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Shockwave Stimulates Oxygen Radical-Mediated Osteogenesis of the Mesenchymal Cells From Human Umbilical Cord Blood

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ABSTRACT: Human umbilical cord blood (HUCB) mesenchymal progenitor cells expressed stro-1 and CD44 (CD29), and subsequently, differentiated toward osteogenic lineage. Physical shockwave treatment increased osteogenic activity of HUCB mesenchymal progenitor cells through superoxide-mediated TGF-β1 induction. Transplantation of shockwave-treated HUCB mesenchymal progenitor cells enhanced healing of segmental femoral defect in severe combined immunodeficiency disease (SCID) mice.

Introduction: Mesenchymal progenitor cells (MPCs) in the bone marrow are precursors to bone development. It remains uncertain whether MPCs are present in human umbilical cord blood (HUCB) and are capable of differentiating into osteogenic cell lineage. Extending from a model of shockwave (SW) promotion of bone marrow stromal cell differentiation toward osteoprogenitors in rats, we further investigated how physical SW mediated biological responses in regulating osteogenic differentiation of HUCB MPCs.

AQ: 1 **Materials and Methods:** HUCB was subjected to ESW treatment at different energy flux densities and impulses. Colony-forming units-stroma (CFU-Stroma), osteogenic activities (Cbfa1/Runx2 expression, bone alkaline phosphatase activity, and bone nodule formation), and bone formation by heterologous transplantation into SCID mice were assessed.

Results: Few $CD34^+$ stem cells (1.3%) and stro-1^+ cells (1.0%) were present in the freshly prepared mononuclear cells (MNCs) from HUCB. The number of stro-1^+ cells, but not $CD34^+$, increased to 72.4% in the adherent cell culture over 6 days. Stro-1^+ cells co-expressed CD44 and CD29 markers and grew into CFU-Stroma that matured into bone nodules. We found that the SW treatment (0.16 mJ/mm^2 energy flux density, 200 impulses) elicited superoxide production and promoted formation of CFU-Stroma, but not of hematopoietic CFU-Mix. SW also enhanced the production of transforming growth factor (TGF)-β1, but not of interleukin (IL)-3 or GM-CSF. Neutralization of TGF-β1 significantly reduced SW-promoted CFU-Stroma formation. Superoxide scavenging by superoxide dismutase blocked SW enhancement of TGF-β1 production and formation of CFU-Stroma. Administration of SW-treated HUCB MPCs to SCID mice with femoral segmental defects facilitated dense, bridging callus and gap closure.

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Conclusion: HUCB MPCs subjected to SW treatment is a potential source for stem cells useful in the treatment of orthopedic disorders. An optimal physical SW treatment enhanced osteogenesis through superoxide-mediated TGF-β1 production. Physical stimulation is an alternative method for extending mesenchymal stem cells of HUCB.

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Key words: mesenchymal progenitor cells, human umbilical cord blood, shockwave, superoxide, growth factor, osteogenesis

INTRODUCTION

Fn* INTEREST IS INCREASING in the role of mesenchymal stem cell system functioning in the support of hematopoiesis and in multipotential differentiation into myoskeletal progenitors.[1,2] Mesenchymal progenitor cells (MPCs) from human bone marrow have generated osteoblasts in immunodeficient mice[3] and have been used in clinical trials to rescue patients with osteogenesis imperfecta.[4,5] Bone marrow MPCs are precursors in bone development.[1–5] It remains uncertain whether MPCs are present in human umbilical cord blood (HUCB) and whether they can differentiate into osteogenic lineage.

Hematopoietic stem cells from bone marrow and HUCB provide have important clinical applications related and unrelated transplantations.[6–8] There is evidence that

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HUCB transplantation permits earlier engraftment and is followed by less graft versus host disease (GVHD) than bone marrow transplantation.[6,7] Although mesenchymal stem cell transplantation using HUCB has been proposed,[9] studies on its potentials and applications lag behind those on the MPC transplantation using bone marrow. Prindull et al.[10] first identified colony-forming unit-fibroblast (CFU-F) from HUCB, but did not clarify their lineage. AQ: 3 Guitierrez-Rodriguez et al.[11] demonstrated that adherent stromal cells from HUCB were mainly $CD1a^+$ dendritic cells, rather than osteoblasts or endothelial cells. Erices et al.[12] have recently reported that stromal cells in the HUCB belonged to osteoclasts- and fibroblast-like cells and that CFU-F colony formation was totally absent in the HUCB. Nieda et al.[13] identified endothelial cell precursors, but not CFU-F colonies in HUCB. Among tissue precursors that have been found in HUCB, it seems that the number of MPCs is low.

Results from the HUCB transplantations for hematological disorders have demonstrated that the HUCB transplantation provides a simple collection of stem cells without purification and is followed by less GVHD.[6-8] This suggests that HUCB hematopoietic stem cells have a higher adaptability of hematopoietic regeneration and totipotential differentiation after transplantation. Although HUCB mesenchymal stem cells (MSCs) are few in number, whether HUCB mesenchymal stem cells possess the capacity for osteochondrogenic adaptability deserves further studies.

To enhance ex vivo expansion of osteogenic tissues from HUCB MPCs, we sought to delineate the growth and differentiation of HUCB MPCs. Studies were also performed to explore whether noninvasive shockwave (SW) therapy enhanced osteogenesis of the HUCB MPCs. SW therapy has induced bone regeneration in nonunion fracture.[14] In two recent studies,[15,16] we used a SW model to promote bone marrow stromal cell differentiation into osteoprogenitors. In this study, we further investigated how physical SW mediated biological responses that regulated growth and differentiation of the HUCB MPCs. We also explored whether HUCB MPCs subjected to SW promoted healing of segmental defects in severe combined immunodeficiency disease (SCID) mice.

