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Repetitive mechanical stretch increases extracellular collagenase activity in vaginal fibroblasts

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Abstract

Objectives—The objectives were 1) to determine whether human vaginal fibroblasts are mechanosensitive and 2) to study the impact of mechanical stretch on these cells in the presence and absence of hormones.

Methods—Fibroblasts obtained from biopsies of full thickness vagina of 3 women were cyclically biaxially stretched at a magnitude of 8 and 16% for 72 hours with or without 17- β -estradiol plus progesterone. Culture media was collected and total collagenase activity was measured in duplicate using a fluorogenic substrate degradation assay. Data were analyzed at the 0.05 level of significance using Student t-test.

Results—Cells remained 90% viable throughout the experiments. Relative to the controls, hormonal treatment alone decreased collagenase activity ($P=0.008$). In the presence of mechanical stretch and in the absence of hormones, collagenase activity was increased (8% elongation, $P=0.04$; 16% elongation, $P=0.001$, respectively). The increase in collagenase activity was linearly correlated with magnitude ($P<0.001$). In the presence of hormones, the increase in enzyme activity by mechanical stretch was suppressed to baseline control levels ($P=0.46$). There was no difference in suppression by hormones by magnitude ($P=0.48$).

Conclusions—Vaginal connective tissue fibroblasts are mechanosensitive with increased collagenase activity in the presence of stretch. This degradative behavior is inhibited in the presence of hormones. The data provide a mechanism by which events that induce vaginal stretch may lead to progression of pelvic organ prolapse, particularly, in the absence of hormones. Further studies are needed to determine whether these events lead to tissue with inferior mechanical properties.

Keywords

collagenases; mechanosensitive; vaginal fibroblasts

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Introduction

One of the primary mechanisms of support to the pelvic organs is provided by the collective action of the vagina and its supportive tissues [1]. Thus, with transient increases in intra-abdominal pressure, the vagina and its supportive tissues orchestrate an equally distributed counter pressure such that pelvic organ support is maintained. Injury to these structures at the time of vaginal childbirth likely alters their load bearing relationships such that increases in pressure are no longer countered by an equal opposing pressure but rather overwhelm the capacity of the support tissues resulting in their downward distension or stretching.

Childbirth is considered the leading risk factor for the development of pelvic organ prolapse since most parous women have some degree of prolapse [2]. While the majority of women never progress to a point where they develop symptoms, those that do, often do not progress to symptomatic prolapse until years often decades later. This observation suggests that other factors such as those that lead to deterioration of tissue quality (eg. menopause and aging) or chronic stretching on the vaginal tissues (eg. obesity, cough and heavy lifting) play a key role in the progression of prolapse from a mild to an advanced form.

Collagens are the primary load bearing proteins of the vagina and its supportive tissues and consequently, the crucial determinant of its biomechanical properties [3]. Collagens are predominantly degraded by collagenases consisting of matrix metalloproteinases (MMPs) -1, -8 and -13 [4], the activities of which are also regulated by estrogen and progesterone. For example, these hormones decrease MMP activity by decreasing conversion of the proenzyme to the active form, increasing the expression of MMP inhibitor and accelerating MMP degradation [5-9]. The net effect is increased collagen deposition and presumably stronger supportive tissue in pelvis [10] that can support increasing loads.

To date, however, there is limited information on the impact of mechanical stretch on the vaginal wall. Here, we hypothesize that repetitive downward distension (stretch) of the vaginal connective tissues previously injured by childbirth eventually results in the breakdown of these structures, causing the vagina and the organs supported by it, to descend more distally into the vaginal canal. We further hypothesize that in the presence of hormones, the quality of the connective tissues is improved such that tissue breakdown is slowed or obviated. However, in the absence of hormones and the presence of repetitive mechanical stretch, the compensatory mechanism is lost, connective tissue degradation accelerates and prolapse progression ensues.

The primary objective of this study was to develop an in vitro cell culture system in which we test the impact of repetitive mechanical loads on the vaginal connective tissue fibroblasts to determine whether or not these cells are mechanosensitive (ie. respond to a mechanical stimulus by altering their environment). In addition, we aimed to determine whether alterations in the cells' environment modify their response to mechanical stress. To this end, we isolated cells from the subepithelial connective tissue of biopsies of full thickness vagina and exposed them to repetitive mechanical stretch. As an indicator of the cellular response, we examined connective tissue degradation by quantitating the collective activity of collagenases and their inhibitors synthesized by the cells using a fibrillar collagen (type I) degradation assay.

