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The role of transforming growth factor-ß (TGF-ß1) in postmenopausal women with pelvic organ prolapse: An immunohistochemical study

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ABSTRACT

Objective: Aim of the study was to investigate the expression of transforming growth factor-β1 (TGF-β1), a key regulator of the extracellular matrix composition, in the uterosacral ligaments (USLs) of women with pelvic organ prolapse (POP) compared with controls. We hypothesized that the expression pattern of TGF-β1 differs between postmenopausal women with or without POP.

Methods: Under ethical approval, USL samples were obtained from postmenopausal women undergoing vaginal hysterectomy for stage two or greater pelvic organ prolapse (cases, n = 70) and from postmenopausal women without pelvic organ prolapse undergoing vaginal hysterectomy for benign indications (controls, n = 30). Immunohistochemical staining was performed from paraffin embedded tissue using anti-TGF-β1 antibodies. The expression of TGF-β1 was evaluated by the pathologist, who was blinded to all clinical data.

Results: The expression of TGF-ß1 was similar in patients with symptomatic POP (89 % positive) and in controls (90 % positive) without any signs of prolapse (p = 0.091). Age-adjusted analysis did not significantly alter these results. Regarding POP-Q stages, TGF-ß1 was significantly more frequently expressed in severe prolapse cases compared to moderate/mild cases (POP-Q stage IV versus POP-Q stage II and III; p = 0.001). No significant association could be detected between TGF-ß1 expression and age, BMI and parity in cases with POP (p > 0.05). As published previously, advanced patients' age as well as early menopausal age remained independent risk factors associated with POP in multiple logistic regression analysis (p = 0.001; p = 0.02).

Conclusion: Although our study detected POP-Q stage related alterations in USL composition and TGF-ß1 expression, there was no significant difference in the expression of TGF-β1 in cases with or without prolapse.

Introduction

Pelvic organ prolapse (POP) is a major health problem affecting the quality of life of many women [1]. With increasing descent of pelvic organs bladder, bowel and prolapse symptoms appear [2,3]. In addition to experiencing physical symptoms, a negative impact on a women's psychological health can also occur [4].

Presently, the lifetime risk of a woman developing POP is estimated to be approximately 30–50 %, with an 11 % lifetime risk of undergoing a single operation [5]. In the US alone around 200,000 women undergo pelvic floor surgical procedures annually [6]. The demand for health care services related to pelvic floor disorders is even estimated to increase at twice the rate of the population itself [7,8], making their prevention a pressing gynaecologic issue.

As POP is a multifactorial disease, its pathophysiology is still not fully understood [9]. Based on a large community based retrospective cohort study, factors that increase the risk of POP are older age, postmenopausal status, parity, elevated intraabdominal pressure

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and overweight [10–12]. Additionally, it seems that a combination of support defects in the anterior, posterior, and apical vaginal segments, abnormalities of connective tissue structure or its repair mechanism might predispose women to the development of POP [10,11,13]. It is well accepted that the USL plays an important role in the pelvic support system, with quantity, structure and organization of the extracellular matrix (ECM) being key elements of the tissue's stability [14]. A growing number of studies have shown an association between POP and alterations of the extracellular matrix. It has been suggested that both collagen and elastin production are altered in POP. It is unclear if these alterations are a result of, or rather a cause of pelvic floor dysfunction [15].

TGF- β1 is a cytokine able of remodelling the ECM by regulating multiple enzymes and components of the ECM [16]. An association between elevated expression of TGF-β1 and pathological conditions, including the loss of fascial support as found in inguinal hernias, has been reported [17]. Additionally, in women with POP an altered expression of TGF-β1 has been shown in fibroblasts and the pubovaginal fascia [18–21]. However, the exact role of TGF-β1 in the etiology of POP is less well studied and the results about its expression and its possible prognostic role in women with symptomatic POP are conflicting.

As TGF-ß1 is a key regulator of the extracellular matrix composition, the aim of this prospective study was to evaluate if the expression pattern of TGF-β1 within the USL of postmenopausal women with or without POP differs. Our hypothesis was that TGF-β1 might be expressed more frequently in patients with symptomatic POP.

Patients and methods

This prospective study was conducted at the department of General Gynecology and Gynecologic Oncology, Medical University of Vienna (MUV, Austria) with recruitment between 2015 and 2019. The study was approved by the ethics committee of Medical University Vienna (EK No.1361/2016) and written, informed consent for the use of removed tissue for research purposes was obtained from every participant preoperatively. Cases were defined as postmenopausal women with planned vaginal hysterectomy and modified USL suspension as part of their standard care for symptomatic POP. Inclusion criteria for the study group included stage 2 or greater prolapse, as assessed by a single physician, and plan for surgery by the same physician to treat the symptomatic prolapse (cases, n = 70). Asymptomatic women without evidence of prolapse (POP-Q prolapse stage 1 or less), who were undergoing vaginal hysterectomy for other benign indications, formed the control group (n = 30).

