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Hydrostatic Pressures Promote Initial Osteodifferentiation With ERK1/2 Not p38 MAPK Signaling Involved

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ABSTRACT

Mechanical stress has been considered to be an important factor in bone remodeling and recent publications have shown that mechanical stress can regulate the direction of stem cell differentiation. The exact mechanobiological effects of pressure on initial osteodifferentiation of mesenchymal stem cells (MSCs) have not been determined. These mechanobiological mechanisms may be important both in biological responses during orthodontic tooth movement and in the development of new mechanobiological strategies for bone tissue engineering. We investigated the effects of static (23 kPa) or dynamic (10–36 kPa and at 0.25 Hz frequency) pressure on MSCs during the initial process of osteoblastic differentiation following treatment with dexamethasone, β-glycerophosphate and ascorbic acid (for 0, 3, and 7 days, respectively). The following parameters were analyzed in the ALPase activity, mRNA level of osteogenesis-related transcription factors (Runx2, Osterix, Msx2, and Dlx5), and phosphorylation of ERK1/2 and p38 MAPK. The results showed that exposure to either dynamic or static pressure induced initial osteodifferentiation of MSCs. Particularly both types of pressure strongly stimulated the expression of osteogenesis-related factors of totally undifferentiated MSCs. ERK signaling participated in early osteodifferentiation and played a positive but non-critical role in mechanotransduction, whereas p38 MAPK was not involved in this process. Furthermore, the undifferentiated MSCs were highly sensitive to pressure exposure; whereas after osteoinduction MSCs reacted to pressure in a lower response state. The findings should lead to further studies to unveil the complex initial biological mechanisms of bone remodeling and regeneration upon mechanical stimuli. J. Cell. Biochem. 107: 224–232, 2009.

KEY WORDS: EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK); MESENCHYMAL STEM CELLS (MSCS); MECHANOBIOLOGY; OSTEOGENIC DIFFERENTIATION

Mechanical stimuli are responsible for the bone remodeling during orthodontic tooth movement. Recent evidences have indicated that mesenchymal stem cells (MSCs) may play a key role in the initiation of this process. Force-subjected MSCs in periodontal ligament may differentiate into osteoblasts and at the same time induce osteoclastogenesis, causing bone apposition and resorption, respectively [Masella and Meister, 2006]. Currently, most studies on the effects of mechanical stress focus on the late stages of osteoblast differentiation examining either osteoblasts or osteoblast-like cells. An important area not yet defined is the role of mechanical stress regulating MSC differentiation into osteoblasts. Evaluating the initial effects of mechanical stress on MSCs could provide insight into the mechanisms both of orthodontic tooth movement and bone regeneration.

It has been well accepted that the culture micro-environment of stem cells has a significant influence on differentiation and phenotypic expression. Mechanical signals can regulate the direction of stem cell differentiation [Altman et al., 2002; Estes

Abbreviations used: ALPase, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MSCs, mesenchymal stem cells; OS, osteogenic supplements; Osx, Osterix; PBS, phosphate-buffered saline.

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et al., 2004; Huang et al., 2004]. Strain alone can induce a significant increase in bone morphogenetic protein-2 (BMP-2) mRNA levels in human MSCs without the addition of osteogenic supplements (OS) [Sumanasinghe et al., 2006]. Undifferentiated human MSCs are highly sensitive to cyclic tensile strain which transcriptionally controls early osteo-chondrogenic response in vitro [Friedl et al., 2007]. However, little is known about the exact mechanobiological effects of dynamic and static pressures on early osteodifferentiation of MSCs.

Essential to cellular commitment to a differentiation lineage is the activation of defined transcription factors. Studies have shed light on the sequential steps in the osteoblast differentiation pathway, and revealed that Runx2 and Osterix (Osx) are two essential transcription factors in this pathway [Komori et al., 1997; Otto et al., 1997; Nakashima et al., 2002, 2003]. Moreover, Msx2 and Dlx5, two early transcription factors upstream of Osx, also regulate osteoblast differentiation [Cheng et al., 2003; Lee et al., 2003].

The mitogen-activated protein kinase (MAPK) pathways are closely related to osteodifferentiation. Runx2 can be phosphorylated and activated by MAPK pathways [Xiao et al., 2000, 2002; Franceschi and Xiao, 2003]. BMP-2 and insulin-like growth factor-I can mediate Osx expression in human MSCs through the MAPK and protein kinase D signaling pathways [Celil and Campbell, 2005].

The objective of this study was to examine the effects of mechanical stress on the initial osteodifferentiation of MSCs. We investigated the effects of static and dynamic pressures on MSCs during the initial process of osteodifferentiation resulting from treatment with OS—dexamethasone, β-glycerophosphate and ascorbic acid (for 0, 3, and 7 days, respectively). The studies were designed to correlate changes in molecular pathways associated with osteodifferentiation of MSCs with different types of pressure.

