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BASIC SCIENCE

Understanding the Role of Adenosine Receptors in the Myofibroblast Transformation in Peyronie's Disease

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ABSTRACT

Background: Peyronie's disease (PD) is a chronic fibrotic disease of the penis affecting a significant number of men worldwide without effective medical treatments. Myofibroblasts are pivotal in the pathogenesis of PD. Adenosine and adenosine receptors have been suggested to be involved in the pathophysiology of fibrosis.

Aim: To understand the role of adenosine receptors in myofibroblast transformation in PD.

Methods: Fibroblasts were isolated from the non-PD tunica albuginea (TA) tissue and PD plaque tissue and were transformed into myofibroblasts using transforming growth factor (TGF)-β1. Quantification of α-smooth muscle actin and adenosine receptors (adenosine receptor A1 [ADORA1], adenosine receptor A2A, adenosine receptor A2B [ADORA2B], and adenosine receptor A3) was performed using immuno-cytochemistry, in-cell enzyme-linked immuno-sorbent assay (ICE), and real-time reverse transcription quantitative polymerase chain reaction. The effect of various adenosine receptor agonists or antagonists on TGF-β1-induced myofibroblast transformation was measured using ICE.

Outcomes: Expression of adenosine receptors in myofibroblasts obtained from human TA and the effect of adenosine receptor ligands on myofibroblast transformation were investigated.

Results: The experiments showed that the protein and messenger RNA levels of α-smooth muscle actin in non-PD TA cells and PD plaque-derived cells were significantly higher in cells exposed to TGF-β1 than those not treated with TGF-β1. 2 of 4 adenosine receptors (ADORA1 and ADORA2B) were found to be expressed in both cell populations. Among various adenosine receptor agonists/antagonist investigated, only ADORA2B agonist, BAY 60-6583, significantly inhibited myofibroblast transformation in a concentration-dependent manner when applied simultaneously with TGF-β1 (IC_{50} = 30 μmol/L).

Clinical Translation: ADORA2B agonists may be clinically efficacious in early-stage PD.

Strengths & Limitations: The strength of this study is the use of primary fibroblasts from human TA. Limitation of the study is the high concentrations of the ligands used.

Conclusion: The effect of an ADORA2B agonist on TGF-β1-induced myofibroblast transformation shows a novel potential therapeutic target for PD if applied during early, non-stable phase of PD. **Mateus M, Ilg MM, Stebbeds WJ, et al. Understanding the Role of Adenosine Receptors in the Myofibroblast Transformation in Peyronie's Disease. J Sex Med 2018;XX:XXX–XXX.**

Key Words: Fibrosis; Transforming Growth Factor; Anti-Fibrotic Therapies; Fibroblast; Cell Culture

INTRODUCTION

Fibrosis can be described as excessive development of fibrous connective tissue, which can occur in various tissue types and organs (eg, kidney, lung, skin, and liver). At a cellular level, tissue resident quiescent fibroblasts and other cells such as endothelial, epithelial cells, and fibrocytes can differentiate into myofibroblasts, which have a crucial role in fibrosis characterized by increased proliferation, increased extra-cellular matrix (ECM) protein production, and contraction.[1,2] Moreover, their persistence (ie, failure to undergo apoptosis) and proliferation

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has been suggested to be one of the hallmarks of chronic fibrosis.[3,4]

Peyronie's disease (PD) is a fibrotic disorder characterized by the formation of plaques within the tunica albuginea (TA) of the penis.[5] Although its etiology is still poorly understood, microvascular trauma has been postulated as the initiating factor.[6] This fibrotic disorder is also characterized by the expression of several cytokines and growth factors, fibrin deposition, and myofibroblast differentiation.[7] In PD, the myofibroblast activity is increased, resulting in increased ECM protein production and eventual plaque formation[7–11] suggesting a pivotal role for myofibroblasts in the pathophysiology of PD.

Inhibition of differentiation of quiescent fibroblasts to profibrotic myofibroblasts has been suggested as a therapeutic approach for fibrosis.[4] Accordingly, we have been investigating potential molecular targets that may be involved in myofibroblast differentiation and small molecule compounds that may inhibit this process.

1 such target that is suggested in pathophysiology of fibrosis is adenosine and its receptors. Adenosine is a ubiquitous purine nucleoside released from cells and tissues under conditions of stress or injury and is generated intra-cellularly and extracellularly from adenine nucleotides, which are then dephosphorylated to adenosine. CD39 and CD73 are 2 cell surface molecules responsible for catalyzing the de-phosphorylation of adenine nucleotides to adenosine in the extra-cellular space.[12,13] Adenosine regulates its effects on tissue re-generation and repair via the interaction with a family of G-protein-coupled receptors: adenosine receptor A1 (ADORA1), adenosine receptor A2A (ADORA2A), adenosine receptor A2B (ADORA2B), and adenosine receptor A3 (ADORA3).[14]

Several studies have shown that adenosine receptors play different roles in acute and chronic injuries. In acute tissue injury, adenosine has been shown to be beneficial, as it is responsible for tissue protection and anti-inflammatory responses[13] (eg, promotion of barrier function and wound healing) in several organs, including kidney,[15] lung,[16] heart,[17] and liver.[18] In contrast to acute states, increased levels of adenosine have been associated with the progression of chronic tissue injuries. In these settings, adenosine has been suggested to promote fibrosis in several organs, such as the heart,[19] skin,[20] liver,[21] lung,[22] penis,[23] and kidney.[24] The adenosine receptors play different roles in the pathogenesis of fibrosis depending on the tissue subtype involved; however, the effects of adenosine are mainly regulated by ADORA2A and ADORA2B.[16] The characterization of these receptors has been investigated in other fibrotic disorders; however, no characterization of these receptors has been carried out in PD. By understanding how adenosine receptors may regulate the response to injury in this specific tissue and by looking at the myofibroblast transformation process, it may provide new insights into the pathophysiology of fibrosis in general and in PD. Furthermore, by targeting the respective pathway and by investigating the effect of selective agonist and antagonist compounds, it may enable avenues to identify potential targets for the treatment of PD and other fibrotic disorders and enhance the resolution of the injury or halt the progression of fibrosis in PD.

