Mechanical Strain Alters Gene Expression in an in Vitro Model of Hypertrophic Scarring

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Abstract: Fibroblasts represent a highly mechanoresponsive cell type known to play key roles in normal and pathologic processes such as wound healing, joint contracture, and hypertrophic scarring. In this study, we used a novel fibroblast-populated collagen lattice (FPCL) isometric tension model, allowing us to apply graded biaxial loads to dermal fibroblasts in a 3-dimensional matrix. Cell morphology demonstrated dose-dependent transition from round cells lacking stress fibers in nonloaded lattices to a broad, elongated morphology with prominent actin stress fibers in 800-mg-loaded lattices. Using quantitative real-time RT-PCR, a dose dependent induction of both collagen-1 and collagen-3 mRNA up to 2.8- and 3-fold, respectively, as well as a 2.5-fold induction of MMP-1 (collagenase) over unloaded FPCLs was observed. Quantitative expression of the proapoptotic gene Bax was down-regulated over 4-fold in mechanically strained FPCLs. These results suggest that mechanical strain up-regulates matrix remodeling genes and down-regulates normal cellular apoptosis, resulting in more cells, each of which produces more matrix. This "double burden" may underlie the pathophysiology of hypertrophic scars and other fibrotic processes in vivo.

Key Words: mechanotransduction, matrix proteins, wound healing

(*Ann Plast Surg* 2005;55: 69–75)

The fibroblast is the major cell type found in the dermis and the principal cell type responsible for wound contraction and scar tissue deposition and remodeling. During wound healing, fibroblasts migrate from the dermis into the wound bed, where they change both form and function in response to mechanical stimuli. Data from previous studies demonstrate that mechanically stimulated fibroblasts in granulation tissue of a wound differentiate into a myofibroblast phenotype.[1,2] These specialized cells are characterized by the presence of a microfilamentous contractile apparatus consisting of actin stress fibers, fibronexus adhesion complexes, and the expression of α-smooth-muscle actin.[3] The contractile forces produced by these cells are believed to approximate the wound edges in normal healing and have also been implicated in pathologic processes, including the development of mobility-limiting joint contractures.

Mechanical stimuli exert a wide range of effects on cell function through the process of mechanotransduction. Mechanical stimuli have been shown to influence fibroblast processes such as cell differentiation, proliferation, survival, and matrix production.[3–6] Yet, the pathogenesis through which mechanical stimuli translate into an altered fibroblast phenotype remains poorly characterized, especially at the genetic level. The physiologic importance of this phenomenon is suggested by the fact that hypertrophic scars are observed clinically to form predominantly in areas of increased skin tension.

Recognizing that fibroblasts are a mechanoresponsive cell type with potential importance in pathologic processes, many labs have developed in vitro models to study the fibroblast response to mechanical stimuli and the mechanisms underlying these changes. Early experiments employed the use of 2-dimensional, elastic silicone membrane models and were successful in demonstrating the response of fibroblasts to varying degrees and frequencies of cellular stretch. However, these models failed to place fibroblasts in an environment which resembled their in vivo milieu.[4,6] This led to the development of the fibroblast-populated collagen lattice (FPCL) model. Placing fibroblasts in a type I collagen lattice allows them to develop 3-dimensional, integrin-mediated interactions with a normal ECM substrate, which more accurately represents cells in vivo.[4,6,7]

Received March 23, 2005; accepted for publication March 24, 2005.

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Presented at the 21st Annual Meeting of the Northeastern Society of Plastic Surgeons, January 19–23, 2005, Naples, Florida.

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ISSN: 0148-7043/05/5501-0069
DOI: 10.1097/01.sap.0000168160.86221.e9

The most widely used FPCL models use self-generated, isometric tension in which fibroblasts contract against a collagen lattice that is restrained by a wire, thread, or adherence to the culture dish, thereby generating the force applied to them.[7–9] The magnitude of force in restrained-lattice models ranges from 40–60 dynes per million cells,[10] but the load placed upon fibroblasts in such models cannot be modulated and is largely controlled by fibroblasts' mechanical homeostatic mechanisms.[11] Utilizing an external source of loading allows the application of exact, graded, reproducible amounts of tension to FPCLs,[12] allowing a more precise examination of the transcriptional response in the cells. In this study, we employ a novel load-bearing apparatus that allows reproducible, graded amounts of biaxial load to be applied to dermal FPCLs in vitro.[13]