MATERIALS AND METHODS

Culture and identification of MPCs from HUCB

Bone marrow stem cells are categorized into adherent and nonadherent progenitors. Adherent cells positive for stro-1 antigen staining are recognized as MPCs; nonadherent stem cells usually develop into hematopoietic cells.[17] Recently, some studies have demonstrated that nonadherent bone marrow cells differentiate into MPCs.[18] Mononuclear cells (MNCs) were harvested from the interface of the Ficoll-Paque density gradient (d = 1.007 g/ml; Pharmacia Biotech AB, Uppsala, Sweden) after centrifugation at 500g for 30 minutes. Cell number and viability were determined using a hemacytometer after staining with 0.4% trypan blue. MNCs (5×10^5 cell/well, 24-well culture plates) were cultured in DMEM containing 10% FBS (Life Technologies, NY, USA) for 6 days in a 5% CO_2, 37°C incubator. After discarding nonadherent hematopoietic cells, adherent MNCs from HUCB were further cultured for 18 days with replacement of one-half culture medium every 3 days to assess growth and differentiation of MPCs. Colony-forming units stroma (CFU-Stroma) formations were identified by positive staining of stro-1 antigen associated with CD44 or CD29 expression in an adherent culture system. Stromal cells were collected on days 0, 1, 3, 6, 9, 12, and 18 for differentiating stro-1^+ stromal cells from other adherent cells and calculating the number of stro-1^+ stromal cells by immunofluorescence assay. Specific CD14 and CD31 antibodies were used to differentiate adherent monocytes and endothelial cells, respectively, from mesenchymal cells.[11,13] Stromal cell colonies with >32 segregated cells positive for human stro-1 and CD44 staining, but not CD34 staining, were identified as CFU-Stroma formations.

Determination of CFU-Stroma cell differentiation

MNCs (5×10^5 cells/well; 24-well plate) harvested from HUCB were respectively cultured in myogenic, osteogenic, chondrogenic, and adipogenic conditional media.[19] Myogenic culture medium used was chick embryo extract (0.5%; Gibco) and 10% FBS in DMEM. Chondrogenic conditional medium contained 10^{-8} M dexamethasone, transforming growth factor (TGF)-β1 (10 ng/ml), and 10% FBS in BGJb medium. Osteogenic culture medium included 10% FBS, 10^{-8} M dexamethasone, ascorbic acid (50 μg/ml), and 10 mM β-glycerol phosphate in DMEM.[15] Adipogenic culture medium initially included 10^{-8} M dexamethasone, isobutyl methylxanthine (0.5 μM), indomethacin (60 μM), insulin (0.01 mg/ml), and 10% FBS in DMEM for 2 days, followed by the condition medium without isobutyl methylxanthine and indomethacin.[19] Cells harvested from these cultures were fixed with methanol on a slide for staining with specific differentiation markers. Horseradish-peroxidase diaminobenzidine (HRP-DAB) immunohistochemical staining of differentiation markers including bone alkaline phosphatase, chondroitin proteoglycans, and myotubulin identified osteogenic, chondrogenic, and myogenic lineage, respectively.[16] Oil red O staining of lipid droplets identified adipogenic lineage as previously described.[16]

SW effects on the CFU-Stroma and CFU-Mix formation

We investigated the effect of various SW energy flux densities on osteogenesis and hematogenesis. MNCs (2×10^5 cells/well; 24-well plate) harvested from HUCB (5 ml) with and without SW treatment (0.16, 0.24, and 42 mJ/mm^2 energy flux density; 200 impulses) were culture in osteogenic and hemtopoietic medium for 12 days, respectively. In some experiments, stromal cell cultures were cultured for 21 days, and bone alkaline phosphatase activities and bone nodule formations were measured as previously described.[15] The CFU-Mix assay was per-

formed using semisolid agar plates containing 0.35% Agar Nobel (Difco) and Iscove's medium supplemented with autologous plasma 20% (vol/vol) for 12 days as modified from our previous description.[20,21] We further determine the influence of various SW impulses on osteogenesis and hematogenesis. MNCs harvested form HUCB with and without SW treatment (0.16 mJ/mm^2 energy flux density; 100, 200, 500, and 1000 impulses) were used to determine CFU-stroma and CFU-Mix. Results were calculated from those colonies that had more than 32 cells aggregating on agar plates.

Detection of osteogenic lineage-specific mRNA expression by RT-PCR

Total RNA was extracted from the 10^6 cells with and without SW treatment and purified by the Tri reagent containing monophasic solution of guanidine thiocyanate and phenol (Sigma Chemical, St Louis, MI, USA).[16] One microgram of total RNA was RT into cDNA, followed by PCR amplification using human gene-specific primers: Cbfa1/Runx2 (forward) (5′-GAATGCTTCATTCGCCTCACA-3′), (reverse) (5′-TGACCTGCGGAGATTAACCAT-3′) (114-bp expected product); collagen type I (forward) (5′-TGACGAGACCAAGAACTG-3′), (reverse) (5′-CCATC-CAAACCACTGAAACC-3′) (156-bp expected product); osteocalcin (forward) (5′-AAGAGACCAAGGCGCTA-CCT-3′), (reverse) (5′-GCCGATAGGCCTCCTTGAAAG-3′) (135-bp expected product); β-actin (forward) (5′-CGCC-AACCGCGAGAAGAT-3′), (reverse) (5′-CGTCACCGG-AGTCCATCA-3′) (168-bp expected product). Parameters for RT-PCR cycling were as follows: RT reaction at 50°C for 2 minutes and 60°C for 30 minutes; PCR reaction 95°C for 5 minutes, followed by 40 cycles of PCR reactions at 94°C for 20 s and 60°C for 1 minute. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by ultraviolet-induced fluorescence.

Oxygen radical production in HUCB treated with SW

Production of oxygen radicals was determined by lucigenin-amplified chemiluminescence as previously described.[22] In brief, 5 ml of HUCB in a conical tube was treated with or without SW treatment (0.16 mJ/mm^2 energy flux density; 0, 100, 200, 500, and 1000 impulses) in the presence or absence of superoxide dismutase (SOD; 500 U/ml). To differentiate which oxidase was responsible for SW induction of superoxide production, HUCB was treated with and without SW in the presence or absence of 30 μM diphenyliodonium (DPI, an NADPH oxidase inhibitor), 50 μM allopurinol (a xanthine oxidase inhibitor), or 50 μM rotenone (a mitochondrial oxidase inhibitor; Sigma Chemicals). After treatment, 0.2 ml blood were placed in a luminometer (1251 LKB; Wallac Co.) for 60 minutes to measure lucigenin-amplified chemiluminescence of oxygen radicals. Results were presented as a total chemiluminescence under the integrated area for each chemiluminescence tracing[22] after SW treatment.

