Changes in extracellular matrix proteins in the cardinal ligaments of post-menopausal women with or without prolapse: a computerized immunohistomorphometric analysis

Ayman A.A.Ewies, Farook Al-Azzawi1 and John Thompson

Leicester and Warwick Medical School, UK

1To whom correspondence should be addressed at: Gynaecology Research Unit, Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK. E-mail: fa2@leicester.ac.uk

BACKGROUND: The precise mechanism of uterine prolapse is poorly understood. There is evidence to suggest that abnormalities of connective tissue structure or its repair mechanism may predispose women to prolapse.

METHODS and RESULTS: This immunohistochemical study was performed on paraffin-embedded sections of the cardinal ligaments in an attempt to evaluate differences in the expression of extracellular matrix (ECM) proteins: collagen I, collagen III, elastin and tenascin, in the cardinal ligaments of prolapsed compared to non-prolapsed uteri. There appear to be discernable differences in the level of expression of ECM proteins in prolapsed compared to non-prolapsed cardinal ligaments. We found that the ligaments of the prolapsed uteri are characterized by a higher expression of collagen III and tenascin, and lower quantities of elastin. It appears that the use of HRT in post-menopausal women reverses some of the changes observed in cases of prolapse. Collagen I expression is directly related to the age and menopausal status rather than to prolapse. CONCLUSIONS: In contrast to collagen I, our findings clearly indicate that collagen III expression is directly related to the presence of prolapse rather than age or menopausal status and is suppressed with the use of HRT. The pattern of change may fit a picture of healing phase of traumatized tissue as evidenced by the raised tenascin expression. The trauma itself may have been initiated by events such as childbirth, and that the lack of estrogen following the menopause results in decompensation. In spite of ameliorating some of the changes such as suppression of collagen III expression, treatment with estrogen falls short of rectifying the expression of other necessary proteins. If these mechanisms can be elucidated, a supplementary drug therapy may help along with estrogens to rebuild these ligaments.

Key words: collagens/elastin/ligament/prolapse/tenascin

Introduction

Pelvic floor dysfunction is a distressing morbidity that affects the quality of women’s life. The prevalence is high in both developed and developing countries, albeit that the main aetiology is different (MacLennan, 2000). It is postulated that pelvic organ support is maintained by complex interactions between levator ani muscles and connective tissues of the urethra, vaginal wall and rectum. Failure of normal levator ani function is an important hallmark of pelvic organ prolapse (Boreham et al., 2002), but the precise mechanism is poorly understood (Bidmead and Cardozo, 1998; Boreham et al., 2002). There is evidence to suggest that abnormalities of connective tissue structure or its repair mechanism may predispose women to prolapse (Norton et al., 1992). The fascia and connective tissues of the pelvic floor may lose strength as a result of ageing and loss of neuroendocrine signalling in pelvic tissues (Smith et al., 1989). The higher incidence in the post-menopausal period suggests that the hypo-estrogenic state is a contributing factor (Mokrzycki et al., 1997).

Ligaments are sheets of connective tissue that consist mainly of collagen fibres, which provide these structures with a high tensile strength (Benjamin and Ralphs, 1997). The majority of cells in ligaments are fibroblasts (Hart et al., 1995). Collagen forms 70–80% of the dry weight of ligaments. Mostly it is type I collagen, the principal tensile-resistant fibre, but smaller quantities of type III, V and VI are also present (Benjamin and Ralphs, 1997). The proper function of a ligament depends on the appropriate type, rate of synthesis, assembly, cross-linking and remodelling of collagen (Cooper and Misol, 1970).

Trauma or pathology may lead to altered responses to mechanical stresses placed on the connective tissue, producing changes in the extracellular matrix (ECM). Alterations in collagen synthesis and collagen types are causally related to...
The molecular mechanisms of human elastin gene regulation under various conditions remain largely unknown. Faulty elastin production, in heritable or acquired diseases, leads to the loss of elastic recoil observed with a variety of connective tissue disorders such as inguinal hernia (Friedman et al., 1993), genito-urinary prolapse and urinary stress incontinence (Ulsten et al., 1987; Versi et al., 1988). It has been suggested that the beneficial effect of estrogens on urethral sphincter function may be mediated by collagen (Falconer et al., 1994) and that large diameter collagen fibrils may provide a greater resistance to tensile loading because of an increase in collagen molecules available to form cross-links (Woo et al., 1991).