MATERIALS AND METHODS

CELL CULTURE

MSCs were isolated from bone marrow of 2-week-old male Sprague–Dawley rats, as reported previously [Friedenstein et al., 1992; Lennon and Caplan, 2006]. Briefly, both femora and tibias were removed and soft tissues were detached. Metaphysis from both ends were resected and bone marrow cells were collected by flushing the diaphysis with 2 ml/bone of Eagle's alpha minimum essential medium (α-MEM; Gibco) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone), 100 U/ml penicillin and 100 mg/ml streptomycin. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a syringe needle (18-gauge). Cells were resuspended in 5 ml of complete medium, plated in a 25 cm^2 glass tissue culture flask (plastic adherent populations isolated from bone marrow are shown to be functionally heterogeneous [Phinney and Prockop, 2007]) and cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. After 2 days, the culture medium and non-adherent cells were removed. The medium was changed two or three times a week. As the culture reached almost complete confluence, cells were subcultured or plated for subsequent experiments. MSCs were identified as CD44(+), CD54(+), and CD34(−); furthermore, MSCs cultured in adipogenic, osteogenic, or chondrogenic media differentiated into adipocytes, osteoblasts, and chondrocytes, respectively (data not shown).

MSCs (passage 2–4) were seeded at approximately 1×10^4 cells/cm^2 on culture dishes (diameter 60 mm, Corning) in a culture medium composed of α-MEM medium plus 10% defined FBS and cultured until subconfluence occurred. After this period, cells were grown in the culture medium alone or in osteogenic medium consisting of the same culture medium with the addition of OS—10 nM dexamethasone, 10 mM β-glycerolphosphate, and 0.05 mM 2-phosphate-ascorbic acid (Sigma). To investigate the effects of mechanical stress on MSCs during their initial osteodifferentiation, we used MSCs that had not yet expressed obvious osteoblastic phenotype such as alkaline phosphatase (ALPase) activity at the stages of 0-, 3- and 7-day-culture (OS-0d, OS-3d and OS-7d) [Koike et al., 2005] (Fig. 1).

DYNAMIC AND STATIC PRESSURE EXPERIMENTS

A custom-made, computer-operated dynamic and static pressure system was designed, fabricated and used in the present study (Fig. 2A). The pressure system exposed cells to mechanical stimulation by increasing the pressure of the gaseous phase above the supernatant media, as well as used by other scholars [Nagatomi et al., 2001]. Briefly, a computer, with software specially written for this system, controlled and maintained a dynamic or static pressure environment inside a sealed chamber, which housed standard tissue-culture plasticware with cells. During experiments with cells, the pressure system (except for the computer and electronic components) was maintained under standard cell culture conditions, that is, a 37°C, humidified, 5% CO_2/95% air environment. MSCs (passage 2–4), seeded at 1×10^4 cells/cm^2 and cultured until subconfluence, were grown in the culture medium alone or in OS for 3 or 7 days. Then OS medium was changed into ordinary culture

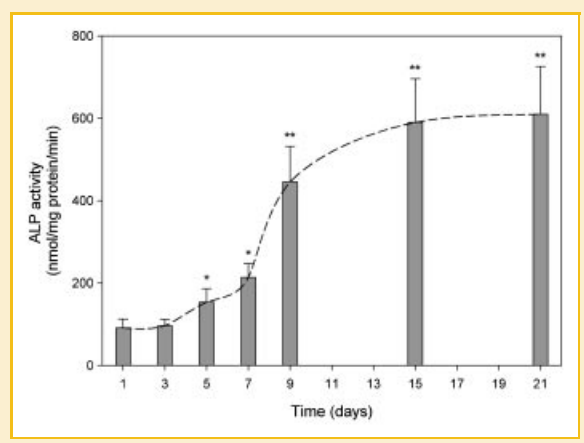

Fig. 1. Time course of alkaline phosphatase (ALPase) activity in osteoinduced rat MSCs. The changing tendency showed as an S-shaped line. During the first 7 days, the ALPase activity had a small rise but remained in low levels. After that, a two- to threefold increase followed. To examine MSCs during their initial osteodifferentiation, we used MSCs at the stages of 0-, 3-, and 7-day-culture (OS-0d, OS-3d, and OS-7d) which expressed low levels of ALPase activity. The values are mean ± SD. There were significant differences of $^*P < 0.05$ and $^{**}P < 0.01$.

