Extracorporeal shock wave promotes growth and differentiation of bone-marrow stromal cells towards osteoprogenitors associated with induction of TGF-β1

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Extracorporeal shock-wave (ESW) treatment has been shown to be effective in promoting the healing of fractures. We aimed to determine whether ESW could enhance the growth of bone-marrow osteoprogenitor cells. We applied ESW to the left femur of rats 10 mm above the knee at 0.16 mJ/mm^2 in a range of between 250 and 2000 impulses. Bone-marrow cells were harvested after ESW for one day and subjected to assessment of colony-forming unit (CFU) granulocytes, monocytes, erythocytes, megakaryocytes (CFU-Mix), CFU-stromal cells (CFU-S) and CFU-osteoprogenitors (CFU-O).

We found that the mean value for the CFU-O colonies after treatment with 500 impulses of ESW was 168.2 CFU-O/well (SEM 11.3) compared with 88.2 CFU-O/well (SEM 7.2) in the control group. By contrast, ESW treatment did not affect haematopoiesis as shown by the CFU-Mix (p = 0.557). Treatment with 250 and 500 impulses promoted CFU-O, but not CFU-Mix formations whereas treatment with more than 750 impulses had an inhibiting effect. Treatment with 500 impulses also enhanced the activity of bone alkaline phosphatase in the subculture of CFU-O (p<0.01), indicating a selective promotion of growth of osteoprogenitor cells. Similarly, formation of bone nodules in the long-term culture of bone-marrow osteoprogenitor cells was also significantly enhanced by ESW treatment with 500 impulses. The mean production of TGF-β1 was 610 pg/ml (SEM 84.6) in culture supernatants from ESW-treated rats compared with 283 pg/ml (SEM 36.8) in the control group.

Our findings suggest that optimal treatment with

ESW could enhance rat bone-marrow stromal growth and differentiation towards osteoprogenitors presumably by induction of TGF-β1.

Extracorporeal shock-wave (ESW) therapy has been shown to have a promising effect on the treatment of tendinopathies and the healing of fractures.[1-5] Experimental studies on cell lines or vessels have indicated that ESW can cause damage.[6,7] Haupt et al[8] first identified that ESW could enhance osteogenic activity, but the mechanism by which it promotes bone healing in fractures remains to be determined. We hypothesised that it may promote the growth and differentiation of bone-marrow mesenchymal cells presumably by the induction of osteogenic growth factors such as transforming growth factor beta 1 (TGF-β1).

It is known that bone-marrow mesenchymal stromal cells have the potential to differentiate into osteoprogenitors and several musculoskeletal-related cell lineages.[9,10] These osteoprogenitors produce bone matrices resulting in a scaffold for bone growth.[11,12] The production of TGF-β1 in the bone-marrow microenvironment has an important role in regulating and stimulating the differentiation of osteoprogenitors during the repair of fractures in vivo[13,14] and in vitro.[15-18] Our aim was to investigate whether ESW could promote bone-marrow osteoprogenitor cell growth in association with the induction of TGF-β1.

Materials and Methods

We used 50 four-month-old male Sprague-Dawley rats (National Experimental Animals Production Centre, Taipei, Taiwan). Eight were used in the pilot study and the other 42 were randomly divided into seven groups of six. They were anaesthetised by an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutal sodium, Abbott Laboratories, Illinois) and placed supine with all four limbs in abducent extensional fixation. ESW treatment at 0.16 mJ/mm^2 (Ossatron HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was applied to the left distal femur 10 mm above the knee. Ultrasound transmission gel (Pharmaceutical Innovations Inc, New Jersey) was used as

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the contact medium between the ESW area and the skin. The seven groups received 0, 250, 500, 750, 1000, 1500 and 2000 impulses of ESW, respectively. After treatment the rats were observed for one day to make sure that there was no visible skin or muscle injury before removing bone-marrow cells. As a control group we used bone-marrow cells from the femora of rats which had not had ESW treatment.