MATERIALS AND METHODS

Cell Culture

Normal human dermal fibroblasts were isolated from skin excised during female breast reduction. All patients were healthy and ranged in age from 18–25 years. Dermal tissue was surgically isolated and placed in a 0.2% collagenase type I solution (Life Technologies, Inc.) for 12 hours. Cells were harvested via centrifugation at 1000 rpm for 5 minutes, suspended in Dulbecco modified Eagle media (DMEM) with 10% FBS, 1% antibiotics and 50 mM ascorbic acid, plated in T150 culture flasks, and grown in a humidified (5% CO_2) incubator at 37°C. Cells were passaged at 90% confluence using 0.05% trypsin and DMEM/10% FBS in a 1:3 split.

Collagen Lattice Preparation

Type I collagen lattices were prepared as previously described.[13] Briefly, the collagen solution consisted of 80% Vitrogen bovine dermal collagen (Cohesion Technologies, Inc.), 10% 0.2 M/pH 9 HEPES buffer (Life Technologies, Inc.) and 10% minimal essential media (Life Technologies, Inc.). Confluent fibroblasts, passage 4 or earlier, were serum starved for 24 hours and harvested using 0.05% Trypsin and DMEM/10% FBS 3:1. After centrifugation at 1000 rpm for 5 minutes, these cells were resuspended in serum-free media; 5×10^6 fibroblasts in 2 mL DMEM were added to 8 mL of collagen solution to give a 5.0×10^5/mL fibroblast, 2.0 mg/mL collagen solution.

Load Application

The loading system was designed to load 3-dimensional FPLCs using small freely hanging weights. The collagen-cell solution was poured into square $4 \times 4 \times 1$ cm molds to a volume of 10 mL each and incubated at 37°C for 3 hours; $20 \times 4 \times 3$ mm polyethylene (Fisher Scientific) bars at the midpoint of each side of the mold were attached to 5-0, monofilament nylon suture. After polymerization, the mold was removed, leaving a 40×40 mm lattice with the polyethylene bars embedded in each side of the gel. The load-bearing apparatus has been previously described[13] (Fig. 1A). Clay weights of masses 0, 50, 100, and 200 mg were attached to the end of the sutures, thereby placing exact and equal loads of 0, 200, 400, or 800 mg to the lattices. Twenty milliliters of DMEM, 10% FBS, 1% Abx, 50 mM ascorbic acid media was added to the culture dish; lattices were released from their attachment to the Petri dish and incubated in a humidified (5% CO_2) incubator at 37°C for 18 hours. The degree of gel contraction was determined, media were collected, samples were harvested for morphologic analysis, and isolation of total RNA from gels was performed to detect changes in matrix protein mRNA levels via quantitative real-time PCR gene analysis.

Gel Contraction Quantification

To quantify the lattice contraction from each load group, lattices were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. They were washed 3 times with PBS and the sides of the gels measured to allow calculation of the surface area of each gel in cm^2 ($n = 6$).

Fluorescence Microscopy

Collagen lattices were fixed in 4% paraformaldehyde for 30 minutes and washed with PBS. Lattices were permeabilized using a 0.5% saponin/0.05% sodium azide solution for 15 minutes. After washing with PBS, lattices were cut into whole-mount sections and stained for 30 minutes using a FITC-labeled, F-actin-staining phalloidin (Sigma). Sections were rinsed with PBS, mounted with vectashield mounting

