

Physical Shock Wave Mediates Membrane Hyperpolarization and Ras Activation for Osteogenesis in Human Bone Marrow Stromal Cells

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Physical shock wave (SW) has shown effectiveness on promotion of bone growth. We have recently demonstrated that SW could promote bone marrow stromal cell differentiation toward osteoprogenitor associated with induction of TGF- β 1. We have further demonstrated that SW-induced membrane hyperpolarization and Ras activation acted an early signal for the osteogenesis in human bone marrow stromal cells. An optimal dose of SW treatment at 0.16 mJ/mm² for 500 impulses induced a rapid membrane hyperpolarization in 5 min, activation of Ras in 30 min, and cell proliferation in 2 days. The SW-promoted cell growth was related to osteogenesis as demonstrated by increase of bone alkaline phosphatase activity in 6 days and osteocalcin mRNA expression in 12 days. In support that SW-induced Ras activation mediated osteogenesis of human bone marrow stromal cells, we further demonstrated that transfection of bone marrow stromal cells with a dominant negative Ras mutant (Asn-17 ras^H) abrogated the SW enhancement of osteogenic transcription factor (CBFA1) activation, osteocalcin mRNA expression, and bone nodule formations. These results suggest that physical SW promotes bone marrow stromal cell differentiation toward osteogenic lineage via membrane hyperpolarization, followed by Ras activation and specific osteogenic transcription factor CBFA1 expression. A link between physical SW and biomembrane perturbation-mediated Ras activation may highlight how noninvasive physical agents could be used to promote fracture healing and to rescue patients with osteoporosis and osteopenic disorders in the future. © 2001 Academic Press

Key Words: shock wave; membrane potential; Ras; osteogenic transcription factor (CBFA1); human bone marrow stromal cells.

Bone marrow stromal cells had a multipotential to differentiate into osteogenic, myogenic, and adipogenic cell lineages (1, 2). It is well clarified that the differentiation and maturation of bone marrow mesenchymal cells into osteoprogenitor is involved in bone regeneration in the fracture healing (3, 4). Application of extracorporeal shock wave (SW) has long been used to disrupt renal stones (5, 6). Recently, we and others have shown that SW treatment has a promising effect on promotion of bone fracture healing and repair of tendinopathies (7–10).

Physical SW is created by high voltage spark discharge under water causing an explosive evaporation of water and producing high-energy acoustic waves. The acoustic waves are focused on a semiellipsoid reflector and therefore can be transmitted into a specific tissue site (11). The mechanism by which SW enhances fracture healing and repair of tendinopathies remained to be determined. The fact that SW treatment enhances both bone and tendon regeneration suggests that SW may induce a certain signal for growth and maturation of the mesenchymal progenitors from bone marrow. We have recently shown that SW treatment did promote bone marrow stromal cell growth and differentiation toward osteogenic cells, presumably through TGF- β 1 induction (12). Since cell membrane acts an important role in signal transduction for activation of human bone cells (13) and chick embryonic myoblasts (14) after stimulation by physical factors. Therefore, we hypothesized that SW treatment promoted osteogenic differentiation of bone marrow stro-

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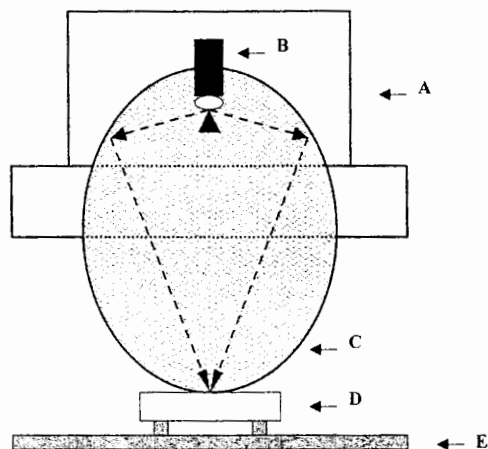


FIG. 1. Schematic diagram of SW apparatus. (A) Shock wave is generated by an OssaTron orthopedic therapy shock wave equipment, which elicits spark discharge underwater from an electrode (B). Degassed water is jacketed with an UV-resistant outer membrane (C). The acoustic waves are reflected in an ellipsoid apparatus and focused into the target tube (D), which is held by a target holder (E).

mal cells via a membrane perturbation-mediated signal transduction. We sought to investigate whether physical SW treatment could alter human bone marrow stromal cell membrane potential, Ras activation, osteogenic transcription factor (CBFA1) expression and bone nodule formations. Furthermore, we also investigated whether transfection of a dominant negative Ras mutant construct could abrogate the SW promotion of osteogenesis.

