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Shockwave Therapy Differentially Stimulates Endothelial Cells: Implications on the Control of Inflammation *via* Toll-Like Receptor 3

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Abstract—Shock wave therapy (SWT) reportedly improves ventricular function in ischemic heart failure. Angiogenesis and inflammation modulatory effects were described. However, the mechanism remains largely unknown. We hypothesized that SWT modulates inflammation *via* toll-like receptor 3 (TLR3) through the release of cytosolic RNA. SWT was applied to human umbilical vein endothelial cells (HUVECs) with 250 impulses, 0.08 mJ/mm^2 and 3 Hz. Gene expression of TLR3, inflammatory genes and signalling molecules was analysed at different time points by real-time polymerase chain reaction. SWT showed activation of HUVECs: enhanced expression of TLR3 and of the transporter protein for nucleic acids cyclophilin B, of pro-inflammatory cytokines cyclophilin A and interleukin-6 and of anti-inflammatory interleukin-10. No changes were found in the expression of vascular endothelial cell adhesion molecule. SWT modulates inflammation *via* the TLR3 pathway. The interaction between interleukin (IL)-6 and IL-10 in TLR3 stimulation can be schematically seen as a three-phase regulation over time.

KEY WORDS: toll-like receptor 3; shock wave therapy; myocardial regeneration; endothelial cells; cytokines.

INTRODUCTION

Inflammatory processes play an important role in post-infarction myocardial remodelling. Adequate repair after loss of a large amount of cardiomyocytes requires a balanced response between inflammatory and regenerative stimuli [1]. Pro-inflammatory response is needed to replace ischemically harmed necrotic tissue. Anti-inflammatory processes are required for limitation of inflammation and initiation of repair. Balanced inflammatory response therefore is prerequisite in myocardial ischemia to enable regeneration and angiogenesis [1].

Shock wave therapy (SWT) has been developed as a standard of care or alternative treatment for a variety of orthopaedic and soft tissue diseases, including ischemic heart disease [2–6]. SWT was described to induce suppression of the pro-inflammatory response in severe cutaneous burn injuries in mice by potently attenuating acute pro-inflammatory cytokine expression and extracellular matrix proteolytic activity at the wound margin [7].

Cardiac shock wave therapy has been repeatedly described to improve left ventricular function in ischemic heart disease [3, 8, 9]. This effect may largely be due to the induction of angiogenesis [10]. In chronic myocardial ischemia in rats, our group showed ameliorated heart function and lower serum levels of BNP after direct

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ABBREVIATIONS: CYP, Cyclophilin; DNA, Deoxyribonucleic acid; dsRNA, Double-stranded ribonucleic acid; HUVEC, Human umbilical vein endothelial cell; IL, Interleukin; PCR, Polymerase chain reaction; Poly(I:C), Polyinosinic:polycytidylic acid; Tie2, Tyrosine kinase with immunoglobulin-like and EGF-like domains 2; TLR3, Toll-like receptor 3; VCAM, Vascular endothelial cell adhesion molecule

epicardial SWT [11]. These beneficial findings could be reproduced in a large animal model in pigs (unpublished data).

However, the mechanism of how the mechanical stimulus of shock waves is translated into a biological response remains unknown [12]. It was suggested that SWT leads to an increase of cell membrane permeability [13]. Thereby, it could cause the release of cytosolic RNA. In the present experiments, we therefore hypothesized that SWT may modulate inflammation *via* stimulation of toll-like receptor 3 (TLR3). TLR3 is part of the innate immune system and involved in the recognition of double-stranded RNA (dsRNA) and fragmented deoxyribonucleic acid (DNA) from viruses [14, 15]. It therefore could be able to detect released cytosolic RNA from neighbouring cells. TLR3 activation is characterized by an early pro-inflammatory phase and a late anti-inflammatory response. This balancing may create the environment for angiogenesis and repair in ischemic tissue [16].

MATERIALS AND METHODS

Cell culture

After obtaining written informed consent of patients, umbilical cords were obtained from Caesarean section at the Department of Gynaecology for isolation of human umbilical vein endothelial cells (HUVECs). Permission was given from the ethics committee of Innsbruck Medical University (no. UN4435). Isolation was performed as described previously [17]. Freshly isolated HUVECs were cultivated in endothelial cell basal medium (CC-3156, Lonza, Walkersville, USA) supplemented with EGM-2 SingleQuots supplements (CC-4176, Lonza). Cells (4×105) were suspended per T25 flask 12 h before treatment. Cells used in these experiments all were in passage 5. Two culture flasks were used for each group. Cells were harvested 2, 4 and 6 h after SWT.