The molecular mechanisms of human elastin gene regulation under various conditions remain largely unknown. Faulty elastin production, in heritable or acquired diseases, leads to the loss of elastic recoil observed with a variety of connective tissue disorders (Uitto et al., 1991), in the ageing skin (Fazio et al., 1988), and has been suspected to contribute to the development of uterine prolapse (Yamamoto et al., 1997). There are no data in the literature regarding the expression of tenascin in the connective tissue of the pelvic floor. Tenascin is sparsely distributed in normal anterior cruciate ligaments, but the expression increases markedly in the ruptured ligaments, suggestive of involvement in the healing process after ligamentous injury (Neurath, 1993).

This immunohistochemical study was performed on paraffin-embedded sections of the cardinal ligaments in an attempt to evaluate differences in ECM protein expression of collagen I, collagen III, elastin and tenascin, in the cardinal ligaments of prolapsed compared to non-prolapsed uteri.

**Materials and Methods**

Thirty-three samples were obtained from Caucasian women with utero-vaginal prolapse; from eight pre-menopausal and 25 post-menopausal women, 10 on HRT and 15 women were not taking HRT. Twenty-five control samples were obtained from Caucasian women without prolapse, as assessed pre-operatively; 15 from pre-menopausal and 10 from post-menopausal women not on HRT. All women with prolapse had had a vaginal hysterectomy (except two cases in the pre-menopausal group who had abdominal hysterectomy and sacral colpopexy), while those without prolapse had had abdominal hysterectomy. All the cases of prolapse included in this study were those with advanced utero-vaginal prolapse where the uterine isthmus reached the introitus. All of the cases had a central defect, with a rectocoele and a small-to-moderate cystocele and none of them used a supportive vaginal pessary or ring prior to surgery. The menopause was defined as 1 year of amenorrhoea in women aged >45 years. All post-menopausal women in this study met this criterion. All HRT users were taking sequential combined HRT (estradiol valerate 2 mg daily and cyclic norethisterone 1 mg per day for 12 days of each 28 day treatment cycle), for >6 months pre-operatively. The demographic data of subjects included in the study are summarized in Table I.

**Table I.** The demographic data of women included in the study groups

<table>
<thead>
<tr>
<th></th>
<th>Prolapse</th>
<th></th>
<th>No prolapse</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PrM</td>
<td>PM</td>
<td>PM/HRT</td>
<td>PrM</td>
</tr>
<tr>
<td>No. of cases</td>
<td>8</td>
<td>15</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Age (years), mean (95% CI)</td>
<td>45.8 (40.6, 50.9)</td>
<td>66.2 (61.5, 70.9)</td>
<td>60.0 (55.4, 64.6)</td>
<td>42.3 (38.0, 46.7)</td>
</tr>
<tr>
<td>Parity (years), mean (95% CI)</td>
<td>2.4 (1.9, 2.8)</td>
<td>2.5 (1.7, 3.3)</td>
<td>2.7 (2.0, 3.3)</td>
<td>2.3 (1.4, 3.1)</td>
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PrM = pre-menopausal; PM = post-menopausal; PM/HRT = post-menopausal on HRT; CI = confidence interval.

**Antibodies**

The primary antibodies used in this study were mouse monoclonal antibodies against collagen I (clone no. COL-1 1:6000; Abcam Ltd, UK), collagen III (clone no. HW1D1 1:50 000; InnoGenex, USA), elastin (clone no. BA-4 1:1600; Novacastra Laboratories Ltd, UK) and tenascin (clone no. BC-24; 1: 100 000; Sigma−Aldrich Company Ltd, UK). The anti-tenascin antibody recognizes all isoforms of human tenasin. To determine the optimal concentration of each antibody, serial dilutions were used in pilot studies on test specimens. The highest dilutions that showed differences in staining patterns, on microscopic examination, between the prolapse and non-prolapsed specimens were used. The secondary antibody, a biotinylated rabbit anti-mouse (Dako A/S; Denmark), was used in all experiments at a dilution of 1:400. Non-specific mouse immunoglobulins (IgG) 1:1000 (Vector Laboratories Inc., USA) was used as a control for the specificity of primary antibody.
Immunohistochemistry

