In vitro mimicking of therapeutic soft tissue stimulation regulates pro-inflammatory cytokines

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Research note

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Abstract

Objective Soft tissue manual therapies such as massage and myofascial release 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 studies established an in vitro model system for mimicking therapeutic soft tissue stimulation of dermal fibroblasts and established that injury-like strain induces the secretion of numerous pro-inflammatory cytokines. Moreover, mechanical strain replicating 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 force profiles intended to mimic repetitive motion strain increases levels of IL-6 in conditioned media. And, we confirm 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. Collectively, 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 mechanical stimulation of soft tissues.

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]. However, the cellular and molecular mechanism(s) mediating these effects are poorly understood.

A series of studies established an in vitro model system for mimicking therapeutic soft tissue stimulation of human dermal fibroblasts (reviewed in [6]). This work demonstrated that injury-like strain of fibroblasts induces the secretion of numerous cytokines; reduces fibroblast proliferation rate; and increases fibroblast apoptosis. Moreover, mechanical strain intended as a surrogate for soft tissue manual therapy reverses numerous aspects of this phenotype [7–10], including reduced secretion of pro-inflammatory interleukin (IL)-6, increased secretion of anti-inflammatory IL-1ra, increased fibroblast proliferation, and reduced fibroblast apoptosis.

We sought to replicate a portion of these findings and, here, 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. We also extend the prior work by reporting that in vitro therapy-like mechanical stimulation 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. Cells were 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, 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 (Fig. 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 (Fig. 2A). For acyclic long-duration strain (ALDS), after a 3-hour rest period, 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 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).

Table 1
Primer pairs utilized for RT-PCR analyses

Target	Forward Primer	Reverse Primer
IL-1β	CATTGCTCAAGTGTCTGAAGC	CATGGCCACAACAACTGACG
IL-6	TCTCCACAAGCGCCTTCG	CTGAGATGCCGTCGAGGATG
IL-8	GCGCCAACACAGAAATTATTGTAAA	TGCTTGAAGTTTCACTGGCATC
IL-10	GGCGCTGTCATCGATTTCTTC	TAGAGTCGCCACCCTGATGT
IL-12A	CAGAAGGCCAGACAAACTCT	GCCAGGCAACTCCCATTAGTTA
Hprt	CCTGCTGGATTACATTAAAGCACTG	GTCAAGGGCATATCCAACAACAAAC
TNF-α	TGTTGTAGCAAACCCTCAAGC	GAGGTACAGGCCCTCTGATG

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 [7, 11, 12], 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 (Fig. 1A), cells were repeatedly stretched between baseline and 10% beyond resting length every 1.6 seconds for eight hours [11, 12]. 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 [11], 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 (Fig. 1B) 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 (Fig. 1A), as previously described [11, 12]. Consistent with a prior report [11], ALDS reduced the levels of IL-6 in the conditioned media (Fig. 1C). ALDS also led to a similar reduction in IL-8 levels in the conditioned media (Fig. 1D).

Next, we examined a different CSDS profile [7] wherein primary human dermal fibroblasts were stretched between 10% beyond resting length and 16% beyond resting length every 1.6 seconds for eight hours (Fig. 2A). In contrast to a prior report [7], 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 [7] – and IL-8 in conditioned media compared to CSDS alone (Fig. 2B-C).

Conclusions

This study was designed to replicate and extend prior work evaluating the impact of therapeutic soft tissue stimulation using a cell-based model (reviewed in [6]). Our findings corroborate the observation that ALDS reduces levels of the pro-inflammatory cytokine IL-6. Additionally, we provide the first evidence that 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. However, there are important limitations to the in vitro model used by us and others. For example, 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. It is also likely that other factors in addition to those analyzed here are regulated by ALDS. Future work is required to address these open questions and advance the mechanistic understanding of therapeutic mechanical stimulation of soft tissues.

Abbreviations

CSDS
cyclic short-duration strain
ALDS
acyclic long-duration strain
IL
interleukin
TNF
tumor necrosis factor

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 and JWL contributed to the writing of this manuscript. All authors read and approved the final manuscript.