Measurement of hematopoietic and osteogenic growth factor production

Hematopoietic growth factors (interleukin [IL]-3 and GM-CSF) and osteogenic growth factors (TGF-β1, bone morphogenetic protein [BMP]-2, and bFGF) were determined using ELISA kits, respectively. All ELISA kits were purchased from Quantikine (R&D Systems, Minneapolis, MN, USA) and performed according to manufacturer's instructions. Briefly, supernatants from the cell cultures were subjected to ELISA assay for 2, 6, 16, 24, 48, and 72 h. To determine whether superoxide was really involved in SW promotion of osteogenic differentiation and growth factor production, SOD (500 U/ml) was added to blood right after SW treatment. Culture supernatants were collected and stored at −70°C until studies. In some experiments, MNCs harvested from HUCB with and without SW were treated with 5 ng/ml monoclonal anti-TGF-β1 antibodies and were assessed for CFU-Stroma after a 12-day culture.

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Bone formation reconstituted by HUCB in SCID segmental defect model

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan. Male SCID mice (22–26 g body weight) were caged in pairs and maintained on rodent chow and water ad libitum. SCID mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutal; Abbott Laboratories, IL, USA). A lateral incision was made along the proximal femurs, and the vastus muscle was divided longitudinally. A transverse, partial osteotomy of the mid-diaphysis of the left femur was made using an oscillating mini-saw. A 0.2-mm intramedullary pin was introduced into the femoral canal through the intercondylar notch for loose fixation. The distal end of pin was existed through a separate incision over the greater trochanter, and the pin tip was bent to prevent the migration. Periosteum at the fracture site was cauterized 2 mm proximally and 2 mm distally. The incision was closed in layers. Chloromycinetin (10 mg/kg, IP) and analgesia (0.02 mg/kg bupenorphine, SC) were given for 2 days postoperatively. Animals were allowed unrestricted weight-bearing and activity as tolerated postoperatively. MNCs (5×10^6 cells/mouse) harvested from HUCB with and without SW treatment (0.16 mJ/mm^2 energy flux density; 200 impulses) were transplanted into the fracture site under flowscope guidance 2 weeks postoperatively. Callus and closure of the osteotomy gap were evaluated using a mammography system (35 kV, 80 mA, film to focus distance 50 cm; Lorad M-IV; Varian, Salt Lake City, UT, USA). Dense callus bridging and closing of the osteotomy gap was radiographically evaluated for a bony union by two radiologists who are blinded to the treatment regimens.

Statistical analysis

Data were analyzed with a nonparametric ANOVA followed by Student's t-test to determine significance between treatments; $p < 0.05$ was considered statistically significant.

TABLE 1. STRO-1$^+$ CELLS IN THE ADHERENT CULTURE OF MNCS OBTAINED FROM HUCB

	Adherent cells (%)				
Culture time	*CD14*	*CD31*	*Stro-1*	*CD44*	*CD29*
0 day	30.2 ± 8.8	8.3 ± 2.1	1.0 ± 0.4	4.0 ± 1.9	5.6 ± 2.5
6 days	10.6 ± 1.9	12.0 ± 3.1	72.4 ± 3.9	78.2 ± 4.7	44.8 ± 8.4
12 days	4.3 ± 1.2	18.3 ± 2.9	76.9 ± 2.4	73.2 ± 5.0	45.6 ± 8.5

MNCs (5×10^5 cells/ml) were cultured in DMEM and harvested for immunohistochemical staining with CD14, CD31, Stro-1, CD44, and CD29 monoclonal antibodies on days 0, 6, and 12. Data presented are mean percentages, calculated from six duplicate experiments.

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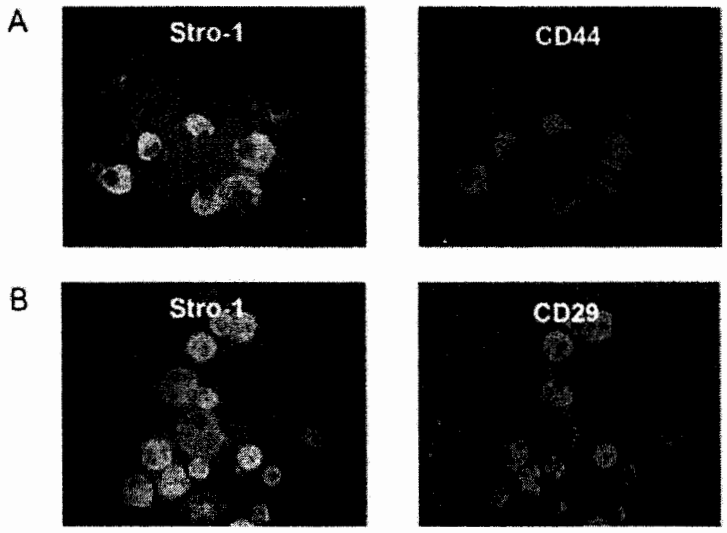

FIG. 1. Co-expression of CD44 and CD29 in stro-1$^+$ stromal cells from adherent cell culture of HUCB. Cells from the adherent cell culture of MNCs (5×10^5 cells/ml) for 6 days were subjected to dual staining of stro-1/CD44 or stro-1/CD29 at 520 and 580 nm wavelength filters. (A) CD44 and stro-1 antigens were simultaneously expressed on stromal cells. (B) CD29 was partially expressed (43.6%) on the stro-1$^+$ stromal cells.

