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● *Original Contribution*

EXTRACORPOREAL SHOCK WAVES STIMULATE OSTEOBLAST ACTIVITIES

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Abstract—The extracorporeal shock wave therapy (ESWT) is an extensively applied treatment for musculoskeletal disorders because it promotes bone repair. The aim of this study was to evaluate the direct effect of ESWT on murine osteoblasts to clarify the cellular mechanism that leads to the induction of osteogenesis. Osteoblasts in culture flasks were treated with ESWT pulses (500 impulses of 0.05 mJ/mm^2) generated by an electromagnetic device. Using western blot analysis 3 h after ESWT, an increased expression of Bax was found, indicating a fast pro-apoptotic effect of treatment on some of the osteoblasts. Activation of the cyclin E2/CDK2 is the complex that regulates the G1-S transition and is essential for cell proliferation. It was evident 24 to 72 h after treatment, indicating a proliferative stimulus. A decreased expression of osteoprotegerin (OPG) and receptor activator NF kappa B ligand (RANKL) 24 and 48 h after ESW, followed by a later increase of OPG, paired with a much smaller increase of RANKL, was evident by real-time polymerase chain reaction (PCR). The decreased RANKL/OPG ratio suggests inhibition of osteoclastogenesis. We can conclude that ESWT induces bone repair through the proliferation and differentiation of osteoblasts and the reduction of their secretion of pro-osteoclastogenic factors. (E-mail: angelanotarnicola@yahoo.it) © **2009 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Osteoblasts, Shockwaves, Osteogenesis.**

INTRODUCTION

Clinical use of shock waves was first applied in therapy for renal stones, where it was casually noted to have an effect on the iliac bone with primary osteocyte damage, followed by osteoblastic stimulation (Graff et al. 1988). Consequently, extracorporeal shock waves therapy (ESWT) was introduced for the treatment of the nonunion of bones with positive results documented by several papers (Rompe et al. 2001). It has been hypothesized that this effect was due to increased regional blood flow and release of prostaglandin E2 (PGE2) and substance P, which would induce osteoblast proliferation (Maier et al. 2002). Studies performed on cell cultures have shown osteoblast stimulation, as indicated by the increase of alkaline phosphatase activity and osteocalcin production (Martini et al. 2003). Other authors have shown an ESW dose-dependent positive effect on viability and maturation of human cancellous bone osteoblasts (Hofman et al. 2008). Animal studies indicated that local delivery of shockwaves stimulated early expression of angiogenesis-related growth factors, including endothelial nitric oxide synthase, vascular endothelial growth factor (VEGF) and proliferating cell nuclear antigen. This resulted in new vessel in-growth that improved blood supply, increasing cell proliferation and accelerating tissue regeneration and healing (Haupt and Chvapil 1990; Wang 2003; Nishida et al. 2004; Meirer et al. 2007). Moreover, it was shown that ESW application caused a hyperpolarization of membranes on the osteoblast population, which leads to the activation of the ras-cascade and to osteoblastic differentiation (Wang et al. 2001).

At present, shock wave therapy is widely applied in the treatment of musculoskeletal disorders, such as the nonunion of long bone fractures, calcifying tendonitis of the shoulder, lateral epicondylitis of the elbow and proximal plantar fasciitis; in addition, the success rate of the application ranges from 65% to 91% (Wang 2003). However, the exact molecular mechanism by which

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ESW promotes bone repair remains largely undetermined. The aim of this study was to evaluate the direct effect of ESW treatment on murine osteoblasts to clarify directly on the cells the mechanism that leads to the induction of bone repair. We found an immediate apoptotic effect on the osteoblasts directly hit by the impulses and a late positive effect inducing proliferation of pre-osteoblasts. Moreover, we observed the stimulation of both early and late osteoblast marker genes and increased production of bone matrix protein. These effects could also influence the osteoclast activity because we found that ESW leads to a decrease of the receptor activator NF kappa B ligand (RANKL)/osteoprotegerin (OPG) ratio, thus, inhibiting bone resorption.

