In Vitro Biophysical Strain Model for Understanding Mechanisms of Osteopathic Manipulative Treatment

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Context: Normal physiologic movement, pathologic conditions, and osteopathic manipulative treatment (OMT) are believed to produce effects on the shape and proliferation of human fibroblasts. Studies of biophysically strained fibroblasts would be useful in producing a model of the cellular mechanisms underlying OMT.

Objective: To investigate the effects of acyclic in vitro biophysical strain on normal human dermal fibroblasts and observe potential changes in cellular shape and proliferation, as well as potential changes in cellular production of nitric oxide, interleukin (IL) 1β, and IL-6.

Design and Methods: Randomized controlled trial. Human fibroblasts were subjected in vitro to control conditions (no strain) or biophysical strain of various magnitudes (10%–30% beyond resting length) and durations (12–72 hours). After control or strain stimuli, fibroblasts were analyzed for potential changes in cell shape, proliferative capacity, nitric oxide secretion, and cytokine (IL-1β, IL-6) secretion.

Results: Low strain magnitudes (<20%) induced mild cellular rounding and pseudopodia truncation. High strain magnitudes (>20%) decreased overall cell viability and the mitogenic response, and induced cell membrane decomposition and pseudopodia loss. No basal or strain-induced secretion of IL-1β was observed. Interleukin 6 concentrations increased two-fold, while nitric oxide levels increased three-fold, in cells strained at 10% magnitude for 72 hours (P<.05).

Conclusion: Human fibroblasts respond to in vitro strain by secreting inflammatory cytokines, undergoing hyperplasia, and altering cell shape and alignment. The in vitro biophysical strain model developed by the authors is useful for simulating a variety of injuries, determining in vivo mediators of somatic dysfunction, and investigating the underlying mechanisms of OMT.

Limited research has allowed osteopathic physicians to make clinical inferences about the cellular mechanisms underlying osteopathic manipulative treatment (OMT). The osteopathic manipulative techniques of strain-counterstrain and muscle energy, for example, are thought to work at the neuromuscular and spinal cord levels, involving intrafusal fibers and Golgi tendon organs. However, the specific biochemical roles that neurotransmitters and other signaling molecules play in these techniques remain elusive.1,2 Osteopathic manipulative techniques of myofascial release, including compression, traction, and rotation maneuvers, are thought to reflexively release restrictions from soft tissue and joint-related structures by mechanically loading areas of tightness.3 However, it is nearly impossible to accurately quantify the complex biophysical forces that such techniques transmit through multiple layers of cell and tissue types. It is also nearly impossible to account for the neurohormonal feedback loops and psychological factors mediating OMT outcome.

Biophysical perturbation of tissues—whether resulting from injury, somatic dysfunction, or OMT—affects range of motion, pain, and local inflammation.3–6 Therefore, somatic dysfunction and OMT are both characterized by various biophysical strains placed on the microenvironment of cells and their surrounding tissue components.

Fibroblasts are a key component of the fascia that are routinely subjected to mechanical forces during normal physiologic movement, as well as in pathologic conditions and OMT. This fact makes fibroblasts uniquely poised to affect the amount and types of extracellular matrix proteins, neuromuscular modulators, cytokines, and vasoactive molecules secreted into the myofascial matrix. These substances regulate the underlying tone of skeletal muscle and are involved in chemotaxis and mitogenesis during the inflammatory response.

Previous studies7–9 have demonstrated changes in a variety of cell types in response to in vitro cyclic strain. For example, applied strain prompts cells to undergo predictable modifications in orientation,7,10 migration,10 gene regulation,7–10 and cytoskeletal protein alignment.7,10 However, there is a lack of controlled studies characterizing acyclically strained fibroblasts. Such studies would be useful for modeling some forms of postural injury and OMT.
In the present study, we sought to use human fibroblasts that were mechanically strained in culture to develop a useful model in which injury and OMT-related strain paradigms could be mimicked in vitro.