RESULTS

Presence of MPCs in the HUCB

Immunohistochemical staining of stro-1 antigen on human MPCs indicated that, 1.3% HUCB MNCs were CD34$^+$ and 1.0% were stro-1$^+$ on the cell surface. Stro-1$^+$ cells were mainly present in adherent cells but not in nonadherent cells during in vitro culture. As shown in Table 1, stro-1$^+$ cells in adherent cells dramatically increased to 72.4% after in vitro culture for 6 days. CD31$^+$ endothelial cells (12.0%) and CD14$^+$ monocytes (10.6%) were detectable in adherent culture for 6 days. Stro-1$^+$ cells in HUCB MNCs co-expressed CD44 (100%) or CD29 (43.6%). Immunohistochemical staining indicated that stro-1$^+$ cells co-expressed CD44 or C29 throughout the culture period (Fig. 1). After in vitro adherent culture for 12 days, 76.9% were stro-1$^+$ cells, 18.3% were CD31$^+$ cells, and 4.9% were CD14$^+$ cells. Stro-1$^+$ cells continued to multiply, with a doubling time every 3 days (Fig. 2), and total stro-1$^+$ cells increased from 1.5×10^3 to 8.5×10^4 cells/ml in 18 days (Fig. 2).

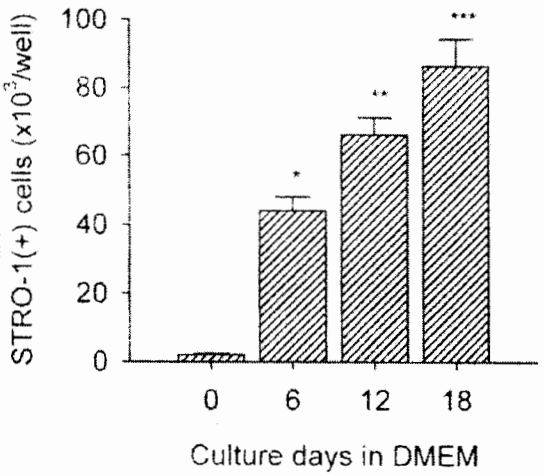

FIG. 2. Growth of stro-1$^+$ stromal cells from HUCB. Total stro-1$^+$ cells in the adherent cell culture of MNCs (5×10^5 cells/ml) from HUCB increased after culture for 6 days. $^*p < 0.001$; $^{**}p < 0.001$; and $^{***}p < 0.001$ indicate significant differences compared with day 0. Results are presented with mean ± SE calculated from six paired triplicate experiments.

CFU-Stroma formations in adherent cell culture of HUCB

HUCB MNCs from the adherent cell culture grew into CFU-Stroma in 12 days (Fig. 3A). Most CFU-Stroma col-

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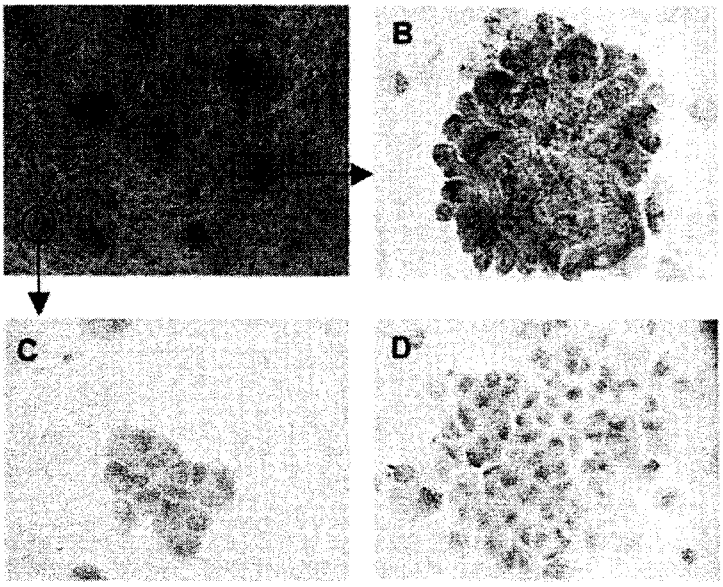

FIG. 3. CFU-Stroma in the adherent cell culture of HUCB. (A) MNCs (5×10^5 cells/well) obtained from HUCB were cultured for 12 days in 24-well culture plates for assessing CFU-Stroma formations. Colonies >32 segregated cells were harvested for cell lineage determination of the stromal cells. (B) Horseradish peroxidase–developed brown DAB immunohistochemical staining demonstrated that most CFU-Stroma formations were stro-1$^+$. (C) A few small colonies were stro-1$^-$. (D) Immunostaining for cell lineages in CFU-Stroma colonies demonstrated that cells were positive for bone alkaline phosphatase, but not for chondroitin proteoglycans, myotubulin staining, or Oil red O staining of lipid droplets.

onies were stro-1$^+$ (Fig. 3B), but a few small colonies were stro-1$^-$ (Fig. 3C). Further studies to determine cell lineages in CFU-Stroma colonies indicated that osteogenic lineage was predominant in stromal colonies. Colonies raised in the osteogenic condition medium were positive for alkaline phosphatase staining (Fig. 3D) and matured into bone nodules (Fig. 5C). There was no positive staining of chondrogenic, myogenic, and adipogenic colonies as respectively determined by chondroitin proteoglycans, myotubulin, and Oil red O staining after culture in respective conditioned medium.

