Fibroblasts from women with pelvic organ prolapse show differential mechano-responses depending on surface substrates

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ABSTRACT

Objective: Little is known about dynamic cell-matrix interactions in the context of pathophysiology and treatments for pelvic organ prolapse (POP). This study seeks to identify differences between fibroblasts from women with varying degrees of prolapse in reaction to mechanical stimuli and matrix substrates in vitro.

Study design: Fibroblasts from the vaginal wall of three patients with POP-Q stages 0, II and IV, were stretched on artificial polymer substrates coated or not with collagen I. Changes in morphology and anabolic/catabolic compounds that affect matrix remodeling were evaluated at protein and gene expression levels. Statistical analysis was performed using one-way ANOVA followed by Tukey-Kramer’s post-hoc test.

Results: POP fibroblasts show delayed cell alignment and lower responses to extracellular matrix remodeling factors at both enzymatic and gene expression levels, compared to healthy fibroblasts.

Conclusion: POP fibroblasts, when compared to healthy cells, show differential mechano-responses on two artificial polymer substrates. This should be taken into account when designing or improving implants for the treatment of POP.

BRIEF SUMMARY

Fibroblasts from women with different degrees of pelvic organ prolapse show in vitro differential mechano-responses to cyclic mechanical loading and two artificial substrates.
INTRODUCTION

Pelvic organ prolapse (POP) is a common multifactorial disease with known risk factors and unclear pathogenesis. POP is characterised by the weakening of the pelvic floor, and is associated with serious inconveniences and a reduced quality of life to almost 50% of women over 50 years of age. Conservative therapies are not always possible or sufficient and reconstructive surgery with native tissue has high failure rates. Of the treated patients 30% will experience recurrent POP within the first two years of treatment. Since the 1970’s, urogynecologists started to use polymeric meshes, originally designed to treat inguinal hernia, in an attempt to restore tissue support in POP patients. However, complications such as mesh contraction, exposure or extrusion caused serious health problems to a point that in July 2011 the FDA issued a safety communication about the use of transvaginal meshes in POP repair. Thus improvement of current therapies is urgently needed but this is hampered by a lack of understanding of the pathophysiology of the disease, along with sparse knowledge of the cause-effect relationships of mesh failures in patient tissues.

In recent years, there has been a growing interest in studying tissue composition of patients with prolapse. Researchers have mainly focused on characterising the extracellular matrix (ECM) of connective tissues that support the pelvic floor such as the vaginal wall, the uterosacral ligaments and the pubocervical fascia. Different outcomes have been reported, but the overall consensus is that the connective tissue of the vaginal wall is abnormal in women with POP.

The vaginal wall is one of the soft tissues that is constantly being remodelled in order to withstand the different forces that are applied to it during a woman’s lifetime. Thus the weakening of the pelvic floor could be caused by an imbalance of its remodeling. The presence of artificial substrates may very well influence this process.

Tissue remodeling is a well-balanced process involving several factors with different roles, and cells as modulators. In the vaginal wall, fibroblasts (FBs) are the mechanosensitive cells responsible for maintaining ECM homeostasis; they produce molecules, and control anabolic and catabolic processes to remodel their surrounding matrix in response to mechanical and biochemical stimuli. Compounds particularly involved in ECM homeostasis include collagens (mainly type I and III), the collagen degrading matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs).
MMPs are involved in both normal and pathological ECM remodeling processes throughout the body, including the pelvic floor. It has been shown that the amounts of active MMP-2\textsuperscript{12,13,16} and/or MMP-9\textsuperscript{7,17} are increased in tissues from patients with POP in comparison to controls. However we were interested to see if these matrix metalloproteinase are also increased when cells are exposed to cyclic mechanical loading. Furthermore it is the question whether this enzymatic activity is affected by the presence of artificial polymeric substrates.

In order to answer these questions, the present study was designed to evaluate in vitro dynamic cell-matrix interactions which are important for understanding the pathophysiology of and treatments for POP. We hypothesize that fibroblasts from women with different degrees of prolapse, display different mechano-responses depending on the substrate encountered. This hypothesis was tested by subjecting fibroblasts from healthy, mild or severe POP women to cyclic mechanical loading mimicking continuous respiration\textsuperscript{18} on artificial polymeric membranes uncoated as well as coated with collagen I. Changes in morphology and anabolic/catabolic compounds that may affect the remodeling of the extracellular matrix were analysed.

