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Fibrotic Protein Expression Profiles in Penile Tissue of Patients With Erectile Dysfunction

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Abstract

Objective—To characterize transforming growth factor beta1 (TGFβ1) and related signaling pathway proteins in a large cohort of human penile tissue (HPT) samples.

Methods—HPT was collected from patients undergoing penile prosthesis implantation (PPI) for erectile dysfunction (ED) and divided into 2 groups: post-radical prostatectomy ED (RP-ED; n=57) or organic ED (O-ED; n=30). HPT from patients undergoing partial penectomy without ED was used as controls (CON; n=6). Western blot analysis was performed to investigate the protein expressions of TGFβ1, thrombospondin 1 (TSP1; an activator of TGFβ1), fibronectin (FN; an extracellular matrix glycoprotein induced by TGFβ1) and a family of transcriptional factors activated by TGFβ1 [Smad2, phospho-Smad2-serine-465/467 (pSmad2), Smad3, phospho-Smad3-serine-423/425 (pSmad3)].

Results—Expressions of TGFβ1 and TSP1 were significantly higher in both RP-ED ($p<0.05$) and O-ED ($p<0.05$) groups compared to that of the CON group, and were not different between either ED groups. Expressions of Smad2, pSmad2, Smad3, pSmad3 and FN were similar among all groups. Within the RP-ED group, a subgroup analysis showed that time from RP to PPI was related to increased expression of pSmad2 ($p<0.05$) and previous history of intracavernosal injection was related to increased expression of TGFβ1 ($p<0.05$).

Conclusion—Our results demonstrate that TSP1 and TGFβ1-dependent fibrotic changes occur in penile tissue in patients with ED regardless of etiology. The unchanged expression of the Smad transcriptional factors may be reconciled by a Smad-independent downstream signaling pathway transmitting TGFβ1 signals.

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Keywords

fibrosis; TGF β 1; human penile tissue; sexual function

INTRODUCTION

Cavernous nerve (CN) injury during radical prostatectomy (RP) is acknowledged to be the primary cause of post-RP erectile dysfunction (RP-ED)¹. Although the precise mechanism is unknown, the surgical procedure is understood to deprive the penis of its nerve supply at least temporarily, leading to atrophic and fibrotic changes, deterioration of corporal smooth muscle and collagen infiltration of erectile tissue^{1,2}. In animal models, cavernosal tissue fibrosis has also been linked to the release of fibrotic cytokines and activation of fibrotic signaling^{3,4}.

Transforming growth factor beta1 (TGF β 1) is a well known profibrotic cytokine that activates fibroblasts and leads to tissue fibrosis. In both animals and humans, acute limited injury to normal tissue is accompanied by only a transient increase in TGF β 1, which leads to normal healing; a sustained increase in TGF β 1 production, however, may lead to the progressive deposition of extracellular matrix and tissue fibrosis³. In animal models, CN injury leads to an increased expression of TGF β 1 in penile tissue⁴, and intracavernosal injection of this cytokine itself alters connective tissue synthesis and the structure of the corpus cavernosum, leading to a decrease in percentage of corporal smooth muscle⁵. Thrombospondin1 (TSP1), a multifunctional glycoprotein, is a major activator of TGF β 1⁶. Increased TSP1 expression is reported to be closely related to hypoxia and the development of fibrosis^{7,8}. The Smad family of proteins are a group of recently identified molecules that function as intracellular signaling mediators and modulators of the TGF family of proteins. Specifically, Smad2 and Smad3, classified as receptor-activated Smads (R-Smad), can transmit TGF β 1 signals from the cell surface into the nucleus⁹. Phosphorylation of Smad2 at serine-465/467 (pSmad2) and Smad3 at serine-423/425 (pSmad3) by the activated TGF β 1 receptor modulates their activity, allowing them to translocate to the nucleus and regulate TGF β 1 target genes^{9,10}. pSmad2 and pSmad3 expressions are increased in rat penile tissue after CN injury¹¹, and men with penile denervation due to spinal cord injury have increased pSmad2 in their cavernous tissue¹².

Fibronectin (FN), which exists among extracellular matrix (ECM) proteins early induced by TGF β 1, is up-regulated after injury and implicated in ECM organization¹³. TGF β 1 increases ECM accumulation through the stimulation of FN and collagen IV production in glomerular epithelial cells, resulting in interstitial fibrosis and glomerular sclerosis¹⁴. In animal models of CN injury and diabetes, FN expression is increased in penile tissue^{11,15}.