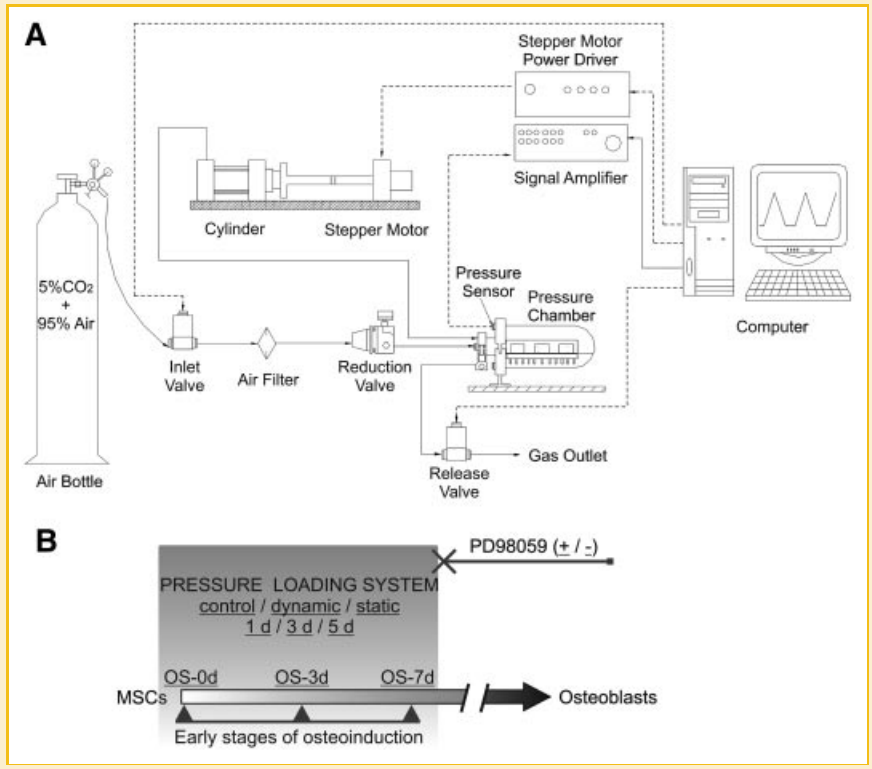

Fig. 2. A: Schematic diagram of the computer-operated pressure system used in this study. During each experiment, the cells were maintained under α-MEM (containing 10% fetal bovine serum) in a tissue-culture dish or plate, which was placed in the sealed pressure chamber. The pressure of the gas phase above the culture medium was monitored via a pressure transducer and controlled by operating inlet and release solenoid valves using computer software specially written for this purpose. In this schematic diagram of the laboratory setup, the electric and pneumatic connections are designated by solid lines; and electrical signals are designated by dash lines. B: Experimental design. OS represents osteogenic supplements.

medium right before OS-3d and OS-7d MSCs were exposed to pressure in order to examine the exact mechanobiological responses of MSCs and exclude the effects of osteogenic agents. Cells were exposed to dynamic (10–36 kPa, at 0.25 Hz frequency and with a sinusoidal wave) or static (23 kPa) pressure, respectively, for 1 h daily for the next one, three and five consecutive days. Control cells were cultured on the same dishes and kept in the similar culture conditions and time periods without loading pressure. During these experiments culture medium was initially changed before loading pressure and subsequently half changed every 2 days in the following time periods. In some experiments, cell cultures were treated with 10 μM PD98059 (Sigma) for 4 h before pressure treatment and afterwards to inhibit ERK activity. Figure 2B schematically outlines the experimental protocol.

ASSAY OF ALPASE ACTIVITY

After exposure to pressure, cells were washed twice with PBS and harvested in 100 μl of PBS by scraping with a rubber policeman. After three consecutive freeze-thaw cycles the cells were disrupted by sonication and centrifuged at 1,500*g* for 5 min at 4°C. The supernatants were then used for ALPase assay and protein determination. ALPase activity was measured using the ALPase test kit (DiaSys. Diagnostic Technology, Shanghai, China), and the absorbance at 405 nm was read on a spectrophotometer. Protein concentration was determined with a BCA protein assay kit (Pierce) with bovine serum albumin as the standard. The enzyme activity was expressed as nanomoles of *p*-nitrophenol produced per minute per milligram of protein (nmol/min/mg protein).

REAL-TIME RT-PCR ANALYSIS

After loading pressures, cells were washed twice with PBS. The total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified, in a spectrophotometer, at an absorbance (A) of 260 nm. The RNA samples had an A_{260}:A_{280} ratio of 2.0 to guarantee high purity. Two micrograms of total RNA from each sample were subjected to reverse transcription using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer's protocol. Each real-time PCR was carried out in triplicate in a total of 20 μl reaction mixture (2 μl of cDNA, 10 μl of SYBR® Premix Ex Taq™, 0.4 μl of ROX Reference Dye II, 0.4 μl of each 10 μM forward and reverse primers, and 6.8 μl of H_2O) in an ABI PRISM 7300 Real-time PCR System. Primers used for real-time PCR analysis are presented in Table I. The PCR program was initiated by 10 s at 95 °C before 40 thermal cycles, each of 5 s at 95°C and 31 s at 60°C. The starting copy numbers of unknown samples were calculated by the 7300 System SDS Software from the standard curve. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was concurrently amplified in each sample as control and was used for normalization. The cDNA of

TABLE I. Real-Time RT-PCR Primers Used in the Experiments

Target	Primer sequence	PCR product (bp)	GenBank acc.
Runx2	Forward: 5′-TCACCTCGTCTCAGACTTCAGG-3′; reverse: 5′-GGGTCTTCCCAGTATGGAGCTA-3′	94	AF325502
Osx	Forward: 5′-CCACCCATTGCCAGTAATCTTC-3′; reverse: 5′-TTCCCAGGGCTGTTGAGTC-3′	172	NM_001037632
Msx2	Forward: 5′-GAGTTCTCCAGCTCTCTGAACCT-3′; reverse: 5′-AGTTGATAGGGAAGGGCAGACT-3′	163	NM_012982
Dlx5	Forward: 5′-AGGCTTATGCGGACTACGGCTAC-3′; reverse: 5′-TTGGTTTACCATTCACCATCCTCA-3′	135	NM_012943
GAPDH	Forward: 5′-GCAAGTTCAACGGCACAG-3′; reverse: 5′-GCCAGTAGACTCCACGACA-3′	143	NM_017008

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

control MSCs untreated of OS induction and pressure normalized to the level of GAPDH mRNA have been ascribed a fold induction of 1. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product (data not shown).

