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Tissue Transglutaminase, Not Lysyl Oxidase, Dominates Early Calcium-Dependent Remodeling of Fibroblast-Populated Collagen Lattices

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Abstract

Cell-populated collagen gels have provided significant insight into the cellular contractile mechanisms and cell-matrix interactions that are necessary for compacting and remodeling extant matrix. Nevertheless, little research has been devoted towards determining how cells entrench these deformations that contribute to establishing a preferred mechanical state. To this end, we examined the roles of two covalent matrix cross-linkers, i.e. tissue transglutaminase and lysyl oxidase, during global remodeling of the free-floating fibroblast-populated collagen lattice. Inhibition of tissue transglutaminase resulted in a reduced rate of compaction compared to controls during early remodeling (up to 2 days). In contrast, inhibition of lysyl oxidase did not alter the early compaction of these lattices, but it reduced the compaction after 2 days of culture. Acute inhibition of different contractile mechanisms suggested further that calcium-dependent contractility may have dominated during the initial remodeling of the collagen lattice before giving way to calcium-independent contractility at later times. In summary, these findings suggest that early remodeling of the free-floating collagen lattice is facilitated by calcium-dependent cell contraction while entrenchment is dominated by a tissue transglutaminase-mediated cross-linking of the extant matrix. As remodeling continues, however, lysyl oxidase increases its contribution, perhaps by consolidating de novo collagen fibrils into fibers to continue the remodeling while the cells transition to a more sustained, calcium-independent contractility. These results promise to influence future tissue engineering studies as well as computational simulations aimed at understanding matrix remodeling in complex in vivo situations.

Keywords

Tissue equivalents; Collagen gels; Matrix remodeling; Tensional homeostasis

Introduction

Since its introduction in 1979 [Bell et al., 1979], the free-floating fibroblast-populated collagen lattice (FPCL) and its variants have been used in hundreds of studies to examine diverse aspects of cell-matrix interactions [Dallon and Ehrlich, 2008; Simon et al., 2012], including the relative effects of different matrix densities, cell concentrations, and cell types (e.g. neonatal vs. mature, dermal vs. cardiac). Complementary work using uniaxially constrained FPCLs has shown further that embedded fibroblasts actively seek to establish, maintain, or restore a preferred mechanical environment through a mechanism referred to as ‘tensional homeostasis’ [Brown et al., 1998]. This endogenous tension appears to arise as the cells actively deform the matrix and then entrench the remodeled collagen, a process that can be repeated until the tension reaches the preferred level. The term ‘residual matrix tension’ has been introduced to describe that part of the overall tension that remains after removing the active cell contribution at any degree of gel compaction [Marenzana et al., 2006]. However, the question remains as to how the cells entrench the matrix deformations and develop this residual tension.

Various reports have directly assessed the contractile mechanisms and downstream signaling pathways that affect the compaction of FPCLs [Dallon and Ehrlich, 2008]. Blocking calcium (Ca^{2+}) ion channels or inhibiting calmodulin can reduce or slow the extent of compaction [Ehrlich et al., 1989], and inhibiting the Rho-kinase pathway can similarly impede compaction [Ina et al., 2007; Bond et al., 2011]. Independently of the specific contractile pathway, entrenching the remodeled matrix would seem to be energetically favorable for the cells. Toward this end, covalently cross-linking the collagen could entrench the induced deformations and thus build-in tensions. Two of the primary enzymatic cross-linkers of collagen are lysyl oxidase (LOX) and tissue transglutaminase (tTG). LOX oxidizes select lysines within collagen to form aldehydes that react to form covalent bonds and stabilize the molecules within fibers [Kagan, 2000]. The absence of LOX-mediated cross-linking in vivo results in weak collagen fibers and fragile collagenous tissues [Avery and Bailey, 2008]. tTG cross-links a number of extracellular matrix proteins, including type I collagen, primarily through the formation of an N ϵ (γ -glutamyl)lysine bond between two molecules [Wang and Griffin, 2012]. tTG is a key player in wound healing and inflammation as well as in pathological states such as fibrosis and arthritis [Griffin et al., 2002].

Although it has long been thought that the free-floating FPCL remains stress free since it is traction free [Tomasek et al., 2002; Grinnell, 2003], we recently showed that a residual-type stress field can exist and explain many prior observations regarding differences in the matrix and cells between the central region and the periphery of the gel [Dallon and Ehrlich, 2008; Simon et al., 2012]. We hypothesize, therefore, that although mechanical restrictions on the free-floating FPCL prevent most of the cells from establishing a homeostatic state, they will nevertheless attempt tensional homeostasis. Hence, this common assay can be used to explore whether LOX or tTG, or both, plays a role in the process of matrix remodeling and subsequent entrenchment.

Methods

Cell Culture and Preparation of Collagen Gels

NIH/3T3 fibroblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and 1% antibiotic/antimycotic (Life Technologies) in an incubator at 37 °C and 5% CO₂. Cells were passaged at 70–80% confluence and cells from passages 4–8 were used for the experiments. Collagen gels were prepared as previously described [Simon et al., 2012]. Briefly, for each gel, 146 µl of concentrated type I rat tail collagen (8.56 mg/ml; BD Biosciences), 200 µl of 5 × DMEM, and 100 µl of a 10× reconstitution buffer (0.1 N NaOH and 20 mM HEPES; Sigma) were combined with 554 µl of the experimental culture medium (described in Experimental Conditions) containing 50,000 cells. The final 1-ml solution contained ~ 50,000 cells and 1.25 mg of collagen; it was then cast into a single well of a 12-well plate and placed in the incubator for 30 min to gel. The casting solution for cell-free gels was the same minus the 50,000 cells. Gels were then suspended in 3 ml of their respective experimental medium and transferred to 35-mm dishes and allowed to float freely in the medium; they were cultured for up to 4 days, with media replaced on day 2.

