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Olmesartan inhibits transdifferentiation of rabbit's valvular interstitial cells into myofibroblast based on α - smooth muscle actin expression

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Abstract--Recent studies revealed that differentiation of valvular interstitial cell (VIC) into myofibroblasts played an important role in valvular remodeling and fibrosis. Study was performed on in vitro cultured of VIC from New Zealand rabbit (*Oryctolagus cuniculus*). Isolated VIC was pretreated using 5 ng/mL of TGF- β 1 and divided into groups of olmesartan (100 nanomol/L) as short 30 minutes exposure duration and as continuous 72 hours exposure duration. Inhibition of myofibroblast differentiation was quantified by the expression of α -SMA levels detected by immunofluorescence staining. Olmesartan administration either, as short 30 minutes exposure duration or as continuous 72 hours exposure duration, were significantly reduced TGF- β 1-induced VIC differentiation into myofibroblast expressed by α -

SMA levels compared to control (short 30 minutes exposure duration: 9.18 ± 2.25 , as continuous 72 hours exposure duration: 10.13 ± 0.69 , and control 22.29 ± 2.78 ; $p < 0.001$), but without significant difference between the two treatment groups ($p = 0.403$). Olmesartan both on short and continuous exposure can inhibit the differentiation of valve interstitial cells into myofibroblasts based on the expression of α SMA, but without a significant difference in the inhibitory effect.

Keywords--valvular interstitial cell, myofibroblast, α -smooth muscle actin, olmesartan

Introduction

Rheumatic heart disease is terminal sequelae of valvulitis in acute rheumatic fever. Current treatment of long-term secondary antibiotic prophylaxis for rheumatic heart disease prevention, has a low compliance and unsatisfactory long-term outcome. Recent studies revealed that differentiation of valve interstitial cells (VICs) into myofibroblasts played an important role in valvular remodeling and fibrosis. VICs populated in all 3 layers of human valve; lamina fibrosa, lamina spongiosa, and lamina ventricularis, with the entire structure covered by valve endothelial cells (VECs).¹ Myocardial fibrosis is a multi-factorial process which includes the progressively increased transdifferentiation of cardiac fibroblasts into myofibroblasts. The phenotype of 'persistent myofibroblast' may lead to increased production of extracellular matrix (ECM), including collagen deposition, enhanced expression and translocation of α -smooth muscle actin (α -SMA) and transforming growth factor beta-1 (TGF- β 1) signalling activation.²

Angiotensin II (Ang II) plays a central role in the regulation of systemic blood pressure and fluid homeostasis. The action of Ang II is mediated by mainly two subtypes of receptors, angiotensin II type 1 (AT1) receptors and type 2 (AT2) receptors, which are distributed in many kinds of organs and tissues. Recently, several lines of evidence have suggested that the rennin-angiotensin system (RAS) plays an important role in the pathogenesis of organ fibrosis.³ In mesangial cells and other cell types, Ang II has been shown to promote the proliferation and collagen synthesis.⁴ Moreover, the expression of TGF- β 1, the key cytokine in the development of cardiac and renal fibrosis, is increased by Ang II. Blockade of the RAS by angiotensin-converting enzyme (ACE) inhibitors or by AT1 antagonists has been shown to improve the progression of organ fibrosis. In this study, we studied the effect of olmesartan as an antagonist of angiotensin II type 1 receptor (AT1) in the inhibition of SMAD dependent and MAPK/ERK pathway involved in VIC differentiation and attenuate valvular fibrosis.