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Physical SW promoted CFU-Stroma formation but not CFU-Mix formation

We have previously demonstrated in a rat bone marrow model that physical SW could enhance bone marrow stromal cell differentiation toward osteoprogenitors.[15,16] Further studies have indicated that SW treatments (0.16, 0.24, and 0.42 mJ/mm^2 energy flux density; 200 impulses) significantly increased CFU-Stroma formation in the stromal cells cultured from HUCB (Fig. 4A). SW treatments did not affect CFU-Mix formation (Fig. 4B). We further demonstrated that SW treatments (0.16 mJ/mm^2 energy flux density; 100 and 200 impulses) significantly enhanced CFU-Stroma formations in the stromal cells cultured from HUCB, whereas a dose >500 impulses suppressed CFU-Stroma formations (Fig. 4C). SW treatments with various impulses did not affect CFU-Mix formation (Fig. 4D). No chondrogenic, myogenic, or adipogenic differentiation was detected by chondrotin proteoglycans, myotubulin, or Oil Red O staining of lipid droplets, respectively. To determine further osteogenic differentiation promoted by SW treatment, we assessed specific osteogenic transcription factor (Cbfa1/Runx2) and bone matrix proteins (collagen type I and osteocalcin expression) after SW treatment. We found that Runx2/Cbfa1, collagen type I, and osteocalcin mRNA expression increased in 1–6 days after SW treatment with 200 impulses (Fig. 5A). Increased bone alkaline phosphatase activity coincided with elevated osteogenic gene expression (Fig. 5B). Enhancement of bone mineralized matrix by SW treatment (0.16 mJ/mm^2 energy flux density, 200 impulses) was visibly demonstrated by an increase in bone nodule formations after culture for 21 days (Fig. 5C).

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SW induced superoxide production in a dose and NADPH-dependent fashion

As demonstrated by lucigenin-amplified chemiluminescence of oxygen radicals,[22] we found that SW treatments (0.16 mJ/mm^2 energy flux density; 100, 200, 500, or 1000 impulses) induced a dose-dependent increase of chemiluminescence in 60 minutes (Fig. 6A). HUCB was subjected to SW treatment (0.16 mJ/mm^2 energy flux density; 1000 impulses) and underwent gross hemolysis (Fig. 6B), and CFU-Stroma formations decreased (Fig. 6A). We further verified whether SW promotion of O_2^- production was linked to mitochondrial, xanthine oxidase, or NADPH oxidase activity. Results indicated that cells pretreated with 30 μM DPI, an NADPH oxidase inhibitor, but not other oxidase inhibitors, significantly suppressed SW-promoted O_2^- production (Fig. 6C) and SW-promoted CFU-Stroma formation (Fig. 6D). Overall, our findings indicate that appropriate SW treatment elicited optimal oxygen radical production for better CFU-Stroma formation from HUCB MPCs.

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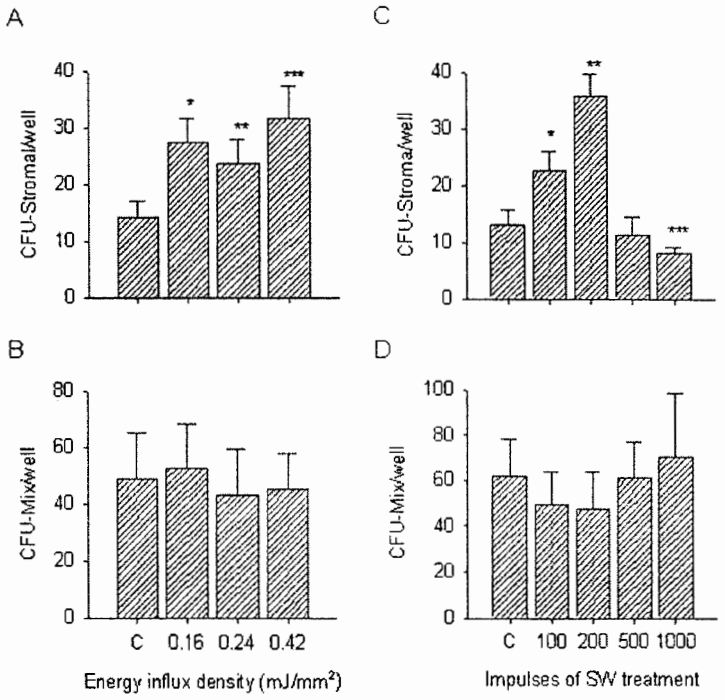

FIG. 4. SW promotion of CFU-Stroma, but not of hematopoietic CFU-Mix, formations in a dose-dependent effect. (A) The SW treatment at 0.16, 0.24, and 0.42 mJ/mm^2 energy flux density for 200 impulses promoted CFU-Stroma formations in the stromal cell culture for 12 days. $*p = 0.015$, $**p = 0.023$, and $***p = 0.011$ indicate significant differences compared with the control without SW treatment. (B) The SW treatments, however, did not significantly affect hematopoietic CFU-Mix formations. (C) The SW treatments at 0.16 mJ/mm^2 energy flux density for 100 and 200 impulses enhanced CFU-Stroma formations in the stromal cell culture for 12 days, whereas doses greater than 500 impulses suppressed the CFU-Stroma formations. $*p = 0.022$, $**p < 0.001$, and $***p = 0.031$ indicate significant differences in comparison with the control without SW. (D) The SW treatments, however, did not significantly affect the hematopoietic CFU-Mix formations in a dose-dependent fashion. Results were presented with mean ± SE calculated from six paired triplicate experiments.