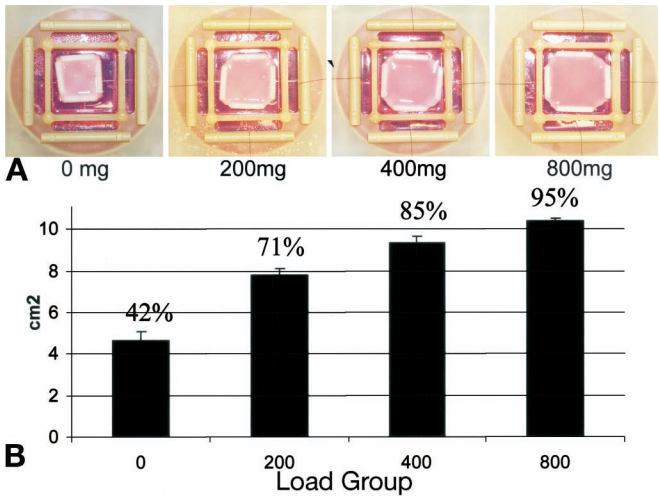

FIGURE 1. A, Fibroblasts were cultured in collagen lattices under gradated strain for 18 hours. Representative photographs of FPCLs after contraction are shown here demonstrating trends observed in surface lattice contraction. B, This graph depicts the changes in surface area (cm^2) for each load group after 18 hours of contraction.

medium with DAPI (Vector Labs, Inc.), and viewed and photographed using an Olympus BX51 microscope.

RNA Extraction and Real-time Quantitative RT-PCR

After 18 hours, the polyethylene bars were removed, and the lattices were placed into 5 mL of Trizol Reagent (Life Technologies, Inc.) and homogenized at 5000 rpm using a Polytron homogenizer (Kinematica). Total RNA was extracted using the Totally RNA extraction protocol (Ambion) and purified with the RNA Easy Mini Kit (Qiagen). The mass and purity of the extracted RNA were determined via spectrophotometry in an Ultraspec 2000 (Pharmacia Biotech).

cDNA libraries were constructed using the RNA PCR Core Kit (Applied Biosystems), and quantitative real-time PCR was performed using the Roche Light Cycler 1.2 instrument and the Lightcycler FastStart DNA Master^{PLUS} SYBR Green I reaction mix. Primers used in this experiment are as follows: human GAPDH F: AACATCATCCCTGCCTCTAC, human GAPDH R: CCCTGTTGCTGTAGCCAAAT, human MMP-1 F: CTGCTTACGAATTTGCCGAC, human MMP-1 R: GCAGCATCGATATGCTTCAC, collagen-1A2 F: ACCGACCTCACCACGTTCA, collagen-1A2R: ATTGGTAACCCTGGCAGAGA, collagen-3A1F: GACTGCCACGTTCACCTTTG, collagen-3A1 R: CTGGTGCTCCTGGACAGAAT, α-smooth muscle actin F: ACAACAGCATCATGAAGTGT, α-smooth muscle actin R: CCAGTAGCCTATTTCAGATT, Bax-F: CATGGAGCTGCAGAGGATGAT, Bax-R: TTGCCGTCAGAAAACATGTCA. A final concentration of 0.5 μm of each primer set was used in each reaction. The identity of all PCR products was confirmed by agarose gel electrophoresis and by DNA sequencing.

MMP-1 ELISA

MMP-1 protein levels were quantified using an enzyme activity assay kit (Biotrak MMP-1 Activity assay system, Amersham Pharmacia Biotech, UK) following the manufacturer's protocol. Lattices were incubated in DMEM, 10% FBS, 1% antibiotics and 50 mM ascorbic acid for 18 hours. After 18 hours, fresh media were exchanged and lattices were incubated for an additional 12 hours. Cell supernatants were harvested at 36 hours and 100 μL loaded into each well. Samples were compared with a serial dilution of standards (assay range 3.13–50 ng/mL). Total MMP-1 levels were determined after addition of 25 μM of *p*-aminophenylmercuric acetate (PAMA) and absorbance was read at 405 nm after 90 minutes.

RESULTS

To demonstrate the effect of externally applied, graded, biaxial mechanical load on fibroblast morphology and gene expression, fibroblasts were cultured in 3-dimensional collagen lattices for 18 hours, bearing 0, 200, 400, and 800-mg loads. Contraction of free-floating lattices against the load began immediately after the detachment from the Petri dish, and peak contraction occurred by 18 hours.

Serum-Induced FPCL Contraction

The percent contraction of these lattices was inversely proportional to the load placed upon them (Fig. 1A). The surface area of the gels immediately after polymerization was approximately 16 cm^2. The surface areas after 18 hours' postpolymerization of 0, 200, 400, and 800-mg-loaded lattices were 4.63 $\pm$ 0.46 cm^2 (42%), 7.8 $\pm$ 0.32 cm^2 (71%), 9.33 $\pm$ 0.34 cm^2 (85%), and 10.4 $\pm$ 0.14 cm^2 (95%), respectively (Fig. 1B).