MATERIAL AND METHODS

Murine clavaria osteoblasts

The frontal and parietal bones from 5- to 6-d old mice (c57bl/6j) were removed by a sterile technique and the periostium was detached by scissors. This was performed in compliance with the protocol established by our local Institutional Care and Use Animal Committee.

The calvaria fragments were digested in 0.5 mg/mL clostridium histolyticum neutral collagenase (Sigma Chemical Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS) at 37 °C for 60 min. After digestion, the calvaria fragments were washed vigorously three times with α-minimal essential medium (α-MEM)and then transferred to a 12.5 cm^2 cell culture flask. The fragments were subsequently cultured in α-MEM, which was supplemented with 10% fetal bovine serum (FBS; Gibco, Uxbridge, UK), 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 50 IU/mL mycostatin (Gibco), at 37 °C in a water-saturated atmosphere containing 5% CO_2. The media were changed every 3 d. Under these conditions, osteoblasts in the fragments proliferated and migrated to the culture surface, reaching confluence within 2 weeks. Cells were then trypsinized and transferred to appropriate dishes for characterization and experiments.

Osteoblast characterization

Murine osteoblasts were characterized according to the well-established parameters of alkaline phosphatase activity, production of cAMP in response to PTH10 − 8 M (Sigma Chemical Co.) and synthesis of osteocalcin in response to 1.25-dihydroxyvitamin D3 10 − 8 M (Sigma Chemical Co.).

Extracorporeal shock waves (ESW) application

Calvaria osteoblasts were seeded in 25 cm^2 at a density of $8 \times 10^3/cm^2$ and cultured at 37 °C in a water-saturated atmosphere containing 5% CO_2. An electromagnetic ESW generator (MINILITH SL1; Storz Medical, Kreuzlinger, Switzerland) as the source of shock waves was used for ESW application. The ESW were focused on the bottom of the flasks containing target cells by ultrasound tracking included inside the electromagnetic generator cylinder. After confluence, some osteoblast flasks were treated with 500 impulses of extracorporeal shock waves (ESW) at an energy density of 0.05 mJ/mm^2 and a 4 Hz pulse repetition rate, docked by means of a water-filled cylinder, whereas the other flasks were used as controls. Several different energy levels and dosages of ESW were tested when the experimental conditions were set for the study. A higher number of impulses did not change the results. However, since the cells are isolated in a culture flask, the treatment with higher energy levels caused large necrotic areas and could not be used. In these *in vitro* cell culture experiments, there were no other tissues that could shield the ESW, except for the plastic surface of flasks and almost all the energy delivered, thus, reached the cell layer and caused its detachment from the surface.

A common ultrasound gel was used as a contact medium between the cylinder and flask. After the shock waves treatment, the culture medium was changed and the flasks were maintained in an incubator under standard conditions, prior to their use in the different experiments.

RNA extraction and reverse transcriptase reaction

Osteoblasts were subjected to RNA extraction, using spin columns (RNAeasy mini kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA with the AccuScript high fidelity 1st strand cDNA synthesis kit (Stratagene, La Jolla, CA, USA). A 10 μL aliquot of the initial mix [1 μg RNA, 1 mM dNTPs, 50 pmol Oligo(dT), DEPC H_2O] was incubated at 65 °C for 5 min and on ice for 1 min and 10 × RT buffer, 25 mM MgCl2, 0.1 M DTT and 1 μL (40 units) of RNaseOUT were subsequently added. After 2 min of incubation at 42 °C, 1 μL (50 units) of SuperScript II RT was added and the incubation at 42 °C resumed for 50 min and then at 70 °C for 15 min. Then 1 μL (2 units) of Rnase-H was added and another 20 min of incubation at 37 °C were performed to complete the reaction.