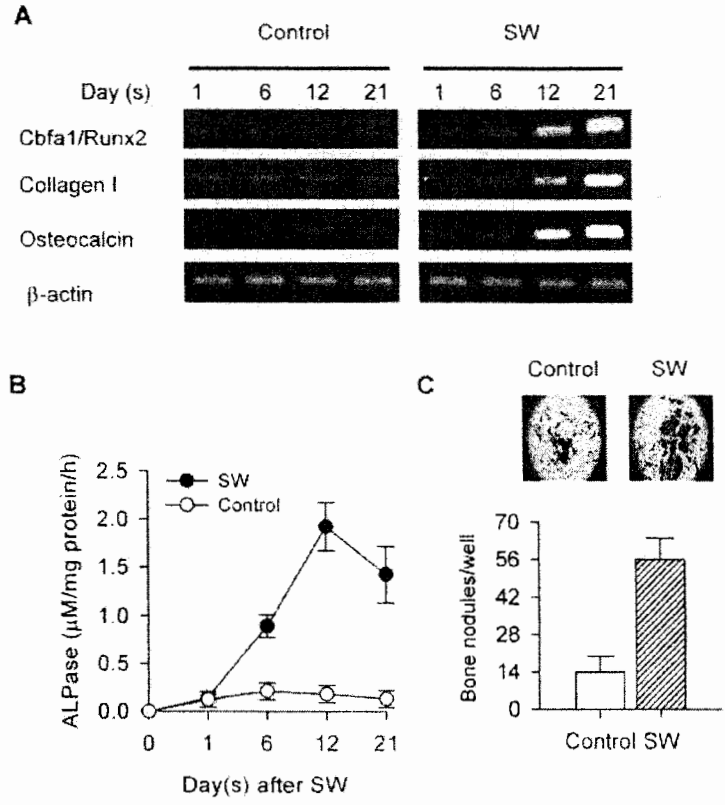

FIG. 5. SW promoted osteogenic differentiation markers of HUCB stomal cells. (A) The SW treatment at 0.16 mJ/mm^2 energy flux density for 200 impulses induced Cbfa1/Runx2, collagen type I, and osteocalcin mRNA as determined by RT-PCR assay in 1–6 days. (B) Increased bone alkaline phosphatase activity coincided with elevated osteogenic gene expression. (C) In a longer culture for 21 days, the SW treatment (0.16 mJ/mm^2, energy flux density, 200 impulses) significantly promoted bone nodule formations as determined by von Kossa silver staining compared with the control without SW treatment. Results were presented with mean ± SE calculated from six paired triplicate experiments.

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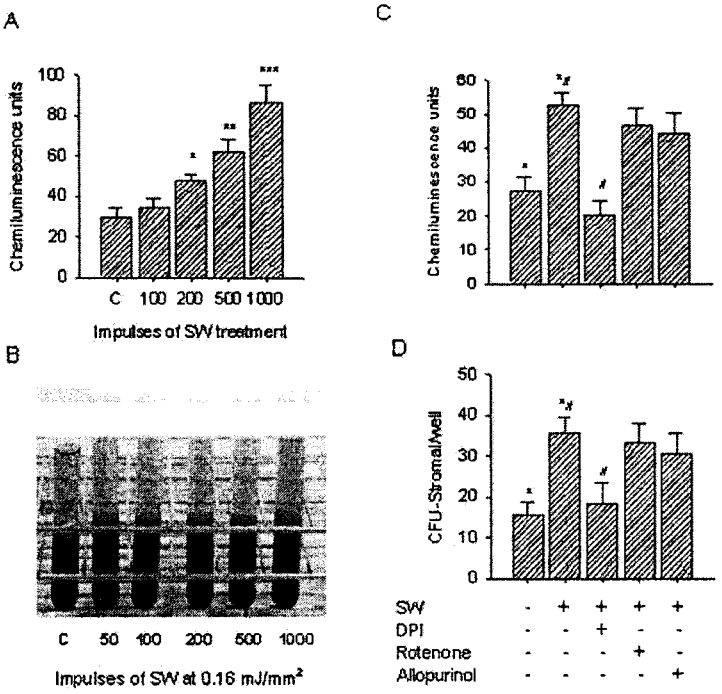

FIG. 6. Effect of SW stimulation on oxygen radical production and osteogenesis of HUCB. (A) SW raised oxygen radical production in a dose-dependent fashion as demonstrated by lucigenin-amplified chemiluminescence. Five milliliters of HUCB was treated with SW at 0.16 mJ/mm^2 energy flux density for 0, 100, 200, 500, or 1000 impulses. After completing SW treatments in 15 minutes, 0.2 ml of blood were placed onto microtiter plates in triplicate for continuous tracing of oxygen radical production by lucigenin-amplified chemiluminescence. $*p = 0.012$, $**p = 0.019$, and $***p < 0.001$ represent a significant difference compared with the control without SW treatment. Results were calculated based on the integrated areas under each chemiluminescence tracing curves from three triplicate experiments. (B) The SW treatment at 0.16 mJ/mm^2 energy flux density for 1000 impulses induced gross hemolysis. (C) Addition of 30 μM DPI, an NADPH oxidase inhibitor, significantly suppressed SW-augmented oxygen radical production. $*p = 0.021$ and $\#p = 0.029$ indicate significant differences between the two groups. (D) DPI pretreatment reduced SW-enhanced CFU-Stroma formations. $*p = 0.015$ and $\#p = 0.018$ indicate significant differences between the two groups. Results were presented with mean ± SE calculated from six paired triplicate experiments.

Scavenge of superoxide by SOD suppressed SW enhancement of TGF-β1 production and CFU-stroma formations

Specific scavenge of superoxide production by SOD (500 u/ml) immediately after SW treatment significantly suppressed lucigenin-amplified chemiluminescence (Fig. 7A) and CFU-Stroma formations (Fig. 7B). This suggested that superoxide acted as an early signal involved in SW promotion of CFU-Stroma formations. Further studies showed that SW-enhanced CFU-Stroma formations was associated with an increase in osteogenic factor TGF-β1 (Fig. 8A), but not in hematopoietic factors, IL-3, and GM-CSF production (Fig. 8B). Scavenge of superoxide by SOD also significantly blocked TGF-β1 production (Fig. 8C). Neutralizing TGF-β1 with monoclonal anti-TGF-β1 antibody significantly suppressed SW-promoted CFU-Stroma formation (Fig. 8D). There was no significant alteration of BMP-2 and bFGF production after SW treatment (data not shown). Our findings indicated that appropriate SW treatment induced osteogenic differentiation of the HUCB MPCs through superoxide-mediated TGF-β1 production.

Heterologous transplantation of SW-treated HUCB promoted bone formation

To show the osteogenic capacity of HUCB in vivo, we transplanted HUCB MNCs with and without SW treatment into segmental femoral defect in SCID mice. Radiographs showed that fractured femurs displayed evident gaps 14 days postoperatively (Figs. 9A and 9B). Fourteen days after transplantation, segmental defect had obvious bridging callus and closure of fracture gap in SCID mice that had received MNC isolated from SW-treated HUCB (Fig. 9C) compared with that without SW treatment (Fig. 9D).