The structural analogue to double-stranded RNA polyinosinic:polycytidylic acid (Poly (I:C) HMW, InvivoGen, San Diego, CA) in a concentration of 200 μg/ml served as a positive control for TLR-3 activation in HUVECs.

Shock Wave Treatment

The electrohydraulic DermaGold® SWT therapy system and the used applicator CG050-P (both TRT LLC, Woodstock, USA produced by MTS Europe GmbH, Konstanz, Germany) were developed for the extracorporeal use of skin lesions. To apply shock waves properly to the cells, the culture flasks were dunked into a water bath. This water bath was built to enable further propagation of shock waves after passing the cell culture as waves would otherwise be reflected at the culture medium to ambient air transition. Reflected waves then would disturb the upcoming ones. In addition, a V-shaped absorber was placed at the back of the bath. The temperature of the water was constantly held at 37 °C using a heater triggered by a temperature sensor.

Referring to our experience in animal models as well as to published data for skin lesions, we used 0.08 mJ/mm^2 energy flux density and applied 25 impulses/cm^2 cell culture flask in a frequency of 3 Hz (pulses per second).

RNA Isolation and PCR

RNA was isolated from homogenized HUVECs using TriReagent solution (Sigma-Aldrich, USA) according to the manufacturer's protocol. cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA). Real-time polymerase chain reaction (PCR) was performed using the StepOnePlus Real-Time PCR (Applied Biosystems, USA) and the following oligonucleotides: huCYPA forward (forw.) GGCCAGGCTCGTGCCG TTTT, reverse (rev.) AAAGGAGACGCGGCCCAAGG; huCYPB forw. AGCTGTCCGGGCTGCTTTCG, rev. CTCATCGGCCGCAGAAGGTCC; huTLR3 forw. ATGCTCCGAAGGGTGGCCC, rev. TGGGACCACCA GGGTTTGCG; huIL-6 forw. ACCCCCAGGAGAAGA TTCCA, rev. CAATTGCTTCTGAAGAGGTGAGT; huIL-10 forw. GAGGCTACGGCGCTGTCAT, rev. CCAGAGCCCCAGATCCGA; huVCAM-1 forw. GCGAGGGTCTACCAGCTCCA, rev. ATCCGGGGT CCAGGGGAGAT; and hu Tie-2 forw. CCAGCCCTGCT GATACCAAA, rev. ATGTGCATGAGGTCCCAAGG. Briefly, after a denaturation step at 95 °C for 10 min, the cycling started. Annealing was performed at 60 °C for 10 s, followed by a synthesis step at 72 °C for 25 s. SYBR Green fluorescence was detected at 78 °C. After 40 cycles, the experiment was finished by running a melting curve with an augmentation of 0.3 to 95 °C followed by fluorescence detection at the end of each augmentation step. The melting curve was used to determine the specificity of the primer pairs [18]. PCRs were performed in duplicate.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism® 5.02 software (GraphPad Software, Inc., San Diego, CA). Results are expressed as means ± standard

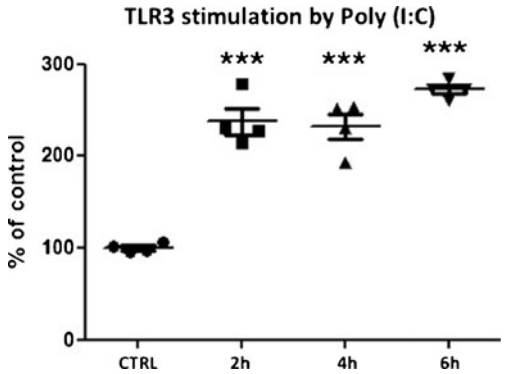

Fig. 1. TLR3 agonist Poly(I:C) stimulates TLR3 on HUVECs. A concentration of 200 μg ml culture medium of Poly(I:C) was used to asses time-dependent stimulation of TLR3 showing an early and highly significant effect after 2 h. This confirms that TLR3 on endothelial cells can be activated by double-stranded RNA and serves as a positive control to shock wave stimulation in the treatment groups. ***$p<0.001$.

error of the mean. Controls were set to 100, and treatment groups are given as percent of control. Statistical significance was calculated using one-way ANOVA followed by appropriate post hoc tests to confirm significance. Statistical significance was set to $p<0.05$.