Real-time PCR

cDNA was amplified with the iTaq SYBR green supermix with ROX kit (Bio-Rad Laboratories, Hercules, CA, USA) and the PCR amplification was performed using the Chromo4 real-time PCR detection system (Bio-Rad Laboratories).

The expression of messengers for OPG, RANK-L, runt-related transcription factor 2 (RUNX2), osteopontin (OPN), bone sialoprotein (BSP), osteocalcin, type 1

collagen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene were evaluated by real-time PCR. The primer sequences, all with a 60 °C annealing temperature (Operon Biotechnologies GmbH, Cologne, Germany), are reported in Table 1.

The amplification process includes three steps: (1) Incubation at 95 °C for 3 min; (2) Incubation at 95 °C for 15 s; and (3) Annealing and extension at 60 °C for 30 seconds. The steps 2 and 3 were repeated 40 times.

After the last cycle, the melting curves analyses were performed on the 55 °C–95 °C interval in increments of 0.5 °C.

The fold change values were calculated by the Pfaffl method (Pfaffl 2001).

Preparation of cellular extracts

The mice calvaria osteoblasts were lysed with RIPA ice cold buffer (20 mM TrisHCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 8 μg/mL leupeptin) added together with 1 mM sodium orthovanadate for 10 min. Consecutive extracts were centrifuged at 14,000 rpm for 15 min at 4 °C to separate the nuclei, while the supernatant was harvested for protein dosage.

The protein extract concentrations were determined by the BCA protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Western blot

Approximately 30 μg of cell proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine extracellular signal-regulated kinase (ERK) phosphorylation, Bax and p-CDK2 variation. Subsequently, the proteins were transferred to nitrocellulose membranes (Hybond; Amersham Pharmacia, London, UK). The blots were blocked by incubation in 5% milk with TBS-T for 1 h at 37 °C and probed overnight at 4 °C with mouse anti-cyclin E2-CDK2 complex (Cell Signaling Technology, Danvers, MA, USA), mouse anti-bax (Cell Signaling Technology), mouse anti-α-actin (Chemicon International Inc.), rabbit anti-phospho-ERK and anti-ERK (Cell Signaling Technology, Billerica, MA, USA).

RESULTS

Several experiments were planned and performed to investigate the early signals and late effects of ESW directly on cells of the osteoblast lineage. Osteoblast-containing culture flasks were treated with pulses of ESW (500 impulses at 0.05 mJ/mm^2). After the treatments, some of the flasks were utilized for morphologic observation, while other flasks were used for RNA or protein extraction after different time lapses.

