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Temporal and spatial expression of bone morphogenetic proteins in extracorporeal shock wave-promoted healing of segmental defect

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Abstract

Extracorporeal shock wave (ESW) is a noninvasive acoustic wave, which has recently been demonstrated to promote bone repair. The actual healing mechanism triggered by ESW has not yet been identified. Bone morphogenetic proteins (BMP) have been implicated as playing an important role in bone development and fracture healing. In this study, we aimed to examine the involvement of BMP-2, BMP-3, BMP-4, and BMP-7 expression in ESW promotion of fracture healing. Rats with a 5-mm segmental femoral defect were given ESW treatment using 500 impulses at 0.16 mJ/mm^2. Femurs and calluses were subjected to immunohistochemistry and RT-PCR assay 1, 2, 4, and 8 weeks after treatment. Histological observation demonstrated that fractured femurs received ESW treatment underwent intensive mesenchymal cell aggregation, hypertrophic chondrogenesis, and endochondral/intramembrane ossification, resulting in the healing of segmental defect. Aggregated mesenchymal cells at the defect, chondrocytes at the hypertrophic cartilage, and osteoblasts adjunct to newly formed woven bone showed intensive proliferating cell nuclear antigen expression. ESW treatment significantly promoted BMP-2, BMP-3, BMP-4, and BMP-7 mRNA expression of callus as determined by RT-PCR, and BMP immunoreactivity appeared throughout the bone regeneration period. Mesenchymal cells and immature chondrocytes showed intensive BMP-2, BMP-3, and BMP-4 immunoreactivity. BMP-7 expression was evident on osteoblasts located at endochondral ossification junction. Our findings suggest that BMP play an important role in signaling ESW-activated cell proliferation and bone regeneration of segmental defect.

Keywords: Bone morphogenetic protein (BMP); Fracture healing; Shock wave; Proliferating cell nuclear antigen (PCNA)

Introduction

Fracture healing is a complex process involving the growth and differentiation of osteogenic progenitor cells, the regulation of inflammatory cytokines, and the synthesis and resorption of the extracellular matrix [1,2]. Whereas, a nonunion fracture is a complicated musculoskeletal disease evidenced by a failure of new bone to bridge the defect or a development of the extracellular matrix into loose fibrous tissues or fibrocartilage [3]. Several biophysical methods, such as low-intensity ultrasound, pulsed electric magnetic field, and mechanical loading, have been identified with the promotion of bone formation and fracture healing [4–6]. Extracorporeal shock wave (ESW) is able to release pulsed acoustic energy and has been shown to be an alternative, noninvasive biophysical strategy to enhance fracture healing [7–9]. ESW treatment has previously been shown to affect local blood flow and bone metabolism in rabbit femur [10]. However, the exact mechanism by which ESW promotes fracture healing remains undetermined.

Differentiation of mesenchymal cells and endochondral ossification into the shape of future skeletal elements has

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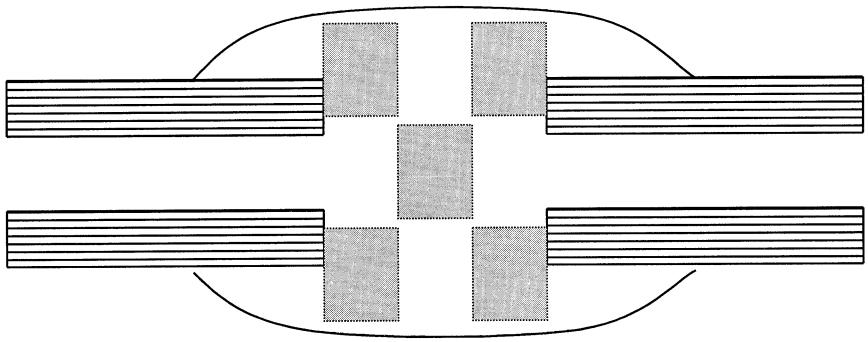

Fig. 1. Schematic view of PCNA and BMP counting area. The number of positive immunostained cells in each of these five areas (shadowed boxes) was counted using 40× magnification.

been implicated as recapitulation of embryonic skeletal development in the repair of bone fracture [11,12]. Bone morphogenetic proteins (BMP) are members of the transforming growth factor (TGF-β) superfamily of signaling molecules and act as morphogens to regulate embryonic development [13,14]. Evidence suggested BMP derived from mesenchymal cells and osteoblasts could exhibit chemotatic properties to stimulate differentiation of mesenchymal cells into osteogenic/chondrogenic lineage and increase expressions of alkaline phosphatase and osteocalcin [15–17]. In addition, BMP also affect bone remodeling through the regulation of osteoclast bone-resorbing activity [18]. BMP have been reported as having a role in the mechanical stimulation of fracture healing and chondrocyte differentiation [19,20]. Four members of the BMP family, BMP-2, BMP-3, BMP-4, and BMP-7, have shown positive effects on facilitating fracture healing and bone formation [21–24]. Thus, we postulated that BMP might be involved in the physical ESW promotion of fracture healing.