WESTERN BLOTTING

To obtain whole-cell extracts, cells that were treated with or without pressures were washed twice with ice-cold PBS and then lysed and sonicated in a lysis buffer (Keygen total protein extraction kit, Keygen Biotech., Nanjing, Jiangsu, China). The cytosolic fraction was collected as the supernatant after centrifugation at 14,000*g* at 4°C for 15 min and assayed it quantitatively with the BCA method. After boiling for 5 min, 20–25 μl of the lysate (50 μg of protein) was applied to SDS–12% PAGE at 120 V for 5 h, and the proteins in the gel were transferred to a PVDF membrane (Millipore). After blocking, the membranes were probed with 1:1,000 dilutions of the anti-phospho-ERK1/2 Thr202/Tyr204 (D13.14.4E), anti-ERK1/2 (137F5), anti-phospho-p38 MAPK Thr180/Tyr182 (3D7), and anti-p38 MAPK (Cell Signaling Technology), followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:6,000) at 37°C for 1 h. Immunoreactive proteins were visualized using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate; Millipore). Band intensities were determined using the ChemiDoc XRS Gel documentation system and Quantity One software (Bio-Rad). Detection of p-ERK1/2 or p-p38 MAPK was performed first. After the targeted bands of p-ERK1/2 or p-p38 MAPK were exposed, PVDF membranes were stripped with eluent (Beyotime Biotech., Haimen, Jiangsu, China). Then the second hybridism was carried out to get the data of ERK1/2 or p38 MAPK, respectively. The band intensity ratio (p-ERK/ERK or p-p38 MAPK/p38 MAPK) was analyzed, respectively.

STATISTICAL ANALYSIS

All experiments were performed at a minimum of three times. Measurements are expressed as mean ± SD. Statistical comparisons were made using factorial analysis of variance (ANOVA), followed by Dunnett's test for comparing treatments from controls. A value of $P < 0.05$ was statistically considered significant.

RESULTS

ALPASE ACTIVITY

ALPase activities of OS-0d MSCs were significantly promoted by dynamic pressure exposure for 1–5 days and by static pressure exposure on the 3rd day. Small increases in ALPase activity were observed in OS-3d MSCs exposed to dynamic pressure for 1 day and to static pressure for 3 days ($P < 0.05$). After OS-7d MSCs were exposed to dynamic pressure for 3 and 5 days and to static pressure for 5 days, ALPase activities had a significant rise (Fig. 3). ALPase activities of both OS-0d and OS-7d MSCs but not OS-3d MSCs were stimulated strongly by dynamic pressure. Exposure to static pressure had a weak effect on ALPase activity in MSCs (OS-0d, OS-3d, and OS-7d). OS-0d MSCs seemed to be highly sensitive to respond while the other two (OS-3d and OS-7d) showed slower and lower amplitude of change. It suggested that the different stages during initial osteodifferentiation of MSCs had various responses to static or dynamic pressure.

PD98059 pretreatment decreased pressure-stimulated ALPase activity, suggesting that ERK1/2 signaling was involved in the transduction of pressure signals. PD98059 also downregulated the ALPase activities in controls. ALPase activities of OS-3d MSCs were affected insignificantly by pressure exposure or plus PD98059 pretreatment for 5 days. The results suggested that pressures might maintain the differentiating status of MSCs despite PD98059 inhibition of osteodifferentiation. Compared to controls, higher ALPase activities of OS-0d and OS-7d MSCs treated with both types of pressures plus PD98059 inhibition supported this as well (Fig. 3).

mRNA EXPRESSION OF OSTEOBLAST-RELATED TRANSCRIPTION FACTORS

Figure 4A–D shows mRNA expression levels of Runx2, Osx, Msx2 and Dlx5 in MSCs (OS-0d, OS-3d, and OS-7d) affected by dynamic

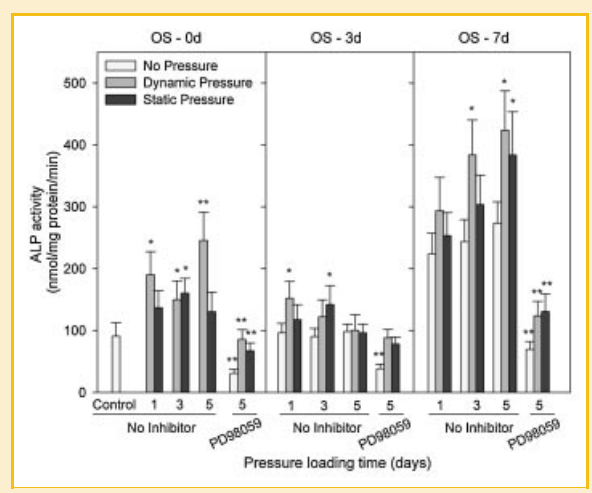

Fig. 3. Expression of ALPase activities affected by dynamic and static pressures for 1–5 days or plus PD98059 pretreatment for 5 days.

