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● *Original Contribution*

SHOCK WAVES ACTIVATE *IN VITRO* CULTURED PROGENITORS AND PRECURSORS OF CARDIAC CELL LINEAGES FROM THE HUMAN HEART

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Abstract—Postischemic cardiomyopathy remains one of the disorders in urgent need of effective noninvasive therapy. It is currently accepted that the isolation, expansion and application of resident cardiac stem cells may hold therapeutic promise for the future. Recently, it has been demonstrated that shock waves (SW) could enhance the expression of vascular endothelial growth factor (VEGF) and its receptor, Flt-1. As the development of angiogenic noninvasive therapy is very important for future therapeutic strategies in cardiovascular diseases, we examined *in vitro*, the effects of SW treatment on adult resident cardiac primitive cells isolated from bioptic fragments of normal human hearts and from explanted pathologic hearts with postischemic cardiomyopathy. This study demonstrates that SW have positive influence on both the proliferation and the differentiation of cardiomyocytes, smooth muscle and endothelial cells precursors, with a more obvious effect being evident in the cells from normal heart than in those taken from pathologic hearts. Our results suggest that SW treatment could inhibit or retard the pathologic remodeling and functional degradation of the heart if applied during the early stages of heart failure. (E-mail: montagna@unina.it) **© 2008 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Cardiac cells, Cell differentiation, Cardiovascular disease, Extracorporeal shock waves.**

INTRODUCTION

Stem cells are capable of generating identical progeny through an unlimited number of cell divisions, while retaining the ability to respond to physiological demands by producing daughter cells committed to differentiation (Wagers and Weissman 2004). Recently, the presence of dividing primitive cells has been observed in organs considered to be terminally differentiated, such as the brain (Uchida et al. 2000) and the heart (Beltrami et al. 2001). Moreover, c-kit positive precursors and progenitors of cardiac cell lineages were identified in the adult human heart, as shown by the expression of certain specific markers. For progenitors, these included transcription factors like myocyte enhancer factor (MEF-2C), GATA-6 and external transcribed spacer (Ets-1), while the cytoplasmic proteins α-sarcomeric actin (α-SA) and ventricular α/β myosin heavy chain (MHC), smooth muscle actin (SMA), factor VIII (FVIII) and vascular endothelial growth factor receptor (VEGFR) were the markers for the precursors for cardiomyocytes, smooth muscle and endothelial cells, respectively (Beltrami et al. 2003; Linke et al. 2005). These findings support the notion that cardiac stem cells provide the adult heart with a growth reserve and contribute to the replacement of cardiomyocytes, vascular smooth muscle cells and endothelial cells that die of apoptosis and necrosis.

Many studies have targeted the regeneration of cardiac tissue damaged by disease. While questions have been raised about the positive effects of injecting bone marrow, peripheral blood or skeletal muscle-derived

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stem cells and mechanisms leading to their homing and differentiation in the heart (Oh et al. 2003; Murry et al. 2004), the cardiac-resident primitive cells seem to be a promising target in the therapy of acute and chronic heart disease, where cardiac regeneration may be accomplished by enhancing the normal turnover of myocardial cells. Ischemic cardiomyopathy, with its high prevalence and poor prognosis, remains one of the disorders in urgent need of effective noninvasive therapy.

The isolation, expansion and application of resident cardiac stem cells may hold therapeutic promise for the future, and the European Society of Cardiology recently established a task force on stem cells and the repair of the heart (Bartunek et al. 2006).

A shock wave is a longitudinal acoustic wave that travels through body tissues. It is a single pressure pulse with a needle-like, positive spike, less than 1 μs in duration and up to 100 MPa in amplitude, which exerts a "cavitation effect" inside and outside the cells (Nishida et al. 2004). It has been recently suggested that shock waves (SW) could enhance angiogenesis *in vitro*. In a porcine model of chronic myocardial ischemia, ESW therapy effectively increased regional myocardial blood flow and normalized myocardial dysfunction (Nishida et al. 2004). Similar results were observed after ESW treatment in the first nonrandomized study of patients with coronary artery disease (Fukumoto et al. 2006). The present study was designed to test whether the application of ESW could have positive effects not only on the endothelial cell population but also on other cardiac primitive early committed cells. With this in mind, we established a method of isolating and expanding the progenitors and precursors of cardiac cell lineages from human biopsy specimens. The quantification of cardiac primitive cells of cardiomyocyte, smooth muscle and endothelial lineage obtained from normal hearts and hearts with postischemic cardiomyopathy was performed *in vitro* on both the SW-treated and untreated cells, as was the analysis of the expression of proteins characteristic for the committed cardiac cells and their mRNA.