Preparation of bone-marrow mononuclear cells. The distal end of the femur was excised 5 mm above the knee. Bone-marrow blood (0.4 ml) was aspirated to an Eppendorf tube by a 20-gauge syringe containing 20 U/ml of heparin and the plasma collected by centrifuging at 800 g for 30 minutes. The bone-marrow mononuclear cells (MNC) were harvested from the interface of the Ficoll-Paque density gradient (d = 1.007 g/ml; Pharmacia Biotech AB, Uppsala, Sweden) at 500 g for 30 minutes. The MNC harvested from each femur ranged from 1.5 to 2.3 $\times$ 10^7 cells per femur. The cell number and viability were determined by a haemocytometer after staining with 0.4% Trypan Blue in phosphate-buffered saline.[19]

Culture of bone-marrow stromal cells. The bone-marrow MNC (2×10^5 cells/well) were cultured in 24-well microplates with Dulbecco's modified Eagle's medium (DMEM) containing 1% antibiotics and antimycotics (1000 U/ml of penicillin G, 1000 U/ml of streptomycin sulphate and 25 U/ml of amphotericin B; Life Technologies, Gaithersburg, Maryland) and 10% fetal bovine serum (FBS; Life Technologies) for 48 hours. After washing out non-adherent haematopoietic cells, total stromal cells were cultured for 12 days in 5% CO_2 at 37°C. Total colonies showing more than 32 segregate cells were counted as colony-forming-units-stromal (CFU-S).[20] The CFU-osteoprogenitor (CFU-O) formations were performed in conditional medium containing DMEM with 10% FBS, 10^{-8}M dexamethasone, 50 µg/ml of L-ascorbic acid and 10^{-2}M β-glycerophosphate (Sigma Chemicals Inc, St Louis, Missouri). Colonies staining positive for bone alkaline phosphatase were recognised as CFU-O colonies after culture for 12 days.[21] We also collected one half of the culture supernatants every three days and replaced them with fresh condition medium. The culture supernatants were subjected to measurement of the production of TGF-β1.

Determination of alkaline phosphatase activity. The cells from the CFU-O colonies were further subcultured for 12 days to confirm the specific character of the osteoprogenitors showing alkaline phosphatase activity. Briefly, cells (1 $\times$ 10^4 cells/well) in each 96-well microplate were incubated with 0.2 ml of substrate buffer containing 50 mM glycine, 1 mM magnesium chloride (pH 10.5), and 2.5 mM p-nitrophenyl phosphate (Sigma Chemicals Inc). The reactions were incubated at 37°C for 30 minutes and stopped with 0.1 ml of 1N sodium hydroxide. The results were read at OD_{405nm} by a microplate reader (Dynex Technologies Inc, Chantilly, Virginia). The alkaline phosphatase activity was expressed as mM p-nitrophenol/well.[22]

Culture of bone-marrow haematopoietic cells. Growth of bone-marrow haematopoietic cells was determined by CFU granulocytes, erythocytes, monocytes and megakaryocytes (CFU-Mix). Bone-marrow cells (2×10^5 cells/well) were cultured in 24-well plates with Iscove's modified Dulbecco's medium (IMDM) containing 10% FBS, 1.0% antibiotics, 10% rat spleen-cell-conditioned medium and 1.2% methylcellulose. The rat spleen-cell-conditioned medium was prepared by incubation of rat spleen cells (1×10^6 cells/ml) with 10 µg/ml of phytohaemagglutinin (Sigma Chemicals Inc) in a T-125 culture flask at 37°C for seven days. The condition medium was filtered through a 0.22 µm filter, aliquoted and stored at -80°C until use.[23] The semi-solid gel of CFU-Mix assay was cultured for 18 days and the CFU-Mix formations were determined by colonies with more than 32 segregate cells.[24]

Measurement of formation of bone nodules. In order to confirm further the osteogenic formation, we also prolonged the stromal cell culture to 21 days and harvested the cells for determination of the formation of bone nodules by von Kossa staining. Bone-marrow stromal cells from rats with and without ESW treatment at 500 impulses were fixed with neutral formaldehyde for five minutes (pH 7.4), rinsed with distilled water and then stained with 0.3 ml of 5% freshly prepared silver nitrate. The reaction was incubated in the dark for 30 minutes, followed by exposure to UV light for 30 minutes, and finally stopped by three washes with distilled water. The number of bone nodules greater than 2 mm^2 showing positive von Kossa staining was counted under an inverted microscope.[25]

Measurement of TGF-β1 production in the culture supernatants. The TFG-β1 levels in the supernatants of bone-marrow stromal cell cultures were determined by an ELISA assay (Quantikine; R&D Systems Inc, Minnesota). Briefly, 0.2 ml of acid-activated culture supernatant were added to each polystyrene microwell precoated with recombinant human TGF-β soluble receptor type II for three hours. The reactions were next incubated with a horseradish peroxidase conjugated TGF-β1 polyclonal antibody for 1.5 hours. After washing they were incubated with a substrate buffer containing 0.1 ml of stabilised hydrogen peroxide and 0.1 ml of stabilised tetramethylbenzidine for 30 minutes before stopping with 0.05 ml of 2 N sulphuric acid. Data were read at OD_{450nm} using a microplate reader. The results were calculated by an interpolation from a standard by a series of standard TGF-β1 concentrations.

