Extracorporeal shock waves increase interleukin-10 expression by human osteoarthritic and healthy osteoblasts *in vitro*

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Abstract

Objective

Extra corporeal shock waves (ESW) have been proposed as additional therapy in bone fracture repair and osteoarthrtitis (OA). However, little is known on the effects of ESW on osteoblast metabolism. The aim of this study was to evaluate phenotype changes of healthy and OA human osteoblasts following ESW treatment.

Methods

Osteoblasts were isolated from subchondral bone of 13 OA patients and 7 healthy donors. Osteoblasts were treated or not with ESW at different levels of energy and impulses. IL-10, TNF-α, CD29/β1 integrin, and CD105/endoglin expression was evaluated by flowcytometry.

Results

Intracellular IL-10 significantly increased using 1000 impulses at 0.055 mJ/mm² in both healthy and OA osteoblasts in comparison with untreated osteoblasts ($p<0.01$). Only in the OA osteoblasts CD29 and CD105 expression significantly increased at 500 impulses and 0.17 mJ/mm² ESW treatment ($p<0.05$).

Conclusions

ESW are capable of modifying IL-10 expression in osteoblasts. There is evidence that IL-10 can play a role in bone remodelling by inhibiting osteoclast differentiation and this suggests that ESW may favour bone growth and healing. This further supports the use of ESW in treating bone fracture to promote callus formation. However, the possible use of ESW in OA therapy needs further studies since in OA, osteoblast metabolism is already enhanced with bone sclerosis and ESW application may further increase bone deposition and osteophyte formation, leading to a subsequent worsening of the disease.

Key words

Osteoarthritis, osteoblasts, endoglin, β1-integrin.

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Introduction

Extra-corporeal shock waves (ESW), first used in the treatment of urine calculi, are expanding their potential applications since the discovery that they can modulate the metabolic activity of different cell types, including osteoblasts (1-4). ESW effects on cell metabolism can be stimulatory or inhibitory depending on both the impulses and energy levels applied and the specific tissue target. We have recently provided evidence that ESW at medium impulses and energy levels down-regulated IL-10 and TNF-α expression by human osteoarthritic chondrocytes "*in vitro*". This might represent a rationale to treat osteoarthritic cartilage damage by ESW (5). Further inhibitory effects are also exerted by ESW on malignant cells such as renal carcinoma (6) and on renal parenchyma of normal and diabetic rats by increasing the cell apoptotic index (7).

Besides these outcomes, ESW seem to be capable of stimulating cell metabolism by interfering with the intracellular pathway of some growth factors. Recently, it has been shown that ESW increased skin flap tissue by inducing VEGF (vascular endothelial growth factor) expression and blood perfusion (8, 9). However, the more extensively studied field of application of ESW is bone repair (10-16). The mechanisms by which ESW promote osteoblast proliferation are still unknown, but it is likely they induce changes of transmembrane cell voltage by increasing K^+ and Ca^{++} cell influxes (12). Increased membrane polarization would stimulate down-stream intracellular events to activate ERK (extracellular signal-regulated kinase) (10, 17) and p38 kinase (10) leading to VEGF and TGFβ_1 (trasforming growth factor β_1) mRNA up-regulation and bone formation (11, 14, 17). These data have been drawn from experiments carried out on rat osteoblasts, osteoprogenitor cells, osteoblast cell lines, or human normal osteoblasts, while the effects of ESW on human osteoarthritic osteoblasts have never been investigated.

During osteoarthritis (OA), osteoblasts of subchondral bone undergo a metabolic boost sustained by a local increase of growth factors such as IGF_1 (insuline growth factor-1), TGFβ_1, BMPs (bone morphogenetic proteins) and others, together with an abnormal expression of integrins regulating osteoblasts/extracellular matrix interactions (18), causing bone sclerosis and osteophytes outgrowth (19).

An emerging role in the pathophysiology of bone remodelling seems to be played by interleukin-10 (IL-10). IL-10 is a pleiotropic immune modulating cytokine that activates a broad range of functional responses in different cell types inducing either inhibitory or stimulatory effects, such as down-regulating IL-1 and TNF-α synthesis by monocytes (20) or promoting the growth and differentiation of B cells (21). Increased expression of IL-10, both at protein and mRNA level, has been detected in OA human cartilage (22), where IL-10 would be act as a growth factor. The role of IL-10 in the pathophysiology of cartilage is not completely understood but it seems to be a protecive factor counteracting the catabolic effect of IL-1 and metalloproteinases (23). Furthermore, IL-10 was critical in inhibiting osteoclastogenesis by preventing differentiation of osteoclast progenitors into preosteoclast-like cells in the rat bone marrow culture system (24).

