EARLY ANGIogenic RESPONSE TO SHOCK WAVES IN A THREE-DIMENSIONAL MODEL OF HUMAN MICROVASCULAR ENDOThelial CELl CULTURE (HMEC-1)

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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 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

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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/mm² 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/mm² 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/mm². 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 1).

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 CO₂) 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
genes representative of 18 different signal transduction pathways.

The cells were harvested, and RNA was extracted using an Array Grade Total RNA Isolation kit (Super Array Bioscience Corp.). RNA purity was evaluated by spectrophotometry, measuring the absorbance rate at 260 and 280 nm. DNA copies (cDNA) were synthesized from the RNA by reverse transcriptase reaction, incubating the mixture at 42°C for 50 mins and at 75°C for 5 mins. Subsequently, biotinylated cDNA was obtained through subsequent reactions and incubation at 37°C for 4 h. The cDNA was then diluted in a suitable buffer and distributed on membranes inside an overnight hybridization oven. Then, after a series of washing cycles, the incubation with avidine-phosphatase alkaline complex and the chromogen produced a chemiluminescent reaction. The resulting patterns were scanned and the images were analysed using densitometry software. The variation in gene expression of the treated cells versus the untreated ones was evaluated normalizing the obtained values on a housekeeping gene expression (glyceraldehyde-phosphate dehydrogenase, GAPDH).

**Statistical analysis**

One-way-ANOVA was performed in order to evaluate the differences in the number of capillary connections between the treated cells and the controls during the *in vitro* angiogenesis, the Dunnnett Test was used as a post-hoc test. The results were expressed as mean±SD. Prism 3.0 software (Graph Pad Software) was used for all statistical analyses. p value <0.05 was considered statistically significant.

**RESULTS**

**Microscopic findings**

The *in vitro* model was developed using a human microvascular endothelial cell line (HMEC-1) cultured in a three-dimensional matrix (BD Matrigel™). HMEC-1 is the first immortalized human microvascular endothelial cell line that retains the morphologic, phenotypic and functional features of normal human microvascular endothelial cells, secreting Von Willebrand factor and forming tubular structures when cultured on solid supports. In particular, when plated on Matrigel, these cells are able to form a three-dimensional network of capillary vessels which is comparable to the structure formed in the final stage of the angiogenic cascade (Kubota et al. 1988).

The cylinder of water placed above the plate ensured that no SW-induced stirring or cavitations were observed that could have affected our results. After 12 hours, the treated cells showed a substantial increase in the number of new vessel-like structures if compared to untreated cells. This morphological differentiation was more remarkable in the samples that were exposed to low energies and limited numbers of shots (Figs. 1 and 2). There was a statistically significant increase in the number of capillary connections (bifurcations) in the cells treated under the E1 condition of lower shock-wave energy (EDF of 0.01 mJ/mm²). Under E1 condition the behaviour of EC was also related to the number of shots applied; indeed, when HMEC-1, plated at a density of 2500 cells/well, received 200 shots, the number of bifurcations was 7.4±1.5 and 2.0±0.4 for treated and untreated cells respectively (p<0.05), whereas, when they received 800 shots the number was not significantly different from the untreated cells (4.1±0.8, p>0.05). Under the same conditions (E1 200 shots) HMEC-1, plated at a density of 5000 cells/well showed 19.1±5, and 10.6±2.1 respectively for treated and untreated cells (p<0.05) and 8.2±5.3 when they received 800 shots. When plated at 10000 cells/well, the cells showed 39.0±1.8 and 22.0±4.4 bifurcations respectively (p<0.05) when they were treated with 200 shots and were left untreated. When cells received 800 shots we observed 37.20±4.0 bifurcations (p<0.05) (Fig. 3).

As reported by other authors (12), we observed a disaggregation of the Matrigel scaffold and a negative effect on the formation of capillary connections at higher energies (data not shown).

**Gene Expression**

In samples showing the most marked increase in number of capillary connections (energy level E1 and 200 shots), we observed a decreased gene expression 3 hours after uSW treatment (Fig. 4). Indeed, cells showed a strong down-regulation of genes involved in the apoptotic process (BAX, anti-apoptotic BCL2LI, GADD45A, PRKCA), also in the cell cycle (CDKN2C, CEBPB, HK2, IRF1, PRKCA), oncogenes (JUN, WNT1), cell adhesion (ICAM-1), and proteolytic systems (CTSD, KLK2, MMP10). Table II illustrates the modulation of the gene expression (fold change) of six genes strongly implicated in the phenomena described. However,
we did not observe any increased expression of receptors for angiogenic agents like endothelial nitric oxide synthase (eNOS) or vascular endothelial growth factor (VEGF).