Material and Methods

Patient demographic data

The study was approved by the Institutional Review Board of Magee-Womens Hospital. For this mechanistic study, we used cells cultured from the subepithelial dense connective tissue

of biopsies of full thickness vagina from 3 patients. Patient 1 was a 42-year-old African American premenopausal woman (gravida 5, para 3) without prolapse; Patient 2 was a 49-year-old white premenopausal woman (gravida 2, para 1) with stage II prolapse; Patient 3 was a 63-year-old white postmenopausal woman (gravida 2, para 2) with stage III prolapse. The biopsies were obtained at time of surgery following informed consent.

Tissue culture

Full-thickness vagina was procured from the vaginal apex of women. We chose the apex to standardize the biopsies and to use a part of the vagina that in most cases would be relatively shielded from confounding secondary effects of prolapse, particularly, ulceration and thickening due to contact with undergarments. After procurement, the tissue specimen was dissected under a dissecting microscope and the epithelium was excised leaving the subepithelium and muscularis for deriving cells. The tissue was minced and incubated in the six-well culture dish with media containing 20% fetal bovine serum (FBS) at 37 °C in a humidified environment of 5% CO₂ until cells were liberated. Cells of fibroblast morphology were then subcultured with the same medium containing 10% FBS (maintenance medium). Cells of passage 3 were used for analyses.

Immunofluorescent staining

To identify the phenotypes of the derived cells, we employed the specific biomarkers for fibroblastic vs myogenic origin via immunofluorescent staining. Cells at 50% confluence cultured in chamber slides were fixed with 2% formaldehyde and treated with 2% Triton X-100. After being washed with PBS and blocked with 1% BSA, the cells were then incubated with a specific biomarker for fibroblasts, mouse anti-human prolyl 4-hydroxylase beta (PHDB, 20µg/ml, Acris Antibodies GmbH, Germany), or with myogenic biomarkers, mouse anti-human smoothelin (1:100, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or H-caldesmon (1:100, Santa Cruz) at 37°C for 1 hour followed by the incubation with secondary antibody [goat anti-mouse IgG (H+L) - FITC conjugated, Chemicon, Temecula, CA] at room temperature in a dark chamber. The primary antibodies have been validated in our previous studies [7,8]. Negative controls were incubated with secondary antibody alone or with mouse IgG₁ conjugated to FITC (Cymbus Biotechnology Ltd. UK). After washing, the slides were mounted and examined using a fluorescence microscope (Zeiss, WEL Instrument Co. Mars, PA)

Mechanical stretching

For experiments, cells were transferred to BioFlex 6-well culture plates pre-coated with collagen type I (FlexCell Int. Corp., Hillsborough, NC) and incubated in maintenance media until they reached 95% confluence. Cells were then cultured in hormone-free media containing 0.2% (w/v) lactalbumin enzymatic hydrolysate (LEH) for 24 hours. Cells were subjected to 72 hours of sinusoidal cyclical loading at a frequency of 1 Hz equibiaxially to 8% or 16% elongation with a FX-4000T Flexercell Tension Plus System (FlexCell Int. Corp.). As there is little data on the in-vivo deformations experienced by the vaginal tissues, the selected loading conditions were based on a study aimed at assessing the mechanosensitivity of tendon fibroblasts from the orthopaedic literature [11] and modified to account for known differences between tendons and the vagina. Unlike tendons, which undergo in-vivo elongations in the range of 5% and are loaded uniaxially, the vagina is a more compliant tissue and therefore likely experiences larger deformations in-vivo that are biaxial in nature due to its anatomic structure. Thus, we selected the upper level of substrate elongation utilized to test tendon fibroblasts, i.e. 16%, and applied the deformation biaxially. Although the frequency and duration employed in the study were less likely reflective of the actual vaginal mechanical loading, the frequency and duration were maintained as the previous studies have shown that they are sufficient to assess the mechanosensitivity of

fibroblasts. The goal of this protocol was to mimic a long duration of high repetitive mechanical stretch in vivo, such as repetitive lifting.

In this system, the cells are grown on custom made tissue culture plates interfaced to a flexible silicone medium. The silicone is deformed by the application of a vacuum, the parameters of which are regulated by a computer. For the treatment of mechanical stretching plus hormones, 17- β -estradiol (E2, 10^{-8} M) and progesterone (P4, 10^{-10} M) were added to the media (LEH, E2 and P4 were from Sigma-Aldrich Co, St. Louis, MO). The LEH, 17- β -estradiol and progesterone were water-soluble and directly dissolved in media. The combination of 17- β -estradiol plus progesterone was chosen based on the results of our previous studies demonstrating suppression of active MMP-1 and active MMP-13 by 17- β -estradiol (10^{-8} M) plus progesterone (10^{-10} M) [7,8]. The conditioned media were collected and immediately frozen in liquid nitrogen and later stored at -80°C until use. Cells cultured in identical conditions but without treatment of mechanical stretching and/or hormones were employed as the control (therefore representing a status of hormone deprivation alone).

