

Transforming Growth Factor- β Type I Receptor Inhibitor Induces Functional and Morphologic Recovery in a Rat Model of Erectile Dysfunction and Cavernous Fibrosis

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= Abstract =

Purpose: To examine the effectiveness of small-molecule inhibitor of transforming growth factor- β (TGF- β) type I receptor, an activin receptor-like kinase 5 (ALK5), on erectile dysfunction (ED) in a rat model of cavernous fibrosis, in which fibrosis was induced by intracavernous injection of adenovirus expressing TGF- β 1 (Ad-TGF- β 1).

Materials and Methods: Four-month-old Sprague-Dawley rats were divided into four groups (n=10 per group): age-matched controls without treatment, age-matched controls receiving intracavernous injection of LacZ adenovirus, and cavernous fibrosis rats receiving an intracavernous injection of saline or ALK5 inhibitor (5 mg/kg). ALK5 inhibitor or saline was administered on day 5 after injection of Ad-TGF- β 1. On day 30, erectile function was assessed by electrical stimulation of the cavernous nerve and the penis was then harvested for histologic studies (n=6 per group) and for the measurement of the hydroxyproline level (n=4 per group).

Results: Ad-TGF- β 1-induced cavernous fibrosis rats treated with saline showed a significant decrease in cavernous smooth muscle and endothelial content, and an increase in collagen deposition, which resulted in profound deterioration of all erectile function parameters, such as the ratios of maximal intracavernous pressure (ICP), total ICP, and slope to mean arterial pressure. ALK5 inhibitor significantly restored erectile function in a rat model of cavernous fibrosis by increasing cavernous smooth muscle and endothelial content, and by blocking cavernous fibrosis.

Conclusions: The results suggest that inhibition of the TGF- β pathway is a promising therapeutic strategy for the treatment of ED related to cavernous fibrosis from various causes.

Key Words: Erectile dysfunction, Fibrosis, Transforming growth factor beta, TGF-beta type I receptor

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Introduction

Erectile dysfunction (ED) is predominately a vascular disease.¹ ED and cardiovascular disease share the same risk factors including hypercholesterolemia, hypertension, diabetes mellitus, dyslipidemia, and smoking.^{2,3} It has been reported that vascular risk factors induce structural changes in the corpus cavernosum tissue, such as a decrease in smooth muscle content and in-

crease in extracellular matrix,^{4,6} which causes derangement in the veno-occlusive mechanism.⁷ Therefore, it is important to identify the factors that alter the functional smooth muscle-connective tissue balance for the development of new therapeutic agents for vasculogenic ED.

Transforming growth factor- β (TGF- β) has been suggested as the most relevant fibrogenic cytokine. We and other investigators have reported that TGF- β 1 expression was up-regulated both in the corpus cavernosum tissue of streptozotocin-induced diabetic rats and of vasculogenic ED patients.^{4,6,8} TGF- β 1 protein dramatically increased collagen synthesis in primary cultured human corpus cavernosum smooth muscle cells.⁹ Moreover, we previously observed that intracavernous injection of NIH3T3 fibroblasts containing the TGF- β 1 gene or adenovirus encoding the TGF- β 1 gene (Ad-TGF- β 1) induced severe cavernous fibrosis and deterioration of erectile function,^{10,11} which further reinforces the etiological role of the TGF- β pathway in the pathogenesis of ED. Therefore, therapies targeted at blocking the TGF- β pathway may be promising therapeutic strategies for the treatment of ED with cavernous fibrosis. It has been reported that small-molecule inhibitor of activin receptor-like kinase 5 (ALK5), a TGF- β type I receptor, induced the regression of tissue fibrosis in the kidney, lung, and liver.¹²⁻¹⁴ We also reported that ALK5 inhibitor successfully promoted the regression of fibrotic responses in animal models of Peyronie's disease (PD) induced by Ad-TGF- β 1¹⁵ and in primary cultured fibroblasts isolated from the fibrotic plaque of PD patients.¹⁶

In this study, we determined the effectiveness of ALK5 inhibitor in promoting the regression of cavernous fibrosis and restoring erectile function in a rat model of cavernous fibrosis induced by intracavernous injection of Ad-TGF- β 1.