MATERIALS AND METHODS

Cell culture. An immortalized human bone marrow stromal cell line (CRL-11882, American Type Culture Center, Manassas, VA) was used for studies. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Gaithersburg, MD) containing 10% FBS at 5% CO₂, 37°C incubator for 6 days. Cells were harvested by trypsinization and resuspended in PBS for studies.

Application of SW treatment. Human bone marrow stromal cells were subjected to SW treatment with modification as previously described (15). An OssaTron orthopedic therapy shock wave equipment (HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was employed for studies. Shock wave was generated by underwater spark discharge from an electrode which was loaded in the first focus of a hemiellipsoid and the de-gassed water was jacketed with a UV-resistant coupling membrane. The propagation wave was focused to the second focus of the ellipsoid where the center of tube containing target cells was positioned on the sample holder as shown in Fig. 1. Cells (1×10^6 cells/ml) were suspended in a 5-ml polystyrene round-bottom tube (Falcon, Becton-Dickson Co., NJ) containing 5 ml PBS at pH 7.4 and exposed to SW at $0.16 \text{ mJ}/\text{mm}^2$ for 0, 250, 500, 1000, 2000, and 3000 impulses. Duration of the SW treatment took 10–20 min depending on the dose applied. The ultrasound transmission gel (Pharmaceutical Innovations Inc., NJ) was used as contact medium between the apparatus and the target tube. The apparatus is covered with an UV-resistant membrane in order to avoid the UV light damage to the environment. After the SW treatment, cell number and viability were determined with a hemocytometer by a 0.4% trypan blue exclusion assay (16).

Measurement of cell growth. Cells (5×10^4 cells/well, 96-well plate) with and without SW treatment were cultured in DMEM with 10% FBS for 24 h before added with $1 \mu\text{Ci}$ [³H]thymidine/well (Amersham-Life Science, Aylesbury, England) for an additional 24-h culture. At the end of culture period, cell culture in each well was released from the plates by trypsinization and proceeded for [³H]thymidine uptake determination by a liquid scintillation counter (Tri-Crab 2100TR, Packard Inc, U.S.A.) (17).

Measurement of TGF- β 1 production in the culture supernatants. Cells (1×10^5 cells/well, 24-well plate) with and without SW treatment were cultured in DMEM with 10% FBS for 72 h. At 0, 3, 6, 12, 24, 48, and 72 h after SW treatment, the cultured supernatants were harvested for measurement of TGF- β 1 by centrifuging at 500g for 5 min and then stored at -70°C until studies. The TGF- β 1 production was determined by an ELISA (Quantikine, R & D Systems Inc., MN) and the cells were collected by trypsinization for assessment of cell number as previously described (12). The TGF- β 1 production of cell culture in each well was normalized with cell number.

Measurement of membrane potential. Cells (1×10^5 cells/ml) were treated with and without SW treatment after the cells were preloaded with 50 nM DiOC₆ (3,3'-diopentylxocarbocyanine; Sigma Chemical Co., St. Louis, MO) for 5 min in the dark. At 5, 15, 30, 60, 120, and 240 min after SW treatment, the cells were washed twice with PBS and re-suspended in 0.5 ml PBS for flow cytometric analysis of cell membrane potential (16). For each sample, 30,000 cells were acquired and analyzed with CellQuest software (Becton-Dickinson, San Jose, CA). A higher arbitrary fluorescence unit at 488 nm excitation and 520 nm is attributed to cells with a higher membrane potential (16). In a pilot study, we made sure that DiOC₆ did not significantly affect the cell viability or mitochondrial function. Cells incubated with DiOC₆ (50 nM) for 180 min had normal cell viability at $96.4 \pm 2.3\%$. Furthermore, we also found that DiOC₆ (50 nM) or SW treatment with 500 impulses did not alter mitochondrial ultrastructure as demonstrated by flow cytometric analysis of 10-*n*-nonyl-acridine orange (NAO) staining.