MATERIALS AND METHODS

Cell Cultures

Cardiac primitive cells were isolated from bioptic fragments of normal human hearts and pathologic hearts. The normal hearts came from donors who had died for reasons other than cardiovascular disease ($n = 16$, mean age of donors 35 ± 12 y, nine males and seven females, death caused by trauma or cerebral haemorrhage). Similar fragments of cardiac muscle were taken from the right ventricle of the hearts explanted due to the terminal heart failure associated with postischemic cardiomyopathy ($n = 8$, mean age 56 ± 5.5 y, five males and three females, mean ejection fraction 25 ± 1%). The investigation conformed to the principles outlined in the Declaration of Helsinki.

The samples were put on culture dishes and covered with sterile glass, in F12K medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 5% horse serum (Invitrogen, Carlsbad, CA, USA), bFGF (Peprotech, London, UK), glutathione (Sigma-Aldrich), penicillin and streptomycin (Invitrogen). The outgrowth of cells from the bioptic fragments was observed after time periods ranging from 2 to 4 wk. Once the adherent cells were more than 75% confluent, they were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and replated. Cells at the second and third passage were used for the experiments. The cells were always split in half and duplicate studies with and without shock waves were performed to evaluate the variability of the data in each set of cardiac cells. Variability never reached 3%.

Shock Wave Treatment

The generator utilized for the *in vitro* application of shock waves was an electromagnetic extracorporeal shock wave machine as used in orthopedics and lithotripsy (Modulith SLK, Storz Medical AG, Taegerwilen, Switzerland). The peak positive pressure and energy flux density of the SW can be varied from 6 MPa to 120 MPa, and from 0.03 mJ/mm^2 to 1.5 mJ/mm^2, respectively. The ESW unit was kept in contact with the culture dish containing adherent cells by means of a water-filled cushion covered with common ultrasound gel. Cells obtained from normal and pathologic hearts and plated at a density of 15×10^3 cells/cm^2 were subjected to 800 impulses of ESW at an energy flux density of 0.1 mJ/mm^2. At this energy flux density, the peak positive pressure is 18 MPa, focal width is 6.5 mm FWHM, 5 MPa focal width is 13 mm, energy in the 6 dB focal width is 3.9 mJ, energy in the 5 MPa focal width is 8.5 mJ, as measured with a fiber optic hydrophone (Steiger et al. 1998). After the treatment, the cells were cultured for 7 d before being analyzed. An identical number of cells was cultured at the same time and served as a control.

Immunofluorescence

The control and treated cells were fixed with 4% paraformaldehyde for protein staining. After blocking with 10% donkey serum, the cells were incubated with one of the following primary antibodies: α-sarcomeric actin, smooth muscle actin, factor VIII (Sigma-Aldrich), ventricular α/β myosin heavy chain (Chemicon, Temecula, CA, USA) or VEGFR-2 (Novocastra, Newcastle, UK). This was followed by incubation with rhodamine or fluorescein-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, USA). The

nuclei of the cells were labeled with DAPI and each sample was evaluated by three independent observers. Signals were visualized with a Leica DMLB fluorescent microscope.

TUNEL Assay

Apoptosis was determined 24 h after the SW application by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay, using an ApopTag Fluorescein *In Situ* Apoptosis Detection kit (Chemicon) according to the manufacturer's instructions. Control and SW-treated cells isolated from both the normal and pathologic hearts were fixed with 1% paraformaldehyde for 10 min. Nuclear DNA was denatured with cooled ethanol 70% at −20°C for 1 h. The cells were rehydrated with equilibration buffer and incubated with TdT working solution at 37°C for 1 h. At the end of incubation, the reaction was stopped and antidigoxigenin-fluorescein conjugate was added for a period of 30 min at room temperature. After washing, the cells were counterstained with DAPI and observed under a Leica DMLB fluorescent microscope.

