Gene expressions of small leucine-rich repeat proteoglycans and fibulin-5 are decreased in pelvic organ prolapse

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Abstract: Few studies are performed on the sustainability of the pelvic floor extracellular matrix important for preventing development of pelvic organ prolapse (POP). Collagens I and III, the elastin-associated proteins fibrillin-1 and fibulin-5 and the small leucine-rich repeat proteoglycans (SLRPs) decorin, lumican and fibromodulin are involved in giving the tissue its mechanical properties. Para-urethral biopsies were obtained from 15 women, 6 pre- and 9 post-menopausal, with POP. Real-time reverse transcription–polymerase chain reaction and immunohistochemistry for collagen I, collagen III, fibrillin-1, fibulin-5, decorin, lumican and fibromodulin were performed and compared with 14 controls, 8 pre- and 6 post-menopausal. Statistical comparisons controlled for age changes in gene expressions. A 16-fold decrease in decorin mRNA expression, \( P = 0.0001 \), and 8-fold in lumican mRNA expression, \( P = 0.001 \), were discovered in premenopausal POP compared with matched controls. In all women with POP, there were lower gene expressions of fibromodulin, \( P = 0.004 \), and fibulin-5, \( P = 0.001 \), compared with all controls. All proteins were detectable by immunohistochemistry, showing a weaker staining for decorin in premenopausal POP. For the first time, we show substantially decreased gene signal for production of SLRPs, regulators of collagen fiber assembly and impairment in elastic fiber assembly by down-regulation of fibulin-5 in POP.

Key words: prolapse / proteoglycan / elastin / collagen / extracellular matrix

Introduction

Pelvic organ prolapse (POP) is a common form of pelvic floor dysfunction leading to impairment of the quality of life. This condition with a prevalence of 8% has often been considered to be related to the senescence, but an epidemiological study on a Swedish population showed that POP-specific symptoms were most common between 50 and 60 years of age, when women in the western world are still very active (Tegerstedt et al., 2005). Furthermore, 30% of the women with POP-specific symptoms were younger than 50, and 14% had symptoms already before 40 years of age. The treatment is often surgical, but there are difficulties in finding adequate methods to ensure good results and avoid recurrences.

Vaginal delivery is considered the main clinical risk factor (Mant et al., 1997; Progetto Menopausa Italia Study Group, 2000), but studies have implicated that some women may have an inherited vulnerability. For instance, it has been demonstrated that there is an increased prevalence of POP in first grade relative to women suffering from POP and that POP is more common in hereditary conditions such as joint hyper mobility and even Ehlers–Danlos syndrome, a mutation in the gene for collagen III (McIntosh et al., 1995; Norton et al., 1995).

The pelvic floor is a structure designed to keep pelvic organs within the body still allowing passage through the urethra, vagina and the anal canal. It consists of striated muscles and fibrous connective tissue distributed within the muscles and surrounding the organs forming fascias and ligaments. The pelvic floor connective tissue has been named the endopelvic fascia and one of its purposes is to maintain the positions of organs adjacent to the vagina, why POP can be considered a hernia in the endopelvic fascia (DeLancy, 1993).

Fibrous connective tissue is defined as extra cellular matrix (ECM), containing mainly fibrous proteins, hyaluronan and proteoglycans, all produced by the sparsely distributed fibroblasts or myo-fibroblasts. The fibrillar collagens I and III are the dominating proteins here and responsible for tensile strength (Gelse et al., 2003).

The elastic fibers enable the tissue to stretch and are composed by the bulking elastin on a framework of microfibrils (Mithieux and Weiss, 2003).
Fibrillin-1 is the most studied microfibril, since mutations in its gene are found in patients with Marfans’ syndrome. Other proteins are of importance for the complex elastic fiber assembly, among them the fibrils. Drewes et al. (2007) showed that fibulin-5 knock-out (KO) mice developed POP already before pregnancy and with increased severity after vaginal delivery.

