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Original Paper

Extracorporal Shock Waves Activate Migration, Proliferation and Inflammatory Pathways in Fibroblasts and Keratinocytes, and Improve Wound Healing in an Open-Label, Single-Arm Study in Patients with Therapy-Refractory Chronic Leg Ulcers

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Key Words
Ulcer • Wound healing • Extracorporal shock wave therapy • ESWT • Keratinocyte • Fibroblast • Cell cycle • Laminin • Skin

Abstract
Background/Aims: Chronic leg ulcers (CLUs) are globally a major cause of morbidity and mortality with increasing prevalence. Their treatment is highly challenging, and many conservative, surgical or advanced therapies have been suggested, but with little overall efficacy. Since the 1980s extracorporal shock wave therapy (ESWT) has gained interest as treatment for specific indications. Here, we report that patients with CLU showed wound healing after ESWT and investigated the underlying molecular mechanisms. ***Methods:*** We performed cell proliferation and migration assays, FACS- and Western blot analyses, RT-PCR, and Affymetrix gene expression analyses on human keratinocytes and fibroblasts, and a tube formation assay on human microvascular endothelial cells to assess the impact of shock waves *in vitro*. *In vivo*, chronic therapy-refractory leg ulcers were treated with ESWT, and wound healing was assessed. ***Results:*** Upon ESWT, we observed morphological changes and increased cell migration of keratinocytes. Cell-cycle regulatory genes were upregulated, and proliferation induced in fibroblasts. This was accompanied by secretion of pro-inflammatory cytokines from keratinocytes, which are known to drive wound healing, and a pro-angiogenic activity of endothelial cells. These observations were transferred “from bench to bedside”, and 60 consecutive patients with 75 CLUs with different pathophysiologies (e.g. venous, mixed arterial-venous, arterial) were treated with ESWT. In this setting, 41% of ESWT-treated CLUs showed complete healing, 16% significant improvement, 35% improvement, and 8% of the ulcers did not respond to ESWT. The induction of healing was independent of patient age, duration or size of the ulcer, and the underlying pathophysiology. ***Conclusions:*** The efficacy

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of ESWT needs to be confirmed in controlled trials to implement ESWT as an adjunct to standard therapy or as a stand-alone treatment. Our results suggest that EWST may advance the treatment of chronic, therapy-refractory ulcers.

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Introduction

Chronic leg ulcers (CLUs) are globally a major cause of morbidity and mortality with increasing prevalence. General factors contributing to the incidence of CLUs are the ageing population and obesity [1, 2]. Moreover, CLUs can be caused by a variety of diseases including diabetes mellitus, infections, peripheral neuropathy, immobility and pressure, rheumatologic diseases, atherosclerosis, and venous dysfunction. Non-healing CLUs are likely a result of multiple factors and comorbid conditions. However, venous disease accounts for the majority of CLUs. Venous hypertension can induce damage of vessel walls and the development of edema, eventually leading to skin breakdown [3]. On the cellular level, possible causes of venous ulcers include inflammatory processes resulting in disturbed microcirculation, leukocyte activation, dysregulated epidermal and dermal cells, endothelial damage, platelet aggregation, and endocrine mechanisms leading to wound formation with a high rate of recurrence [4-6]. CLUs negatively affect the quality of life and productivity of the patients [7-9]. Venous CLUs affect between 500,000 and 2 million persons annually in the USA and account for over 50% of leg ulcers [10, 11]. The current standard therapy for venous CLUs includes compression of the lower limbs, debridement, infection control and local ulcer care with various wound dressings, which heals 50–60% of venous leg ulcers [12]. Venous ulcers are cared for by a variety of medical practitioners including internists, dermatologists, family practitioners, surgeons, certified wound nurses, and physical therapists using different interventions [13]. Thus, the public health burden and direct costs for care of CLUs including their complications amount to approximately 25 billion dollars per year in the US [14].

As a high percentage of CLUs do not adequately heal and/or quickly relapse with standard treatments, additional therapeutic modalities are used, termed "advanced wound care therapies." However, clinical evidence of efficacy of most of the commonly applied therapies and advanced wound care therapies in terms of induction of healing or shortening the healing time is limited. In fact, there is little to no evidence that any given topical agent or wound dressing positively affects the healing of (venous) CLUs [15]. Considering (i) the high number of topical (and systemic) therapies routinely applied by medical practitioners throughout the world mostly lacking evidence in the healing of CLUs, (ii) the tremendous financial burden to the public health systems, and (iii), most importantly, the amount of suffering imposed on the millions of chronically afflicted patients world-wide by a delayed healing, novel therapeutic approaches are desperately needed for the treatment of chronic leg ulcers independent of the underlying pathophysiology.

During the past years, the application of extracorporal shock wave therapy (ESWT) has been steadily increased. ESWs are defined as a sequence of acoustic pulses characterized by ultrafast pressure rise within several nanoseconds, a high peak pressure (100 MPa), and short duration of several microseconds. The greater the difference in impedance the more energy is released in form of mechanical energy. ESWs are conveyed to a specific target area by an appropriate generator with an energy density in the range of 0-3 mJ/mm^2. ESWT employs medical high-energy focused shockwaves such as generated by the electro-hydraulic CellSonic® Medical machine. Some evidence suggests that ESWT might play a role as advanced wound care in therapy-resistant CLUs [16-18]. However, the biological mode of action in the induction of wound healing by ESWT on a molecular level remains largely unknown.

In the present study, we first analyzed the effects of SWT (CellSonic®) on primary human fibroblasts, keratinocytes, and microvascular endothelial cells. We hypothesized that all three cell types are individually influenced by SWT. In particular, we aimed to analyze morphological changes, cellular motility, cell cycle regulation and resulting early differences

in gene- and protein expression 24 hours after SW treatment of the cells. Since our *in vitro* analyses clearly demonstrated a comprehensive induction of wound healing on the molecular and the morphological levels by SWT in all three cell types analyzed, in a second step we went "from bench to bedside". Applying the same treatment modalities as for the *in vitro* experiments 60 patients with 75 CLUs with various underlying diseases (e.g. venous, mixed arterial-venous, arterial, pressure) were treated with ESWT (CellSonic®) during ambulant routine visits in the hospital. In summary, 41% of ESWT-treated CLUs showed complete healing (100% size reduction), 16% significant improvement (>75% size reduction), 35% improvement (20-75% wound size reduction), while 8% of the ulcers did not improve under ESWT (<20% wound size reduction). The induction of healing by ESWT was independent of patient age, duration of the ulcer, size of the ulcer, and underling pathophysiology. Our results strongly encourage the routine application of ESWT in the treatment of chronic ulcers, independent of their pathophysiology.

Materials and Methods

Cell culture and application of shock waves in vitro

The use of human skin tissues (source: human foreskin from routine circumcision operations) in this study was approved by the medical ethical committee of the University of Tuebingen and was performed in accordance with the Declaration of Helsinki principles. Patients gave their written informed consent. HaCaT keratinocytes (immortalized human skin keratinocyte cell line) and primary human fibroblasts (isolated from human foreskin) were cultivated in Dulbecco's modified Eagle medium (DMEM) or RPMI-1640 (Lonza; PAA), supplemented with 10% fetal calf serum (Biochrom AG) and 1% Penicillin/Streptomycin. Isolation and cultivation of primary human keratinocytes from human foreskin was performed as described before [19]. Primary keratinocytes and primary fibroblasts from different donors were used up to passage 4. The human dermal microvascular endothelial cell line CDC/EU-HMEC (HMEC) was kindly provided by Prof. Günter Eissner, University College Dublin, and was originally provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA). HMEC were cultured in MCDB131 medium, supplemented with 15% fetal calf serum, 1 µg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (EGF; Sigma) and antibiotics. All cell cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere at 100% humidity. Digital images were taken with the light microscope (Olympus CK40) with 4X objective and analyzed with TS View Digital Imaging Software.