This study attempted to elucidate the effect of ESW treatment on cell proliferation and histomorphological changes in the healing of segmental defect. We also sought to clarify the spatial and temporal expression of BMP-2, BMP-3, BMP-4, and BMP-7 in the ESW promotion of bone regeneration of segmental femoral defects.

Materials and methods

Segmental defect model

Three-month-old Sprague–Dawley rats (National Experimental Animals Production Center, Taipei, Taiwan) were used in this study. Animals were caged in pairs and maintained on rodent chow and water ad libitum. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutal sodium, Abbott Laboratories, IL, USA). A four-hole AO/ASIF miniplate was positioned on the anteromedial femoral shaft after stripping of the periosteum. The proximal and distal holes were drilled and tapped with 1.5-mm bicortical screws. A midshaft femoral fracture was created by resection of a 5-mm-long full-thickness disk of cortical and cancellous bone from the middle of the diaphysis using a saline-cooled burr. The fixation plate was further stabilized using a 0.45-mm Kirschner wire that was passed through the hole and the diaphysis and was engaged with the trabecular bone in the proximal femoral diaphysis. After removal of the bone disk, the wound was closed in layers. Chloromycinetin (10 mg/kg) was given intraperitoneally for 2 days postoperatively. Animals were allowed unrestricted weight-bearing and activity as tolerated postoperatively.

ESW treatment

Each rat with the segmental defect was reanesthetized 6 weeks postoperatively and placed in a supine position with all four limbs stabilized in extension. The ESW treatment at 0.16 mJ/mm^2 for 500 impulses (Ossatron HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was employed at the fracture site. The ultrasound transmission gel (Pharmaceutical Innovations Inc, NJ, USA) was used as contact medium between the ESW apparatus and skin. Anterposterior radiographs were taken using a mammography system (35 kV, 80 mAs, film to focus distance 50 cm; Lorad M-IV, Varian Inc., Salt Lake, UT, USA) postoperatively at 2-week intervals. The callus and closure of the osteotomy gap was evaluated. Dense callus bridging and closing of the osteotomy gap was radiographically evaluated for a bony union by a orthopedic surgeon and a radiologist who were blinded to the treatment regimen.

Experimental design

Eighty rats with a segmental defect were randomly divided into ESW and control groups. Forty rats with a segmental defect received ESW treatment at 0.16 mJ/mm^2 for 500 impulses. Ten rats were killed with an overdose of intraperitoneal pentobarbital sodium 1, 2, 4, and 8 weeks after ESW treatment. The remaining 40 rats with a segmental defect received no ESW treatment and were used as controls. Ten rats were killed at the same time intervals as

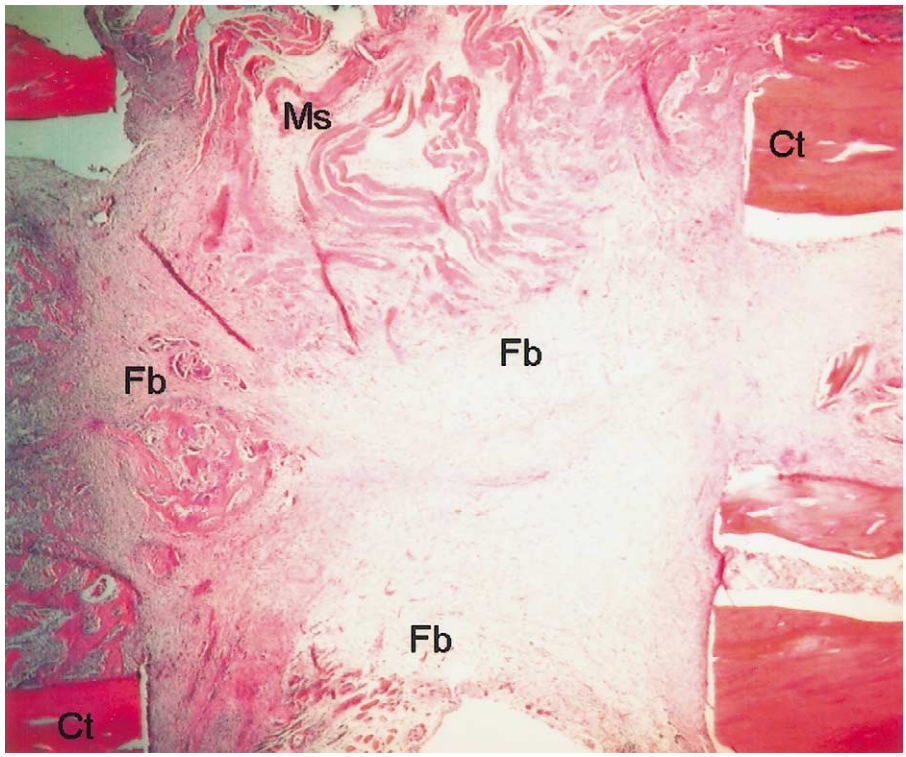

Fig. 2. Low-power photograph of a segmental femoral defect. The segmental defect is filled with fibrous tissue and muscular tissue (5× magnification). Ms, muscle tissue; Fb, fibrous tissue.

the ESW group. Each group of six femurs was dissected to harvest callus for determining BMP mRNA expressions. The remaining four femurs were dissected for histological assessment. Callus was harvested by dissection clean of surrounding tissue, snap-frozen in liquid nitrogen, and then stored at −80°C until RNA extraction.