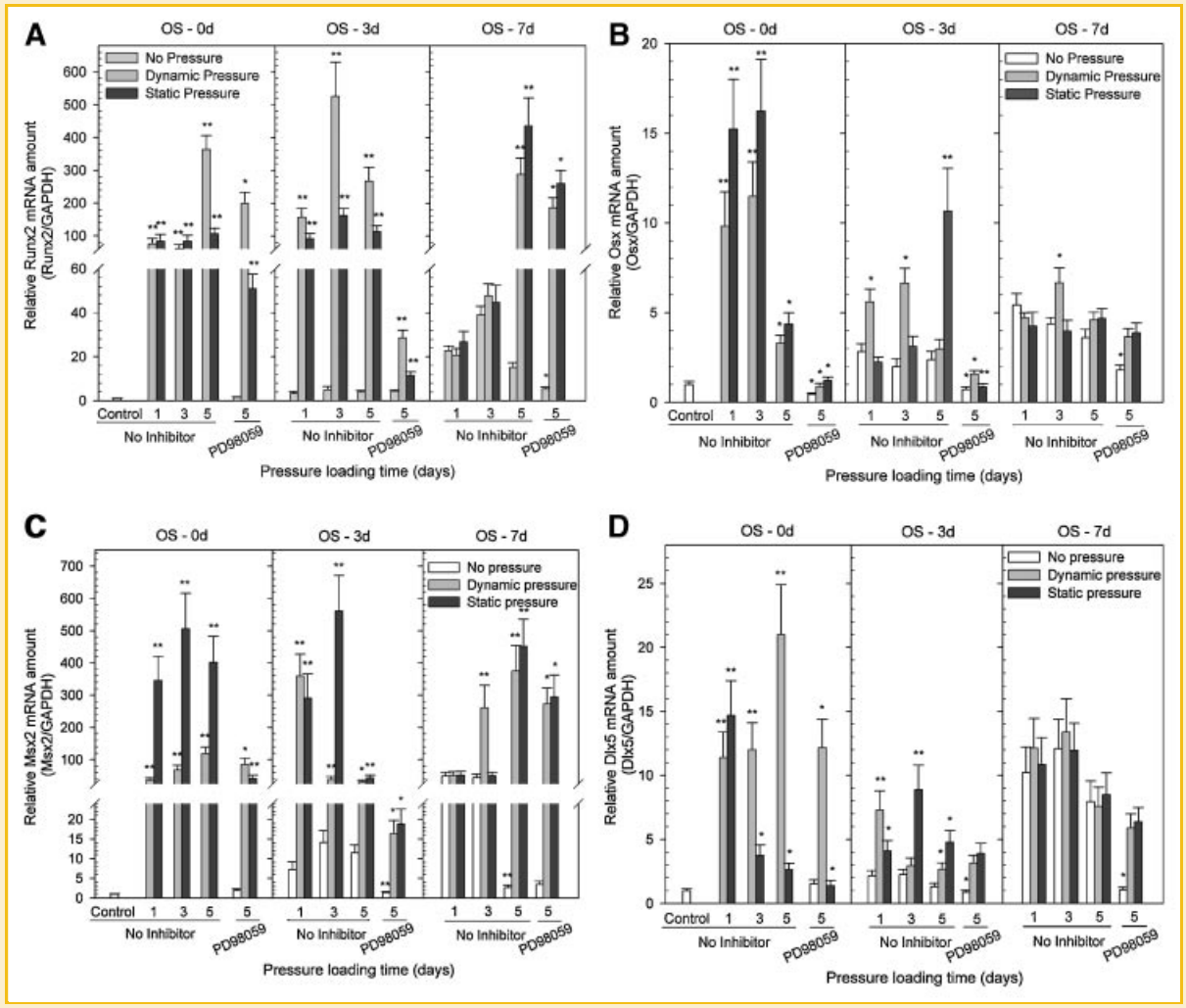

Fig. 4. Expression of osteogenesis-related transcription factors affected by dynamic and static pressures for 1–5 days or plus PD98059 pretreatment for 5 days. mRNA levels of (A) Runx2, (B) Osx, (C) Msx2, and (D) Dlx5. The values are mean ± SD. There were significant differences at $^{*}P < 0.05$ and $^{**}P < 0.01$.

or static pressure for 1–5 days or plus PD98059 pretreatment for 5 days. No significant differences between both pressure treatments were indicated in the mRNA expression of osteoblast-related transcription factors.

For control cells, mRNA levels of Runx2, Osx, Msx2 and Dlx5 significantly increased over the time of osteoinduction (0–7 days) and mRNA levels of Runx2 and Msx2 in control OS-7d MSCs were noted to decrease significantly on the 5th day after the OS medium was changed. Runx2 and Msx2 mRNA levels increased greatly ($P < 0.01$ or $P < 0.05$) when OS-0d and OS-3d MSCs were exposed to both types of pressures for 1–5 days. For OS-7d MSCs exposed to either type of pressure for 5 days, significant increase of Runx2 and Msx2 mRNA was also observed. After exposure of MSCs (OS-0d, OS-3d and OS-7d) to pressure, the peaks of Runx2 and Msx2 mRNA expression had 19- to 364-fold increase and 40- to 505-fold increase compared to controls, respectively (Fig. 4A,C). Usage of PD98059 downregulated Runx2 mRNA level in control OS-7d MSCs and Msx2 mRNA level in control OS-3d MSCs. PD98059 pretreatment decreased pressure-induced Runx2 and Msx2 mRNA levels which, however, remained at much higher levels than controls (Fig. 4A,C).