The shock wave applications were performed as follows: 6-well-plates containing the respective cells were removed from the incubator and placed directly onto the ultrasound gel-covered (Aquasonic® 100, Parker) shock head, which pas positioned between the knees of the person performing the experiment (IA and/or CB). Since the surface of the shock head was almost the size of a single 6-well (approximately 7.5 cm^2), it was very easy and reproducible to shock one 6-well at a time. Directly after application of the shocks (for detailed technical conditions see below), the 6-well dishes were replaced into the incubator or further processed for the experiments described in the following paragraphs.

Tube Formation Assay

HMEC cells were grown to confluence and seeded the next day onto 6-well dishes, which were then SW-treated using the CellSonic® device (0, 375, 750, and 1500 shocks per well at energy level 4 (Energy Flux Density of 0.136 mJ/mm^2), corresponding to 0, 50, 100, and 200 impulses per cm^2). Subsequently, HMEC cells were detached counted and prepared for the tube formation assay. The ability of shocked HMEC cells to form endothelial-like tube structures on Matrigel® (BD) in serum-free media was assessed using a µ-slide Angiogenesis Assay (Ibidi) according to the manufacturers' instructions. One image per well was taken after 1, 2, 4, and 5 h, imaged by the Olympus CK40 microscope and tube length was analyzed with Image J.

Proliferation/ Vitality assay

For analysis of proliferation of HaCaT keratinocytes and fibroblasts, 2.5-5x10^3 SW-treated cells (0, 375, 750, and 1500 shocks at energy level 4) were seeded into 96-well plates and cultured for either 24 or 48 h. After incubation periods cell supernatant was removed, washed and replaced with 4-methylumbelliferyl

heptanoate in PBS (100 µg/ml) for 1 h at 37°C. Microplates were measured in a fluorescence microplate reader (Berthold, Germany) with Ex355/Em460 nm. Metabolic activity of cells was compared at 24 h and 48 h post treatment relative to untreated cells, and calculated as proliferation of cells (%).

Wound Scratch Assay

For wound scratch assays, HaCaT cells and primary fibroblast cells were seeded onto 6-well tissue dishes in DMEM supplemented with 10% FCS and cultivated till they nearly reached confluence (~80%). SWT was applied (0, 375, 750, 1500 shocks per well at energy level 4) and the cells were cultivated for another 18-24 h. Afterwards scratch wounds were created in confluent monolayers by using a sterile 0.1–10 µl pipette tip. We chose to apply SWT before setting the scratch wounds, and not vice versa, since SWT led to detachment of the cells with subsequent random re-attachment, which would have made a correct measurement of wound closure technically impossible, if the cells had been shocked after generating the scratch wounds. The time of scratching of the wound was designated as time 0 h. Migration of cells into the wound was recorded 24 h post scratch under a phase contrast microscope with a 5 x phase objective at 37°C and 5% CO_2. For analysis, the same fields of the wound margin were photographed and interspaces were measured using the using the Axiovision software (Carl Zeiss, Oberkochen, Germany).

RNA extraction and RT-PCR

Shocked or control fibroblasts / HaCaT keratinocytes (treated as above) were harvested and total RNA was isolated with the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The quality and quantity of RNA were assessed using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE). Integrity of RNA samples was assessed by using Agilent 2100 Bioanalyzer (Santa Clara, CA).

1-1.5 µg RNA was reverse-transcribed using random hexamer primers. cDNA corresponding to 25 ng RNA served as a template for the following real-time PCR. A quantitative real-time-PCR (q RT-PCR) analysis was used to analyze the expression of CDK1, CCNA2, CCNB1, CCNB2, 18S rRNA, LAMA3, Vimentin, Vinculin (for all online suppl. material, see www.karger.com/doi/10.1159/000460503, for primer sequences see Table S2). Gene expression was analyzed using KAPA SYBR FAST LightCycler 480 (Peqlab, Erlangen, Germany). All experiments were performed in duplicate and repeated with at least three independent biological replicates. Quantification was determined by using the comparative 2-ddCT-method using 18S rRNA as a reference gene.

Flow cytometric cell cycle analysis (FACS)

SW-treated cells (as above) were harvested 24 h after treatment, washed with cold PBS, fixed with 70% ethanol and incubated at 4°C overnight. Cells were then centrifuged and washed twice in cold PBS. Intracellular DNA was labelled with propidium iodide solution [final concentration propidium iodide 50 µg/ml (Sigma-Aldrich, St. Louis, Missouri, USA) and RNase 100 µg/ml (Thermo Scientific) in PBS] and incubated at 4°C for at least 20 min in the dark. Cell cycle was analyzed by using 100,000 cells per treatment group for flow cytometry and FACSDiva software (BD Biosciences, Heidelberg, Germany).

Transcriptomics

For each sample, 250 ng of total RNA was processed using the Affymetrix Gene Chip Whole Transcript Sense Target Labeling Assay. The analysis was performed by the core facility of the European Molecular Biology Laboratory (EMBL) at Heidelberg, Germany.

Western blotting

SW-treated or untreated fibroblasts and keratinocytes were harvested. Cells were next washed with PBS and lysed for 20 min on ice with lysis buffer (10 mM Tris- HCl pH7.4, 200 mM NaCl, 1 mM EDTA, 10% Triton). Cell lysates were cleared by centrifugation for 5 min at 13.000 g at 4°C and 20-30 µg protein subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes. After blocking in PBS/0.1% Tween-20 /5% dry milk for 1 h at RT, the blots were incubated overnight with the primary antibodies directed against anti-vimentin (1:1000; Santa Cruz Biotechnology), anti-vinculin (1:2000; Sigma-Aldrich), anti-fibronectin (1:250; Santa Cruz Biotechnology), anti-GAPDH (1:1000; Cell Signaling Technology), and anti-laminin α3 (1:3000; kindly provided by Patricia Rousselle; University of Lyon) in PBS/0.1%

Tween-20/5% dry milk, washed with PBS and incubated with secondary HRP-conjugated antibody (Cell Signaling Technology). After washing with PBS for 3 x 10 min, the ECLplus detection reagent (GE Healthcare, Munich, Germany) was used for detection and exposure to X-ray film (Eastman Kodak, Rochester, NY, USA).

Application of ESWT (CellSonic®) on patients with chronic leg ulcers

The CellSonic® medical devise has a CE certificate and is approved for the treatment of patients. For clinical application, 75 consecutive patients with chronic therapy-refractory leg ulcers treated at the ulcer clinic (Department of Dermatology and Allergology, University Medical Hospital, Tuebingen, Germany) were offered ESWT (CellSonic®) as part of routine clinical care during their ambulant visits. All patients were thoroughly instructed and gave written informed consent after the nature and possible consequences of the ESWT were explained. One of the 75 patients treated with ESWT was a minor. In this particular case, written informed consent was obtained both from the patients` mother and from the patient himself. The corresponding author (CB) was both the prescribing and the treating physician (ESWT and ulcer care in general). Since the treating physician (CB) collected the clinical data from ulcer patients, these primary data were not anonymized. However, for retrospective analysis and depiction of the results (Table 2) the data were anonymized by CB. Thus, the other authors had neither contact to the ulcer patients nor any personal information thereof. ESWT was performed as follows: After removal of the wound dressing and cleansing of the wound and its edges using Octenisept® (Schuelke), ultrasound gel (Aquasonic® 100, Parker) was directly applied onto the entire wound, and the wound was closed using sterile Opsite® foil (Smith&Nephew, London, UK), which was also covered with ultrasound gel. The disinfected silicone membrane of the CellSonic® ESWT application device (with a surface of 7.5 cm^2, corresponding to the surface of a 6-well plate) was then applied onto the wound, and 100 impulses per cm^2 (corresponding to the application of 750 impulses onto the cells as described above) + 200 impulses at energy level 4 (Energy Flux Density of 0.136 mJ/mm^2) were homogenously applied on the entire surface of the wound and its edges at a frequency of 4 impulses per second (compare Fig. 5A). In cases of wounds exceeding 20 cm^2, a maximum number of 2200 impulses were homogenously distributed on the wound and its edges. ESWT was usually performed 4 times once every 3-4 weeks, or less. After ESWT, the Opsite® foil was removed, and surgical debridement was performed if necessary, followed by standard wound care (stage-specific (antiseptic) dressing, protection of wound edges, compression bandage or stocking, regular manual lymph drainage, etc., according to the underlying pathophysiology). At each visit, the wound size was measured, and the wound was photo-documented. Two dimensions of the wounds were usually measured (largest length and width); in case of deep wounds, the depth was also additionally measured. In case of large wounds, during the course of the healing phase, sometimes a wound split up into two or three different, smaller wounds that subsequently individually healed. In such cases, all different aspects (length, width, depth, combined wound surface) was taken into consideration. Since a high percentage of the shock wave treated wounds showed an impressive healing, the classification of the healing for statistical analysis (no change (<20% size reduction), improvement (20-75% size reduction), significant improvement (>75% size reduction), complete healing (100% size reduction)) was easy to determine and reproducible.