Measurement of osteogenic differentiation. To elucidate the osteogenic activities in the human bone marrow stromal cells, cells (1×10^5 cells/well, 24-well plate) with and without SW treatment were initially cultured in DMEM containing 10% FBS, 10^{-8} M dexamethasone, 10^{-2} M glycerophosphate and 50 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma Chemical Co.) for 6 days, followed by change of fresh medium every 3 days. Cell culture in each well was fixed with neutral buffered formaldehyde for 5 min (pH 7.4), rinsed with distilled water, and subjected to assessment of bone alkaline phosphatase activity for osteogenic activity (12). In some experiments, cells (1×10^5 cells/well, 24-well plate) were also cultured for 12 and 21 days before fixed with neutral formaldehyde and subjected to assessment of osteoprogenitor colony formation and bone nodule formation. Plating efficiency of cell growth was determined by colony formations showing aggregation of more than 32 cells after inoculation of 10^3 cells/well for a 12-day culture. Colony forming-units-osteoprogenitor (CFU-O) were assessed according to instruction of a Sigma bone alkaline phosphatase cytochemistry assay kit (Sigma Chemical Co.) and were defined by colonies showing more than 32 segregate cells with positive bone alkaline phosphatase staining. Lineages of myogenic and adipogenic differentiation were determined by positive myotubulin and lipoprotein lipase staining (Developmental Studies Hybridoma Bank, University of Iowa, IA), respectively (2). Bone nodule formations greater than 2 mm^2 were determined by von Kossa staining and were counted under an inverted microscope (12).

Detection of lineage-specific mRNA expression by RT-PCR. Total RNA was extracted and purified by the Tri reagent containing monophasic solution of guanidine thiocyanate and phenol (Sigma Chemical Co.) from the 10^6 cells with and without SW treatment. One microgram of total RNA was reverse transcribed (RT) into cDNA, followed by PCR amplification using human gene-specific