Materials and Methods

1. Adenoviral vectors

Replication-deficient adenoviruses encoding porcine TGF- β 1 were generated by homologous recombination. Gene expression was driven by a cytomegalovirus pro-

motor/enhancer. LacZ adenovirus (Ad-LacZ) was used as a parallel control during gene transfection. Viruses were propagated in 293 cells, which were purified and titered by use of a standard method and then stored at -80°C until used.

2. Animals and treatment

Four-month-old male Sprague-Dawley rats were used in this study. The experiments performed were approved by the Institutional Animal Care and Use Subcommittee of our university. Rats were classified into 4 groups (n=10 per group): group 1, age-matched controls without treatment (untreated control); group 2, age-matched controls receiving an intracavernous injection of Ad-LacZ (day 0; 1×10^{10} particles/0.1 ml); group 3, cavernous fibrosis rats receiving an intracavernous injection of saline (day 5; 0.1 ml); and group 4, cavernous fibrosis rats receiving an intracavernous injection of ALK5 inhibitor (day 5; 5 mg/kg in 0.1 ml saline). Cavernous fibrosis was induced by a single intracavernous injection of Ad-TGF- β 1 (day 0; 1×10^{10} particles/0.1 ml) as previously described.¹¹ Treatment of cavernous fibrosis with ALK5 inhibitor or saline was done on day 5, when the fibrotic responses begin to be noted in the corpus cavernosum tissue (data not shown).

On day 30, erectile function was assessed by electrical stimulation of the cavernous nerve (n=6 per group). The penis was then harvested for Masson's trichrome staining or immunohistochemical staining for smooth muscle α -actin and factor VIII (n=6 per group). Cavernous specimens from a separate group of animals were used for the measurement of the hydroxyproline level (n=4 per group).

3. Measurement of erectile function

Rats from each group (n=6 per group) were anesthetized with chloral hydrate (20 mg/kg) intraperitoneally, and a carotid artery was cannulated to measure systemic arterial pressure. Bipolar platinum wire electrodes were placed around the cavernous nerve. Stimulation parameters were 5 V at a frequency of 12 Hz, a pulse width of 1 ms, and a duration of 1 min. During tumescence, the maximal intracavernous pres-

sure (ICP) and slope for the ICP to reach 80% of maximal ICP were recorded. The total ICP was determined by the area under the curve from the beginning of cavernous nerve stimulation until the ICP returned to baseline or prestimulation pressure. The ratios of maximal ICP slope (S80), and total ICP to mean arterial pressure (MAP) were calculated to normalize for variations in systemic blood pressure.

4. Histologic examinations

The midportion of each penile segment was harvested and immediately fixed in 10% formalin phosphate-buffered solution before paraffin embedding. The specimens were cut (4 μ m) and stained with Masson's trichrome (n=6 per group). For immunohistochemistry (n=6 per group), paraffin block sections (4 μ m) were incubated with antibody to smooth muscle α -actin (Sigma-Aldrich, St. Louis, MO, USA; 1 : 100), followed by the Histostain[®]-Plus Bulk Kit (Zymed Laboratories, South San Francisco, CA, USA). For fluorescence microscopy (n=6 per group), frozen tissue sections (8 μ m) were incubated with antibody to factor VIII (DakoCytomation, Glostrup, Denmark; 1 : 50) at 4°C overnight. After washes with phosphate-buffered saline 3 times, the sections were incubated with rhodamine-conjugated rabbit antibody to immunoglobulin G for 1 h at room temperature. Signals were visualized and digital images were obtained with an Apotome microscope (Zeiss, Gottingen, Germany). Quantitative analysis of smooth muscle and endothelium in cavernous tissue was performed with an image analyzer system (National Institutes of Health [NIH] Image J 1.34, <http://rsb.info.nih.gov/ij/index.html>).