Methods
Human fibroblast cultures were randomly selected to receive strain stimuli or to be control cells, which did not receive strain stimuli. All cells were grown in culture in flexible collagen I-coated wells and allowed to adhere to the well surfaces and multiply for 24 hours. Cells were then kept in an inactive state for 24 additional hours.

Selected cells were next strained for indicated magnitudes (10%-30% beyond resting length) and times (12-72 hours). Control cells were not strained. During experiments in which magnitude or time were altered, replicate representative wells were removed for analysis while the remainder of replicates were subjected to strain stimuli in a nonrepeated measures format.

After control or strain periods were completed, the fibroblasts were analyzed for potential changes in cell shape, proliferative capacity, cellular protein content, protein-deoxyribonucleic acid (DNA) ratio, nitric oxide secretion, and cytokine (interleukin [IL] 1β, IL-6) secretion.

The number of experiments varied between 4 and 6 per experimental paradigm. For each paradigm, 3 to 6 replicate wells were analyzed per group. All cells from each experiment were derived from a single culture.

Human Fibroblast Cultures
In all steps of the present study, normal human dermal fibroblasts (Cambrex Corp, East Rutherford, NJ) were used. The fibroblasts were cultured in growth medium (Fibroblast Growth Medium 2; Cambrex Corp, East Rutherford, NJ) at 37°C (98.6°F), 5% carbon dioxide (CO2), and 100% humidity. They were supplied every other day with fresh growth medium. When confluent (usually 5–7 days), the cells were passed by removing them from the culture dishes, washing them, and reseeding them at lower densities in new dishes. For each experiment, passage number was matched. Thus, all cells from each experiment were derived from a single culture.

Strain Apparatus
A computer-assisted vacuum strain, or stretch, apparatus (Flexercell FX-2000; Flexcell International Corp, Hillsboro, NC), was used to deliver programmed strain regimens to fibroblasts in flexible wells attached to a base plate on the apparatus. The base plate contained six flexible wells (Flexwells) with collagen I-coated elastomer surfaces. Cells adhered to these surfaces and were strained under negative pressure. However, the cells themselves did not experience the negative pressure and were only subjected to a prescribed deformation caused by the vacuum acting upon the flexible collagen elastomer.

Throughout the duration of the strain procedure, the base plate remained in a tissue culture incubator. The investigator programmed the magnitude, duration, and frequency of strain. Valves located in the base plate were adjusted to allow selected culture plates to be strained while control plates remained unstrained.

Strain Paradigms
Fibroblasts were seeded (40,000–70,000 cells/well) in the collagen I-coated Flexwells. For each experiment, two or three Flexwell plates were used for control and strained groups alike. Each plate contained six replicate wells for a total of 12 to 18 wells per experiment.

After the cells were approximately 50% to 60% confluent (usually 24–48 hours postseeding), the growth medium was replaced with reduced-serum medium for 24 additional hours. On the day of each experiment, fresh reduced-serum medium was substituted. The fibroblasts were then acyclically strained for 0.25 hour to 72 hours at a magnitude of 10% to 30% over their initial resting lengths.

Assays, Photomicrography, and Immunohistochemistry
For all the strained and control groups, cellular concentrations of cytokines, DNA, and total protein were measured fluorometrically, and photographs of cell cultures and stained cytoskeletal components were analyzed for potential morphologic and viability changes. Double-stranded DNA (dsDNA) from freeze-thawed cells was measured using the FluorReporter Blue Fluorometric dsDNA Quantitation Kit (Invitrogen Corp, Eugene, Ore), while total protein was quantified colorimetrically using the Pierce BCA (bicinchoninic acid) Protein Assay (Pierce Biotechnology Inc, Rockford, Ill).

In the cell processing procedure used in the present study, adherent cells were lysed with deionized water. Then the cells were frozen at a temperature of −80°C (−112°F) for 1 hour and subsequently thawed to room temperature. The dsDNA and protein from the destroyed cells were released into the surrounding fluid and assayed without the need for further processing.