All patients underwent a thorough history and physical examination before surgery. The examination included an urogynecologic exam to check for genital prolapse according to ICS (International Continence Society) POP-Q–system [22–24] and assessment of pelvic floor strength by the Oxford Grading Scale [27]. Exclusion criteria for cases and controls included unclear menopausal status, cancer, severe endometriosis or severe pelvic inflammatory disease or inability to read and sign informed consent. Menopause was defined as blood-free interval of at least 12 months and menopausal status was confirmed by serum FSH > 20 U/l.

Tissue collection

At the time of hysterectomy samples of approximately 5 mm^3 were obtained from uterosacral ligament of all patients. The biopsies of the uterosacral ligament were all taken from an identical anatomical site, lateral to the corpus of the uterus, and all samples were confirmed by histological analysis. The samples were formalin-fixed and paraffin-embedded immediately after biopsy. The immunohistochemical analysis for TGF-β1 was performed on sections from this fixed and embedded samples. All samples were analyzed by histological examination by a gynecopathologist.

Immunohistochemistry

The expression of TGF-β1 was investigated in all samples by using immunofluorescence techniques. Serial sections (2 μm) from archived histological samples (biopsy of uterosacral ligament tissue) were prepared using a microtome (HM355; Microm) and deparaffinized. Antigens were retrieved in PT Module Buffer 1 (citrate buffer, pH 6; Thermo Fisher Scientific) by KOS Microwave Station (Milestone SRL, Sorisole, Italy). Subsequently, sections were blocked in blocking buffer (1 × PBS containing 5% FBS and 0.3% Triton™ X-100) and incubated with primary antibodies against TGF-β1 (#GTX76527; GeneTex, Irvine,CA, USA; dilution: 1/500) and von Willebrand Factor (#A008229-2; Agilent Dako, Santa Clara, CA, USA; dilution: 1/100) overnight at 4 °C. Following, slides were washed three times in PBS and incubated for 1 h with secondary antibodies (Alexa Fluor; Thermo Fisher Scientific, Waltham, MA, USA; concentration: 2 μg/ml). Nuclei were stained with 1 μg/ml DAPI. Images were acquired on a fluorescence microscope (Olympus BX50) and digitally photographed (F-View soft imaging system digital camera, CellP software; Olympus, Hamburg, Germany). To make immunofluorescence double stainings visible, corresponding colors were added to the original black-and-white images and overlays were constructed using Adobe Photoshop software.

Interpretation of slides

Interpretation of immunohistochemistry (IHC) was performed by a pathologist who was blinded to the clinical data. The staining for TGF-β1 of the fibrous tissue, smooth muscle and the section in its entirety (global) were quantified at 200 x magnification. Components such as vessels and endothelial cells were not analyzed. For each sample, intensity of TGF-β1 expression was categorized as low (+), intermediate (++) and high (+++) and the area of positive staining (1–100 %) was assigned by the study pathologist. Immunolabeling was analyzed using a microscope equipped for fluorescence (Zeiss, Germany) and photomicrographs (x200) were taken on a Kodak Ektachrome 400 film. No fluorescence labeling was observed in the negative controls.

Patient's characteristic

Clinical information and follow-up data of every participant was obtained from the database of the department of General Gynecology and Gynecologic Oncology of the Medical University of Vienna. All participants were sequentially numbered, and their records were pseudo-anonymized prior to analysis.

Statistical analysis

Chi-square tests were used to compare the frequency distributions of TGF-ß1 between the analyzed groups and to compare the frequency distributions of binary outcome variables between TGF-ß1 positive and TGF-ß1 negative POP cases. Student's *t*-test was used for continuous variables. Correlation analysis was analyzed using Pearson test for continuous variables. A p-value less than 0.05 was considered statistically significant. The SPSS system (IBM, Armonk, NY, USA, Version 23) was used for the calculations. Multivariate stepwise logistic regression was performed to identify risk factors associated with POP.

Results

Clinical characteristics

One hundred postmenopausal women were included in this study: 70 postmenopausal women with symptomatic POP and 30 postmenopausal women without any signs and symptoms of POP. The clinical characteristics of the study population are shown in Table 1. The mean age as well as the postmenopausal age differed significantly between cases and controls (p = 0.001; p = 0.02). Cases underwent vaginal hysterectomy and modified USL suspension as part of their standard operative care for symptomatic POP. Controls underwent hysterectomy due to benign indications as follows: menorrhagia (n = 20), postmenopausal bleeding (n = 2), chronic pelvic pain (CPP) with additional fibroids (n = 6) and carcinoma in-situ (n = 2).