5. Hydroxyproline assay

Collagen protein levels were estimated by hydroxyproline determination (n=4 per group) as previously described.¹⁷ Briefly, aliquots of standard hydroxyproline or penis samples were hydrolyzed in alkali. The hydrolyzed samples were then mixed with a buffered chloramine-T reagent, and the oxidation was allowed to proceed for 25 min at room temperature. The chromophore was then developed by the addition of Ehrlich's reagent, and the absorbance of the reddish

purple complex was measured at 550 nm with a spectrophotometer. Absorbance values were plotted against the concentration of standard hydroxyproline, and the presence of hydroxyproline in penis tissue extracts was determined from the standard curve.

6. Statistical analysis

Results are expressed as means \pm standard deviations. Statistical analysis was performed by using one-way ANOVA followed by Scheffé multiple-comparison tests. Probability values less than 5% were considered significant.

Results

1. ALK5 inhibitor restores erectile function in an Ad-TGF- β 1-induced cavernous fibrosis model

Representative intracavernous tracings after stimulation of the cavernous nerve (5 V, 12 Hz, 1 ms) for 1 min in an untreated control, an Ad-LacZ-treated control, and cavernous fibrosis rats treated with saline or ALK5 inhibitor are shown in Fig. 1A. During electrical stimulation of the cavernous nerve, the ratios of maximal ICP, total ICP, and slope to MAP were significantly lower in the saline-treated cavernous fibrosis rats than in the untreated or Ad-LacZ-treated controls. A single intracavernous injection of ALK5 inhibitor restored all erection parameters, which reached up to 90% of untreated or Ad-LacZ-treated control values (Fig. 1B~D).

2. ALK5 inhibitor promotes the regression of cavernous fibrosis in an Ad-TGF- β 1-induced cavernous fibrosis model

A single intracavernous injection of Ad-TGF- β 1 induced profound cavernous fibrosis 30 days after injection. Cavernous fibrosis rats treated with ALK5 inhibitor showed a significant, but not complete, regression of fibrosis in the corpus cavernosum tissue. The control rats receiving Ad-LacZ did not show any histologic changes (Fig. 2A).

Total collagen content in the corpus cavernosum tissue was determined by measuring the amount of hydroxyproline. The hydroxyproline contents in the