Reverse transcription polymerase chain reaction assay

Each callus was ground with a mortar and pestle under liquid nitrogen in a RNase-free condition. Total RNA was extracted with a Tri reagent containing monophasic solution of guanidine thiocyanate and phenol (Sigma Chemical Inc. St. Louis, MI, USA). A total RNA at 1 μg was reversely transcribed (RT) using 0.5 mg/ml oligo(dT) primer, reverse transcription buffer, 10 mM dNTP mix, and AMV reverse transcriptase (Promega Corporation, Madison, WI, USA), followed by PCR using rat gene-specific primers: BMP-2 (sense) (5′-GAC GGA CTG CGG TCT CCT AAA G-3′), BMP-2 (antisense) (5′-TCT GCA GAT GTG AGA AAC TCG CA-3′) (258-base pair expected), BMP-3 (sense) (5′-TGC TGT GGC TCT ATG ACA GG-3′), BMP-3 (antisense) (5′-TGG TGT TAC CCA ATT CTC CA-3′) (216-base pair expected), BMP-4 (sense) (5′-CGC CGT CAT TCC GGA TTA CAT-3′), BMP-4 (antisense) (5′-GGC CCA ATC TCC ACT CCC TT-3′) (312-base pair expected), BMP-7 (sense) (5′-CGC CGT CAT TCC GGA TTA CAT-3′), BMP-7 (antisense) (5′-GGC CCA ATC TCC ACT CCC TT-3′) (342-base pair expected), β-actin (sense) (5′-AGT ACC CCA TTG AAC ACG GC-3′), β-actin (antisense) (5′-TTT TCA CGG TTA GCC TTA GG-3′) (168-base pair expected). The RT-PCR cycling parameters were set as follows: the RT reaction at 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min; followed by 40 cycles of PCR reactions at 94°C for 20 s and 60°C for 1 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. All signals were quantified by scan densitometry and the final value was obtained by calculating the BMP/β-actin ration value. The fold of promotion was calculated as the increase over the value of its corresponding control sample.

Immunohistochemistry

Femurs were fixed in 4% buffered paraformaldehyde for 48 h and then decalcified in PBS-buffered 10% EDTA. Decalcified tissues were embedded in paraffin. Bone specimens were cut longitudinally into 5-μm-thick sections and transferred to polylysine-coated slides for conventional hematoxylin–eosin staining (Sigma Chemicals Inc, St. Louis, MO, USA). Immunoreactivity in specimens was demonstrated using horseradish peroxidase (HRP)–3′-,3′-diaminobenzidine (DAB) cell and tissue staining kit (R & D Systems, Inc. Minneapolis, MN, USA), in accordance with manufacturer's instructions. After blocking endogenous peroxidase and nonspecific