Expression pattern of TGF-β1 in cases and controls

Expression of TGF-ß1 within the USL of prolapse cases were compared to control subjects (Table 2). Expression pattern was similar between the two groups, as TGF-β1 was detected in nearly all samples collected from cases and controls. Fig. 1 shows representative sections of TGF-ß1 staining in the USL of postmenopausal cases and controls. In detail, TGF-ß1 was expressed in 62/70 (89 %) prolapse cases and in 27/30 (90 %) control cases and comparison of TGF-β expression showed no significant difference between these two groups (p = 0.091). Furthermore, age-adjusted analysis of the data was performed which did not significantly influence the results (p = 0.098). The frequency and intensity of TGF-β1 expression in cases and controls is shown in Table 2.

Correlation between TGF-ß1 expression and POP-Q stage

Patients with severe prolapse (POP-Q Stage IV) showed a significantly stronger immunoreactivitiy to TGF-ß1 specific antibody compared to patients with prolapse POP-Q stage II and III (p=) (Table 3).

Multiple logistic regression analysis

Multiple logistic regression analysis was performed to identify the impact of different risk factors on POP. The presence of POP was defined as the dependent variable. Independent variables included in the model were age, menopausal age, BMI, parity, smoking and TGF-ß1 expression. As published previously, also for this larger study population, the strongest factors associated with POP remained early menopausal age and advanced patients' age (p = 0.001; p = 0.02 (Table 4).

Discussion

The exact pathophysiology of prolapse is hard to define, as the risk factors for POP are multifactorial. Some authors have found that defects in urogenital tissue contribute to prolapse development and a tissues' strength deficit is connected to changes in synthesis and degradation of different types of ECM proteins [14,25]. Therefore, a predisposition to POP may be found in women with abnormalities in their ECM [26,27]. Previous studies have shown that patients with POP express increased matrix metalloproteinase (MMP) activity in their ECM resulting in an increased degeneration of collagen content compared to non-prolapse patients and thus leading to tissue with an impaired mechanical strength [28–30]. TGF-β1 is involved in the synthesis of ECM and the inhibition of matrix metalloproteinases (MMPs). Increased mechanical strain can reduce the expression of TGF-β1 [28,31]. Furthermore, sustained elevations of TGF-β1 have been associated with multiple other pathological conditions, such as pulmonary fibrosis, keloid formation, coronary artery restenosis, and acute respiratory distress syndrome (ARDS) [31]. Pascual et al. also described increased TGF-β1 expression in the fascia of inguinal hernias [17], an interesting finding as the pathophysiology of inguinal hernias might be similar to that of POP, since both are associated with a loss of fascial support.

Multiple studies have analyzed the changes in expression of MMP and its signaling pathways, some including TGF-β1, in the pelvic floor connective tissue of patients with and without POP [14,29,32]. However, findings still remain inconclusive as for example Mijerink et al. detected a positive correlation between TGF-β1 expression and POP in the vaginal wall [21], whereas Qi and colleagues described a negative correlation between TGF-β1 expression and POP in the pubo-cervical fascia [18]. The discordancy of these findings could be explained by a different expression of TGF-β1 in different parts of the pelvic floor. Our study showed similar TGF-ß1 expression pattern in patients with or without POP and no statistically significant difference could be detected between the two groups.

The hypothesis of our study was that TGF-β1 expression in the USL differs between postmenopausal women with and without POP. The selection of the USL as biopsy site was made, as in our opinion this ligament plays a major role in the pelvic support system. Since age and menopause are postulated to be strong risk factors in the development of POP [11,12], we decided to investigate only postmenopausal women. Our results failed to show that the uterosacral ligaments of the prolapsed uterus are characterized by higher immunoreactivity for TGF-ß1 compared to non-prolapsed uterus in postmenopausal women. However, patients with severe/advanced POP (POP-Q stage IV) had a significantly stronger TGF-ß expression compared to mild-moderate prolapse cases. Similarly to our findings, Leegant et al. also found no difference in the expression of TGF-β1 and MMP-9 in the USL of subjects with prolapse versus controls [15].

In our opinion clinical demographic factors such as a woman's menopausal status and age play a much larger role in the development of POP than the expression of TGF-β1 in the USL.

Table 1
Clinical characteristics of study population (n = 100).