The aim of this study was to assess the possible phenotypic changes of human OA osteoblasts following ESW treatment "*in vitro*". We investigated the effects of ESW on IL-10 intracellular levels, on CD105/endoglin, and CD29/β_1 integrin membrane expression on human normal and osteoarthritic osteoblasts "*in vitro*".

Patients and methods

Osteoblast isolation

Human subchondral bone was obtained from 13 patients (9 females, 4 males, age 68±8 years) with primary knee OA, undergoing total joint replacement and from 7 healthy donors (HD) (5 females, 2 males, age 38±21 years) with joint traumatic fracture. All patients gave their written informed consent and the study was approved by the local ethics committee. Trabecular subchondral bone sampling was performed by needle byopsies. Bone specimens

were washed twice in sterile polysaline buffer solution to eliminate medulla and incubated in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with penicilline and 0.1% collagenase (Sigma, St. Louis, MO, USA) at 37°C and 5% CO_2 overnight. After collagenase removal, bone samples were incubated (37°C, 5 % CO_2) in 25 cm_2 culture flasks in DMEM supplemented with 10% fetal calf serum (FCS) until osteoblasts came out and adhered to bottom flasks (range 7-13 days). Osteoblastic lineage of adherent cells was confirmed by assessing alkaline phosphatase production by spectrophotometry (Perkin Elmer, Waltham, MA, USA).

ESW treatment

The osteoblasts were detached before confluence with 1% trypsin (Sigma, Milan, Italy) and then re-suspended in serum-free DMEM, supplemented with antibiotics and placed in 1,8 ml cryogenic vials (Nunc, Denmark) at 4-5 x 10^5/cells/ml. The ESW electromagnetic generator system (MINILITH SL1, STORZ MEDICAL) was used in this study. The test-vial was placed in a special cylindrical support on the shockwave generator, which enabled the focal depth probe to be directed correctly to the vial containing the cells. The osteoblasts were subdivided into 5 populations: 4 specimens underwent ESW treatment at different parameters of impulses, energy levels and energy fluxes, as shown in Table I, while an untreated vial was kept as control sample, but underwent the same laboratory processing. ESW dosages were selected according to current clinical applications (3). At the end of the treatment, cellular vitality was evaluated with the Trypan blue dye exclusion test, and the osteoblasts were cultured in 24-well plate in serum-free DMEM for 6-8 hours on a rotating plate to prevent cell adhesion prior to flow-cytometry.

Osteoblast phenotype

After incubation, the osteoblasts were re-suspended in PBS containing 0.1% sodium azide and 0.2% bovine serum albumin, and blocked by incubating with 2% normal human serum (Advanced Protein Products, UK). After fixation with paraformaldehyde and permeabilization with saponin (Fix & Perm Cell Permeabilization Kit, Caltag Lab., Burlingame, CA), the osteoblasts were incubated (20 min. at 4°C) with 5 µl of FITC/anti-human IL-10 mAb or FITC/anti-human TNF-α mAb (Serotec, Oxford, UK). β_1 integrin (Serotec) surface expression was assessed with the same procedure without cell membrane permeabilization. Control samples were incubated with rat IgG1-FITC/IgG2-PE (DAKO, Denmark). Stained cells were analysed on a FACScan (Cell Quest, Becton Dickinson, Mountain View, CA). The FACS setting was identical throughout all the study.

Statistical analysis

The results are expressed as mean values ± 1 standard deviation (SD). Statistical analysis was performed using the analysis of variance (ANOVA) with post hoc LSD (least square difference) test and the level of significance was set at $p<0.05$.

Table I. The panel of ESW treatment of osteoblasts.

Chondrocytes	E mJ/mm^2	Level	Impulses
A1	0.055	2.5	500
A2	0.055	2.5	1000
B1	0.17	5	500
B2	0.17	5	1000

Table II. Osteoblast viability following ESW treatment.

Viability	E mJ/mm^2	Level	Impulses
82% ± 8	0.055	2.5	500
79% ± 9	0.055	2.5	1000
87% ± 12	0.17	5	500
84% ± 10	0.17	5	1000
88% ± 6	Untreated	_	_

Results

Cell viability

The Trypan blue dye exclusion test showed that ESW application slightly affected osteoblast viability. Cell viability was lower in ESW treated osteoblasts than in the controls, both in OA and in the healthy osteoblasts, although these differences did not reach the significance level. Relevant changes in chondrocyte viability were not seen according to the different ESW energy levels and pulses applied and were similar in healthy donors and OA patients (Table II).

