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ORIGINAL RESEARCH

AT_1 expression in human urethral stricture tissue

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Background: Urethral stricture has a high recurrence rate. There is a common doctrine stating that "once a stricture, always a stricture". This fibrotic disease pathophysiology, pathologically characterized by excessive production, deposition and contraction of extracellular matrix is unknown. Angiotensin II type 1 (AT_1) receptor primarily induces angiogenesis, cellular proliferation and inflammatory responses. AT_1 receptors are also expressed in the fibroblasts of hypertrophic scars, whereas angiotensin II (AngII) regulates DNA synthesis in hypertrophic scar fibroblasts through a negative cross talk between AT_1 and angiotensin II type 2 (AT_2) receptors, which might contribute to the formation and maturation of human hypertrophic scars.

Objective: This study was conducted to determine the expression of AT_1 receptors in urethral stricture tissues.

Methods: Urethral stricture tissues were collected from patients during anastomotic urethroplasty surgery. There were 24 tissue samples collected in this study with 2 samples of normal urethra for the control group. Immunohistochemistry study was performed to detect the presence of AT_1 receptor expression. Data were analyzed using Mann–Whitney *U* test, and statistical analysis was performed with SPSS version 20.

Results: This study showed that positive staining of AT_1 receptor was found in all urethral stricture tissues (n=24). A total of 8.33% patients had low intensity, 41.67% had moderate intensity and 50% had high intensity of AT_1 receptors, while in the control group, 100% patients had no intensity of AT_1 receptors. Using the Mann–Whitney *U* test, it was found that urethral stricture tissue had a higher intensity of AT_1 receptors than normal urethral tissue with a p-value = 0.012.

Conclusion: The results showed that AT_1 receptor had a higher intensity in the urethral stricture tissue and that AT_1 receptor may play an important role in the development of urethral stricture.

Keywords: angiotensin, AT_1 receptor, urethral stricture

Introduction

Urethral stricture is defined as a narrowing of a segment of urethra that is enveloped by corpus spongiosum and specifically underwent spongiofibrosis process. Accumulation of fibrotic tissue will eventually lead to disturbance of voiding process and consequently affect the quality of life. The etiology of urethral stricture disease is traumatic, inflammatory, iatrogenic and idiopathic (Table 1). Every process that causes injury of urethral epithelium or corpus spongiosum could eventually lead to urethral stricture disease.[1]

Until now, urethral stricture disease is still a challenging problem, and its management has been still developing. From 16th century until now, the methods for overcoming urethral stricture have constantly changed from metal dilator, blind inter-

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Table 1 AT_1 receptor expression in urethral tissue

AT_1 expression	Urethral stricture	Normal urethra	*p*-value
(–) negative	0 (0.0%)	2 (100.0%)	0.012
(+) mild	2 (8.33%)	0 (0.0%)	
(+) moderate	10 (41.67%)	0 (0.0%)	
(+) strong	12 (50.0%)	0 (0.0%)	

Abbreviation: AT_1, angiotensin II type 1.

nal urethrotome in the 18th century, direct vision internal urethrotomy (DVIU) to urethroplasty surgery now.[2]

One challenging problem of the urethral stricture is its high recurrence rate. There is a common doctrine stating that "once a stricture, always a stricture". A study by Zehri et al[3] in 2009 revealed that the median duration between optical urethrotomy and recurrence was 4.5 months and the recurrence rate was 34%.

Another study by Han et al in 2015 found that 26% of all patients had a recurrence at a mean follow-up of 62 months. The recurrence rate after anastomotic urethroplasty was 18%, compared with 31% after substitution urethroplasty. The mean time to recurrence was 34 months.[4]

The mechanism of this fibrotic disease, pathologically characterized by excessive production, deposition and contraction of extracellular matrix, is still unclear. Recent studies showed the role of renin–angiotensin system (RAS) in fibroblast proliferation and extracellular matrix deposition. Shirazi et al[5] in 2007 showed a decrease in urethral stricture recurrence after DVIU using intraurethral captopril gel compared to the placebo group.

Another study by Nababan et al (unpublished, 2013) observed an increasing expression of angiotensin II (AngII) receptor in injured urethra of Wistar mice.

All the study mentioned earlier showed us that RAS may have a role in urethral stricture formation and recurrence. To demonstrate the relationship of RAS with urethral stricture formation in human beings, we conducted this study.