The immunohistochemical protocols followed in this study have been established in our laboratories. Sections were de-waxed in xylene (Genta Medical, UK), rehydrated in grades of industrial methylated spirit (IMS; Genta Medical) in distilled water and then subjected to trypsin (Difco Laboratories, USA) digestion for 30 min. Endogenous peroxidase activity was quenched using hydrogen peroxide (6% in distilled water) for 10 min. Sections were blocked for 20 min with normal rabbit serum in 3% bovine serum albumin in phosphate-buffered saline (PBS) to minimize non-specific reactivity. The slides were incubated overnight at 4°C with the primary antibody in a humidified chamber. Sections were incubated with biotinylated rabbit anti-mouse for 30 min, treated with Vectastain ABC peroxidase (Elite; Vector Laboratories Ltd, UK) for another 30 min, and then bound antibodies were visualized with 0.05% diaminobenzidine (DAB) in 0.05 mol/l Tris±HCl, pH 7.4, and 0.01% hydrogen peroxide, according to the supplier’s instructions (Vector Laboratories Inc.). Specimens were washed with PBS for 20 min between the steps. Sections were then submerged in CuSO4/NaCl (16 mmol/l:123 mmol/l) solution for 5 min to enhance DAB stain, and counter-staining with haematoxylin, Gill’s formula (Vector) for 1 min, was performed. Sections were dehydrated through graded alcohols, cleared with xylene and permanently mounted using XAM® neutral mounting medium (Waters and Rogers Van International Ltd, UK). Specificity of immunostaining was confirmed using non-specific mouse IgG in all stains. To control for the secondary antibody, sections were produced by omission of the primary antibody and did not show any staining while the positive control slides were stained with the non-specific mouse IgG and there was no background reaction.

Image analysis

Images of tissue sections were captured using an Axioplan microscope (Carl Zeiss, UK), a colour video camera (Sony CCD/RGB) and KS 300 image analysis programme (Imaging Associates Limited, UK). From each slide, 10 randomly selected fields (×200) (Hamilton, 1995) were captured. The areas of smooth muscles and blood vessels were excluded. Within each field, the stained area of ECM was outlined, measured, and the percentage of the stained area was processed by the software and transferred to Microsoft Excel programme for statistical analysis (Wahab et al., 1999). The examiner was blinded to the different groups during image capture and analysis.

Statistical analysis

P-Values and confidence intervals (CI) for the percentage of positive areas were analysed by a generalized linear model (GLM) with gamma errors and a log link, also adjusted for clustering of repeat readings within women. The statistical analysis was performed using the GLM command of Stata software (StataCorp 2001 Stata Statistical Software Release 7.0 College Station, TX: Stata Corp.).

Results

Collagen I

The percentage of positive areas for the no prolapse group was 7.1% (95% CI: 2.3, 21.6) and 41.9% (26.7, 65.8) for pre- and post-menopausal women respectively. For the prolapse group, the percentage of positive areas was 9.7% (3.8, 17.4), 41.3% (29.4, 58.0) and 23.3% (11.0, 49.1) in pre-menopausal, post-menopausal not taking HRT and post-menopausal women on HRT respectively. These findings demonstrated that collagen I was higher in post-menopausal not taking HRT when compared with pre-menopausal women. The differences were significant for both no prolapse (P = 0.004) and prolapse (P < 0.001) groups. There was suggestion of an increase with age in the post-menopausal women, but this does not explain the differences between pre- and post-menopausal women (Figure 3a and Figure 4a).

Collagen III

The percentage of positive areas for the no prolapse group was 11.5% (95% CI: 7.9, 16.6) and 17.9% (11.3, 28.6) for pre- and post-menopausal women respectively. For the prolapse group, the percentage of positive areas was 48.5% (36.0, 65.2), 35.8% (26.2, 49.0) and 12.0% (6.8, 21.2) in pre-menopausal, post-menopausal not taking HRT and post-menopausal women on HRT respectively. In pre-menopausal women, the percentage of collagen III staining in the prolapse group was significantly higher than that in the no prolapse group (P < 0.001). Similarly, in post-menopausal women not taking HRT, the percentage was significantly higher in the prolapse group (P = 0.02),

Figure 2. Haematoxylin and eosin staining of cardinal ligament (magnification ×200). (a) Prolapsed ligament, (b) non-prolapsed ligament. There is no discernable histological difference between prolapsed ligaments and control.
Collagen III expression in post-menopausal women was significantly suppressed by HRT ($P = 0.001$) with no indication for the effect of ageing (Figure 3b and Figure 4b).