Viability Assay

To specifically evaluate the cell viability under mechanical stretch, we adapted a method to quantify the “percent viability” of the cells via a non-destructive luciferase based bioluminescent cytotoxicity assay (Lonza Rockland Inc. Rockland, ME) used to measure toxicity in cells in culture. The assay quantitatively measures the release of adenylate kinase (AK), an intracellular enzyme present in all cells, which is released into the media when damage occurs to the plasma membrane. The AK reacts with the ADP substrate producing ATP, and luciferase is used to catalyze the formation of light from the ATP and luciferin. The emitted light intensity is expressed as the relative light unit (RLU), which is linearly related to the AK activity in the media.

Upon evaluation, 10 microliters (μL) of media were allowed to equilibrate to room temperature and transferred into each well on a 96 well plate, in addition to 100 μL of the AK detection reagent. The samples were allowed a reaction time of 5 minutes and the chemiluminescence was predicted at 600 nm with a SpectraMax M2 system (Molecular Devices, Sunnyvale, CA). Percent viability of the tested specimens was calculated based on a standard curve of viability that was produced by measuring the RLUs of samples containing a series of known percentages of lysed cells. All samples were assayed in triplicate.

One limitation of the above assay is a failure to distinguish the real nonviable cells from viable cells whose cell membrane integrity may be compromised, yet the cell may be able to repair itself to become fully viable. Thus, we performed a confirmatory assay of trypan blue exclusion test in parallel. Briefly, the cells were trypsinized, suspended in fresh medium and then spun down. The cell pellet was then resuspended in serum-free media and 20 μL of the cell supernatant was diluted in phosphate buffered saline (Cellgro, Mediatech Inc., Manassas, VA) at a ratio of 1:5. Twenty μL of this solution was then added to 20 μL of 0.4% trypan blue dye (Cellgro, Mediatech Inc.). A reaction time of 2 minutes was allowed at room temperature, and live cells (cells not incorporating the dye) were then counted with respect to total number of cells to determine percent viability. All counts were performed in duplicate.

Endogenous collagenase activity assay

Cell media were collected, centrifuged to remove debris and the amount of total protein in each specimen was determined in duplicate with a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Total collagenases activity per 180µl of media was measured using a collagenase activity assay (Chondrex Inc. Redmond, WA). The measurement was performed in duplicate following the manufacture's protocol except that the procollagenase activator was not added to the tested samples. In brief, after the sample was added into a 1.5ml tube, the FITC-labeled soluble type-I collagen substrate was added to samples. After an incubation of the mixture at 35°C for 60 minutes, an organic solution was added to stop the reaction and the fluorescence intensity indicative of the enzyme activity was determined at $E_m / E_x = 520\text{nm} / 490\text{nm}$ with a SpectraMax M2 system (Molecular Devices). Recombinant human MMP-8 was used as a positive control. Since collagenase activator was not utilized in this assay, the assay determined solely the enzyme activity of endogenous total active collagenases present in these samples. In this way, the sum of collagenase activity measured reflected the balance of active collagenases and their inhibitors. The enzyme activity was expressed as units/milliliter (units/ml) and was normalized to the total protein concentration of the media. The 1 unit of collagenolytic activity is defined as the cleavage of 1 milligram of collagen per minute.

Statistical analysis

All statistical analyses in this study were performed by a statistician who was blinded to the study aims, using SPSS 17.0. statistical software (SPSS Inc., Chicago, IL) and statistical tests were evaluated at the 0.05 significance level. The primary outcome of the study was to investigate if the vaginal cells were mechanosensitive, and the values of the skewness and kurtosis statistics did not indicate a significant departure from a normal distribution; therefore data was combined in this report and analyzed using Student's t-tests. Since these were pre-planned analyses, no adjustment for multiple comparisons was made. Linear regression was used to assess whether increased elongation correlated with increase in enzyme activity.

Results

As shown in Figure 1, immunofluorescent labeling of the cultured cells with a biomarker specific for fibroblasts, prolyl 4-hydroxylase beta (PHDB), demonstrated the identity of the cells to be fibroblasts. In parallel, the negative staining results, both with smoothelin and H-caldesmon, excluded the possibility of myogenic origin.