Statistics

Quantitative RT-PCR data were analyzed using the Kruskal-Wallis Test (GraphPad Prism 6.00, GraphPad Software, San Diego, CA). Differences were considered statistically significant with $p<0.05$. For flow cytometry and wound closure the unpaired two-tailed Mann-Whitney test was performed to determine statistical significance in treated and control cells (GraphPad Prism version 4.03 for Windows; $p<0.05$). Patient data were evaluated by using the T-Test; differences were considered statistically significant with $p<0.05$.

Results

Shock waves influence the cellular morphology of human fibroblasts, keratinocytes and dermal microvascular endothelial cells

Mechanical forces can influence the orientation and the spatial distribution of fibers [20], and structural reorganization of cells in normal skin. The effect of SWT on morphology

of dermal fibroblasts and primary keratinocytes has not been studied yet in detail. Therefore, we microscopically visualized epidermal and dermal cells. SWT (CellSonic®) was applied after cells reached approximately 80% confluence. Overall, SWT negatively influenced cellular viability only to a minor extent and produced only marginal cell debris (data not shown). Pictures were taken 24 h after application of 0 (untreated), 375, 750 or 1500 shocks at energy level 4 (Fig. 1). Primary fibroblasts immediately detached upon SWT from the plastic surface and re-organized after 24 h to attach comparably to untreated cells. In detail, fibroblasts re-organized in a radial and star-shaped clustered manner and had large "stress" fibers. Cluster formation of fibroblasts was dose dependent (Fig. 1A).

Primary human keratinocytes also detached directly upon SWT, however, after 24 h the cells had attached to confluence again, even to a larger extent when compared to untreated cells (Fig. 1B). Since HaCaT cells (immortalized keratinocytes) are more homogenous and are easier to cultivate than primary human keratinocytes, we decided to use HaCaT cells in further experiments.

To examine the effect of SWT on angiogenesis, we measured capillary formation of human dermal microvascular endothelial cell (HMEC) in Matrigel -coated chambers (Fig. 1C). Capillary network formation increased upon SWT over time (0-5 h), measured as tube area in % compared to untreated cells and was impulse-independent; the lowest impulse rate applied (375) was already sufficient to increase the number of capillary structures after approximately 2 h (Fig. 1C). Capillary formation is depicted as microscopic images from the endpoint measurement at 5 h after SWT (Fig. 1D).

Shock waves activate cell migration in fibroblasts and keratinocytes

To investigate the suggested impact of ESWT on wound healing, we now focused on human primary fibroblasts and HaCaT keratinocytes as the two major cell types present in the dermal-epidermal layer. To analyze cellular motility, cell migration was monitored in a wound closure experiment 24 h after application of SWT in a dose-dependent manner (Figs. 2A and B). Both primary human fibroblasts and keratinocytes showed increased wound closure upon SWT. The highest shock rate of 1500 significantly increased migration of cells displayed as wound closure in % for both cell types tested.

To analyze if the increased wound closure of fibroblasts and keratinocytes 24 h after SWT was in part due to augmented proliferation, a proliferation assay was conducted 24 h after SWT (Figs. 2C and D). We found no increase in cellular proliferation between the conditions tested after 24 h in both cell types. In detail, HaCaT cells even showed a slight decrease of proliferation (Fig. 2D), which however was not significant. These results indicate that the observed increased wound closure 24 h upon SWT was exclusively caused by an enhanced migratory capacity.

Shock waves induce expression of cell cycle regulatory genes and proteins

To investigate how SWT affects gene expression, we used the Affymetrix GeneChip® Human Gene 2.0 ST Array to compare the transcriptomes of primary fibroblasts and HaCaT keratinocytes with and without SWT at a single dose of 750 impulses. To analyze differentially expressed genes (DEG), filtering was performed in gene sets with absolute fold changes ≥ 1.5. Filtering produced a list of 67 genes for keratinocytes and 652 genes for fibroblasts differentially regulated compared to the untreated control cells (see supplementary material, Table S1), suggesting that the response to SWT was more intense in fibroblasts. Most notably, the majority of the DEG were up-regulated in both cell types. To identify functional groups enriched by the DEG, they were entered into the Database for Annotation, Visualization and Integrated Discovery (DAVID). DAVID-based functional analysis of fibroblast-derived genes showed significant enrichment for the Biological Process (BP) terms – mitotic cell cycle (32%), DNA repair (22%), meiotic cell cycle (17%), cytoskeleton organization (13%), chromatin organization, DNA repair and RNA splicing, indicating considerable rewiring in core cellular processes responsible for genome stability (Fig. 3A). Functional analysis of keratinocyte genes revealed significant relevance of immune response (36%), stress

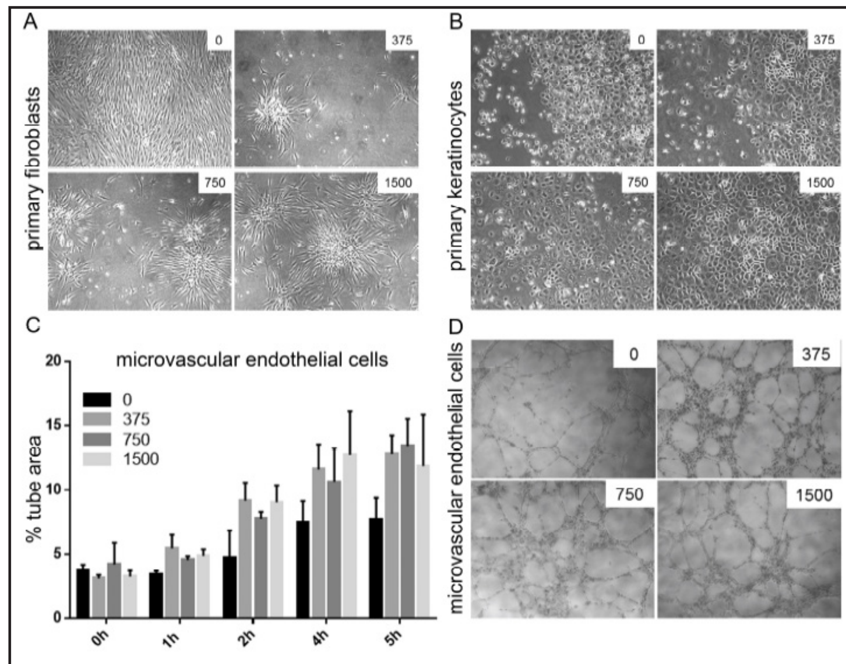

Fig. 1. Shock waves influence cellular morphology of human fibroblasts, keratinocytes and dermal microvascular endothelial cells. Shock waves (0, 375, 750 or 1500 shocks) were applied to fibroblasts or keratinocytes, pictures were taken after 24 h. Fibroblasts detached upon SWT from the plastic surface and re-organized after 24 h in a dose-dependent radial and star-shaped clustered manner (A). Primary human keratinocytes detached directly upon SWT. After 24 h the cells had a more epithelial, cobblestone-like morphology compared to untreated cells (B). Capillary formation of human dermal microvascular endothelial cells, assessed in Matrigel-coated chambers. Shock waves increased capillary network formation over time (0-5 h), measured as tube area in % compared to untreated cells (C). Capillary formation depicted as microscopic images from the endpoint measurement at 5 h after application of shock waves (0, 375, 750 or 1500 shocks (D)).