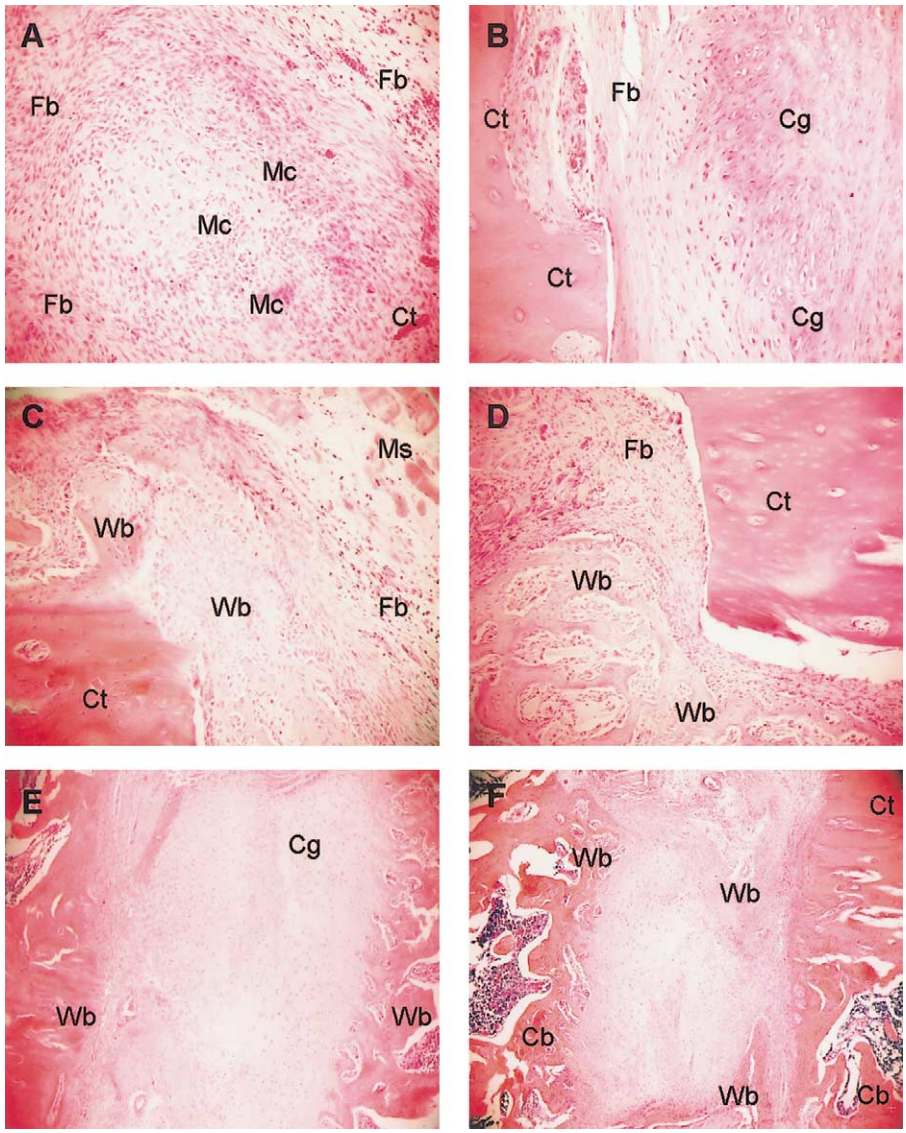

Fig. 3. Histomorphologic changes in a segmental defect treated by ESW treatment. (A) Mesenchymal cell aggregation appeared in the fracture defect at 1 week after treatment. (B) Round shaped chondral cells and cartilageous tissue at the front of the fractured bone cortex appeared 2 weeks after treatment. (C) Intramembrane ossification of periosteum and (D) endochondral ossification of endosteum was observed 2 weeks after treatment. (E) Fracture defect bridged by newly formed cartilage and osseous tissue observed at 4 weeks after treatment. (F) Cartilage is replaced with newly developed osseous tissue resulting in healing of fracture. Ct, cortex; Wb, woven bone; Fb, fibrous tissue; Mc, mesenchymal cells; Cb, cortical bone; Cg, cartilage.

binding, sections were incubated overnight with the antibodies at 4°C. Monoclonal antibodies used for immunohistochemistry were anti-BMP-2, BMP-3, BMP-4, and BMP-7 (Research Diagnostics Inc, Flanders, NJ, USA) and anti-proliferating cell nuclear antigen (PCNA; Upstate Biotechnology, Lake Placid, NY, USA). Sections were further incubated with biotinylated anti-mouse IgG and then incubated with streptavidin conjugated to HRP. Immunoreactivity was determined by incubating the sections in a chromogen solution containing DAB and 0.1% hydrogen peroxide in the dark, followed by counterstaining with hematoxylin. Dehydrate sections were mounted with mounting medium. Those without primary antibodies were enrolled as negative controls for the immunostaining.

Histomorphologic assessment

Five areas of segment defect (Fig. 1) were selected from three sections of four rats to quantitatively assess the number of cells expressing positive PCNA, BMP-2, BMP-3, BMP-4, and BMP-7 cells, using Image-Pro Plus image-analysis software (Media Cybernetics, Sliver Spring, MD, USA). Fibroblasts, chondrocytes, and osteoblasts were identified morphologically. A pathologist

Table 1
Number of fibroblastic, chondral, and osteoblastic cells exhibiting positive PCNA expression of segmental defects in ESW treatment and control groups

Week	Fibroblastic cells		Chondrocytes		Osteoblasts	
	ESW	Control	ESW	Control	ESW	Control
1	1032 ± 187	132 ± 48	353 ± 64	113 ± 32	216 ± 46	94 ± 29
2	632 ± 78	157 ± 36	773 ± 84	127 ± 27	386 ± 67	121 ± 35
4	332 ± 89	165 ± 34	753 ± 83	136 ± 35	654 ± 96	142 ± 64
8	222 ± 45	156 ± 26	453 ± 93	157 ± 31	438 ± 86	157 ± 43

Note. Data are means ± standard error calculated from five areas of segmental defect from three sections of four rats with or without ESW treatment.

blinded to the treatment regimen performed measurement of all sections under 40× magnification.

Statistical analysis

Data were expressed as means ± standard error and analyzed using a nonparametric one-way analysis of variance followed by Student's *t* test to determine the significance between treated and untreated groups. $P < 0.05$ was considered statistically significant.

Results

Histomorphologic changes in segmental defect following ESW treatment

Histological observation demonstrated segmental defects with little or no new bone formation. The central regions of the defects were filled with fibrous tissue and skeletal muscle that had collapsed into the defect. The fractured cortex ends were surrounded with fibroblast, although no bone matrix was produced (Fig. 2). There was also no callus formation around the defects.