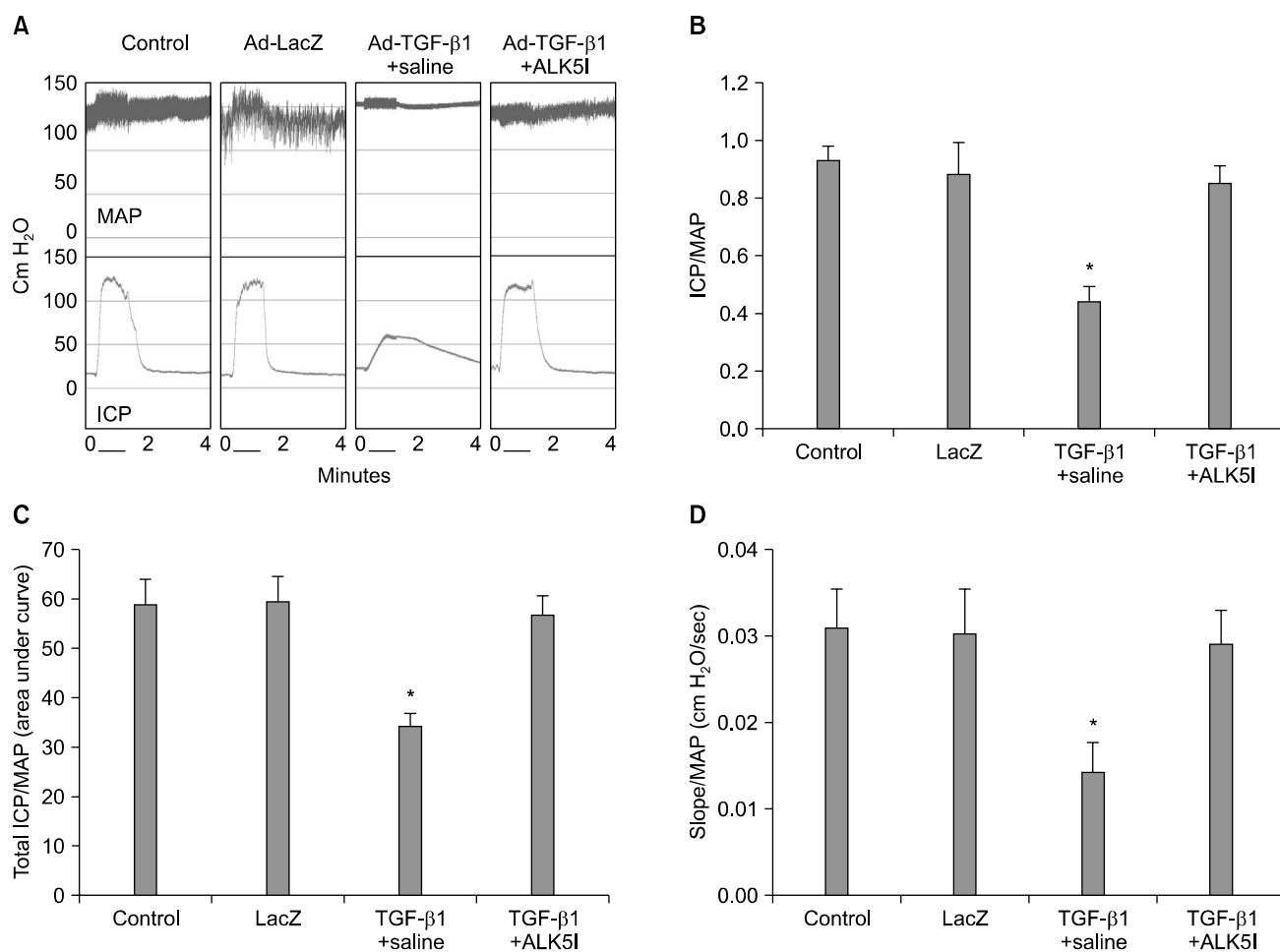


Fig. 1. Activin receptor-like kinase 5 (ALK5) inhibitor restores intracavernous pressure (ICP) elicited by electrical stimulation of the cavernous nerve in an adenovirus expressing TGF- β 1 (Ad-TGF- β 1)-induced cavernous fibrosis model. (A) Representative ICP responses for the untreated control, LacZ adenovirus (Ad-LacZ)-treated control, and cavernous fibrosis model receiving intracavernous injection of saline or ALK5 inhibitor. (B~D) Ratios of the mean maximal ICP, total ICP (area under the curve), and slope to mean arterial pressure (MAP) were calculated for each group. Each bar depicts the mean±standard deviations from n=6 animals per group. *p<0.01 compared with other groups. ALK5I, small-molecule inhibitor of activin-like receptor kinase 5.

penile tissue of cavernous fibrosis rats treated with saline increased to 137% and 131%, respectively, of that of untreated or Ad-LacZ-treated control rats. ALK5 inhibitor significantly reduced the hydroxyproline content in cavernous fibrosis rats to amounts comparable with those in untreated or Ad-LacZ-treated control rats (Fig. 2B).