A subset of cells (1–2 wells from each of 12–18 control and strained groups per experiment) were fixed in formaldehyde for 10 minutes and washed (phosphate buffered saline) three times. These cells were then preincubated with bovine serum albumin, washed three additional times, and permeabilized with the nonionic surfactant Triton X-100 (Sigma Chemical Co, St Louis, Mo). After permeabilization, cells were washed and incubated again for 20 minutes. Rhodamine-conjugated phalloidin fluorescent staining solution was added to the cells and washed and incubated again for 20 minutes. The cells were then stained and washed and treated with Vectashield mounting medium (Vector Laboratories, Burlingame, Calif) to help prevent photobleaching. Phase contrast and fluorescence digital images were captured using an inverted fluorescent microscope (Olympus America Inc, 158 • JAOA • Vol 106 • No 3 • March 2006
ferences in population means among the respective groups. Population means were considered to be significantly different if $P < 0.05$. All statistical data were analyzed using the InStat software suite (Version 4.00; GraphPad Software Inc, San Diego, Calif).

Results

Major Cell Changes From Graded Acyclic Strain

The application of graded acyclic strain—specifically strain at magnitudes in excess of 10%—induced major alterations in fibroblast shape and partial or complete loss of cell viability.

Figure 1 illustrates morphologic alterations of cultured fibroblasts in the presence of acyclic strain, compared with no morphologic alterations in the absence of strain (controls). Cells in both the strain and control groups were photographed at 12, 24, and 36 hours after the experiment began. These times corresponded to strain magnitudes of 10%, 20%, and 30% for the cells in the strain group.

Throughout the 36 hours of this phase of the experiment, control cells displayed the spindle-shape appearance and well-defined pseudopodia typical of healthy, cultured human fibroblasts. Strained cells photographed after 12 hours of 10% strain displayed morphologic characteristics similar to those of healthy cells, though slight softening of the pseudopodia bor-

![Figure 1. Phase contrast photomicrographs of human fibroblasts acyclically strained to 10%, 20%, and 30% magnitudes above resting length at 12 hours, 24 hours, and 36 hours after beginning of strain, compared with controls at corresponding times. Arrows denote morphologic changes ranging from cellular rounding to blatant cell membrane damage (original magnification ×100).]
ders was noted in some of the strained cells.

Subsequent to these measurements, the strain magnitude was increased to 20% with the Flexercell strain unit; the strain remained at that magnitude for 12 hours. At the end of this time, a subpopulation of strained cells displayed rounding with complete absence of pseudopodia (Figure 1). Other cells, however, appeared unchanged.

Next, the strain magnitude was increased and maintained at 30% for 12 additional hours. At the end of this time, nearly all strained cells displayed either rounding or complete destruction, as noted by lack of an intact membrane structure (Figure 1). Exclusion data (not shown) based on tests using Trypan blue dye confirmed loss of cell viability in nearly 75% of cells strained in this manner.

Fibroblast growth measures were obtained at the same data points that cells were photographed in Figure 1. At each point (10%, 20%, and 30% strain magnitudes), representative wells were processed for quantification of dsDNA (a measure of cellular hyperplasia), cellular protein, and protein-to-DNA ratio (a measure of cellular hypertrophy).

Although unstrained control cells showed no significant changes in any of these parameters, strained cells displayed significant decreases in dsDNA at all strain magnitudes tested ($P < .05$), indicative of cell loss (Figure 2). Furthermore, at the 30% strain magnitude, strained cell cultures displayed significant

**Figure 2.** Proliferation index, as assessed with double-stranded DNA (dsDNA), in human fibroblasts acyclically strained to 10%, 20%, and 30% magnitudes at 12 hours, 24 hours, and 36 hours after beginning of strain, compared with controls at corresponding times. Data represent six experiments, each measured in duplicate.

Although unstrained control cells showed no significant changes in any of these parameters, strained cells displayed significant decreases in dsDNA at all strain magnitudes tested ($P < .05$), indicative of cell loss (Figure 2). Furthermore, at the 30% strain magnitude, strained cell cultures displayed significant

**Figure 3.** Cellular protein content in human fibroblasts acyclically strained to 10%, 20%, and 30% magnitudes at 12 hours, 24 hours, and 36 hours after beginning of strain, compared with controls at corresponding times. Data are based on minimum of 180 representative cells and maximum of 720 representative cells.