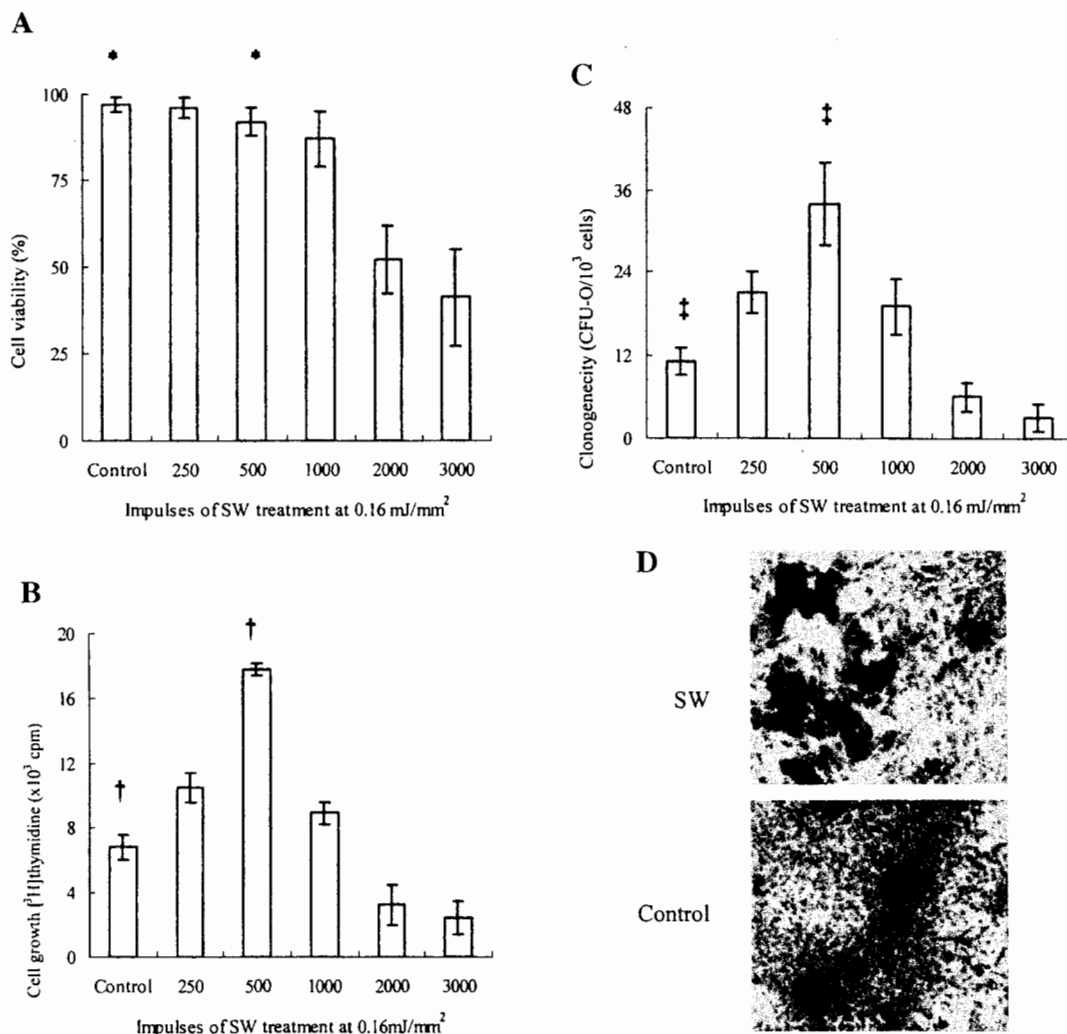


FIG. 2. Effects of SW on cell viability and cell growth. (A) Physical SW treatment at 0.16 mJ/mm² for 250, 500, or 1000 impulses did not affect cell viability as determined by trypan blue exclusion, but doses higher than 1000 impulses significantly decreased cell viability. (B) The SW treatment at 0.16 mJ/mm² for 250 and 1000 impulse had a minimal enhancement on cell growth, whereas doses more than 1000 impulses suppressed the cell growth as determined by [³H]thymidine incorporation. The optimal dose of 500 impulses had the best enhancement on cell growth. (C) The plating efficiency of CFU-O formations was determined by colonies showing more than 32 aggregated cells after inoculated 1×10^3 cell/well for 12-day culture (D). * $P = 0.64$, † $P < 0.001$, and ‡ $P < 0.001$, respectively, represent significant differences between both groups indicated. Results were presented with mean values \pm standard error calculated from six paired triplicate experiments.

primers: collagen type I (forward) (5'-T GA CGA GAC CAA GAA CTG-3'), (reverse) (5'-CCA TCC AAA CCA CTG AAA CC-3') (156-base pair expected); osteocalcin (forward) (5'-AAG AGA CCA AGG CGC TAC CT-3'), (reverse) (5'-GCC GAT AGG CCT CCT TGA AAG-3') (135-base pair expected product); β -actin (forward) (5'-CGC CAA CCG CGA GAA GAT-3'), β -actin (reverse) (5'-CGT CAC CGG AGT CCA TCA-3') (168-base pair expected). The parameter for RT-PCR cycling were set as follows: the RT reaction at 50°C for 2 min and 60°C for 30 min; and PCR 95°C for 5 min; followed by 40 cycles of PCRs at 94°C for 20 s and 60°C for 1 min. The PCR products were run on a 1.5% agarose gel electrophoreses containing ethidium bromide and visualized by UV-induced fluorescence.

Preparation of cytosol and nuclear extract. Cells were harvested and lysed with 200 μ l of ice-cold buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.7% NP-40 on ice

for 10 min and centrifuged at 500g for 5 min. The nuclear pellets were further lysed with buffer containing 40 mM Tris, pH 7.9, 350 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μ M DTT, 2 μ g/ml leupeptin and 1 μ g/ml aprotinin (Sigma Chemical Co.) on ice for 20 min and harvested by centrifugation at 12,000g, 4°C for 10 min. Protein concentrations in cytosolic and nuclear extracts were determined by a Bio-Rad assay kit (Bio-Rad Laboratories, U.S.A.) (18).