The collagens and elastic fibers are cross-linked and the small leucine-rich repeat proteoglycans (SLRPs) seem to be involved in this process. SLRPs cover the surface of the collagens where they contribute to optimal fibril formation. Among these, decorin, lumican and fibromodulin were reported to dramatically influence the collagen fiber assembly and they appear to co-operate in this process as lumican and fibromodulin compete for the same binding site on collagen (Danielson et al., 1997; Chakravarti et al., 1998; Svensson et al., 1999, 2000). Furthermore, decorin and fibrillin-1 interactions make up a part of elastic fiber assembly (Trask et al., 2000; Schaefer et al., 2007).

We hypothesize that a constitutionally impaired quality of the pelvic floor ECM in women developing POP makes their tissues less sustainable to vaginal delivery. Furthermore, these alterations may differ with regard to the age at which the symptoms appear. This was shown in an earlier study by our group revealing a 30% decrease of collagen concentration in women younger than 53 suffering from POP, but not among the older women (Soderberg et al., 2004). To analyze this more extensively, we investigated the gene expressions and tissue localizations of collagens I and III, the elastic fiber associated proteins fibrillin-1 and fibulin-5 and the SLRPs decorin, lumican and fibromodulin in the pelvic floor ECM in women developing POP compared with controls matched for age by including the participants in pre- and post-menopausal subgroups.

Materials and Methods

Patients

Fifteen patients were included during surgery for POP, six were premenopausal and nine post-menopausal. Selection criterion was POP stage II or more according to the POP-Q staging system (Bump et al., 1996). Fourteen women served as controls, when undergoing surgery for other benign conditions. Eight were premenopausal and six had passed menopause. None of the participants in the POP or control groups suffered from stress urinary incontinence. Table I shows age, parity, use of hormonal contraceptives (HC)/hormone replacement therapy (HRT), surgical conditions and surgeries performed in the respective groups.

The local Ethics Committee at the Karolinska Hospital approved of this study (D-nr 99-176) and the included women gave their informed consent.

Biopsies

During surgery, punch biopsies, 6 mm in diameter and 10 mm of depth, were collected from the para-urethral ligaments 5 mm from the urethral orifice on either or both sides. The mucosa was excised from the underlying ECM, which has earlier been considered being a representative part of the endopelvic fascia (DeLancey, 1994). It is notable that the biopsies were not in any patient taken from a site involved in the actual prolapse, for avoiding discussions whether possible changes could be mechanical effects of the prolapse itself.

The biopsies used for the real-time reverse transcription–polymerase chain reaction (RT–PCR) were immediately fixed in RNA-later® (Ambion, TX, USA) and stored frozen in −70°C. Biopsies prepared for immunohistochemistry were fixed in 4% formaldehyde, followed by dehydration in 70% ethanol and paraffin embedded.

Real-time RT–PCR

After the biopsies were homogenized, the total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was controlled for by an OD\textsubscript{260}/OD\textsubscript{280} ratio of > 1.7 (Eppendorf AG, Hamburg, Germany) and by electrophoresis on 1.5% agarose gels. The reverse transcription (RT) to cDNA was performed using SuperscriptTM RNase H-Reverse Transcriptase (Invitrogen) according to the standardized protocol provided by the manufacturer.

In order to quantify the levels of mRNA encoding fibrillin-1, fibulin-5, decorin, lumican, fibromodulin, collagen I and collagen III by real-time RT–PCR, the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was employed. All determinations were analyzed in triplicates in Taqman Universal PCR Master Mix.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Age, parity, use of HC/HRT, surgical conditions and surgeries performed</th>
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<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>POP all</td>
<td>15</td>
</tr>
<tr>
<td>POP premenopausal</td>
<td>6</td>
</tr>
<tr>
<td>POP post-menopausal</td>
<td>9</td>
</tr>
<tr>
<td>Control all</td>
<td>14</td>
</tr>
<tr>
<td>Control premenopausal</td>
<td>8</td>
</tr>
<tr>
<td>Control post-menopausal</td>
<td>6</td>
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IUD, intrauterine device; D&C, dilatation and curettage; PMP, post-menopausal; HC, hormone contraceptives; HRT, hormone replacement therapy.
Reduced extracellular matrix gene expressions in prolapse