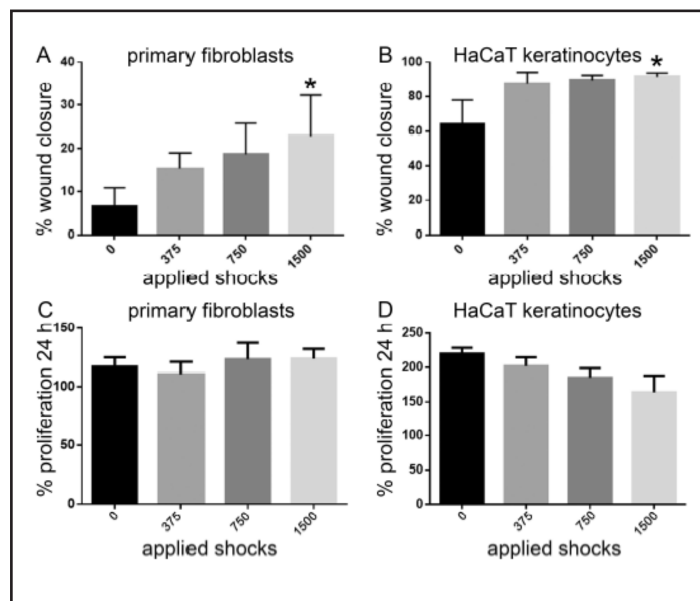

Fig. 2. Shock waves activate cell migration in fibroblasts and keratinocytes. Fibroblasts (A) and HaCaT keratinocytes (B) were used to analyze the impact of shock waves on cell migration 24 h after application. Fibroblasts and keratinocytes showed increased wound closure upon exposure to shock waves. 1500 applied shocks significantly increased migration of cells displayed as wound closure in % for both cell types tested. Proliferation assay of fibroblasts (C) and HaCaT keratinocytes (D) 24 h after exposure to shock waves. No change in proliferation was observed after 24 h in both cell types. HaCaT cells showed a slightly reduced proliferation. *$p<0.05$.

response (28%), and response to wounding (15%) (Fig. 3B). Selected genes differentially regulated and significantly enriched in the functional class of mitotic cell cycle (fibroblasts) or immune response (keratinocytes) are depicted in Table 1.

Whereas after 24 h no change in the proliferation of fibroblasts was detected (Fig. 2C) the upregulated cell cycle genes in fibroblasts upon SWT corroborated with an increase of proliferation after 72 h (Fig. 3C). Hence, we further analyzed the role of the cell cycle in SW-treated fibroblasts by flow cytometric cell cycle analysis (Fig. 3D). In primary fibroblasts SWT

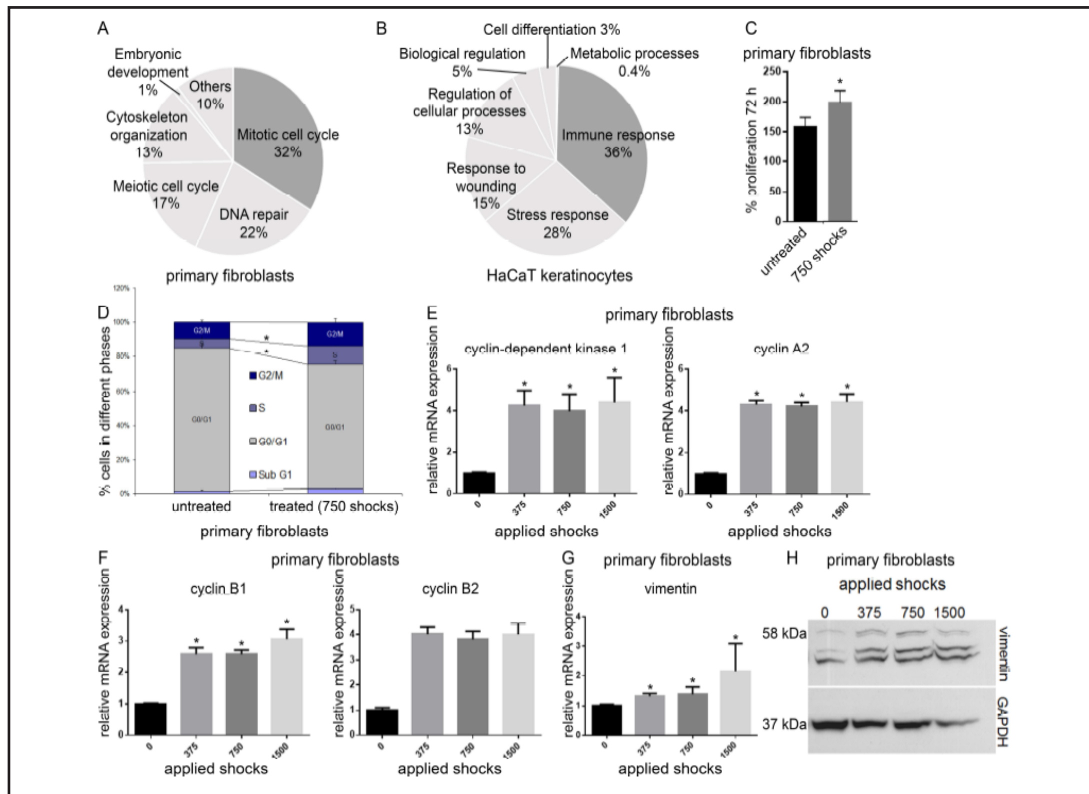

Fig. 3. Shock waves induce proliferation in human fibroblasts via alteration of expression of cell cycle regulatory genes and proteins. Affymetrix GeneChip® Human Gene 2.0 ST Array of primary fibroblasts (A) and HaCaT keratinocytes (B) 24 h after exposure to 750 shock waves. Depicted are functional groups enriched by the differentially expressed genes compared to untreated cells (compare Table 1 and see supplementary material, Suppl. Table 1). Proliferation of fibroblasts increased 72 h after exposure to 750 shock waves (C). FACS cell cycle analysis (D) demonstrating an increased number of cells in S phase (10.8%) and in G2/M phase (13.7%) after 24 h in fibroblasts compared to untreated cells (5.4% and 9.6%, respectively). qRT-PCR showing a ~4-fold increase of cyclin-dependent kinase 1 and cyclin A2 expression (E), and of cyclin B1 and cyclin B2 (F) in fibroblasts 24 h upon exposure to shock waves compared to untreated cells. Vimentin expression was significantly up-regulated on mRNA (G) and protein (H) levels 24 h upon shock waves compared to untreated fibroblasts. *$p<0.05$

significantly increased the number of cells in S phase (10.8%) and in G2/M phase (13.7%) after 24 h when compared to untreated cells (5.4% and 9.6%, respectively, $p<0.05$). The percentage of cells in the G0/G1 phase was consequently significantly decreased in ESW-treated fibroblasts compared to untreated cells (72.7% vs 83.5%, respectively, $p<0.05$), indicating that SWT induced entry to S phase and G2/M, and reduced G0/G1 phase, thus starting the mitotic machinery as early as 24 h after application and resulting in enhanced proliferation after 72 h.