Experimental Conditions

The culture media used for the experiments consisted of DMEM with 10% fetal bovine serum (HyClone), 10% porcine serum (Life Technologies), ascorbic acid (50 µg/ml; Sigma), CuSO₄ (3 ng/ml; Sigma), proline (50 µg/ml; Sigma), alanine (50 µg/ml; Sigma), glycine (50 µg/ml; Sigma), HEPES (0.01 M), basic fibroblastic growth factor (10 ng/ml; R&D Systems), platelet-derived growth factor (10 ng/ml; R&D Systems), and 1% antibiotic/antimycotic [Solan et al., 2003]. The additional amino acids constitute significant portions of the collagen structure while ascorbic acid is required to facilitate the hydroxylation of proline and lysine [Murad et al., 1981]. The additional growth factors are known to stimulate collagen production [Ivarsson et al., 1998] and the CuSO₄ provides copper, which is necessary for LOX activity [Kagan, 2000]. Control gels were maintained in this medium formulation (n = 30 for day 0, n = 22 for all other times).

To inhibit LOX, the medium was supplemented with β-aminopropionitrile (BAPN, 100 or 1,000 µM; Sigma; n = 12 for each treatment at each time) [Redden and Doolin, 2003]. To inhibit tTG, the medium was supplemented with either the nonspecific, competitive inhibitor cystamine (100, 316, or 1,000 µM; Sigma; n = 9 for each treatment at each time) [Bakker et al., 2005] or the irreversible active site inhibitor Boc-DON (6 µM; Zedira; n = 10 at each time) [Schaertl et al., 2010]. Another group was treated with both 1,000 µM BAPN and 6 µM Boc-DON to examine the effects of simultaneous inhibition of LOX and tTG (n = 6 at each time). Cell-free gels were also maintained in control media or media containing either 150 ng/ml LOX (Origene; n = 10 at each time) [Baker et al., 2013] or 67 mU/ml tTG (Sigma; n = 10 at each time) [Bakker et al., 2005] to determine the effects of cross-linkers alone. Gels were imaged at 24-hour intervals from 0 to 4 days (fig. 1a).

Cell Contractility and Matrix Entrenchment

Possible effects of matrix entrenchment and cell contractility on gel compaction were assessed by acutely removing active cell stresses. Cell-seeded gels were prepared and maintained as described above. At 24-hour intervals from 1 to 4 days, images were taken of the gels to determine radii before eliminating the deformations maintained actively by the cells. Specifically, TritonX-100 was added to the culture media to a final concentration of 1% (v/v) to lyse the cells [Zhao et al., 2013]. After 30 min, another image of each gel was taken and the measured radii were compared to those before exposure to determine the percent radial dilation (fig. 1b). Other control gels were exposed for 30 min to the calcium chelator ethylene glycol tetra-acetic acid (EGTA, 4 mM; Calbiochem), the myosin II inhibitor blebbistatin (30 µg/ml; Sigma), or the Rho-kinase inhibitor Y-27632 (20 µM; Sigma) to assess the roles of, respectively, Ca²⁺-dependent contractility, myosin II activity, and Rho-kinase-dependent contractility in the active compaction of the collagen gels by the cells (n = 8 for all cases at each time). Although tTG is a calcium-dependent enzyme, the EGTA was only used acutely to sequester calcium during the assessment of contractility, not during the full duration of culture. Note, too, that Ca²⁺ is not required for cell adhesion to type I collagen, and thus EGTA should not have caused cells to detach from the matrix [Martz, 1980; Grzesiak et al., 1992]. While EGTA can also chelate magnesium, which is required for fibroblast adhesion to collagen, it has a significantly higher affinity to Ca²⁺, and EGTA-treated 3T3 fibroblasts actively contract uniaxial collagen gels, thus indicating a maintained cell-matrix adhesion [Nobe et al., 2000]. Following the administration of EGTA, blebbistatin, or Y-27632, images were similarly taken to assess radial dilation, and then TritonX-100 was added to eliminate any remaining active cell contraction with further radial dilation measured, if any (n = 5 for each group). Matrix entrenchment and cell contractility were also assessed in gels treated with BAPN, Boc-DON, or cystamine via subsequent exposure to 1% TritonX-100 (n = 8 for all treatments at each time). After eliminating cell contractility, the gels were either used for a residual stress test (fig. 1c; described below) or fixed for additional imaging.

Matrix entrenchment was assessed as the percent overall radial Green strain, relative to day 0, that remained following the elimination of cell contractility via treatment with TritonX-100; it was calculated as:

$$\%Entrench = \frac{\frac{1}{2} (\lambda_{Triton}^2 - 1)}{\frac{1}{2} (\lambda_{Before}^2 - 1)} \times 100 \quad (1)$$

where λ_{Before} is the radial stretch ratio of the gel before TritonX-100, relative to day 0, and λ_{Triton} is the radial stretch ratio of the same gel 30 min after exposure to TritonX-100, also relative to day 0. This measure can thus be thought of as the percent overall radial strain relative to day 0 that remained after eliminating cell contractility. Note that, for this metric of entrenchment, the circumferential Green strain could be employed as well because it is equal to the radial Green strain in the case of uniform radial compaction.

Residual Stress Test

As shown previously [Simon et al., 2012], introduction of a radial cut in a collagen gel can be used to assess the presence of a residual-type state of stress. A radial cut from the center to the outer edge was introduced using a new No. 10 scalpel blade in control and treated gels following the elimination of cell contractility using TritonX-100 ($n = 8$ for all cases at each time). Gels were imaged 15 min after the cut, with opening angles quantified using ImageJ (fig. 1c).