Although the process of cytokine release that activates fibrotic signaling has been described in animal models, it is poorly understood in humans. Our hypothesis is that ED following RP is associated with fibrotic signaling in the penis: once activated by TSP1, TGF β 1 then binds to TGF β 1 receptors, which phosphorylate the Smad family of transcriptional factors which in turn upregulate specific genes in the nucleus, leading to an increase in expression of fibrotic proteins, such as FN. The aim of this study was to evaluate changes in

expressions of fibrotic proteins (TSP1, TGF β 1, Smad2, pSmad2, Smad3, pSmad3 and FN) in a large cohort of penile tissue collected from men with ED associated with prior RP in comparison with that representing other organic ED etiologies.

METHODS

Tissue collection

Tissue collection and clinical history review were performed with approval by the Institutional Review Board of the Johns Hopkins Medical Institutions. Human penile (corpus cavernosal) tissue (HPT) weighing approximately 500 mg was obtained from patients who underwent penile prosthesis implantation (PPI) due to ED and divided into two groups: post-RP ED (RP-ED; n=57) and organic ED (O-ED; n=30). HPT obtained from patients who underwent penectomy for penile or urethral cancer with no related ED were used as controls (CON; n=6). HPT from this group was unassociated with malignancy. We excluded patients with histories of pelvic radiation, androgen deprivation, Peyronie's disease or priapism. All HPT was immediately placed in cold tissue culture media following surgery and stored in -80°C as described until use¹⁶.

Western blot analysis

HPT samples were homogenized in lysis buffer and processed as described¹⁶. Briefly, 60 μg of total proteins per sample was loaded on a 4-20% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA, USA), separated by electrophoresis, transferred to a polyvinylidene fluoride membrane, and incubated overnight at 4°C with the following primary antibodies (dilutions indicated): TSP1 (1:600; Abcam Inc, Cambridge, MA), TGF β 1 (1:2000; Abcam Inc), Smad2 (1:10,000; Abcam Inc), pSmad2 at serine 465/467 (1:1000; Cell Signaling Technology, Danvers, MA), Smad3 (1:1000; Cell Signaling Technology) pSmad3 at serine 423/425 (1:1000; Cell Signaling Technology), β -actin (1:10000; Sigma-Aldrich, St. Louis, MO). The membranes were then incubated for 1 hour at room temperature with goat anti-mouse or goat anti-rabbit IR-Dye 670 or 800cw secondary antibodies (1:20,000; Li-Cor Biosciences, Lincoln, NE). The membranes were analyzed and quantified using LiCor Odyssey Fc Imaging Systems (Li-Cor Biosciences). The values were normalized to β -actin and expressed as a ratio of a reference penile tissue, which was run on every blot.

Statistical analysis

All data were expressed as arithmetic mean values $\pm$ standard deviation (SD). Comparisons between groups were performed using Chi square tests or Fisher's exact test for categorical data, and analysis of variance (ANOVA) or Kruskal-Wallis test for continuous data. The level of significance was set at .05 in all analyses, except in analyses in which a Bonferroni adjustment for multiple comparisons was applied, in which case the significance level was .05 divided by the number of comparisons. Non-parametric analysis of covariance (ANCOVA) and two way ANOVA were used to test the group differences when adjusted for other variables. Because of the limited sample size and unbalanced group size, it was not possible to adjust for more than 2 variables and the results only show tests of group differences adjusted for 1 variable. Quade's distribution-free test was used when adjusted for age and body mass index (BMI). Friedman two-way analysis of variance was used when

adjusted for clinical characteristics. Tests within the RP-ED group were based on nonparametric Wilcoxon sum rank test. All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC).

RESULTS

Patient demographic and clinical characteristics

All three groups were similar for age, BMI and race distribution (Table 1). The prevalence of diabetes was significantly higher in O-ED patients compared to RP-ED and CON patients ($p<0.001$). No significant differences were observed between the groups for prevalence of hypertension, hypercholesterolemia or current tobacco use. O-ED and RP-ED patients had an average Sexual Health Inventory for Men (SHIM) score of 3.9 and 3.4, respectively.