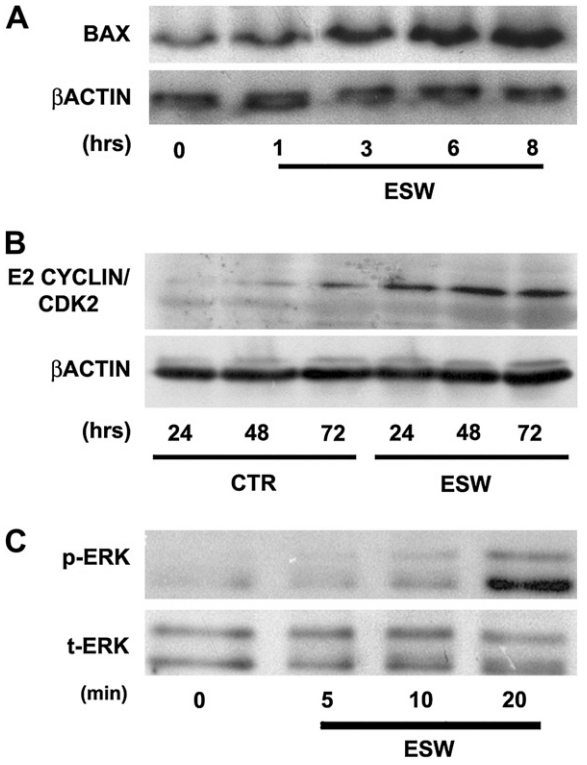

Fig. 1. (A) Western blot analysis of extracts from mouse osteoblasts using the Bax antibody. After 3 h from ESW application, an initial pro-apoptotic effect was observed. (B) Western blot of osteoblast samples treated with extracorporeal shock waves for 24, 48 and 72 h and their respective controls. A stimulatory effect on cell proliferation, as reflected by the increase of a G(1)-S phase marker, was observed. In fact, we found that 24, 48 and 72 h after ESW treatment, a stronger association of cyclin E2 and Cdk2, forming active cyclin E-Cdk2 kinase, compared with untreated cells at the same times. (C) ERK phosphorylation in response to ESW treatment. At 10 min after the ESW stimulation, the phosphorylation of ERK was observed. (CTR: control; ESW: extracorporeal shock waves.)

Bax expression

Three hours after the ESW pulses, morphologic observation showed a necrotic area localized in the center of the flasks in all the experiments, where the cells were directly hit by shock waves. Thus, we investigated whether ESW treatment could induce not only an immediate lysing of the cells directly hit, but also induce the activation of an apoptotic cascade in the area surrounding the directly affected cells in a time dependent manner. Western blot analyses were performed on cell lysates and an increased expression of Bax was observed, where Bax is a key component for apoptosis induced through mitochondrial stress, detectable 3 h after ESW stimulation and not present in untreated controls. This result indicated a fast pro-apoptotic effect of ESW treatment for the osteoblasts directly reached by ESW (Fig. 1A).

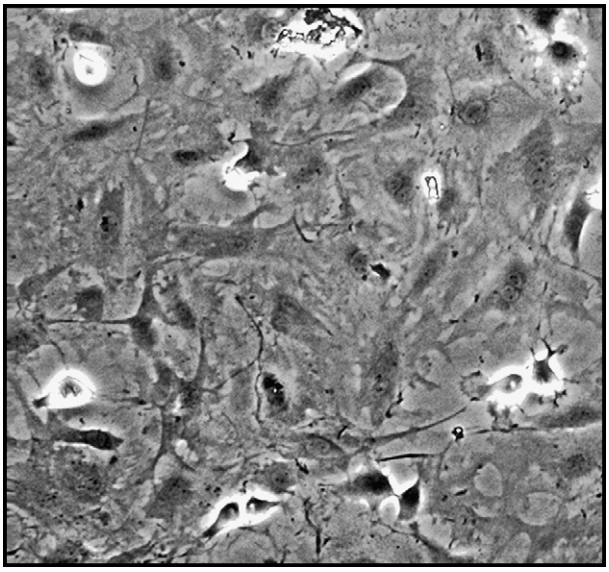

Fig. 2. Morphologic observation of osteoblast cultures at 24 h after ESW treatment. An unusual number of mitotic cells were evident (magnification ×200).

Cell cycle

Morphologic observations of the cultures at a later time (24 h after ESW treatment) revealed an unusual number of cells undergoing mitosis (Fig. 2). Thus, we investigated if the cell cycle was affected by shock waves. Western blot on the ESW-treated osteoblasts demonstrated the activation of the cyclin E2/CDK2, already detectable 24 h after ESW application and well-evident up to 72 h after, compared with the respective untreated controls (Fig. 1B). The E2/CDK2 complex regulates the G1-S transition, essential for cell proliferation, indicating that ESW treatment stimulates the osteoblast precursor proliferation.

Effect on RUNX2

As it was seen that ESW at low energy stimulated cell proliferation, we investigated if the differentiation markers of the osteoblastic lineage were also affected. The master gene for osteoblast differentiation is the transcription factor RUNX2 and, thus, we investigated if its expression was modified by the treatment. By real-time PCR, we found increased RUNX2 RNA expression 24 to 48 h after ESW stimulation (Fig. 3A).