Cell Viability

Cells were seeded at subconfluent (10,000 cells/well) or confluent (50,000 cells/well) levels in collagen I-coated 96-well plates (BD Biosciences) and incubated for 4 h to allow the cells to attach. After 4 h, the culture medium was aspirated off and replaced with control media or media containing BAPN, cystamine, or Boc-DON and then cultured for an additional 24 h. Cell viability was determined using the XTT Cell Viability Assay (Biotium), which is reduced by metabolically active mitochondria. Cells maintained in control media alone served as positive controls (PCs); those maintained in control media but exposed to 1% TritonX-100 for 30 min served as negative controls (NCs) as all of the cells were lysed and rendered metabolically inactive. To ensure that differences in absorbance readings were not due to the chemical content of a given medium formulation, the culture medium from all wells was aspirated and replaced with fresh control media before adding the XTT solution and activation reagent. The plate was then incubated for 4 h before measuring the absorbance at 490 nm and the background absorbance at 655 nm ($n = 12$ for all cases at each seeding density). The background absorbance was subtracted from the measurement absorbance to correct for any nonspecific absorbance. These corrected absorbance measures were then normalized as:

$$Abs_{Norm} = \frac{Abs - \overline{NC}_{10k}}{\overline{PC}_{50k} - \overline{NC}_{10k}}, \quad (2)$$

where Abs_{Norm} is the normalized absorbance, Abs is the background corrected absorbance for a sample, $\overline{NC}_{10k}$ is the mean background corrected absorbance of the NC in subconfluent wells (initially containing 10,000 cells), and $\overline{PC}_{50k}$ is the mean background corrected absorbance of the PC in confluent wells (initially containing 50,000 cells). Hence, $Abs_{Norm} \in [0, 1]$, with a value of 1 indicated an absorbance equal to that of the PC of 50,000 cells maintained in control media and a value of 0 indicated no living cells.

Note that comparing subconfluent versus confluent cultures helped to delineate possible confounding effects of cell proliferation in contrast to metabolic activity alone. Moreover, the PC for the normalization was selected as the PC at the confluent density as it gave the largest measured absorbance of the two PCs due to its higher cell density at 24 h in culture. The NC for the normalization was selected as the low-density NC as it had the lowest measured absorbance.

Statistical Analysis

All statistical analyses were performed using Minitab 16 (Minitab). ANOVA accounting for interactions was performed on radial compaction, cell contractility, entrenchment, and opening-angle data to determine the effects of treatment and time, and on cell viability data to determine the effects of seeding density and treatment. For the effect of cross-linkers on entrenched radial compaction, the data were divided to focus on two primary comparisons: control versus LOX inhibitors and control versus tTG inhibitors. If the interaction of treatment and time was determined to be significant, an ANOVA comparing treatments with the covariant of time was performed to ensure the significance of treatments while correcting for the effects of time. After ensuring significant differences, a one-way ANOVA was performed for a single treatment across all times or for a single time across all treatments. For the viability data, an ANOVA was performed for each seeding density across treatments. Significance ($p < 0.05$) was determined using a Bonferroni correction on the confidence interval ($\alpha = 0.05$). Significance is indicated within times for treatment versus control. All other comparisons are noted in the text and on the figures. Results are reported as means $\pm$ SEM.

Results

Effects of Exogenous LOX and tTG on Compaction

Cell-free collagen gels maintained in control media showed little to no change in radial dimension (i.e. compaction) over 4 days of culture. The measured radii for LOX-treated (150 ng/ml) and tTG-treated (67 mU/ml) cell-free gels were normalized by their same-day control values, which revealed modest levels of compaction ($<3\%$) that did not change significantly from 1 to 4 days of culture. Specifically, normalized values at day 4 were 0.989 ± 0.005 for LOX treatment and 0.972 ± 0.003 for tTG treatment (day 1 values shown in fig. 2). The tTG-treated group was nevertheless significantly different from both the control and the LOX-treated groups, thus suggesting that, though minimal, tTG alone can compact collagen in the absence of cells, though not to the degree seen in cell-populated gels (fig. 1a).

Cell Viability under Treatment

Cell viability was examined to determine whether treatment with inhibitors had a potential cytotoxic effect that could prevent gel compaction by impairing the cells rather than inhibiting the cross-links. Figure 3 reveals for the subconfluent cells that all of the cystamine-treated groups exhibited significantly lower absorbance levels compared to the PCs, thus suggesting either reduced metabolic activity or proliferation, or possibly both. The 100 μM cystamine alone was significantly different from the NCs, however, thus suggesting a measurable cytotoxicity only at 316 and 1,000 μM cystamine. All other treatments at the subconfluent density were either comparable to the subconfluent PC, thus indicating normal cellular activity, or had significantly higher absorbance levels, thus suggesting an increase in metabolic activity or proliferation, or both. Figure 3 reveals further for the confluent seeding density that only the 1,000 μM cystamine group showed a significantly lower absorbance compared to the PC. This group was significantly different from the NC, thus suggesting that cells maintained some metabolic activity but likely experienced cytotoxic effects from this level of cystamine treatment.