**Figure 4.** Protein-to-DNA ratio in human fibroblasts acyclically strained to 10%, 20%, and 30% magnitudes at 12 hours, 24 hours, and 36 hours after beginning of strain, compared with controls at corresponding times. Data are based on minimum of 180 representative cells and maximum of 720 representative cells.
loss of cellular protein ($P < .05$; Figure 3). These cells also displayed marked increases in their protein-to-DNA ratios, compared with unstrained controls, for all strain magnitudes tested ($P < .05$; Figure 4).

**Cell Changes at Moderate Strain Magnitude**

The application of acyclic strain at moderate (10%) magnitude induced alterations in fibroblast shape, growth measures, and cytokine production over the course of 72 hours.

*Figure 5* illustrates morphologic alterations of cultured fibroblasts in the presence of 10% acyclic strain, compared with no morphologic alterations in the absence of strain (controls). Cells were photographed at 6, 24, 48, and 72 hours after the experiment began.

Throughout the 72-hour period, cells of the control group displayed the spindle-shaped appearance with well-defined pseudopodia that is typical of healthy, cultured fibroblasts. Strained cells photographed after both 6 hours and 24 hours of 10% strain displayed morphologic characteristics similar to those of the control cells, though slight rounding of some of the strain cells' borders was noted at 24 hours (*Figure 5*). At 48 and 72 hours of 10% strain, more pronounced cell rounding was noted in the strained cells. Partial or complete absence of pseudopodia was also noted.

Despite these observed cellular modifications, no changes were noted in cell viability at 10% strain magnitude after 72 hours.

*Figures 6–8* illustrate the effects of 10% acyclic strain on fibroblast proliferation index (as assessed by dsDNS), nitric oxide secretion, and IL-6 secretion. Compared with unstrained control cells, those cells strained at 10% magnitude displayed a significant proliferative response at 48 and 72 hours ($P < .05$; *Figure 6*). Significant increases in levels of nitric oxide were observed in the strained cells at 24, 48, and 72 hours ($P < .05$; *Figure 7*). Similarly, these cells secreted significantly increased levels of IL-6 after 48 and 72 hours of strain ($P < .05$; *Figure 8*).

Interleukin 1β was assayed in conditioned media from all experiments conducted in the present study. However, its presence was not detected under any control or strain paradigm tested.

**Moderate Strain and Intracellular Actin Localization**

*Figure 9* illustrates intracellular actin localization in unstrained fibroblasts (controls) at 48 hours after the beginning of an experiment in which other fibroblasts were strained at 10% magnitude (*Figure 10*). The control cells display intracellular actin filaments arranged along several intersecting axes. Actin in these cells concentrated at the periphery of numerous, elongated pseudopodia. Strained cells display an increased clustering and a general lack of elongated pseudopodia compared with control cells.

Data represented by *Figure 9* and *Figure 10* suggest that strained cells aligned in response to applied strain (ie, the strain vector was along a vertical line). However, the long axis
orientation of control cells appeared random (ie, the orientation was between 0 and 90 degrees relative to the strain vector).

**Comment**

The present study reveals that human fibroblasts respond to acyclic strain by altering cell shape and alignment, undergoing hyperplasia (ie, abnormal multiplication of cells), and increasing IL-6 and nitric oxide secretion. At strain magnitudes nearing 30%, cellular destruction becomes prevalent. However, at moderate strain magnitudes (10%), strain-induced proliferation is observed at 48 hours with little loss in cell viability. Although IL-1β secretion was not detected from fibroblasts exposed to acyclic strain, basal secretion of IL-6 increased markedly after 48 hours of strain. Nitric oxide secretion increased two-fold as early as 24 hours after application of strain. The increased secretion of IL-6 and nitric oxide may provide insight into the pathophysiology of fibrosis and tissue texture changes seen with injury and OMT.

