Shock Wave Therapy Applied to Rat Bone Marrow-Derived Mononuclear Cells Enhances Formation of Cells Stained Positive for CD31 and Vascular Endothelial Growth Factor

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Background This study tested the hypothesis that shock wave (SW) therapy applied to bone marrow-derived mononuclear cells (BMDMNCs) enhances the formation of vascular endothelial growth factor (VEGF) and positively stained CD31 (CD31+) cells, an endothelial phenotype.

Methods and Results The BMDMNCs (~1.2×10⁶ cells/2 femoral bones) were obtained from adult male Sprague-Dawley rats and SW therapy was applied once to BMDMNCs in group I (140 SW: defined as 140 shots in total, given at 0.09 mJ/mm²), group II (280 SW), and group III (560 SW). Group IV was not treated by SW and served as the control group. Six experiments were done in each group. The BMDMNCs were cultured following SW therapy and the supernatants were collected on days 1, 3, 7 and 14 for assessment of VEGF levels. Immunocytochemical staining and flow cytometric measurement were performed on days 0 and 14. Experimental results demonstrated that VEGF levels were significantly higher in groups I–III than in group IV, and in group II than in group I at all intervals, and in group II than in group III on day 14 (all p values <0.005). Additionally, the number of positively stained VEGF cells on days 1, 3 and 14 and the number of newly formed CD31+ cells on day 14 were significantly higher in group II than in group IV (all p values <0.001).

Conclusions These data suggest that application of SW to BMDMNCs significantly enhanced VEGF production and promoted differentiation of BMDMNCs into endothelial phenotype cells. (Circ J 2008; 72: 150–156)

Key Words: Bone marrow cells; Endothelial phenotype cells; Shock wave; Vascular endothelial growth factor

Atherosclerosis manifests clinically as coronary artery disease, stroke, transient ischemic attack and peripheral vascular disease, so it is a single pathologic entity that affects different vascular territories. Patients with diffuse atherosclerosis usually have high morbidity and/or mortality because most are unsuitable candidates for either surgical or catheter-based interventions. Additionally, medical responses are usually poor in most of these patients. So the currently available treatment options remain unsatisfactory and development of a new, effective, safe and noninvasive therapy for these high-risk patients is imperative!

Increasing recent evidence has demonstrated that stem cell therapy is safe and can provide additional benefits for improving ischemic legs and ischemia-induced myocardial dysfunction, as well as cardiac regeneration of postinfarcted myocardium. Of the stem cells, bone marrow-derived stem cells (BMDSCs) seem to be highly advantageous for cell therapy. Bone marrow contains a substantial amount of progenitor cells that can be expanded ex vivo and cultured under appropriate conditions to differentiate into various cell types, including myogenic cells and endothelial cells, and enhance angiogenesis. The mechanisms of improving cardiac function following stem cell therapy have been debated extensively and suggested to be the results of angiogenesis, vasculogenesis, myogenesis and the effects of cytokines. Thus, the significance of any single mechanism may be less possible as an explanation of cardiac function improvement.

In vitro studies have demonstrated that shock wave (SW) therapy can enhance mRNA expression of vascular endothelial growth factor (VEGF) in cultured human umbilical vein endothelial cells (HUVECs). Accordingly, animal models of extracorporeal cardiac SW therapy have been used recently to investigate the effects of extracorporeal real cardiac SW therapy, and the experimental results show that applying appropriate energy to the ischemic myocardium can induce angiogenesis, thereby improving ischemic cardiac function. However, the impact of SW on increasing the production of VEGF from BMDSCs, which are the most potential candidate source of stem cell therapy in the near future, is currently unclear.

Therefore, this in vitro study tested the hypothesis that SW therapy applied to bone marrow-derived mononuclear cells (BMDMNCs) enhances VEGF production and accel-
erates new formation of VEGF positively stained (VEGF+) cells and CD31 positively stained (CD31+) cells, an endothelial phenotype.