**MATERIALS AND METHODS**

**Patient selection, tissue processing, and cell culture**

Retrieval of biopsies was approved by the medical ethical committees of two hospitals in the Netherlands: VU University Medical Centre (Amsterdam) and Kennemer Gasthuis Hospital (Haarlem), and informed consent was obtained. Full thickness biopsies (1cm\textsuperscript{2}) of the anterior vaginal wall were obtained during vaginal hysterectomy of one patient with mild (with POP-Q stage II) and one patient with severe POP (with POP-Q stage IV). A third woman, who was operated for benign gynecological reasons, was selected as a healthy control. For ethical reasons the biopsy site of the latter patient was the precervical region of the anterior vaginal wall. Tissues were collected in PBS at 4°C and cells were isolated within 24h under sterile conditions. Fascia was scraped, cut into little pieces, and digested with Liberase TM (final concentration: 0.3U/ml; Roche Diagnostics, Mannheim, Germany) for 3h at 37°C and constant agitation. After filtration with a 100µm cell strainer (BD Falcon, Franklin Lakes, NJ, USA), the pellet was re-suspended in culture medium (Dulbecco’s modified Eagle’s medium-DMEM) supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, and 250µg/ml amphotericin-B. FBS was obtained from HyClone (South Logan, UT, USA), the
other culture components were obtained from Gibco-Life technologies (Paisley, UK) and Sigma (St. Louis, MO, USA). Subsequently, cells were seeded on 6-well plates, and grown in an incubator at 37°C, 95% humidity and 5% CO₂ until they reached 60% confluence. At that point, the cells were considered to be at passage 0. Fibroblasts from passage 3-5 were used for loading experiments.

**Cyclic mechanical loading**

Forty-eight hours before mechanical loading, fibroblasts were seeded at a density of 150,000 cells/well on two artificial polymer substrates: 6-well uncoated or collagen I-coated silicone Bioflex® plates (BioFlex, Flexcell International Corp., McKeesport, PA, USA). Just before loading, the cells were refreshed using culture media containing 1% FBS. The loading regime was applied using a Flexercell FX4000 system (Flexcell International Corp.), and consisted of 24 or 48 hours of continuous cyclic mechanical loading (CML) mimicking continuous respiration as described by Blaauboer and colleagues (parameters: sinusoidal wave, frequency of 0.2 Hz and maximum elongation of 10%). The Flexercell is a device that is widely used and has a vacuum pump that pulls down the elastic membrane of the bioflex plates stretching the cells that are seeded on top accordingly. Fibroblasts cultured under the same conditions but without loading served as static controls. After the loading period, the cells were imaged using the bright field of an inverted Leica DMIL microscope with a DFC320 digital camera (Leica Microsystems, Wetzlar, Germany) and samples were collected for F-actin staining, MMP activity, protein content and gene expression.

**F-actin staining**

For immunocytochemical staining of F-actin, cells were fixed using 4% formaldehyde, stained for F-actin with Alexa Fluor 488 phalloidin (Molecular Probes, Leiden, The Netherlands) and imaged using an inverted Leica DMIL microscope (Leica Microsystems) as previously described.¹⁹

**Enzymatic activity**

After mechanical loading, conditioned media was collected and the gelatinolytic activity of MMP-2 and MMP-9 was evaluated by zymography using Novex zymogram gels (10% zymogram gelatin gel, Life Technologies) following manufacturers protocol. Dark bands of gelatinolytic activity were visualized using an eStain protein staining device (GeneScript, Piscataway, NJ, USA). Images were acquired with Biospectrum AC Imaging System (UVP, Cambridge, UK) and zymogram quantification of the density of the bands was performed using Image J 1.44p software (National Institutes of Health, USA). Quantitative data was normalised to
total protein content (section below).