Western Blot

Protein extracts were prepared from the cultures of treated and untreated cells derived from the normal and pathologic hearts, as previously described (Linke et al. 2005). Proteins (80 μg) were loaded on 8% SDS-polyacrylamide gel and, after electrophoresis, transferred onto nitrocellulose membrane by semidry electroblotting (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with one of the following primary antibodies: α-sarcomeric actin, smooth muscle actin, Factor VIII (Sigma-Aldrich), VEGFR-2 (NeoMarkers, Fremont, CA, USA) and α-actinin. After this, they were probed with HRP-labeled secondary IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To visualize the bands, we used chemiluminescence (Pierce, Rockford, IL, USA), followed by autoradiography and then measured their intensity on scanned films with ImageJ software (NIH, USA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by lysing the cells in Trizol solution (GIBCO BRL, Life Technologies, Rockville, MD, USA). After confirming the yield and integrity of each RNA sample, 5 μg of total RNA were reverse-transcribed by using the first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) with the random hexamer primers. The same volume (2 to 5 μl) of products from each sample was used for subsequent PCR amplification with the primers prepared for the target genes and the GAPDH housekeeping gene. Samples without cDNA served as negative controls for the PCR amplifications. The PCR reactions were obtained by using the following cycle conditions: denaturation for 1 min at 94°C, annealing for 1 min for MHC and FVIII at 61°C and 51°C, respectively, extension for 1 min at 72°C and a final extension at 72°C for 10 min.

The primers used for PCR and the expected size of their amplification products were:

(a) α/βMHC (Nakao et al. 1997) 5′-AGCAGAAGCG-CAACGCAGAGT-3′ (forward) and 5′-TGCTGCAC-CTTGCGGAACTTG -3′ (reverse primer) 217 bp;
(b) FVIII 5′-CAGCCTCTACATCTCTCAGTT-3′ (forward) and 5′-ATGCGAAGAGTGCTGCGAATG-3 (reverse primer), 210bp;
(c) GAPDH 5′-CACCATCTTCCAGGAGCGAG-3′ (forward) and 5′-TCACGCCACAGTTTCCCGGA-3′ (reverse primer), 372 bp.

The specific primers for FVIII were designed with the assistance of Oligo4 software.

The amplified products were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination. The levels of target cDNA were estimated by densitometric scanning and normalized against GAPDH loading controls. Densitometric analyses of the PCR products were performed with NIH Image software version 1.62F.

Statistical Analysis

Summary values are given as a mean ± standard deviation of the mean. The unpaired Student's *t*-test was used for statistical analysis. Data were considered to be statistically significant at a value of $p < 0.05$.

RESULTS

Cells obtained *in vitro* from bioptic fragments of normal and pathologic hearts with postischemic cardiomyopathy were characterized by immunofluorescence, whether the cells had been treated with SW or not (Fig. 1). SW treatment was associated with cell detachment over an area of about 0.2 mm^2, which in our settings equals 0.007% of the total area of culture dish. After the treatment, no significant increase in apoptosis was detected by the TUNEL assay. The rate of apoptosis was as low as 0.59% and 0.54% in the untreated cells from normal and pathologic hearts, respectively, raising to 0.91% and 0.61% after treatment (Fig. 2).

Cells expressing ventricular α/βMHC and/or α-SA, corresponding to primitive cardiomyocytes, constituted 7.3 ± 1.2% and 20.5 ± 1.7%. After SW treatment, their rate