Measurement of Ras activation by a specific Raf-1 agarose conjugate. Cell lysate was precleared with glutathione agarose, followed by incubation with specific Raf-1 RBD agarose conjugate (Upstate Biotechnology, NY) for 30 min at 4°C. After incubation, the immune complexes were harvested by centrifugation at 12,000g for 1 min. The immunoprecipitate (20 μ g) was incubated

TABLE 1

Shock Wave Enhancement of Osteogenic but Not Myogenic or Adipogenic Colony Formations in Human Bone Marrow Stromal Cells

	Control		SW treatment	
	Colonies	%	Colonies	%
CFU-O	11 ± 3*	68.8	35 ± 5*	92.1
CFU-M	5 ± 2	31.2	3 ± 2	7.9
CFU-A	0	0	0	0

* Human bone marrow stromal cells (10^4 cells/well, 24-well plate) with or without SW treatment for 500 impulses were cultured for 12 days. Colonies greater than 32 aggregated cells were recognized as colony formations. CFU-O, CFU-M, and CFU-A formations were, respectively, determined by bone alkaline phosphatase, myotubulin, and lipoprotein lipase staining.

* Significant difference between both groups indicated ($P < 0.001$). Results were presented with mean values ± standard errors calculated from six paired triplicate experiments.

with the Laemmli buffer (200 mM Tris, pH 6.8, 10% glycerol, 4% SDS, 50 μ M DTT, and 0.05% bromophenol blue) for 5 min at 95°C. The mixtures were subject to Western blot assay. The activated Ras protein on the blot was recognized by a mouse anti-Ras antibody (Upstate Biotechnology, NY) at 1:500 dilution, followed by goat anti-mouse horseradish peroxidase-conjugated IgG (1:2000) as the second antibody. The activated Ras protein levels were visualized with chemiluminescence agents (SuperSignal; Pierce Co., Rockford, IL) (19).

Measurement of CBFA1 activation. The nuclear extracts were incubated with anti-CBFA1 antibody (1:100; Santa Cruz Biotechnology Inc., CA) for 1 h at 4°C. After incubation, the immune complexes were precipitated with protein A (Sigma Chemical Co.). The immunoprecipitate (20 μ g) was mixed with Laemmli buffer for 5 min at 95°C. The mixtures were subject to Western blot assay. The total CBFA1 on the blot was recognized by a rabbit anti-CBFA1 antibody at 1:500 dilution, followed by goat anti-rabbit horseradish peroxidase conjugated IgG (1:2000) as the second antibody. The phosphorylated CBFA1 on the blot was further recognized by a specific mouse anti-phosphotyrosine (1:1000) antibody, followed by goat anti-mouse horseradish peroxidase conjugated IgG (1:2000) as the second antibody. The CBFA1 activity was reflected on the phosphorylated CBFA1 visualized with chemiluminescence agents.

Transient transfection of a dominant negative Ras construct. Bone marrow stromal cells (5×10^5 cells/well, 6-well plate) were transfected with 3 μ g dominant negative Ras mutant cDNA plasmid (Asn-17 ras^H) as previously described (20) using FuGENE 6 transfection reagent (Roche Diagnostic Corp., Indianapolis, IN) according to manufacturer's recommendation. The dominant negative Ras mutant construct is a gift from Dr. Shiao-Shen Liao, Department of Microbiology and Immunity, Cheng Kong University (Taiwan, Republic of China). After removal of medium and wash with PBS, cells stably transfected with the plasmid were selected in medium containing 400 μ g/ml G418 (Life Technologies, Gaithersburg, MD). Transfected cells were subjected to 0.16 mJ/mm² SW treatment for 500 impulses and culture for assessment of osteogenesis as mentioned above.

Statistical analysis. Data were analyzed with a nonparametric one-way analysis of variance followed by Student's *t* test to determine significance between treatments. $P < 0.05$ was considered statistically significant.