**Western blot analysis**
Conditioned media after 48h of loading were used to detect protein levels of TIMP-2 by western blot. Samples were concentrated 2x by freeze drying, denatured for 5min and reduced with dithiothreitol. Samples were separated by electrophoresis on a NuPAGE® Novex 4-12% Bis-Tris gel and transferred to iBlot® PVDF membrane (Life Technologies). For protein detection, monoclonal antibody anti-TIMP-2 mouse (mAb T2-101; Calbiochem®, Merck Millipore, Darmstadt, Germany) was used at a concentration of 1:500. Blots were blocked for 1h at room temperature with a blocking buffer (PBS with 0.5% Tween-20 and 1% bovine serum albumin), then incubated with primary antibody in blocking buffer for 1h at room temperature, and followed by an overnight incubation at 4°C. Bound antibodies were visualized with a horseradish peroxidase-conjugated antibody goat anti-mouse (1:10000) and Supersignal west pico chemiluminescence kit (Thermo Scientific, Rockford, IL, USA). Images were acquired with Biospectrum AC Imaging System (UVP) and quantification of the density of the bands was performed using Image J 1.44p software (NIH).

**Total protein quantification**
For total protein quantification cells were lysed in buffer containing 50mM Tris, pH 7.5, 150mM NaCl, 1mM sodium orthovanadate, 1% Nonidet P-40, 0.1% sodium deoxycholate, and EDTA-free protease inhibitor mixture (Sigma-Aldrich). The total protein content was determined using Pierce BCA Protein Assay kit (Thermo Scientific) following the supplier’s specifications. Quantification was performed spectrophotometrically between 540-590nm and using a 1420 multilabel counter VICTOR2 (WALLAC, Turku, Finland).

**Gene expression**
For gene expression cells were lysed in a solution (1:100) of β-mercaptoethanol (Sigma-Aldrich) and RA1 buffer (Macherey-Nagel, Bioke, Leiden, The Netherlands). According to the manufacturers instructions, total RNA was isolated using NucleoSpin TriPrep kit (Bioke) to a final concentration of 250 ng/ml, and was reverse-transcribed using SuperScript VILO cDNA synthesis kit (Life technologies). Gene expression of Col 1α1, Col 3α1, MMP-2, TIMP-2 and the housekeeping genes Ywhaz and hUBC were evaluated using the primers listed in Table 1 (Life technologies), the SYBR Green Reaction Kit following suppliers’ specifications (Roche) and measured by RT-PCR in a Light Cycler 480 device (Roche). Gene expression levels were normalized using a factor derived from the equation √(Ywhaz x hUBC). Crossing points were
assessed using the Light Cycler software (version 4) and plotted versus serial dilutions of cDNA derived from a human universal reference total RNA (Clontech Laboratories Palo Alto, CA, USA).

Statistical analysis
Three independent experiments were performed in duplicate and data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer’s post-hoc test (Prism version 5.02, GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at 5% level (p < 0.05).

**Table 1.** Primer sequences used for RT-PCR.

| Target gene   | Oligonucleotide sequence      | Annealing temperature (°C) | Product Size (bp) |
|---------------|-------------------------------|---------------------------|-------------------|
| Col 1α1       | 5’ TCCAACGAGATCGAGATCC 3’     | 57                        | 191               |
|               | 5’ AAGCCGAATTCCTGGTCT 3’      |                           |                   |
| Col 3α1       | 5’ GATCCGTCTCTGCGATGAC 3’     | 56                        | 279               |
|               | 5’ AGTTCTGAGGACCAGTGGG 3’     |                           |                   |
| MMP-2         | 5’ GGCAGTGCAATACCTGAACA 3’    | 56                        | 253               |
|               | 5’ AGGTTGTTAGCCAATGATCT 3’    |                           |                   |
| TIMP-2        | 5’ CTGAAACCACAGGTACACCAGAT 3’| 63                        | 237               |
|               | 5’ TGCTTATGGGGTGTCCTGATG 3’   |                           |                   |
| Ywhaz         | 5’ GATGAAAGCCATTGCTGAACCTG 3’| 56                        | 229               |
|               | 5’ CTATTGTGGGGACAGCATGGGA 3’  |                           |                   |
| hUBC          | 5’ GCCTGGAACCCCGATATTAT 3’    | 56                        | 202               |
|               | 5’ TTTGGCCTGACATTTCGATGGG 3’  |                           |                   |