Methods

Tissue samples

Tissue samples were obtained from urethral stricture segment that had been excised from the patients during anastomotic urethroplasty surgery for patients with anterior urethral stricture. The segment that was excised should also contain normal urethral tissue. In total, we obtained 24 urethral tissue samples from patients with urethral stricture and 2 normal urethra from cadaveric bodies. The urethral stricture tissue samples were obtained under written informed consent from the 24 patients who underwent anastomotic urethroplasty procedure for research purpose. This study was approved by the ethical committee of Hasan Sadikin Hospital, Bandung. The normal urethra tissue samples were obtained from cadaveric tissues that had deceased less than 24 hours. The cadaveric tissues were also obtained for education and research purpose and had been approved by the ethical committee of Hasan Sadikin Hospital, Bandung, and Department of Forensic Medicine of Hasan Sadikin Hospital, Bandung.

Immunohistochemical staining of AngII type 1

Immunohistochemistry (IHC) examination was done using angiotensin II type 1 (AT_1) with 100 times dilution and ultra vision/HRP system with three-step anti-polyvalent detection system conjugated with secondary antibody.

The tissue samples were fixed with 10% formalin and embedded in paraffin. The samples then were cut as thick as 4–5 µm using microtome and stored in a 38–40°C incubator. Sections then were deparaffinized with xylol and sequentially rehydrated in a graded ethanol series for 5 min each.

The sections were dipped inside 90%, 80% and 70% alcohol solution and then washed using Aquadest and dipped inside boiled buffer citrate solution for 5 minutes and cooled at room temperature. After that, the sections were washed using phosphate-buffered saline (PBS) for 3 times, 5 minutes each. Blocking serum was added, and the sections were then incubated in a closed room for 10 minutes and washed using PBS for 3 times, 5 minutes each.

AngII was added to the sections, and the sections were then incubated in room temperature for 60 minutes and washed using PBS for 3 times, 5 minutes each. Biotinylated universal secondary antibody was added to the sections and then incubated in a closed room for 10 minutes and washed using PBS for 3 times, 5 minutes each. After that, diaminobenzylene chromogenic solution was added, incubated in the closed room for 5 minutes and washed using running water for 5 minutes. Counterstaining was done using Mayer's hematoxylin, incubated for 2 minutes and then washed using running water.

Sequential dehydration was done by dipping the sections in 70%, 80% and 90% alcohol solutions for 5 minutes each and 100% ethanol solution for 2 times, 5 minutes each. The sections were then dried using filter paper, dipped into xylol for 3 minutes and observed and analyzed using light microscope.

AT_1 receptor expression measurement procedure was done in Pathological Anatomy Department's laboratory, Hasan Sadikin Hospital, Bandung. All sections were exam-

ined by experts in pathology. AT$_1$ expression was measured by observing immunoexpression in the fibrotic tissue of the urethra.

Statistical analysis

To determine the expression of AT$_1$ in urethral stricture tissue compared to normal urethral tissue, we used Mann–Whitney *U* test with 95% confidence level (α=5%). Statistical analysis was performed using SPSS version 20.0.

Result of the study

Descriptive analysis was done to d

etermine the AT$_1$ receptor in urethral stricture tissue compared to normal urethral tissue. The AT$_1$ expression was graded as negative, mild positive, moderate positive and strong positive (Figure 1).

From a total of 24 samples of urethral stricture tissues, 2 (8.33%) samples were categorized as mild positive, 10 (41.67%) samples were categorized as moderate positive, and 12 (50%) samples were categorized as strong positive. None of the samples were categorized as negative. From the normal urethra group, none of the samples expressed the AT$_1$ receptor in their tissues.

Using Mann–Whitney *U* test, we concluded that AT$_1$ receptor expression in urethral stricture tissue was significantly higher than normal urethral tissue (p=0.012; Table 1).

Discussion

Urethral stricture disease is a fibrotic condition of a segment of urethra due to spongiofibrotic process of the urethra and corpus spongiosum (Figures 2 and 3). Damage to urethral epithelium or corpus spongiosum would eventually lead to urethral stricture formation. Pathological process in urethral stricture formation showed a change in pseudostratified columnar epithelium that lines the urethra toward squamous metaplastic epithelium.[7] Further damage to the metaplastic region can cause urine extravasation that leads to fibrotic process of the corpus spongiosum. This process will lead to narrowing of the urethral caliber and cause one to develop lower urinary tract symptoms and eventually urinary retention.