Changes in Cell Morphology Occur in a Load-Dependent Fashion

Previous studies describing the effects of mechanical strain on fibroblast morphology have largely used isometric tension models in which cells were either exposed to isometric tension (stressed) or no load (free floating).[14] Fibroblasts in free-floating lattices have a round morphology and lack stress fibers and focal adhesions, whereas isometrically stressed fibroblasts have a broad, elongated cell shape and develop actin stress fibers and focal adhesions.[6,7,15,16] We used our model to determine if these changes occur as an "all-or-none" phenomenon or in a load-dependent manner. Whole-mount sections of fixed lattices were stained with a FITC phalloidin, which binds f-actin, to examine the development of actin stress fibers in the microfilamentous contractile apparatus. We observed a load-dependent transition in cell morphology from the 0- to 800-mg groups. As the load increased, cells adopted a more elongated and broad cell shape, with increasing numbers and size of cell processes. Interestingly, while the morphology of 400-mg-loaded gels began to approach that of the 800-mg-loaded group, actin stress fibers were relatively sparse in 400-mg lattices and abundant in 800-mg-loaded gels (Fig. 2).

Load-Dependent Changes in Gene Expression

We chose to examine the effect of externally applied, graded tension on the pretranslational regulation of matrix metalloproteinase-I (collagenase), type 1 collagen (seen in mature scars and dermis), type 3 collagen (found predominantly in immature, remodeling matrix), and α-smooth-muscle actin to determine if the regulation of these matrix molecules is controlled by load-sensitive mechanoresponsive elements. Total RNA was extracted from FPCLs exposed to graded tension, and quantitative real-time PCR analysis was performed (Fig. 3).

We appreciated a 2.8-fold induction of collagen-I mRNA transcription in the 800-mg lattice in comparison to the 0-loaded lattice ($P < 0.01$). This was consistent with previous publications noting excess collagen I in human