Table II Probes and primers used in real-time RT–PCR

<table>
<thead>
<tr>
<th>Probe/primer for</th>
<th>Assay ID</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillin-1</td>
<td>Hs00973197_m1</td>
<td>NM0001383</td>
</tr>
<tr>
<td>Fibulin-5</td>
<td>Hs01056635_m1</td>
<td>NM0063292</td>
</tr>
<tr>
<td>Decorin</td>
<td>Hs00370385_m1</td>
<td>NM1335032, NM1335042, NM1335052, NM0019203</td>
</tr>
<tr>
<td>Lumican</td>
<td>Hs00158940_m1</td>
<td>NM0023453</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Hs00157619_m1</td>
<td>NM0020233</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Hs00164004_m1</td>
<td>NM0000883</td>
</tr>
<tr>
<td>Collagen III</td>
<td>Hs00943816_m1</td>
<td>NM0000903</td>
</tr>
</tbody>
</table>

(Applied Biosystems) on 96-well optical PCR-plates. Appropriate primers and probes were purchased from commercially available Taqman® gene expression assays (Applied Biosystems, Table II). Ribosomal 18S rRNA (Human 18S rRNA-4310893) was used as an internal standard. The real-time PCR reaction was performed involving 40 cycles of denaturation-annealing. The threshold cycles (Ct) correspond to a low mRNA regulation, the ΔCt values are presented inverted as 10/ΔCt. A gene expressing mRNA with a Ct > 40 was considered undetectable.

Immunohistochemistry

The biopsies were sectioned, mounted on glasses and stained applying the MACH3™ Mouse/Rabbit-Probe HRP Polymer Kit (Biocare Medical, CA, USA) according to the manufacturer’s protocol. After incubation with appropriate mouse monoclonal antibodies or rabbit polyclonal antibodies, diluted as shown in Table III, the sections were stained with Betazoid DAB (Biocare Medical) and counterstained with 10% Mayer’s hematoxylin. The blinded samples were examined in toto by two independent researchers who subjectively evaluated the immunostaining intensity and distribution in the ECM.

Statistics

The ANOVA test or the ‘simple main effects test’ was used for calculating group differences of mRNA expression. By analyzing the interaction of age concerning every variable in the respective pre- and post-menopausal groups, using univariate tests of significance comparing inclinations of parallel lines and the ANCOVA test with age as co-variable, appropriate age groups were compared with each other.

Results

The mRNA expressions of all the analyzed molecules decreased by age significantly in the control groups, reflecting the effect of aging in this tissue. In the POP groups, there was a parallel age effect except concerning decorin and lumican for which the expressions were low regardless of age. This interaction of age regarding decorin and lumican was verified by the univariate test of significance and the ANCOVA. Accordingly, calculations for case–control differences were performed in separate pre- and post-menopausal groups for decorin and lumican, but regardless of menopausal status for fibulin-5, fibromodulin and collagens I and III.

The most prominent finding was a 16-fold lower mRNA expression of decorin in biopsies from premenopausal women with POP compared with premenopausal controls, \( P = 0.0001 \), Fig. 1. In addition, an 8-fold decrease of lumican mRNA expression was detected in the premenopausal subgroup suffering from POP, \( P = 0.001 \), Fig. 1. There was no significant difference when comparing the postmenopausal groups regarding decorin or lumican. The expression of fibromodulin mRNA, however, was decreased in all women suffering from POP compared with all controls, \( P = 0.004 \), Fig. 2. Of the elastic fiber-associated proteins, fibulin-5 mRNA expression was lower in the total POP group, \( P = 0.001 \), Fig. 2.