The Osx mRNA level increased significantly when OS-0d MSCs were exposed to both types of pressures for the first 3 days and then fell but remained at higher levels than control on the 5th day. The Osx mRNA level of OS-3d MSCs was promoted by dynamic pressure for the first 3 days ($P < 0.05$) and static pressure treatment did not show significant effect until the 5th day ($P < 0.01$). When OS-7d MSCs were exposed to dynamic pressure for 3 days, a small increase of Osx mRNA was observed but no significant pressure-stimulated Osx mRNA was found in other OS-7d MSCs groups. OS-0d MSCs had a higher peak level of Osx mRNA than OS-3d and OS-7d MSCs. PD98059 pretreatment inhibited Osx mRNA levels in individual controls ($P < 0.05$), similar to the change of ALPase activities. Pressure-stimulated Osx mRNA levels of OS-0d and OS-3d MSCs were downregulated by PD98059. Osx mRNA of OS-7d MSCs was affected insignificantly by pressure exposure or plus PD98059 pretreatment for 5 days ($P > 0.05$), suggesting that pressure signals might maintain the differentiating status of OS-7d MSCs via ERK-

independent pathways (Fig. 4B). Pressure-stimulated Dlx5 mRNA levels were similar to the changes of Osx mRNA. But for OS-0d MSCs, Dlx5 mRNA was more sensitive to dynamic pressure than static one; whereas Osx mRNA was more sensitive to static pressure. PD98059 pretreatment inhibited Dlx5 mRNA levels in control OS-3d and OS-7d but not OS-0d MSCs.

PHOSPHORYLATION OF ERK1/2 AND P38 MAPK

When MSCs (OS-0d, OS-3d, and OS-7d) were exposed to both types of pressures for 1 h for one to five consecutive days, ERK1/2 was significantly activated ($P < 0.01$ or $P < 0.05$) at different levels. After dynamic pressure treatment, ERK1/2 phosphorylation reached peaks on the first or third day, whereas static pressure had slower effects (Fig. 5A,C). Figure 5D demonstrates the peak levels of activated ERK1/2. When exposed to dynamic pressure, OS-0d MSCs (exposed for 1 day) showed significantly lower level of ERK1/2 activation than OS-3d MSCs (exposed for 1 day) and OS-7d MSCs (exposed for 3 days) ($P < 0.05$); but there was no significant difference between the latter two. For cells exposed to static pressure for 5 days, peak levels of ERK1/2 activation increased over the time of osteoinduction ($P < 0.05$). PD98059 pretreatment (10 μM) effectively blocked pressure-induced ERK activity. Figure 5E illustrates that ERK activities were effectively inhibited by PD98059 when OS-0d and OS-7d MSCs were exposed to both types of pressures for 5 days.

Phosphorylation of p38 MAPK was not found in MSCs (OS-0d, OS-3d, and OS-7d) exposed to either dynamic or static pressure. Figure 6 illustrates phosphorylation level of p38 MAPK when OS-0d cells exposed to static or dynamic pressure.