Col1α1, α1(II)procollagen; Col3α1, α1(III)procollagen; MMP-2, matrix metalloproteinase 2; TIMP-2, tissue inhibitor of metalloproteinases 2; hUBC, human ubiquitin C; Ywhaz, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide.
RESULTS

After 48 hours of cyclic mechanical loading, morphological analysis showed cell alignment perpendicular to the force that was facilitated by collagen-coated surfaces with no apparent differences between healthy (Fig.1b and d) and POP fibroblasts (Fig.2c and d). At an earlier time point (24h) load-induced rearrangement of F-actin fibres was completed for mild but not for severe POP fibroblasts (Fig.2a vs. b). Static control cells displayed random distribution independent of the surface (Fig.1a and b).

Figure 1. Cell attachment and alignment is facilitated by collagen I substrates. Representative images of healthy fibroblasts after 48h of cyclic mechanical loading (CML) on uncoated (a and c) or collagen I-coated (b and d) plates. Morphology shows random cell distribution under static conditions (-CML, a and b); whereas stretching induced cell alignment perpendicular to the force (F), especially on collagen I-coated plates (+CML, c and d). Images were acquired with 10x objective of a Leica microscope, bar is 100µm.
To evaluate the effects of the different experimental conditions on the capacity of the fibroblasts to remodel the extracellular matrix, secreted MMP-2 and MMP-9 enzymatic activity were evaluated at two time points (24h and 48h). Total levels of released MMP-2 by POP fibroblasts at 24h were lower than healthy control cells independent of the surface substrate (Fig.3a). At the same time point, but only on uncoated plates, cyclic mechanical loading induced activation of MMP-2 which was more pronounced by healthy fibroblasts. Under static conditions, activation of MMP-2 was only observed in healthy control cells on uncoated plates (Fig.3a left blot). There was no apparent activation of MMP-2 on collagen I-coated plates, with the exception of a very faint band of active MMP-2 on stretched mild POP fibroblasts (Fig 3a middle blot).

Figure 2. On collagen I-coated plates, rearrangement of F-actin fibres was completed after 24h of loading for mild but not for severe POP fibroblasts. Representative images of POP fibroblasts F-actin fibres showing cell alignment perpendicular to the force (F) on collagen I-coated plates after 24h (a and b) or 48h (c and d) of loading. F-actin fibres stained with phalloidin, bar is 100µm.
Chapter 5

| CML | Healthy | Mild | Severe | Healthy | Mild | Severe | Positive | Negative |
|-----|---------|------|--------|---------|------|--------|----------|----------|
|     | -       | +    | -      | +       | -    | +      | pro MMP-9 | pro MMP-9 |
|     | 24 hours|      |        |         |      |        | pro MMP-2 | active MMP-2 |
|     |         |      |        |         |      |        | pro MMP-9 | pro MMP-9 |
|     |         |      |        |         |      |        | pro MMP-2 | active MMP-2 |
| c)  | Uncoated|      | Collagen I | Controls |      |        | TIMP-2  | 21 kDa |

48 hours

| d)  | pro MMP-2 |
|-----|------------|
|     | Uncoated   | Healthy | Mild | Severe |
|     |            | 20     | 40   | 60     |
|     |            |         |      |        |

| e)  | pro MMP-2 |
|-----|------------|
|     | Collagen I | Healthy | Mild | Severe |
|     |            | 20     | 40   | 60     |
|     |            |         |      |        |

Ratio MMP-2 active/inactive

| f)  | Uncoated   | Healthy | Mild | Severe |
|-----|------------|---------|------|--------|
|     |            | 10     | 30   | 50     |
|     |            |         |      |        |

TIMP-2

| g)  | Collagen I | Healthy | Mild | Severe |
|-----|------------|---------|------|--------|
|     |            | 20     | 40   | 60     |
|     |            |         |      |        |
At a later time point (48h), levels of released pro-MMP-2 were similar on collagen I surfaces and no active MMP-2 was found (Fig.3b middle blot). On uncoated plates, stretching of fibroblasts induced activation of MMP-2 that increased from 24h to 48h. Such activation was lower in POP fibroblasts when compared to healthy controls (Fig.3b and b, left blot). Western blot analysis at 48h revealed the presence of TIMP-2 only in the case of cells exposed to collagen coated plates without any apparent regulation by loading (Fig.3c). Released MMP-9 was below detection levels.

