JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS Vol. 26, no. 1, 29-37 (2012)

EARLY ANGIOGENIC RESPONSE TO SHOCK WAVES IN A THREE-DIMENSIONAL MODEL OF HUMAN MICROVASCULAR ENDOTHELIAL CELL CULTURE (HMEC-1)

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Received July 11, 2011 - Accepted November 9, 2011

The exact nature of shock wave (SW) action is not, as yet, fully understood, although a possible hypothesis may be that shock waves induce neoangiogenesis. To test this hypothesis, a three-dimensional (3D) culture model on Matrigel was developed employing a human microvascular endothelial cell line (HMEC-1) which was stimulated with low energy soft- focused SW generated by an SW lithotripter. After 12 hours we observed a statistically significant increase in capillary connections subsequent to shock-wave treatment in respect to the control group and a marked 3-hour down-regulation in genes involved in the apoptotic processes (BAX, BCL2LI, GADD45A, PRKCA), in cell cycle (CDKN2C, CEBPB, HK2, IRF1, PRKCA), oncogenes (JUN, WNT1), cell adhesion (ICAM-1), and proteolytic systems (CTSD, KLK2, MMP10). Our preliminary results indicate that microvascular endothelial cells *in vitro* quickly respond to SW, proliferating and forming vessel-like structures, depending on the energy level employed and the number of shocks released. The early decreased expression in the analysed genes could be interpreted as the "first reactive response" of the endothelial cells to the external stimuli and the prelude to the events characterizing the neo-angiogenic sequence.

Over the last few decades the clinical use of shock waves (SW) has widened from its urological application of lithotripsy for gallstones to the treatment of both inflammatory and degenerative orthopaedic diseases in tendons, ligaments and soft tissues. In bone pathology, building on the early experience of Valchanou and Michailov (1), SW have been successfully employed in the treatment of non-union and in bone vascular diseases (2, 3). More recently, they have been proposed for wound care management, owing to the tissue trophic effect generated by the largest distribution of the high pressure focused acoustic waves into low energy soft focused or unfocused shock waves (uSW) (4).

Although the physical principles and the effects of SW on tissues have been broadly investigated, some cellular and biochemical aspects have not yet been completely clarified. *In vivo* studies have demonstrated the angiogenic activity of SW (5, 6). Experimental data suggest that one of their main biological effects is the production of nitric oxide (7), which has been shown to be effective in promoting

Key words: shock waves, endothelial cells, neo-angiogenesis, three dimensional cultures

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0393-974X (2012)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

angiogenesis (8). Angiogenesis, the formation of new blood vessels from pre-existing capillaries, is indeed essential for tissue healing and is regulated by a variety of growth and angiogenic factors.

Current opinion suggests also that mechanical stimulation induced by SW produces a direct effect on the extracellular matrix (ECM) which then triggers cytoplasmatic and nuclear reactions that vary with the experimental model, the energy level, the number of impulses and the cell type (9). As has been demonstrated for specific bio-mechanical stimuli, SW could induce a biochemical reaction in responsive cells that affects growth, development, differentiation, apoptosis regulation and gene expression via signal transduction pathways (10)

The aim of this study is to verify the ability of uSW to induce new vessel proliferation (neoangiogenesis) and, at the same time, to investigate the initial response of endothelial cells to acoustic stimulation. For this purpose we employed an *in vitro* system consisting of a matrix support seeded with microvascular endothelial cells which resembles, as closely as possible, the structure of the natural tissues.

MATERIALS AND METHODS

3D cell culture

A human microvascular endothelial cell line (HMEC-1, Invitrogen) was used in this experiment. Cell cultures were established in the central well of 24-multiwell plates containing BD Matrigel™. BD Matrigel™ is derived from a protein mixture containing structural elements which are analogous to the components of ECM. It provides an excellent environment *in vitro* for the promotion, differentiation and proliferation of endothelial cells (11). Cultures were established at different cell densities (2500, 5000, and 10,000 cells/well respectively), for both untreated and uSW treated cells, with 5 wells prepared for each different density. Cells were cultured in MCDB 131 Medium (Invitrogen) without endothelial growth factors supplemented with 2% fetal bovine serum (FBS, Invitrogen). A control culture for each cell density was left untreated, whilst the other cultures, when they had reached 70-80% of confluence, were treated with a Dermagold™ 100 shock-wave electro-hydraulic SW-device (MTS Europe GmbH, Konstanz, Germany), designed to be applied cutaneously.

Device description and shock wave stimulation

In brief, in an electro hydraulic device, the discharged high voltage produced by two submerged opposite electrodes, emits a surrounding spherical SW which expands in the watery medium from the original first focal point (F1) to the walls of an ellipsoid reflector. Because of the geometric shape of the reflector, SW are conveyed to a second focal point (F2) which corresponds to the target tissue. In Dermagold™ 100 the paraboloid reflector generates soft focused (un-focused) SW.

Two different energy levels were used for treating the cells: level E1, with an Energy Flux Density (EFD) value of 0.01 mJ/mm^2 and Focus Total Energy (FTE) of 0.12 mJ at -6 dB, corresponding respectively to 83.5 mm of focal length and 8 mm of focal diameter; level E2, with an EFD value of 0.02 mJ/mm^2 and FTE of 0.40 mJ at -6 dB, corresponding to 83 mm of focal length and 8.7 mm of focal diameter. EFD conventionally represents the amount of acoustic energy delivered to, and flowing through, a distinct square area of the focus, perpendicular to the plane of wave propagation, and is defined in mJ/mm^2. The -6dB focus corresponds to the focus area where pressure is greater or equal to half the value of the maximum energy. Moreover, for each energy level (E1-E2), 200 and 800 impulses were applied at a frequency of 3 Hz (Table I).

The SW-device membrane was spread with ultrasound conduction gel, and then multiwells were placed on the membrane using a coupling device. Only the five central wells were seeded with cells, whereas the outer wells contained only the culture medium, to avoid acoustic interference. The plate was positioned on the coupling SW-device membrane, ensuring that all the wells containing cells lay on the membrane. Moreover, in order to avoid the creation of air interface and the risk of cavitation and stirring, a cylinder filled with water was positioned over the plate.

Angiogenic evaluation

After the treatment, plates were incubated at 37°C (in the presence of 5% of CO_2) for 12 h. Then all the samples were observed under an inverted microscope (Leica Microsystems) connected to a charged coupled device camera. The capillary density was evaluated by counting the number of connections formed by the endothelial cells in each microscopy field at 40X of magnification (10 fields were counted for each sample) with the aid of a specific software program (ImageJ, Wayne Rasband, NIH, USA, http://rsb.info.nih.gov/ij/download.html)

Gene expression analysis

The most responsive group in terms of numbers of capillary connections underwent gene expression analysis using the Super Array kit-Signal Transduction Pathway Finder (SABiosciences, Qiagen), able to profile 84 key