Statistical analysis. Data were analysed using a non-parametric one-way analysis of variance followed by Student's t-test to determine the significance between treated and untreated groups. A p value of < 0.05 was considered to be statistically significant.

Results

Dose-dependent effects of ESW treatment on the viability of bone-marrow cells. Gross observation showed that

various doses of ESW treatment from 250 to 2000 impulses did not cause any skin or muscle injury. Microscopic findings showed that bone-marrow cells obtained from rats which had had ESW treatment had normal viability unless the ESW dosage was higher than 750 impulses (Fig. 1).
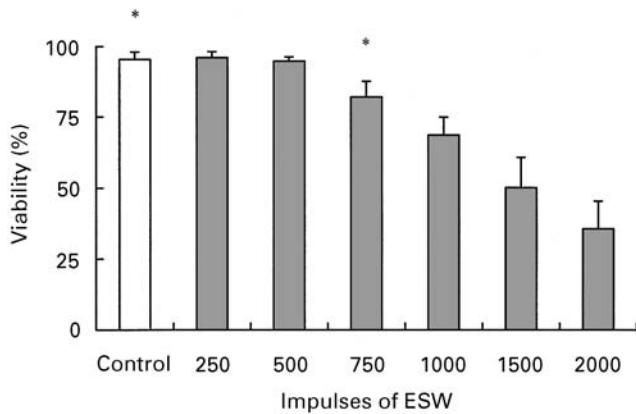

Fig. 1

Graph showing the effect of ESW on the viability of rat bone-marrow MNC. There is a significant difference between the effect of treatment with 750 impulses and the control group (*p = 0.024).

ESW treatment promoted bone-marrow stromal, but not haematopoietic, cell growth. We found that ESW treatment at 0.16 mJ/mm^2 for 500 impulses significantly enhanced CFU-S formation in comparison with the untreated group (p < 0.001; Fig. 2). By contrast, the CFU-Mix in the ESW treatment group was not significantly different from that in the control group (p = 0.557; Fig. 3). Our results suggest that ESW selectively promoted rat bone-marrow stromal cells, but not haematopoietic cell growth.

ESW induced a dose-dependent effect on formation of CFU-O and induction of TGF-β1. Using a variety of ESW doses, we found that 500 impulses had the best promoting effect on the CFU-O formation as shown in Figure 4a and that 250 impulses also produced some effect. CFU-O formation was suppressed when the dosage was higher than 750 impulses. In addition, we also found that CFU-O formation at different ESW treatments was significantly correlated with production of TGF-β1 in the supernatants (Fig. 4b) (R^2 = 0.984). These results suggest that ESW promotion of CFU-O formation may be related to the induction of TGF-β1.

ESW enhanced not only osteoprogenitor growth but also the formation of bone nodules. To determine whether
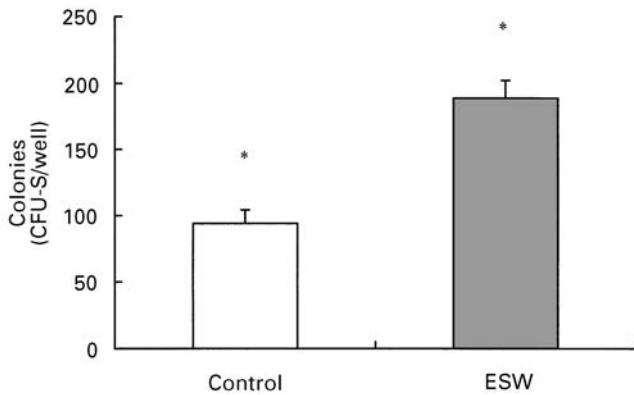