Effect on bone extracellular matrix protein expression

As RUNX2 is a key regulator of the osteoblast-specific gene expression implicated in the secretion of bone matrix, such as bone sialoprotein (BSP), type I collagen (COLL1) and osteopontin (OPN), we investigated if the expression of those genes was also affected by the ESW treatment. The real-time PCR experiments showed increased expression of BSP, OPN and COLL1 (Fig. 3B, C and D). We also determined the expression of osteocalcin, a specific late marker of osteoblast differentiation, and, by real-time PCR, we found an increase in the osteocalcin gene expression 72 h after ESW stimulation (Fig. 3E).

Table 1. Primer sequences

OPG/S	AGTCTGAGGAAGACCATGAG
OPG/A	AAACAGCCCAGTGACCATTC
GAPDH/S	TGCGACTTCAACAGCAACTC
GAPDH/A	CTTGCTCAGTGTCCTTGCTG
RUNX2/S	CGTCAGCATCCTATCAGTTC
RUNX2/A	CCGTCAGCGTCAACACCATC
OSTEOCALCIN/S	TCTCTGACCTCACAGATCCC
OSTEOCALCIN/AS	CCTTATTGCCCTCCTGCTTG
COLLAGEN1/S	GGCTCCTGCTCCTCTTAG
COLLAGEN1/AS	ACAGTCCAGTTCTTCATTGC
OSTEOPONTIN/S	ATCTCAGAAGCAGCCTCTCC
OSTEOPONTIN/AS	ATGGTCATCATCGTCGTCC
BSP/S	AGCAGCACCGTTGAGTATGG
BSP/AS	TTCTGACCCTCGTAGCCTTC
RANKL/S	GCTCCGAGCTGGTGAAGAAA
RANKL/AS	CCCCAAAGTACGTCGCATCT

A: adenosine; C: cytosine; G: guanine; T: tymine.

Intracellular signal

Considering that the described findings, such as the induction of increased gene expression of RUNX2 and of bone matrix proteins, are relatively late effects of ESW, we searched for the early signal responsible for the activation of the intracellular cascade. Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine/threonine protein kinases involved in many cellular programs, such as cell proliferation, differentiation, motility, and death. The ERK1/2 signaling pathway can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors and cytokines (Meloche et al. 2007) and is an important target in the diagnosis and treatment of cancer (Roberts and Der 2007). We verified whether the ERK1/2 was phosphorylated in response to ESW by western blot. In Figure 1C, ERK phosphorylation 10 min after ESW treatment is evident.

OPG and RANKL expression

Clinical treatment with ESW is utilized in several different pathologies, but it is mainly used to reactivate areas where repair after trauma seems to be slow. Remodeling of bone and collagen matrix is expected to occur, as well as proliferation of new cells and new matrix formation. Osteoclasts are the cells responsible for bone matrix degradation, and their formation is regulated by osteoblasts *via* the secretion of relevant cytokines RANKL and OPG. RANKL promotes osteoclast maturation and activation. OPG is the decoy receptor for RANKL that blocks the RANKL action and thus inhibits osteoclast differentiation and function. Therefore, it is the balance