RESULTS

TLR3 Agonist Poly(I:C) Stimulates TLR3 on HUVECs

Polyinosinic:polycytidylic acid (Poly (I:C)) serves as a synthetic, structural analogue to dsRNA. A concentration of 200 μg ml culture medium was used to asses time-dependent stimulation of TLR3 showing an early and highly significant effect after 2 h lasting for 6 h (agonist group 237.7±14.1 (2 h); 231.9±14.1 (4 h); 272.4 ±4.7 (6 h) *vs.* control, $p<0.001$) (Fig. 1). This confirms that TLR3 on endothelial cells can be activated by dsRNA and represents a positive control to shock wave stimulation for this experiment.

Cellular Uptake of Nucleic Acids and TLR3 Stimulation After SWT

Cyclophilin B (CYP B) is responsible for the uptake of nucleic acids into cells. In the cytosol, it can bind to the TLR3 receptors, which are located on endosomes. Treated cells showed an immediate up-regulation of CYP B after SWT (SWT 597.39±59.84 (2 h), $p<0.001$; 527.84±68.16 (4 h), $p<0.001$; 424.73±67.13 (6 h), $p<0.01$ *vs.* control) (Fig. 2a). An increased amount of the transporter protein CYP B is necessary to accomplish the cellular uptake of nucleic acids. CYP B expression decreases again as indicated after 6 h representing the depletion of the uptake process.

In line with CYP B up-regulation, the expression of TLR3 mRNA was found to be significantly increased after SWT. As TLR3 up-regulates its expression by an auto-loop, after 6 h, the difference between untreated controls and therapy group was even more significant (SWT 123.78± 6.56 (2 h), $p>0.05$; 165.68±10.61 (4 h), $p<0.05$; 328.15± 19.33 (6 h), $p<0.001$ *vs.* control) (Fig. 2b).

Initiation Phase: Pro-Inflammatory Response Mediated by Cyclophilin A and Interleukin 6

The TLR3 pathway is characterized by an early pro-inflammatory response mainly of interleukin 6 (initiation phase). It is mediated by cyclophilin A (CYP A), which further promotes the production of the pro-inflammatory cytokine interleukin 6 [19]. Interleukin (IL)-6 itself serves

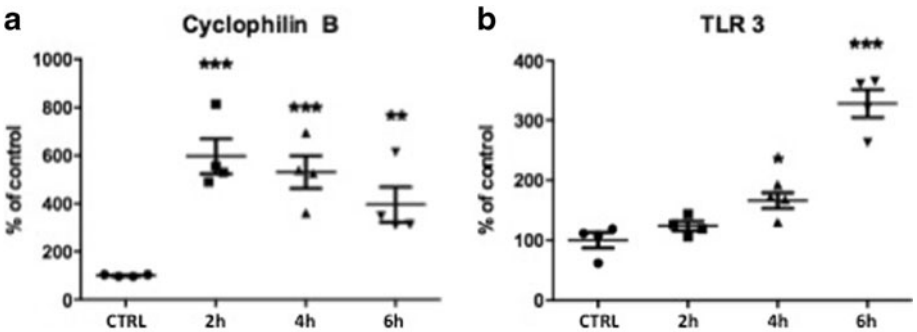

Fig. 2. Cellular uptake of nucleic acids and TLR3 stimulation after SWT. **a** Cyclophilin B shows an early response to SWT that indicates nucleic acid uptake to the cells. **b** In line with an increase of cyclophilin B expression, TLR3 increases over time reaching its peak after 6 h. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

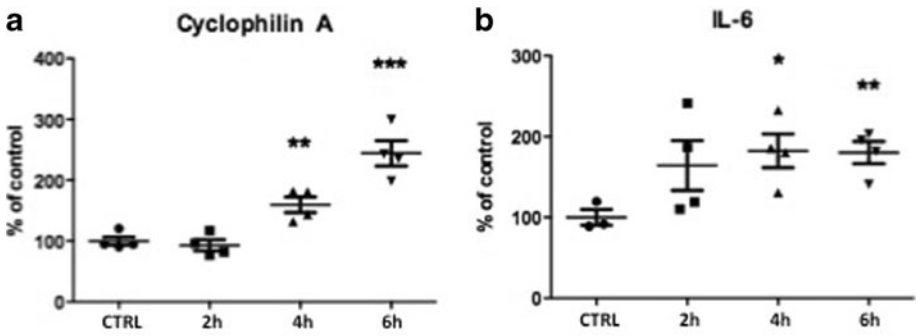

Fig. 3. Initiation phase: early pro-inflammatory response. **a** Cyclophilin A that serves as a mediator for IL-6 is steadily increasing after SWT thereby further promoting the already increased IL-6 expression. **b** The initiation phase is marked by the pro-inflammatory cytokine IL-6 that shows an early increase directly after SWT. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