Effects of Cross-Linker Inhibition on Compaction

Cell-seeded gels maintained in control media showed the largest degree of compaction during the first day of culture (normalized radius of 0.656 ± 0.023 on day 1 vs. 1.00 ± 0.005 on day 0), with the rate of compaction slowing over the next few days but yielding a final normalized radius of 0.440 ± 0.026 on day 4 (fig. 4). Recall that some gels were treated with BAPN to inhibit LOX throughout the 4-day study period. BAPN at $100 \mu\text{M}$ did not significantly change the extent of the overall compaction compared to the control group at any time (fig. 4a). In contrast, the $1,000 \mu\text{M}$ BAPN group progressively compacted until day 2, after which it appeared to reach a near steady level of compaction (normalized radius of 0.545 ± 0.19 on day 2 and 0.527 ± 0.022 on day 4; fig. 4b); however, the day 4 gels alone were statistically different from the control. These findings are consistent with prior work showing that LOX inhibition by BAPN only affects gel compaction after 2–3 days [Redden and Doolin, 2003].

Cell-seeded gels were also treated with cystamine and Boc-DON to inhibit the activity of tTG throughout the 4-day study period. The $316 \mu\text{M}$ (fig. 5c) and $1,000 \mu\text{M}$ (similar, not shown) concentrations of cystamine prohibited essentially any compaction of the collagen gels, consistent with possible cytotoxicity. In contrast, compaction levels on days 1 and 2 were significantly different from the control for both the $6 \mu\text{M}$ Boc-DON (fig. 5a) and the $100 \mu\text{M}$ cystamine (fig. 5b) treatments. By days 3 and 4, however, the normalized radial values for these two groups were not statistically different from the corresponding control values. These day 3 and 4 results suggested, therefore, that neither the $6 \mu\text{M}$ Boc-DON nor the $100 \mu\text{M}$ cystamine treatment inhibited the cells' later ability to compact the gels, hence suggesting that collagen cross-linking by tTG played a significant role during the early remodeling and compaction of the collagen gels.

Simultaneous inhibition of LOX and tTG (via treatment with $1,000 \mu\text{M}$ BAPN and $6 \mu\text{M}$ Boc-DON) resulted in reduced compaction compared to the control group on all days (fig. 5d). In addition, when compared with the $1,000 \mu\text{M}$ BAPN group, the dual-treatment group was statistically different on days 1 to 3, but not on day 4; when compared with the $6 \mu\text{M}$ Boc-DON group, the dual-treatment group was not different on days 1 or 2, though the dual-treatment gels tended to compact less. Hence, while tTG appeared to dominate at early times, LOX appeared to contribute some to this compaction. Finally, note that the dual treatment did not inhibit compaction completely. The cells were still able to compact the gels slightly, perhaps via simple geometric rearrangement of the extant collagen fibers (not quantified).

Cell Contractility and Matrix Entrenchment

A 30-min exposure to 1% TritonX-100 resulted in mostly uniform radial dilations of cell-seeded control gels at all times (from a minimum of $7.84 \pm 0.13\%$ on day 2 to a maximum of $9.63 \pm 0.35\%$ on day 4; fig. 6a). The dilation caused by a 30-min exposure to the calcium chelator EGTA was maximal on day 1 ($5.35 \pm 0.29\%$; fig. 6a) and decreased to a minimum on day 4 ($0.06 \pm 0.20\%$). In contrast, a 30-min exposure to the Rho-kinase inhibitor Y-27632 resulted in a minimal dilation of control gels on day 1 ($0.76 \pm 0.45\%$; fig. 6a) but a maximal dilation on day 4 ($7.97 \pm 1.08\%$). Exposure to the myosin II inhibitor blebbistatin resulted in

a minimal dilation on day 1 ($2.33 \pm 0.13\%$; fig. 6b) and a maximal dilatation on day 4 ($7.67 \pm 0.36\%$). Subsequent exposure of these treated gels to TritonX-100 resulted in final radial dilations that were not significantly different from those treated with TritonX-100 alone (not shown). These findings are consistent with prior reports [Ehrlich et al., 1991; Tomasek et al., 2002; Grinnell, 2003; Dallan and Ehrlich, 2008] and suggest that early (up to day 2) compaction may be driven primarily by rapid, Ca^{2+} -dependent contractions, while later cell contractility may be primarily Ca^{2+} -independent and more sustained via Rho-kinase-dependent pathways.

Gels treated with cross-link inhibitors were also exposed to TritonX-100 at each time to assess that part of the compaction that was maintained by active cell contraction. The 316 μM cystamine group showed effectively no dilation when the cells were lysed (fig. 6c), which was expected since little compaction was seen for this group (fig. 5c). The other experimental groups (1,000 μM BAPN and 6 μM Boc-DON) showed no significant difference in dilation when compared to the same-day controls, though the day 2 Boc-DON dilations trended to be smaller. The day 2 Boc-DON value was significantly different from Boc-DON-treated values at all other times, and similarly the minimal values of dilation for all groups trended smaller on day 2.

Matrix entrenchment among the control, 1,000 μM BAPN, and 6 μM Boc-DON groups was not statistically different on any day, though the Boc-DON value trended lower than the control and BAPN values on days 1 and 2 (fig. 7). The percent entrenchment for each group tended to increase from day 1 to day 2 or 3, with day 1 values for the control ($85.4 \pm 2.6\%$) and 1,000 μM BAPN ($80.7 \pm 2.8\%$) groups being significantly different from the values on days 2 through 4 ($93.3 \pm 1.1\%$ for the control and $89.9 \pm 2.3\%$ for BAPN on day 4), and the day 1 Boc-DON value ($72.3 \pm 5.6\%$) being significantly different from the values on days 3 and 4 ($94.4 \pm 0.6\%$ on day 4). These results suggested that tTG inhibition may reduce the rate of matrix entrenchment compared to controls.