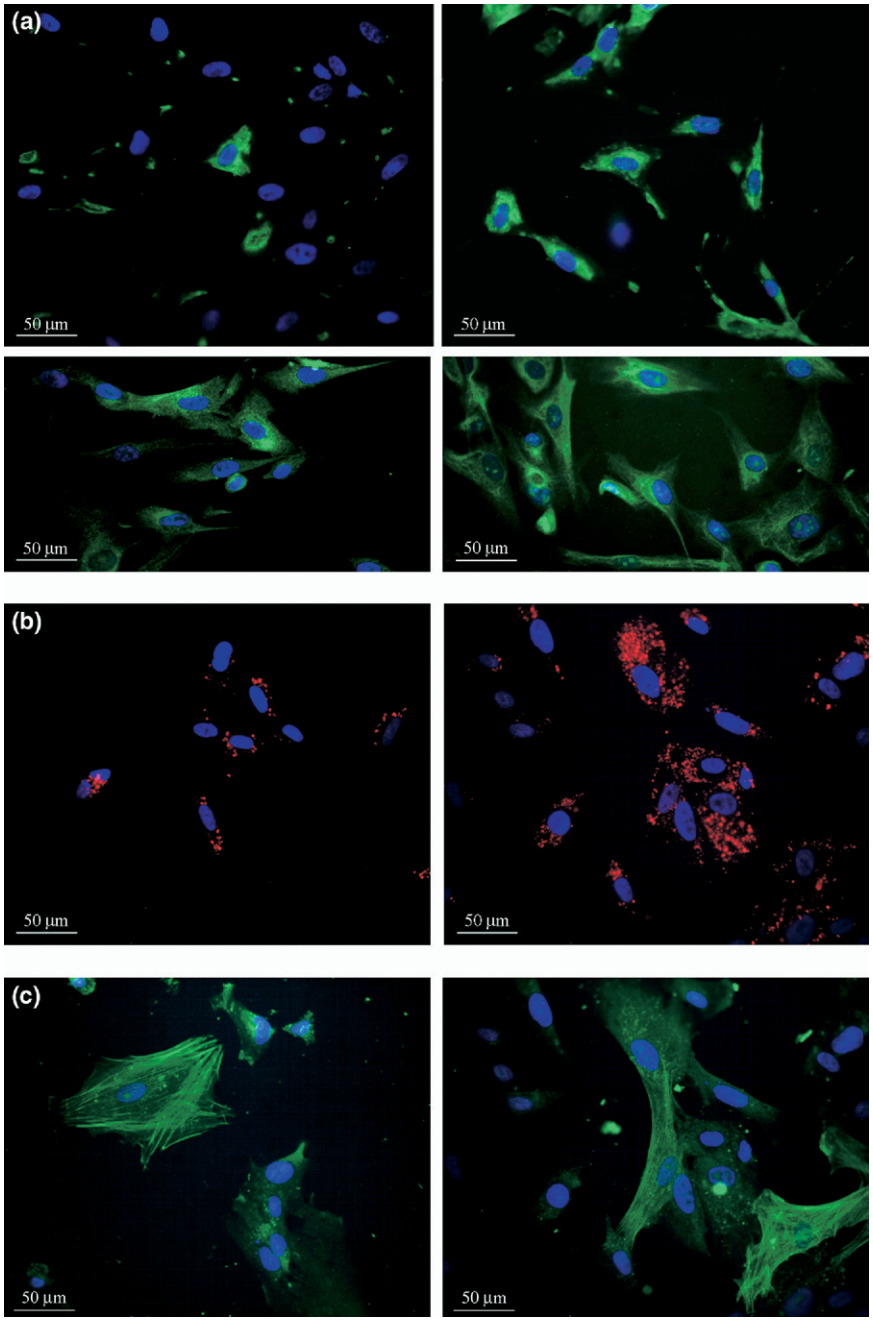

Fig. 1. Immunostaining of proteins characteristic of primitive cells of cardiac lineages *in vitro* (on the left) and after they were subjected to ESW (on the right). The figure presents merged pictures of cytoplasmic proteins and nuclei stained with DAPI. (a) Precursors of cardiomyocytes were identified on the basis of MHC (upper panel) and α-SA expression (lower panel) (green fluorescence); (b) Endothelial precursors express FVIII (red fluorescence); (c) SMA was detected in smooth muscle cell precursors (green fluorescence). Magnification 400×.

increased to 13.8 ± 2.3% ($p = 0.01$) and 24.8 ± 2.3% ($p = 0.06$) in the normal and pathologic hearts, respectively. Primitive endothelial cells identified by the expression of FVIII made up 6.1 ± 0.6% of the cells in the normal heart and 10.03 ± 0.9% of the cells in the pathologic heart. After exposure to SW, their rate increased to 8.6 ± 1.0% ($p = 0.02$) and 13 ± 1.3% ($p = 0.03$) in the normal and pathologic hearts, respectively. Smooth muscle cells were quantified on the basis of SMA expression. Exposure to SW increased their rate in the normal hearts from 7.8 ± 0.6% to 10.8 ± 1.5% ($p = 0.04$) and from 23.8 ± 1.3% to 30.8 ± 2.8% ($p = 0.03$) in the hearts with postischemic cardiomy-

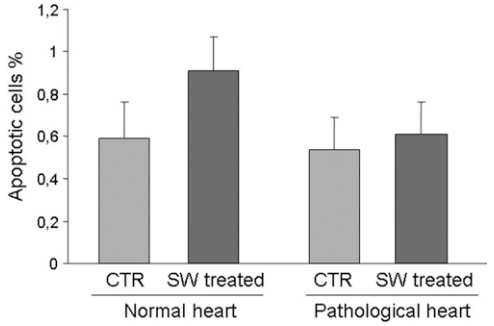

Fig. 2. The effects of SW treatment on apoptosis of cardiac cells isolated from normal and pathologic hearts. Results are expressed as mean percentages ±SD, $n = 10$. No statistically significant difference was observed between the treated and untreated cells of both groups.

opathy. Variability never reached 3%. These data are summarized in Fig. 3.