The aim of this study was to understand the involvement of adenosine receptors in the myofibroblast differentiation in PD by characterizing the myofibroblast transformation process in TA-derived fibroblasts to identify potential, novel targets for anti-fibrotic therapies.

METHODS

Sample Acquisition

TA tissue samples were acquired from patients undergoing corrective surgery for PD (to be referred to as PD plaque tissue) or invasive penile cancer (to be referred to as non-PD TA tissue) at University College London Hospital (UCLH), United Kingdom. Patients aged between 18–75 years, listed for surgical treatment of PD or penile cancer at UCLH, and able to understand the patient information sheet and to give consent were included in this study. Ethical approval was obtained from local independent research ethics committees (East of England Essex [12-EE-0170] and North of Scotland [15-NS-0051]).

The non-PD TA tissue was obtained from penis of patients with penile cancer (N = 3; average age = 72 ± 10 years). The TA tissue was removed from the proximal side away from the tumor and the tumor had negative margins on histology examination.

The PD TA tissue was obtained from penis of patients with chronic PD (N = 3; average age = 61 ± 6 years). The tissue used in this group was the plaque tissue that was surgically removed and would have otherwise been discarded.

Isolation of Fibroblasts From TA Tissue

Tissue samples were dissected into small pieces, submerged in culture media in 6 well plates and incubated at 37°C, 5% carbon dioxide in a humidified atmosphere for 5–7 days. This method is known as "explant technique" by which pure fibroblast cultures can be obtained.[25] Tissues were removed from the 6 well plates, once cells were observed growing out of the tissue. Cells were then washed 3 times with PBS and fresh, warm medium was added to each well. Cells were incubated at 37°C until they reached 50–70% confluence, after which old medium was removed and cells were washed with PBS. Cells were detached with 0.25% trypsin/EDTA (Thermo Fisher Scientific, Altrincham, United Kingdom) and neutralized with culture media. The cell suspension was transferred to T75 flasks (Thermo Fisher Scientific) and the cells were propagated and maintained up to passage 10.

Real-Time RT-q-Polymerase Chain Reaction

Cells were seeded onto 6 well plates (Thermo Fisher Scientific) at 1.0×10^5 cells/well and incubated in DMEM-F12 medium

(Thermo Fisher Scientific) supplemented with 10% FCS (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific). After overnight incubation, fibroblasts were incubated with or without transforming growth factor (TGF)-β1 (Sigma-Aldrich, Gillingham, United Kingdom) (10 ng/mL) for 72 hours. Total RNA was extracted from cells using the RNeasy mini kit (QIAGEN, United Kingdom) according to the manufacturer's instructions and a DNAse digestion step was included to eliminate genomic DNA contamination (RNAse-free DNAse set, QIAGEN). Complementary DNA (cDNA) was generated from 500 ng of RNA by reverse transcription using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, United Kingdom), according to manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using the QuantiTect SYBR Green PCR kit (QIAGEN), following the manufacturer's instructions and the CFX Connect real-time PCR detection system (Bio-Rad, Watford, United Kingdom). Gene-specific primer pairs for genes encoding α-smooth muscle actin (SMA) (NM_001613, 98 bp) were purchased from Sigma-Aldrich; and ADORA1 (NM_000674.2, 96 bp), ADORA2A (NM_000675.5, 93 bp), ADORA2B (NM_000676.2, 117 bp), and ADORA3 (NM_000677.3, 139 bp) were purchased from Primerdesign, Southampton, United Kingdom. The primers for EIF4A2 (NM_001967, 87 bp), TOP1 (NM_003286, 89 bp), vimentin (NM_003380), and desmin (NM_001927) were purchased from QIAGEN. PCR reactions were carried out by performing an initial activation step for 15 minutes at 95°C, followed by 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. All samples and negative controls (no template control and no reverse transcriptase enzyme control) were run in triplicate and in addition, a melting curve was generated at the end of the PCR cycle to ensure amplification of a single product. Relative messenger RNA (mRNA) levels were calculated using the $2^{-\Delta\Delta Ct}$ method[26] and obtained as fold-change of the target gene in the test samples relative to the calibrator sample and normalized to the expression of the reference genes (EIF4A2 and TOP1).

Immuno-Cytochemistry

Fibroblasts obtained from TA tissue samples were seeded onto coverslips (Thermo Fisher Scientific) at 2.5×10^5 cells/well and were incubated with or without TGF-β1 (10 ng/mL) for 72 hours. After incubation, cells were washed with PBS (Thermo Fisher Scientific) and fixed in ice-cold methanol (−25°C) (Thermo Fisher Scientific) for 10 seconds. Cells were then washed with PBS and 10% donkey serum (EMD-Millipore, United Kingdom) in PBS was added for 1 hour at room temperature. Blocking solution was then replaced by primary antibody solution (1:1,000 dilution of mouse monoclonal anti-α-SMA antibody [Sigma-Aldrich]; 1:1,000 dilution of mouse monoclonal vimentin [Abcam, Cambridge, United Kingdom]; or 1:100 mouse monoclonal desmin [Abcam]) diluted in PBS for 2 hours at room temperature in a humidified chamber. Cells were washed 3 times with PBS, after which, they were incubated with the donkey anti-mouse secondary antibody conjugated with fluorescein dye (Millipore) at 1:250 dilution in PBS for 2 hours at room temperature in a humidified chamber in the dark. After the incubation period, the secondary antibody solution was washed 3 times with PBS and the coverslips were mounted in a glass microscope slide with cells facing down with Vectashield mounting medium with propidium iodide (Vector Laboratories, Peterborough, United Kingdom). Cells were observed and images were captured using the Zeiss confocal microscope (LSM 510; Carl Zeiss, Cambridge, United Kingdom). The fluorescence images were taken in at least 3 random areas in each coverslip from each cell line acquired from 3 different patients (per group) (PD and non-PD). The number of α-SMA-positive cells was determined by counting the number of α-SMA-positive cells in 3 random fields per coverslip and the total number of cells was also determined by counting the number of nuclei stained.