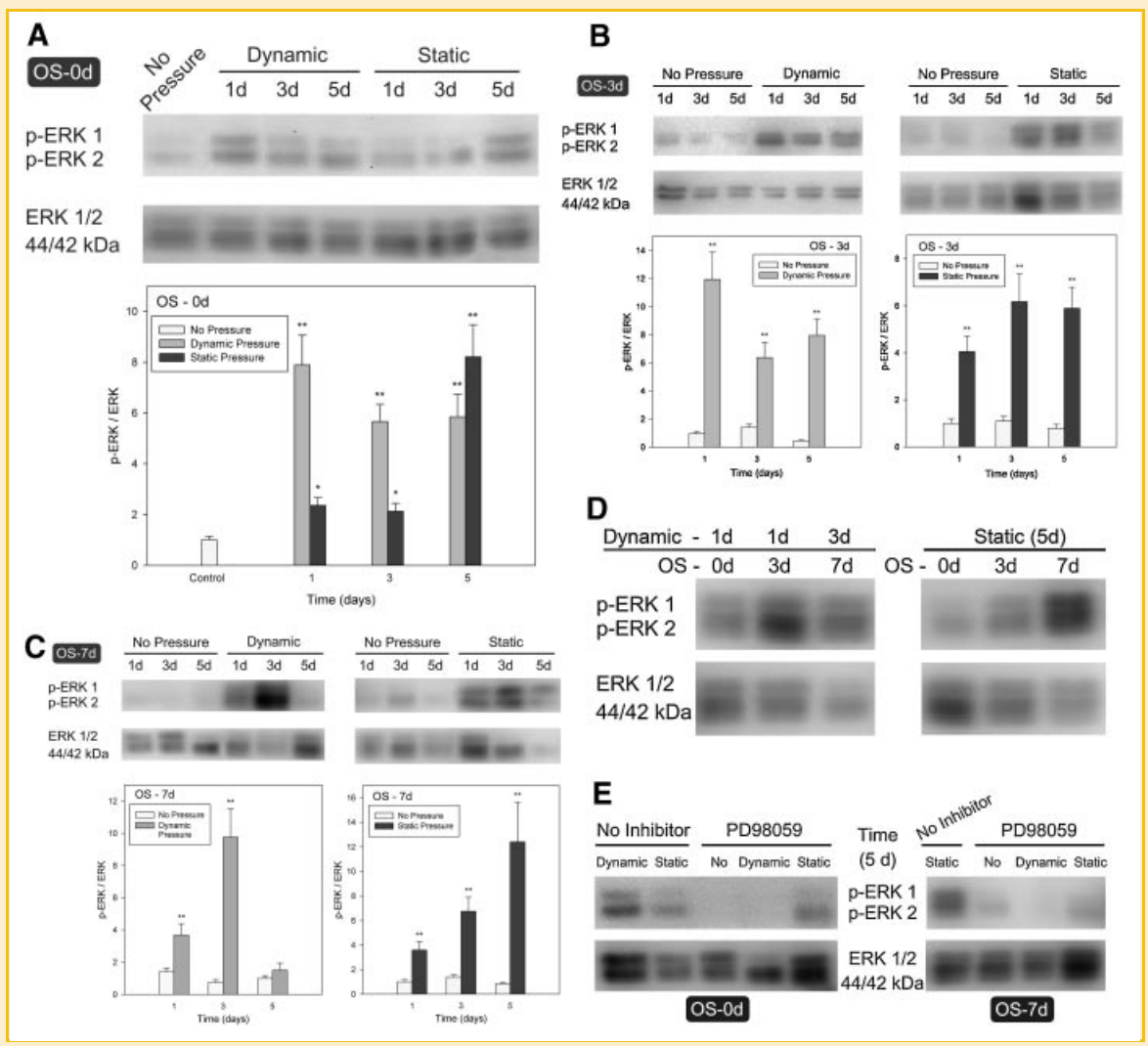

Fig. 5. Activation of ERK1/2 induced by dynamic and static pressures or PD98059 pretreatment. ERK1/2 activation of (A) OS-0d MSCs, (B) OS-3d MSCs, and (C) OS-7d MSCs. D: Peak levels of activated ERK1/2 of OS-0d, OS-3d, and OS-7d MSCs in response to both types of pressures. E: OS-0d and OS-7d MSCs treated with PD98059 (10 μM) and both types of pressures for 5 days (note that ERK activities were effectively inhibited by PD98059). The blots shown are representative of three independent experiments. The values are mean ± SD. There were significant differences at $^{*}P < 0.05$ and $^{**}P < 0.01$.

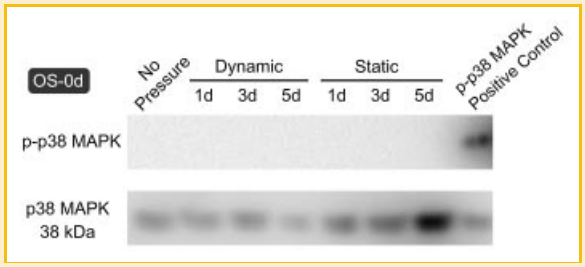

Fig. 6. Phosphorylation of p38 MAPK after exposure of OS-0d MSCs to dynamic or static pressure. The blot shown is representative of three independent experiments.

DISCUSSION

One of the most important findings of the present study was that exposure to both dynamic and static pressures could induce further osteogenesis of MSCs at the initial stage of osteodifferentiation. Especially, the expressions of osteogenesis-related factors were all strongly stimulated in totally undifferentiated MSCs by exposure to both types of pressures. Based on the present study, we could not make a simply conclusion of the comparison between the effect of dynamic pressure and the one of static pressure. Recently, some studies reported that stretch [Koike et al., 2005] and pulsed ultrasound [Sant'Anna et al., 2005] regulated Runx2 expression during the osteogenic differentiation of MSCs. Dramatically increased of Runx2 and Msx2 mRNA levels were noted in the present study, possibly because MSCs during their initial osteodifferentiation express low levels of Runx2 and Msx2 mRNA and pressure exposure induces a de novo elevated expression of their genes. Runx2 and Msx2 mRNA levels were highly sensitive to pressure signals, suggesting that these two genes were regulated as key factors when pressure signals induced osteodifferentiation. These conclusions were also supported by the fact that PD98059 pretreatment decreased pressure-induced Runx2 and Msx2 mRNA levels but the levels remained at much higher levels than controls. Thus ERK signaling might not play a key role in the mechano-signal transduction though involved.

ERK1/2 pathway had been shown to participate in cellular mechanotransduction, turning mechanical signals into intracellular biological signals to regulate cell proliferation and differentiation [Fan et al., 2006; Liu et al., 2006; Kanno et al., 2007; Ward et al., 2007]. We demonstrated that both types of pressures activated ERK1/2 signaling at different levels during initial osteodifferentiation of MSCs.

Jansen et al. [2004] had investigated the effect of mechanical loading on SV-HFO (a human osteoblast cell line) and ERK1/2 signaling in relation to osteodifferentiation. Their study demonstrated that the extent of ERK activation depended on the differentiation stage of the osteoblast. However, their study did not include the mechanoresponse of MSCs during initial osteodifferentiation. The present study showed that after exposure to static pressure for 5 days the peak levels of ERK activation increased over the time of osteoinduction. When treated with dynamic pressure, OS-3d and OS-7d MSCs also expressed higher peak levels of ERK1/2 activation than OS-0d MSCs but at different loading times. Generally, dynamic pressure groups reached the peak levels sooner than static pressure groups.