Baskin et al in 1993 showed us that there were no differences in the amount of collagens in urethral stricture tissue compared to that in normal urethra, but they have found a change in the ratio between collagen types that could explain the fibrotic and scar formation process. In corpus spongiosum of normal urethral tissue, the ratio between collagen type I and type III was 75%:25%, whereas in urethral stricture tissue, the ratio was 16%:84%.[6]

RAS is an endocrine system cascade, which consists of angiotensinogen (α-glycoprotein), that is released from the liver into the circulation and transformed by renin, which is produced by the juxtaglomerular apparatus of the kidney, into angiotensin I (AngI). AngI will further transform

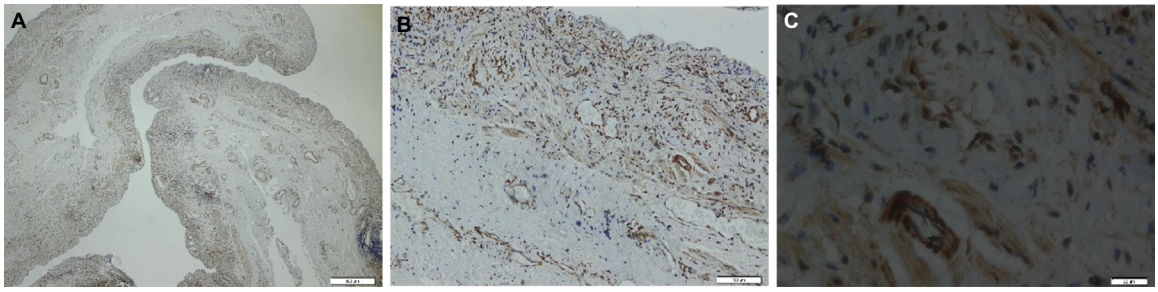

Figure 1 AT$_1$ expression in urethral stricture tissue: (**A**) mild positive, (**B**) moderate positive and (**C**) strong positive.

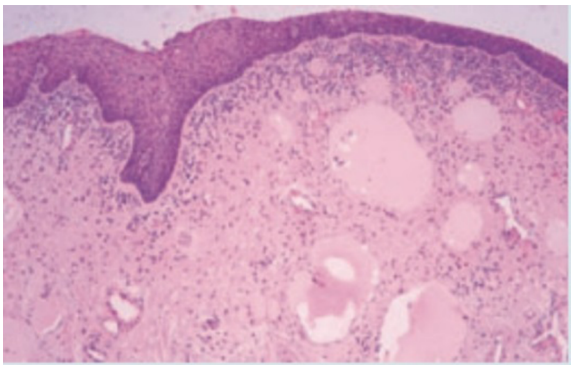

Figure 2 Normal urethral tissue (100× magnification).

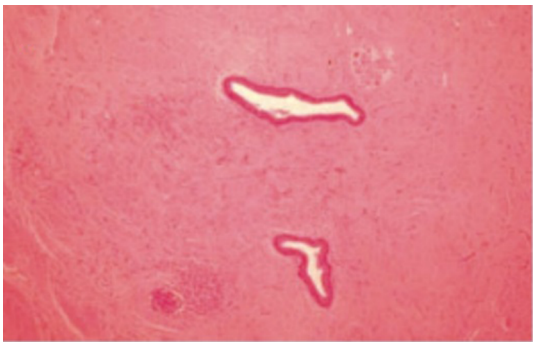

Figure 3 Urethral stricture tissue (100× magnification).

into more potent AngII by angiotensin-converting enzyme (ACE; Figure 4). AngII is the potent form that produces vasoconstriction of vascular wall or stimulates androgen production from adrenal cortex through angiotensin receptor.[7–11]

AngII receptor

AngII has 2 kind of receptors, AT_1 receptor and angiotensin II type 2 (AT_2) receptor. AT_1 is a G protein receptor that mediates several cascades, including phospholipase A, C and D activation, adenylate cyclase inhibition, tyrosine phosphorylation mediation and mediation of second messenger transduction, such as MAP kinase and phosphatidylinositol 3-kinase (PI3K).[10] Most of AT_1 mediate physiologic and pathophysiologic processes, including growth factors, vasoconstriction, aldosterone secretion and sympathetic outflow (Figure 5).[13–17]

The AT_1 distribution and location in the tissues have been widely studied on human tissues or animal studies. Dominantly, AT_1 has been found in the brain, adrenal glands, blood vessels and kidneys.[12,18,19,20]

Recent studies stated that there is expression of AT_1 in human prostate, which is concentrated in the periurethral area and stromal tissue. This finding showed that AngII has its role in cellular growth and sympathetic activity of the prostate and is related to voiding stream.[21]

AngII stimulates the AT_1 receptor in blood vessels that cause vasoconstriction. It also causes an increase in systemic vascular resistance. AT_1 receptor in cardiac cells was known for its chronotropic and inotropic functions. AngII was also known to stimulate proliferation of cardiac cells and fibroblasts and has role in the formation of various growth factors such as fibroblast growth factor, transforming growth factor and platelet growth factor.