In-Cell Enzyme-Linked Immuno-Sorbent Assay

The expression of α-SMA protein was quantified in 96 well plates using a technique called "in-cell enzyme-linked immunosorbent assay" (ICE), which involves fixation of the cells on the plate surface, permeabilization of the cells, staining of the proteins with an antibody ligated with infra-red dyes and visualizing the dyes using an infra-red imaging system.[27] Cells were seeded onto 96 well optical flat-bottom black microplates (Thermo Fisher Scientific) at 5.0×10^3 cells/well and left overnight at 37°C, 5% carbon dioxide in a humidified incubator. Media was replaced with fresh media with and without agonists and antagonists of adenosine receptors (all purchased from Tocris, Abingdon, United Kingdom) in co-incubation with or without TGF-β1 (5 ng/mL) for 72 hours. N6-cyclopentyladenosine (CPA) and BAY 60-6583 were used as ADORA1 and ADORA2B agonists, respectively. SLV 320 and MRS 1754 were used as ADORA1 and ADORA2B antagonists, respectively. The compounds were dissolved in 100% DMSO to 100-mmol/L stock concentration and further diluted to 100-μmol/L final assay concentration. After incubation, media was removed, and cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. Fixing solution was then removed and cells were washed 3 times with 0.1% Triton X-100 in PBS. A 5% donkey serum plus 0.1% Triton X-100 in PBS solution was incubated with cells for blocking and permeabilization for 90 minutes at room temperature. After removing the buffer, a primary antibody solution diluted in PBS (1:3,000 dilution of mouse monoclonal anti-α-SMA antibody, 1:500 dilution of rabbit monoclonal anti-ADORA1, or 1:500 dilution of rabbit polyclonal anti-ADORA2B) was added and incubated for 2 hours at room temperature. Cells were then washed with 0.1% Tween 20 in PBS 3 times, and a solution containing both the secondary

antibody (donkey anti-mouse or donkey anti-rabbit at 1:500, which emits at 800 nm) (IRdye 800CW; Li-Cor, Cambridge, United Kingdom) and a nuclear counter-stain at 1:1,000 that emits at 700 nm (DRAQ5; Biostatus, Loughborough, United Kingdom) diluted in PBS was incubated for 1 hour at room temperature in the dark. Cells were washed 3 times with 0.1% Tween 20 in PBS and the plate was scanned using an infra-red imaging system (Odyssey CLx imager; Li-Cor) at both 700-nm and 800-nm wavelengths.

Statistical Analysis

Data analyses and graphs were plotted using software (Excel; Microsoft, Redmond, WA) and presented as mean ± SEM since the data were normally distributed according to Shapiro-Wilks test. Statistical differences were determined by Student t test for un-paired means (2-sided) with a P value less than .05 considered statistically significant. All experiments were carried out in triplicate using samples from at least 3 patients (N = 3).

RESULTS

Characterization of Cells Isolated From Non-PD TA Tissue and PD Plaque Tissue

In order to ascertain that the cells we have isolated from human TA were fibroblasts, we investigated the expression of vimentin and desmin since fibroblasts are known to be vimentin-positive and desmin-negative.[28] Vimentin was expressed at both protein and mRNA levels in cells obtained from PD plaque tissue and non-PD TA tissues. Treatment of the cells with TGF-β1 did not alter the expression of vimentin (Figure 1).

The cells established from non-PD TA tissue and PD plaque tissue were desmin-negative; this was not affected by TGF-β1 treatment (Figure 1). These results confirm that the cells isolated from TA were indeed fibroblasts.

Cells Derived From Non-PD TA Tissue and PD Plaque Tissue Express α-SMA

Myofibroblasts are known to be vimentin-positive, desmin-negative, and α-SMA-positive.[29] The expression of α-SMA was investigated in cells isolated from non-PD TA tissue and PD plaque tissue. The mRNA levels of α-SMA significantly increased in both cell populations when exposed to TGF-β1 for 72 hours (Figure 2E). In addition, α-SMA-positive cells were observed in both cell populations in the presence of TGF-β1 (Figure 2C, D), whereas, in the absence of TGF-β1, the presence of α-SMA-positive cells was rare or non-existent in cells established from non-PD TA tissue and PD plaque tissue (Figure 2A, B). The number of α-SMA-positive cells in each cell population exposed to TGF-β1 was significantly higher compared to un-treated cells (Figure 2F). In order to quantify the protein levels in an objective and higher throughput manner, the ICE method was used to test the effect of TGF-β1 on non-PD TA cells and PD plaque-derived cells. Similar to the immuno-cytochemistry results, treatment with TGF-β1 elicited a significant increase in expression of α-SMA protein in cells obtained from either non-PD TA or PD plaque tissues (Figure 3).

Expression of Adenosine Receptors in Non-PD TA Cells and PD Plaque-Derived Cells

The expression of 4 adenosine receptors (ADORA1, ADORA2A, ADORA2B, and ADORA3) was investigated in cells derived from non-PD TA tissue and PD plaque tissue. Both cell populations expressed low levels of ADORA2A and ADORA3 (Figure 4), whereas, ADORA2B was expressed in both cell groups isolated from healthy and fibrotic tissues and ADORA1 was expressed in cells derived from PD plaque tissue. ADORA1 and ADORA2B were significantly higher in cells established from PD plaque tissue than from non-PD TA tissue (Figure 4).

Effect of TGF-β1 in ADORA1 and ADORA2B in Cells Derived From Non-PD TA Tissue and PD Plaque Tissue

The transcriptional levels of ADORA1 were significantly reduced in cells treated with TGF-β1 compared to un-treated cells that were derived from non-PD TA tissue and PD plaque tissue. However, no statistically significant difference was observed between non-PD TA cells and PD plaque-derived cells (Figure 5A). Contrary to mRNA levels, the protein levels of ADORA1 were significantly increased when exposed to TGF-β1 in cells isolated from non-PD TA tissue and PD plaque tissue (Figure 5B).