Opening Angles

The presence of a residual stress field was assessed qualitatively by introducing a radial cut, from the center to the outer edge of a gel, and measuring any appreciable opening angle following the elimination of cell contractility using TritonX-100 (i.e. to assess cell-independent, residual matrix tensions). Day 0 gels showed small opening angles ($4.5 \pm 1.0^\circ$; fig. 8), with the majority of this angle likely being due to the width of the scalpel blade. This observation suggests that there is little to no residual stress present within gels due to the initial casting. Gels treated with 316 μM cystamine had opening angles on all 4 days that were not significantly different from the day 0 controls, again consistent with possible cytotoxicity. In contrast, the day 1 control gels exhibited significant opening angles ($22.6 \pm 1.2^\circ$), which suggests early development of a tensile circumferential stress at the outer edge and compressive stress within the central region of the gels. The opening angles tended to decrease slightly from day 1 to day 4 ($17.3 \pm 1.9^\circ$), with day 3 and 4 values being significantly different from day 1 values. Finally, gels treated with 1,000 μM BAPN and 6 μM Boc-DON had opening angles that tended to be lower on days 1 and 2, with day 2 values being significantly different from the control value ($20.6 \pm 0.6^\circ$ for the control vs. 15.2

$\pm 0.8^\circ$ for BAPN and $17.2 \pm 1.1^\circ$ for Boc-DON). Results from the BAPN and Boc-DON groups were not different from controls on days 3 and 4. These results suggest that, even for instances of reduced matrix compaction compared to controls, residual stresses still developed.

Discussion

The development, maintenance, remodeling, or repair of collagenous tissues results from de novo deposition of new matrix, remodeling of extant matrix, or both. Both of these mechanisms require cells to actively work on the matrix via actomyosin activity and integrin engagement. As shown many years ago, however, fibroblasts tend not to synthesize matrix when compacting initially stress-free collagen gels [Paye et al., 1987]. Rather, these cells appear first to actively deform the matrix and then ‘lock-in’ or entrench such deformations in an incremental fashion. Such entrenchment can change the overall geometry and material properties of the matrix, which dictates the local mechanical environment in which the cells reside. With regard to in vitro studies using the FPCL, extensive work has revealed the intracellular mechanisms [Bell et al., 1979; Ehrlich and Rittenberg, 2000; Ehrlich et al., 2000; Redden and Doolin, 2006; Dallon and Ehrlich, 2008] and cell-matrix interactions via integrins [Schirotto et al., 1991; Langholz et al., 1995; Cooke et al., 2000; Kelynnack et al., 2000; Travis et al., 2001; Grundström et al., 2003] are necessary for cells to deform their surrounding matrix. An important question, however, has been how the cells, after compacting the matrix, entrench the induced deformations and thereby develop or attempt to develop a preferred mechanical environment that does not require continued actomyosin activity and thus energetic expenditures to maintain.

Direct application of tissue tTG or LOX to cell-free gels showed that tTG, but not LOX, was capable of modestly, yet significantly, compacting the collagen gels in the absence of cells. This finding suggested that the initial collagen matrix had available binding sites for tTG but not LOX, or else that LOX-induced changes require cell mediation. This latter possibility is consistent with the primary role of LOX in supporting the consolidation of collagen molecules and fibrils into fibers following new deposition by cells [Kagan, 2000], noting that the initial collagen used in the gel seeding was already at a fiber diameter. Though it was not tested, one could hypothesize that LOX would affect cell-free gel compaction if pepsin-degraded collagen were used, which might allow LOX-aided fibril consolidation. Our focus, however, was on the remodeling of extant matrix consisting of collagen fibers.

The roles of tTG in the remodeling of collagenous tissue have been examined in vivo using diverse animal models. Of note is small artery remodeling following a ligation-induced reduction in flow, which was studied using either tTG inhibition in normal rats [Bakker et al., 2005] or a lack of expression in a tTG^{-/-} mouse [Bakker et al., 2006]. As expected, low-flow controls exhibited extensive remodeling and had reduced vessel diameters after only 2 days. Both inhibition of tTG and a lack of tTG expression resulted in vessel diameters comparable to normal-flow controls despite the low-flow conditions, thus suggesting the importance of tTG in early arterial remodeling and entrenchment to restore preferred wall shear stresses. Our results are consistent with these findings for, in the absence of cytotoxicity (i.e. high cystamine concentrations), tTG inhibition slowed early matrix

remodeling within the gels on days 1 and 2 (fig. 5). Note, too, that it has also been shown that tTG^{-/-} vessels nevertheless remodel after 7 days to levels similar to those of the low-flow controls, thus revealing a longer-term (compensatory) mechanism to reestablish the preferred flow conditions independently of tTG [Bakker et al., 2006]. Our 3- and 4-day results showed a similar compensation as the measured radii for the tTG-inhibited gels were comparable to controls on those days. Noting that endogenous LOX was not inhibited in the tTG-inhibited gels, the results achieved with dual inhibition (1,000 μ M BAPN and 6 μ M Boc-DON; fig. 5d) were not significantly different from the results for tTG-inhibited gels on days 1 and 2, again suggesting a dominance of tTG in matrix entrenchment during early compaction.

tTG activity has also been shown to play roles in some cell-matrix interactions [Griffin et al., 2002; Zemskov et al., 2006]. In its closed confirmation, extracellular tTG can attach to fibronectin and aid in cell adhesion to these substrates through α_v integrins. With regard to type I collagen, however, there has not been any report of tTG aiding or being necessary for fibroblast adhesion via β_1 integrins. Conversely, it has been shown that proteolysis of extracellular tTG may increase the binding affinity of $\alpha_1\beta_1$ integrins to type I collagen [Zemskov et al., 2006]. It appears, therefore, that inhibition of tTG in collagen lattices should not induce cell detachment, which could slow or inhibit gel compaction.