In this study, one may argue that the mechanical forces applied can compromise the cell integrity and cause intracellular enzyme to leak into the surrounding media. We therefore employed a bioluminescent cytotoxicity assay to investigate the cell viability. As demonstrated in Figure 2, the viabilities (%) were not found to be significantly different among the various experimental conditions (overall $P=0.68$). The cell viability was further verified by the Trypan blue exclusion assay demonstrating > 90% viability throughout the experiments.

Cultures from each of the 3 women were treated and analyzed independently; however, as described in the statistics section, all of the cultures responded similarly to hormone independent of patient age, the presence or absence of prolapse or hormonal status. Therefore, in the results that follow the data from all cultures was combined.

Repetitive mechanical stretching increased the activity of collagenases

As shown in Figure 3, relative to the control (cells without treatment of mechanical stretching and/or hormones), in the absence of hormones, endogenous collagenase activity was increased by both the 8% elongation (76%, $P=0.04$) and the 16% elongation (188%, $P=0.001$). The collagenases activity was higher with 16% elongation than that with 8%

elongation ($P=0.02$). Linear regression modeling revealed that the increase in collagenase activity significantly correlated with the magnitude of elongation (stretch) applied ($P<0.001$).

Addition of hormones decreased the activity of collagenases

The addition of hormones (17- β -estradiol plus progesterone) to mechanically stretched cells decreased the amount of collagenase activity (*8% and 16% elongation combined*) to the control level ($P=0.46$). However, there was no difference in the magnitude of suppression by hormones in cells stretched to 8% elongation vs 16% elongation ($P=0.48$) (see Figure 3).

The collagenase activity in the presence of hormones (17- β -estradiol plus progesterone) but in the absence of mechanical stretching was 71% decreased compared to control ($P=0.008$) (Figure 4). Thus, although the addition of hormones to mechanically stretched cells suppressed the enzyme activity to control level, it did not lower the activity to the level of hormonal treatment alone ($P=0.02$) (Figure 4), indicating an effect of mechanical stretching independent of the presence or absence of hormones. Mechanical stretching in the absence of hormones had an 86% higher collagenase activity than in the presence of hormones ($P=0.013$) (Figure 4).

Discussion

In this paper, we tested the hypothesis that, in the presence of repetitive stretching, the vaginal tissues of women previously injured from vaginal birth remain relatively stable due to the tropic effects of estrogen and progesterone on the supportive structures. In the absence of hormones, however, this compensatory effect is lost resulting in tissue breakdown. In the present study, we investigate our hypothesis by developing a cell culture model under the condition of repetitive mechanical stretching. We examined extracellular collagenase activity using a substrate degradation assay. By these methods, we showed that fibroblasts of vaginal connective tissue are mechanosensitive. Specifically, repetitive mechanical stretching of the cells resulted in the global cellular response of increased extracellular collagenase activity. While the addition of hormones mitigated this degradative response, it did not reduce collagenase activity to the level of cells with hormone in the absence of stretch indicating that hormones are not entirely protective in the presence of these magnitudes of stretch. Importantly, we performed viability assays to exclude the possibility that the increases of enzyme activity were secondary to cell death under the experimental conditions.

Previous studies have demonstrated that an increased intra-abdominal pressure is directly transmitted to vaginal wall [12] and increased its mechanical load to the vagina results in tissue stretch [13]. Mechanical loading has been shown to change the remodeling of tissues by inducing matrix metalloproteinases produced by the cells, including mesenchymal stem cells [14], osteoblastic cells [15] and rat periodontal ligament fibroblasts [16] – a phenomenon referred to as mechanotransduction. Although mechanotransduction has been demonstrated in multiple load bearing tissues throughout the body and the vagina is clearly a load bearing structure, to date, little is known of the impact of repetitive stretch on the behavior of vaginal fibroblasts. In this study, we measured the collective enzyme activity of collagen degrading enzymes-collagenases (including MMPs-1, -8 and -13) as well as their endogenous inhibitors. Collagenases are capable of degrading the major elements of extracellular matrix (ECM), including interstitial collagens (I, II and III), gelatin, aggrecan and perlecan, etc. [17]. Therefore, a net accelerated degradative activity in the presence of repetitive mechanical stretch may provide an intriguing explanation for the risk factors associated with the progression of vaginal prolapse, including chronic cough, repetitive

heavy lifting, obesity and chronic constipation [2] all which may increase intra-abdominal pressure and induce vaginal stretch [12,13]. Indeed, such repetitive mechanical stretching on the vagina and supportive tissues previously injured following vaginal birth may eventually lead to a deterioration in their supportive capacity due to a net loss of key structural proteins such as collagen and elastin. In addition, we demonstrate that even in the presence of a relatively small amount of stretch (8%), there is a rapid increase in collagenase activity not counteracted by inhibitors. It is unclear whether this increase persists or represents a transient response. Future studies at later time points are needed to resolve this issue.