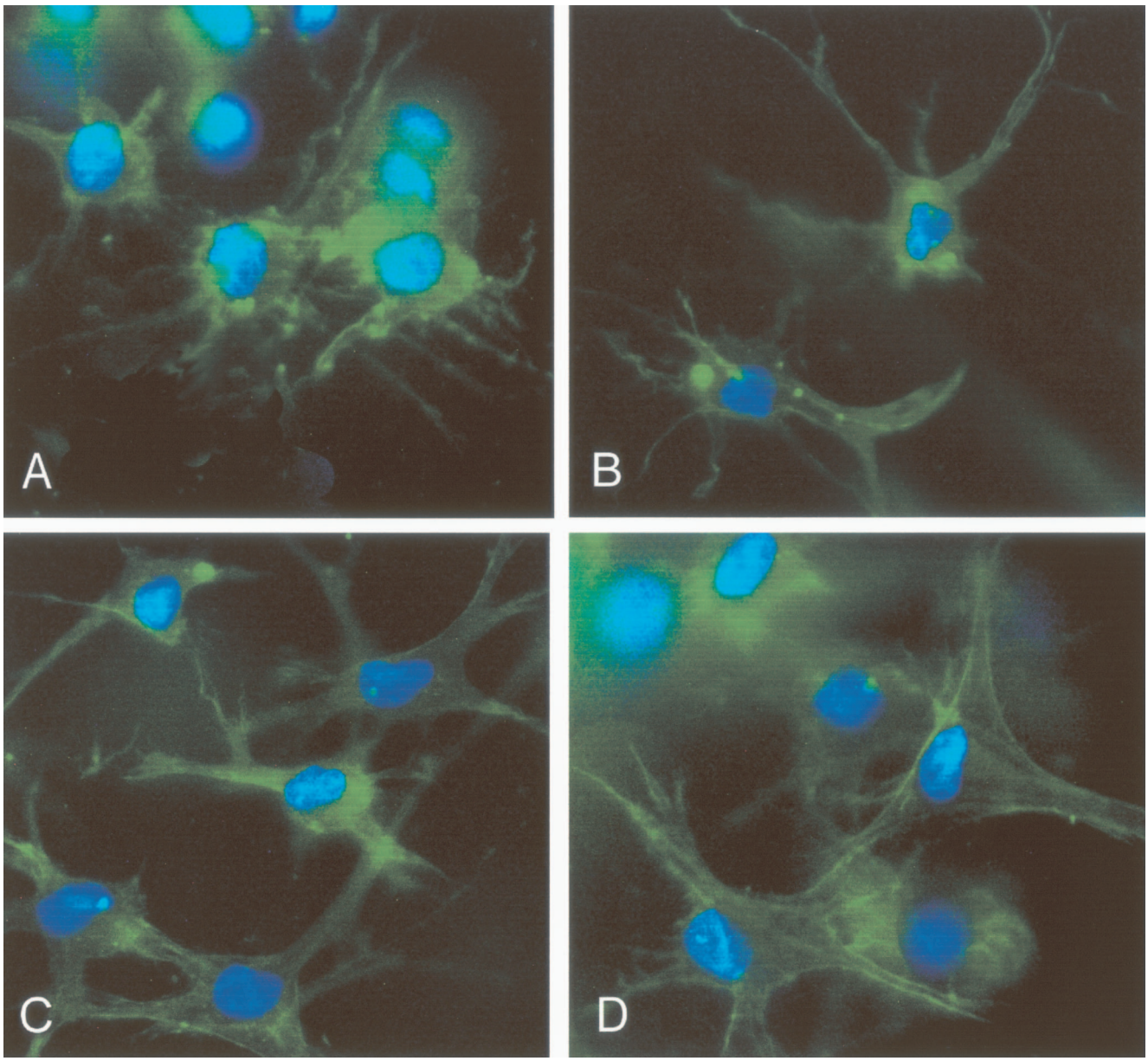

FIGURE 2. Fibroblasts were cultured in 0-, 200-, 400- and 800-mg-loaded FPCLs for 18 hours, fixed, and stained with FITC-phalloidin and DAPI nuclear stain, as described. The overlay images in A–D are representative of the morphology observed in 0-, 200-, 400-, and 800-mg-loaded lattices, respectively.

hypertrophic scars[5,9] (Fig. 3A). In conjunction, the mRNA expression of collagen III increased proportional to the load placed upon the lattice, with a 3-fold increase in the 800-mg lattice as compared with the 0-loaded lattice ($P < 0.01$) (Fig. 3B).

Previous studies using restrained FPCL models reported that self-generated tension caused a down-regulation of MMP-1 as compared with unrestrained, free-floating FPCLs. Based upon these findings, we hypothesized that MMP-1 mRNA levels would decrease with increasing tension in a load-dependent fashion.[5,9] Surprisingly, we found that externally applied, passive tension led to an increase in FPCL MMP-1 mRNA levels. This up-regulation occurred in load-dependent fashion, where the mRNA level was directly proportional to the load placed upon the lattice. There was a 2.5-fold difference in MMP-1 mRNA between the 0-loaded and 800-mg-loaded lattices, as determined by the relative number of mRNA transcripts ($P < 0.01$) (Fig. 3C).

In addition, we chose to look at the expression of α-smooth-muscle actin mRNA, a marker for myofibroblast