DISCUSSION

Endothelial cells (ECs) are mechano-sensitive cells which physiologically react to flow shear stress. Particular regions of the cell membrane seem to be involved in the recognition of the different features of the laminar flow (13-15) which are subsequently transferred to the cytoskeleton (16, 17). Those cellular compartments engaged with selective sites of eNOS are thought to mediate the angiogenic (18) and anti-apoptotic effect (19) of the shear stress.
Fig. 3. Count of capillary connections (bifurcations) in HMEC-1 seeded at different cell concentrations, 12 h after low energy uSW treatment (E1, 0.01 mL/mm²) with different number of shots (200 shots, light grey bars; 800 shots, dark grey bars). Untreated HMEC-1 are shown as negative control (white bars). Data are expressed as mean ± SD. Under E1 condition the behaviour of EC was related to the number of shots applied: indeed at all densities, cells receiving 200 shots showed a significant increase in the number of bifurcations in comparison to untreated cells (7.4±1.5 vs 2.0±0.4 at 2500 cells/well; 19.1±5 vs 10.6±2.1 at 5000 cells/well; 39.0±1.8 vs 10000 cells/well; all p<0.05). On the other hand, E1 uSW treatment with 800 shots was able to induce a significant increase in the number of bifurcations just in HMEC-1 cells plated at high density (10000 cells/well) in comparison to untreated cells (37.20±4.0 vs 22.0±4.4, at p=0.05).

In clinical practice, several vascular pathologies are characterized by an intrinsic EC dysfunction and a diminished production of growth factors (GF) (20). Hence, new therapeutic options attempt to correct this sort of “biologic imbalance” by inducing neovascularisation, a process which can be achieved by supplementing the VEGF either via gene therapy or transplanting endothelial progenitor cells (20, 21).

On the other hand, SW stimulation represents an alternative, and innovative, therapeutic approach in those conditions where a strong angiogenic impulse is required – for example in severe skin wounds (4) or in myocardial ischemic lesions (6). Moreover, recent experimental studies suggest that the treatment of ischemic tissue with low energy SWs improves the recruitment of circulating endothelial progenitor cells (EPCs) due to enhanced expression of specific chemo-attractant factors (22).

Although the late neo-angiogenic response has been documented adequately, much less attention has been given to the very early changes induced by mechanical stimulus. Our study was established to investigate the early effects of unfocused shock waves on HMEC-1 cultured in a three-dimensional Matrigel model, where cells were stimulated using a source of low energy unfocused shock waves (soft-focussed) with a pulsation frequency of 3 Hz per second. The experimental Matrigel model, as with any in vitro model, is obviously not a perfect replica of the biological tissues. However, by reproducing the architecture of a mechano-sensitive structure such as the capillary network, it is possible to provide a valid model for analyzing the behaviour of ECs when submitted to an acoustic signal characterized
Fig. 4. Gene expression analysis of HMEC-1 treated at lower energy level (E1, 0.01 mJ/mm²) with 200 shots after 3 h from treatment (a) and of untreated HMEC-1 (b). Downregulated genes are circled (from the top WNT1, CDKN2C, ICAM-1 (double circled), TANK, CEBPB and BAX).

by regular impulse and larger focal extension. The employed multiwaves device generates shock waves in which the larger energy distribution area corresponds to a lower depth of penetration, due to the geometric properties of the paraboloid reflector, which causes the transmission of the acoustic wave front in a large area, nearly parallel (4) and almost laminar.

HMEC-1 are different from the lining EC of large vessels, and are thought to be involved in angiogenesis and in wound healing. We demonstrated that unfocused shock waves induced a quick morphological response (12 hours), characterized by a significant increase of vessel-like structures formation. The difference between treated and not-treated cells was statistically significant at lower shock-wave energy levels (E1, 0.01 mJ/mm² EDF) with a limited number of shots (200), thus demonstrating that the number of shots and cell concentration seem to affect the cell response to uSW.

Although derived from a different experimental model, analogous morphological results have been reported in an in vivo study by Zimpfer et al. (23). They described an increased microvascular density assessed by quantitative histology at 6 and 14 weeks, following a single uSW epicardial stimulation in myocardial infarction in a rat model. Stojadinovic et al. (24) reported an enhanced vascularisation after 4 and 7 days from treatment in a murine model of ischemic skin, and an up-regulation of pro-angiogenic genes after 6 hours. Additionally, Keji et al. (25) reported an increased capillary density in ischemic skeletal muscle 28 days after SW stimulation.