Adipocytes and osteoblasts are two major lineages differentiating from pluripotent MSCs and the relationship between these two is reciprocal [Jaiswal et al., 2000; David et al., 2007]. Evidence shows that commitment of human MSCs into osteogenic or adipogenic lineage is governed by activation or inhibition of ERK, respectively [Jaiswal et al., 2000]. The present study indicated that PD98059 blockade of ERK activity downregulated the expression of osteogenesis-related factors in MSCs with no pressure exposure. These results coincided with the findings of Jaiswal et al. [2000], supporting that ERK1/2 pathway was involved in the initial osteodifferentiation of MSCs. Moreover, our study showed that pressure signals could maintain the status of osteodifferentiation of MSCs despite PD98059 inhibition of both the osteodifferentiation and the transduction of pressure signals partially. Recently, David et al. [2007] reported that cyclic stretch partially overcame the induction of adipogenesis and was able to favor osteodifferentiation. Zayzafoon et al. [2004] indicated that modeled microgravity inhibited the osteodifferentiation of human MSCs and induced the development of an adipocytic lineage phenotype. These results support that mechanical stimuli may promote osteogenesis whereas inhibit adipogenesis. Msx2 may shift cell fate toward the osteoblast lineage by promoting osteogenesis and suppressing adipogenic differentiation of multipotent mesenchymal progenitors [Cheng et al., 2003; Ichida et al., 2004]. We found that mRNA levels of Msx2 were sensitively promoted after exposure of MSCs to both types of pressures. Our study only investigated osteogenesis promoted by pressure after PD98059 pretreatment.

Simmons et al. [2003] investigated the effect of cyclic substrate deformation on the proliferation and osteodifferentiation of human MSCs and their results suggested an inhibitory role for p38 signaling in the modulation of strain-induced osteodifferentiation. The present study provided evidence that p38 MAPK signaling was not involved in the mechanobiological response of MSCs during their early osteodifferentiation, which contradicted the finding of Simmon et al. The relation between the p38 MAPK pathway and mechano-induced osteogenic differentiation requires further investigation.

Our findings showed that different points of initial osteodifferentiation of MSCs had varying responses to either type of pressure. The expression of osteogenesis-related factors in OS-0d MSCs was highly sensitive to pressure exposure; whereas OS-3d and OS-7d MSCs responded slowly to pressure. We hypothesize that with increased expression of osteogenesis-related factors after OS induction, MSCs could go into a state of low response to the osteoinduction by pressure. Furthermore, cells might have different responses over the pressure loading time. Take OS-0d MSCs as an example, the cells were sensitive to pressure and so Osx gene increased quickly. Then the cells might go into a low- or no-response state and consequently the Osx decreased. It suggests the complexity of the mechanism of the initial bone remodeling and regeneration upon pressure. Further studies about initial osteodifferentiation are needed to unveil the complex mechanisms.

MAPK inhibitor was able to suppress Runx2 levels only on OS-d3 but not OS-d0 and OS-d7. PD98059 might inhibit both the osteodifferentiation and the transduction of pressure signals partially. Furthermore, our findings showed that different points of initial osteodifferentiation of MSCs had varying responses to either type of pressure. The expression of osteogenesis-related factors in OS-0d MSCs was highly sensitive to pressure exposure; whereas OS-3d and OS-7d MSCs responded slowly to pressure. We hypothesize that the Runx2 levels of OS-0d MSCs were not suppressed because OS-0d MSCs were much more sensitive to pressure. And though both OS-3d and OS-7d MSCs were less sensitive to pressure, OS-3d MSCs might remain in a reversible differentiation state which could be significant suppressed by PD98059 inhibition of osteodifferentiation.

To examine the exact mechanobiological responses of MSCs and exclude the effects of osteogenic agents, we used the ordinary culture medium instead of the OS medium right before OS-3d and OS-7d MSCs were exposed to pressure. So our study established individual controls for the different pressure exposure times in OS-3d and OS-7d MSCs to exclude the possible bias since the differentiating status of OS-3d and OS-7d MSCs might change over time after that. However, the differentiating status of control OS-0d MSCs did not change over time. So we used the mean value of the OS-0d controls and the standard deviation (SD) was very small.

In our study, the magnitudes (dynamic pressure 10–36 kPa and static pressure 23 kPa) and duration (1 h per day) of either type of pressure were chosen according to the macroscopic level physiological values reported in the literature for daily activities of humans and the stress analysis of the periodontal ligament under various orthodontic loadings, considering the side effects of pressure on culture conditions as well [McGuinness et al., 1991; Nagatomi et al., 2001].

CONCLUSIONS

In summary, we show that exposure to either dynamic or static pressure can induce initial osteodifferentiation of MSCs. In particular, both types of pressures strongly stimulate the expression of osteogenesis-related factors of totally undifferentiated MSCs. The undifferentiated MSCs were highly sensitive to pressure exposure; whereas after osteoinduction MSCs reacted to pressure in a lower response state. ERK pathway participates in early osteodifferentiation and plays a positive but non-critical role in mechanotransduction, whereas p38 MAPK is not involved in this process. The findings should lead to further studies to unveil the complex initial biological mechanisms of bone remodeling and regeneration upon mechanical stimuli.

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