Major morphologic changes in segmental defects were observed 1 week after ESW treatment. Mesenchymal cells at the segmental defect showed aggregation and the production of bone matrix (Fig. 3A). Small, round chondral cells and cartilaginous tissue appeared at the fracture gap within 2 weeks after treatment (Fig. 3B). Newly formed bone matrix and woven bone morphology also appeared along the periosteum and endosteum toward the fracture gap (Figs. 3C and D). Newly formed cartilage filled the fracture gap and soft callus had developed 4 weeks after ESW treatment (Fig. 3E). Intensive intramembrane/endochondral bone formation gradually replaced the cartilage and the hard callus completely bridged the segmental defect. Woven bone at the front of cortical bone showed remodeling and newly formed bone-marrow cavity appeared 8 weeks after ESW treatment (Fig. 3F). Dense callus bridging and closing of the osteotomy gap was radiographically observed in the ESW group. Nevertheless, evident fracture gaps remained in fractured femurs that received no ESW treatment (not shown).

ESW treatment promoted cellular proliferation

Experiments were conducted to elucidate the influence of ESW treatment on cell proliferation. Proliferating cells were immunostained for PCNA, a protein associated with the S-phase of a dividing cell [25]. The nuclei of cells with positive PCNA expression showed brown color. Proliferating cells were concentrated at the fracture gap. Aggregated mesenchymal cells adjacent to newly formed bone matrix revealed intensive PCNA expression 1 week after ESW treatment (Fig. 4A). Fibroblastic osteoblasts at newly developed woven bone and round-shaped chondrocytes at hypertrophic cartilage showed evident PCNA expression 2 weeks after treatment (Figs. 4B and C). Osteoblasts at the junction of the cartilage and newly developed osseous tissue continued to proliferate and showed evident PCNA expression (Fig. 4D). However, PCNA expression of mature chondrocytes with large lacuna was slight at 4 weeks after treatment. We compared the number of proliferating cells in the fractured femur with and without ESW treatment and found ESW treatment could stimulate a significant increase ($P < 0.05$) in cell proliferation (Table 1). Furthermore, PCNA expression intensity appeared to depend on the differentiation potential of osteogenic and chondrogenic cells in the temporal sequence of bone regeneration.

BMP-2, BMP-3, BMP-4, and BMP-7 mRNA expression in ESW promotion of callus formation

We further conducted experiments to determine whether ESW promotion of osteogenic/chondrogenic cell proliferation and bone regeneration is linked to BMP induction. Calluses were subjected to RT-PCR assay to determine BMP-2, BMP-3, BMP-4, and BMP-7 mRNA expression. Significant increases ($P < 0.05$) in BMP-2, BMP-3, BMP-4, and BMP-7 mRNA expression of ESW groups were noted within 4 weeks after ESW treatment. BMP-2 was identified as revealing the greatest promotion among the BMP mRNA expression. Calluses of the control group exhibited weak BMP mRNA expression (Fig. 5).

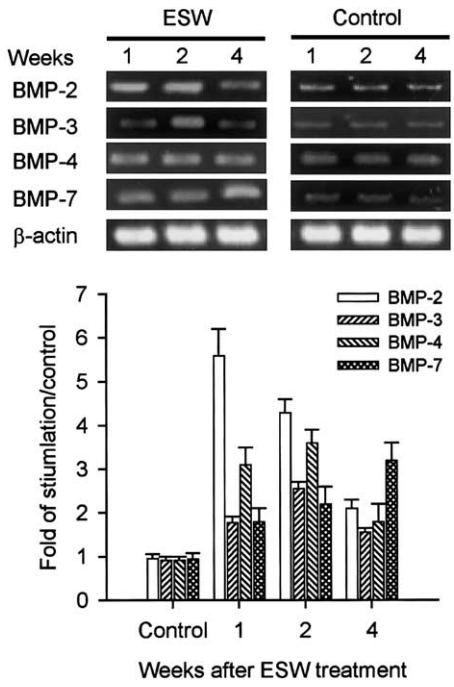

Fig. 5. Kinetic expression of BMP-2, BMP-3, BMP-4, and BMP-7 mRNA in callus. Significant increases in four BMP expressions were noted in ESW group during the healing process. BMP-2 showed the greatest elevation. The levels of callus BMP mRNA expression in the fractured femurs with and without ESW treatment were determined using RT-PCR assay.