To further verify the role of cell cycle regulatory genes and mitosis-specific genes in ESW-treated fibroblasts, cyclin-dependent

Table 1. Genes differentially regulated in HaCaT keratinocytes and primary fibroblasts 24 h after application of 750 shock waves

Differentially up-regulated genes in	
HaCaT keratinocytes	Primary fibroblasts
1. IL1α	1. CDK1
2. IL7R	2. CCNB1, CCNB2
3. IRF7	3. CDCA2, CDCA3
4. IFI6	4. CCNE2
5. IFI44	5. CDC6,7
6. IFI44L	6. KIFs
7. IFI27	7. POLQ
8. C3	8. E2F (1,7,8)
9. VEGFA	9. PLK1,4
10. CCL5	10. CDK2

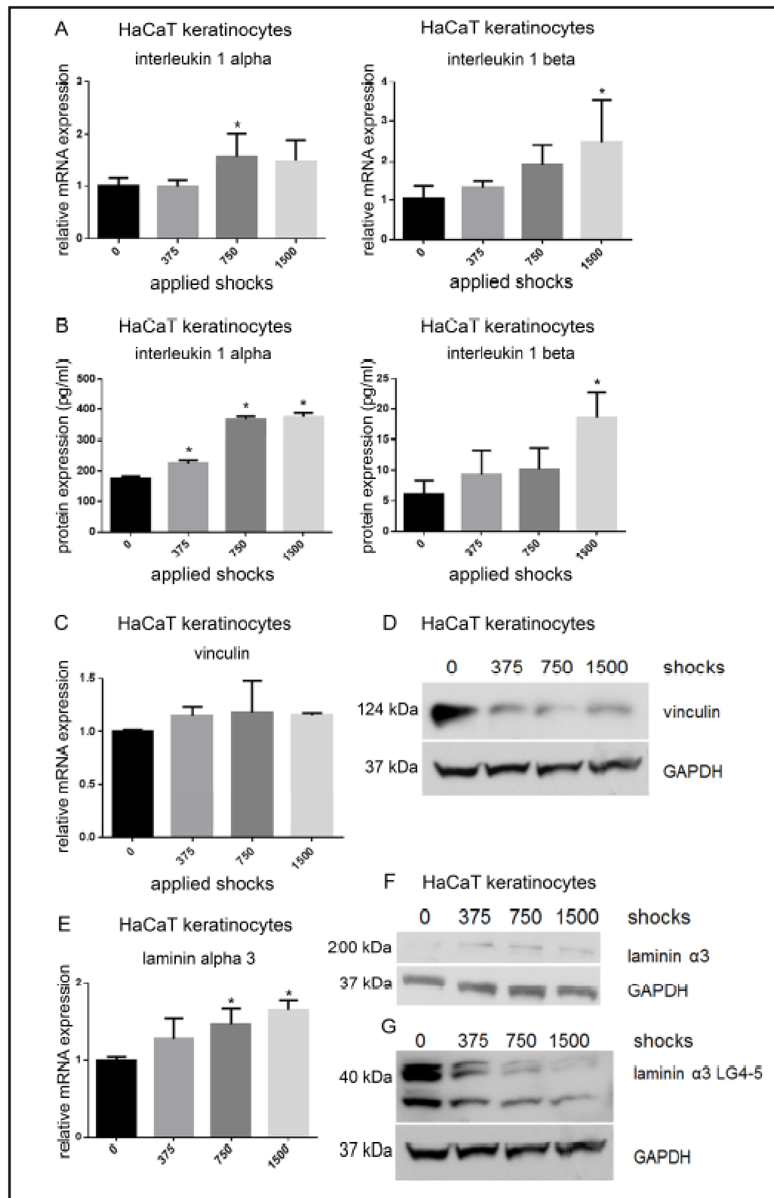

Fig. 4. Shock waves activate immune response factors in human keratinocytes. Increase of mRNA- (A) and protein (B) expression of interleukin 1 alpha and beta 24 h upon exposure to 750 shock waves in primary keratinocytes. Dose-dependent expression of vinculin on mRNA (C) and protein (D) levels in HaCaT keratinocytes. Increase of the laminin alpha 3 chain on mRNA (E) and protein (F) levels in HaCaT keratinocytes 24 h upon exposure to shock waves. The processed and secreted laminin alpha 3 LG4-5 tandem module decreased in a dose-dependent manner (G). *$p<0.05$.

protein kinases (CDKs) and corresponding cyclins were analyzed. Microarray data showed that in SW-treated fibroblasts, CDK1 was 2.7-fold up-regulated and significantly enriched in the respective gene subset (see Fig. 3B). Since CDK1 is essential for G1/S and G2/M phase transitions of the eukaryotic cell cycle, we aimed to confirm the up-regulation of CDK1. qRT-PCR confirmed a ~4-fold increase of CDK1-expression within 24 h in fibroblasts upon SWT when compared to untreated cells (Fig. 3E). In this experiment, the CDK1 mRNA level upon ESWT was even more pronounced than in the microarray test results.

To further validate the microarray data and the role of cell cycle regulatory genes, we confirmed up-regulation of CCNA2 (Cyclin A2), which functions as regulator of CDK and thus promotes cell cycle G1/S and G2/M transitions (Fig. 3E). Similarly, CCNB1 (G2/Mitotic-Specific Cyclin B1), and CCNB2 (G2/Mitotic-Specific Cyclin-B2) involved in mitosis showed a significant increase of expression (>2-fold) upon SWT, as analyzed by qRT-PCR (Fig. 3F).

Shock waves alter the expression of cytoskeletal proteins in fibroblasts

A striking morphological observation in fibroblasts upon SWT was the visible reorganization of the cytoskeleton. We chose vimentin as major protein comprising the

cytoskeleton for further analysis. Indeed, vimentin expression was significantly up-regulated on mRNA level upon SWT when compared to untreated fibroblasts (Fig. 3 G). Western blot analysis confirmed the up-regulation of vimentin on protein level 24 h after shock treatment in a dose-dependent manner (Fig. 3H).

Shock waves activate immune response factors in human keratinocytes

In contrast to fibroblasts, keratinocytes showed a significant enrichment of genes related to immune response upon SWT (compare Fig. 3B). Among those, pro-inflammatory cytokines were mainly induced upon SWT. We screened for possible overlapping gene expression pathways (cell-type independent) upon ESWT between fibroblasts and keratinocytes. Only four overlapping differentially expressed genes were found (KCNQ5-IT1, RN5S150, EGR1, FOSL1), the latter two of which are involved in cell proliferation and mitogenesis. To corroborate the results of the microarray analysis, we selected IL-1α and IL-1β, two inflammatory cytokines playing a central role in the regulation of immune responses and response to wounding, for a more in-depth analysis. To this end, we performed quantitative real-time PCR on SW-treated primary keratinocytes with rising numbers of impulses as above 24 h after application. A significant increase of mRNA expression was observed for IL-1α upon SWT at 750 impulses, and for IL-1β at 1500 impulses (Fig. 4A). The increased expression levels of IL-1α and IL-1β mRNA upon SWT in primary keratinocytes were next confirmed by ELISA analysis on protein level. Here, we also detected dose-dependent increasing levels of both IL-1α and IL-1β upon SWT (Fig. 4B).

Next, we aimed to study possible changes in keratinocyte cell adhesion/migration after SWT. Focusing on the cytoskeleton, we chose to investigate vinculin as cytoskeletal protein that promotes directionally persistent cell migration and tension-dependent ECM remodeling in complex 3D environments [21]. In SW-treated keratinocytes we detected a dose-dependent expression of vinculin on mRNA (Fig. 4C) and protein (Fig. 4D) levels in HaCaT keratinocytes. Another important protein of the extracellular matrix, which mediates cell motility and adhesion in keratinocytes and thus promotes wound healing via collagen VII, is laminin-332 [22]. Upon SWT, in HaCaT keratinocytes we measured an increase of the laminin α3 chain on mRNA (Fig. 4E) and protein (Fig. 4F) levels. Laminin-332 is proteolytically processed and its extracellular mature form lacks the α3 chain C-terminal globules 4 and 5 (LG4-5). The LG4-5 tandem has keratinocyte adhesion and migration properties [23] and antimicrobial activity [24]. Interestingly, 24 h after SWT, in lysates of HaCaT keratinocytes, LG4-5 tandem modules were detected at decreasing concentrations in a dose-dependent manner (Fig. 4G), suggesting that SWT had both increased the expression of laminin-332 and the extracellular secretion of LG4-5, thus promoting wound healing.