Strained fibroblasts display increased clustering, a fusiform cell shape, and a general lack of elongated pseudopodia associated with intracellular actin. In addition, strained cells may align themselves (aided by actin mobilization) partly in response to applied strain.

These data highlight the potential utility of the in vitro biophysical strain model developed in this study to examine the...
cellular mechanisms underlying clinical signs and symptoms of strain-induced injury and OMT-directed counterstrain.

**Validation of the Fibroblast as an Appropriate Cell Model**

The present study focused on human fibroblasts because of the role these cells play as the main component of the myofascial architecture supporting various tissues, including bone, ligament, lymphatic, nerve, tendon, and vascular tissue. Fibroblasts secrete collagen, fibronectin, plasminogen, vitronectin, and other extracellular matrix proteins, which act as multifunctional adhesion substances that form the necessary scaffolding for tissue and cellular support and promote cellular migration to sites of injury.\(^{12,13}\)

This extracellular matrix is constantly affected by biophysical strain from both normal and injurious strain patterns. It also acts as an efficient conduit for the transmission of strain to the cell matrix through interactions of extracellular matrix ligands, intracellular actin, and integrin molecules. Such matrix interactions allow for bidirectional communication between the cytoplasm and the extracellular fluid in regard to cytoskeletal architecture, cellular alignment, and cellular migration.\(^{14}\)

Previous studies\(^{15-20}\) have shown that biophysical strain regulates the synthesis and secretion of autocrine molecules and extracellular matrix proteins from fibroblasts, smooth muscle, and other tissue types. Although human fibroblasts are as diverse in function as they are in their orientation throughout the body,\(^{21}\) we believe they represent an excellent cell model to study strain-induced alterations in injury and OMT. Investigating the effects of other cell types (eg, myofibroblasts) with this strain model will likely yield additional information about strain- and counterstrain-induced tissue remodeling.

**Biophysical Perturbations to the Cellular Microenvironment**

Biophysical strain regulates cellular proliferative capacity, production of extracellular matrix molecules, gene expression patterns, and the contractile state of fibroblasts through the actions of transmembrane mechanoreceptors.\(^{22}\) In vitro strain models have proven to be a reliable standard for studying the effects of these mechanical forces on many types of cells.\(^{8,10,15,17}\) Therefore, one of our earliest objectives was to establish minimum and maximum strain thresholds, which would affect physiologic change and loss of cellular viability, respectively. Two quantifiable measures of such change are cellular shape and viability in response to various strain magnitudes and durations. The data collected in the present study correlate well with other in vitro studies that have found increased cellular response to strain magnitudes of less than 30%, as well as to in vivo studies that have identified strain...
dimensional strain system. In certain in vitro studies of three-dimensional architecture increases cellular sensitivity to strain. Thus, to more closely mimic the in vivo environment, we plan to investigate cellular alterations in response to lower magnitude strain in a three-dimensional system.

Despite this study’s limitations, programming appropriate counterstrain paradigms to determine potential reversal of its findings will be challenging—given clinician variability and difficulty in assessing what fraction of applied strain is transferred to deep tissue.

Tissue injury and tissue repair each involve a complex and coordinated set of cellular mechanisms of a proliferative and an apoptotic (ie, programmed cell death) nature.6 Upon injurious strain, both hyperplastic and hypertrophic responses occur, leading to in vivo fibroblast growth. These increases in cell size and number aid the osteopathic physician in palpat ing the tissue texture changes. Outcomes of the application of strain in the present study’s in vitro model parallel these in vivo responses.

The mitogenic response likely leads to further deposition of extracellular matrix proteins, contributing to vascular extravasation, capillary fluid slowing, and tissue congestion.14 There are many factors regulating this deposition and congestion process, one of which is the well-known mechanotransduction signaling pathway involving extracellular matrix adhesion molecules and stretch-activated membrane channels. Although the primary mechanosensor is still unknown, studies have linked mechanical signals to the enhanced activation of intracellular mitogenic signaling pathways.10,22,26,27

We are currently conducting studies designed to investigate cellular proliferation rates over longer strain durations than those examined in the present study. We are also investigating proliferation rates after cessation of strain or induction of appropriate counterstrain to determine if the increases in these rates are reversible.