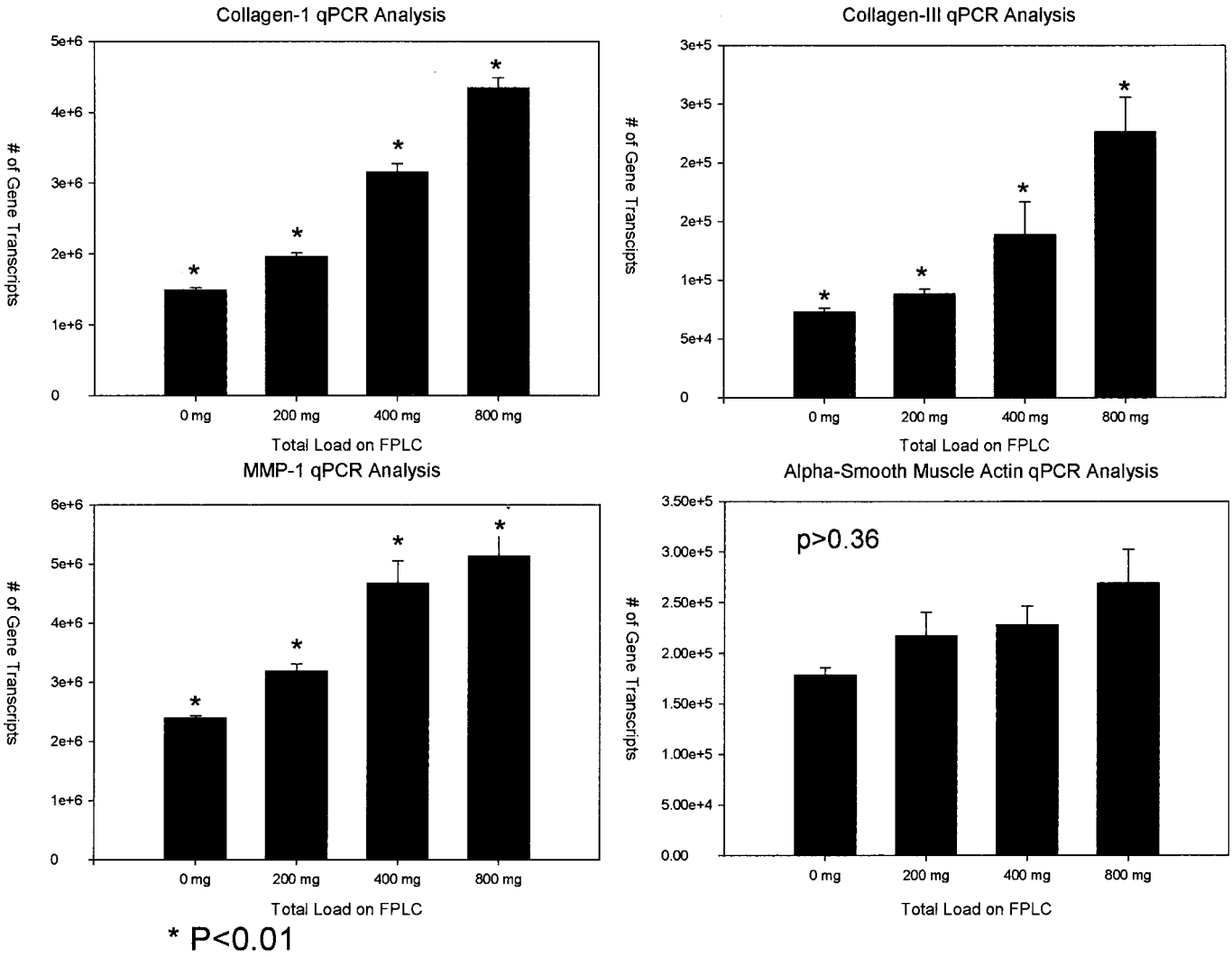

FIGURE 3. Fibroblasts were cultured under graded strain for 18 hours. mRNA from 0- to 800-mg-load lattices was analyzed via quantitative real-time RT-PCR as described. This figure shows representative total gene copy numbers (normalized to GAPDH expression) demonstrating expression levels of MMP-1, COLα2, COLIIIα1, and α-SMA for each load group.

differentiation, in our FPLCs. Previous studies have demonstrated that, in the absence of TGF-β supplementation, fibroblasts must be exposed to greater than 3 days of elevated mechanical stress for detectable levels of α-smooth-muscle actin to be produced and the transition from protomyofibroblast to myofibroblast phenotype to occur.[5,6,17–19] We demonstrate here that only very low levels of α-SMA mRNA were detected in our lattices at 18 hours ($P > 0.05$) (Fig. 3D).

Load-Dependent Changes in MMP1 Secretion

To confirm the unexpected finding of an increase in MMP-1 mRNA levels with increasing tension loads, we quantified the levels of MMP-1 protein secreted into the media during the loading period. FPCLs were exposed to gradated load in the presence of serum-supplemented media for 18 hours to allow for equilibration of gene expression and protein production. MMP-1 protein expression by ELISA demonstrated a load-dependent increase that mirrored our mRNA findings. MMP-1 concentrations were 0.55 $\pm$ 0.02, 0.80 $\pm$ 0.07, 0.99 $\pm$ 0.02 and 1.08 $\pm$ 0.02 ng/mL in 0-, 200-, 400-, and 800-mg-load media, respectively (Fig. 4).