With regard to LOX, it has been reported that inhibition via 1,000 μ M BAPN treatment had little effect on matrix compaction by adult human lung fibroblasts during the first 2 days of treatment [Redden and Doolin, 2003]. After day 2, however, the gel compaction either stalled or the gels dilated before compaction resumed by day 4. It was thus concluded that the matrix compaction was bimodal, with later (after day 2) compaction requiring de novo collagen synthesis facilitated by LOX activity. Our results for a different cell type (3T3 fibroblasts) followed a similar trend for the same concentration of BAPN, with treated gels being significantly larger than controls on day 4. While our results with regard to LOX inhibition coincide with this prior study, our results related to tTG inhibition suggest further that tTG-mediated cross-linking contributes to early remodeling, which is not due to cell contraction alone. In both cases, consistent with prior suggestions, it would seem that a sequential process of active compaction and passive entrenchment enables the cells to achieve such dramatic remodeling of the gels (fig. 1a).

We wanted to ensure cell viability for all of our experimental conditions. Of greatest concern was the treatment of cells with cystamine [Schaertl et al., 2010]. At low seeding densities, all concentrations of cystamine showed a significantly lower signal in the XTT Cell Viability Assay compared to the PC (fig. 3). Because differences in cell number can influence the overall signal measured, the low values of absorbance may have only indicated an attenuation of cell proliferation by cystamine. Results for the higher seeding density would seem to support this possibility as the absorbance signals for the low and middle concentrations of cystamine were statistically the same as the PC. This finding is concerning with respect to some reports in the literature, however, as the use of cystamine to inhibit tTG either in vivo or in vitro may confound the desired results. Cells treated with high levels of cystamine may stop proliferating, have reduced metabolic activity, or be induced to undergo apoptosis. Such effects would result in fewer active cells compared to control groups and

thus make it difficult to determine whether differences between controls and cystaminetreated groups are due to tTG inhibition or just a reduced cell number or cell activity. In contrast, Boc-DON did not cause any significant cytotoxicity, and hence the associated results were preferred as indicators of tTG inhibition. BAPN similarly did not cause any significant cytotoxicity.

Cell contractility plays a key role in the compaction of FPCLs. Before the matrix can be cross-linked in a new geometry, it must be actively deformed by the resident cells. We used 30-min exposures to 1% TritonX-100 to lyse the cells (online suppl. fig. 1 and 2; see www.karger.com/doi/10.1159/000381015 for all online suppl. material) and thereby to assess contributions to gel compaction by the actively applied cell tension. Regardless of the treatment, dilations following the administration of TritonX-100 were not significantly different across experimental groups at any time. Note that it has been observed experimentally [Discher et al., 2005] and shown theoretically [Simon et al., 2012] that the stress actively applied by a cell to a matrix tends to be proportional to the stiffness of the matrix. It is thus possible herein that the gel stiffness was comparable, at least within most regions, regardless of the degree of entrenchment achieved and so too for the degree of cell contractility that was eliminated by the TritonX-100. Alternatively, if the bulk stiffness differed at different levels of compaction, the applied cell stresses may also have differed in a way that the actively generated deformations remained similar. For example (fig. 7), the 6 μM Boc-DON-treated gels showed less overall compaction on day 1 compared to the control and 1,000 μM BAPN gels, indicating less remodeling and, possibly, a lower bulk stiffness. Releasing a lower level of applied stress in a less stiff gel could thus result in applied cell stresses that are statistically equal.

We also examined the mode of cell contractility via acute exposure to EGTA, which sequesters Ca^{2+} , blebbistatin, which inhibits myosin II, or Y-27632, which inhibits Rho kinase. Results from these experiments suggested a temporal variation in the primary mode of cell contractility. While day 1 appeared to be dominated by Ca^{2+} -dependent activity, days 3 and 4 appeared to be dominated by a Ca^{2+} -independent pathway, perhaps mediated by Rho kinase as suggested by others [Ina et al., 2007; Bond et al., 2011] since the day 4 data showed essentially no acute effects of removing Ca^{2+} whereas Y-27632 treatment resulted in dilations similar to those in TritonX-100 controls. Results from day 2 are consistent, however, with an interplay between Ca^{2+} -dependent and Ca^{2+} -independent modes of contraction, consistent with a prior report [Castella et al., 2010]. That is, it has been shown that a single myofibroblast in 2-D culture can utilize both modes of contraction: Ca^{2+} -independent contraction to maintain tension on the culture surface and Ca^{2+} -dependent contraction to apply small deformations to the surrounding matrix. The model these authors proposed suggests that sustained contraction via the Rho-kinase pathway allows the cell to locally unload the matrix tension whereas Ca^{2+} -dependent contractions allow the cell to reorganize the unloaded matrix or orient newly deposited material. Our results similarly suggest that both modes of contractility may be present, perhaps with one or the other dominating at different times during compaction, though we did not directly examine the associated signaling pathways via particular phosphorylation events.

Recalling that, by day 4, the removal of Ca^{2+} had no appreciable effect with regard to the removal of cell contractility while inhibition of myosin II and Rho kinase was statistically the same as that for TritonX-100, multiple factors may have affected this result. For example, possible apoptosis has been suggested previously [Zhu et al., 2001; Tian et al., 2002], likely in the central compressive region of the gel [Simon et al., 2012]. Conversely, the cells at the outer edge of the gel may begin to differentiate into myofibroblasts [Ehrlich, 1988; Ehrlich and Rajaratnam, 1990; Dallon and Ehrlich, 2008; Simon et al., 2012], as seen in online supplementary figure 2, which maintain contractility via the Rho-kinase pathway [Tomasek et al., 2006].