AT_2 receptor is 30% homologous with AT_1 receptor, even though its relation with G protein is still controversial. The most common AT_2 expression was found in fetal tissue, and it downregulated after birth. AT_2 receptor was believed to have a counterregulatory mechanism to protect kidney from damage that was caused by AngII.[13,14,16,17,22,23]

Some clinical studies have showed the nonhemodynamic effects of RAS.[23] The involvement of AngII in fibrosis forma-

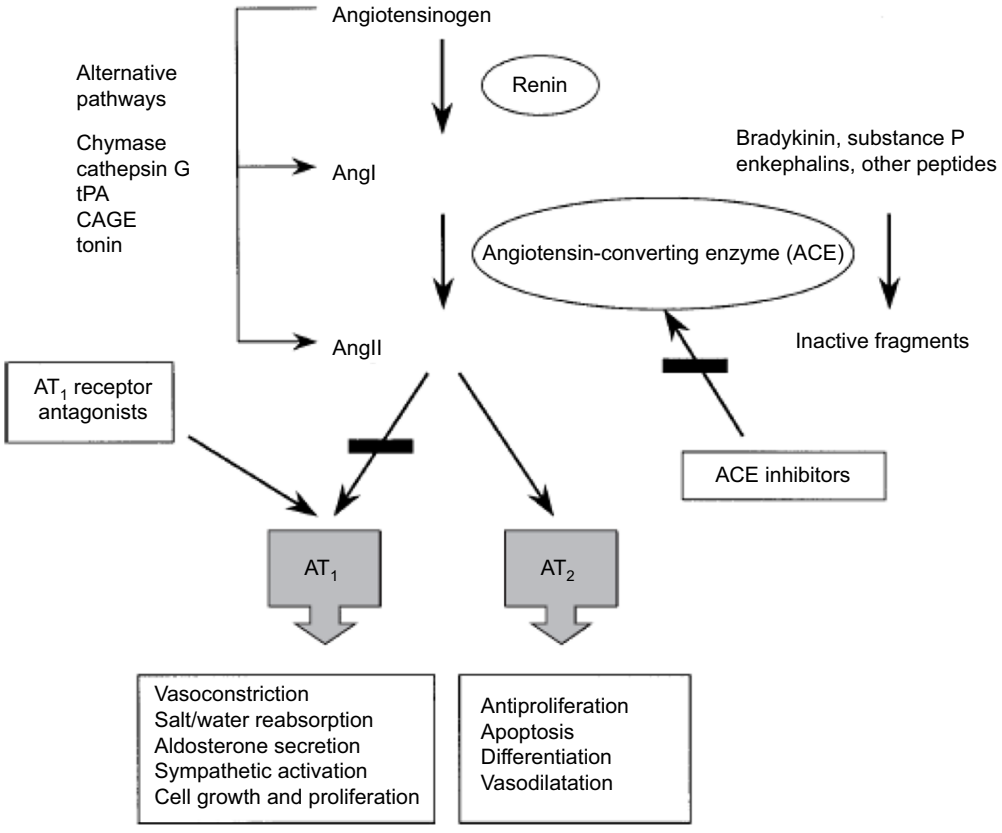

Figure 4 The RAS cascade.

Notes: ACE will convert AngI to more potent AngII. The AT_1 receptor in tissues has a role in modulation of vasoconstriction, sympathetic activation and including cell growth and proliferation.

Abbreviations: RAS, renin–angiotensin system; AngI, angiotensin I; AngII, angiotensin II; AT_1, angiotensin II type 1; AT_2, angiotensin II type 2; ACE, angiotensin converting enzyme; CAGE, chymostatin-sensitive angiotensin II converting enzyme; tPA, tissue plasminogen activator.