The phenotype of normal osteoblasts is shown in Figure 1. The percentage of normal osteoblasts bearing CD29/β_1 integrin was 14.2±9 at baseline and ESW treatment did not significantly change its expression (A_1 13.8±8, A_2 13.4±6, B_1 13.9±8, B_2 12.7±7). CD105/endoglin membrane expression was similar in the control osteoblasts and the ESW treated osteoblasts (Control 8.3±7, A_1 8.9±8, A_2 8.2±6, B_1 8.5±7, B_2 6.7±5). Also, intracellular levels of TNF-α were not modified under ESW application in the normal osteoblasts (Control 2.4±0.8, A_1 2.2±1, A_2 2.4±0.8, B_1 1.9±0.7, B_2 2.1±1). Conversely, the ESW treatment affected IL-10 intracellular levels and at 0.055 mJ/mm_2 and 1000 impulses (A_2) a significant increase of IL-10 in comparison with the untreated osteoblasts ($p<0.01$) was detected (Control 11.1±7, A_1 15.8±6, A_2 18.3±5, B_1 16.4±5, B_2 12.9±7).

In OA (Fig. 2), CD29/β_1 integrin expression was detected in 14.1±5 of the untreated osteoblasts and after ESW treatment it progressively decreased at A_1 (13.3±4), A_2 (11.7±4), and B_2 (11.4±4) without reaching a statistically significant difference. However, the percentage of positive OA increased to 16.4±8 at B_1 treatment and the difference was significant when compared to A_2 and B_2 ($p<0.05$) but not to baseline. Likewise, CD105 positive osteoblasts

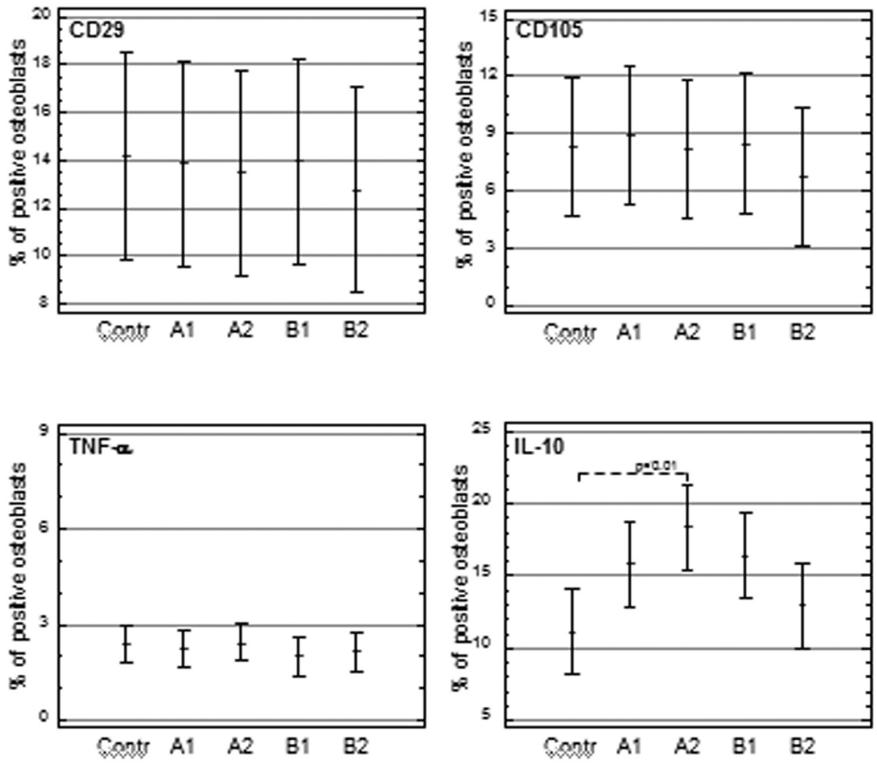

Fig. 1. ESW effects of different ESW treatments on the expression of CD29/β_1 integrin, CD105/endoglin, TNF-α, and IL-10 in healthy osteoblasts. On the *y* axis, the percentage of positive osteoblasts, and on the *x* axis, ESW treatments (see Table 1 for the panel of ESW) are shown. Bars represents mean ± 1 standard deviation.