as a chemoattractant to monocytes. Thereby, they get directed to the site of inflammation. In our experiment, we could find an up-regulation of CYP A (SWT 92.98±7.44 (2 h), $p>0.05$; 159.75±10.43 (4 h), $p<0.01$; 244.35±17.05 (6 h), $p<0.001$ *vs.* control) as well as of IL-6 (SWT 164.3± 25.19 (2 h), $p>0.05$; 182.2±17.06 (4 h), $p<0.05$; 180.23± 11.4 (6 h), $p<0.01$ *vs.* control) after SWT indicating the activation of an early pro-inflammatory response of the TLR3 pathway in the initiation phase (Fig. 3a, b).

Middle Phase: Suppression of Inflammation

Vascular cell adhesion molecule (VCAM) is a surface protein responsible for the mediation of leucocyte adhesion and is therefore an indicator for prolonged inflammation [20]. Although pro-inflammatory cytokine IL-6 is increased, VCAM is not up-regulated in treated cells compared to untreated controls (SWT 119.31±17.23 (2 h), $p>0.05$; 102.63±7.17 (4 h), $p>0.05$; 111.78±3.33 (6 h), $p>0.05$ *vs.* control) (Fig. 4a). This fact indicates that IL-6 may not cause inflammation in treated tissue after SWT. We therefore hypothesize that it rather serves as a chemoattractant to monocytes. Thereby, it reveals the modulation of TLR3-mediated inflammatory response that results in a middle phase with beginning suppression of inflammation.

Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie2) represents a protein, which is expressed on endothelial cells only. Up-regulated Tie2 mRNA shows significantly enhanced proliferation in endothelial cells after SWT (SWT 154.25±16.39 (2 h), $p<0.05$; 137.23±8.52 (4 h), $p<0.05$; 166.68±2.15 (6 h), $p<0.01$ *vs.* control) (Fig. 4b). This finding shows that endothelial cells are in a physiologic condition and it therefore further supports the hypothesis of a balanced inflammatory response. It is in line with the nonsignifi-

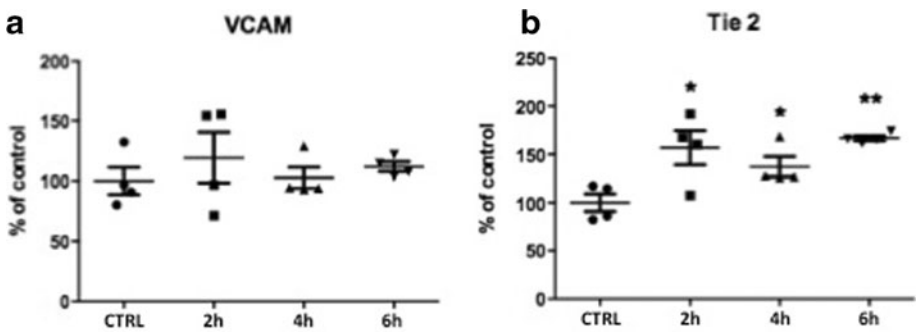

Fig. 4. Middle phase: suppression of inflammation. **a** No up-regulation of VCAM could be observed. This indicates no prolonged inflammation, but IL-6 in the initiation phase being rather up-regulated for monocyte recruitment than tissue inflammation. **b** Up-regulated Tie2 mRNA indicates enhanced proliferation in treated endothelial cells compared to untreated controls. The up-regulation of Tie2 being higher at later time points clearly marks the middle phase of suppression of the inflammatory response. *$p<0.05$, **$p<0.01$.

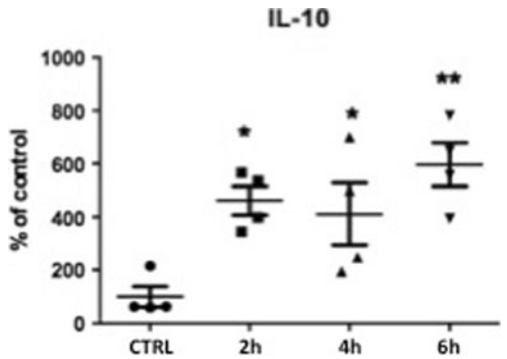

Fig. 5. Limitation phase: late anti-inflammatory effect. TLR3 response is characterized by the late production of IL-10 marking an anti-inflammatory limitation phase and thereby creating an environment for regeneration and repair. *$p<0.05$, **$p<0.01$.

cant expression of VCAM at all time points. Moreover, the suppression of inflammation may be indicated by Tie2 expression being slightly higher at later time points.