Methods

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Chang Gung Memorial Hospital (CGMH) and performed in accordance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

Animals, Protocol and Procedure

Adult male Sprague-Dawley (SD) rats, weighing 325–350 g (Charles River Technology, BioASCO Taiwan Co Ltd, Taiwan) were used. The rats were anesthetized by intraperitoneal injection of chloral hydrate (35 mg/kg). Both thighs were shaved, and the rat was placed on a warming pad at 37°C. Prior to separating the cross ligament over the patella, local anesthesia with 1 ml of 2% xylocaine was injected into the area. After careful separation of the ligament from the patella, a hole with a diameter of 0.8 mm was drilled straight into the femoral bone from the distal end of the femoral bone fossa and a sterile 22-gauge syringe was used to aspirate the bone marrow.

Preparation of BMDMNCs

Bone marrow cells from each rat, in 10 ml of culture medium (IMDM+10% fetal bovine serum), were centrifuged at 400 g for 5 min, digested for 40 min with 0.01% collagenase B and DNase1, and then filtered through 30-μm nylon mesh. Next, the bone marrow cells in Ficoll solution were centrifuged at 400 g for 30 min. Finally, the interphase of the BMDMNCs was collected and washed twice in phosphate-buffered saline (PBS); these BMDMNCs were then centrifuged at 400 g for 5 min and approximately 1.2×10⁶ BMDMNCs were obtained from each rat (both femoral bones).

Rationale of Energy Dose of SW Therapy Delivered to BMDMNCs

Based on the results of a previous study, we wanted to identify the most suitable energy dose of SW therapy, so we designed the following experimental study. SW was applied once to BMDMNCs on day 0 before cell culture was performed (in group I, 140 SW (defined as 140 shots in total, given at 0.09 ml/mm²); in group II, 280 SW (defined as 280 shots in total, given at 0.09 ml/mm²); and in group III, 560 SW (defined as 560 shots in total, given at 0.09 ml/mm²)). Group IV (without SW treatment) served as the control group. For the purpose of statistical analysis, 6 experiments and 6 groupings were performed in each group. These BMDMNCs were then cultured in DMEM culture medium for 14 days and cell viability was assessed by Trypan blue exclusion (cell viability >95.0% was noted in each group).

Our preliminary results showed that 280 SW therapy was the most effective energy dose for mRNA and protein expressions of VEGF.

Real-Time Quantitative PCR Analysis

Real-time PCR was performed using SYBR Green technology with a LightCycler rapid thermal cycler (Roche). The primers for VEGF were: (1) Sense 5'-GACACCCAC-GACAGAAGGGGA-3' and (2) anti-sense 5'-TCACCGC-CTTGGCTTGCACAT-3' (product sizes, 497 bp for 120-aa isoform and 629 bp for 164-aa isoform). Sense and anti-sense primers were designed from different exons of the target gene sequence, eliminating the possibility of amplifying genomic DNA. For each set of primers, a basic local alignment search tool ensured specific sequence homology for the target gene. To confirm the specificity of the amplification, the PCR product was subjected to a melting curve analysis. PCR amplification was performed in duplication in a total reaction volume of 20 μl. The reaction mixture consisted of 1 μl diluted template, 1.5 μl FastStart DNA Master SYBR Green I kit, 3 mmol/L MgCl₂ and 0.5 μmol/L sense and antisense primers. After an 8-min activation of Taq polymerase, amplification was allowed to proceed for 30–40 cycles, each consisting of denaturation at 95°C for 10 s, annealing at 65°C for 5–10 s and extension at 72°C for 5–24 s, depending on the target gene.

Semi-Quantitative Reverse Transcription-PCR

Total cellular RNA was isolated by a single-step method using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and 10 μl of total RNA for the reverse transcriptase reaction. cDNA was prepared and PCR was performed as described. The reverse transcription-PCR products were visualized on 2.0% agarose gels electrophoresed in 1X TAE buffer. After 30 min, the gels were put in solution containing 0.5 μg/ml ethidium bromide and then into a UV trans-illuminator (BioDoc-It Imaging System, AH diagnostics, Helsinki, Finland).