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Figures

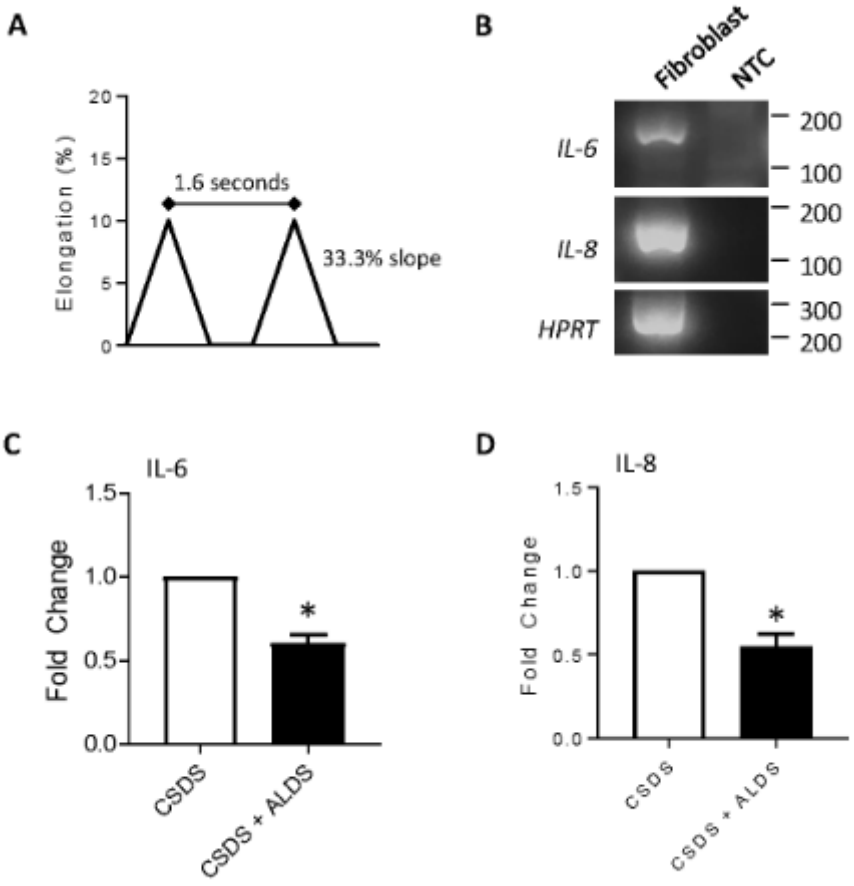

Figure 1

A: Schematic representation of cyclic short-duration strain (CSDS) profile number 1. B: 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. C-D: Determination of IL-6 (C) and IL-8 (D) 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=3 per condition. * indicates $p<0.05$ against CSDS by paired t-test.

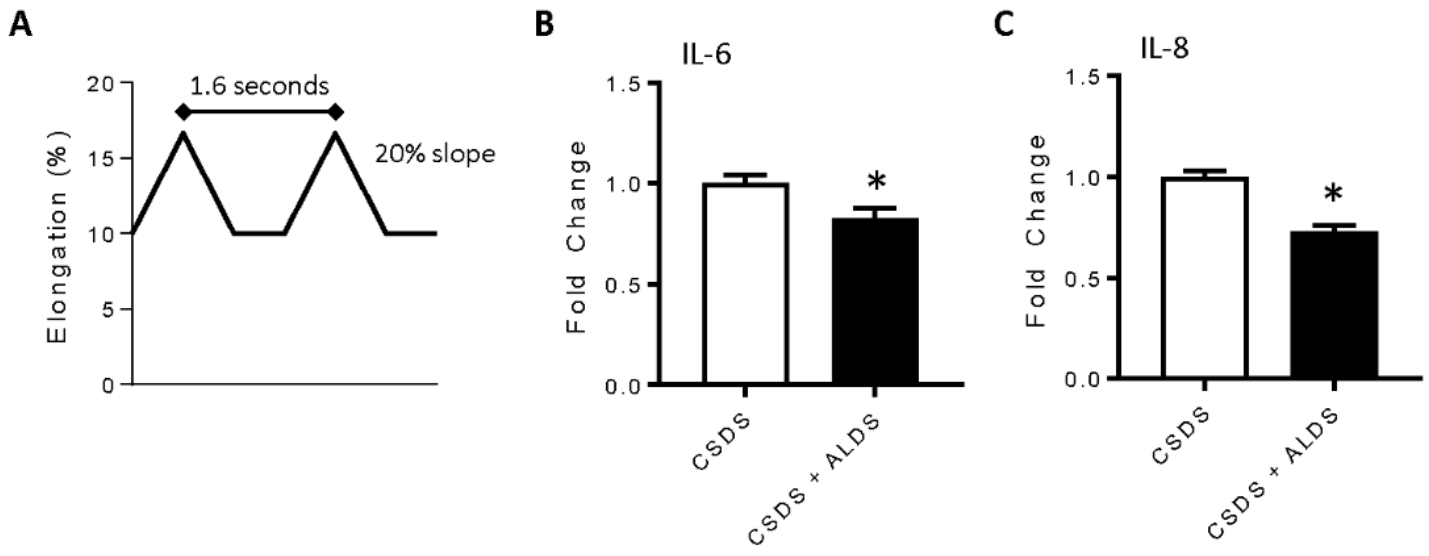

Figure 2

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.