The mRNA levels of ADORA2B were significantly decreased in cells exposed to TGF-β1 compared to cells not exposed to TGF-β1 (Figure 6A). Although, no statistically significant difference was observed in the protein levels of ADORA2B when treated with TGF-β1, significance was achieved between non-PD TA cells and PD plaque-derived cells (Figure 6B).

Effect of ADORA1 and ADORA2B Agonist and Antagonist on TGF-β1-Induced Myofibroblast Transformation

To further investigate the change of ADORA1 and ADORA2B expression and their ability to inhibit myofibroblast transformation, agonists and antagonists were used. N6-CPA and BAY 60-6583 were used as ADORA1 and ADORA2B agonists, respectively. On the other hand, SLV 320 and MRS 1754 were used as ADORA1 and ADORA2B antagonists, respectively.

ADORA1 agonist (CPA) showed inhibition of TGF-β1-induced myofibroblast transformation only at 100 μmol/L in either cell populations. At the same high concentration, the cell viability was also reduced (Figure 7A). ADORA2B agonist (BAY 60-6583) significantly inhibited TGF-β1-induced myofibroblast transformation in a concentration-dependent manner in both non-PD TA cells and PD plaque-derived cells (IC_{50} = 30 μmol/L)

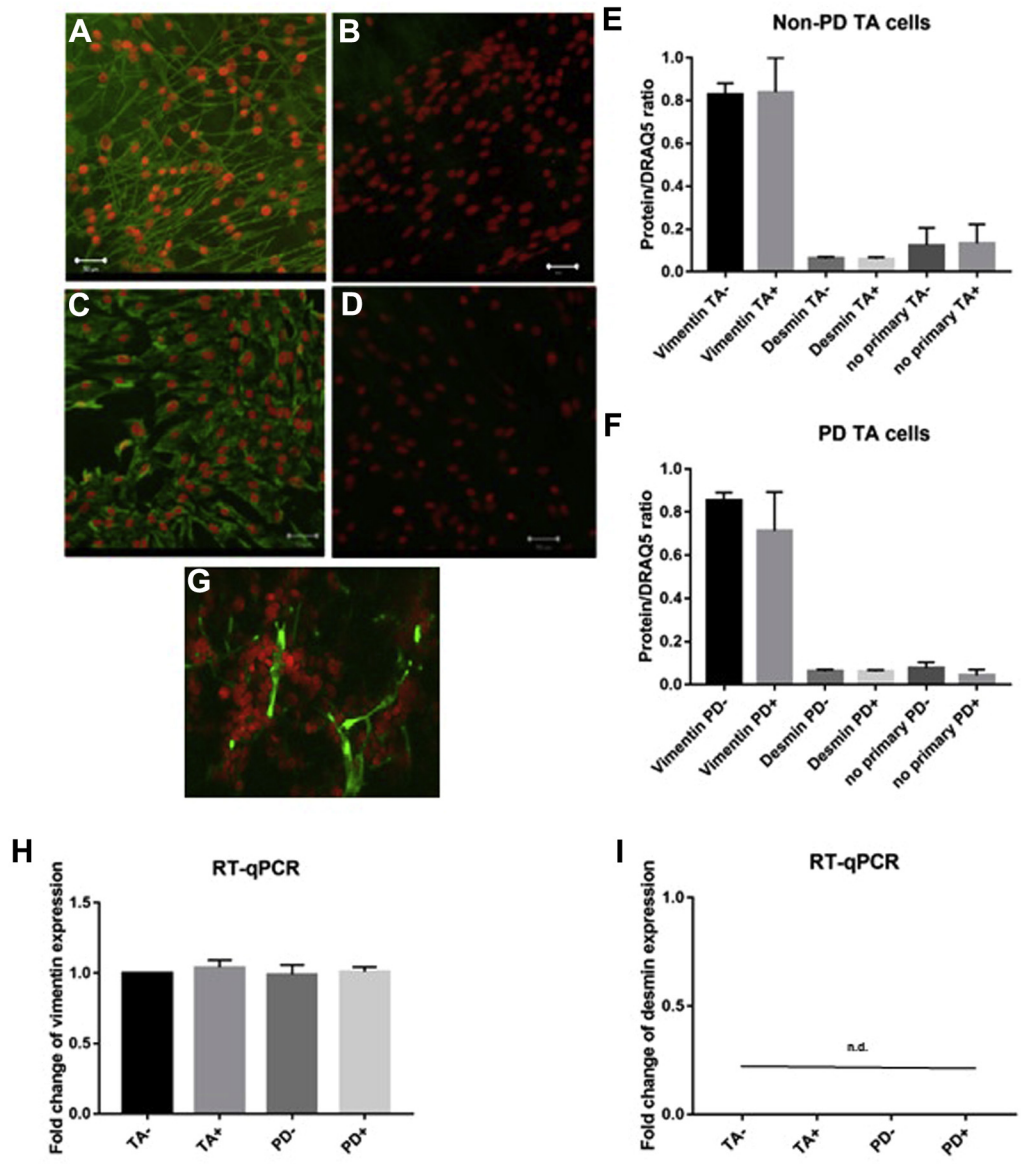

Figure 1. Vimentin and desmin quantification in cells derived from patients with or without Peyronie disease (PD). Fibroblasts were exposed to transforming growth factor (TGF)-β1 (10 ng/mL) for 72 hours. Representative images of: vimentin-stained non-PD tunica albuginea (TA)-derived cells exposed to control conditions (A); desmin-stained non-PD TA-derived cells exposed to control conditions (B); vimentin-stained non-PD TA-derived cells exposed to TGF-β1 (C); desmin-stained non-PD TA-derived cells exposed to TGF-β1 (D). Images were captured at $\times$200 magnification. Scale bars represent 50 μm. E, Quantification of vimentin/desmin expression non-PD TA-derived cells using in-cell enzyme-linked immuno-sorbent assay (ICE). F, Quantification of vimentin/desmin expression PD TA-derived cells using ICE. Data points were plotted as mean $\pm$ SEM, N = 3 patients for each group. G, Representative image for desmin staining of rhabdomyosarcoma cell line RD. H, Quantification of vimentin messenger RNA (mRNA) expression of non-PD and PD TA-derived cells using q-polymerase chain reaction (PCR). I, Quantification of desmin mRNA expression of non-PD and PD TA-derived cells using qPCR. Fibroblasts were exposed to TGF-β1 (10 ng/mL) for 72 hours before RNA isolation. Data points were plotted as mean $\pm$ SEM, N = 3 patients for each group. n.d. = not detectable.