**Chemical Mediators of Cellular Strain**

Interleukins and other cytokines have been associated with fibroblast-based tissue changes and in the mediation of extracellular matrix protein secretion during cyclic strain.8,17,19,28–30 Nitric oxide, a potent vasodilator and apoptotic molecule, has been shown to orchestrate wound healing25 and to play a major role in decreasing collagen deposition while maintaining a cytostatic state during injury and repair.31,32 The data in the present study strongly support the implication of IL-6 and nitric oxide in these processes, because the rates at which they were secreted increased during strain-induced growth responses.

Although we have not yet conclusively shown a cause-and-effect relationship between these mediators and the growth responses, it is interesting to note from previous reports33 that nitric oxide seems to be stimulated by both strain and interleukin activity. Furthermore, increases in nitric oxide secretion due to cyclic strain—and the subsequent decreases in IL-6 secretion in cytokine-treated fibroblasts—appear to confirm cross-communication between pro-inflammatory/mitogenic pathways and apoptotic pathways.

Previous reports,33 along with our preliminary data showing an increase in IL-6 and nitric oxide in response to acyclic strain, support our proposal that the need for balance between cell growth and cell death may be grossly shifted in patients with somatic dysfunction. The balance between the proliferative and apoptotic pathways may be tipped toward the side of proliferation during the early phase of strain-induced injury. Later, when this balance is reversed, damaged cells are ushered out, signaling the end of the reparative state.25

**Biophysical Strain: Heterogeneity and Cessation**

Previous studies34,35 have determined that cyclic strain induces cell alignment and migration as soon as 3 hours after injury, and that this alignment and migration persists for a short period following the cessation of strain. These findings suggest that cells respond in vitro to dynamic strain in order to create the most energetically efficient architecture. Moreover, cells lose this ability when there is a cessation of cyclic strain. Thus, the misalignment of fibroblasts and their associated extracellular matrix proteins during acyclic strain may explain, in part, the mechanisms underlying fibrosis and decreased range of motion.

The data collected in the present study are unique in that we used acyclic strain to observe the same kinds of proliferative responses previously reported for cyclic strain.7–9 Although the cellular alignment response to acyclic strain appears attenuated when compared with the cyclic strain regimen, data suggest that cells migrate in response to the biophysical stimulus of acyclic strain (Figure 9 and Figure 10). Therefore, while “static” acyclic strain appears to be a weaker stimulus for cell
growth and alignment, it can nevertheless be effective, especially if the strain stimulus persists for a long time.

If the reversibility of these biophysical effects can be documented upon cessation of strain or upon appropriately applied counterstrain maneuvers, such documentation would support the idea of fascia exhibiting a “memory” of cellular/extracellular matrix protein orientation and gene expression from the pre-injury state.

Conclusion

Evidence that fibroblast-derived inflammatory and anti-inflammatory cytokines, as well as nitric oxide, play important roles in injury and OMT is persuasive. We plan to continue investigating strain in terms of an injury-and-repair process in order to examine the effects of placing appropriately programmed counterstrain and compressive forces on strained cells. Our goals are to identify the potential reestablishment of normal cytokine expression; any reversible changes to fibroblast cytoskeletal structure, proliferation, or shape; and any reversible changes in inflammatory cytokine expression. Programming such counterstrain paradigms will require further analysis of clinician-directed biophysical maneuvers to best mimic similar biophysical manipulations in vitro.

The Flexercell FX-2000 strain apparatus is adaptable to studying an infinite variety of acyclic and cyclic strain magnitudes, frequencies, and durations of interest to osteopathic medical researchers. Therefore, the in vitro biophysical strain model developed in the present study holds great promise for unraveling the cellular and molecular mechanisms underlying strain-regulated injury and OMT.

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