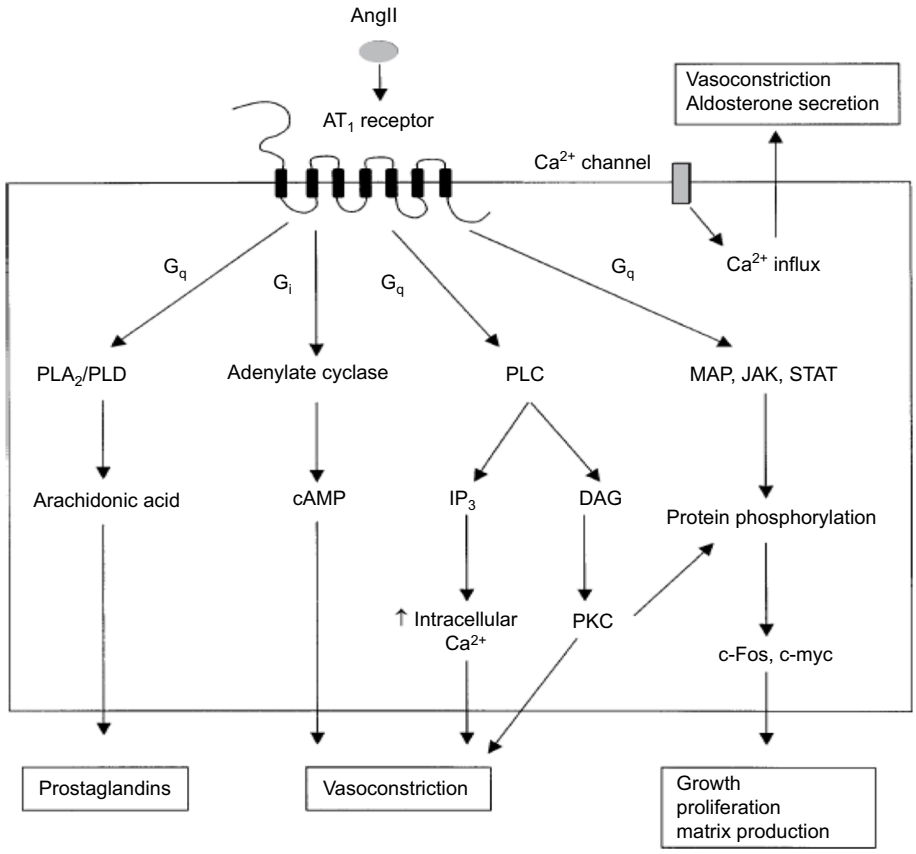

Figure 5 Signal transduction mechanism of AT$_1$ receptor.
Note: AngII through MAP, JAK and STAT pathway stimulates tissue growth, cell proliferation and extracellular matrix deposition.
Abbreviations: AT$_1$, angiotensin II type 1; AngII, angiotensin II; JAK, Janus kinase; STAT, signal transducer and activator of transcription; DAG, diacyl glycerol; cAMP, cyclic adenosine mono phosphate; PLC, phospholipase C.

tion process was seen with the overexpression of renin and angiotensinogen which synergically induce extracellular matrix formation. AngII induces collagen and fibronectin expression.[24] AngII also increases the proliferation of fibroblast and induces TGF-$\beta_1$expression.[25,26] Connective tissue growth factor (CTGF) presented as profibrotic cytokine and functioned as an important mediator of TGF-β_1 and as a profibrotic component that stimulates proliferation and activation of fibroblast and induces epithelial cell transition to become fibroblast.[27]

In some other studies, there were a decrease in CTGF expression and fibrotic process in samples that give ACE inhibitor and AT$_1$ blockers. Beside overexpression of extracellular matrix, disturbance of extracellular matrix degradation also leads to fibrosis process.[28] AngII induces fibrosis not only by increasing extracellular matrix synthesis but also by decreasing the turnover of extracellular matrix. AngII, through AT$_1$ receptor, induces PAI-1 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) to inhibit metalloproteinase to slow the degradation of extracellular matrix and produce fibrosis.[28]

In this study, we found a significant difference in AT$_1$ expression between urethral stricture tissue and normal urethra tissue. Despite some limitations in this study, such as lack of tissue samples and inequality between urethral stricture sample and normal urethra group, this study showed us another insight of fibrotic formation in urethral tissue and the probability of AT$_1$ receptor involvement in fibrosis formation in human urethral tissue.

Study limitation

The cadaveric urethral tissue in this study was taken from the dead body less than 24 hours and was fixated using formaldehyde. We recognize the limitation of this sample that decomposing process might affect the AT$_1$ expression in the urethral tissue. However, normal urethral tissue was impossible to collect unless we used cadaveric tissue. We excluded cadaver that decreased caused by urogenital cause, trauma or other conditions that may affect the urethra and collected the urethral tissue as soon as possible after the permission was granted from the ethical committee.

Disclosure

The authors report no conflicts of interest in this work.

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