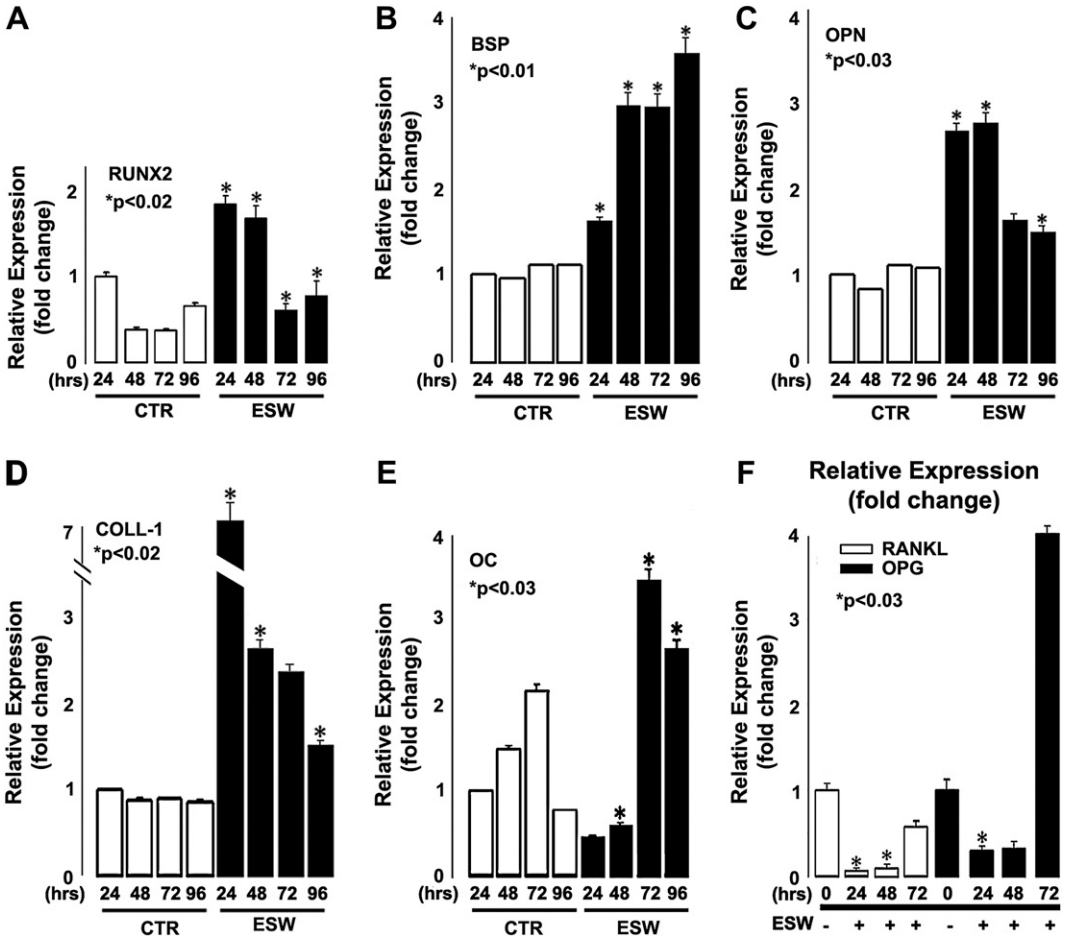

Fig. 3. (A) Real-time PCR for Run ×2 transcription factor. An increase in RNA expression at 24 h after ESW stimulation was observed. (B), (C) and (D) Effect of ESW application on bone extracellular matrix protein RNA expression. The real-time PCR showed an increase of BSP, COLL type 1 and OPN expression. (E) Increase in the expression of osteocalcin, a specific late marker of osteoblast differentiation, mRNA at 72 h after ESW stimulation. (F) ESW modulates the pattern of expression of the RANKL/OPG system in osteoblast cultures. Expression of mRNA for RANKL or OPG was measured by real-time PCR. The decrease in the RANKL/OPG ratio was observed 48 h after ESW treatment. (CTR: control; ESW: extracorporeal shock waves)

between the expression of RANKL and OPG that determines the extent of osteoclast activity and subsequent bone resorption. We investigated if ESW stimulation could determine OPG and RANKL gene expression variation in osteoblasts. Quantitative real-time PCR detection was performed at several time points after ESW. The results showed decreased expression of OPG and RANKL 24 and 48 h after ESW, followed by a later increase of OPG, paired with a much smaller increase of RANKL (Fig. 3F). This indicates a decrease of the RANKL/OPG ratio that suggests the inhibition of osteoclastogenesis, at least at the time points evaluated.

