Mechanical stimulation of human dermal fibroblasts regulates pro-inflammatory cytokines: potential insight into soft tissue manual therapies

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Research note

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Abstract

Objective Soft tissue manual therapies are commonly utilized by osteopathic physicians, chiropractors, physical therapists and massage therapists. These techniques are predicated on subjecting tissues to biophysical mechanical stimulation but the cellular and molecular mechanism(s) mediating these effects are poorly understood. A series of previous studies established an in vitro model system for examining mechanical stimulation of dermal fibroblasts and established that repetitive strain, intended to mimic overuse injury, induces the secretion of numerous pro-inflammatory cytokines. Moreover, mechanical strain intended to mimic soft tissue manual therapy reduces strain-induced secretion of pro-inflammatory cytokines. Here, we sought to partially confirm and extend these reports and provide independent corroboration of prior results.

Results Using cultures of primary human dermal fibroblasts, we confirm mechanical forces intended to mimic repetitive motion strain increases levels of IL-6 and that mechanical strain intended to mimic therapeutic soft tissue stimulation reduces IL-6 levels. We also extend the prior work, reporting that therapy-like mechanical stimulation reduces levels of IL-8. Although there are important limitations to this experimental model, these findings provide supportive evidence that therapeutic soft tissue massage may reduce inflammation. Future work is required to address these open questions and advance the mechanistic understanding of therapeutic soft tissue stimulation.

Introduction

Soft tissue manual therapies such as massage and myofascial release are commonly utilized by osteopathic physicians, chiropractors, physical therapists and massage therapists [1-4]. These techniques are predicated on subjecting tissues to biophysical mechanical stimulation [5, 6]. While the precise cellular and molecular mechanism(s) mediating these effects are poorly understood, the limited available evidence suggests that soft tissue manual therapy may reduce inflammation [6]. For instance, a series of studies established an in vitro model system for examining therapy-informed mechanical stimulation of human dermal fibroblasts, which are a cell type that resides in close approximation to vasculature and lymphatics and are recipient of strain from soft tissue manual therapy (reviewed in [7]). This in vitro work demonstrated that strain intended to mimic repetitive, overuse injury of fibroblasts induces the secretion of numerous cytokines; reduces fibroblast proliferation rate; and increases fibroblast apoptosis. Moreover, mechanical strain intended to mimic soft tissue manual therapy reverses numerous aspects of this phenotype [8-11], including reduced secretion of pro-inflammatory interleukin (IL)-6, increased secretion of anti-inflammatory IL-1ra, increased fibroblast proliferation, and reduced fibroblast apoptosis. These findings are generally consistent with a preliminary study in healthy, young adult males which found that soft tissue manual therapy applied to the low back reduces serum levels of several pro-inflammatory cytokines and circulating neutrophil levels (MTL, data forthcoming).
We sought to provide further insight and replicate a portion of the previous *in vitro* findings examining mechanical stimulation of dermal fibroblasts. Here, we provide independent corroboration that mechanical force profiles intended to mimic repetitive motion strain increases levels of IL-6 in conditioned media from dermal fibroblasts. Moreover, we confirm that mechanical strain intended to mimic therapeutic soft tissue stimulation reduces IL-6 levels and extend the prior work by reporting that it also reduces levels of the pro-inflammatory cytokine IL-8.