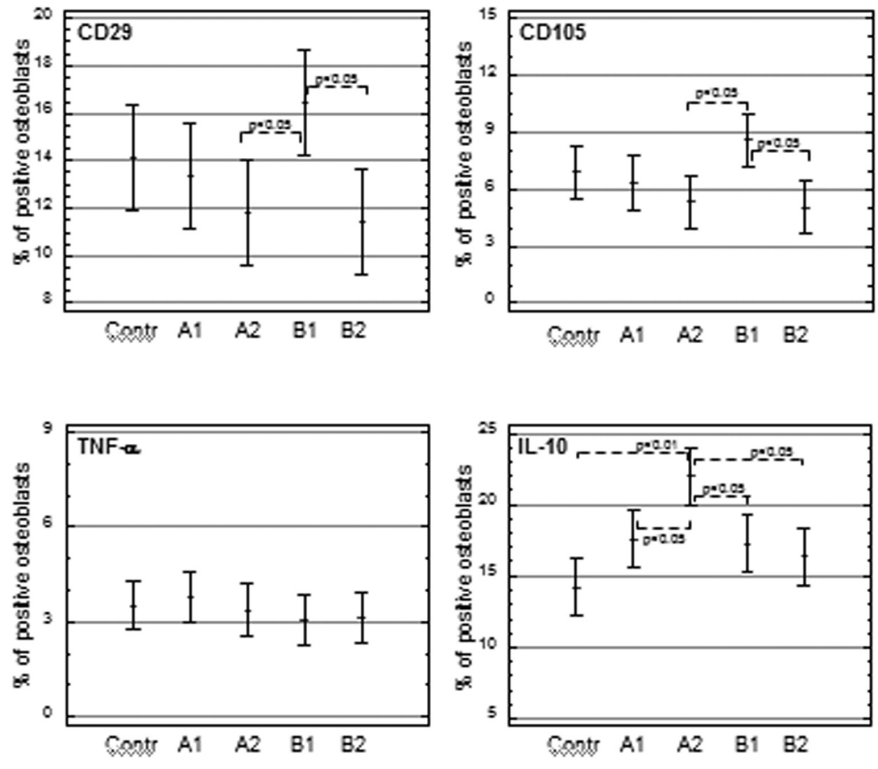

Fig. 2. ESW effects of different ESW treatments on the expression of CD29/β_1 integrin, CD105/endoglin, TNF-α, and IL-10 in OA osteoblasts. On the *y* axis, the percentage of positive osteoblasts, and on the *x* axis, ESW treatments (see Table I for the panel of ESW) are shown. Bars represents mean ± 1 standard deviation.

were similar at baseline (6.9±3) and at A_1 (6.3±3), A_2 (5.3±2), and B_2 (5.0±1) treatments, respectively, whilst at B_1 (8.6±5) they were signicantly higher than at A_2 and B_2 (p<0.05). Intracellular levels of TNF-α did not vary following ESW treatment (Control 3.5±2, A_1 3.7±2, A_2 3.4±1, B_1 3.0±1, B_2 3.1±2). ESW treatment induced a significant increase of IL-10 intracellular levels at 0.055 mJ/mm$_2$ and 1000 impulses (A_2 22.0±5) in comparison with baseline (14.1±5, p<0.01) and the other ESW treatments (A_1 17.6±7, A_2 17.2±4, B_2 16.3±5, p<0.05).

Discussion

There is experimental evidence that ESW are capable of promoting bone growth and stimulating osteoblast anabolism. ESW, using 500 impulses at 0.16 mJ/mm^2, promoted healing of segmental femoral defect in rats and immunoisthochemistry showed up-regulation of BMP-2, BMP-3, and BMP-4 in mesenchymal cells and immature chondrocytes, while BMP-7 reactivity was prevalent in enchondral osteoblasts (16). These effects seemed to be mediated through the intracellular signal ERK, which has been found phosphorylated in osteoblasts of the new bone filling the femoral gaps (10). Using the same experimental model, it also been shown that ESW enhanced the recruitment and differentiation of mesoblasts, and increased TGFβ_1 and VEGF expression in regenerated bone (11).