(Figure 7B, E). The compound did not affect cell viability at any concentration tested in TGF-β1-induced myofibroblasts (Figure 7B) or non-stimulated fibroblasts (Supplementary Figure 1).

ADORA1 antagonist, SLV 320, inhibited TGF-β1-induced myofibroblast transformation only at 100 μmol/L, without affecting the cell viability (Figure 7C). The ADORA2B antagonist, MRS 1754, also failed to inhibit TGF-β1-induced myofibroblast transformation in either cell population significantly; the inhibition that was observed at only high concentrations was due to decreased cell viability (Figure 7D).

DISCUSSION

Although adenosine and its receptors have been studied in other fibrotic diseases, there is a lack of studies reporting the

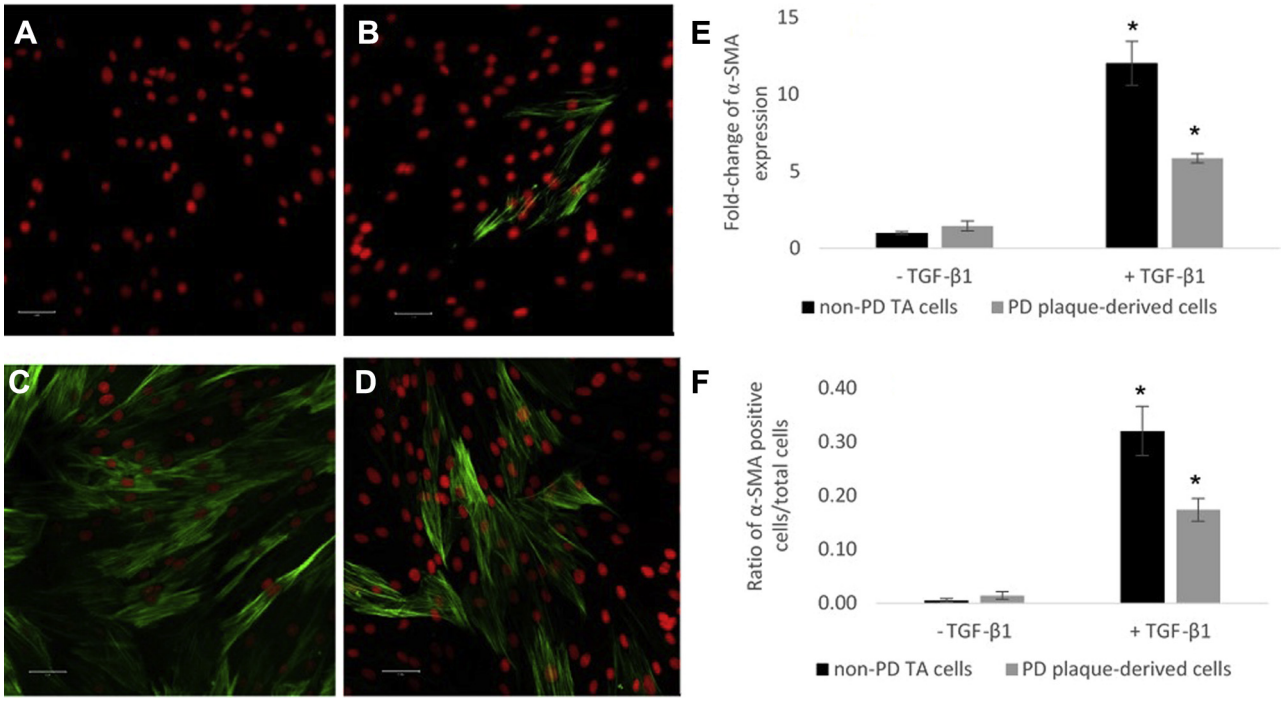

Figure 2. Expression of α-smooth muscle actin (SMA) in cells established from non-Peyronie disease (PD) tunica albuginea (TA) tissue and PD plaque tissue. Representative illustrations of α-SMA staining in: un-treated non-PD TA cells (A), un-treated PD plaque-derived cells (B), non-PD TA cells treated with transforming growth factor (TGF)-β1 (C), and PD plaque-derived cells treated with TGF-β1 (D). The nucleus of the cells was stained with propidium iodide, a red nuclear counter-stain, whereas the α-SMA-positive cells were stained in green, which is conferred by the FITC conjugated secondary antibody. Confocal microscope at $\times$200 magnification. Bar in the corner of each image represents 50 μm. E, α-SMA messenger RNA levels in cells derived from non-PD TA tissue and PD plaque tissue exposed to 10 ng/mL of TGF-β1 for 72 hours. F, Ratio of α-SMA-positive cells in cells derived from non-PD TA tissue and PD plaque tissue exposed to TGF-β1 at 10 ng/mL for 72 hours, after which, the number of myofibroblasts was calculated by counting the number of α-SMA-positive cells in each field and then divided by the number of total cells. Data points were plotted as mean $\pm$ SEM, N = 3. *$P < .05$ tested by Student *t* test vs un-treated cells.

characterization and effects of adenosine receptors in PD. Therefore, the aim of this study was to investigate the role of adenosine receptors in myofibroblast transformation in PD.