Enzyme-Linked Immunosorbent Assay for VEGF

 Supernatants were collected at 24 h, 72 h, and on days 7 and 14 for the measurement of VEGF levels after SW therapy. The VEGF level was determined in duplicate using a standard ELISA and a commercial kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Intra-individual variability of VEGF levels was assessed. Mean intra-assay coefficients of variance for VEGF were <5%.

Immunocytochemical Staining

The BMDMNCs were centrifuged and loaded on to slides at a concentration of 2×10⁶ cells per slide and fixed in ice-cold acetone for 10 min. The cells were then rinsed twice with PBS buffer and deperoxidased with 7% H₂O₂ for 10 min. After being washed again twice with PBS buffer for 3 min each, the nonspecific potentially reactive antigens were blocked with Blocking Image iT-FX kits (Invitrogen) and incubated at room temperature for 30 min. The cells were then washed twice with PBS buffer for 3 min each. Primary antibody (polyclonal rabbit anti-rat VEGF, Lab Vision RB-222-P, Lab Vision Corp, CA, USA) at a concentration of 1:50 was added to the cells and they were incubated at room temperature for 30 min. The cells were then washed by dipping in PBS buffer twice for 7 min, which was followed by the addition of HRP anti-rabbit secondary antibody (Zymed superpiTure polymer detection kit) to the cells and then incubated at room temperature for 60 min. The cells were then washed by dipping in PBS buffer twice for 7 min, which was followed by the addition of DAB chromogen, followed by HRP anti-rabbit secondary antibody (Zymed superpiTure polymer detection kit) to the cells and then incubated at room temperature for 6 min. Following washing with PBS buffer 3 times for 2 min each, DAB chromogen was added to the cells, which were incubated at room temperature for 5 min. The cells were then rinsed twice with distilled water for 1 min each, followed by hematoxylin treatment for 4 min. After the final wash with distilled water for 1 min, the cells were air-dried for 60 min before the slides were mounted.
Measurement of CD31+ Cells by Flow Cytometry

To identify the numbers of CD31+ cells in cell culture, aliquots of 2×10^5 BMDMNCs were labeled with monoclonal antibody against FITC-conjugated rat CD34 and the phycoerythrin-conjugated rat CD31. Isotype-identical antibodies (IgG) served as controls. After staining, the cells are fixed with 1% paraformaldehyde. Quantitative 2-color (CD31/CD34) flow cytometric analyses were performed using a fluorescence-activated cell sorter (FACSCalibur™ system; Becton Dickinson, NJ, USA). Each analysis includes 10,000 cells per sample. Assays for CD31 expression in each sample were performed in duplicate, with the mean level reported.

Statistical Analysis

Data are expressed as means (±SD). The significance of differences between groups was evaluated with a t-test or ANOVA followed by Tukey’s multiple comparison test. Statistical analyses used SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC, USA). A probability value <0.05 was considered statistically significant.

Results

Effect of SW Therapy on mRNA Expression of VEGF in BMDMNCs

Figs 1A,B shows the mRNA expression of VEGF at 0, 30, 60, and 120 min following 280 shock wave (SW) therapy. Fig 1A shows the mRNA expression of VEGF at 0, 30, 60, and 120 min following 280 shock wave (SW) therapy. By 120 min: ‡ vs †, p=0.03; ‡ vs *, p=0.007.

Serial Change in the Supernatant VEGF Levels

Fig 2 shows the serial changes in the supernatant levels of VEGF following different energies (0.09 mJ/mm^2) of SW therapy to bone marrow-derived mononuclear cells (BMDMNCs). Means of the groups with significant difference (on days 0, 3 and 14, p<0.0001; on day 7, p=0.002) by one-way ANOVA; Means of difference (p<0.05) within each group of different days by repeated measure of ANOVA; Symbols (*, †, ‡) indicate significant difference (at 0.05 level) by Tukey’s multiple comparison procedure.
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therapy. In the control group (group IV), without SW therapy, VEGF was slowly secreted by BMDMSCs in cultured medium and a significant increase was only detected on day 14 following culturing. SW treatment rapidly enhanced secretion of VEGF from cultured BMDMSCs on day 1 and significantly further enhanced VEGF secretion from cultured BMDMSCs thereafter. Our results also showed that VEGF levels were significantly higher in SW-treated groups than in the control group on day 1 and more substantially higher in SW-treated groups than in the control group thereafter. Comparing the serial changes in the VEGF levels among the SW-treated groups, the maximum effect of SW therapy was observed for 0.09 mJ/mm² with a total of 280 shots.

