The tyrosine phosphorylation enhancement of proteins shown in Figures 1 and 2, and activation of ERK1/2, caused us to speculate that Shc, a key protein in signal transduction [van Biesen et al., 1995], is tyrosine-phosphorylated. A Shc immunoprecipitate showed enhancement of Shc tyrosine phosphorylation after shaking in cells cultured in 10% FBS containing media (upper panel in Fig. 4). This enhancement did not occur in non-FBS-containing media (lower panel in Fig. 4). Time-dependent

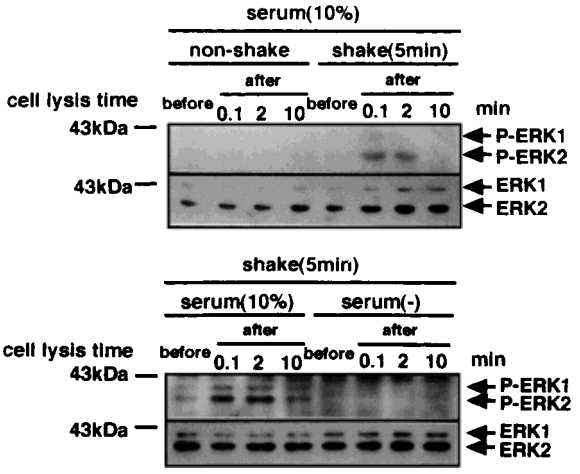

Fig. 3. Western blotting with anti-active form ERK1/2 antibodies. MC3T3E1 cells were cultured, loaded, and treated as described in Figure 1 **(upper panel)** or Figure 2 **(lower panel)**, except that a 10% gel was used. Immunoblotting was performed with anti-active form ERK1/2 antibodies (P-ERK1 and P-ERK2), then the blots were stripped and re-immunoblotting was performed with anti-ERK1 antibodies, which cross-react with ERK2.

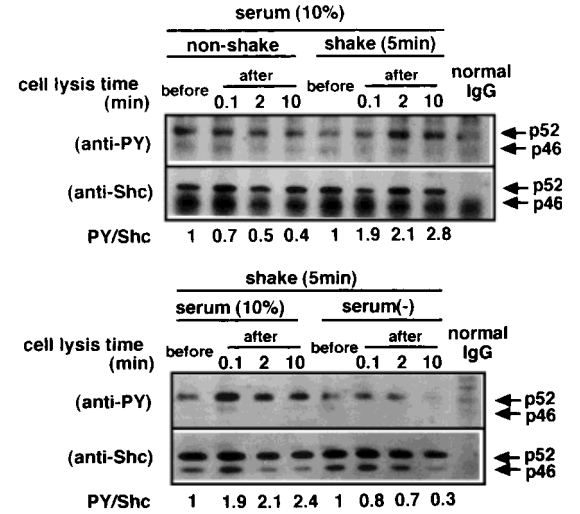

Fig. 4. Western blotting of anti-Shc antibody immunoprecipitates with anti-phosphotyrosine antibodies. The cells were treated as described in Figure 1 **(upper panel)** or Figure 2 **(lower panel)**. Immunoprecipitation with anti-Shc polyclonal antibodies of the cell lysates was performed. The immunoprecipitate was fractionated with 8% gel-SDS-PAGE and electroblotted onto a membrane, followed by immunoblotting with anti-phosphotyrosine antibodies (anti-PY). After the blots were stripped, re-immunoblotting was performed with anti-Shc monoclonal antibodies. The ratios of the density of bands (p52) blotted with anti-PY to that of bands (p52) blotted with anti-Shc were normalized with the value before shaking and are shown at the bottom of each figure.

decreases were observed in Shc tyrosine phosphorylation in 'non-shake' group (upper panel) and 'serum (-) group' (lower panel) in Figure 4. Although the reason is unclear, the tendency suggests that the Shc tyrosine phosphorylation effect of shaking may be larger than values shown in Figure 4.