ESWT (CellSonic®) induces healing of chronic leg ulcers

Due to the mechanistic results gained above on the cellular level in human fibroblasts, keratinocytes and endothelial cells, it became highly plausible to step from "bench to bedside" and to apply the same SWT treatment modality on patients suffering from therapy-refractory, chronic leg ulcers. We chose the ulcer clinic of the Department of Dermatology and Allergology, Medical University Hospital, Tuebingen, Germany, as adequate treatment location for the clinical application. Daily, in this specified environment 10-15 ulcer patients (out-patients) are treated by certified wound nurses, dermatologists, and receive high-level standard care ulcer treatment and diagnostics according to the German wound care guidelines (standard care: wound cleansing, surgical debridement, (antiseptic) wound dressing, antibiotic therapy in case of clinical infection, compression (venous ulcers), regular manual lymph drainage (venous ulcers); standard diagnostics: venous and arterial duplex ultrasound, bacterial smear). In case of complicated wounds, an interdisciplinary team of specialists (vascular surgeon, plastic surgeon, angiologist, radiologist, cardiologist, nephrologist) is additionally consulted for further diagnostics and therapy.

During daily medical practice ESWT (CellSonic®) was offered as supplemental therapy additional to standard care. Within this setting 75 consecutive patients with non-healing

Table 2. Ulcer patient characteristics and details of applied ESWT. PTS: postthrombotic syndrome

Patient	Pathophysiology	No. of ulcers	Duration (y)	Shocks at 1st ESWT	No. of ESWT	Clinical result
1	venous	1	2	500	4	complete healing
2	mixed	1	2	400	5	improvement
3	mixed	1	23	1700	4	improvement
4	traumatic	1	3	700	4	improvement
5	calciphylaxis	1	2	300	4	improvement
6	venous	1	1	600	1	complete healing
7	biopsy, venous	1	0.25	600	5	complete healing
8	venous	1	2	1200	4	improvement
9	mixed	1	2	500	1	improvement
10	traumatic	1	1	500	1	complete healing
11	livedovasculopathy	1	20	400	4	no change
12	venous	1	5	1400	4	complete healing
13	venous, PTS	1	4	2200	3	no change
14	traumatic	1	3	320	3	significant improvement
15	venous, PTS	2	17	1800	4	complete healing
16	morphea	2	2	600	3	complete healing
17	venous, PTS	1	1	1000	4	improvement
18	mixed	1	5	2000	4	no change
19	venous	1	40	600	4	improvement
20	mixed	1	11	1800	4	improvement
21	venous	1	20	800	4	improvement
22	venous	2	0.75	1000	3	complete healing
23	venous	4	10	1100	4	complete healing (3) / impr. (1)
24	mixed	1	1	2000	1	significant improvement
25	pressure	2	0.33	500	3	complete healing (1) / impr. (1)
26	venous	1	4	400	5	significant improvement
27	mixed	1	4	800	1	complete healing
28	venous	1	0.5	1000	4	complete healing
29	venous	1	9	1400	4	significant improvement
30	venous	1	4	900	4	significant improvement
31	fasciitis, venous	1	4	1500	4	improvement
32	venous	1	50	1800	4	complete healing
33	traumatic	1	1	800	3	complete healing
34	pressure	2	1	400	1	complete healing
35	mixed	1	3	1500	6	improvement
36	arterial	1	1	2000	3	significant improvement
37	venous	1	3	400	4	no change
38	venous, PTS	1	0.25	900	3	complete healing
39	mixed	2	10	1400	4	complete healing (1) / impr. (1)
40	venous	1	4	2000	2	improvement
41	venous	1	1.5	600	1	complete healing
42	venous	1	2	300	1	improvement
43	venous	1	1	250	4	no change
44	venous	2	1 / 0.25	1400	1 / 4	complete healing (1) / sign. impr. (1)
45	venous, PTS	1	0.6	1200	1	complete healing
46	venous	3	1	1000	4	improvement
47	venous, PTS	1	1.5	1500	4	significant improvement
48	venous	1	18	1500	4	complete healing
49	calcinosis cutis	1	2	400	4	significant improvement
50	traumatic	1	1.5	300	3	improvement
51	venous, PTS	4	0.5	1500	3x4 / 1x5	complete healing
52	venous	1	1	1400	3	significant improvement
53	venous, PTS	1	0.5	800	4	significant improvement
54	mixed	1	3.5	700	3	improvement
55	calcinosis cutis	1	0.75	1500	2	improvement
56	venous	1	10	2000	2	improvement
57	ulcus hypertonicum	1	6	2000	2	no change
58	ekthymata	1	0.25	700	2	significant improvement
59	morphea	1	0.75	500	2	improvement
60	venous, PTS	1	17	500	2	improvement
n=60		n=75				

chronic ulcers with duration of 3 months to 50 years and various etiologies were included and analyzed. All patients were carefully instructed and gave informed consent. Three patients could not stand the treatment due to pain already after a few seconds of treatment, so the treatment was immediately abandoned and the patients continued to receive solely

standard ulcer care. During the course of the treatments, the impact of ESWT alone on ulcer healing could not be evaluated in another 12 patients (with n=12 ulcers) due to various reasons (4 received either additional vacuum therapy and/or a skin graft, 2 ulcers became infected 3 weeks after treatment and ESWT was therefore stopped, 2 patients did not present anymore after the first ESWT, 3 ulcers continued to progress after the first treatment and were therefore not treated a second time, and one patient with a long history of repetitive arterial embolisms developed a new arterial embolism in the leg two weeks after the first ESWT treatment). The 60 remaining patients (for clinical details refer to Table 2) with a total of n=75 leg ulcers that were evaluated in the following had a mean age of 68.3±14.6 years (range: 12 – 93 years); 60% were male and 40% female; the mean duration of the ulcers was 5.9±9.3 years (range: 3 months – 50 years). Of the evaluated 75 ulcers, 43 were "venous" (57%), 11 were "mixed" arterial-venous (15%), and 21 (28%) had "other", rare pathophysiologies (posttraumatic, calciphylaxis, livedovasculopathy, scleroderma/morphea, pressure, fasciitis, arterial, calcinosis cutis, hypertonic ulcer, ecthymata).

ESWT was well tolerated and usually performed four times, once every 3-4 weeks, or depending on the healing of the ulcer (11 x 1 treatment, 7 x 2 treatments, 14 x 3 treatments, 38 x 4 treatments, 4 x 5 treatments, and 1 x 6 treatments). The average number of ESWT was 3.3±1.2 (Table 2). The number of pressure impulses applied per treatment was calculated according to the size of the ulcer: 100 impulses per cm^2 + 200 impulses (mean impulses per ulcer: 996±562, range: 250 - 2200). The pressure impulses were homogeneously applied to the entire surface of the ulcer plus the edges of the wound; in case of ulcers exceeding 20 cm^2, a maximum number of 2200 impulses were similarly applied. The mean size of the ulcers was 8 cm^2, ranging from 1 cm^2 – 100 cm^2.

By comparing the three groups of underlying pathophysiologies (1. "venous", 2. "mixed", 3. "other"), we detected that "other" were significantly younger than "venous" and "mixed": Venous: 70.6±10.7 years, range: 53 – 93 years; mixed: 75.3±9.2 years, range: 55 – 91 years; other: 60.5±19.4 years, range: 12 – 84 years; p=0.022 (other vs. venous), p=0.033 (other vs. mixed), p=0,217 (venous vs. mixed), T-Test.