There was no significant difference concerning Fibrillin-I or collagen I or III. By immunohistochemistry, all proteins were expressed in the ECM, Fig. 3. A weaker staining for decorin was clearly demonstrated in the premenopausal women with POP compared with premenopausal controls, Fig. 4. For the other studied proteins, distributions in the ECM and the staining intensities varied randomly even within the biopsies, why semi-quantitative estimation of group differences was not feasible.

Discussion

We have shown that the gene signal for the production of the three studied SLRPs and fibulin-5 is substantially decreased in the supportive ECM in women suffering from POP. These mRNA differences were seen in a tissue not stretched by the prolapse, so that they may be considered a pre-requisite for the development of POP. Furthermore, the alterations seem to differ whether POP appears in the younger years before menopause or in the post-menopausal period.

Table III Antibodies and dilutions used in immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody for</th>
<th>No., manufacturer</th>
<th>Type</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Fibrillin-1</td>
<td>ab3090, Abcam, Cambridge, UK</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>Fibulin-5</td>
<td>ab36611, Abcam, Cambridge, UK</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Decorin</td>
<td>270425, Seikagaku, Tokyo, Japan</td>
<td>Mouse monoclonal</td>
<td>1:2000</td>
</tr>
<tr>
<td>Lumican</td>
<td>Gift from Dr K. Rubin, Uppsala, Sweden</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Gift from Dr D. Heinegård, Lund, Sweden</td>
<td>Rabbit polyclonal</td>
<td>1:300</td>
</tr>
<tr>
<td>Collagen I</td>
<td>C2456, Sigma-Aldrich, MO, USA</td>
<td>Mouse monoclonal</td>
<td>1:4000</td>
</tr>
<tr>
<td>Collagen III</td>
<td>C7905, Sigma-Aldrich, MO, USA</td>
<td>Mouse monoclonal</td>
<td>1:8000</td>
</tr>
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</table>
There was a considerable decrease in mRNA expression of decorin, which was supported by immunohistochemistry showing a corresponding decrease in protein expression in the premenopausal POP group. Together with the slighter, but significant, down-regulation of lumican mRNA, the findings suggest that POP appearing in the early ages may be a more severe condition due to additional impairment in the pelvic floor ECM compared with POP among elderly. Fibromodulin on the other hand seems to be affected in both pre- and postmenopausal POP.

The potential impact of SLRP shortage could affect properties associated with both the collagen and elastin. The three SLRPs in this study bind to the collagen fibrils and are of importance for normal collagen fibrillogenesis (Danielson et al., 1997; Chakravarti et al., 1998; Svensson et al., 1999). When deleting the genes in a mouse for the respective SLRP, it affects the shape of the collagen fibrils and the phenotypes express properties associated with the Ehlers–Danlos syndrome, in which POP is more common (Danielson et al., 1997; Jepsen et al., 2002). Of the SLRPs, decorin has shown an additional interaction with elastinogenesis both via fibrillin-1 and α-elastin (Trask et al., 2000; Itabashi et al., 2005; Schaefer et al., 2007).

In contrast to our study, Song et al. (2007) found a significant increased expression of decorin mRNA in premenopausal women with POP compared with controls. The biopsies were obtained...
Figure 3  Immunohistochemical staining in pelvic floor ECM showing (a) negative monoclonal control, (b) fibrillin-1, (c) fibulin-5, (d) decorin, (e) collagen I, (f) collagen III, (g) negative polyclonal control, (h) fibromodulin and (i) lumican. Light microscope magnification 1 × 200, magnification bar 100 μm.

Figure 4  Immunohistochemical staining of decorin in (a) a premenopausal control and (b) a pre-menopausal woman with POP in which the immunostaining is clearly weaker. Light microscope magnification 1 × 200, magnification bar 100 μm.
from the prolapsed anterior vaginal wall, and the stretch could have damaged the tissue, so that the decorin over-expression could be a marker of secondary inflammation (Hakkinen et al., 1996; Provenzano et al., 2005).