As described above, the pro-angiogenic effects of SW are most likely mediated by VEGF and NO. In our study, we did not observe either VEGF or eNOS modulation 3 hours after SW stimulation, whereas other authors demonstrated the production of VEGF and the increased expression of the specific angiogenesis pathway after 6 hours (24, 26). Instead, we observed a significant down-regulation of genes
Table I. Experimental setting: each condition was tested in a well of 24-multiwell (n=5).

<table>
<thead>
<tr>
<th></th>
<th>5000 cells/well</th>
<th>10000 cells/well</th>
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<tbody>
<tr>
<td>E1 200 shots</td>
<td>E1 200 shots</td>
<td>E1 200 shots</td>
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<tr>
<td>E1 800 shots</td>
<td>E1 800 shots</td>
<td>E1 800 shots</td>
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<tr>
<td>E2 200 shots</td>
<td>E2 200 shots</td>
<td>E2 200 shots</td>
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<tr>
<td>E2 800 shots</td>
<td>E2 800 shots</td>
<td>E2 800 shots</td>
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$E_1 = 0.01 \text{ mJ/mm}^2; \ E_2 = 0.02 \text{ mJ/mm}^2$

Table II. Most relevant gene expression down-regulation (fold change) induced by uSW treatment (energy level $E_1$ and 200 shots) and revealed 3 hours after treatment by gene array.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Bank</th>
<th>Function</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAX</td>
<td>NM_004324</td>
<td>Apoptosis regulation</td>
<td>-8.0</td>
<td>0.042</td>
</tr>
<tr>
<td>CDKN2C</td>
<td>NM_0078626</td>
<td>Cell cycle regulation</td>
<td>-10.0</td>
<td>0.030</td>
</tr>
<tr>
<td>CEBPB</td>
<td>NM_0078626</td>
<td>Transcription regulation</td>
<td>-8.0</td>
<td>0.046</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>NM_00201</td>
<td>Adhesion molecule</td>
<td>-10.0</td>
<td>0.009</td>
</tr>
<tr>
<td>WNT1</td>
<td>NM_005430</td>
<td>Signal transduction</td>
<td>-8.0</td>
<td>0.034</td>
</tr>
<tr>
<td>TANK</td>
<td>NM_004180</td>
<td>Signal transduction</td>
<td>-7.0</td>
<td>0.008</td>
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The gene expression was normalized on the expression of glyceraldehyde-phosphate dehydrogenase (GAPDH).

involved in the apoptotic process (BAX, anti-apoptotic BCL2LI, GADD45A, PRKCA), in cell cycle (CDKN2C, CEBPB, HK2, IRF1, PRKCA), in cell adhesion (ICAM-1), and in proteolytic systems (CTSD, KLK2, MMP10), even if some genes (like PRKCA, IRF-1 and the proteolytic genes) are involved in many other processes and so their specific role in endothelial cells cannot be determined by gene-expression analysis alone. However, this SW-induced modulation, which is more significant for the antiapoptotic genes, could represent the “early reactive response” of HMEC-1 to the physical impulse induced by the uSW. These observations compare favorably with Kim and Von Recum (27) who remarked that mechanical stimulus induced by shear stress could improve the differentiation EPCs in ECs, regulating the expression of several genes involved in apoptosis and inducing EPCs to form capillary-like networks in 3D cultures.

A possible limitation of this study could be the presence of air between the cylindric holes of the well plates which could have partially affected the wave propagation. Another limitation lies in the fact that the central cell-seeded well received the maximum energy whilst the four peripheral ones received a slightly lower energy. What we observed is a sort of ‘average’ effect of the shock waves. However, this replicates the action of shock waves
in vivo, where the areas nearer the focal point receive a higher energy dose than those surrounding them.

In conclusion, our results seem to confirm that some aspects of the early gene response of ECs to uSW stimulation are comparable to those of the laminar shear stress flow, mainly characterized by an anti-apoptotic effect. We observed that, under specific experimental conditions, uSW were able to induce neovascularisation after 12 hours from a single stimulation. After 3 hours, the EC did not show specific pro-angiogenic activity, but just preparatory signals, such as the downregulation of the genes involved in cell cycle and cell adhesion, probably correlated to an upcoming detachment of endothelial junctions.

Further experimental studies are necessary to validate these hypotheses and to investigate whether the biological response of EC to SW stimulation involves the same intercellular pathways and regulatory mechanisms that characterise other types of biophysical stimuli. At the same time, new research into the gene expression could shed light on the triggering of the angiogenic process when acoustic stimulation is applied.

REFERENCES