**Materials And Methods**

*Fibroblast culture and strain*

Primary human dermal fibroblasts (#PCS-201-012) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and cultured as directed by the vendor. Cells were free of mycoplasma contamination as confirmed by the MycoProbe Mycoplasma Detection Kit (R&D Systems, Minneapolis, Minnesota, USA) used as directed by the manufacturer. As detailed in Figure 1A, cells were combined from separate flasks then seeded at 120,000 cells per well on 6-well flexible collagen I-coated membranes (Flexcell International, Burlington, North Carolina, USA) and, the next day, cells were left unstrained (control) or mechanical stimulation was performed on a Flexcell FX-6000 according to two previously reported strain profiles [6]. Briefly, for the first cyclic short-duration strain (CSDS) profile, fibroblasts were subjected to an 8-hour cycle with 1.6 second bouts of deformation increasing at 33.3%/second starting from rest to a maximum of 10% beyond resting length, followed by decreasing strain to baseline at 33.3%/second (Figure 1A). For the second CSDS profile, fibroblasts were subjected to an 8-hour cycle with 1.6 second bouts of deformation increasing at 22%/second starting at a baseline strain of 10% and a maximum of 16.6%, followed by decreasing strain to baseline at 22%/second (Figure 2A). For acyclic long-duration strain (ALDS), after a 3-hour rest period following CSDS, cells were subjected to a single 60 second bout of stretch at 6% beyond resting length at a loading rate of 3%/second followed by release at 1.5%/second until return to resting length. Conditioned media was collected 96 hours after the strain protocols and stored at -80°C.

*Cytometric Bead Array*

Conditioned media was analyzed with the Human Pro-Inflammatory Cytokine Cytometric Bead Array kit (BD Biosciences, Franklin Lakes, New Jersey, USA) as directed by the manufacturer using a BD Accuri C6 Flow Cytometer. Since concentrations for IL-6 and IL-8 varied between runs, data were normalized to CSDS strain profile.

*RT-PCR*
For expression analyses, primary human dermal fibroblasts were cultured on flexible collagen I-coated membranes as described above and, the next day, cells were scraped using a cell lifter and RNA was collected using the RNEasy Plus kit (QIAGEN). cDNA was synthesized using the SuperScript III First Strand Synthesis kit (ThermoFisher) and PCR was performed using KeenGreen Taq Polymase (IBI Scientific) with primer pairs (IDT DNA) detailed in Table 1 on a miniPCR Thermal Cycler (miniPCR). All primer pairs were designed to cross exon:exon junctions and sample containing no template DNA served as negative control. Amplicons were analyzed by agarose gel electrophoresis and imaged using a blueBox transilluminator (miniPCR).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 as described in each respective figure legend or in the text. A p-value of < 0.05 was considered significant.

Results

To replicate conditions of prior reports [8, 12, 13], primary human dermal fibroblasts were cultured on collagen I-coated flexible membranes and subjected to two different mechanical force profiles intended to mimic repetitive motion strain (i.e., cyclic short-duration strain, CSDS). In the first CSDS profile (Figure 1B), cells were repeatedly stretched between baseline and 10% beyond resting length every 1.6 seconds for eight hours [12, 13]. Conditioned media was collected 96 hours later and subjected to analysis using the human pro-inflammatory cytokine bead array, which targets IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α. Consistent with a prior report [12], this strain profile led to increased levels of IL-6 in conditioned media (4.7±0.4511 fold change, p=0.0145 by paired t-test, n=3 each condition). Additionally, this strain profile led to increased levels of IL-8 in conditioned media (3.2±0.1986 fold change, p=0.0081 by paired t-test, n=3 each condition). We did not detect IL-1β, IL-10, IL-12p70, or TNF-α in conditioned media from either unstrained or strained cells; consistent with this, these cells express IL-6 and IL-8 under unstrained conditions (Figure 1C) but not the other cytokines detected by the cytometric bead array (data not shown).

We subjected primary human dermal fibroblasts to this CSDS profile followed by mechanical strain intended to mimic therapeutic soft tissue stimulation (i.e, acyclic long-duration strain, ALDS) such as massage or myofascial release (Figure 1B), as previously described [12, 13]. Consistent with a prior report [12], ALDS reduced the levels of IL-6 in the conditioned media (Figure 1D). ALDS also led to a similar reduction in IL-8 levels in the conditioned media (Figure 1E).
Next, we examined a different CSDS profile [8] wherein primary human dermal fibroblasts were stretched between 10% beyond resting length and 16% beyond resting length every 1.6 seconds for eight hours (Figure 2A). In contrast to a prior report [8], this CSDS profile did not lead to increased levels of IL-1β or IL-6. However, ALDS following CSDS reduced the levels of IL-6 – as previously reported [8] – and IL-8 in conditioned media compared to CSDS alone (Figure 2B-C).