Method

Cell Isolation and Culture

Valve endothelial cells (VICs) were isolated from the aortic valve leaflets of 12-13-week-old New Zealand rabbit (*Oryctolagus cuniculus*) by collagenase digestion as previously described.² Isolated valve leaflets were placed in Dulbecco's modified

Eagle's medium (DMEM, Fisher Scientific, Waltham, MA, USA) containing 100 U/mL of collagenase type II (Invitrogen, Carlsbad, CA, USA) at 37°C and agitated to remove valve endothelial cells (VECs). The reaction was stopped by the addition of heat-inactivated foetal bovine serum (FBS, Fisher Scientific). Cells were then washed and seeded onto six-well plates in DMEM supplemented with 100 mg/mL penicillin, 100 U/mL streptomycin (Fisher Scientific), and 10% heat-inactivated FBS culture media to adhere and grow to confluence for 5 – 7 days at 37°C in an atmosphere of 95% air and 5% CO₂. All experiments were performed with cells in passages 1-2. After the cells were 80% confluent, the medium was replaced by serum-free DMEM the day before and was treated with olmesartan (OLM) 100 nmol/l for 30 minutes as pre-treatment or OLM 100 nmol/l for 72 hours as continuous treatment, and exposed to TGF- β 1 5 ng/ml for 72 hours.

Immunofluorescence quantification and analyses

The VICs was immunostained to monitor phenotypic markers with vimentin. VICs that undergo phenotypic changes into myofibroblasts express the contractile protein, α -SMA. The expression levels of α -SMA were detected by immunofluorescence staining. VICs were treatment with OLM (100 nmol/l) as pre-treatment 30 minutes or as continuous treatment 72 hours, and stimulated by TGF- β 1 (5 ng/ml) for 72 hours, the cells were washed by PBS, fixed with 2% paraformaldehyde and permeabilized with 1% Triton X-100. α -SMA was detected by avidin biotinylated enzyme. Endogenous peroxide was quenched with 3% H₂O₂ after the cells were previously blocked with 2% BSA/PBS then incubated with rabbit monoclonal antibody against SMA (Sigma) for 60 minutes, followed by incubation with biotinylated secondary antibody (goat anti-mouse IgG; Sigma) and 20 minutes with extra Avidin-conjugated peroxidase (Sigma). Cells were then rinsed with PBS three times for 5 min after each incubation phase with antibodies. The cells were visualized under fluorescence microscope. Fluorescence intensity of cells was examined and quantified by the specialized measurement incorporated within imaging software. Fluorescence intensities could be compared between regions of the same set of stress fibres and between cells of different treatments. The data were analysed by the mean fluorescence intensities from 10 random selected regions, each with a standard 50- μ m² circular region.

Statistical analysis

Data are expressed as mean $\pm$ SD. After confirming that all variables were normally distributed using the Kolmogorov-Smirnov test, statistical differences between groups were evaluated by ANOVA followed by the post hoc Games-Howell test. $p < 0.05$ was accepted as statistically significant.

Results

Isolation and Expression of Valve Interstitial Cell Markers

The characterization of valve interstitial cells was carried out with positive markers of vimentin through immunocytochemical examination. Immunocytochemical analysis showed that there was a positive expression (luminescence above 95% of cell luminescence per field of view) of vimentin.

Immunocytochemical staining results showed positive markers for vimentin which is a marker of valve interstitial cells.

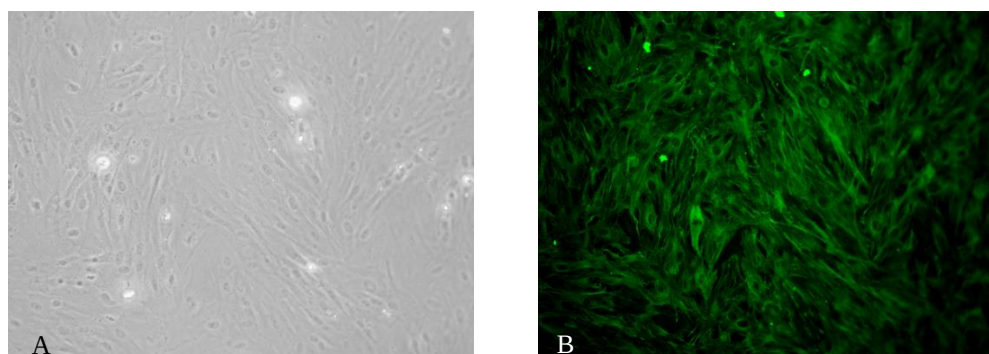


Figure 1. Valve Interstitial Cells (VICs) at 10 x 10 magnification (A) phase-contrast microscopy image; (B) fluorescent labelling with vimentin

Myofibroblast Marker Expression

VICs transform into myofibroblasts after being exposed to TGF- β 1. Myofibroblasts have characteristics of irregular surfaces and the cytoplasm was filled with stress fibers. Immunocytochemical staining after exposure to TGF- β 1 showed a significant increase in α -SMA expression (fluorescence above 95% luminescence of cells per field of view) when compared to controls. This indicates the process of differentiation of valve interstitial cells into myofibroblasts.