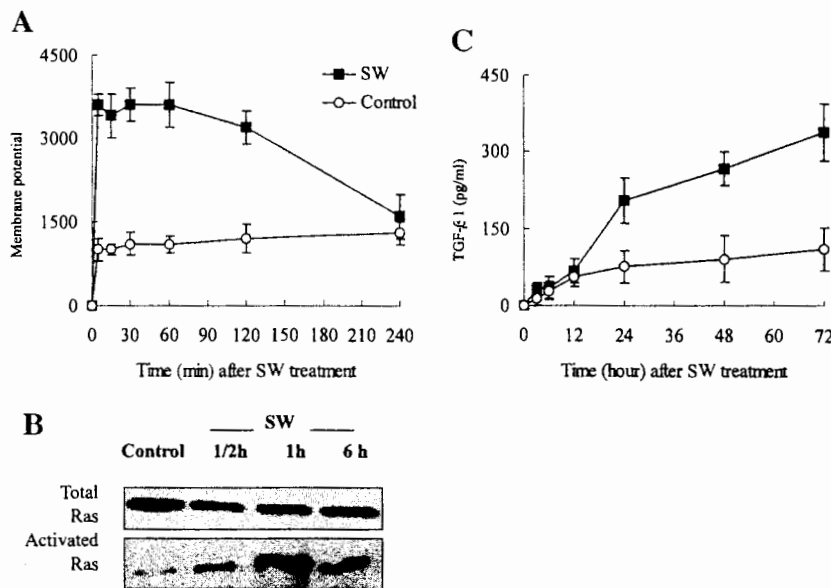


FIG. 3. Kinetic changes of SW-induced membrane potential, Ras activation and TGF- β 1 production. (A) The SW treatment at 0.16 mJ/mm² for 500 impulses induced a rapid membrane potential hyperpolarization in 5 min. Membrane potential changes were determined by DiOC₆-staining with flow cytometric assays. Results were presented with mean values ± standard errors calculated from six paired triplicate experiments. (B) The SW treatment at 0.16 mJ/mm² for 500 impulses induced Ras activation in 30 min. Ras activation was determined by specific Raf-1 RBD immunoprecipitate on Western blot assay. (C) The SW treatment at 0.16 mJ/mm² for 500 impulses increased osteogenic growth factor, TGF- β 1, production in 24 h as determined by an ELISA. Results were presented with mean values ± standard errors calculated from six paired triplicate experiments.

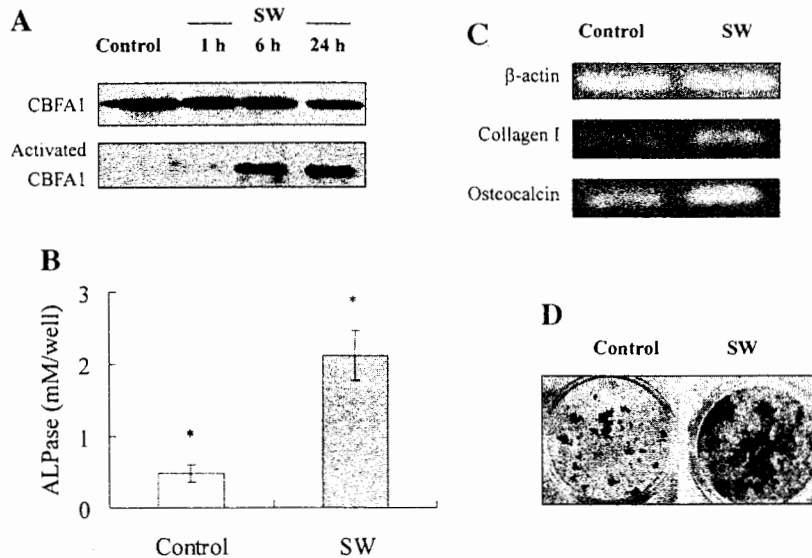


FIG. 4. SW promoted osteogenic differentiation of marrow stromal cells. Cells (1×10^3 cells/well, 24-well plate) were cultured in DMEM containing 10% FBS, 10^{-8} M dexamethasone, 10^{-2} M β -glycerophosphate, and 50 μ g/ml ascorbic acid. (A) The SW treatment at 0.16 mJ/mm^2 for 500 impulses induced osteogenic transcription factor (CBFA1) expression in 6 h. Activated CBFA1 was determined by anti-phospho-CBFA1 antibody on Western blot. (B) The SW treatment at 0.16 mJ/mm^2 for 500 impulses promoted bone alkaline phosphatase activity in 6 days. *Significant difference between both groups ($P < 0.001$). (C) The SW treatment at 0.16 mJ/mm^2 for 500 impulses promoted collagen I mRNA expression in 6 days and osteocalcin mRNA expression in 12 days as determined by RT-PCR assay. (D) The SW treatment at 0.16 mJ/mm^2 for 500 impulses promoted bone nodule formation as determined by von Kossa staining. Results were presented with mean values \pm standard errors calculated from six paired triplicate experiments.