**Conclusions**

This study was designed to replicate and extend prior work using an in vitro model to examine the impact of mechanical stimulation of a cell type that is recipient of therapeutic soft tissue stimulation (reviewed in [7]). Our findings corroborate the observation that ALDS, intended to mimic soft tissue therapy such as myofascial release, reduces levels of the pro-inflammatory cytokine IL-6. Additionally, we provide the first evidence that therapy-informed ALDS reduces levels of IL-8, which is also a pro-inflammatory cytokine. We were unable to replicate the prior result that ALDS reduces expression of pro-inflammatory IL-1β as this cytokine was not detected in any of our assays.

**Limitations**

Collectively, these findings provide supportive evidence that therapeutic soft tissue massage may reduce inflammation and will assist in designing future mechanistic studies in this area. However, there are important limitations to the *in vitro* model used by us and others that temper the generalization of these results. For instance, while a cell-based approach may provide insight into the underlying mechanisms of soft tissue massage, our uniculture model examines only one soft tissue cell type as opposed to the complex tissue-level interactions that likely occur *in vivo*. Similarly, we are unable to speculate how our findings might compare to a three-dimensional cell culture model as opposed to a cell in a monolayer. Additionally, soft tissue manual therapy likely involves several factors beyond mechanical stimulation, including sensory, cognitive, thermal, neurovascular, lymphatic, autonomic, neuro-hormonal-endocrine, psychosocial, and emotional components. Ultimately, the complex integration of multiple elements determines the biological response, functional outcomes, subjective pain perception, and sense of well-being associated with soft tissue manual therapies in clinical care. Future work is required to address these open questions and advance the mechanistic understanding of therapeutic mechanical stimulation of soft tissues.

**List Of Abbreviations**

CSDS: cyclic short-duration strain

ALDS: acyclic long-duration strain

IL: interleukin
Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

All authors contributed to the design, generation, and analysis/interpretation of the data presented in this manuscript. AA, MTL, and JWL contributed to the writing of this manuscript. All authors read and approved the final manuscript.

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Table 1. Primer pairs utilized for RT-PCR analyses

| Target | Forward Primer       | Reverse Primer                        |
|--------|----------------------|---------------------------------------|
| IL-6   | TCTCACAAGCGCCCTTCG   | CTGAGATGCCGTCGAGGATG                  |
| IL-8   | GCGCCAACACAGAAATTATTG| TGCTTGAAGTTTCACTGGCAT                |
| Hprt   | CCTGCTGGATTACATTAAGC | GTCAGGGGCATATCCAACAAC               |

Figures
Figure 1
**Figure 1**

A: Schematic representation of cell culturing method designed to allow matched comparison between unstrained cells (Control) and cells subjected to cyclic short-duration strain (CSDS) or CSDS combined with acyclic long-duration strain (ALDS). All cells were collected simultaneously 96 hours after the onset of the CSDS strain profile. 

B: Schematic representation of CSDS profile number 1. 

C: RT-PCR analyses for interleukin (IL)-6 and IL-8 from unstrained primary human dermal fibroblasts relative to HPRT housekeeping control. Molecular weight and sample lacking template DNA (no template control, NTC) served to confirm specificity of amplicon. 

D-E: Determination of IL-6 (D) and IL-8 (E) levels in conditioned media collected from primary human dermal fibroblasts 96 hours following CSDS or CSDS combined with ALDS. Data are mean±SEM normalized to CSDS; n=3 per condition. * indicates p<0.05 against CSDS by paired t-test.
Figure 2
Figure 2

A: Schematic representation of cyclic short-duration strain (CSDS) profile number 2. B-C: Determination of IL-6 (B) and IL-8 (C) levels in conditioned media collected from primary human dermal fibroblasts 96 hours following CSDS or CSDS combined with acyclic long-duration strain (ALDS). Data are mean±SEM normalized to CSDS; n≥8 per condition. * indicates p<0.05 against CSDS by unpaired t-test.