The opening angles indicated that residual stresses developed whenever there was radial compaction of the gel. At all times, the BAPN-and Boc-DON-treated groups had similar opening angles even though the overall degrees of compaction may have differed. For example, the opening angles for each group on day 2 were similar despite the overall compaction being different. This finding does not necessarily mean that both groups had similar stress fields. To determine what may give rise to this phenomenon, 2-day gels that were cultured in control media and gels treated with either BAPN or Boc-DON were examined histologically and stained with picrosirius red. The gels were imaged under circularly polarized light to show only birefringent collagen. Day 2 gels for both treatment groups and controls showed similar levels of collagen alignment in the outer region (online suppl. fig. 4). The picrosirius red images also revealed that the fibers in the control and BAPN gels were thicker and denser at the outer edge compared with the Boc-DON group, which likely led to a more anisotropic and possibly stiffer material behavior. A similar matrix alignment may allow different groups to have similar opening angles even when the degree of compaction and bulk material properties are different.

Based on our general observations (fig. 9), we propose a possible timeline for free-floating FPCL remodeling. Cells were initially cast in a dilute collagen gel with little to no mechanical stress. As previously noted, prior studies at both the tissue [Brown et al., 1998] and the cell [Mizutani et al., 2004] level suggest that mesenchymal cells seek to establish or maintain a preferred mechanical state via a process referred to as 'tensional homeostasis'. Initially being in a zero-stress state, the cells likely sought quickly to develop stress within the matrix, which in turn could stress the cells themselves. Following a lag during which cells were initially adhering to and spreading within the matrix, they began active contractions to stress the surrounding matrix, which began the process of compaction because of the traction-free boundary conditions. Ca^{2+} -dependent contractility may have been utilized during this early, rapid deformation and reorganization of the collagen; the Rho-kinase pathway could also have been engaged to allow the cells to sustain the local deformation while utilizing Ca^{2+} -dependent contractions to reorganize the surrounding collagen [Castella et al., 2010]. To entrench the incrementally achieved deformations, the fibroblasts appeared to use α 1(I)TG early on to covalently cross-link the remodeled collagen fibers. Upon entrenchment, the cells could relax and possibly spread out or reorient yet again. Such a process could allow the cells to repeatedly contract, entrench, and spread to apply small, repetitive deformations that led over time to the global compaction of the FPCL.

As cells contract and entrench the gel, a residual-type stress field develops within the matrix [Simon and Humphrey, 2012; Simon et al., 2012]. The central region is mechanically characterized as in equibiaxial compression while a tensile circumferential stress develops at the outer edge. Cells at or near the outer edge may find this mechanical environment to be tolerable or preferred. Nearby cells may thus begin to migrate toward the outer edge, possibly driven by durotaxis (in response to the radially changing matrix stress or stiffness) or chemotaxis (in response to cytokines produced by cells in the more favorable environment). Such a migration could contribute to the increased cell density seen near the outer edge of compacted gels [Ehrlich, 1988; Simon et al., 2012]. Because the cells in the central region would nevertheless remain in a nearly equibiaxial compressive stress environment, they may begin to undergo apoptosis, which could contribute to the overall reduced Ca^{2+} -dependent contractility at later times.

The tensile environment of the outer edge could also lead to differentiation of the fibroblasts in this region into myofibroblasts, which has been previously reported [Ehrlich, 1988; Ehrlich and Rajaratnam, 1990; Dallon and Ehrlich, 2008; Simon et al., 2012]. As these cells establish a more preferred level of stress, their primary mode of contraction may switch from Ca^{2+} -dependent to a sustained contractile phenotype supported by the Rho-kinase pathway. If the cells could achieve their homeostatic target (unlikely in the free floating gel), they could then switch from a state of remodeling extant matrix to a state of tissue maintenance.

At later times (3–4 days), the effects of tTG inhibition appeared to have diminished while LOX inhibition appeared to stall further FPCL compaction. Though not measured in this study, this may have been due to new collagen deposition. When fibroblasts achieve a preferred or tolerable level of stress, they may begin to deposit new collagen either to maintain or to develop further their preferred mechanical environment. Inhibition of LOX would prevent the transition of newly produced soluble collagen molecules into insoluble fibrils and fibers [Kagan, 2000; Kagan and Ryvkin, 2011]. Also, by days 3 and 4, the tTG activity may become minimal as available cross-linking sites on the initial collagen fibers become bound. New collagen deposition may be necessary to further alter the organization of the FPCL [Redden and Doolin, 2003].

To understand more fully the nature of FPCL remodeling, more work is needed to characterize well the evolving mechanical environment. Until recently, the mechanical state of the compacting gel has been largely ignored or assumed to be negligible [Tomasek et al., 2002; Grinnell, 2003]. However, new insights into the mechanics of the free-floating FPCL have suggested that the cells develop a residual-type stress field (compression in the center and tension at the outer edge) through matrix remodeling and the development of a radially varying matrix organization [Simon and Humphrey, 2012; Simon et al., 2012]. As the cells contract and remodel the matrix, differences in local mechanical environments could lead to heterogeneous rates and methods of remodeling as well as variations in gene expression and cell viability. Here, we have characterized the global mode of remodeling. Further work is needed not only to characterize potential regional variations within the FPCL but also to correlate cell activity with any variations in the local mechanical environment. To this end, we need a better understanding of the evolving mechanical environment of the FPCL.

In summary, tTG, not LOX, appears to play a dominant role in the early remodeling and entrenchment of extant fibers in the free-floating FPCL, with Ca^{2+} -dependent contractility dominating early on as cells attempt to quickly remodel their surrounding matrix. LOX is not necessary for, though it contributes modestly to, the early remodeling of the matrix as new matrix production is likely minimal. At later times, however, LOX may be required as new collagen is produced, either to continue remodeling the matrix or to begin the process of tissue maintenance if the cells have established a homeostatic mechanical state. Similarly, our results suggest that a Ca^{2+} -independent contractility dominates at later times, with cells switching to a state of sustained contractility that is a hallmark of the myofibroblast phenotype.