To examine whether the up-regulation of tyrosine phosphorylation was caused by media flow or by other forces, we shook the culture flasks filled with media in order to prevent medium movement by shaking. In cells cultured in culture flasks with 10% FBS containing media of 2.5 ml, activation of ERK1/2 was observed after shaking (lanes 1–3 in Fig. 5), while in cells cultured in culture flasks filled with 10% FBS-containing media of 42 ml, ERK1/2 activation after shaking was not observed (lanes 4–6 in Fig. 5). These results suggest that the responses after shaking described above are induced through medium flow.

Addition of EGF or bFGF to Serum-Free Media Restores the Cell Responses to Fluid Flow

The results shown in Figures 1 to 5 show that fluid flow activates signaling pathways similar to those of growth factors. Therefore, to examine whether EGF participates in fluid flow signaling, we added EGF (1 ng/ml) to serum-free media. When the cells cultured in EGF-containing serum-free media were subjected to flow for 1 or 5 min, enhancement of tyrosine phosphorylation and activation of ERK1/2 were observed

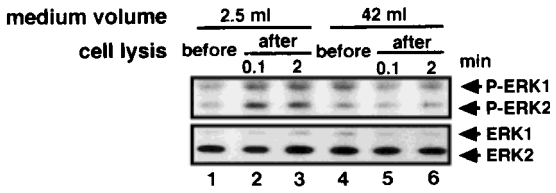

Fig. 5. Effect of fluid flow on ERK1/2 activation. The cells were cultured in culture flasks (culture area of 12.5 cm^2) with αMEM of 2.5 ml containing 10% FBS, where media were moved by shaking **(lanes 1–3)**, or in culture flasks (culture area of 12.5 cm^2) with αMEM of 42 ml containing 10% FBS, where media did not move due to the fullness of the flasks with media **(lanes 4–6)**, for 5 h on a shaker. A flask in each group was lysed immediately before shaking (lanes 1 and 4). After that, the remaining flasks were shaken for 5 min and a flask in each group was lysed successively 0.1 and 2 min after shaking. The cell lysates were fractionated with 8% gel-SDS-PAGE and electroblotted onto a membrane. The membrane was immunoblotted as described in Figure 3.

to be similar to those observed in cells cultured in FBS-containing media. As we noted that there was a time lag between proteins when tyrosine phosphorylation levels peaked after shaking for 1 min, we showed the results from 1 min flow of serum-free media containing EGF in Figures 6 and 7. The most striking protein was p42, in which tyrosine phosphorylation enhancement was not observed until 3 min after shaking. Activation of ERK1/2 took also 3 min. This was slower in comparison with the other occurrences of tyrosine phosphorylation which were induced immediately after shaking. Addition of EGF also restored up-regulation of egr-1 mRNA by fluid flow, which did not occur in serum-free media, especially clearly at an EGF concentration of 0.1 to 3 ng/ml (data not shown), although we had previously shown that fluid flow-induced egr-1 mRNA up-regulation did not occur in the presence of 10 ng/ml EGF [Ogata, 1997].

As only EGF participating in the cell response to fluid flow was unlikely, we examined the effect of basic-FGF (b-FGF) on fluid flow-induced tyrosine phosphorylation and ERK1/2 activation. As shown in the right four lanes in Figure 8, tyrosine phosphorylation enhancement and ERK1/2 activation were induced by a flow of bFGF-containing serum-free media, which seemed to be stronger than those in media containing EGF at the same concentration shown in the left four lanes.