Comparison of fibrotic signaling protein expressions in patient groups

Western blot analysis was performed to determine the expressions of fibrotic signaling proteins including TSP1, TGF β 1, Smad2, pSmad2, Smad3, pSmad3 and FN. TSP1 (Figure 1A) and TGF β 1 (Figure 1B) expressions were significantly increased in RP-ED and O-ED groups compared to CON ($p<0.01$ and $p<0.05$, respectively). The analyses of outcomes (expression levels of the fibrotic signaling proteins) among the 3 patient groups adjusted for variables considered as risk factors for ED confirmed that TSP1 and TGF β 1 expression were still significantly different after adjusting for confounding variables: age ($p<0.01$ and $p<0.01$) respectively, BMI ($p<0.01$ and $p<0.001$), race ($p<0.05$ and $p<0.01$), hypertension ($p<0.01$ and $p<0.01$), hypercholesterolemia ($p<0.01$ and $p<0.01$), diabetes ($p<0.05$ and $p<0.01$) and smoking status ($p<0.01$ and $p<0.01$). Smad2, Smad3, pSmad2, pSmad3 and FN were not significantly different among RP-ED, O-ED and CON groups (Table 2).

Analyses of fibrotic signaling protein expressions in sub-groups of RP-ED patients

Within the RP-ED group, patients had an average pre-RP SHIM score of 21.3 (SD: 4.6; range: 8-25) and average time between RP and PPI of 54.1 months (SD: 40.2; range: 9-180). TGF β 1 expression was significantly higher ($p<0.05$) in patients with previous use of intracavernous injection (ICI; $n=30$) compared with the patients without previous ICI ($n=25$) (Figure 2A). HPT of patients who underwent PPI >2 years after RP ($n=44$) had significantly increased expression of pSmad2 ($p<0.05$) compared with those treated <2 years from RP ($n=13$) (Figure 2B). Subgroup analysis also showed that outcomes were not different based on age (>65 vs. <65 years), nerve-sparing RP status (bilateral vs. non-nerve-sparing), or preoperative SHIM score (>22 vs. <22).

COMMENT

Our study supports the concept that penile fibrotic changes occur in patients with ED, and provides further evidence regarding a possible molecular mechanism for these changes at a clinically relevant level in a large cohort of HPT samples. HPT was obtained from a group of patients categorized as RP-ED, O-ED and CON (no ED), which had overall similar demographics and clinical characteristics except significantly increased prevalence of diabetes in the O-ED group. A comparison of molecular markers among the experimental

groups demonstrated that the main fibrotic cytokine TGF β 1 and one of its main extracellular activators TSP1 were upregulated in HPT from patients with ED, compared with that of CON patients, regardless of RP history or any other confounding factors, without a significant difference in expression of any other markers. We also showed that within the RP-ED group, the expression of the activated form of the intracellular transcriptional factor pSmad2 was higher in HPT from patients in whom >2 years had elapsed since RP. We further showed that TGF β 1 expression was increased in HPT from patients with a previous history of ICI.

Changes in penile structure as a consequence of aging, social habits or pathological conditions can be associated with ED¹⁷. A study comparing histologic features of penile corpora cavernosa in patients with ED and controls without ED showed a statistically significant reduction in the percentage of elastic fibers in the former¹⁸. Age, a known risk factor for ED, was also found to be related to an increased expression of collagen and decreased expression of smooth muscle in human cavernosal tissue¹⁹. Arteriogenic ED is related to an increased expression of TGF β 1 mRNA, and correlates with a reduced smooth muscle to collagen ratio in cavernosal tissue from rats with bilateral internal iliac artery ligation²⁰. TGF β 1 expression was also shown to be increased in cavernosal tissue of the rat penis after CN injury, leading to collagen synthesis and tissue fibrosis²¹. Moreover, histologic studies in penile biopsies from patients with RP-ED demonstrate a decrease in interstitial elastic fibers and an increased accumulation of collagen². Thus, our study further confirmed the increase in TGF β 1 in ED of various etiologies.

The main regulatory mechanism of TGF β 1 activity occurs extracellularly, by controlling conversion of the latent complex to the active molecule⁶. One of the best known activators of TGF β 1 is TSP1^{6,22,23}. Expression of TSP1 is highly predictive of the development of interstitial fibrosis in glomerulonephritis, is associated with TGF β 1 expression in areas of tubulointerstitial injury⁸, and acts as a TGF β 1 activator in diabetic nephropathy in vivo²⁴. Expressions of TGF β 1 and TSP1 are also increased significantly in the cavernous tissue of diabetic rats²⁵. In a rat model of CN injury, TSP1 expression is significantly increased 7 days after crush¹¹, supporting an interaction of TSP1 with the TGF β 1 fibrotic pathway as a mechanism that may be associated with ED. Our findings are consistent with these data.