Proapoptotic Gene Bax

Based on preliminary data from a microarray of our novel in vivo murine hypertrophic scar model (manuscript in progress), we analyzed the effect of mechanical strain on the expression of the proapoptotic gene Bax. Mechanical strain decreased the expression of the proapoptotic gene Bax up to 4-fold in comparison to the unstrained control ($P < 0.01$, Fig. 5). Physiologically, an antiapoptotic process may lead to the

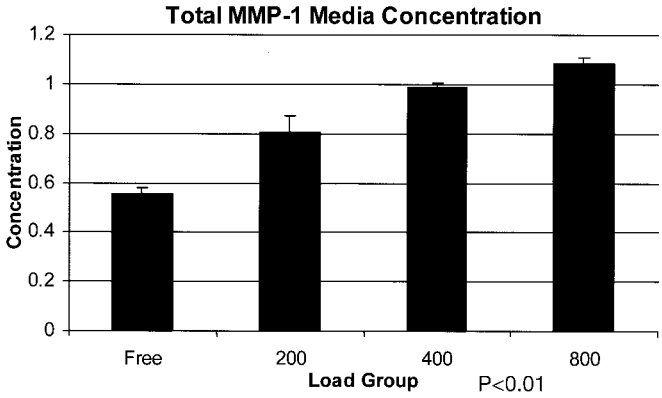

FIGURE 4. Fibroblasts were cultured in 0-, 200-, 400-, and 800-mg-loaded FPCLs for 18 hours. Fresh media were exchanged and lattices were cultured for an additional 12 hours. Total MMP-1 concentration in the conditioned media of 0- to 800-mg-loaded lattices was measured via ELISA. This graph depicts the total MMP-1 concentration for each load group.

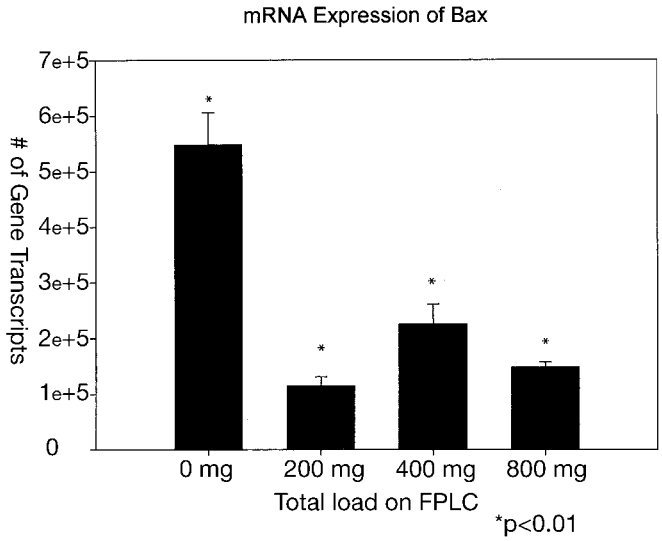

FIGURE 5. Real-time qPCR gene expression analysis of the proapoptotic gene Bax. Mechanical strain reduces expression of Bax up to 4-fold over the unloaded control.

persistence of fibroblasts that normally undergo apoptosis in the organizing scar, allowing for increased numbers of cells contracting and producing ECM substrate. This cycle may be perpetuated by the continuous level of tension sustained by the activated fibroblasts and wound environment, and thus tension may serve as a key mediator in the pathogenesis of hypertrophic scar formation.

DISCUSSION

The purpose of this study was to determine if there is a load-dependent effect on fibroblast morphology, mRNA levels, and protein production. Our in vitro model consisted of fibroblasts embedded in a type I collagen lattice, to which gradated tension was applied using a novel system for the study of dermal fibroblasts. The data presented here indicate that externally applied, graded tension generates reproducible load-dependent changes in fibroblast morphology, matrix protein mRNA levels and MMP-1 secretion.