**Collagen I:collagen III ratio**

Collagen I:collagen III ratio (CI:CIII ratio) for the no prolapse group was 1.56 (95% CI: 0.34, 7.11) and 2.68 (1.40, 5.14) for pre- and post-menopausal women respectively. For the prolapse group, the ratio was 0.18 (0.08, 0.41), 2.16 (0.86, 5.43) and 4.24 (1.80, 10.00) in pre-menopausal, post-menopausal not taking HRT and post-menopausal women on HRT respectively. In the group of women with a prolapse, the ratio was significantly higher in post-menopausal women not taking HRT when compared with pre-menopausal women ($P < 0.001$).

**Figure 3.** The percentage expression (mean and 95% confidence interval) in tissue sections of the cardinal ligaments of extracellular matrix proteins: collagen I (a), collagen III (b), collagen I:III ratio (c), elastin (d), tenascin (e). HRT = hormone replacement therapy; PrM = pre-menopausal; PM = post-menopausal; P = prolapse; NP = no prolapse; *$P < 0.05$, **$P < 0.001$. 

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In pre-menopausal women, the no prolapse group expressed significantly higher ratio than the prolapse group \((P = 0.02)\). In post-menopausal women not taking HRT, the ratio was higher in the no prolapse group compared to the prolapse group, but this was not statistically significant. There was a suggestion of an age effect especially in pre-menopausal women (Figure 3c).

**Elastin**

There was a consistently lower expression of elastin in the prolapsed ligaments. The percentage of positive areas was 4.8\% (95\% CI: 2.3, 10.0), 2.5\% (1.0, 6.4) and 1.4\% (0.7, 3.0) in pre-menopausal, post-menopausal not taking HRT and post-menopausal women on HRT respectively. This was in marked contrast to the no prolapse group where the percentage of positive areas was 11.7\% (7.4, 18.5) and 17.6\% (9.2, 33.5) for pre- and post-menopausal women respectively. In post-menopausal women not on HRT, the percentage of elastin staining in the prolapsed ligaments was significantly lower than that in the no prolapse group \((P < 0.001)\). Similarly, in pre-menopausal women, the percentage was significantly lower in the prolapse group \((P = 0.02)\). There was no indication of an age effect in any group (Figure 3d and Figure 4c).

**Tenascin**

The percentage of positive areas for the no prolapse group was 0.3\% (95\% CI: 0.1, 2.4) and 3.5\% (1.6, 7.7) for pre- and post-menopausal women respectively. For the prolapse group, the percentage of positive areas was 26.6\% (15.6, 45.4), 26.5\% (19.7, 35.6) and 40.0\% (27.3, 58.5) in pre-menopausal, post-menopausal not taking HRT and post-menopausal women on HRT respectively. These findings demonstrated that tenascin expression is significantly higher in the prolapse group regardless of the menopausal status \((P < 0.001)\). In the no prolapse group, the percentage of tenascin staining was also significantly higher after the menopause \((P = 0.03)\). There was no indication of an age effect in any group (Figure 3e and Figure 4d).

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**Figure 4.** Immunohistochemical staining of sections of the cardinal ligaments for extracellular matrix proteins (magnification ×200). (a) Collagen I, (b) collagen III, (c) elastin, (d) tenascin. HRT = hormone replacement therapy; PrM = pre-menopausal; PM = post-menopausal; P = prolapse; NP = no prolapse; CI = collagen I; CIII = collagen III; E = elastin; t = tenascin.
Discussion

Our study showed that the ligaments of the prolapsed uterus are characterized by a higher expression of collagen III and tenascin, and lower quantities of elastin. It appears that the use of HRT in post-menopausal women reverses some of the changes observed in cases of prolapse. In contrast to collagen I, our findings clearly indicate that collagen III expression is directly related to the presence of prolapse rather than age or menopausal status and is suppressed with the use of HRT. To our knowledge, this is the first immunohistochemical report that evaluates the difference in expression of ECM proteins in the cardinal ligament of women with utero-vaginal prolapse versus those with no prolapse.