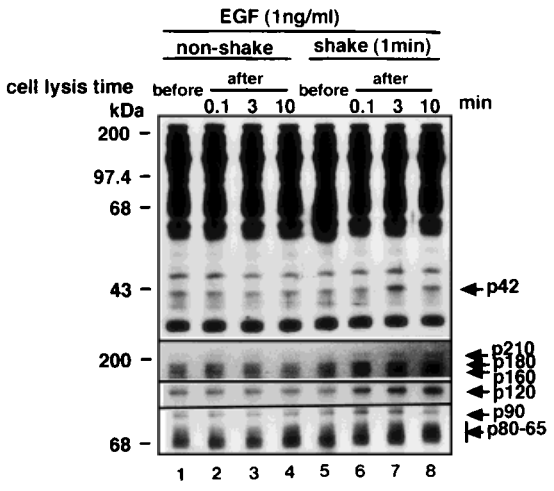

Fig. 6. Western blotting of the cells cultured in EGF-containing media with anti-phosphotyrosine antibodies. The cells were cultured in αMEM containing 1 ng/ml EGF for 5 h on a shaker for loaded cells **(lanes 5–8)**, or on an upper shelf for non-loaded cells **(lanes 1–4)** for 5 h. Immediately before shaking, a dish in each group was lysed (lanes 1 and 5). After that, the dishes on the shaker were shaken for 1 min and a dish in each group was lysed successively 0.1, 3, and 10 min after shaking. The lysates were treated as described in Figure 1.

The Cell Response to Fluid Flow Restores Within 1 h After EGF Addition and the Inhibitor of EGF Receptor Kinase Activity Blocks the Response

To explore the role of these growth factors in the cell response, we examined how long it takes to restore the response after EGF addition to serum-starved cells. Thirty minutes and 1 h after the addition of EGF, when the activated state of ERK1/2 by the EGF addition had been retained (compare lanes 1 and 3 with lane 5 in Fig. 9), activation of ERK1 was observed after shaking (lanes 2 and 4), although the rate of the activation was a little, similarly in two separated experiments. ERK2 activation by shaking 1 h after the EGF addition was observed more clearly (lane 4). Fifteen minutes after the addition of EGF, up-regulation of ERK1/2 activity was not observed (data not shown). However, this may be a result of EGF addition disturbing the response to shaking rather than conditions for the response not being primed within 15 min. In any case, as it takes at least 1 h to synthesize de novo proteins after growth factor treatment [Alberts, 1994], restoration within 1 h after EGF addition suggests that de novo protein synthesis due to EGF addition is not involved in the restoration of the response after shaking.

To confirm the participation of the EGF signaling pathway in the cell response to fluid flow, we examined whether the cells treated with AG1478, an inhibitor of EGF receptor kinase activity [Osherv et al., 1994], were stimulated by a flow of EGF-containing serum-free media. As shown in Figure 10, in the cells treated with dimethyl sulfoxide (DMSO) alone (lanes 1–4), tyrosine phosphorylation of p210, p180, p120, p90, and p75–65 and activation of ERK1/2 increased after shaking, although the decrease of the tyrosine phosphorylation enhancement seemed to be faster and tyrosine phosphorylation of the 160 kilodalton protein was not detected, perhaps due to DMSO. On the other hand, in the cells treated with AG1478 (lanes 5–8), these responses were not induced. The egr-1 mRNA up-regulation by fluid flow likewise was blocked by AG1478 (Fig. 11). The specificity of AG1478 for the EGF receptor was confirmed by lack of effect of AG1478 on ERK1/2 activation after shaking in b-FGF containing serum-free media (lower panel in Fig. 10).

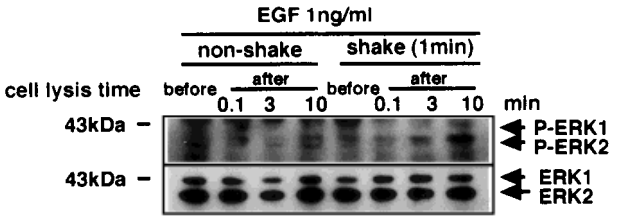

Fig. 7. Western blotting of the cells cultured in EGF-containing media with anti-active form ERK1/2 antibodies. The cells were treated as described in Figure 6. Western blotting, stripping, and re-immunoblotting were performed as described in Figure 3.