Total number	Cases 70	Controls 30	p-value
Age (years) - *mean (SD)*	697 (7,9)	593 (5,1)	0,001*
Menopausal Age (years) - *mean (SD)*	501 (2,6)	535 (2,6)	0,020*
BMI (kg/m^2) - *mean (SD)*	280 (2,5)	277 (4,1)	0.089
Parity - *mean (SD)*	22 (0,6)	20 (0,8)	0.099
Tobacco - *number (%)*	35 (35 %)	11 (36 %)	0,240

SD = standard deviation; BMI = body mass index; * p < 0,05; statistically significant.

Table 2
Frequency and intensity of TGF-ß in cases and controls.

Total number	POP 70 Number (%)	Control 30 Number (%)	p-value
TGF-ß			
positive	62 (89 %)	27 (90 %)	0.091
negative	8 (11 %)	3 (10 %)	
Staining intensity for TGF-ß			
– (0 % staining)	8 (11 %)	3 (10 %)	
+ (weak)	6 (10 %)	5 (19 %)	
++ (moderate)	44 (71 %)	16 (59 %)	
+++ (strong)	12 (19 %)	6 (22 %)	

POP = pelvic organ prolapse.

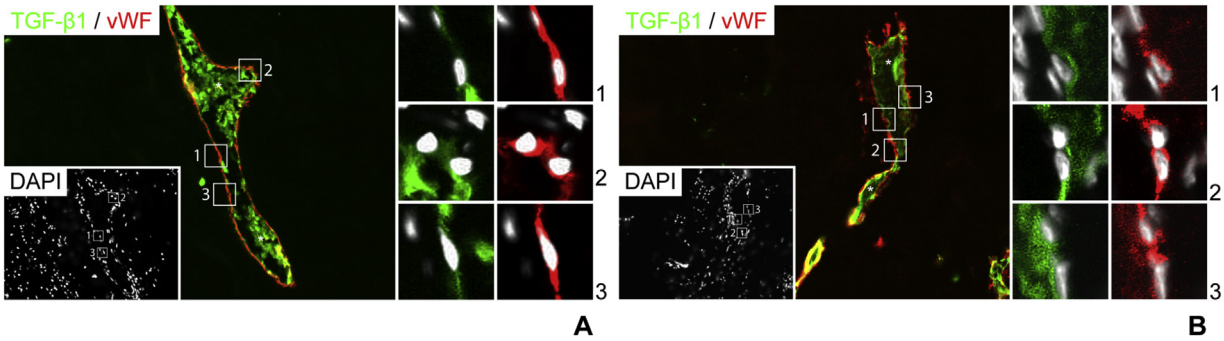

Fig. 1. Immunofluorescence of serial sections of uterosacral ligament using an antibody against TGF-β1 (green) and von Willebrand Factor (red). Respective DAPI staining is shown. Pictures were taken at a 200 fold magnification (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
A . . . study.
B . . . control.
* . . . Autofluorescence of erythrocytes.

Table 3
Correlation between TGF-ß expression and POP-Q stages.

	TGF-ß staining intensity				p-value
	0	+	++	+++	
POP-Q stage					0.001
Stage II	8	3	0	0	
Stage III	0	23	22	0	
Stage IV	0	0	0	14	

POP-Q = pelvic organ prolapse quantification system.

Table 4
Multiple logistic regression analysis to identify the impact of different risk factors on pelvic organ prolapse cases (n = 70).

Variable	OR	95% Confidence Interval	p-value
Menopausal Age	−,310	−,459 to −,213	0,001*
Age	0,812	0,289–1,201	0,001*
BMI	−,059	−,293 to −,260	0,620
Parity	−1,123	−2,222 to 0,59	0,073
TGF-ß	−,177	−1,061 to 0,729	0,722
Tobacco	−,481	−2,188 to 1,245	0,599

* statistically significant; $p < 0.05$; OR = Odds ratio.

Limitations of the study

We are aware of the limitations of our study. Since this was a clinical case-control study, there are limits in determining a cause and effect relationship. Only associations between immunohistochemistry and prolapse occurrence can be reported, while no conclusions on the underlying causality can be made.

On the other hand, this study includes a large population with 100 included cases and significantly enlarges the scarce literature regarding POP and TGF beta.

Conclusion

ECM proteins such as collagen, MMP and TGF-β seem to play some part in the pathogenesis of POP, but their exact role is still not entirely clear. Our results showed no difference in the expression of TGF-ß1 in cases with prolapse versus controls, but a significantly higher immunoreactivity to TGF-ß1 in severe prolapse stages compared to mild -moderate stages. In our opinion, the similar expression pattern in patients with or without prolapse suggests that TGF-ß1 does not play a major role in the development of POP in postmenopausal women,

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Ethics

Ethical approval was obtained from ethics committee of Medical University Vienna, reference number 1361/2016.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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