Proteins characteristic of early committed primitive cells of cardiac lineages were evaluated by immunoblotting (Fig. 4). After treatment, the expression of α-SA was about 1.6-times higher in the cells from normal hearts and 1.3-times higher in the cells from diseased hearts. SW treatment resulted in a significant increase in VEGFR-2 expression (about a 5.6-fold increase in the normal hearts and a 1.5-fold increase in the pathologic hearts). Similarly, an increase in SMA was noted after the treatment (about 1.5-fold for normal hearts and 1.1-fold for pathologic hearts).

To investigate the effects of SW on the transcriptional activity of cardiac cell specific genes, the cDNA

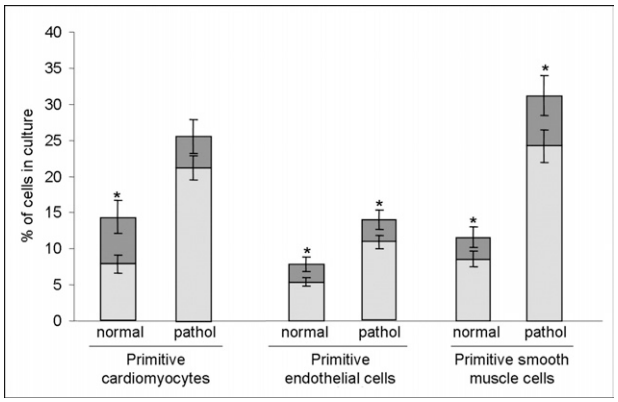

Fig. 3. The effects of SW treatment on cardiac primitive cardiomyocytes, endothelial and smooth muscle cells identified on the basis of the expression of α-SA, FVIII and SMA, respectively. The increase in the relative number of positive cells in the SW-treated culture (light plus dark gray column) culture is shown with respect to untreated cells (light gray). The data are the means ± SD of ten experiments. $*p < 0.05$ between untreated and treated cells.