These data strongly support the use of ESW in the treatment of bone fractures in humans, while little is known about their application in the therapy of human OA. Few data have been provided in animals, in which ESW were used in the cure of OA in horses and dogs (25, 26). We have recently shown that ESW down-regulate intracellular levels of IL-10 and TNF-α in human OA chondrocytes "*in vitro*" (5). As IL-10 and TNF-α are increased in human OA and are believed to play a critical role in the induction of cartilage damage (19), we speculated that ESW might be a useful approach to treat OA. However, the effects of ESW on human OA subchondral osteoblasts are unknown.

In this study, for the first time, we as-

sessed the effects of ESW application on healthy and OA subchondral osteoblasts "*in vitro*" by evaluating changes of IL-10, CD105, CD29, and TNF-α expression. We found a significant increase of IL-10 intracellular levels in healthy and OA osteoblasts following ESW treatment. These changes occurred by applying 1000 impulses at 0.055 mJ/mm^2 and were markedly relevant in OA osteoblasts, in which IL-10 progressively increased up to 22.0% to decrease down to basal levels at higher levels of energy and impulses. These results were surprising because IL-10 expression was previously found to be down-regulated in human OA chondrocytes, in the same experimental settings (5). However, this behaviour is consistent with IL-10 function, which is a pleiotropic cytokine that promotes both inhibitory (20) and stimulatory responses (21) and it might also be differently modulated in cartilage and bone by the same stimuli. Furthermore, it has been shown that ESW can stimulate different intracellular signals in cartilage and bone as they preferentially induced intracellular ERK phosphorylation in osteoblasts and p38 activation in chondrogenic cells in rats (10). Then, it is conceivable that ESW, by activating different intracellular downstream events, may differently regulate IL-10 expression in chondrocyte and osteoblasts "*in vitro*". Additionally, methodological differences should be taken into account as the osteoblasts were cultured before undergoing ESW treatment, while the chondrocytes were freshly stimulated following isolation. The functional outcomes of these findings are unknown owing to our poor knowledge of the role of IL-10 in bone physiology. It has been shown that IL-10 can inhibit osteoclast formation by preventing differentiation of nonadherent bone marrow cells, which are osteoclast progenitors, into preosteoclasts (24). Herein, it is conceivable that IL-10 may contribute to bone remodelling by hampering bone resorption and that ESW-induced IL-10 up-regulation may favour bone growth and healing. These findings would provide the theoretic rationale to use ESW in treating bone fracture to promote callus formation, but our data also suggest that ESW therapy in OA can be detrimental since in OA subchondral bone undergoes tissue remodelling leading to excessive bone eposition and IL-10 up-regulation may further imbalance bone metabolism toward sclerosis and osteophytosis.

It is well established that TGFβ$_1$ plays a key role in bone growth and differentiation, and ESW increased TGFβ$_1$ production in rat osteoblast long term cultures (14) and mRNA TGFβ$_1$ expression in bone tissue in the rat model of healing fractures (11). CD105/endoglin is a co-receptor of multiple kinase receptor complexes of the TGFβ superfamily, including TGFβ$_1$, TGFβ$_3$, and BMP$_2$ (27) and we wondered if ESW could modulate CD105 expression on surface osteoblasts. CD105 was poorly expressed on both healthy and OA osteoblasts at baseline, but its levels increased following ESW treatment only on OA osteoblasts. Interestingly, these effects were seen at a different ESW exposition, 0,17 mJ/mm^2 and 500 impulses, from that observed for IL-10. These findings suggest that ESW might modulate osteoblast sensitivity and response to the anabolic effects of TGFβ and BMPs.

We also evaluated CD29/β$_1$ integrin modulation by ESW on osteoblasts. CD29 is the common chain of a large family of dimeric transmebrane glycoproteins, named β$_1$ integrin family, which modulates cell interactions with extra-cellular matrix (ECM) proteins, such as as collagen, fibronectin, vitronectin and laminin, and cooperates with TGFβ, IL-4, and IGF receptors to activate intracellular anabolic processes (28). Subchondral OA osteoblasts expressed significantly less CD29 than healthy osteoblasts (18), suggesting that changes of cell/ECM signalling occurs in OA. In our experiments, ESW increased CD29 expression only on OA osteoblasts at 0,17 mJ/mm^2 and 500 impulses, although significant differences were reached only among different ESW treatments.

Our study provided evidence that ESW treatment induces osteoblast changes and enhances the expression of factors, such as IL-10 and CD105 that seem to be involved in bone growth. This strongly supports the use of ESW in the therapy of bone fractures to promote callus formation (4), while the possible use of ESW in OA therapy needs further studies since they may increase osteophyte outgrowth that could have a detrimental effect on joint function.

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