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LABORATORY STUDY

Aldosterone Blockage in Proliferative Glomerulonephritis Prevents Not Only Fibrosis, but Proliferation as Well

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Studies performed recently have determined that aldosterone has not only a major role in electrolyte and water balance and K excretion, but it also modulates myofibroblast growth in the heart and blood vessels and causes fibrosis. This study investigated the effects of aldosterone blockers in rats with anti-thy 1.1 nephritis, both on proliferation and fibrosis, by comparing it to an angiotensin receptor inhibitor valsartan. Rats with anti-thy 1.1 nephritis were randomly allocated to one of the three following groups of treatment: the control group (group 1); those treated with the aldosterone receptor blocker spironolactone (group 2); and those treated with the ATRB valsartan (group 3). On day 7, the parameters of glomerular fibrosis [transforming growth factor beta, TGF staining areas %], proliferation (Ki-67), and renal damage scores were determined. The TGF-β and Ki-67 levels of control group were significantly more than the other two groups ($p<0.01$). The TGF staining areas percentages were significantly decreased compared to control group. The artery, glomerular, and renal injury scores evaluated between the groups were found to be significantly decreased compared to control group. In line with previous studies, this study found that in anti–thy 1.1 mesangioproliferative glomerulonephritis, aldosterone blockage affected proliferation and fibrosis.

Keywords TGF beta, anti-thy 1.1, Ki-67, aldosterone antagonism, AT receptor blocker, glomerulosclerosis

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INTRODUCTION

End-stage renal disease is an important public health problem with increasing rates of incidence and prevalence.[1] With these increases, many have thought that the current therapeutic approaches are not adequate and that new therapeutic interventions are needed. The key therapeutic goal in patients with renal parenchymal loss is to prevent renal fibrosis and glomerulosclerosis—in other words, renal remodeling. The best available treatment method may be the blockage of various neurohumoral pathways. Although the role of angiotensin is documented clearly in progressive renal disease, the results of recent studies indicate that aldosterone also can be an important pathogenic factor. Aldosterone is the main mineralocorticoid released from the adrenal cortex and has a major role in electrolyte transport via epithelial mineralocorticoid receptors. It has recently been shown to be synthesized from the endothelium, heart, vascular smooth muscle, and brain.[2–4] The current studies determined that aldosterone not only has a major role in electrolyte and water balance and K excretion, but it also modulates myofibroblast growth in the heart and blood vessels and causes fibrosis by stimulating the plasminogen activator inhibitor-1 (PAI-1), transforming growth factor beta (TGF β), and reactive oxygen species.[5–7]

According to the traditional view, angiotensin II is the leading actor in RAAS system. However, it has recently been accepted that aldosterone also has a role in this system and contributes to the detrimental effects of angiotensin. This is an important issue because the inhibitory effect of ACE inhibitors on aldosterone levels is weak,

variable, and inconstant in heart failure. In many chronic heart failure (CHF) patients treated chronically by ACE inhibitors and AT II receptor blockers, aldosterone and AT II levels are increased.[8–10] The clinical importance of this is that even in the presence of ACE inhibitors and AT II receptor blockers, aldosterone escape can not be prevented, something that can cause major health problems. The determination of the effects of aldosterone in nonepithelial tissues like the brain and heart, as well as the introduction of the cardiac remodeling effect of antialdosterone treatment (which was shown by RALES and EPHESUS studies) to the heart failure guidelines, led researchers to believe that the inhibition of aldosterone can also be effective in nephropathies.[11,12] Preclinical evidence supports the relationship between hypertensive nephropathy and vascular remodeling and aldosterone.

Greene at al. first demonstrated that plasma aldosterone concentration was markedly elevated in the remnant kidney model. They went on to show that the angiotensin receptor blocker (ARB) losartan and the angiotensin-converting enzyme (ACE) inhibitor enalapril reduced renal injury. When rats received an aldosterone infusion to maintain very high plasma aldosterone levels, however, losartan and enalapril were not effective in preventing or reversing renal injury. These results further support an independent pathogenetic role for aldosterone as a mediator of progressive renal disease.[13] In monitoring the effect of aldosterone inhibition on the progression of renovascular damage in hypertensive rats, it was observed that proteinuria was decreased and survival was increased.[14]

This study sought to investigate the effects of aldosterone blockers in rats with anti-thy 1.1-induced mesangioproliferative glomerulonephritis, both on proliferation and fibrosis, by comparing it to an angiotensin receptor inhibitor valsartan.

METHOD

Animals

Eighteen Sprague Dawley rats weighing 150–200 g were used in this study. Rats were kept in metabolic chambers in a room with 12:12 h light-dark cycle and with an ambient temperature of 20 ± 1°C. Standard rat chow and tap water were available ad libitum. Experiments were performed in accordance to the guidelines of our local ethical committee.