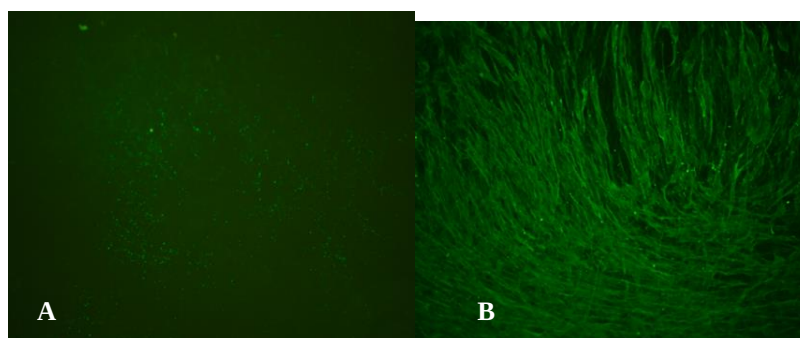


Figure 2. Myofibroblast cell morphology and expression of α -SMA on immunocytochemistry (A) control group; (B) with TGF- β 1 exposure.

Olmesartan inhibit TGF- β 1-induced myofibroblasts transdifferentiation

Olmesartan as an antagonist of angiotensin II type 1 receptor (AT1) was known for the ability to inhibit SMAD dependent and MAPK/ERK pathway involved in VIC differentiation and attenuate valvular fibrosis. In this study, olmesartan administration either, as short 30 minutes exposure duration or as continuous 72 hours exposure duration, were significantly reduced TGF- β 1-induced VICs differentiation into myofibroblasts expressed by α -SMA levels compared to control.