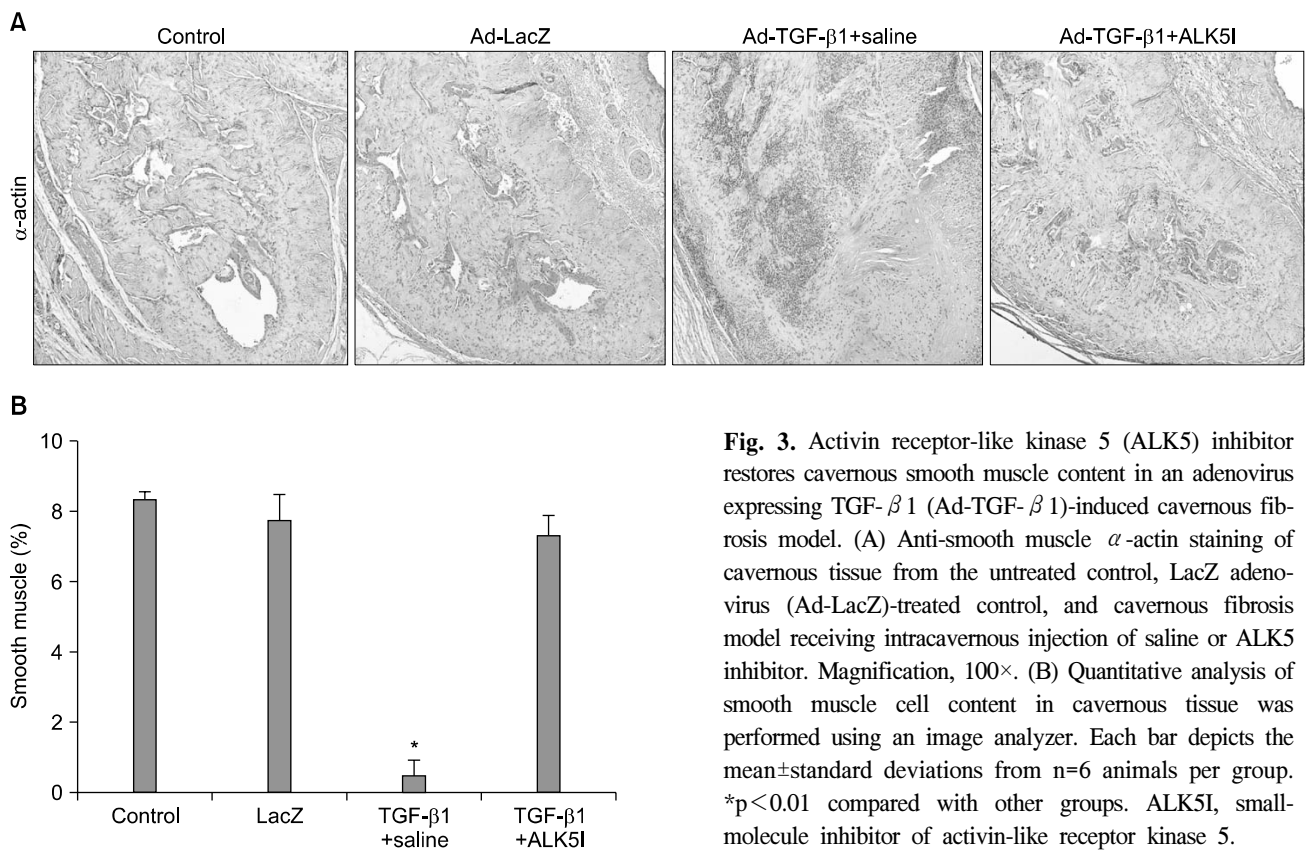
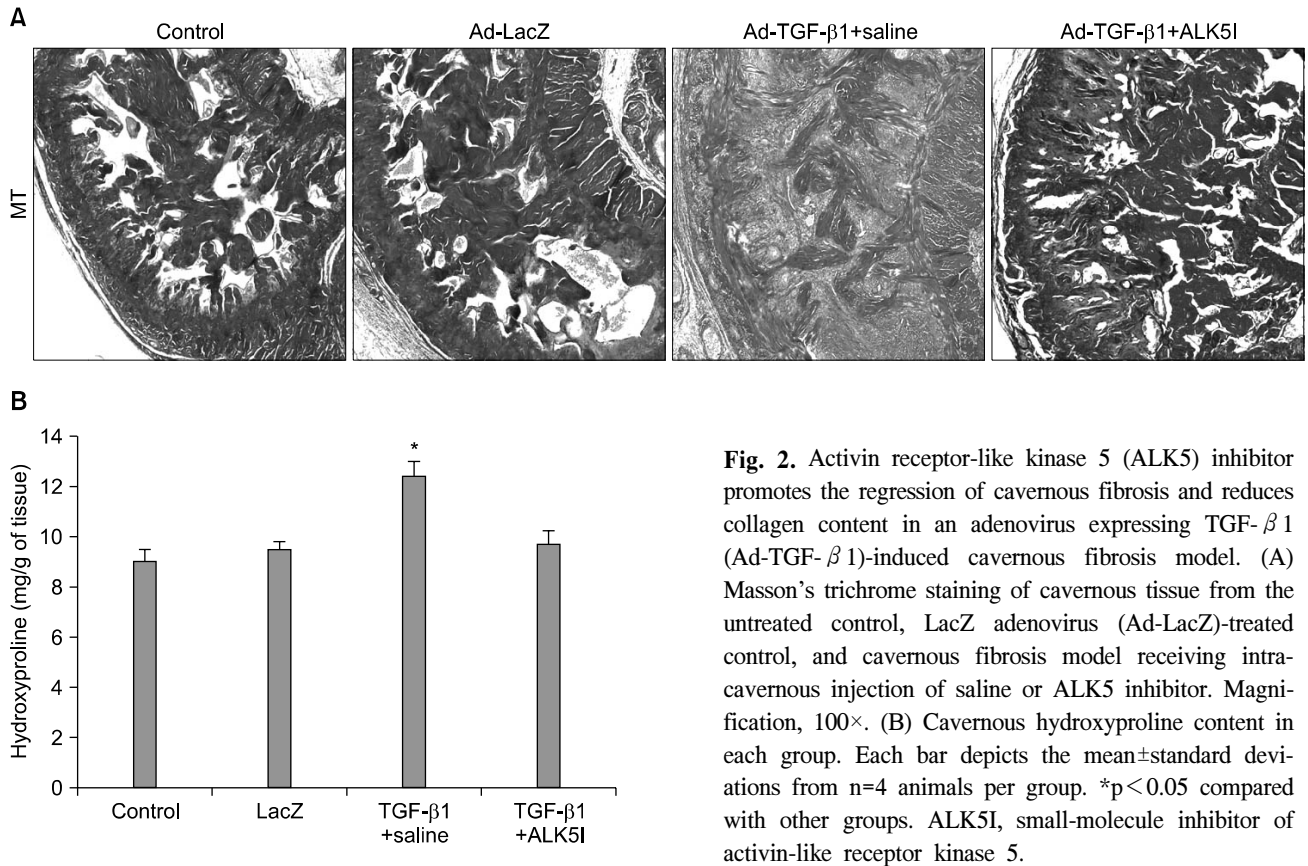
3. ALK5 inhibitor restores cavernous smooth muscle and endothelial content in an Ad-TGF- β 1-induced cavernous fibrosis model