There was no difference in duration of the ulcers between the three groups (1. "venous", 2. "mixed", 3. "other"): Venous: 7.4±11.3 years, range: 3 months – 50 years; mixed: 6.5±6.7 years, range: 1 – 23 years; other: 2.8±4.5 years, range: 3 months – 6 years; p=0.107 (other vs. venous), p=0.095 (other vs. mixed), p=0.811 (venous vs. mixed), T-Test. In terms of size of the ulcer and thus numbers of impulses applied, we found that "other" received significantly less impulses than "mixed" ulcers: Venous: (1028±511 impulses, range: 250 – 2200; mixed: 1280±623 impulses, range: 400 – 2000; other: 779±565 impulses, range: 320 – 2000; p=0.113 (other vs. venous), p=0.039 (other vs. mixed), p=0.199 (venous vs. mixed), T-Test.

We classified the impact of ESWT (Fig. 5A) on ulcer healing according to four criteria: 1. no change (<20% size reduction); 2. improvement (20 – 75% size reduction); 3. significant improvement (>75% size reduction); 4. complete healing (100% size reduction). Clinical examples (n=4) of the four different healing categories are depicted in Figs. 5 B-E. At each clinical visit the ulcers were measured and photo-documented. Of the 75 ESW-treated ulcers, 31 (41%) showed complete healing, 12 (16%) significantly improved, 26 (35%) improved, and 6 (8%) showed no change. In total, 92% of all ESWT-treated ulcers independent of the underlying pathophysiology improved during ESWT. For further statistical evaluation we defined group 1 ("no change") to "bad response", and combined groups 2, 3 and 4 ("improvement", "significant improvement" and "complete healing") to "good response". We found no difference in terms of age, duration of the ulcer, and size of the ulcer between the two groups ("good response" vs. "bad response"; p=0.154, p=0.826, p=0.342, respectively, T-Test).

We further detected no difference in healing through ESWT by comparing the three different groups of underlying pathophysiologies (1. "venous", 2. "mixed", 3. "other", Table 3): For venous ulcers (n=43), 21 showed complete healing (49%), 7 showed significant improvement (16%), 12 improved (28%), and 3 did not improve (7%). For mixed ulcers (n=11), a similar result was obtained: 2 (18%) completely healed, 1 significantly improved

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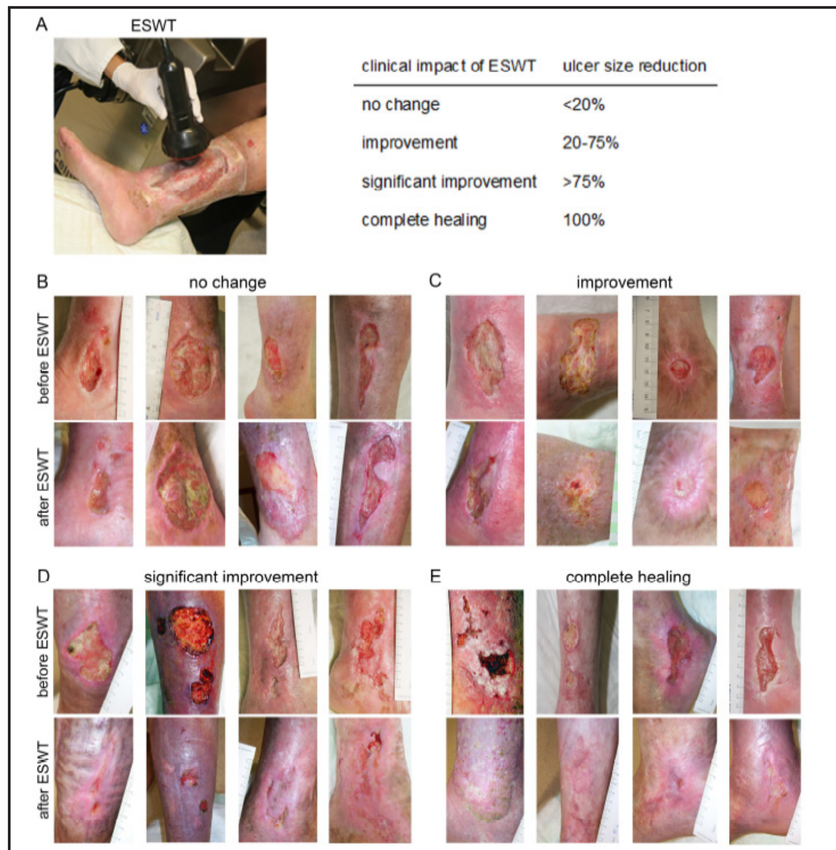

Fig. 5. Clinical impact of extracorporal shock wave treatment on chronic leg ulcers. ESWT was performed on therapy-refractory chronic leg ulcers (n=75). Application of ESWT on a leg ulcer using the CellSonic® medical device (A). Exemplary images (n=4) of the four different categories of healing upon ESWT used for statistical evaluation (no change (B), improvement (C), significant improvement (D), and complete healing (E), compare Table 3).

Table 3. ESWT treatment response of the 75 treated ulcers.

	Ulcer pathophysiology		
Outcome	Venous (n=43)	Mixed (n=11)	Other (n=21)
Complete healing	49%	18%	38%
Significant improvement	16%	9%	19%
Improvement	28%	64%	33%
No change	7%	9%	10%
Overall ESWT response	93%	91%	90%

(9%), 7 improved (64%), and 1 did not improve (9%). In the third group ("other", n=21), 8 completely healed (38%), 4 significantly improved (19%), 7 improved (33%), and 2 did not improve (10%).

Discussion

The complex process of cell regeneration that leads to healing of wounds is associated with epithelial cell migration and an orchestrated interaction of various cell types, the extracellular matrix, and an interplay of specific cytokines [25]. Cytokines stimulate fibroplasia and angiogenesis, and thus support blood vessel formation into a microvascular network throughout the granulation tissue, to ensure nutrient transport and sustain cell metabolism [26]. A second physiological process that leads to healing of wounds is the process of cell repair. Damaged specialized tissue is then replaced by new fibrous tissue. In case of a dysbalanced cooperation of the latter mechanisms and cell types, tissue damage cannot properly be restored and chronic wounds arise.

In this study, we clinically demonstrated that ESWT induced healing of CLUs, demonstrating that SWT positively modulated the process of cell regeneration and tissue repair. To investigate the underlying biological mechanisms, we thoroughly analyzed the three major cell types involved in physiological wound healing, keratinocytes, fibroblasts, and endothelial cells *in vitro* after application of SWT. A distinct morphological transformation

of all three studied cell types after SWT was our initial observation. In a previous study on fibroblast morphology during wound healing, fibroblasts were categorized into normal dermal fibroblasts, which showed elongated and spindle shape morphology, and "wound fibroblasts" which grew more slowly and displayed a large, star-shaped form and cytoplasmic stress fibers [27]. In line, SW-treated fibroblasts in the current study displayed morphological similarities with the latter wound fibroblasts, suggesting a possible positive influence on wound closure in ulcer patients. Additionally, we analyzed morphological changes of epidermal keratinocytes after SWT. Our results indicated that upon SWT, the keratinocytes changed motility and formed confluent sheets of cells. We also observed a morphological shape change from round cells to a cobblestone pattern, indicating that SWT accelerated epidermal (keratinocyte) wound closure. Finally, we observed a stimulating effect of SWT on human dermal microvascular endothelial cells in terms of capillary network formation, suggesting an induction of (paracrine) angiogenic signals. Together, SWT potently induced morphological shape transformations in all cell types tested, which highlighted a possible efficacy in the induction of the complex wound healing process.