Immunohistochemistry of BMP in ESW-promoted healing of fracture defect

Fibroblastic cells located at fibrous tissue in fracture defects that had not undergone ESW treatment exhibited slight BMP-2 expression during experiments. ESW treatment stimulated increase in BMP expression in the segmental defect, with BMP-2, BMP-3, BMP-4, and BMP-7 expression appearing throughout bone regeneration. Immunohistochemical observation showed intensive BMP-2, BMP-3, and BMP-4 expression in aggregated mesenchymal cells at the fracture gap 1 week after ESW treatment (Fig. 6). Chondrocytes at the hypertrophic cartilage and osteoblasts adjacent to the newly formed woven bone showed intensive expression of the four BMP during the 2 and 4 weeks posttreatment (Fig. 6). Mature chondrocytes and osteoblasts showed weak BMP-2, BMP-3, and BMP-4 expression during later stages of the healing process. Osteoblasts in the area of endochondral/intramembrane ossification exhibited strong BMP-7 expression (Fig. 6). Intensities of BMP expression varied with the process of bone regeneration and cell morphology. Table 2 summarizes the number of fibroblasts, chondrocytes, and osteoblasts that exhibited positive BMP expression during ESW-promoted fracture healing. Results show that cells which displayed BMP-2 BMP-3, and BMP-4 expression were mainly mesenchymal cells and chondrocytes during the early stages of healing. The number of chondrocytes and osteoblasts exhibiting evident BMP-7 expression was increased with healing process.

Discussion

In this study, we demonstrate that ESW treatment of segmental femoral defects promoted cell proliferation and resulted in a cascade of bone regeneration that induced healing of the fracture gap. Interestingly, significant increases in BMP-2, BMP-3, BMP-4, and BMP-7 mRNA expression and intensive BMP immunoexpression of callus were noted during the fracture healing process. While a number of physical treatments have been employed for cartilage and bone repair, little has been done to define the role of BMP expression in repair processes. Our research here provides the first evidence that ESW-promoted healing of segmental defects is linked to BMP expression. The findings of this study further evidence that physical ESW treatment may provide an effective noninvasive method by which to initialize bone regeneration in the treatment of bone fracture.

The mechanical or physical impact on tissues exposed to ESW is dependent on tissue impedance. As the impedance of bone tissue is higher than that of soft tissue, the former is more susceptible to damage [26]. ESW, by focusing energy and high pressure, is able to compress and stretch the surface of hard materials [26]. A controversial postulation regarding ESW promotion of fracture healing had attributed the phenomenon to “ESW-induced microfractures.” In animal models, ESW treatment caused periosteal hematoma and periosteum detachment in canine femurs [27]. Femurs of rabbits, after exposure to high-energy pulses of ESW, showed fracture foci and displacement of bony trabeculae in the medullary cavity [28]. Nevertheless, as such studies were performed on normal bones, their hypotheses should not be extrapolated to explain healing of segmental defects. Other reports note that ESW therapy for fractured rat humeri and canine tibia did not cause necroses or hemorrhages at the fracture defect [7,9]. In our study of ESW treatment at the fracture gap, which is filled with muscular and fibrous tissues, histological observation did not show any ESW-induced cracks or microfractures. We suggest that ESW-augmented bone regeneration be attributed to elevation of acoustic energy-sensitive osteogenesis, rather than to damage to the bone architecture. Further research by our group supports this hypothesis, as we found proliferation and differentiation of mesenchymal cells in segmental defects after ESW treatment. Our previous studies *ex vivo* and *in vitro* also support the hypothesis that ESW treatment promotes mesenchymal cell growth and osteogenic differentiation [29–31].

Histomorphological results from the present study demonstrated that segmental defects treated with ESW treatment resulted in mesenchymal cell proliferation, as indicated by intensive PCNA expression, and underwent subsequent typ-