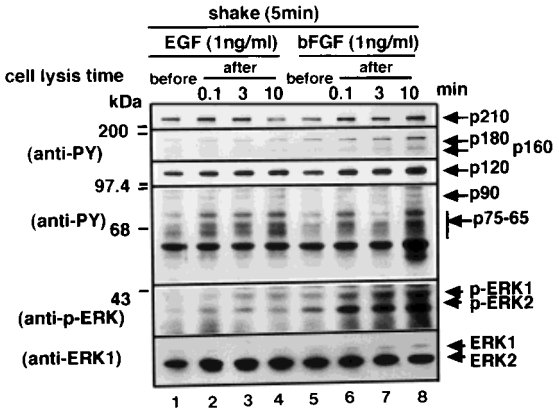

Fig. 8. Fluid flow-induced tyrosine phosphorylation and ERK1/2 activation in the cells cultured in bFGF-containing media. The cells were cultured in αMEM containing 1 ng/ml EGF **(lanes 1–4)** or 1 ng/ml bFGF **(lanes 5–8)** for 5 h on a shaker. Immediately before shaking, a dish in each group was lysed (lanes 1 and 5). After that, the remaining dishes were shaken for 5 minutes and a dish in each group was lysed successively 0.1, 3, and 10 min after shaking. The lysates were fractionated with 8% gel-SDS-PAGE and electroblotted onto a membrane. The membrane was cut at 43 kilodaltons. The upper part was immunoblotted with anti-phosphotyrosine antibodies and the lower part was immunoblotted with anti-active form ERK1/2 antibodies and anti-ERK1/2 antibodies as described in Figure 3.

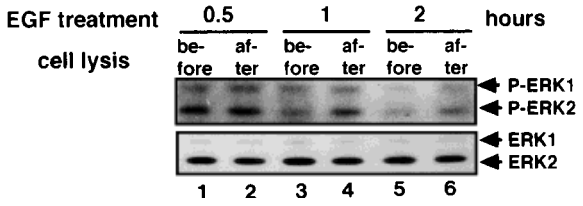

Fig. 9. Time course of ERK1/2 activation by shaking after EGF addition to serum-starved cells. After the cells were cultured in αMEM for 18 h, culture media were changed to αMEM containing 5 ng/ml EGF 0.5, 1, and 2 h before shaking and put on a shaker. A dish in each group was lysed immediately before shaking and the remaining dishes were lysed 2 min after shaking for 5 min. Western blotting of the cell lysate was performed with anti-active form ERK1/2 antibodies and anti-ERK1 antibodies as described in Figure 3.

Repetition of Fluid Flow Repeatedly Up-Regulates egr-1 mRNA

From the results described above we speculated that cells could be stimulated repeatedly through the repetition of flow without new addition of growth factor. As we had previously found that fluid flow-induced egr-1 mRNA elevation peaked 30 min after shaking and returned to the basal level after 60 min [Ogata, 1997], we subjected cells to 1 min shaking at hourly intervals, repeatedly. The egr-1 mRNA level increased repeatedly 30 min after each shaking (Fig. 12). Although this up-regulation seemed to decrease gradually with repetition, the decrease may be reduced by changing the shaking conditions, because this desensitization may be due to the same mechanism as egr-1 mRNA decrease by shaking for 120 min [Ogata, 1997].

DISCUSSION

In this study, to explore the mechanism by which mechanical loading on bone stimulates bone cells, we stimulated clonal murine osteoblast-like cells, MC3T3E1, using media flow generated by shaking culture dishes, because fluid flow is a candidate for forces subjected on bone cells by mechanical loading. Since we had previously found that egr-1 mRNA is up-regulated by the shaking, and that the regulation involves tyrosine kinase, we examined which proteins are tyrosine-phosphorylated by shaking. The results showed that this stimulation induces enhancement of tyrosine phosphorylation of p210, p180, p160, p120, p90, p80–65, and Shc, as well as activation of ERK1/2 (Figs.1, 3, and 4). Besides fluid flow, shaking culture dishes may affect cells through vibration, acceleration or other factors. However, non-response to shaking in cells cultured in culture flasks filled with media, where media did not move, suggested that the force inducing these responses is fluid flow (Fig. 5). The flow generated