We went on to demonstrate in a wound scratch assay that SWT-induced migration of fibroblasts and keratinocytes was mediated without proliferative effects within 24 hours after treatment. In particular, epithelial keratinocytes demonstrated a remarkable motility during the *in vitro* process of wound healing upon SWT. Earlier studies reported that extracellular matrix proteins such as fibronectin mediated epithelial cell adherence of cultured cells; however, migrating epithelial cells lack such anchoring proteins [28]. Cell adhesion (the physical interaction of a cell with another cell or with the extracellular matrix (ECM)) is essential for cell migration and tissue integrity. In this aspect, in the current study it was intriguing, that the whole protein of the ECM component laminin α3 was increased upon SWT, while its C-terminal LG4-5 modules dose-dependently diminished in fibroblasts, suggesting that an increased protease activity released the LG4-5 fragment. This corresponds to our previous findings, which demonstrated enhanced expression and processing of the LG4-5 modules of laminin α3 in keratinocytes after infection and in chronic leg ulcers in which the level of expression and further processing of the LG4-5 module positively correlated with wound healing [19]. In line with laminin, the expression of vinculin, which belongs to the focal adhesion complex and links actin with ECM molecules [29], was dose-dependently regulated by SWT in our study. From this data, we speculate that the secreted vinculin or laminin α3 LG4-5 proteins could promote cytoskeleton reorganization and thus cell migration in the ECM periphery. These latter observations concerning ECM and anchoring proteins contribute to a hypothesis on how ESWT could induce and/or promote wound healing *in vivo*, since the migratory behavior was increased in keratinocytes, fibroblasts and endothelial cells after SWT *in vitro*.

In addition to promoting cell motility, we observed that SWT also lead to significant expression of cell cycle regulatory genes in fibroblasts, which are involved in mitosis. It is widely acknowledged that restoration of normal epidermal architecture during wound healing results from both mitosis and migration of epidermal cells [30] and that mitosis occurs in these adjacent epidermal cells to replace the cells lost as a result of the injury. This implies that the damaged tissue is restored, more or less as it was, by the process of cellular and tissue regeneration, which is the ideal form of healing giving good cosmetic and functional results. In our experiments SWT induced mitosis and proliferation in fibroblasts and thereby initiated a cell regeneration cascade. Among the up-regulated regulatory genes, CDKs are key regulators of cell cycle progression, which integrate positive as well as negative growth factor-mediated signals [31]. CDKs also play a necessary role in the regulation of G1- to S-phase transition during the cell cycle. In mammalian cells, the transient rise in levels of cyclins subsequently leading to activation of CDKs is critical, as cyclin-CDK complexes trigger the transition to the mitotic phase (G1/S) of the cell cycle. Our gene expression analysis yielded that CDK1 and CCNA2 (Cyclin A2) were significantly up-regulated on mRNA level upon SWT in human fibroblasts. Cyclin A2–Cdk1 is critical regulators of S-phase programs in mammalian cells. Moreover, it was reported that besides the importance of cyclin A2 in the

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initiation of mitosis, two other cyclins, cyclin B1 and B2 were important for the maintenance of the mitotic arrest [32]. The increased expression of cyclin B1 and cyclin B2 observed in both our microarray analysis and the confirmatory qRT-PCR experiments corroborated our FACS data analyzing the cell cycle. Cyclin B1 for instance is predominantly expressed during G2/M phase and mitosis, and its activity is also increased after serum stimulation, however low in G1 phase or serum starvation [33]. Cyclin B1 and B2 levels are very low in G1 phase, increase in S and G2 phases, and peak at mitosis, and are involved in the regulation of the mitotic spindle checkpoint [34]. A recently published study showed that certain bacteria negatively influence re-epithelialization during wound healing, which is associated with down-regulation of cell cycle genes including CDK1 and others that are critical for the G1/S transition [35]. The latter report supports our hypothesis that S-phase progression in eukaryotic cells could have a positive impact on wound healing *in vivo*, which was suggested by the SW-treated primary fibroblasts in our experiments.

Taken together, the *in vitro* experiments elucidate how the different cell types, important for an efficient wound healing, responded to SWT. Nevertheless, we are aware that epidermal cells, dermal fibroblasts, endothelial cells and the surrounding immune cells form a functional unity, which they orchestrate, and trigger each other during the tissue regeneration process. Therefore, many biological processes are involved in tissue repair, including production of ECM, secretion of proteases and protease inhibitors, cellular migration, chemotaxis, and proliferation of macrophages, fibroblasts of the granulation tissue, keratinocytes, and capillary endothelial cells. In line, we could demonstrate, that SWT positively influenced some of these latter mentioned interacting processes required for wound healing. Therefore, in a second approach, we transferred our pre-clinical *in vitro*-results, which had accumulated in our hypothesis that ESWT could indeed induce the wound healing process in CLUs by the mechanisms described above. Consequently, we treated 60 consecutive patients suffering from 75 CLUs that presented at the ulcer clinic of our hospital with EWST in addition to standard ulcer care. Since we hypothesized from the *in vitro* results that the induction of wound healing via ESWT should be independent of the underlying pathophysiology (e.g. venous ulcer, arterial ulcer, etc.), we included ulcers with different pathophysiological backgrounds in our *in vivo* application of ESWT. Most strikingly, we clinically observed and documented a high percentage of chronic ulcers that responded to this simple treatment regimen: 41% of ESWT-treated CLUs showed complete healing, 16% a significant improvement, 35% an improvement, while only 8% of the ulcers did not improve during the treatment. In this respect, it is important to acknowledge that the duration of the ulcers was three months to fifty years, that we treated ulcers independent of their pathophysiology, and that the patients age ranged from 12 – 93 years. Of the evaluated 75 ulcers, 43 were venous, 11 were mixed arterial-venous, and 21 had other, rare pathophysiologies. Despite the different etiologies of the ulcers, a treatment response of > 90% was achieved for all three groups. This clearly demonstrates that ESWT seems to be a simple non-invasive and promising tool for the induction of the wound healing. Interestingly and importantly, the treatment response was independent of the patient age, duration, and size of the ulcer. Consequently, ESWT is continuously and successfully applied as routine ulcer therapy by the treating physician (CB). In the meantime, the frequency of treatments was condensed, and ulcers are now treated once weekly with ESWT whenever possible. Using this condensed protocol, the ulcers seem to heal even faster and to a greater extent. We acknowledge a technical difference between the *in vitro* and the *in vivo* application of the shock waves: *In vitro*, the shocked cells were kept in liquid (cell culture medium) and not in gel. Since most chronic ulcers do secrete considerable amounts of liquid, we believe that in the clinical setting a comparable milieu was achieved by the addition of ultrasound gel. However, we are aware that the plastic bottom of the cell culture dishes (6-well plates) did possibly influence (decrease) the physical impact of the shock waves. Therefore, in the clinical application, the loss of energy during transmission of the shocks onto the wound was probably less pronounced, and their impact might therefore even be more potent than in the *in vitro* situation.

Although the presented clinical data were not collected during a randomized, controlled, prospective clinical trial, the results obtained are nevertheless intriguing and extremely promising, considering the current lack of effective non-invasive treatment modalities for patients with chronic wounds. Furthermore, the fast and easy application of ESWT, the general applicability on any kind of CLUs, and the low number of required treatments make ESWT the ideal subsidiary ulcer treatment in addition to the classical treatment (dressing, debridement, compression, etc.), which by itself often fails to achieve complete wound closure [36]. Together, we present for the first time a large body of evidence suggesting that application of shock waves is potently capable of inducing the wound healing process *in vitro* in the respective cellular models, and also *in vivo* in patients afflicted with CLUs, via induction of a complex biological cascade of cellular events. However, the clinical efficacy of ESWT needs to be confirmed in controlled, prospective, randomized clinical trials to implement ESWT as an adjunct to standard therapy or as a stand-alone treatment. At this point our results strongly suggest that EWST may advance the treatment of chronic, therapy-refractory leg ulcers.

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Disclosure Statement

The authors declare no conflict of interest.

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