Immunocytochemical Staining Results

Figs 3A,B presents the findings of immunocytochemical staining (×200). Very few VEGF+ cells were found in group IV (control group) on days 1, 3 and 14 (Figs 3A-a,c, respectively), whereas the number of VEGF+ cells was significantly higher in group II (280 SW-treated group) (Figs 3A-d,e) than in group IV on days 1 and 3 (Figs 3A-a,b) following 280 SW therapy. Additionally, the number of VEGF+ cells was markedly increased in group II (Figs 3A-d-f) compared with group IV (Figs 3A-c) on day 14 following 280 SW therapy. Moreover, BMDMNCs in group II (Figs 3A-d-f) had more strongly positive staining of VEGF than those in group IV (Figs 3A-c) on day 14 following 280 SW therapy.

Flow Cytometry and Immunofluorescence Staining

Fig 4A shows the percentage of CD31+ cells in group II (280 SW-treated group) and group IV (control group) on day 14 following 280 SW therapy, according to flow cytometry.
No CD31+ cells existed in group IV or II on day 1 following 280 SW therapy. Additionally, CD31+ cells were scarce in group IV on day 14. However, the number of CD31+ cells was significantly increased in group II on day 14 than on day 1, and in group II than in group IV on day 14, following 280 SW therapy (all p<0.001).

Figs 4B,C shows the results of immunofluorescence on day 21 following culture. CD31+ cells were infrequently observed in group IV on day 21. However, they were clearly observed in group II on day 21 following 280 SW therapy. These findings indicate that SW therapy accelerated the formation of BMDMNCs into CD31+ cells, an endothelial phenotype cell.

**Discussion**

The results of this experimental study, which applied SW therapy to BMDMNCs, has several striking implications. First, although BMDMNCs have an inherent ability to secrete VEGF, it is weak and slow in primary cell culture. Second, SW therapy applied to cultured BMDMNCs speeded up and markedly enhanced VEGF production. Third, this therapy accelerated the differentiation of BMDMNCs into VEGF+ cells and CD31+ cells, an endothelial phenotype. Finally, 280 SW therapy was the most effective energy dose for the in vitro study.

How to best treat severe diffuse atherosclerosis of the coronary arteries, cerebral arteries or peripheral arteries remains a formidable challenge to physicians. Most of these diseases have a poor response to conventional medical therapy or surgical intervention. Recently, stem cell therapy to ischemia-induced organ dysfunction has been reported mostly in either animal models or from clinical observational studies, which have shown that...
stem cell therapy is a safe and effective method for improving ischemic-induced organ dysfunction. Thus, stem cell therapy may be a promising novel therapeutic strategy in the near future for various ischemic diseases. Notably, although reports are promising, the mechanisms responsible for the improvement in ischemic organ function following stem cell therapy remain poorly defined. Some studies have suggested that it may be related to the effects of cytokines secreted by BMDSCs and angiogenesis in the ischemic/infarct area. Other studies suggest that a paracrine effect may account for the benefits of stem cell therapy. Thus, the significance of any single mechanism is unlikely. One important finding in the present study was that although BMDMNCs slowly secrete VEGF, the VEGF level significantly increased on day 14 following primary cell culture, which corroborates an in vitro study and further confirms results from other in vivo studies, indicating that BMDMNCs participate in angiogenesis.

Although BMDSCs have been suggested to have the ability to differentiate into endothelial cells and participate in angiogenesis, little is known about the speed and concentration of VEGF that can be produced following implantation of BMDSCs into an ischemic or in culture medium. Another important finding from the present study is that although the BMDMNCs produced VEGF in culture medium, the speed of VEGF production was relatively slow and the levels of this angiogenic growth factor were low and did not significantly increase in culture medium until day 14. Therefore, our experimental findings extend those obtained by other recent studies. Additionally, this finding raises the need to consider more effective tools for accelerating the production and increasing the concentration of VEGF during application of stem cell therapy.