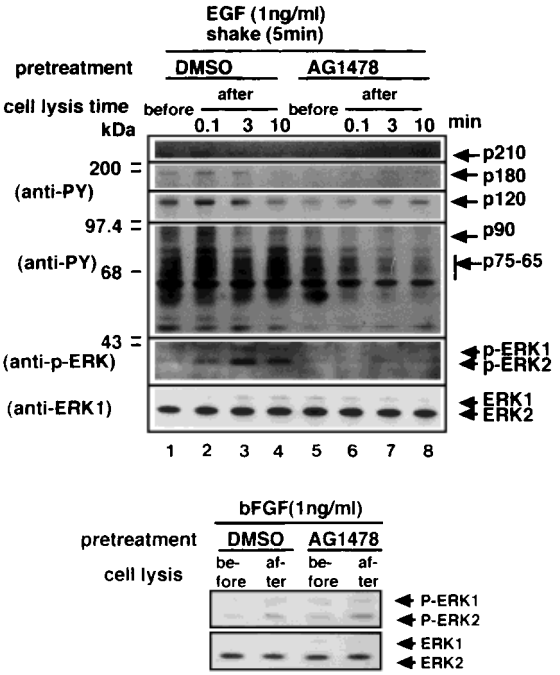

Fig. 10. The effect of EGF receptor kinase activity inhibitor, AG1478, on tyrosine phosphorylation and ERK1/2 activation. The cells were cultured in αMEM containing 1 ng/ml EGF **(upper panel)** or 1 ng/ml bFGF **(lower panel)** and AG1478 (0.5 μM) or vehicle (DMSO) for 5 h on a shaker, and treated as described in Figure 8 or 9.

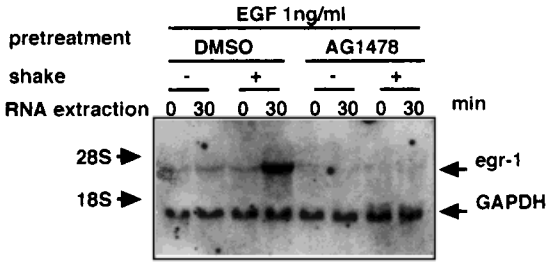

Fig.11. The effect of EGF receptor kinase activity inhibitor, AG1478, on egr-1 mRNA up-regulation. The cells were cultured in αMEM containing 1 ng/ml EGF and AG1478 (0.5 μM) or vehicle (DMSO), on a shaker for loaded cells (indicated with shake +), or on an upper shelf for non-loaded cells (indicated with shake −), for 5 h. Immediately before shaking, total RNA was extracted from a dish in each group (indicated with RNA extraction 0). After that, the dishes on the shaker were shaken for 1 min. Then, 30 minutes after shaking, total RNA was extracted from a dish in each group (indicated with RNA extraction 30). AP labeling was performed for Northern blotting.

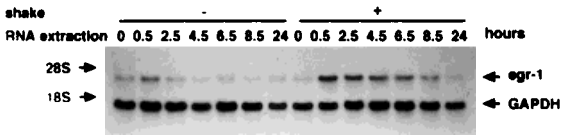

Fig.12. The effect of repeated fluid flow on egr-1 mRNA level. The cells were cultured in αMEM containing 10% FBS for 16 h on a shaker for loaded cells (indicated with shake +), or on an upper shelf for non-loaded cells (indicated with shake −). Shaking was performed nine times at hourly intervals for 1 min each time. Immediately before the first shake, total RNA was extracted from a dish in each group (RNA extraction 0). Thirty minutes after the first shake, total RNA was extracted from a dish in each group (RNA extraction 0.5). Although the second shake was performed 1 h after the first shake, RNA was not extracted. Thirty minutes after the third shake, which was 2 h after the first shake, RNA was extracted from a dish in each group (RNA extraction 2.5). In the same manner, shaking was further performed 3, 4, 5, 6, 7, and 8 h after the first shake and RNA was extracted 30 min after shaking at 4, 6, and 8 from a dish in each group (RNA extraction 4.5, 6.5, 8.5). Twenty-four hours after the first shake, RNA was extracted without shaking. The same treatment was performed at the same time for both loaded cells and non-loaded cells. ^{32}P labeling was used for Northern blotting.