RESULTS

Optimal Dose of SW Treatment Promoted Bone Marrow Stromal Cell Growth

We first investigated whether SW treatment could affect human bone marrow stromal cell viability and growth. Cells exposed to different doses from 250 to 1000 impulses of the 0.16 mJ/mm^2 SW treatment revealed normal viability, whereas doses more than 1000 impulse significantly suppressed cell viability (Fig. 2A). After SW treatment, cells were cultured for 2 days and subjected to measurement of cell proliferation as determined by [^3H]thymidine incorporation. It was found that the SW treatment with doses from 250 to 1000 impulses promoted the bone marrow stromal cell proliferation. The SW treatment with 500 impulses had the best promotion, whereas the treatments with more than 1000 impulses exerted a suppressing effect (Fig. 2B). Similarly, the SW treatment at 500 impulses promoted CFU-O formations (Fig. 2C) and turned into osteogenic differentiation as demonstrated by bone alkaline phosphatase positive colonies (Fig. 2D). The doses greater than 1000 impulses, however, suppressed the CFU-O formations (Fig. 2C). The increase in CFU-O by SW treatment was mainly osteogenic but not myogenic or adipogenic colonies (Table 1).

SW Induced a Rapid Membrane Hyperpolarization and Ras Activation

Since SW is an high energy acoustic waves, we first sought to investigate whether cell membrane potential of bone marrow stromal cell was affected by the physical SW. Results showed that the SW treatment with 500 impulses significantly induced cell membrane hyperpolarization in 5 min. The hyperpolarization returned to normal state in 240 min (Fig. 3A). Change of cell membrane potential is usually associated with Ras activation and growth factor induction (21). Experiments were further to elucidate whether SW-induced membrane potential change was linked to induction of Ras activation and osteogenic growth factor, TGF- β 1 induction. It was found that the SW treatment with 500 impulses induced Ras activation in 30 min and reached the peak in 1 h (Fig. 3B). The enhancement of TGF- β 1 production was found 24 h after SW treatment (Fig. 3C).

Determination of the Osteogenic Differentiation Induced by SW Treatment

To further determine the osteogenic differentiation induced by SW treatment, we assessed induction of specific osteogenic transcription factor, bone matrix proteins (collagen type I and osteocalcin) expression, and bone nodule formation. We found that specific os-

teogenic transcription factor (CBFA1) was induced by the SW treatment with 500 impulses in 6 h (Fig. 4A). Bone alkaline phosphatase activity and collagen type I mRNA were significantly ($P < 0.001$) increased in 6 days (Figs. 4B and 4C), and osteocalcin mRNA expression was induced in 12 days after the SW treatment (Fig. 4C). The enhancement of bone mineralized matrix by SW treatment was definitely demonstrated by increase of bone nodule formations after a 21-day culture (Fig. 4D).

Transfection of Dominant Negative Ras Mutant Abrogated SW Promotion of CBFA1 Activation, Osteogenic Activity, and Bone Nodule Formation

To confirm whether Ras activation is critical in SW promotion of osteogenic differentiation of marrow stromal cells, we transfected an immortalized bone marrow stromal cell line with dominant negative Ras mutant to verify the signal transduction pathway. Results showed that SW could induce membrane hyperpolarization in the mutant-Ras-transfected cells (Fig. 5A), but transfection of the mutant-Ras completely blocked the SW-induced Ras activation and suppressed CBFA1 activation (Fig. 5B). The osteocalcin mRNA expression (Fig. 5C) and bone nodule formations (Fig. 5D) were also significantly suppressed in the mutant-Ras-transfected cells. These results suggested that SW could perturb membrane potential and transduced osteogenic differentiation signal through Ras activation pathway.

DISCUSSION

In this study, we showed that physical SW treatment could elicit membrane perturbation and Ras activation resulting in induction of nuclear osteogenic transcription factor (CBFA1), collagen type I mRNA expression and osteocalcin mRNA expression, and terminal bone nodule formations. This is first evidence to show that physical SW transduces biological responses from membrane potential changes, Ras activation to osteogenesis.