Since levels of released MMP-2 were higher after 48 hours, these data were quantified and normalised to total protein content. Quantitative data revealed that under static conditions and regardless of the type of substrate, most cells released similar amounts of pro-MMP-2 into the extracellular environment (Fig.3d and e). On uncoated plates, mechanical loading induced activation of MMP-2 by all cell populations as the ratios between active and inactive (pro) MMP-2 forms were always greater than 1 and an ANOVA showed that such increment was significant only in the case of healthy fibroblasts (p < 0.01) (Fig.3f). On collagen I-coated plates, quantitative data revealed similar expression profiles of pro-MMP-2 and TIMP-2 in all groups (Fig.3e and g).

To detect differences at gene expression level, the ECM remodeling related genes Col 1α1, Col 3α1, MMP-2 and TIMP-2, were chosen to evaluate the effects of cyclic mechanical loading and surface substrate after 48 hours of treatment. On uncoated plates, both mild and severe POP fibroblasts showed significantly lower
gene expression of Col 1α1 and Col 3α1 when compared to healthy controls (Fig.4a and b). On the same substrate, gene expression of Col 1α1 was down-regulated by mechanical loading in healthy but not in POP fibroblasts. MMP-2 expression levels were similar in all fibroblasts independent of loading conditions (Fig.4c and d). On collagen I-coated plates, mild but not severe POP fibroblasts showed lower gene expression levels of Col 1α1, Col 3α1 and MMP-2 when compared to healthy controls (Fig. 4e, f and g). TIMP-2 expression levels were similar in all fibroblasts independent of loading or coating conditions (Fig.4h).

Figure 4. Relative expression of extracellular matrix remodeling related genes. Fibroblasts from healthy, mild or severe POP patients were subjected, or not subjected, to cyclic mechanical loading (+/- CML) on uncoated (a, b, c and d) or collagen I-coated plates (e, f, g and h). Each column represents a different gene: Col 1α1 (a) and (e); Col 3α1 (b) and (f); MMP-2 (c) and (g); TIMP-2 (d) and (h). Values are normalized to housekeeping genes (Ywhaz and hUBC), expressed as a percentage of healthy control and represent the mean ± SD. *p<0.05, **p<0.01, ***p<0.001; compared to the first bar unless indicated otherwise.
DISCUSSION

In pelvic organ prolapse tissue strength is lost, stiffness is increased\(^5\)\(^\text{-}^\text{11}\), and quality of the extracellular matrix is compromised.\(^1^5\) Consequently, fibroblasts might be exposed to an abnormal matrix. The biomechanical environment may be further compromised if during pelvic reconstructive surgery stiff, non-resorbable polymeric meshes are used to replace tissue function. We evaluated the possible roles of cyclic mechanical loading and two surface substrates on the functionality of healthy as well as mild and severe POP fibroblasts using an in vitro dynamic model. We found variations of fibroblast responses at morphological, enzymatic and gene expression levels.

Cells respond to mechanical stimuli by remodeling their actin cytoskeleton.\(^2^0\) Our results show that all fibroblasts were mechano-responsive as their actin cytoskeleton aligned perpendicular to the force after 48 hours of cyclic mechanical loading, especially in the presence of collagen I. This finding is consistent with previous reports that fibroblasts from the pelvic floor completely align after being stretched for 48 hours.\(^2^1\)

Differences between cell populations were seen after 24 hours of loading on collagen I substrates, when visualization of actin filaments revealed that alignment of severe POP fibroblasts appear delayed in comparison to their mild counterparts, and released MMP-2 was lower in fibroblasts from POP patients compared to healthy control cells. These effects seemed to disappear with time since after 48 hours of stretching there were no apparent differences in cell alignment, there was no activation of MMP-2, TIMP-2 protein levels corresponded to pro MMP-2, and there were no differences in gene expression of MMP-2 or TIMP-2.