Immunohistochemical staining of cavernous tissue with an antibody to smooth muscle α -actin and factor

VIII was performed in each group of animals. We found significantly lower cavernous smooth muscle and endothelial cell content in saline-treated cavernous fibrosis rats than in untreated or Ad-LacZ-treated controls. ALK5 inhibitor significantly restored the cavernous smooth muscle and endothelial cell content in cavernous fibrosis rats to amounts comparable with those in untreated or Ad-LacZ-treated control rats (Fig. 3, 4).

Discussion

In the present study, we showed that a single intracavernous injection of ALK5 inhibitor induced re-



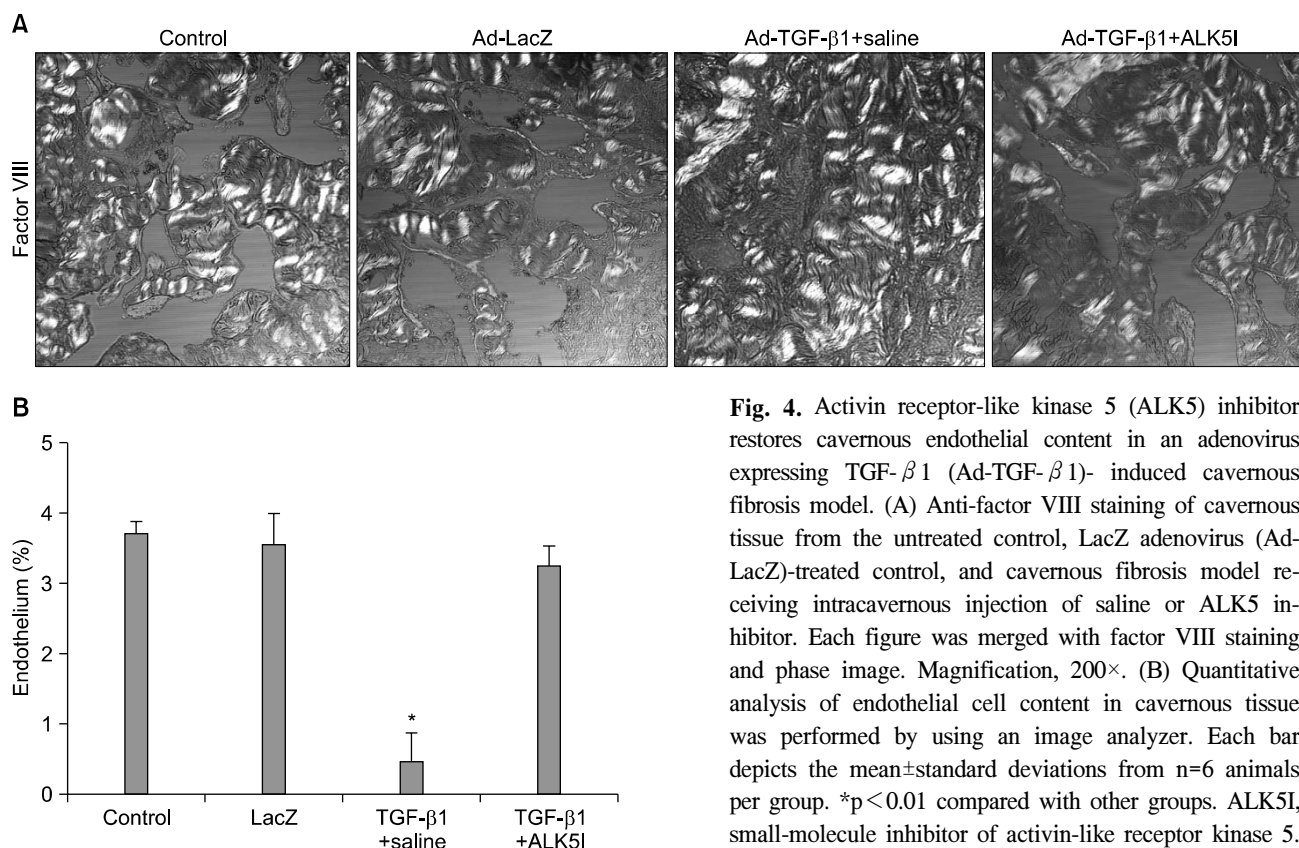


Fig. 4. Activin receptor-like kinase 5 (ALK5) inhibitor restores cavernous endothelial content in an adenovirus expressing TGF- β 1 (Ad-TGF- β 1)- induced cavernous fibrosis model. (A) Anti-factor VIII staining of cavernous tissue from the untreated control, LacZ adenovirus (Ad-LacZ)-treated control, and cavernous fibrosis model receiving intracavernous injection of saline or ALK5 inhibitor. Each figure was merged with factor VIII staining and phase image. Magnification, 200 \times . (B) Quantitative analysis of endothelial cell content in cavernous tissue was performed by using an image analyzer. Each bar depicts the mean \pm standard deviations from n=6 animals per group. *p<0.01 compared with other groups. ALK5I, small-molecule inhibitor of activin-like receptor kinase 5.

gression of cavernous fibrosis and restored erectile function. These effects were demonstrated by measurement of hydroxyproline content and by tissue pathology, including reduced deposition of connective tissue and restoration of smooth muscle and endothelial content in the corpus cavernosum tissue, which resulted in physiologically relevant changes in erectile function.