Fig. 2

Graph showing ESW enhancement of the formation of total stromal cell colonies (CFU-S). The difference was significant compared with the control group (*p < 0.001).
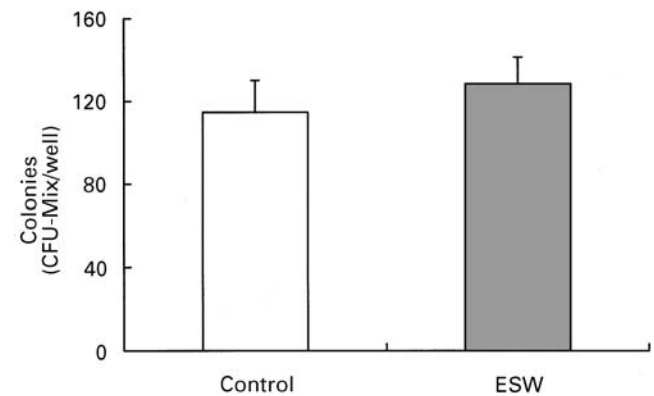

Fig. 3

Graph showing the effect of ESW on the formation of CFU-Mix. This was not significant (p = 0.557).
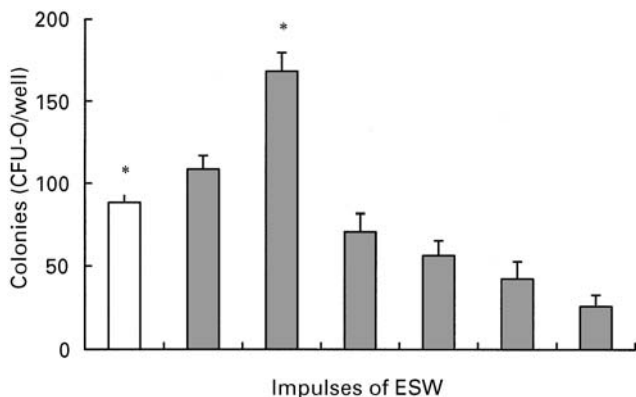

Fig. 4a

Fig. 4b

Graph showing the effect of ESW on the formation of a) CFU-O and b) TGF-β1. There was a significant difference for both groups compared with the control group (*p < 0.001).
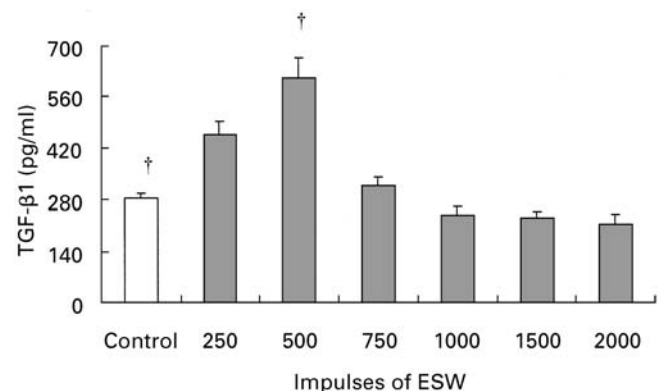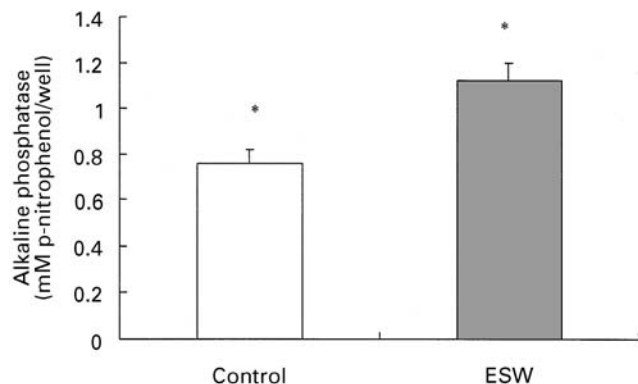
Fig. 5

Graph showing the effect of ESW on the activity of alkaline phosphatase in cells from CFU-O colonies. There was a significant difference compared with the control group (*p < 0.01). Results are presented with the mean values and standard errors calculated from experiments with six rats.
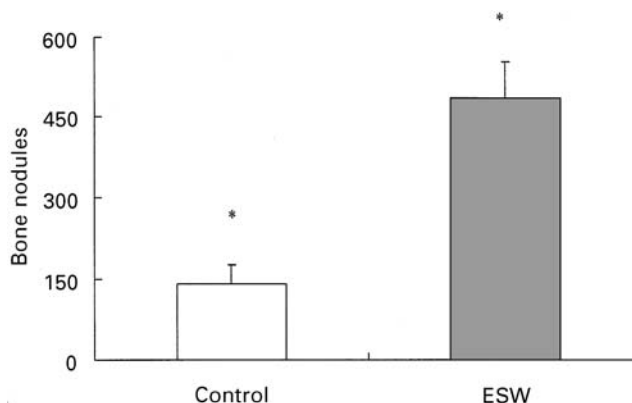

