Low-intensity vibrations accelerate proliferation and alter macrophage phenotype in vitro

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Abstract

Macrophages are essential for the efficient healing of various tissues. Although many biochemical signaling pathways have been well characterized in macrophages, their sensitivity to mechanical signals is largely unexplored. Here, we applied low intensity vibrations (LIV) to macrophages to determine whether macrophages could directly transduce LIV signals into changes in the expression of genes and proteins involved in tissue repair. Two different LIV signal frequencies (30Hz or 100Hz) were combined with two acceleration magnitudes (0.15g or 1g) to generate four distinct LIV signals that were applied to cultured murine macrophages. All four LIV signals significantly increased macrophage number after 3d of stimulation with the combination of the smallest acceleration and the highest frequency (0.15g at 100 Hz) generating the largest response. Compared to non-LIV controls, gene expression of the pro-healing growth factors VEGF and TGF- β increased with all four LIV signals (Day 1). LIV also decreased protein levels of the pro-inflammatory cytokines IL-6, IFN- γ , and TNF- α (Days 1&3). These data demonstrate the sensitivity of macrophages to high-frequency oscillations applied at low intensities and may suggest that the benefit of LIV for tissue repair may be based on reducing inflammation and promoting a pro-healing macrophage phenotype.

1. Introduction

Macrophages play a critical role in the healing of various tissues (Duffield et al., 2005; Lucas et al., 2010; Mirza et al., 2009; Summan et al., 2006; van Amerongen et al., 2007). Tissue repair consists of overlapping phases of inflammation, proliferation, and remodeling, and macrophages are present in all phases. As tissue repair progresses, macrophages exhibit transitions in phenotype and function, although the precise factors regulating these transitions have not been fully elucidated (Novak and Koh, 2013a, b). During efficient healing, macrophages appear to orchestrate transition from one phase to the next, contributing to phagocytosis of necrotic tissue, cell proliferation, angiogenesis, collagen deposition and matrix remodeling. However, dysregulation of macrophage function can contribute to failure to heal or fibrosis in several pathological situations (Mirza and Koh, 2011; Mirza et al., 2013; Sindrilaru et al., 2011; Villalta et al., 2011). Thus, therapies that modulate macrophage phenotype and function may provide an avenue to promote tissue repair.

Accumulating evidence demonstrates that mechanical stimulation via low-intensity vibration (LIV; $\leq 1g$ acceleration magnitude of the oscillating plate) can be anabolic and/or anti-catabolic in musculoskeletal tissues including bone (Garman et al., 2007; Gilsanz et al., 2006) and enhance neuromuscular control (Fu et al., 2013). LIV has also been shown to accelerate bone regeneration (Hwang et al., 2009) and skin wound healing (Weinheimer-Haus et al., 2014) but the underlying mechanism(s) remains to be elucidated. While LIV has the potential to influence many cell types involved in tissue repair, macrophages are sensitive to mechanical signals as both cyclic and static stretch, albeit at much greater magnitudes than LIV, rapidly induced gene expression in monocyte/macrophages (Wehner et al., 2010; Yang et al., 2000). Thus, the aim of this study was to determine whether macrophages could directly transduce LIV signals into changes in the expression of genes and proteins involved in tissue repair. We also tested whether LIV frequency and magnitude may play a role in modulating macrophage response.

2. Materials and Methods

2.1 Cell culture

Murine macrophages (J774A.1, ATCC, Manassas, VA) were cultured in RPMI 1640 (Gibco, <http://www.invitrogen.com>) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS, Gibco 10082-147) and 1% penicillin/streptomycin and incubated at 37°C, 5% CO₂.

2.2 Mechanical stimulation

Vibration was applied horizontally as described elsewhere (Uzer et al., 2012). Briefly, cells were exposed to either a non-LIV sham control, or one of the following four signal combinations of LIV frequency and acceleration: 30Hz and 1g, 100Hz and 1g, 30Hz and 0.15g, and 100Hz and 0.15g. Vibrations were applied for 20 min/session, 2x/day; sessions were separated by a 2 hour rest period. Non-LIV control cell culture plates were handled identically to experimental plates except that vibrations were not applied.

2.3. Cell number

Macrophages were seeded at a density of 7,500 cells/cm². After allowing internal equilibrium for 24 hours, cells were exposed to LIV or sham treatment (n=9 each) on each of three consecutive days. Cell number was determined via XTT cell proliferation assay (ATCC). On Day 3 of stimulation, immediately after LIV treatment, XTT reagent was added to the samples and incubated for 3 hours prior to measuring absorbance. Cell number was calculated via comparison with a standard curve. Cell viability was checked with a live/dead cell assay (Invitrogen, NY) and visualized under a microscope (Zeiss, Germany).