DISCUSSION

In contrast to studies showing that $CD1a^+$ dendritic cells or $CD14^+$ osteoclasts were the major MPCs in HUCB,[11–13] we have demonstrated that stro-1/$CD44^+$ and/or $CD29^+$ MPCs are present in HUCB. Moreover, HUCB MPCs grew into CFU-Stroma formations that differentiated toward osteogenic, but not chondrogenic, myogenic, or adipogenic lineage. This has greatly increased the possibility of applying the MPCs of HUCB for repair of bony dysgenesis or defect by using pharmacologic modulation or genetic transfer. More interestingly, our findings also indicate that physical SW therapy induced by a spark charge under water, a noninvasive method used to enhance bone growth[14–16] and disrupt renal stones,[23] promoted differentiation of MPCs toward osteogenic cell lineage through superoxide-mediated TGFβ production. We have provided the first in vivo evidence that physical SW-stimulated HUCB MPCs significantly promoted healing of femoral segmental defect in SCID mice. This suggests that physically modulation of HUCB MPCs may have a significant role in the treatment of osteopenic disorders.

Results from studies with animal bone marrow cells have provided information that leads to promising approaches for treating many malignancies and degenerative diseases[2–8]

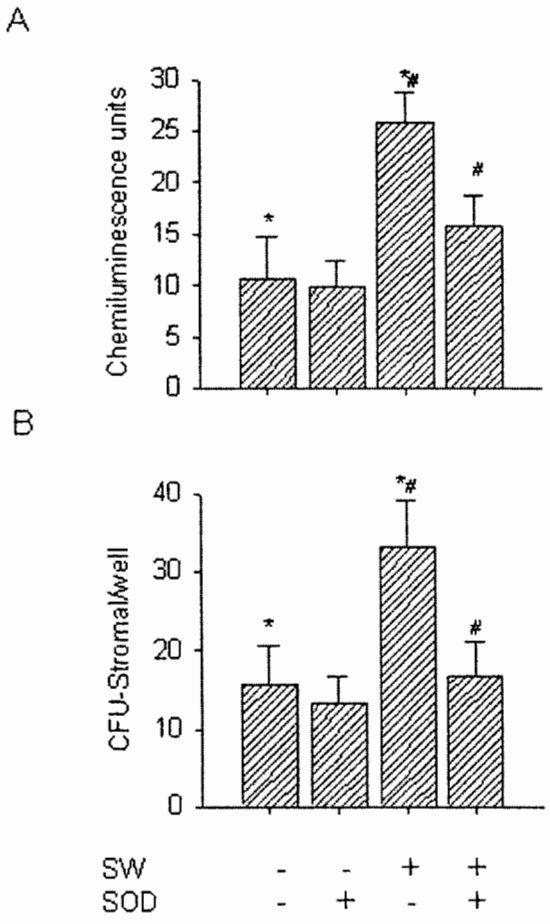

FIG. 7. Superoxide dismutase scavenged SW-induced oxygen radical chemiluminescence and suppressed SW-promoted CFU-Stroma formations. (A) Addition of SOD (500 U/ml) significantly suppressed SW-induced lucigenin-amplified chemiluminescence. $*p = 0.022$ and $\#p = 0.014$ indicate significant differences between the two groups. (B) Addition of SOD (500 U/ml) significantly suppressed SW-promoted CFU-Stroma formations. $*p = 0.017$ and $\#p = 0.011$ indicate significant differences between the two groups. Results were presented with mean ± SE calculated from six paired triplicate experiments.

and for providing neuronal cell replacement.[24] Experiences in the HUCB transplantations for different diseases have shown that HUCB transplantation permitted earlier engraftment and less GVHD than bone marrow transplantation, although HUCB has significantly few stem cells than bone marrow.[6–8] These findings have suggested that HUCB stem cells may be better than bone marrow stem cells for engraftment and may promote faster growth after transplantation. However, because all this information has been accumulated from the hematopoietic cell experiences, it is not known whether mesenchymal stem cells, particularly those in HUCB, possess properties that allow for easier engraftment and faster growth than those from the BM cells.

There is increasing evidence to support that it is possible to transplant MPCs for different purposes after ex vivo modulation for specific lineage differentiation by genetic or biological modulation in ceramic carriers.[24–26] In previous studies, we found that physical SW promoted rat bone marrow stromal cell differentiation toward osteoprogenitors.[15,16] As an extension study of the study, we have now demonstrated that SW promoted osteogenic differentiation of HUCB MPCs and increased in vivo bone formation in an animal model based on femoral segmental defects in SCID mice. To our knowledge, this study is the first to show that application of low energy of SW promoted growth and differentiation of CFU-Stroma formations, but did not affect hematopoiesis, as demonstrated by CFU-mix formation in HUCB. We also found SW-induced optimal superoxide production by NADPH oxidase, but not by xanthine oxidase or mitochondrial oxidase, was involved in inducing TGF-β1 production and mesenchymal cell growth and differentiation. In contrast to other studies showing that superoxide-induced oxidative stress cause cell injury,[27,28] we noted that SW-induced superoxide activated anabolic responses of osteogenic cells by increasing osteogenic and angiogenic factors.[29] Previous studies have indicated that low-level generation of reactive oxygen radicals in nonphagocytic cells participated in a diverse number of normal physiological or cell signaling.[30,31] Our findings support the general concept that a family of NAD(P)H-dependent oxidoreductases may be present in nonphagocytic cells that function as generators of redox signal in response to various growth factor and physical stimulation.