After TGF β 1 binds to its specific receptor, an accessory protein named SARA (Smad anchor for receptor activation) interacts with inactive R-Smad, promoting Smad phosphorylation, at C-terminal serines⁹. Elevated Smad expression has been described in different pathological conditions, including hypertension, atherosclerosis, and cardiac damage¹³ and also in a rat model of CN injury¹¹. However, we did not find significant differences statistically in pSmad2 and pSmad3 expressions among the ED groups compared to the control group, only observing a trend toward increased expressions. This could be explained by the small sample size of the control group. Moreover, non-Smad pathways also participate in signaling of TGF β , which acts as a second messenger and convergence locus for crosstalk with other major signaling pathways, such as members of the mitogen-activated protein kinase (MAPK) family, the Rho/Rho-kinase pathway, and reactive oxygen species (ROS)^{9,13}.

FN is an essential molecule of the ECM with a role in the development of fibrosis¹³. In a rat model of CN injury, FN is increased in penile tissue at one week after injury¹¹. In our study, FN protein expression was not different among groups. This could be explained by the uncontrolled duration of the disease in our study. Since the tissue was collected a long time after surgery (RP-ED group) or after a long history of ED (O-ED), collagen synthesis may have already subsided and FN expression may have returned to basal levels, similar to CON levels. It is possible that other fibrotic endpoints such as collagen type I and IV or laminin expressions could better represent long-term fibrotic tissue changes^{14,15}.

Using relevant clinical factors that may influence erectile recovery, such as age, nerve-sparing status, pre-RP SHIM and other associated factors (time since RP and previous history of ICI) that could relate to tissue derangement and fibrosis, we analysed the outcomes within the RP-ED group. Our results showed no particular correlations. HPT was collected when all patients in the study similarly had developed ED, which offers an explanation for the lack of any distinct correlation. However, considering the time from RP to PPI, we found that this outcome correlated significantly with increased expression of pSmad2. This finding suggests a correlation between the time of the disease in RP-ED patients, designating prostate surgery as a constant variable for this group, and the increased expression of a transcriptional factor involved in the TGFβ1 fibrotic pathway. Canguven et al showed in a rat model of CN injury that pSmad2 protein expression is more increased than pSmad3 expression in the fibrotic pathway¹¹. Early TGFβ1 fibrotic responses, such as induction of connective tissue growth factor and down-regulation of E-cadherin are critically dependent on Smad3²⁶. Smad2 is involved in delayed events such as induced expressions of matrix-metalloproteinase-2 and α-smooth muscle actin, the latter requiring both Smad2 and Smad3²⁶. Furthermore, it has been observed that Smad2, and to a lesser extent Smad3, bind to SARA, which is needed to recruit and present these proteins to TGFβ1 receptors, in human mesangial cells²⁷. This difference in binding may account for the divergence between Smad2 and Smad3 activation, and/or the TGFβ1 response in different cell types.

In the RP-ED group, 54.5% of the patients had previously employed ICI as ED treatment. We found that HPT of patients who previously reported ICI use had increased expression of TGFβ1. Long-term ICI is associated with potential structural and fibrotic alterations in penile tissue. Although retrospective, uncontrolled clinical studies have shown that the incidence of fibrotic changes is low and frequently limited to the injection site only²⁸, injectable vasoactive agents have been shown to induce significant cytotoxicity in cavernosal smooth muscle cells in vitro^{29,30}. This observation suggests that the use of intracavernous pharmacotherapy, especially regimens containing papaverine, may contribute to intracorporal fibrosis and loss of smooth muscle and endothelial cell content^{29,30}. Considering TGFβ1 to be a profibrotic agent, our findings imply a correlation between the use of vasoactive agents and the development of cavernosal tissue fibrosis. We did not stratify patient data by the type of medication used because of the relatively small size of the study population. An alternative explanation for our results may be that patients with the most severe penile fibrosis were those in whom oral agents failed and who required the use of ICI for ED treatment. Controlled studies must be conducted to define if ICI use has a causal correlation with cavernosal fibrosis.