Interestingly, when fibroblasts were exposed to artificial polymeric substrates (uncoated plates), clear differences were seen in the production and activation of MMP-2, TIMP-2 could not be detected and mechanical loading promoted activation of MMP-2 over time. After 48 hours of loading, MMP-2 activation was significant only in healthy, and not in POP fibroblasts. Moreover, cells from women with prolapse showed differential gene expression of anabolic but not catabolic compounds: collagens I and III were lower in women with POP and mechanical loading down-regulated collagen I, but only on healthy fibroblasts. Changes seen with catabolic secreted proteins were not correlated at gene expression level. Such discrepancy highlights the importance of using different evaluation parameters because changes at gene expression do not necessarily reflect changes at protein
levels and they can occur at different times.

Taken together our data suggest that, although fibroblasts from POP patients seem to have lower mechano-responses, in the presence of collagen I substrate the system eventually reaches a balance. The latter confirms 48 hour data from Zong and colleagues\textsuperscript{22}, who used collagen I-coated plates in their in vitro dynamic model with similar experimental conditions (sinus wave; amplitudes: 8% and 16%; frequency: 1Hz), and did not find differences between healthy, mild and severe POP vaginal fibroblasts.

However, it appears that when cells are exposed to artificial polymeric substrates and stress is imposed, this balance is not reached. Fibroblasts from women with POP seem preconditioned by the abnormal prolapsed matrix. Unable to respond in the same way as control cells, POP fibroblasts might not be able to restore ECM homeostasis when artificial polymeric substrates are added to their microenvironment. In such circumstances tissue remodeling might not be restored but instead, may in turn provide a negative feed-back loop that deteriorates the ECM even further leading to an additional loss of strength, increased stiffness and eventually more tissue damage. Such implications at the micro level are in line with findings at macro level. Several studies have reported increased stiffness in prolapsed tissues when assessing biomechanical properties of vaginal wall from patients with POP compared to healthy controls\textsuperscript{17-19}. Moreover, it has also been shown that polymeric meshes used in genital prolapse surgery are stiffer than the native tissue.\textsuperscript{23} Feola and colleagues\textsuperscript{24} recently showed correlation between mesh stiffness and tissue deterioration in a non-primate animal model. Therefore, changes in the vaginal wall at cellular level could be good indicators of tissue behaviour and should be taken into account when treating patients with polymeric meshes. Since surface substrate affects cellular behaviour, and cell-matrix interactions seem to be impaired in POP fibroblasts, improving mesh surface characteristics could enhance implant integration.

Conclusions from the present study should be treated with care: results were obtained in an in vitro set up which allowed us to control certain parameters, but does not fully reflect the in vivo situation. Furthermore, we are aware that our patient population is too small to draw conclusions about all women with POP. The experiments have been repeated three times confirming the first results suggesting our model to be valid. We are currently in the process of expanding our sample size.
To improve pelvic floor treatments, further studies evaluating the effect of different ECM proteins to fibroblasts in dynamic in vitro systems could provide important clues to improve mesh designs before using animals for in vivo studies and humans for clinical trials. This approach should provide better clinical outcomes by using information from bench to bed side to restore support and improve host-implant integration in treatments for pelvic organ prolapse.

In summary: this study provides a model to evaluate dynamic interactions of fibroblasts from the pelvic floor with artificial substrates in vitro. Unlike previous models, we chose a continuous physiological stretch regimen to compare the fibroblasts’ mechano-responses to artificial collagen I-coated and uncoated substrates. We thereby show clear differences between POP and healthy fibroblasts on artificial polymer substrates. This highlights the importance of evaluating cell-matrix interactions with different surroundings to better understand the influences of proteins from the ECM on vaginal fibroblast behaviour in dynamic environments. Such outcomes may provide important clues on how to design biomeshes that mimic the ECM environment appropriately, since it seems that the addition of collagen coating helps to restore the vaginal wall metabolic balance. This new approach may enable the improvement of treatments for pelvic organ prolapse.
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