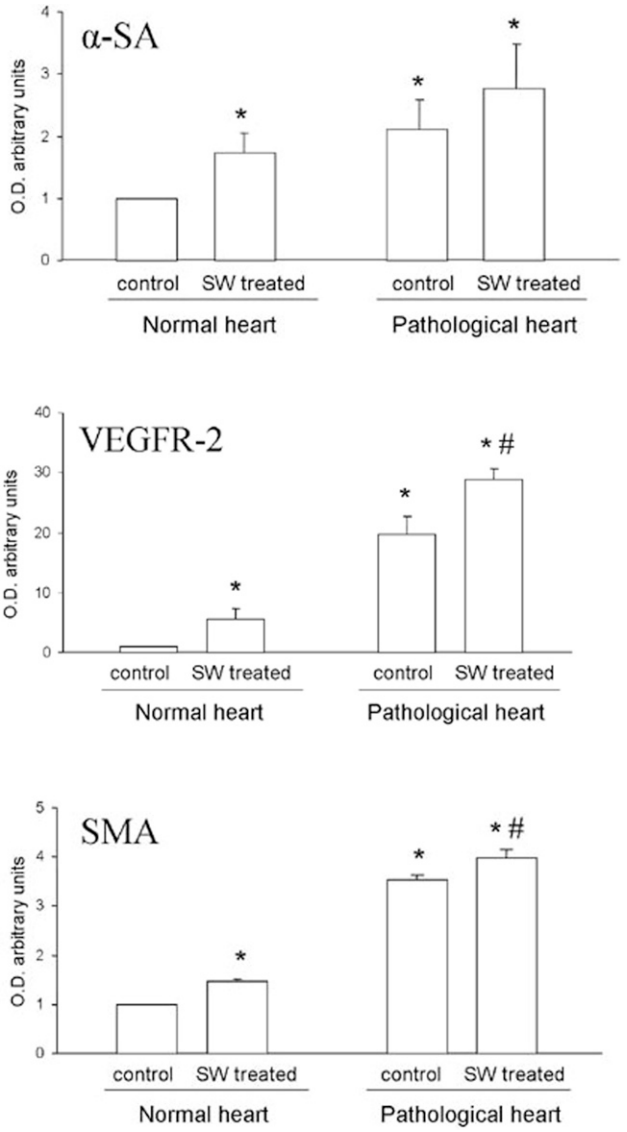

Fig. 4. Densitometric analysis of western blot for α-SA, SMA and VEGFR-2 expression in the untreated and SW-treated cells isolated from normal and pathologic hearts. The results are normalized to normal untreated cells (control). Values are means ± SD, $n = 10$. $*p < 0.05$ vs. normal control. $\#p < 0.05$ between pathologic control and SW-treated cells.

synthesized from total mRNA of the control and treated cells was subjected to PCR, using primers specific for human α/βMHC, FVIII and the GAPDH housekeeping gene. SW treatment upregulated the expression of mRNA for FVIII and α/βMHC in cells derived from both normal and pathologic human hearts (Fig. 5).

DISCUSSION

The cell cultures obtained in this study from bioptic fragments of normal and pathologic hearts with postisch-

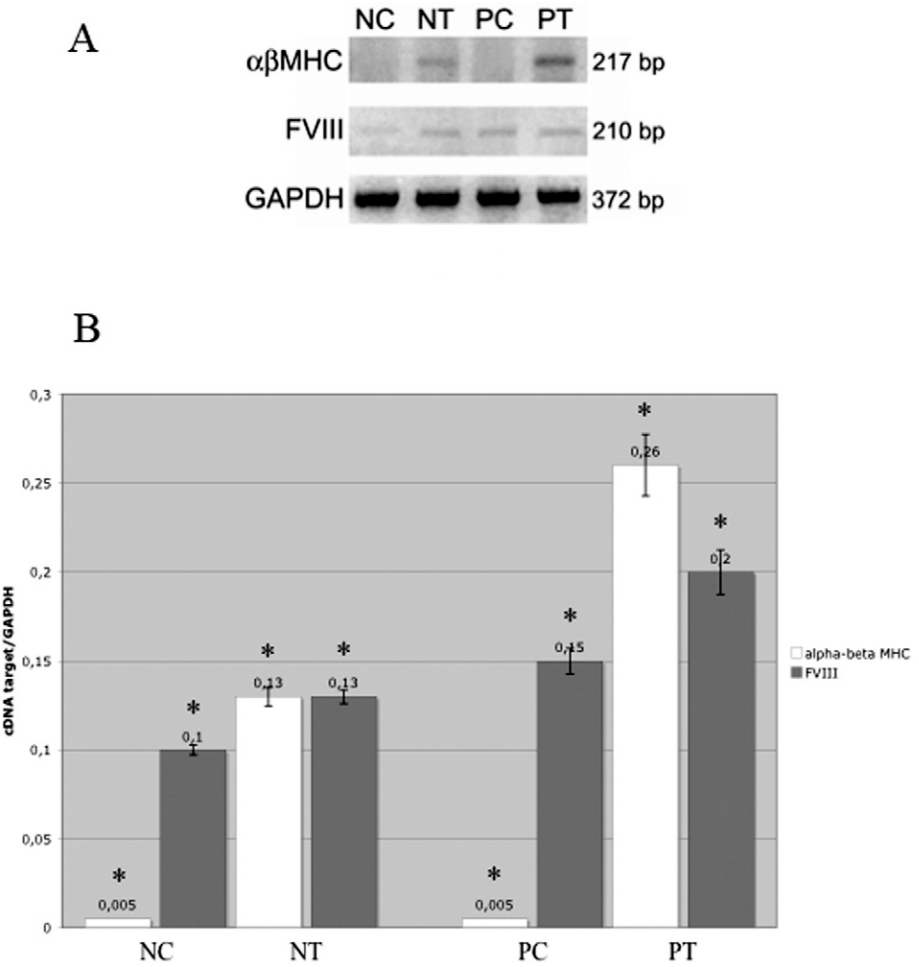

Fig. 5. The effects of SW on the expression of mRNA for α/βMHC and FVIII were analyzed by RT-PCR; GAPDH was used as control in each reaction. NC = normal control cells; NT = normal SW-treated cells; PC = pathologic control cells; PT = pathologic SW-treated cells. (A) Representative RT-PCR results for α/β MHC and FVIII are shown. (B) Relative expression of α/βMHC and FVIII. The data are the means ± SD, $n = 10$. *$p < 0.05$.