First, the cells isolated from non-PD TA tissue and PD plaque tissue were characterized. The results showed that both cell groups were positive for vimentin and negative for desmin suggesting that these cells were fibroblasts. It was also observed that treatment with TGF-β1 did not alter the expression of these specific markers, but significantly increased the expression of α-SMA confirming myofibroblast phenotype.[29]

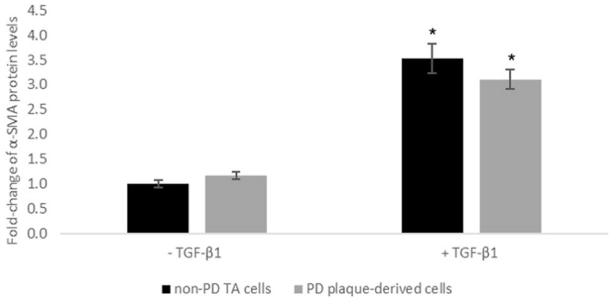

Figure 3. α-Smooth muscle actin (SMA) protein levels in cells derived from non-Peyronie disease (PD) tunica albuginea (TA) tissue and PD plaque tissue. In-cell enzyme-linked immuno-sorbent assay was performed to assess the α-SMA protein levels in fibroblasts exposed to transforming growth factor (TGF)-β1 at 10 ng/mL for 72 hours. Data points were plotted as mean $\pm$ SEM, N = 3. *$P < .05$ tested by Student *t* test vs un-treated cells.

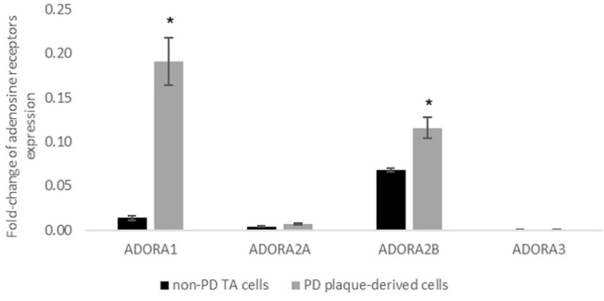

Figure 4. Adenosine receptor (ADORA) mRNA levels in cells derived from non-Peyronie disease (PD) tunica albuginea (TA) tissue and PD plaque tissue. The expression of ADORA mRNA was determined using the $2^{\Delta Cq}$ method, where the result obtained corresponds to the fold change of each ADORA mRNA relative to the expression of reference genes (EIF4A2 and TOP1). Each sample was run in triplicate. Data points were plotted as mean $\pm$ SEM, N = 3. *$P < .05$ tested by Student *t* test vs non-PD TA cells.

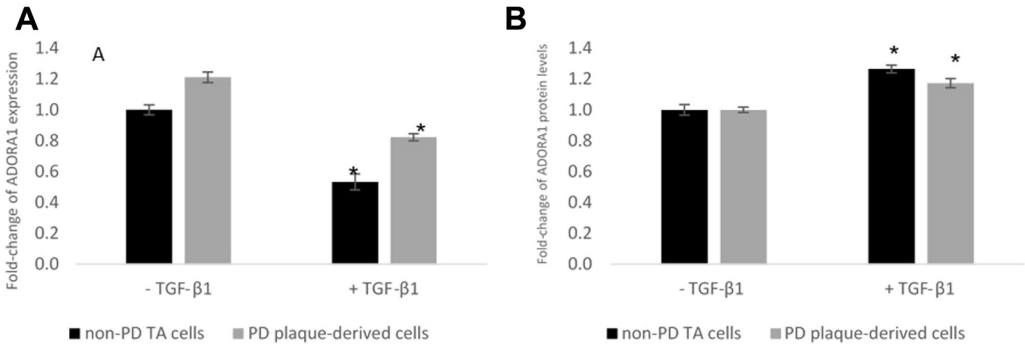

Figure 5. Expression of adenosine receptor A1 (ADORA1) in cells derived from non-Peyronie disease (PD) tunica albuginea (TA) tissue and PD plaque tissue. ADORA1 messenger RNA (A) and protein (B) levels. Fibroblasts were treated with transforming growth factor (TGF)-β1 at 10 ng/mL for 72 hours. Data points were plotted as mean ± SEM, N = 3. *$P < .05$ tested by Student *t* test vs un-treated cells.

Myofibroblasts have a crucial role in pathophysiology of fibrosis, as these cells are not only responsible for contraction but also for production of several cytokines and abundant ECM proteins. Myofibroblast transformation is well established in the literature as being an important contributor in the pathophysiology of several fibrotic disorders including PD. A key feature of myofibroblasts is their α-SMA expression, which can be targeted to investigate the myofibroblast differentiation. Both mRNA and protein levels of α-SMA were assessed by exposing cells derived from non-PD TA tissue and PD plaque tissue to TGF-β1. In both cell groups, a significant increase of α-SMA mRNA levels was observed when treated with TGF-β1. The effect of TGF-β1 on mRNA levels was mirrored by α-SMA protein expression, that is, both cell populations showed an increase in α-SMA-positive cells in the presence of TGF-β1. These results are supported by previously published reports that have shown that both α-SMA mRNA and protein levels increase in the presence of TGF-β1 in fibroblasts obtained from PD tissue.[10,11]

Interestingly, α-SMA-positive cell numbers and α-SMA mRNA and protein levels in un-stimulated cells obtained from PD tissue were similar to those from TA tissue suggesting that basal myofibroblast numbers were similar in cultures derived from the 2 tissue types. Although we did not aim to investigate specifically whether PD tissue would yield more myofibroblasts than TA tissue, this was a surprising and un-expected finding. We believe that this could be a result of the choice of method we employed to isolate cells from the tissue samples. During the explantation of the tissue and subsequent cell passaging, more fibroblasts than myofibroblasts might have migrated from the tissue or myofibroblasts might have reverted in their phenotype during cell passaging. Another possibility is that the PD cells could have already been exposed to TGF-β1 in the tissue during the formation of the plaque; they may have developed resistance to TGF-β1, hence their lower responsiveness to TGF-β1 than the TA cells. Nevertheless, our results confirmed that both tissue types yielded sufficient number of fibroblasts, which can be transformed to myofibroblasts using TGF-β1 and can be further utilized in myofibroblast transformation assay.