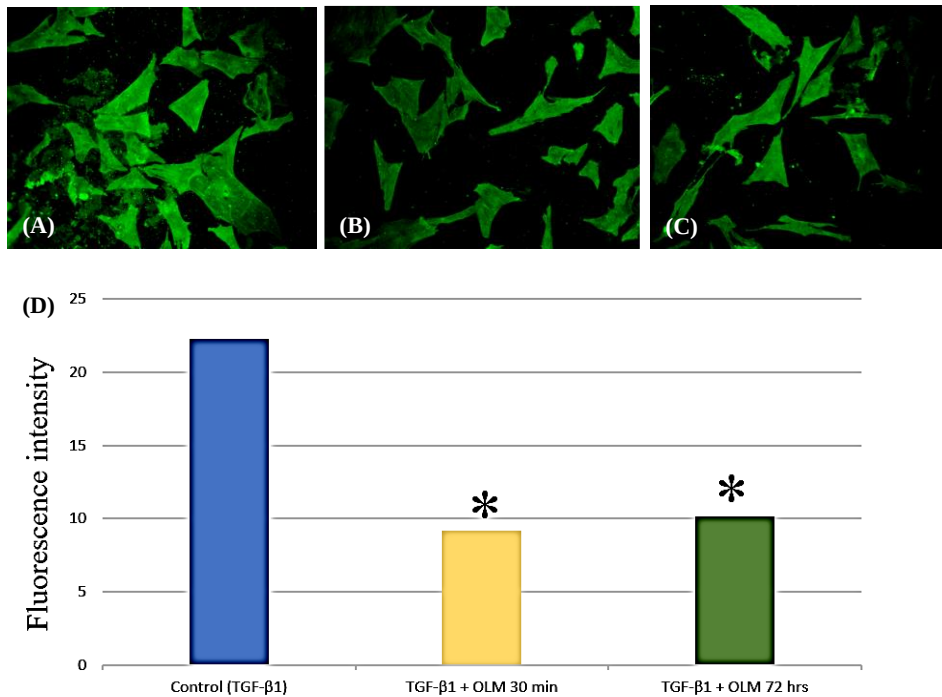


Figure 3. Myofibroblast were detected by the expression of α -SMA immunofluorescence from different treated group (A) control group; (B) olmesartan as pre-treatment 30 minutes; (C) olmesartan as continuous 72 hours treatment. (D) quantification of mean fluorescence intensity of stress fibers in myofibroblasts with α -SMA staining. *indicates $p < 0.05$ versus TGF- β 1-induced group

Discussion

This study used valve interstitial cells with vimentin labelling isolated from the rabbit valve. Exposure of valve interstitial cells to the cytokine TGF- β 1 caused the differentiation of cells into myofibroblasts which was characterized by a significantly increased expression of α -SMA when compared to valve interstitial cells that were not exposed to TGF- β 1. TGF- β 1 can activate the small-mothers-against-decapentaplegic (Smad) transduction pathway⁵ and initiate transcription of myofibroblastic pro-differentiation genes.⁶ TGF- β 1 has been shown to play a role as a key profibrogenic cytokine in in vitro and in vivo studies,⁷⁻¹⁰ as well as an increase in TGF- β 1 levels in the fibrotic heart.¹¹⁻¹³

The process of fibrogenesis in valve interstitial cells also involves the molecule angiotensin II which is produced by endothelial cells surrounding inflammatory cells, macrophages, and paracrinely by valve interstitial cells in response to inflammation. Through its binding to the angiotensin II type 1 receptor (AT1R), it causes activation of the Janus Kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT) pathway, Ras-MAPK, and the Smad 2-3-4 pathway. These

three pathways have the effect of amplifying the inflammatory response and fibrogenesis that have been triggered by TGF- β 1.

Olmesartan as one of the AT1R inhibitor was postulated to have therapeutic effects on inflammatory conditions. Nishio et al. [29] previously demonstrated that olmesartan could improve the LV hypertrophy, fibrosis and diastolic dysfunction though attenuation of inflammatory response in a mouse model of diastolic heart failure. Studies in rabbits that induced valve fibrogenesis by giving 1% high cholesterol diet for a week, showed that olmesartan significantly decreased macrophage accumulation and osteopontin expression. In the valves of rabbits that received a high-cholesterol diet, there was a significant increase in the levels of angiotensin converting enzyme and SMA which were statistically significantly different compared to the group of rabbits that received olmesartan 1mg/kg/day. This suggests the potential of olmesartan in maintaining endothelial integrity and inhibiting the differentiation of valve fibroblasts into myofibroblasts.¹⁴

The results of this study showed that olmesartan, both as pre-treatment 30 minutes or as 72 hours continuous treatment, exerted an inhibitory effect on the differentiation of rabbit valve interstitial cells induced by TGF- β 1 into myofibroblasts based on α -SMA expression compared to controls. But there was no significant difference in the inhibitory effect between olmesartan as pre-treatment 30 minutes compared to 72 hours continuous treatment on the differentiation of valve interstitial cells into myofibroblasts. The inhibitory effect of olmesartan, although only given as a pre-treatment with a short exposure of 30 minutes, still provided a stable inhibitory effect until the end of the 72-hour incubation period. This is possible because the inverse agonism effect of olmesartan causes changes in the structure of the AT1 receptor to become inactive and the condition of this inactive receptor remains stable during the incubation period of 72 hours, although at the same time there is still TGF- β 1 continuous exposure.¹⁵

Conclusion

Olmesartan, both as pre-treatment 30 minutes or as 72 hours continuous treatment, exerted an inhibitory effect on the differentiation of rabbit valve interstitial cells induced by TGF- β 1 into myofibroblasts based on α -SMA expression compared to controls.

Acknowledgments

Not applicable.

Conflict of interest

None declared.

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