Physiologic penile erection is a mainly vascular phenomenon requiring interaction between endothelial and smooth muscle cells in the corpus cavernosum tissue. Functional and structural derangements in these cells play a crucial role in the pathophysiology of ED.^{6,8,18-20} Similar to the results of previous studies in diabetic animals,^{6,8,18} quantitative loss of smooth muscle cells and endothelial cells of the corpus cavernosum tissue was noted in the cavernous fibrosis model induced by Ad-TGF- β 1.

The TGF- β signaling pathway is involved in tissue fibrosis by activating Smad2 and Smad3, key intracellular signaling mediators for TGF- β -mediated

fibrosis. ALK5 is a type I TGF- β receptor most specific for active TGF- β . When TGF- β binds to ALK5, serine/threonine residues in ALK5 are phosphorylated. Activated ALK5 subsequently phosphorylates the downstream signaling molecules Smad2 and Smad3. When phosphorylated, Smad2/3 form a heteromeric complex with Smad4, which translocates into the nucleus and regulates the transcription of TGF- β -responsive genes, thereby inducing fibrosis-related changes.²¹ In the present study, significant recovery of cavernous smooth muscle and endothelial content was noted after local delivery of ALK5 inhibitor into the penis of cavernous fibrosis rats. Although we did not present the exact mechanisms by which ALK5 inhibitor restores smooth muscle and endothelial content, there is some evidence supporting this finding. TGF- β 1 is known to inhibit proliferation of smooth muscle cells through modification of the cell cycle, such as extension of the G2 phase or arrest in the late G1 phase.^{22,23} Furthermore, the TGF- β 1-Smad2 pathway

is involved in apoptosis of vascular smooth muscle cells induced by pioglitazone, a peroxisome proliferator-activated receptor γ ligand.²⁴ TGF- β is also known to inhibit endothelial cell proliferation via the ALK5-Smad2/3 pathway,²⁵⁻²⁷ whereas inhibition of ALK5 facilitates endothelial cell proliferation.^{26,27}

We also investigated whether ALK5 inhibitor can induce regression of cavernous fibrosis. ALK5 inhibitor suppressed deposition of collagen as determined by hydroxyproline measurement and Masson's trichrome staining. This finding is similar to the findings of previous studies that showed a significant reduction in hydroxyproline content by ALK5 inhibitor in the fibrotic plaque of a PD model¹⁵ and in kidneys with unilateral ureteral obstruction.²⁸

The limitation of this study is that we used an inhibitor of the TGF- β signaling pathway, ALK5 inhibitor, in an animal model of cavernous fibrosis induced by Ad-TGF- β 1. However, treatment with ALK5 was started on day 5 after intracavernous injection of Ad-TGF- β 1, when the fibrotic process had already begun and developed well. Further studies are needed to examine whether inhibition of the TGF- β pathway would induce structural and functional recovery in animal models of vasculogenic ED, such as a diabetic ED model characterized by induction of the TGF- β 1 gene and fibrosis in the corpus cavernosum tissue.

Conclusions

We showed that a single intracavernous injection of small-molecule inhibitor of ALK5 induced the regression of cavernous fibrosis and restored erectile function in rats with Ad-TGF- β 1-induced cavernous fibrosis. The results suggest that inhibition of the TGF- β pathway is a promising therapeutic strategy for the treatment of ED related to cavernous fibrosis from various causes.

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