emic cardiomyopathy contained a small number of stem cells and a relatively larger population of cardiac progenitors and precursors, with the most numerous population being composed of fibroblasts. Worth noticing is the increase in the relative number of cardiac primitive cells isolated from the explanted human hearts compared with normal hearts. This observation reflects the activation of the regenerative potential of the human heart in postischemic heart failure. A similar conclusion was drawn from a study of hypertrophied myocardium in patients with chronic aortic stenosis, in which the growth and differentiation of cardiac stem cells into cardiomyocytes was markedly enhanced (Urbanek et al. 2003).

It has been recently observed that SW treatment applied to cultured human umbilical vein endothelial cells upregulated mRNA expression of VEGF and its receptor Flt-1(fms-like tyrosine kinase) *in vitro* (Nishida et al. 2004). Possible mechanisms of SW action include nonenzymatic nitric oxide synthesis (Gotte et al. 2001), the activation of intracellular protein kinases in the absence of growth factor receptor ligands (Wang et al. 2001), the induction of intercellular gaps (Seidl et al.1994) and membrane permeabilization (Berger et al. 2005). Whatever the mechanism may be, it is doubtful that the positive effects observed after the application of ESW in heart disease can be related only to neoangiogenesis.

To address this issue, we have treated the cardiac primitive cells forming a monolayer on a standard adherent culture dish. The pressure pulse transmission of the culture dish is ≥95%. At the low energy setting used in our study the waveform is not fully shocked but almost symmetrical (Fig. 6). Even with this limitation, the treatment of adherent cells is favourable for the study of biological activity and differentiation of primitive cells, allowing to preserve intercellular contacts, as well

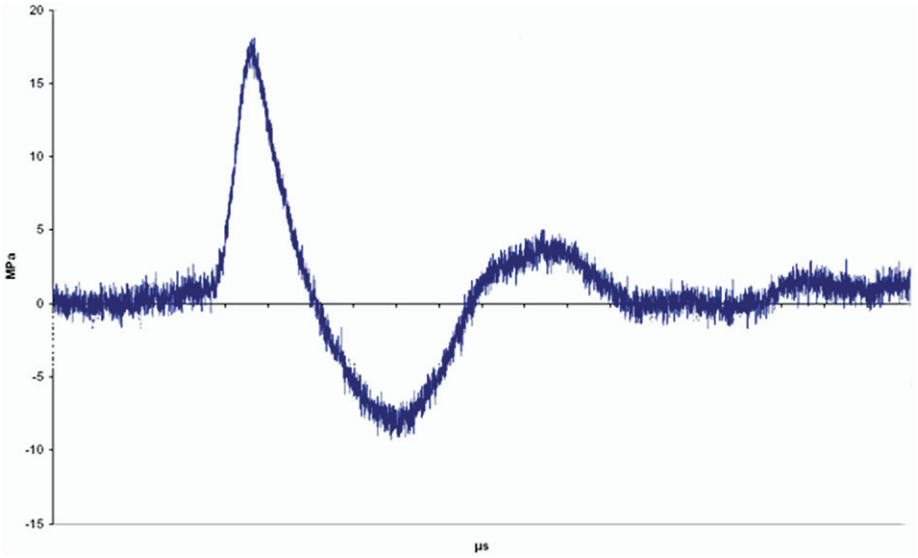

Fig. 6. Shock waveform and pressure at the energy flux density 0.1 mJ/mm^2, as measured with a fiber optic hydrophone. The reflection of the shock wave at the liquid-air interface, although present during adherent cell treatment *in vitro*, was not taken into consideration when performing this measurement.

as contacts with extracellular matrix, and outside-inside signaling. The results of the present study confirm that SW treatment can have a positive effect on the proliferation and differentiation of endothelial cells, as our data confirm the increase in the number of mature endothelial cells (FVIII positive) as well as endothelial cells involved in neo-angiogenesis (VEGFR-2 positive). At the same time, SW can improve the number of primitive cardiomyocytes and smooth muscle cells without any significant pro-apoptotic effect, apparently decreasing the fibroblast growth rate. We are carrying out further investigations to clarify this point.