The principal finding in this study was that the mRNA expression of VEGF following SW therapy was quickly increased and was significantly higher in SW-treated groups than in the control group. Additionally, the supernatant level of VEGF on day 1 following SW therapy was substantially higher in the SW-treated groups than in the control group. Furthermore, the supernatant level of VEGF was markedly elevated following day 1 of SW therapy. This study also identified that the effect of once-only SW therapy was persistent and extended more than 14 days. Nishida et al recently demonstrated that applying SW therapy to cultured HUVECs significantly upregulated mRNA expression of VEGF. It is well known that endothelial cells have an inherent ability to secrete VEGF, so it may not be surprising that SW treatment applied to cultured HUVECs enhanced mRNA expression of VEGF. However, Nishida et al did not show whether VEGF was produced or whether the VEGF level changed after SW therapy. In the present study, application of SW therapy to BMDMNCs, which are believed to be the most suitable candidate for stem cell therapy in the near future in various clinical settings, markedly enhanced the mRNA expression of VEGF in vitro. Furthermore, serial changes in the VEGF levels were clearly delineated during the study period. Accordingly, this study reinforces and extends the findings obtained by Nishida et al. Interestingly, a more recent study showed that SW treatment significantly upregulated the mRNA expression of the chemoattractant, stromal cell-derived factor 1 (SDF-1), and the number of VEGF+ cells in chronic hand limb ischemia. Thus, the results from the current and recent studies indicate that SW therapy, in addition to enhancing VEGF formation from BMDMNCs, can also improve the recruitment of circulating VEGF+ cells into the site of chronic ischemic injury. Endothelial progenitor cells are well endowed with receptors for VEGF (VEGF receptor 2) and SDF-1 (CXCR4), which allows them to sense their way through the circulation to sites of acute ischemic injury. Therefore, our experimental results and those of the recent study highlight the potentially clinical implication of SW therapy on improving ischemic organ dysfunction.

The exact mechanism of the enhancement of VEGF production by BMDMNCs after SW therapy remains uncertain. The VEGF-Flt system has been suggested to be essential in initiating vasculogenesis and/or angiogenesis. An animal study demonstrated that SW induces angiogenesis of the Achilles tendon-bone junction in dogs. Nishida et al have also demonstrated that SW therapy increases angiogenesis and ameliorates ischemia-induced myocardial dysfunction in pigs and Fukumoto et al have shown that SW therapy ameliorates myocardial ischemia in patients with severe coronary artery disease. Taking these findings into consideration, recruitment of circulating endothelial progenitor cells to the site of ischemia, activation of the VEGF-Flt system and upregulation of SDF-1 in the ischemic area are believed to be the mechanisms of angiogenesis/vasculogenesis following SW therapy. The most important finding from the present study is that SW therapy markedly enhanced BMDMNCs expression of CD31+ cells, which indicates that SW therapy accelerated the differentiation of BMDMNCs into endothelial phenotype cells. Experimental studies have demonstrated that bone marrow contains abundant multipotent progenitor cells that can be expanded ex vivo with under appropriate culture conditions, differentiate into various cell types, including endothelial cells, and enhance angiogenesis. Additionally, SW therapy has been suggested to induce the shear force on the cell membrane which could result in induction of gene expression. Therefore, the current and recent findings could, at least in part, explain some of the mechanisms of the effect of SW therapy on increasing VEGF production from BMDMNCs.

Study Limitation

Although the results of this in vitro study are impressive, without an in vivo experiment using an animal model, we cannot address whether BMDMNC transplantation plus SW therapy would provide an additional benefit for improving ischemic-induced organ dysfunction.

In conclusion, SW therapy applied to BMDMNCs significantly enhanced VEGF mRNA expression, increased VEGF production, and accelerated differentiation of BMDMNCs into an endothelial phenotype. These experimental findings raise the possibility that combined BMDMNC transplantation and SW therapy could provide an additional benefit to ischemia-induced organ dysfunction in the near future.

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