DISCUSSION

In the literature, the success of shock wave therapy is reported in the clinical applications for the treatment of delayed unions. The cellular mechanisms by which ESW, although largely used in therapy, enhances tissue healing and repair have not yet been completely clarified. The researchers have investigated the effects on cells *in vitro* and *in vivo* and have reported an immediate cytodestructive effect that wears off within 24 h and is followed by a tissue-repair-stimulating effect of the shockwaves based on an increased blood flow (Kaulesar Johannes et al. 1994). Experimental evidence has shown that shock waves could promote growth and differentiation of bone-marrow stromal cells toward osteoprogenitors (Wang et al. 2002). In subsequent experiments, it was realized that SW act on several genes critical for osteoblast differentiation and function. In fact, SW up-regulate PTHrP, prostaglandin E2-receptor EP3, BMP-2 inducible kinase, chordin, cartilage oligomeric matrix protein and matrillin (Hofmann et al. 2008). Moreover,

ESW induces positive effects on proliferation in primary human cancellous osteoblasts (Hofmann et al. 2008) and in collagen type 1 expression in osteoblast-like cells (Martini et al. 2003). No other data are available about ESW effects on human or murine primary osteoblasts.

In the present work, we studied in depth the interaction of apoptotic and proliferation signals after a SW application on the osteoblasts. Thus, we studied the effect of ESW treatment on the proliferation and differentiation of primary osteoblasts obtained from calvarias of newborn mice. At first, we performed experiments on ESW utilizing human primary osteoblasts. In addition, the mouse cells exhibited similar results as the human osteoblasts. Mice constitute an excellent experimental model to study the activity of bone cells because of the higher reproducibility of the results. Human cells are obtained from patients of different age and genetic background, and the results are not always convincing.

Previous studies have demonstrated that in osteoblast cultures one of the most important aspects to be considered is not the total number of impulses used, but rather the energy level of the shock waves; thus, this confirms that ESWT has a dose-dependent effect on cells (Martini et al. 2003). In addition, Martini demonstrated that the factors most affecting the osteoblast activity involve both the device and the level of EFD energy flux density (EFD) selected, and they must be considered together. The use of the electromagnetic device and a level of EFD lower than 0.40 mJ/mm^2 would appear to induce fewer immediate cytodestructive effects and better stimulate subsequent proliferation and the synthetic activity of MG63 (Martini et al. 2006). Dose-dependent new bone formation has also been found in clinical applications (Tischer et al. 2008). In the choice of SW protocol, we considered that, in clinical use, it is preferable to apply a low number of shockwaves with a high energy density (Kaulesar Johannes et al. 1994). On the other hand, a cytocidal effect was obtained by applying high-energy shock waves (Yao et al. 1994). After testing several energy levels, we finally selected the highest energy level that could be both effective in stimulating cells and compatible with cell survival.

In our experiment, we found a dual and apparently contradictory effect, which was the stimulation of apoptosis in one part of the culture, while the remaining cells were actively proliferating and differentiating toward the osteoblastic lineage. This contradiction can be easily explained by considering that the same device utilized for therapy was used for the cell treatment. The dosage of ESW was higher in the center of the culture and progressively less intense toward the edges. In the literature, the necrotic effect of high energy ESW on the cells is well reported (Kaulesar Johannes et al. 1994, Martini et al. 2003). Therefore, while the cells in the middle, which received a high dosage, were irreversibly damaged and the surrounding elements eventually underwent apoptosis, the other osteoblasts were positively stimulated to proliferation and differentiation. In a poorly reacting tissue, the death of old cells can also be positive, if they are replaced by a newly formed, more active tissue. The apoptosis in our experiments was demonstrated by Bax activation 3 h after ESW treatment compared with the untreated controls. Bax is a key component for cellular induced apoptosis through mitochondrial stress; upon apoptotic stimulation, Bax forms oligomers and translocates from the cytosol to the mitochondrial membrane (Wei et al. 2001). Through interactions with pore proteins on the mitochondrial membrane, Bax increases membrane permeability, which leads to the release of cytochrome c from the mitochondria, activation of caspase-9 and initiation of the caspase activation pathway for apoptosis (Marzo et al. 1998; Narita et al. 1998).