It is worth mentioning that we demonstrate that the increase in collagenase activity in the presence of stretch is linearly correlated with the magnitude of the mechanical force applied, it is in accordance with a previous findings by Yang et al. [11] in which magnitude of stretch has been shown to alter the cellular response, using human patellar tendon fibroblasts under lower magnitudes of 4% and 8%. On the other hand, mechanical stretching may not always be detrimental, it is likely that a physiological stretch (smaller magnitude) has a beneficial effect since all tissues may require some degree of mechanical loading to maintain their structure. For example, Sun et al. demonstrated that, in human synovial cells, 2% stretching decreased collagenases (MMPs-1 and -13) at both mRNA and enzymatic levels [18]. Whether a similar mechanism applies to human vaginal fibroblasts of this study is not determined yet. In this study, we were primarily mimicking a high repetitive mechanical stretch in vivo therefore chose higher magnitudes of 8% and 16%, however, we are in the process of testing a serial of mechanical stretch with various magnitudes and frequencies.

Sex hormones have previously been demonstrated to inhibit active MMPs [5-9]. However, to date, there has been limited research defining the interplay between mechanical stretch and hormones on MMPs in human tissues. We found that hormones (17- β -estradiol plus progesterone) suppressed collagenase activity both in the absence and presence of mechanical stretching, but the amount of inhibition by hormones was directly dependent on the presence or absence of mechanical stretch, indicating that hormones are not completely protective against the deleterious effects of repetitive stretch. It is also noteworthy that, although the increase in enzyme activity by mechanical stretching in the absence of hormones was linearly related to the scale of the stretch applied, in the presence of hormones, we did not find a difference between enzyme activities by the magnitude of the applied force. Such an on/off effect of hormones is in agreement with our previous studies, in which we identified that the inhibitory effect on MMPs by 17- β -estradiol and progesterone (in doses of 10^{-7} - 10^{-10} M, whether in combination or not) occurred by a similar mechanism [7,8]. Further studies are needed to investigate whether increased concentrations of hormones can completely counteract mechanical stretch or whether less hormones would fail to counteract the effect of stretch. Regardless, however, the suppressive effect of hormones demonstrated in this study suggests that hormones play a key role in mitigating the impact of repetitive stretch.

There are limitations to the present study. First, the study was designed as a mechanistic study and therefore, it was based on cells. It is unclear whether cells specifically derived from women with prolapse would behave differently from women with normal support or vary by age. Although our initial analyses suggested that these factors did not impact the cellular response, more data is needed to confirm this finding. Indeed, we are currently in the process of testing cells from more patients with serial doses of hormones and broader range of levels of mechanical loads. A second shortcoming is that the data in this paper were generated in vitro under highly controlled conditions that may not reflect in vivo events. This study, however, will serve as a basis for future studies where our hypothesis will be tested using animal models in which the vagina and supportive tissues are exposed to cyclical stretch. Evidently, however, based on the findings in this study, future studies on

enzymes involved in tissue remodeling in the pelvic floor should take into account the effects of mechanical stretch.

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Figure 1.

Immunofluorescence labeling of the cells derived from the vaginal wall demonstrating intracellular location of prolyl 4-hydroxylase beta (PHDB) (x100). Cells were grown to 50% confluence and labeled with a marker for fibroblasts – PHDB and markers for myocyte - smoothelin or H-caldesmon.

Figure 2. Percent viability of cells among various experimental conditions. H: 17- β -estradiol (10^{-8} M) plus progesterone (10^{-10} M). Control: cells cultured without H and mechanical stretching. The error bar represents standard deviation (SD). Overall P value was from ANOVA.

Figure 3.

Collagenase activity in response to different treatments. H: 17- β -estradiol (10^{-8} M) plus progesterone (10^{-10} M). *The error bar represents standard deviation (SD).* Control: cells cultured without H and mechanical stretch. *: compared to control; **: comparison between 8% vs 16% stretch.

Figure 4.

Collagenase activity among different treatments. H: 17- β -estradiol (10^{-8} M) plus progesterone (10^{-10} M). The error bar represents standard deviation (SD). Control: cells cultured without H and mechanical stretching. Stretch: combined data of 8% and 16% stretching. *: compared to control; **: compared to H alone; ***: comparison between stretch in the presence vs absence of H.