2.4 Gene expression

To determine whether macrophages can respond within hours to the application of LIV with increased transcriptional levels of two pro-healing genes, cells were plated at a density of 18,000 cells/cm². 48 hours after plating, cells were exposed to LIV or sham treatment (n=9 each). Immediately after the second LIV treatment, cells were lysed (TRIzol, Ambion, TX) and total RNA was isolated (RNeasy, Qiagen, CA). RNA concentration and quality were determined (NanoDropND-1000, Thermo Scientific, NY). cDNA was synthesized using standard protocols (High Capacity RNA to cDNA kit, Applied Biosystems, CA) and RT-PCR was performed (StepOne, Applied Biosystems) using Taqman primer (Applied Biosystems) probes for vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)- β . Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the endogenous control. Results were reported relative to non-vibrated control.

2.5 Flow cytometry

To test whether LIV induced changes in cell proliferation were accompanied by changes in protein levels of pro-inflammatory cytokines or whether LIV could decrease their production, flow cytometric analysis was performed for interleukin (IL)-6, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α . Cells were plated at 18,000 cells/cm² 48 h prior to the start of the experiment and were then exposed to LIV at 0.15 g and 100 Hz or sham treatment (n=6 each) for 3 days. The acceleration magnitude of 0.15 g at a frequency of 100 Hz was selected for this experiment because this LIV combination was most effective at increasing cell number and altering VEGF expression. Immediately after the second experimental session on Day 1 or 3, cells were collected and fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton, and stained with fluorescently-labeled antibodies against IL-6, IFN- γ , or TNF- α (Abcam, Cambridge, UK). Live cells and dead cells were identified using calcein AM and ethidium homodimer (Invitrogen). Stained cells were analyzed by flow cytometry (FACScan, BD) and FlowJo

software (Tree Star Inc. OR); percent cell populations were gated on the cells positive for these fluorescent antibodies.

2.6 Statistics

Data were presented as mean $\pm$ SD. All samples were run as triplicates and statistics were computed on averages of the triplicates, preserving the samples sizes described above. Differences between groups were identified by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests (SPSS 22.0, IBM, New York, NY). P-values of less than 0.05 were considered significant.

3. Results

3.1 Cell number

Fluorescence microscopic images showed predominantly viable macrophages under LIV and non-LIV conditions on Day 3 (Figure 1). Potential differences in cell morphology were not assessed quantitatively. Flow cytometric analysis revealed that the percent live macrophages was not different across LIV treated cells and non-LIV controls ($>99.7 \pm 2\%$; data not shown). All four LIV signal combinations were effective in increasing cell number. Averaged across all LIV signals and compared to non-LIV controls, LIV increased cell number by 82% ($p < 0.001$) on Day 3 (Figure 1). At 30 Hz, increasing acceleration magnitude from 0.15g to 1g increased cell number whereas at 100 Hz, cell number was greater at 0.15 g than at 1 g. The signal combination of 0.15 g at 100 Hz provided an increase in cell number that was significantly greater than for the other three LIV signals.

3.2 Transcriptional changes

To assess whether macrophages can directly transduce LIV signals into changes in the expression of genes involved in tissue repair, transcriptional levels of the pro-angiogenic growth

factor VEGF and the healing-associated growth factor TGF- β were determined immediately after the second LIV treatment. Averaged across frequency signals, LIV increased ($p<0.001$) VEGF expression by 82% at 30 Hz and by 131% at 100 Hz (Figure 2). At 30 Hz, increasing acceleration magnitude increased VEGF expression whereas, at 100 Hz, increasing acceleration magnitude reduced VEGF transcriptional levels. Similar to data on cell proliferation, the 0.15 g LIV signal applied at 100 Hz provided the greatest increase in VEGF expression (156%), significantly greater ($p<0.001$) than any other LIV signal.

LIV also upregulated the expression of TGF- β by 46% when averaged across all LIV signals (Figure 2). The increase in TGF- β was qualitatively similar across the four LIV signals, however, the increase was significantly greater with the 30 Hz signals than the 100 Hz signals (52% vs. 40%, $p<0.001$).

3.3 Changes in protein levels

We tested whether macrophages can convert the high-frequency low-intensity mechanical signal into changes in protein levels. Using the LIV signal that was most successful in altering cell proliferation and transcriptional levels (100Hz-0.15g), protein expression of several cytokines were measured in macrophages after treatment on days 1 and 3 of the protocol. LIV reduced the percentage of IL-6+, IFN- γ +, and TNF- α + cells on both days 1 and 3. The reduction was similar across the three cytokines and amounted to 12-16% on Day 1 and to 43-54% on Day 3 (Figure 3).

4. Discussion

LIV may provide a novel therapeutic approach for enhancing tissue repair and has the potential to influence several cell types involved in tissue repair, including macrophages. The major finding of this study is that macrophages are sensitive to LIV and are able to transduce LIV signals into changes in cell number and in the expression of genes and proteins involved in

tissue repair. Specifically, LIV increased the expression of the growth factors VEGF and TGF- β and reduced the production of three pro-inflammatory cytokines was compared to non-LIV controls. With the exception of raising TGF- β levels, macrophages showed the greatest response to the LIV signal combination that employed the smallest amplitude at the highest frequency. Collectively these findings suggest that macrophages have the ability to differentiate between individual LIV signals based on frequency and acceleration magnitude and that the promotion of tissue repair by LIV may be mediated by changes in macrophage phenotype.