The expression of mRNA and proteins characteristic of cardiac muscle cells (α-SA and MHC, VEGFR-2 and FVIII, and SMA for the precursors of cardiomyocytes, endothelial cells and smooth muscle cells, respectively) always increased after the cells were exposed to SW. In most cases, these changes were more prominent in the normal heart cells than in those taken from pathologic hearts. However, the untreated cells from pathologic hearts showed a higher expression of proteins compared with the untreated cells from normal hearts, indicating that the former are activated during the progression of a disease in response to chronic pathologic conditions. It is possible that these cells reach a level of activation that can only be increased slightly more, while the cells from normal hearts reach levels of protein expression similar to those found during the reaction to the pathologic process only after SW are applied.

Heart failure is one of the most frequent causes of death in developed countries. Even if properly individualized pharmacotherapy leads to the relief of symptoms, only a few pharmaceutics can inhibit the progression of pathologic heart remodeling. Access to the life saving treatment of heart transplantation is limited by the scarcity of donors and the ethical problems associated with the donation of organs. For all these reasons, new treatment options should be studied *in vitro* and verified in randomized clinical trials.

In this study, the application of SW *in vitro* has led to the activation of proliferation and differentiation in cardiac primitive cells. The study was performed on specimens of hearts in two extreme conditions: organs with no sign of cardiovascular disease and those with the cardiomyopathy that leads to terminal insufficiency. In regard to the less significant influence of SW on cells from explanted hearts, this is probably due to the marked activation of primitive cardiac cells during the early progression of the disease, leading in some way to their pool in the heart being exhausted in the chronic pathologic state. It is probable that inflammation and fibrosis at the sites of ischemic injury inhibit the reconstitution and spatial arrangement of myocytes and coronary vessels *in vivo* (Anversa et al. 2006). In this respect, the anti-inflammatory action of extracorporeal SW mediated by nitric oxide production (Ciampa et al. 2005), together with the activation of cardiac primitive cells observed in the present study, may overcome this obstacle and promote tissue repair. Further studies *in vivo* are needed to verify whether the observed effects of SW on cardiac primitive cells *in vitro* can prevent pathologic remodeling and functional degradation by positively influencing the process of cardiac regeneration during the early

stages of heart failure. At the same time, ESW treatment could well open new doors as regards the development of angiogenic noninvasive therapy for the failing heart while also modulating the differentiation of cardiac cells.

Limitations of the Study

Several limitations should be aknowledged for our study. First, the physical characteristics of the shock waves applied *in vitro* are not comparable with those present during an extracorporeal cardiac shock wave treatment. The presence of liquid-air interface in cell culture dish induces the reflection of shock wave which interferes with the primary wave. This does not occur in the tissue *in vivo*. However, the choice of the treatment of attached cells seems justified by the possibility of the preservation of cell to cell and cell to extracellular matrix contacts, which are totally lost when treating cells in suspension. A correct interaction of extracellular matrix proteins with their receptors on the cell membrane has been found to be critical in heart development and remodeling (Jane-Liese et al. 2000). The adherent growth of cardiac primitive cells *in vitro* is the optimal way of mimicking these complex interactions of extracellular matrix proteins present on the cell culture dish and growth factors and cytokines released by the cells in the medium and in the matrix.

Second, the calibration of the shock wave kinetics in the cell culture dish filled with medium is not precisely known. Nevertheless, it does not diminish the significance of our results indicating that the application of SW *in vitro* leads to the activation of proliferation and differentiation of cardiac primitive cells. Additional *in vitro* and *in vivo* experiments are warranted to explain the physical and biological mechanisms of such effects.

SUMMARY

Our study confirms that SW treatment can have a positive effect on endothelial cells proliferation and differentiation as well as on the number of primitive cardiomyocytes and smooth muscle cells *in vitro*. In most cases, more obvious effects were achieved in the cells taken from normal hearts than in those from pathologic hearts, whose activation had already been increased during the progression of disease in response to chronic pathologic conditions. Nevertheless, a further increase in their level of activation was also observed.

Further studies *in vivo* are needed to verify whether ESW can modulate fibrosis by reducing the growth of fibroblasts at the sites of ischemic injury and by activating neoangiogenesis and cardiac primitive cell differentiation. Should this be the case, treatment with ESW might well prevent pathologic remodeling and functional degradation by positively influencing the process of cardiac regeneration in the early stages of heart failure. At the same time, ESW treatment could also offer new prospects for the development of angiogenic noninvasive therapy for heart failure as well as modulating the differentiation of cardiac cells.

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