Adenosine receptors have been associated with the progression of chronic tissue injuries when the levels of adenosine are increased, suggesting that adenosine may promote fibrosis. To the best of the author's knowledge, this is the first time that the expression of adenosine receptors has been investigated in cells derived from non-PD TA tissue and PD plaque tissue. In order to demonstrate the involvement of adenosine receptors in myofibroblast transformation in PD, the expression of these receptors

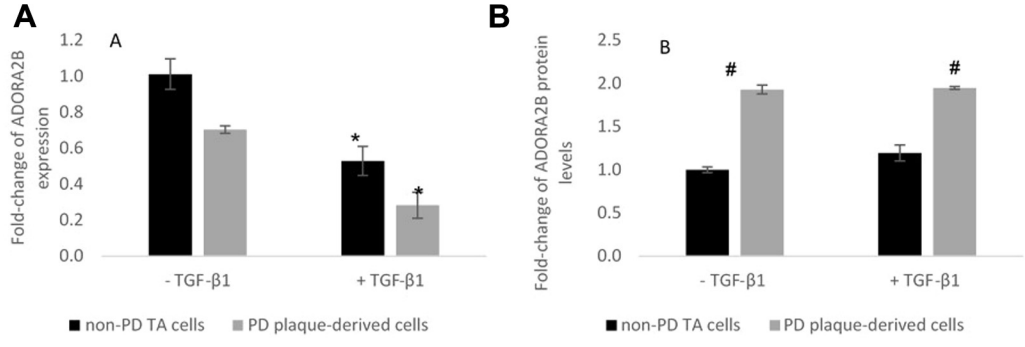

Figure 6. Expression of adenosine receptor A2B (ADORA2B) in cells derived from non-Peyronie disease (PD) tunica albuginea (TA) tissue and PD plaque tissue. ADORA2B messenger RNA (A) and protein (B) levels. Fibroblasts were treated with transforming growth factor (TGF)-β1 at 10 ng/mL for 72 hours. Data points were plotted as mean ± SEM, N = 3. *$P < .05$ tested by Student *t* test vs un-treated cells and #$P < .05$ tested by Student *t* test vs non-PD TA cells.

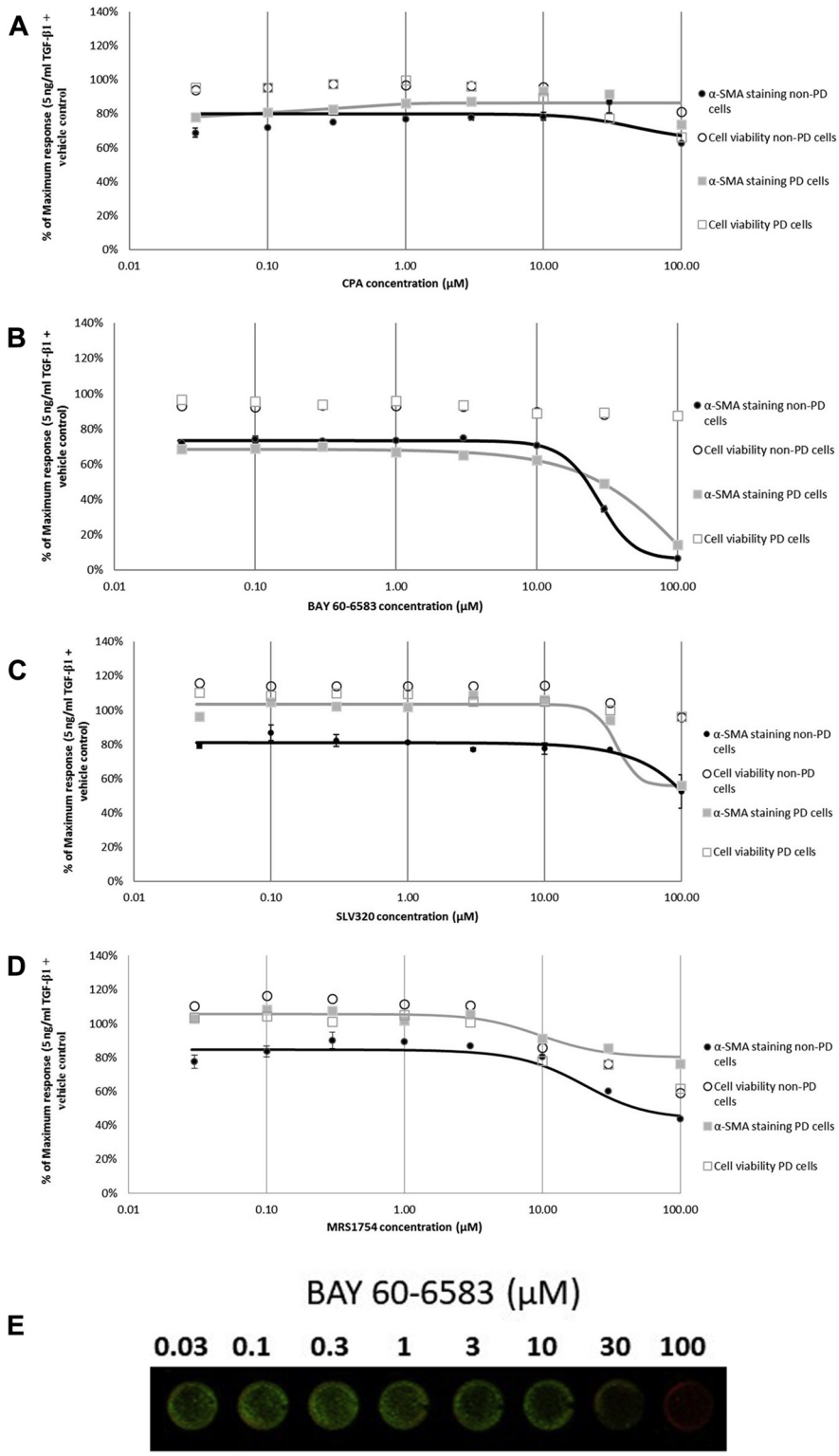

Figure 7. Effect of adenosine receptor ligands on transforming growth factor (TGF)-β1-induced myofibroblast transformation. Effect of cyclopentyladenosine (CPA) (adenosine receptor A1 [ADORA1] agonist) (A), BAY 60-6583 (adenosine receptor A2B [ADORA2B] agonist) (B), SLV 320 (ADORA1 antagonist) (C), and MRS 1754 (ADORA2B antagonist) (D) on TGF-β1-induced myofibroblast transformation and cell viability. Cells derived from non-Peyronie disease (PD) tunica albuginea tissue and PD plaque tissue were exposed to a range of concentrations of the ligands between 0.03—100 μmol/L and applied in co-incubation with TGF-β1 for 72 hours. Data points were plotted as average ± SEM of the percentage of maximum response of either the α-smooth muscle actin (SMA)/DNA staining ratio (measure of myofibroblast transformation) or DNA staining (measure of cell numbers and viability), N = 3. E, Representative images of individual wells in an in-cell enzyme-linked immuno-sorbent assay plate obtained from Odyssey infra-red imager in the presence of TGF-β1 and increasing concentrations of BAY 60-6583.