The ability of macrophages to respond to mechanical signals has been reported previously (Martin et al., 1995; Pugin et al., 1998; Wehner et al., 2010; Yang et al., 2000). In these studies, cells were stretched at magnitudes of greater than 1%, in great contrast to the oscillatory signal used here that induced deformations at least three orders of magnitude smaller (Uzer et al., 2012; Uzer et al., 2014). In rat peritoneal macrophages, static strain of 20% induced gene expression of the pro-inflammatory genes inducible nitric oxide synthase, cyclooxygenase-2, IL-6 and IL-1 β (Wehner et al., 2010), while cyclic strain of great than 1% induced gene expression of the M-CSF receptor and various early response genes, which are involved in cell proliferation, differentiation and survival, in human blood monocytes/macrophages (Yang et al., 2000). In contrast to the pro-inflammatory effect of a large static stretch on rat peritoneal macrophages, we report that the dynamic extremely low magnitude LIV signal (<0.001% deformation) had a predominantly anti-inflammatory effect on a mouse macrophage cell line. The differences in the response are likely associated with the stark differences in the mechanical signal being delivered.

During normal tissue repair, macrophages transition from a pro-inflammatory phenotype to a pro-healing phenotype. However, a sustained pro-inflammatory phenotype has been associated with impaired tissue repair (Mirza and Koh, 2011; Mirza et al., 2013; Sindrilaru et al., 2011; Villalta et al., 2011). LIV increased expression of growth factors associated with healing and decreased expression of pro-inflammatory cytokines, indicating that LIV may promote a

pro-healing macrophage phenotype. We previously reported that LIV increased VEGF levels in wounds of diabetic mice, and this was associated with increased angiogenesis and accelerated wound closure (Weinheimer-Haus et al., 2014). Perhaps, the LIV induced increase in transcriptional levels of pro-healing genes and decrease in pro-inflammatory protein levels observed here may be most beneficial in situations (e.g., diabetes) where sustained inflammation impairs healing. The future exploration of precise mechanisms and pathways by which LIV can enhance healing of soft and hard (Gao et al., 2016; Hwang et al., 2009) tissues will be critical for the design of improved mechanical interventions and may also reveal novel pharmacologic targets.

It is not clear what physical parameters drive the cellular response to vibration. Using an in vitro model in which acceleration and fluid shear can be controlled independently during vibrations, we previously demonstrated that fluid shear does not regulate vibration-induced proliferation and osteogenic commitment of mesenchymal progenitor cells (Uzer et al., 2013). Instead, it is possible that cells sense LIV through an out-of-phase oscillating nucleus that converts the mechanical information to biochemical signals through inside-out signaling, in contrast to low-frequency signals like fluid flow or cellular stretch that ostensibly act through outside-in signaling (Uzer et al., 2015). The overall preference of macrophages for the 100 Hz, 0.15 g signal is similar to data collected from mesenchymal progenitor cells (Uzer et al., 2013) and may suggest a similar LIV mechanotransduction mechanism for these two cell types. Therefore, the macrophage response to LIV observed here was unlikely to be modulated by fluid shear stress.

The current investigation is limited in that the signaling pathways that transduce the LIV signal into cellular responses were not investigated. Another limitation is that only one time point was considered for mRNA expression and only two for protein levels. Further, it is unclear whether the observed differences in transcriptional levels, in particular TGF- β , will translate into altered protein levels. Previous studies have indicated that multiple transduction pathways

may participate in converting mechanical signals into biochemical signals, including stretch-activated ion channels, integrins, and tyrosine kinases (Banes et al., 1995; Martin et al., 1995; Takahashi et al., 1997). We previously reported that LIV increased gap junctional intracellular communication between osteocytes and this was dependent on Akt activation (Uzer et al., 2014). Future studies investigating the effects of LIV on these signaling pathways utilizing a series of time points will provide further insight into the mechanisms through which mechanical signals improve tissue repair.

In summary, we investigated the mechanosensitivity of macrophages to LIV signals in vitro. LIV increased cell number and the expression of healing-associated markers, while decreasing production of pro-inflammatory cytokines. Extrapolated, these results suggest that LIV may be beneficial for tissue repair by reducing inflammation and promoting a pro-healing macrophage phenotype in conditions such as diabetes.

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Figure Captions

- Figure 1.** (A-B) Representative images of cells double stained with calcein AM (green; live cells) and ethidium homodimer (red; dead cells) following exposure to (A) non-LIV sham or (B) LIV (100Hz-0.15g) treatments. (C) Cells were exposed to one of four LIV signals or sham and cell number was determined after 3 consecutive days of stimulation. Labeled means without a common letter differ, $P \leq 0.05$.
- Figure 2.** Gene expression of pro-angiogenic growth factor VEGF and growth factor TGF- β immediately following treatment with one of four LIV signals or non-LIV control. Labeled means without a common letter differ, $P \leq 0.05$.
- Figure 3.** Protein expression of pro-inflammatory cytokines IL-6, IFN- γ , and TNF- α following 1 or 3 days of LIV (0.15 g at 100 Hz) or sham treatment (Con). * $P \leq 0.05$ vs Con.

Figure 1

Figure 2

Figure 3