We acknowledge that this is an observational study with some limitations. HPT was collected similarly from patients with likely severe ED, regardless of ED etiology, possibly accounting for similar tissue characteristics in both ED groups. Moreover, statistical analyses were limited by the small number of control HPT (collected from penile cancer patients, a low incidence disease). Although future studies applying a larger number of healthy or less severely diseased HPT may resolve the matter, such tissue is usually scarce for these investigations. The current study was focused on the most described pathway of fibrosis and did not include other alternative molecules and pathways that may also be involved in this condition. Other fibrotic markers and signaling proteins may be investigated in future studies.

CONCLUSION

To our knowledge, this is the first time that protein expression changes characterizing the initial steps of the fibrotic pathway were related to ED in a large cohort of HPT samples. Our results are consistent with previous animal studies and indicate that fibrotic cytokine upregulation is involved in the molecular mechanism of ED regardless of ED etiology. A better understanding of the pathways that lead to structural changes in HPT of patients with ED, including RP-ED patients, can be expected to provide new scientific targets for the treatment of ED.

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Figure 1.

Changes in TSP1 (A) and TGFβ1 (B) protein expressions. In both RP-ED and OED groups, expressions of TSP1 and TGFβ1 were significantly increased compared to CON group. ** $p < 0.01$ vs CON. Top panels: representative immunoblots; bottom panel: densitometry data; bars represent means $\pm$ SD.

Figure 2.

Subgroup analyses of the fibrotic protein expressions within the RP-ED group. The expression of TGFβ1 (**A**) was significantly increased in patients who reported a past history of ICI therapy [(+) ICI], compared to patients who did not [(-) ICI] * $p < 0.05$ vs (-) ICI. The expression of pSmad2 (**B**) was significantly increased among patients who had a longer time (> 2 yrs) from RP to PPI than patients whose time since RP was shorter (<2 yrs). * $p < 0.05$ vs >2 yrs. Top panels: representative immunoblots; bottom panel: densitometry data; bars represent means $\pm$ SD.

Table 1

Demographic and clinical presentations of patients

	CON (n=6)	O-ED (n=30)	RP-ED (n=57)	<i>p-value</i>
Age (year)				
Mean (SD)	61.5 (12.5)	61.3 (13.3)	63.4 (6.2)	0.960
range	48-77	19-77	50-76	
BMI (kg/m²)				
Mean (SD)	30.5 (8.4)	29.7 (5.7)	27.8 (2.7)	0.894
range	23.5-47.0	24.3-45.6	22.5-36.6	
Race				
Caucasian	5 (83.3%)	20 (66.7%)	44 (77.2%)	0.538
African American	1 (16.7%)	10 (33.3%)	13 (22.8%)	
Hypertension				
Yes	4 (66.7%)	19 (63.3%)	33 (57.9%)	0.889
No	2 (33.3%)	11 (36.7%)	24 (42.1%)	
Hypercholesterolemia				
Yes	2 (33.3%)	13 (43.3%)	31 (54.4%)	0.463
No	4 (66.7%)	17 (56.7%)	26 (45.6%)	
Diabetes				
Yes	1 (16.7%)	15 (50.0%)	8 (14.0%)	0.001
No	5 (83.3%)	15 (50.0%)	49 (86.0%)	
Current smoker				
Yes	1 (16.7%)	7 (24.1%)	14 (25.9%)	0.880
No	5 (83.3%)	22 (75.9%)	40 (74.1%)	

Table 2

Fibrotic protein expressions

	CON (n=6)	O-ED (n=30)	RP-ED (n=57)	<i>p-value</i>
Smad2				
Mean (SD)	0.87 (0.25)	1.27 (0.73)	1.17 (0.63)	0.314
range	0.59-1.16	0.39-3.72	0.13-3.53	
pSmad2				
Mean (SD)	1.05 (0.37)	1.43 (1.19)	2.68 (4.34)	0.559
range	0.34-1.37	0.08-4.15	0.05-26.95	
Smad3				
Mean (SD)	1.11 (0.67)	1.21 (0.99)	1.05 (0.96)	0.609
range	0.20-2.05	0.19-4.72	0.10-6.50	
pSmad3				
Mean (SD)	0.85 (0.39)	1.12 (0.47)	1.32 (0.67)	0.128
range	0.43-1.42	0.28-1.98	0.31-4.02	
FN				
Mean (SD)	1.36 (0.46)	1.40 (0.53)	1.33 (0.59)	0.751
range	0.65-1.88	0.69-3.10	0.17-3.68	