Limitation Phase: Late Anti-Inflammatory Effect Mediated by IL-10

TLR3 response is characterized by the late production of IL-10 marking an anti-inflammatory limitation phase. The expression of anti-inflammatory cytokine IL-10 was significantly enhanced after SWT (SWT 460.9±43.72 (2 h), $p<0.05$; 410.83±95.55 (4 h), $p<0.05$; 595.88±66.66 (6 h), $p<0.01$ *vs.* control) (Fig. 5). It seems to be responsible for limitation of the inflammatory regulation thereby creating the environment for tissue repair.

DISCUSSION

Low-energy shock wave treatment is well known to induce tissue regeneration and angiogenesis in ischemic myocardium. It has been proven in numerous animal models as well as in human pilot trials [4, 8–11]. Nevertheless, the underlying mechanism remains largely unknown. Modulation of inflammation is prerequisite for regeneration and angiogenesis as shown in a burn injury model in mice in which SWT potently attenuates cytokine expression at the wound margin [7].

In the present *in vitro* experiments, we hypothesized that SWT may modulate inflammation *via* stimulation of TLR3. TLR3 is part of the innate immune system and involved in the recognition of dsRNA and fragmented DNA from viruses [14, 15]. TLR3 activation is characterized by an early pro-inflammatory and a late anti-inflammatory response. This balancing creates an environment for repair and angiogenesis in ischemic tissue [16].

We first proved that TLR3 activation on endothelial cells is possible by using the TLR3 agonist polyinosinic:polycytidylic acid (Poly(I:C)). Then, we exposed the cells to low-energy SWT and performed analysis of the main inflammatory cytokines. Thereby, we show that the complex interaction between the two main cytokines IL-6 and IL-10 after TLR3 stimulation can be schematically seen as a three-phase regulation over time (Fig. 6). The different phases are of course overlapping. After an early pro-inflammatory initiation phase mediated by IL-6, a middle phase with beginning suppression of inflammation can be seen. It finally results in a late anti-inflammatory limitation phase of IL-10.

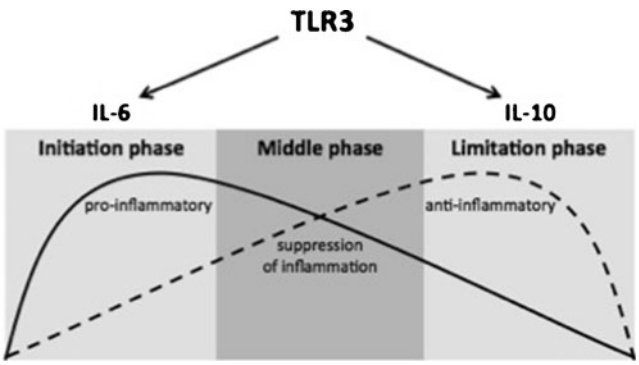

Fig. 6. TLR3 stimulation leads to three phases of inflammatory modulation. The complex interaction between the two main cytokines IL-6 and IL-10 in TLR3 stimulation can be schematically seen as a three-phase regulation over time. After an early pro-inflammatory initiation phase mediated by IL-6, a middle phase showing suppression of inflammation can be seen before the late anti-inflammatory limitation phase of IL-10 results. This modulation of the inflammatory response is prerequisite for angiogenesis and repair in ischemic tissue.

Preclinical studies show beneficial effects of anti-inflammatory treatment after myocardial infarction by decreasing the infarct size-to-area-at-risk ratio [21, 22]. However, these studies remain experimental as none of them have been translated into clinic. Therefore, a safe treatment option that modulates the inflammatory response after myocardial infarction is of high therapeutic interest.

The results of our present study suggest that the tissue regenerative effect of shock wave therapy is at least in part mediated by TLR3 stimulation.

Nevertheless, further experiments are needed and a trial with TLR3 knockout mice is on its way to reproduce our current findings *in vivo* and prove the hypothesis.

In conclusion, we for the first time show that the effects of myocardial regeneration by low-energy shock wave treatment are at least in part by creating an environment for regeneration and angiogenesis through modulating inflammation *via* toll-like receptor 3 stimulation.

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Conflicts of interest. None

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