was investigated in cells isolated from non-PD TA tissue and PD plaque tissue. Among the 4 receptors, the expression of ADORA1 and ADORA2B was significantly higher in PD plaque-derived cells than in non-PD TA cells; however, this increase was dramatically higher in ADORA1 transcripts levels. The expression of ADORA2A and ADORA3 was below the detection limits, therefore these 2 receptors were not studied further. Zhong et al[30] (2005) found that primary human lung fibroblasts expressed high mRNA levels of ADORA2B and the expression of this receptor at protein levels was confirmed by immuno-fluorescence. These authors also showed that the activation of the ADORA2B by adenosine promoted myofibroblast transformation. In our study, the effect of TGF-β1 on mRNA levels of both adenosine receptors was also assessed, leading to a significant decrease of ADORA1 and ADORA2B mRNA levels in both cell groups. Even though the mRNA levels were significantly decreased, it may not represent a biological change in the transcript levels, as according to MIQE guidelines, to show biological changes a 2-fold (recommended cut-off value) increase or decrease should be observed.[31] The protein levels of ADORA1 and ADORA2B in both cell populations was assessed using the ICE assay, showing that a significant increase of ADORA1 was observed in cells treated with TGF-β1; however, no difference was observed between non-PD TA cells and PD plaque-derived cells.

In addition, Wen et al[23] (2010) showed that primary corpus cavernosal fibroblasts from mice expressed ADORA2B, which was suggested to be responsible for adenosine-mediated penile fibrosis. Furthermore, 2 other studies have also shown that deaminase-deficient mice had an increment of adenosine levels and ADORA2B activation in the penis, suggesting an essential mechanism for the progression of priapism in these mice.[23,32]

Receptor expression results therefore suggest that ADORA1 and ADORA2B were expressed in both fibroblasts and myofibroblasts derived from TA. It would be worth investigating in the future how these 2 receptors and their down-stream pathways may interact in physiological and pathological conditions.

The role of ADORA1 and ADORA2B was assessed in TGF-β1-induced myofibroblast transformation using ADORA1 and ADORA2B agonists and antagonists. CPA and BAY 60-6583 were utilized as ADORA1 and ADORA2B agonists, respectively, while SLV 320 and MRS 1754 were used as ADORA1 and ADORA2B antagonists, respectively. ADORA1 agonist, CPA, showed an inhibitory effect on TGF-β1-induced myofibroblast transformation only at high concentrations while affecting the cell viability; therefore, we conclude that the inhibitory effect is most probably due to cytotoxicity at the high concentrations. ADORA2B agonist BAY 60-6583 significantly inhibited the transformation in a concentration-dependent manner with an IC_{50} value of 30 μmol/L and had no cytotoxicity either on fibroblasts or myofibroblasts. No inhibition of TGF-β1-induced myofibroblast transformation was observed with the ADORA1 antagonist (SLV 320) and ADORA2B antagonist (MRS 1754). We are particularly surprised to see no effect by ADORA2B antagonist since we would have expected inhibition of myofibroblast transformation with this compound. However, we have observed an inhibition by ADORA2B agonist suggesting that endogenous adenosine may have anti-fibrotic effect under these conditions, which obviously warrants further research.

We conclude that among the 4 agonists/antagonists tested, ADORA2B agonist BAY 60-6583 has proven to be the most successful one and further suggest that ADORA2B agonism may be a potential therapeutic approach for PD. This is further supported by our finding of ADORA2B expression in cells derived from human TA with or without PD. It would be worthwhile testing this compound in animal models of PD.

To our knowledge, BAY 60-6583 has not progressed to clinical development and remains to be the only ADORA2B agonist that has been developed.[33,34] Further development of similar compounds would be beneficial in future fibrosis research.

One of the limitations of our study is being limited to in vitro findings. Although we have used primary fibroblasts isolated from fresh human tissue, there is always the risk of our findings not being able to be translated to in vivo. Similarly, the IC_{50} of BAY 60-6583 is relatively high; it is un-likely that such a concentration can be reached at tissue level in an in vivo situation. Further work would be required to understand the pharmacology of ADORA2B agonists in PD. Another limitation is the choice of non-PD TA tissue being obtained from patients with penile cancer. Although we made sure that the tissue obtained was away from the tumor and the tumor margins were negative in histology examination, we cannot exclude the possibility of cancer genetics influencing the fibroblast phenotype.

CONCLUSION

Cell culture models from both non-PD TA tissue and PD plaque tissue were successfully established, with confirmed fibroblast identity and differences regarding TGF-β1-induced myofibroblast transformation, mainly increased α-SMA mRNA and protein levels in cells isolated from PD plaque tissue.

The expression of 2 adenosine receptors (ADORA1 and ADORA2B) was demonstrated in both cell groups in which the ADORA2B agonist BAY 60-6583 significantly inhibited TGF-β1-induced myofibroblast transformation in the 2 cell types investigated.

Inhibition of differentiation of quiescent fibroblasts to profibrotic myofibroblasts has been suggested as a therapeutic approach for fibrosis.[4] The effect of BAY 60-6583 on TGF-β1-induced myofibroblast transformation may suggest a novel potential therapeutic target for PD if applied during early, non-stable phase of PD and in other fibro-proliferative diseases, although we did not test the compounds on cells derived from the acute phase of PD. We suggest that such compounds may be

useful in early stages of the disease to prevent formation of the plaque.

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SUPPLEMENTARY DATA

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