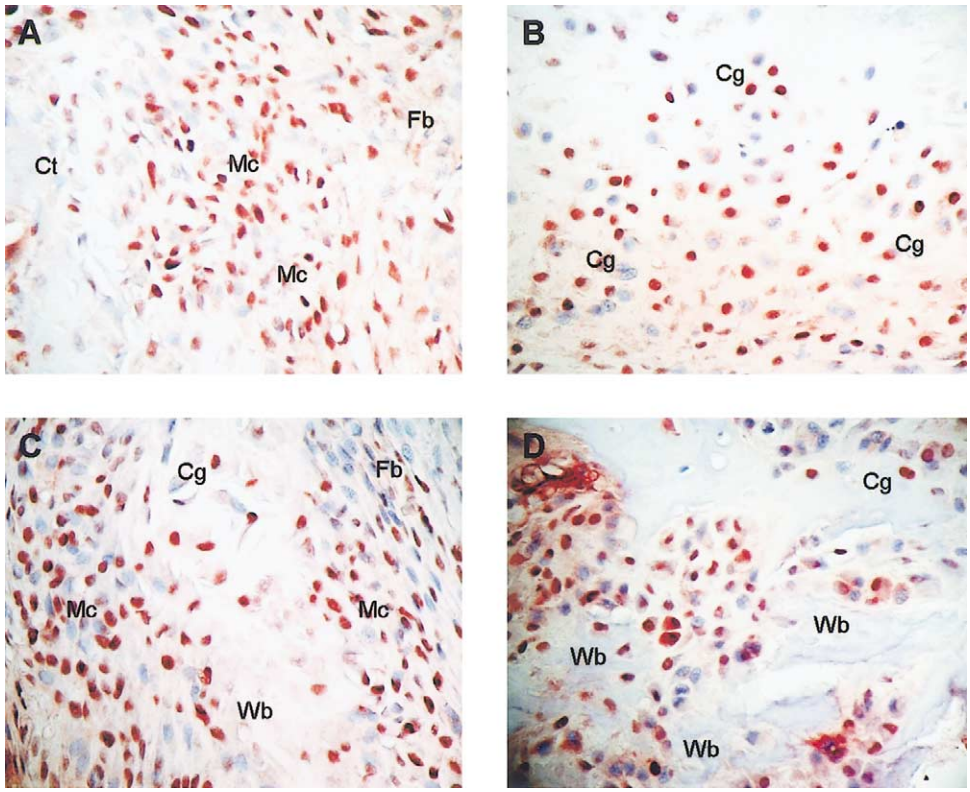

Fig. 4. PCNA expression in ESW promotion of bone regeneration. (A) Aggregated mesenchymal cells show intensive PCNA staining. (B and C). Osteoblastic and chondral cells at the junction of the hypertrophic cartilage exhibits significant PCNA staining. (D) Osteoblasts adjacent to newly formed bone reveal marked PCNA expression. The positive PCNA-stained cells showed brown immunostaining in the nuclei. Fb, fibrous tissue; Cg, cartilage; Wb, woven bone.

ical bone formation as indicated by hypertrophic cartilage and endohondral/intramembrane ossification. This indicates that physical ESW treatment potentially raises the mitogenic response of bone tissue, which enhances osteogenic and chondrogenic precursor cell growth and differentiation. Mesenchymal cell aggregation (condensation) and differentiation into committee chondrogenic and osteogenic progenitor cells has been implicated as an initial phase of skeletal development [32,33]. BMP are regarded as essential osteogenic factors for bone formation. They exhibit proliferative and chemotactic properties to stimulate mesenchymal cell growth [18,34]. Results of the present study suggest that intensive BMP expression in mesenchymal cells, chondrocytes, and osteoblasts potentially promotes progressive chondrogenesis and osteogenesis during bone repair. Elevating BMP production of the callus microenvironment in segmental defects accelerates fibrous tissue remodeling into new cartilage and bone tissue.

It has been implicated that the mitogenic effect of BMP on bone development depends on the developmental potential of osteogenic and chondrogenic progenitor cells [35]. BMP have a strongly stimulating effect on mesenchymal or preosteoblast progenitor cells, although they have little effect on mature osteoblasts or chondrocytes [34]. BMP-2/-4 participated in promotion of callus formation of fractured rabbit tibia and membrane bone healing of rat [36,37]. Furthermore, BMP-2/-4 have shown positive effects on the promotion of collagen I and osteocalcin mRNA during the early stage of distraction osteogenesis. BMP-7 has been reported to act predominantly in the initial phase of endochondral ossification [38,39]. Our observations showed intensive BMP-2, BMP-3, and BMP-4 expression in mesenchymal cells at the segmental defect and in chondrocytes and osteoblasts at newly formed cartilage and bone. We also observed osteoblasts at hypertrophic cartilage and endochondral ossification exhibiting evident BMP-7 expression. Results indicate an important role for BMP-2, BMP-3, and BMP-4 in the early phase of bone regeneration and a key role for BMP-7 in bone maturation and remodeling. We cannot exclude the possibility that certain members of the BMP family including BMP-1, BMP-5 and BMP-6, might be involved in bone regeneration of segmental defects. Our research findings suggest that several members of the BMP family are actively involved in fracture healing. Each has a distinct temporal expression pattern and potentially unique role in healing of segmental defects.

The mechanical adaptive modeling response that promotes bone tissue formation is thought to be beneficial to fracture healing. It has been indicated that bone tissue can convert physical stimulation into biochemical signals *via* release of anabolic molecules or cytokines of bone tissue [40,41]. Other techniques of physical stimulation demonstrated increases in BMP expression after mechanical distraction and mechanical loading treatment for bone forma-