in this study was irregular. The average flow rate was estimated to be 10 mm/sec with a distribution between 0 and 30 mm/sec, using a video connected to a stereoscope [Ogata, 1997]. This average rate is about 10 times the flow rate in the Haversian canals [Dillaman et al., 1991]. This rate was used on the basis of the limitations of our apparatus, though preliminary experiments suggest that a slower rate and flow using other methods also can induce these responses.

Although these responses were not observed in cells cultured in serum-free media (Fig. 2), addition of EGF to serum-free media restored fluid flow-induced tyrosine phosphorylation enhancement, ERK1/2 activation, and egr-1 mRNA accumulation (Figs. 6, 7, and 11). The enhancement of tyrosine phosphorylation of p210, p180, p160, p120, p90, and p80–65 was induced immediately after shaking for 1 min, but the tyrosine phosphorylation enhancement of p42 and the activation of ERK1/2 took 3 min. Furthermore, the peak of the egr-1 mRNA up-regulation occurred 30 min after shaking [Ogata, 1997]. These results suggest that fluid flow signals sequentially, through tyrosine phosphorylation of proteins including Shc and then MAP kinase activation. This signal is probably transduced to nuclear events, although the relationship between MAP kinase activation and egr-1 mRNA up-regulation remains unclear. Since b-FGF also enables fluid flow to induce tyrosine phosphorylation enhancement and ERK1/2 activation (Fig. 8), various growth factors in serum are presumed to be involved in this response.

The mechanism of the EGF effect remains unclear. The first possibility is that EGF primes cells to respond to fluid flow. Although de novo protein synthesis by EGF addition is speculated not to participate, judging by our result that within 1 h after EGF addition ERK1/2 activation by fluid flow is observed (Fig. 9), localization, or aggregation of cytoskeletal molecules or signal transduction-related molecules, which occur within a few minutes after growth factor stimulation, may be necessary for these responses. The second is that fluid flow accelerates the transportation of EGF to EGFR. If internalization of EGF-binding EGFR is faster than EGF diffusion in the media, fluid flow may up-regulate EGF signaling, such as the relationship between ATP and ATP receptors in fluid flow-induced Ca elevation in endothelial cells [Dull and Davies, 1991; Mo et al., 1991]. The third is that shear stress generated by fluid flow has an effect. As EGFR is an actin binding protein [den Hartigh et al., 1992; Gronowski and Bertics, 1995], aggregates with integrin and collaborates with integrin in intracellular signaling [Miyamoto et al., 1996], changes induced by shear stress in the cell shape or in binding state between integrin and extracellular matrix may stimulate EGF signaling. The fourth is that, as the cell membrane is dynamic and flexible, fluid flow may change the conformation or aggregation of EGFR, which is dimerized and activated by EGF binding, followed by the dissociation of the dimer and the formation of a new secondary dimer with an EGFR family member [Gamett et al., 1997; Graus-Porta et al., 1997]. The fifth is that, as fluid flow induces increases in NO release [Johnson et al., 1996; Klein-Nulend et al., 1995b], prostaglandin release [Klein-Nulend et al., 1995b, 1997; Reich and Frangos, 1991], cAMP levels [Reich et al., 1990], and IP_3 levels [Reich and Frangos, 1991], such mediators may stimulate the EGF signaling pathway.

Enhancement of tyrosine phosphorylation of many proteins including Shc, activation of ERK1/2 and up-regulation of egr-1 mRNA induced by fluid flow are similar to cell responses to growth factors. Although these signalings by growth factor addition are transient, repetition of fluid flow repeatedly up-regulated egr-1 mRNA without new addition of growth factors (Fig. 12). Such events may also occur in bone under mechanical loading.

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