Fig. 6

Graph showing the effect of ESW on the formation of bone nodules. The difference was significant compared with the control group (*p < 0.001).

the promotion of CFU-O growth by ESW could propagate into mature osteogenic differentiation, we measured the alkaline phosphatase activity in the second passage of the CFU-O colonies, and the formation of bone nodules. As shown in Figure 5, the alkaline phosphatase activity in the CFU-O colonies was dramatically increased by ESW treatment with 500 impulses. In addition, formation of bone nodules was significantly higher after ESW treatment in 500 impulses (Figs 6 and 7).

Discussion

The mechanism by which ESW enhances the healing of fractures is not clear. It has been suggested that the ESW-induced acoustic streaming effect may facilitate cell growth or enzymic activities.[26] Furthermore, it has been postulated that ESW-promoted cavitation and cell permeability may result in vascular and bony regeneration.[27] To our knowledge, there has been no study which suggests that treatment by ESW enhances the proliferation and maturation of bone-marrow osteoprogenitors. Our study has shown that optimal ESW treatment promotes CFU-O growth and the formation of bone nodules in bone-marrow stromal cells, providing new evidence that bone-marrow mesenchymal stem cells may be involved in the promotion of bone repair by ESW.

It has been shown that bone-marrow stromal cells express soluble and membrane factors which mediate intercellular interaction and promote haematopoiesis.[28] By contrast, there is also evidence that osteogenesis can be stimulated by co-culture of bone-marrow stromal cells with bone-marrow haematopoietors in vitro.[21,29] The fact that ESW did not significantly affect haematopoiesis but selectively promoted the formation of CFU-O indicates that ESW may have a unique application in the treatment of disorders such as tendinopathies and nonunion. Data accu-
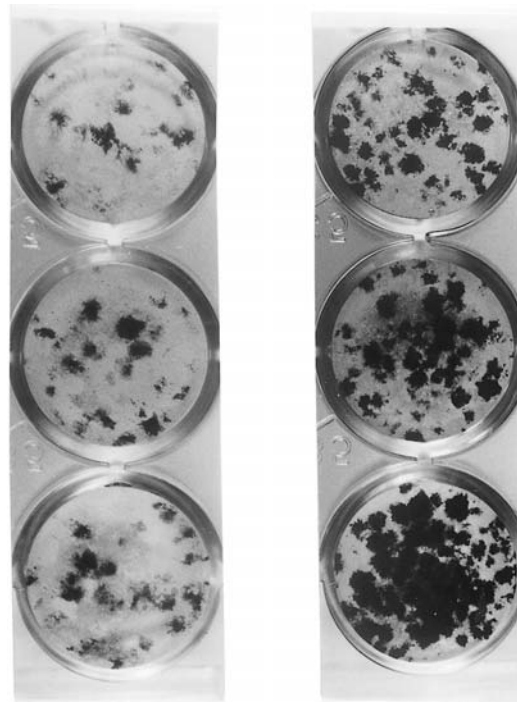

Fig. 7

The formation of bone nodules in a) the untreated and b) the ESW-treated groups (von Kossa).

mulated from treatment by ESW for renal calculi and gallstones have shown that side-effects from treatment by ESW are dependent on the energy and impulses of the treatment.[30] Higher doses may cause the production of heat-induced free radicals or disturbance in the homeostasis of cellular calcium, resulting in cell and tissue damage.[31,32] High-energy ESW treatment also produces aseptic necrosis and damage of osteocytes in rat bone marrow.[33] We have used a variety of doses to test its effect on bone marrow

stromal cell growth and maturation. An optimal ESW dosage enhanced but did not damage the cell growth. This result has further provided evidence to indicate that ESW treatment could be used to treat certain orthopaedic disorders.[34,35] Production of TGF-β1 was significantly associated with the formation of CFU-O by ESW. Further studies are required to determine how TGF-β1 is induced by ESW treatment and which signal is responsible for the promotion by TGF-β1 of the growth and maturation of selective osteoprogenitors.

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