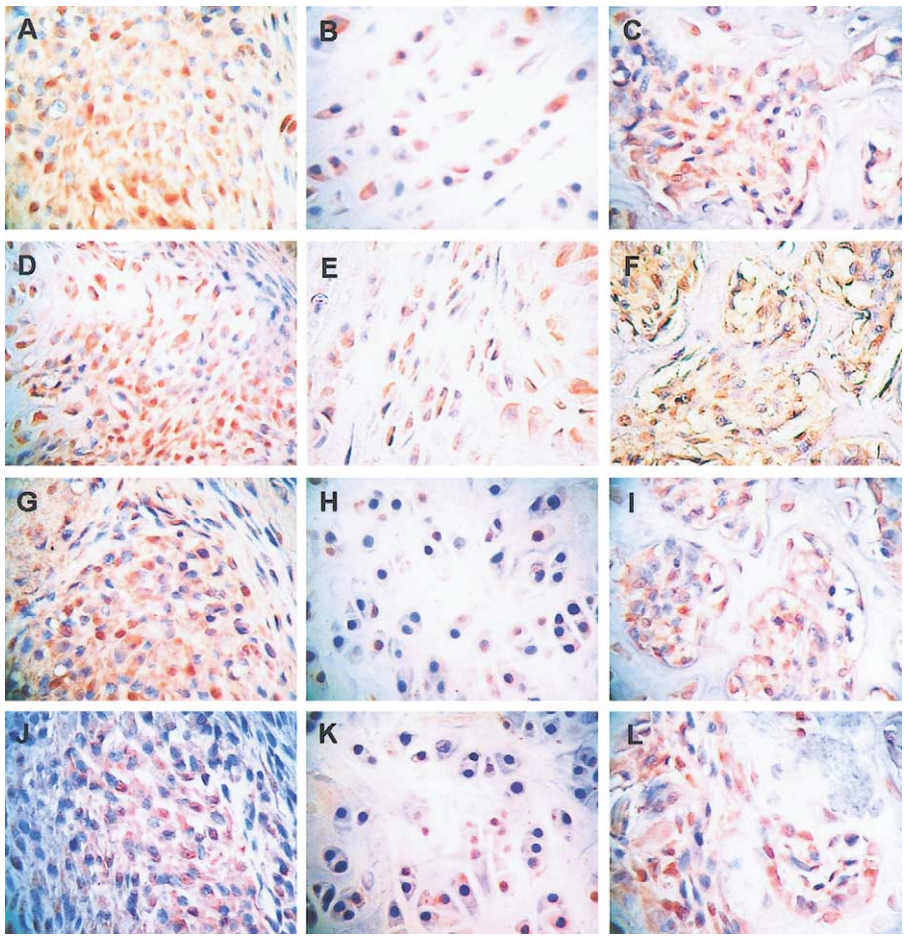

Fig. 6. Immunohistochemical photographs of ESW-promoted fracture healing. (A–C) BMP-2, (D–F) BMP-3, (G–I) BMP-4, (J–L) BMP-7. BMP expression in mesenchymal cells is shown in A, D, G, and J; BMP expression in chondrocytes is exhibited in B, E, H, and K; BMP expression in osteoblasts is shown in C, F, I, and L. The positive BMP-stained cells showed brown immunostaining.

tion and fracture healing [19,38,42]. We also show here that segmental defects receiving ESW treatment display intensive BMP expression, suggesting that BMP are likely important osteogenic mediators in the regulation of new bone formation. The temporal BMP expression in segmental defects suggests that BMP production might be directly and indirectly promoted by the physical stimulation provided by ESW treatment. It is unknown at present how acoustic energy and high pressure released by ESW is translated into biological signals and what mechanoreceptor or signaling pathway may be responsible for these biological reactions. Further studies are needed to explore how cell proliferation is induced by pulsed acoustic energy and pressure of ESW and to determine which cellular signal is responsible for promotion of BMP expressions in bone regeneration of segmental defects in vivo.

Several experimental tissue and genetic engineering strategies, including bone grafts, mesenchymal cell implantation, and gene therapy employing BMP-2-producing bone-marrow cells, for healing of segmental defects are currently undergoing trials [43,44]. However, safety, time, and costs are issues of concern with all these strategies. The

Table 2
Number of fibroblastic, chondral, and osteoblastic cells exhibiting positive BMP-2, BMP-3, BMP-4, and BMP-7 expression of segmental defects after exposure to ESW treatment

Protein	Week	Fibroblastic cells	Chondrocytes	Osteoblasts
BMP-2	1	965 ± 178	312 ± 93	117 ± 31
	2	484 ± 86	627 ± 107	334 ± 87
	4	247 ± 79	679 ± 85	378 ± 76
	8	165 ± 32	411 ± 79	235 ± 82
BMP-3	1	423 ± 41	284 ± 38	157 ± 31
	2	456 ± 52	378 ± 46	292 ± 42
	4	225 ± 38	114 ± 21	158 ± 42
	8	125 ± 25	159 ± 25	181 ± 45
BMP-4	1	785 ± 151	342 ± 73	147 ± 51
	2	413 ± 92	727 ± 147	294 ± 82
	4	285 ± 87	714 ± 133	348 ± 79
	8	213 ± 47	437 ± 92	197 ± 82
BMP-7	1	201 ± 39	236 ± 54	203 ± 47
	2	137 ± 24	375 ± 65	342 ± 63
	4	101 ± 21	568 ± 91	576 ± 81
	8	56 ± 19	382 ± 67	425 ± 92

Note. Data are means ± standard error calculated from five areas of segmental defect from three sections of four rats after ESW treatment.

results of our studies suggest that a noninvasive physical ESW treatment provides a promising regimen to promote fracture healing. This treatment could alter cellular and morphologic changes in fracture defects, with BMP playing an important role in signaling ESW biophysical stimulation of bone regeneration activities of segmental defects.

Acknowledgments

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