In our system, we observed that changes in lattice area were inversely proportional to the applied load, with minimal decreased area evident in 800-mg-loaded lattices (Fig. 1). This likely represents a critical load that exceeds the collective contractile potential of these fibroblasts. Likewise, load-dependent changes in cell morphology were observed under microscopic examination of whole-mount lattice sections, where cells had a broader, more elongated shape with increasing load. Whereas 0-mg- and 200-mg-loaded lattices demonstrated a paucity of actin stress fibers, scattered actin stress fibers were present in cells of 400-mg lattices, and 800-mg lattice fibroblasts contained many, prominent stress fibers, consistent with the findings of isometric-tension studies.[2–4] Despite the presence of such prominent contractile filaments, 800-mg-loaded lattices exhibited limited ability to contract after 18 hours of culture.

The in vivo environment of dermal fibroblasts is mechanically dynamic, and one would expect that these cells would have compensatory mechanisms to adjust to constant changes in load. Indeed, the ability of dermal fibroblasts to maintain mechanical homeostasis in collagen lattices has been well established.[10–12,20] Upon stretch or relaxation of a FPCL, fibroblasts rapidly increase or decrease contraction until baseline tension levels have been restored. Since these cells have the ability to sense and adjust to changes in the surrounding mechanical state, isometric tension models are only able to produce a level of tension within the fibroblasts' homeostatic limits. Thus, to subject fibroblasts to loads consistent with wounds under tension, it is necessary to use an external source of tension that exceeds their range of force generation.

The changes in gene transcription (mRNA) observed in our model follow a logical pattern with regard to normal, physiologic processes, as well as pathologic conditions. We hypothesized that the levels of collagen I and collagen III mRNA would mirror the excessive matrix deposition seen in dysfunctional wound healing processes such as hypertrophic scarring. In accordance with our hypothesis, we found that the application of mechanical strain generated an increase in the mRNA levels of genes integral to ECM remodeling, such as the type 3 collagen found in immature wounds[3,8,21] and type 1 collagen found in organizing wounds.[22] Previous studies[8] have suggested that a down-regulation of MMP's supports a "synthetic phenotype." Our results indicate that load linearly induces collagen III and collagen I, as well as MMP-1 (collage-

nase) gene expression, suggesting that the overall process of matrix remodeling is augmented by load. The up-regulation of MMP-1 gene transcription was confirmed at the protein level because it contradicts existing published data.[8,23] Hypertrophic scar formation may occur when the equilibrium of ECM formation and degradation is disturbed by mechanical tension, thus slanting towards ECM production and matrix accumulation.

Clearly, the prolonged time course of scar formation suggests that subtle imbalances in matrix production and breakdown are more likely responsible for hypertrophic scar formation. The propensity for hypertrophic scars to form on areas of the body where the skin is under greater tension correlates clinically with our findings that mechanical tension modulates ECM production. Our model allows us to determine on the molecular level the effect of pathologic tension on fibroblast mechanosensitive gene regulation.

We believe that our model provides an attractive system in which to study the effect of graded tension on the regulation of fibroblast gene expression that accurately models in vivo processes. Data from our model suggest that mechanical strain disrupts the normal steady-state equilibrium that exists between ECM formation and degradation, as well as producing aberrant fibroblast cell survival and excessive ECM matrix synthesis. These data are corroborated with our murine in vivo model of hypertrophic scar formation (data not shown, manuscript in preparation) and are consistent with clinical findings. Using microarray techniques, FPLCs, and our murine in vivo model, we will be able to identify mechanoresponsive genes underlying the fibroblast response to normal and pathologic tensions. These genes will be obvious targets for the development of new treatments to decrease the incidence of hypertrophic scarring and likely will be used to prevent the development of fibrosis involved in other types of disease, such as pulmonary fibrosis, hepatic fibrosis, and scleroderma.

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OPEN DISCUSSION

Jamie P. Levine, MD (New York, NY): Dr. Bhatt, this is phenomenal work, and hopefully opening up the doors of understanding hypertrophic scars and scarring abnormalities. In some of the strain work in vitro, the signals are turned on quite early. When do you think this process becomes irreversible, or when does it start into gear and will continue no matter what you do?

Dr. Bhatt: A very good question. From the data we showed here and some of our other data we haven't presented, it seems as though everything starts to increase around 2 weeks, and then it continues on, but the process itself occurs earlier. We don't see the gross manifestations until 2 weeks and onwards. But from the histologic data and some of the protein data, we see that by the first week onward, it starts having lasting effects.