Evidence previously showed that SW propagated in a fluid phase revealed a demolitive effect at the interface between solid fragment and fluid (22). The biological effect of the SW treatment on cells or tissues may be mediated by bubble-cavitation (23). The acoustic cavitation could induce membrane potential change as demonstrated in rat papillary muscle (24). We demonstrated in this study that physical SW treatment could induce membrane hyperpolarization of human bone marrow stromal cells in 5 min. A study with embryonic stem cells also showed that change of electrical fields could induce embryonic stem cell membrane potential change and cell differentiation (25). Similarly, studies with rat marrow cells also showed that change of mem-

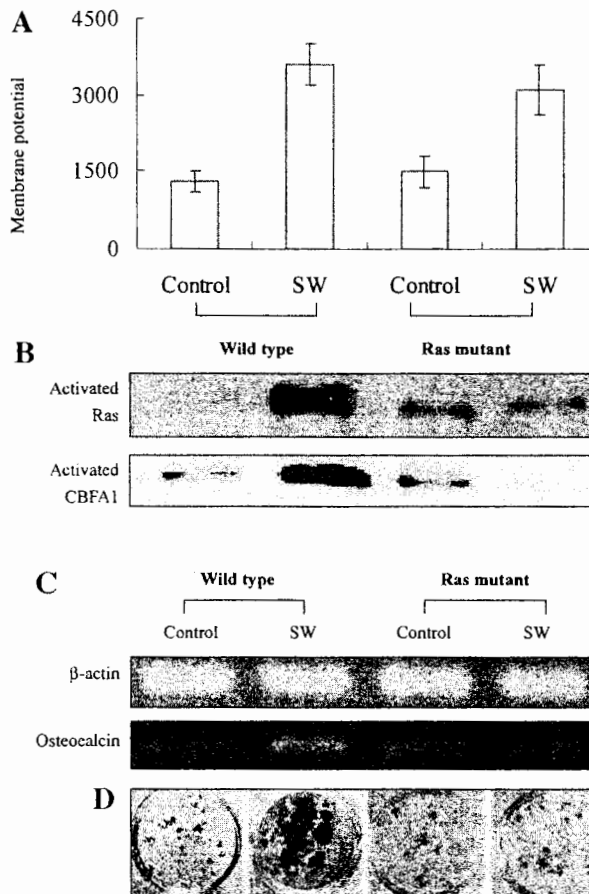


FIG. 5. Transfection of mutant Ras expression suppressed SW-enhanced osteogenesis, but not SW-induced membrane hyperpolarization. (A) SW treatment induced membrane hyperpolarization in both wild-type and mutant Ras-transfected cells. (B) Transfection of mutant Ras expression eliminated SW-induced CBFA1 activation as determined by Western blot assay. (C) Transfection of mutant Ras expression suppressed SW-induced osteocalcin mRNA expression as determined by RT-PCR assay. (D) Transfection of mutant Ras abrogated SW-promoted bone nodule formations as determined by von Kossa staining.

brane potential could induce osteoprogenitor cell differentiation (26). In contrast to growth factor induced membrane hypopolarization in PC12 cells (21), we demonstrated that SW treatment induced membrane hyperpolarization of bone marrow stromal cells. Studies with articular chondrocytes or bone cells also demonstrated that membrane hyperpolarization could be induced by autocrine/paracrine molecules (27, 28). Moreover, evidence has previously shown that SW treatment could cause increase of membrane permeability and influx of biological substance (29), this may be also related to membrane hyperpolarization. Results from the present study have further demonstrated that SW-promoted membrane hyperpolarization is accompanied with a rapid activation of Ras signaling transduction and later osteogenic differenti-

ation of human bone marrow stromal cells. It remains unknown why the SW-mediated membrane perturbation and Ras activation directs osteogenesis but not myogenesis or adipogenesis. There are many small G proteins incorporated in plasma membranes (30, 31) and involved in mechanical promotion bone formation (32, 33). Further studies are needed to clarify whether certain membrane associated G protein activation mediates different lineage differentiation of human mesenchymal stem cells.

In summary, we have demonstrated that a noninvasive physical SW treatment provides a promising regimen for regulation of osteogenesis. The SW treatment may be not only applicable for enhancement of osteogenesis in fracture healing but may also provide an alternatively noninvasive method for control of bone remodeling such as osteoporosis and osteopenia.

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