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Abbreviations used in this paper

BAPN	β -aminopropionitrile
DMEM	Dulbecco's modified Eagle's medium
EGTA	ethylene glycol tetra-acetic acid
FPCL	fibroblast-populated collagen lattice
LOX	lysyl oxidase
NC	negative control
PC	positive control
tTG	tissue transglutaminase

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Fig. 1.

Representative images of collagen gels illustrating three of the empirical manipulations. **a** Seeding the gels with 3T3 fibroblasts resulted in significant compaction over the 4 days of observation (right) relative to day 0 (left). **b** Exposure of a BAPN-treated (1,000 μ M), 4-day gel to TritonX-100 lysed the cells and resulted in dilatation of the gel (right) relative to the state prior to treatment (left). **c** Introduction of a radial cut within a 2-day control gel released residual stresses within the gel as evidenced by an 'opening up' of the cut (right), relative the state prior to the cut (left). Scale bar = 5 mm (**a**); all images are at the same magnification.

Fig. 2.

Introduction of exogenous LOX and tTG into cell-free collagen gels caused only modest compaction after 1 day. Data are shown for 1-day gels, but the results remained essentially unchanged over the 4-day study period. Bars indicate the standard error of the mean. * Significant difference ($p < 0.05$) compared to the day 0 untreated control.

Fig. 3.

Normalized absorbance values from an XTT cell viability assay for subconfluent (10,000 cells) and confluent (50,000 cells) seeding densities in 96-well plates, not within collagen gels. † Significant difference compared to the same seeding density NC, i.e. cells lysed with TritonX-100. * Significant difference compared to the same seeding density PC, i.e. untreated cells. Decreased values for the confluent groups suggested varying degrees of treatment-induced cell death. Decreased values in the subconfluent group (from ~0.55) suggested either cell death or reduced proliferation, the latter of which can be entertained based on the increased values in normalized absorbance in 2 cases relative to their PC. Results calculated using equation 2.

Fig. 4. Time course of fibroblast-mediated compaction of collagen gels (i.e. outer radii normalized relative to day 0 starting values) over the 4-day study period for two concentrations of the LOX inhibitor BAPN: 100 μM (a) and 1,000 μM (b). Error bars denote the standard error of the mean. * Significant difference compared to the same-day control mean. BAPN had no effect on cell-mediated compaction over the first 3 days and only a modest effect on day 4 at the higher concentration.

Fig. 5.

Similar to figure 4, except for the time course of cell-mediated gel compaction over the 4-day study period for two inhibitors of tTG, one of which was administered at two concentrations: 6 μ M Boc-DON (**a**), 100 μ M cystamine (**b**), and 316 μ M cystamine (**c**). compaction on days 1 and 2, relative to day 0 controls, though the gels achieved control values on days 3 and 4. In contrast, the high concentration of cystamine inhibited cell-mediated compaction on all days. **d** Comparison of the control and gels treated with 6 μ M Boc-DON and 1,000 μ M BAPN to those treated with both 6 μ M Boc-DON and 1,000 BAPN μ M (BAPN/Boc-DON).

Fig. 6.

The percent radial dilatation (i.e. partial release of compaction relative to same-day controls) is shown on each of the 4 days for two protocols. **a** Control gels were exposed to EGTA to chelate the calcium, Y-27632 to inhibit Rho kinase, or TritonX-100 to lyse the cells. **b** Control gels were exposed to blebbistatin to inhibit myosin activity. TritonX-100 results are shown to provide a comparison. **c** Control gels and gels treated with either BAPN (to inhibit LOX) or Boc-DON or cystamine (to inhibit tTG) were exposed to TritonX-100. * Significant difference compared to the same-day control mean (TritonX-100 for **a** and **b**). [†] Significant difference compared to the 1-, 3-, and 4-day Boc-DON means.

Fig. 7.

Time course of the percent gel compaction that was entrenched (i.e. not recovered upon removal of active cell tension by lysing the cells with TritonX-100) on each of the 4 days of study for 3 groups: control, BAPN treated, and Boc-DON treated. Entrenchment was quantified as the percent change in the total radial Green strain due to TritonX-100 treatment relative to day 0. [†] Significant difference compared across all other times for the same experimental group treatment; [‡] Significant difference compared to the 3-and 4-day times within the Boc-DON group. Note that the 316 μ M cystamine group was not included as little to no compaction was measured for this group compared to day 0 gels (fig. 5c).

Fig. 8.

Time course of radial-cut related opening angles (fig. 1c) over the 4-day study period for 4 groups: control, LOX-inhibited (BAPN) and tTG-inhibited (both Doc-DON and high-concentration cystamine) gels. * Significant difference compared to the same-day control. ‡ Significant difference for the control group between day 1 (high) and days 3 and 4 (low). Surprisingly, treatments with BAPN and Boc-DON had similar effects at all times; in contrast, treatment with 316 μ M of cystamine disallowed any matrix remodeling and associated development of residual stress. The initial value of 5 degrees for the opening angle likely represents the width of the cut alone and can be considered to be an experimental offset.

Fig. 9.

Schematic drawing of some of the primary metrics of interest illustrated for day 1 results; specific results were different on other days but are qualitatively represented here. Note, in particular, that inhibition of tTG (i.e. Boc-DON treatment), but not LOX (BAPN treatment), reduced both early matrix compaction and entrenchment. Nevertheless, active cell-mediated compaction was similar across all groups (indicated by the ~8.5% dilatation following exposure to TritonX-100) independently of the degree of entrenchment.