It remains unknown what bioactive molecule(s) initiate(s) the osteogenic and proliferative response HUCB subjected to SW treatment. In our study, we found that physical SW raised superoxide production in a dose-dependent fashion. Optimal dose for enhancement of CFU-Stroma was 200 impulses. A dose higher than 500 impulses, which brought about huge superoxide production, suppressed CFU-Stroma formations. SW treatment at 0.16 mJ/mm^2 energy flux density for 1000 impulses even induced blood cell damage, as indicated by gross hemolysis. In support of this hypothesis, Suhr et al.[32] have shown that higher energy SW treatment induced cavitation and free radicals, resulting in tissue damage. We suggest that an appropriate release of oxygen radicals at a critical time may transmit a signal for mesenchymal cell growth and differentiation, whereas a higher level of oxygen radicals induces a damage signal. Although conclusions drawn from our in vitro study cannot directly apply to in vivo situations or clinical applications, they did highlight the possibility of modulating the growth and differentiation of MPCs by regulation of redox reactions. Certain studies have demonstrated that modulation of redox reactions can promote cell growth or differentiation,[33–35] depending on different cell systems. Moreover, osteogenic cells are found to convert physical stimulation into biochemical signals through release of anabolic or cytokine molecules.[36,37] SW has been found to disturb cell homeostasis and the influx of bioactive radicals.[38] In an earlier study, we found evidence of ESW-induced membrane hyperpolarization and membrane-bound mitogenic signaling.[16] We suggest that physical SW treatments rap- AQ: 8

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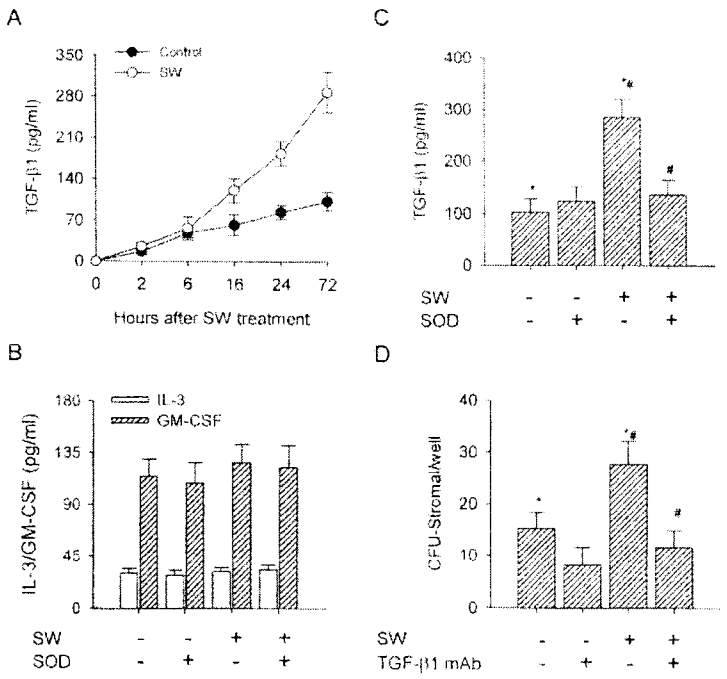

FIG. 8. Superoxide mediated SW promotion of TGF-β1 production, but not of IL-3 or GM-CSF production. (A) Kinetic changes of TGF-β1 production. MNCs (5×10^5 cells/ml) obtained from HUCB with and without SW treatment at 0.16 mJ/mm^2 for 200 impulses in the presence or absence of SOD (500 U/ml) were cultured in 24-well culture plates. Culture supernatants harvested at 0, 2, 6, 16, 24, and 72 h. Enzyme-linked immunoassay indicated significant production of TGF-β1 in 3 days. (B) IL-3 and GM-CSF productions were not affected. (C) SW-enhanced TGF-β1 production was inhibited by scavenging of superoxide by SOD. $*p < 0.001$ and $\#p = 0.017$ indicate significant differences between the two groups. (D) Addition of monoclonal anti-TGF-β1 antibody significantly reduced SW-promoted CFU-Stroma formations. $*p = 0.029$ and $\#p = 0.018$ indicate significant differences between the two groups. Results were presented with mean ± SE calculated from six paired triplicate experiments.

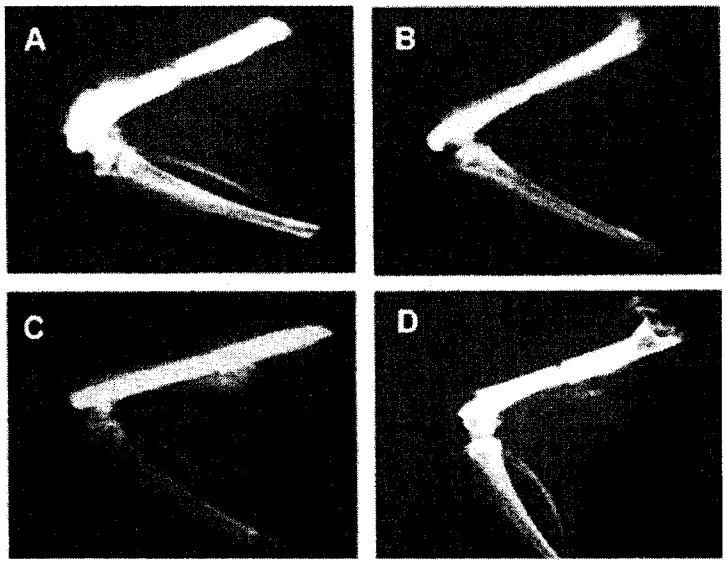

FIG. 9. Radiographs of segmental defects in SCID mice after heterologous transplantation of HUCB MNCs, with and without SW treatment. (A and B) Segmental defects displayed evident gaps. (C) Segmental defect showed dense and bridging callus 14 days after transplantation of MNCs with SW treatment. (D) Segmental defect in the control group without SW treatment displayed radiolucent callus and fracture gap remained clearly evident. Findings are from one of three representative experiments with reproducible experiments.

idly transmit acoustic energy and pressure that perturb cell membranes and alter membrane potentials. Membrane perturbation then induces superoxide and TGF-β1 synthesis and initializes a cascade of mitogenic and osteogenic signaling for osteogenic differentiation of HUCB. In summary, we have shown in this study that MPCs were present in HUCB and were able to differentiate into osteogenic lineage, but not chondrogenic, myogenic, or adipogenic lineages. Physical SW stimulation of HUCB enhanced formation of CFU-Stroma and maturation into presumably through superoxide-mediated induction of osteogenic growth factor, but not of hematopoietic factors. Our findings indicated that physical stimulation is an alternative method for extending osteogenic precursors in HUCB.

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