Our earlier finding of reduced collagen in premenopausal POP has earlier been confirmed by other studies, but altered cross-linking and increase in collagen, especially collagen III has also been found (Jackson et al., 2002; Ewies et al., 2003; Wong et al., 2003; Soderberg et al., 2004; Moalli et al., 2005). We did not see a corresponding decrease in mRNA expression of collagens reflecting a reduced collagen synthesis in POP. However, the decrease of SLRP mRNA, crucial for collagen fibril formation, may contribute to impaired quality and perhaps changed collagen III ratio and increased degradation thus resulting in reduction of collagen concentration. This is supported by earlier studies revealing increased expression of pelvic floor matrix metalloproteinases (MMPs) in POP (Jackson et al., 1996; Moalli et al., 2005; Connell et al., 2008).

The elastic fibers have early been subjects of studies in this field. Elastin has been studied in women with POP as desmosine, unique to mature cross-linked elastin. The desmosine content in these studies was unchanged in vaginal wall or decreased and broken in ligaments involved in the prolapse (Jackson et al., 1996; Ewies et al., 2003; Klutke et al., 2008).

Although elastin is extremely long-lasting with a half-life of 70 years under undisturbed conditions, there are situations where adult elastogenesis is required. An increased production of the elastin precursor tropoelastin is then followed by the assembly of the elastic fiber, a complex chain of events involving not only the scaffolding microfibrils fibrillin-I and -2, but also other proteins, among them fibulin-5 (Mithieux and Weiss 2005; Wagenseil and Mecham, 2007). An important example in this respect is the remodeling of the mouse vagina during pregnancy, where Drewes et al. (2007) showed a decreased elastogenesis reflected as a decrease of tropoelastin and fibulin-5 content, followed by a burst of these proteins 12–14 h after delivery. In the assembly, pathways interactions between fibulin-5 and fibrillin-1 as well as fibrillin-1 and decorin are described (Trask et al., 2000; Freeman et al., 2005; Hirai et al., 2007; Schaefer et al., 2007).

Our study, showing significantly decreased fibulin-5 mRNA expression in pelvic floor ECM of women with POP, is in concordance with fibulin-5 KO mice developing POP (Drewes et al., 2007). On the other hand, Klutke et al. (2008) observed an increase in fibulin-5 mRNA in the utero-sacral ligaments of women with POP. Again, as a speculation, this may be a part of elastic fiber assembly as an effect of tissue damage caused by the stretching of the POP rather than an explanatory mechanism.

We conclude that POP before menopause is associated with decreased gene signals for the three investigated SLRPs, important for the cross-linking of collagen and for the elastic fiber associated fibulin-5. The considerably reduced expression of decorin mRNA was reflected at the protein level. In post-menopausal women with POP, there was a reduction in fibromodulin and fibulin-5 mRNA expression, but not for decorin or lumican. These results imply that POP in younger years represents a more severe form of the condition not only from a clinical point of view, but also at the tissue level.

Since the biopsies were collected from the pelvic floor ECM unaffected by the stretch of the prolapse, we speculate that this potentially compromised ability to synthesize the respective proteins important for tissue resilience may contribute to the development of POP. Either these possible alterations are constitutional, bringing about a weaker tissue less resistant to the effects of pregnancy and vaginal delivery. If a remodeling of the pelvic floor ECM during pregnancy, delivery and the post-partum period takes place in humans, such as in mice, impairment in this process would be another possibility.

Further studies are needed to clarify pelvic floor ECM processes in pelvic floor dysfunctional conditions as well as during the physiological phases related to childbirth in humans. These studies should comprise not only, as in this study, synthesis of the involved parameters, but also quantification of proteins, degradation and signs of inflammatory reactions and other interactions. Immunohistochemistry, a semi-quantitative method, did not in this study allow verification of any group differences but for decorin, and methods such as western blot or proteomics may be considered for quantification. Studies on polymorphism in genes associated with POP would also be appropriate.

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References


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