Conversely, morphologic observations of the cultures at a later time (24 h from ESW treatment) showed an unusual number of cells undergoing mitosis (Fig. 2), and we demonstrated the activation of the cyclin E2/CDK2 complex, which is essential for cell proliferation, because it regulates the G1-S transition. This result indicates that ESW treatment stimulates the osteoblasts that do not undergo apoptosis to proliferate after ESW application (Fig. 1B).

To obtain active osteoblasts from proliferating cells, specific transcription factors are induced in sequence, and the master gene for osteoblast differentiation is RUNX2. This protein plays a critical role in osteoblast differentiation and function and stimulates the expression of the components of bone extracellular matrix, like BSP, OPN and type I collagen (Karsenty 2001; Kern et al. 2001). We found an increase of RUNX2 RNA expression 24 h after ESW stimulation (Fig. 3F), indicating that ESW positively affects osteoblast activity and extracellular matrix secretion. Osteocalcin, a late marker of osteoblast differentiation, was also increased 72 h after ESW treatment.

As the results discussed are all relatively late effects of ESW, we searched for the early signal responsible for the osteoblast activation, and we found the phosphorylation of ERK 1/2 in response to ESW application. The ERK1/2 signaling pathway can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, and cytokines (Meloche and Pouyssegur 2007). ERK has been found to be activated in the growth factor stimulation of osteoblast proliferation (Lou et al. 2000; Xiao et al. 2000; Lai et al. 2001), as well as being a mediator for stimuli such as mechanical strain, fluid flow and hypergravity (Gebken et al. 1999; Jessop et al. 2002).

Bone repair is the result of the osteoblast and osteoclast activities regulation. We investigated if ESW could induce an osteoblast-mediated osteoclast regulation. RANKL is a type II homotrimeric transmembrane protein that is expressed as a membrane-bound and/or secreted protein, the latter deriving from the membrane form as a result of either proteolytic cleavage or alternative splicing (Ikeda et al. 1999). RANKL expression is stimulated in osteoblast/stromal cells by most of the factors that are known to stimulate osteoclast formation and activity. RANKL-induced osteoclast differentiation - activation may regulate progenitor recruitment as a part of homeostasis and host defense, linking bone remodeling with regulation of hematopoiesis. OPG is expressed in many tissues apart from osteoblasts, including heart, kidney, liver, spleen and bone marrow (Wada et al. 2006). Its expression is regulated by most of the factors that induce RANKL expression by osteoblasts. Although there are contradictory data, in general, up-regulation of RANKL is associated with downregulation of OPG or at least lower induction of OPG, such that the ratio of RANKL to OPG changes in favor of osteoclastogenesis. Many reports have supported the assertion that the RANKL/OPG ratio is a major determinant of bone mass (Rogers and Eastell 2005).

We investigated if ESW stimulation could determine a change in OPG and RANKL gene expression in osteoblasts, and we found a decrease in the RANKL/OPG ratio that suggests the inhibition of osteoclastogenesis, at least at the time points evaluated. However, this result needs to be supported by co-culture experiments that validate the real relevance of the change in protein ratio.

In summary, in this research, we demonstrate that the positive effects of ESW on bone forming cells are dependent upon the activation of an intracellular signaling cascade that, through a series of phosphorylations starting from ERK 1/2, induces the expression of relevant genes in the nucleus that lead to osteoblast proliferation and differentiation. The osteogenic effects of ESWT have been shown at the tissue, cellular and molecular levels. Clinical trials should be performed to convert the dosage of ESWT from the present study on cells to clinical applications in human trials.

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