The connective tissue cells synthesize a variety of ECM components that act not only to underpin the specific biomechanical and functional properties of tissues, but also regulate a variety of cellular functions (Culav et al., 1999). It was suggested that disorders of pelvic support might be due to an intrinsic abnormality in collagen synthesis or an imbalance between synthesis and degradation (Yamamoto et al., 1998). The supportive function of a ligament depends on the appropriate type, rate of synthesis, assembly, cross-linking and remodelling of collagen (Cooper and Misol, 1970). The amount of collagen bundles and the individual types of collagen influence the ability of the tendon to withstand loading (Liu et al., 1995). Collagens I and III have distinctive physical properties and their relative proportions influence tissue function (Savvas et al., 1993). It is also known that collagen I imparts a great mechanical strength to connective tissues, whereas collagen III appears to play a role in tissue elasticity and extensibility (Liu et al., 1995). Therefore, it is generally agreed that a larger CI:CIII ratio in the ligament is indicative of greater strength, whereas a lower ratio may be characteristic of tissue laxity (Laros et al., 1971). It is difficult to be certain that the reduced ratio in the prolapsed cardinal ligaments observed in our study is the cause of prolapse. A very interesting observation made in this study was that HRT significantly reduced collagen III expression in the prolapsed ligaments to levels similar to those of normal ligaments, a finding which might suggest a beneficial role for estrogen in maintaining or restoring the strength of these ligaments. In a histopathological assessment of the connective tissue of the pelvic ligaments, Kókcu et al. (2002) reported a higher expression of collagen, but the type was not defined, and fewer fibroblasts assessed by nuclear count. These changes were deemed significant in cases of uterine prolapse compared to control. However, there was no significant difference in the expression of elastin between the groups.

It has been reported that oral estrogen and progesterone therapy significantly reduced procollagen I production in bone cells of post-menopausal women (Hassager et al., 1991), but estrogen alone caused a dose-dependent increase in the production of collagen I mRNA in human osteoblasts (Ernst et al., 1989; Majeska et al., 1994). Similarly, there was an increase in total body collagen III content with daily administration of estrogen (Hassager et al., 1990). In a tissue culture model, Yu et al. (1999) found that human anterior cruciate ligament fibroblast proliferation and procollagen I synthesis showed a dose-dependent decrease with increasing estradiol concentrations. In contrast, no significant differences in procollagen III synthesis were observed.

We found that elastin expression was three to four times lower in women with prolapse when compared with those without prolapse. This association was more pronounced in post-menopausal compared with pre-menopausal women, but this loss of elastic fibres has not been rectified by the administration of HRT. Elastin plays a major functional role in the maintenance of the integrity of the ligaments, but the factors involved in elastic fibre formation are generally unknown (Chadwick and Goode, 1995). The alteration of elastin in uterine prolapse was unclear until Yamamoto et al. (1997) found that steady-state elastin mRNA levels and elastin synthesis were significantly down-regulated in quiescent cultured fibroblasts derived from prolapsed cardinal ligaments compared to controls. The lack of regeneration of functional elastic fibres in adults is a major problem, and once this ability to regenerate is lost, the restoration of normal function is not possible (Cleary, 1996). Adult tissues synthesize elastin in response to cyclic stretching, injury, UV radiation (Bernstein et al., 1994), and in many diseases including emphysema (Pierce et al., 1995). Adults, however, cannot rebuild the elastic fibre assembly mechanisms, and consequently, function is not restored.

We report here a significantly increased expression of tenascin in the prolapsed ligaments regardless of the menopausal status. In the no prolapse group, there was a significant increase in the values after the menopause in comparison with the pre-menopausal state. Tenascin is a large glycoprotein, which is present transiently in the ECM of cells and is involved in morphogenetic movements, tissue patterning, and tissue repair (Liu et al., 1995). It is generally found in adult tissues undergoing active remodelling such as healing wounds and in tumours (Wallner et al., 1999). Our findings of increased tenascin in the prolapsed ligaments are plausible because prolapse can be viewed as a form of tissue trauma.

There appear to be discernable differences in the level of expression of ECM proteins in prolapsed compared to non-prolapsed cardinal ligaments. The pattern of change may fit a picture of healing phase of traumatized tissue as evidenced by the raised tenascin expression. The trauma itself may have been initiated by events such as childbirth, and the lack of estrogen following the menopause may have caused the tissue to decompensate. Treatment with estrogen may ameliorate some of the changes such as suppression of collagen III expression but estrogen action falls short of rectifying the expression of other necessary proteins. If these mechanisms can be elucidated further, then a supplementary drug therapy may help along with estrogen to rebuild these ligaments.

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References


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