Protocol

Anti-thy 1.1 nephritis was induced through the intravenous injection of 20mg/100mg body weight OX7, a monoclonal antibody against the thy1.1-like antigen on the surface of rat mesangial cells (Probetex, Inc., Experimental Pathology Laboratories, San Antonio, Texas, USA). One day after induction of anti-thy1 glomerulonephritis, rats were randomly allocated to one of three groups of treatment: the control group (group 1, n: 6), those treated with the aldosterone receptor blocker spironolactone (group 2, 20 mg/kg/day by gavage), and those treated with the ATRB valsartan (group 3, 20 mg/kg/day by gavage).[15,16] Each rat was anesthetized with ketamine (25mg/kg) and sacrificed on day 7 of the experiment. Blood was collected for biochemical evaluation during this procedure. Urinary protein concentration was determined by the sulfosalicylic acid turbidity method. Plasma aldosterone concentration was determined by radioimmunoassay.

Immunohistochemistry

TGF β and Ki-67 Immunostaining

For Ki-67 and TGF β, the following immunohistochemical staining procedure was completed. The slides were kept in 3% hydrogen peroxide solution in methanol for 30 minutes for blocking the endogenous peroxidase activity. Afterwards, the slides were sunk in 500 ml of 0.01 M sodium citrate buffer (pH = 6) and exposed to microwave for 20 minutes. They were washed with a phosphate-buffered saline solution (pH = 7.4) prior to immunohistochemical staining. They were then incubated at room temperature with a monoclonal antibody solution for 1 hour (1/100 dilution, rabbit polyclonal antibody Ki-67 antigen [RB-15R7], Lab Vision Corporation, Fremont, California, USA, and Mouse monoclonal antibody TGF β (NCL-TGFB) Novocastra, Newcastle, United Kingdom). The presence of immunoperoxidase was inspected using an avidin-biotin complex method and diaminobenzidine substrate. Counter staining was accomplished with hematoxylin-eosin. Ki-67 positive cells were distinguished by the brown stained nuclei, and the TGF β positive area was distinguished by the brown stain.

Renal Histopathology

Following immunohistochemical staining, tissue cell counts were performed by an A-Plan objective Carl Zeiss microscope with a magnification of 40/0.65×10. Histological examination was conducted in a blinded fashion, and glomerular and arteriolar injury scores were calculated as described previously.[17–19] Glomerular injury score (GIS) was graded from 0 to 3+, in which 0 was no injury, 1+ was injury of up to one-third (≤1/3) of the

glomerulus, 2+ was one-third to two-thirds glomerular injury, and 3+ was an injury of more than two-thirds (≥2/3) of glomerular involvement. The arteriolar injury score (AIS) was also graded from 0 to 3+, in which 0 was no injury at all, 1+ demonstrated hyalinosis of the arteriolar wall up to 50% of the mural circumference, 2+ demonstrated hyalinosis between 50% and 100% of the wall circumference but without luminal narrowing, and 3+ was complete mural hyalinosis with luminal encroachment. The overall nephrosclerosis score was calculated by adding the GIS and AIS for each rat. These scores were obtained by independent study by two investigators, and scoring of all tissue was conducted in a blinded manner. The tubulointerstitial damage score were graded as follows: grade 0, no change; grade I, lesion affecting less than 25%; grade II, lesion involving 25–50%; grade III, lesion involving more than 50%; and grade IV, involving the whole area.

Nucleus counting for Ki-67 was also performed, beginning with the region with the densest Ki-67 coloring. In all cases with mesangio proliferative glomerulonephritis MMPGN), the cells of glomerular capillary were counted without endocapillary or extracapillary separation, and tubular cells were not included to the count. The signed nuclei were counted regardless of the intensity of dyeing. On each sample, 1000 cells were counted, and the number of cells dyed with Ki-67 was recorded. The pictures of samples dyed with Ki-67 are shown in Figure 1.

For TGF β images, at least 20 systematically sampled glomeruli in each animal were digitized from the light microscope with a video camera and a computer-based image analysis system (Scion image, Frederick, Maryland, USA). Glomerular cross-sections were displayed on the computer screen. The mean TGF β staining areas were measured by using an image analyzer scion image for each animal. Immunostaining for TGF β was assessed with a semiquantitative score as follows: 0, little or no positive staining; 1+, <25% of total glomerular area stained; and 2+, 3+, and 4+: 25–50, 50–75, and 75–100% positive staining of glomerular area of at least 20 cortical glomeruli, respectively.

Statistical Analysis

Results are expressed as mean ± standard deviation. Student's t-test was performed to compare the three groups. The correlations among biochemical and pathological parameters in all rats were determined using linear regression analysis. A p value less than 0.05 was considered statistically significant.

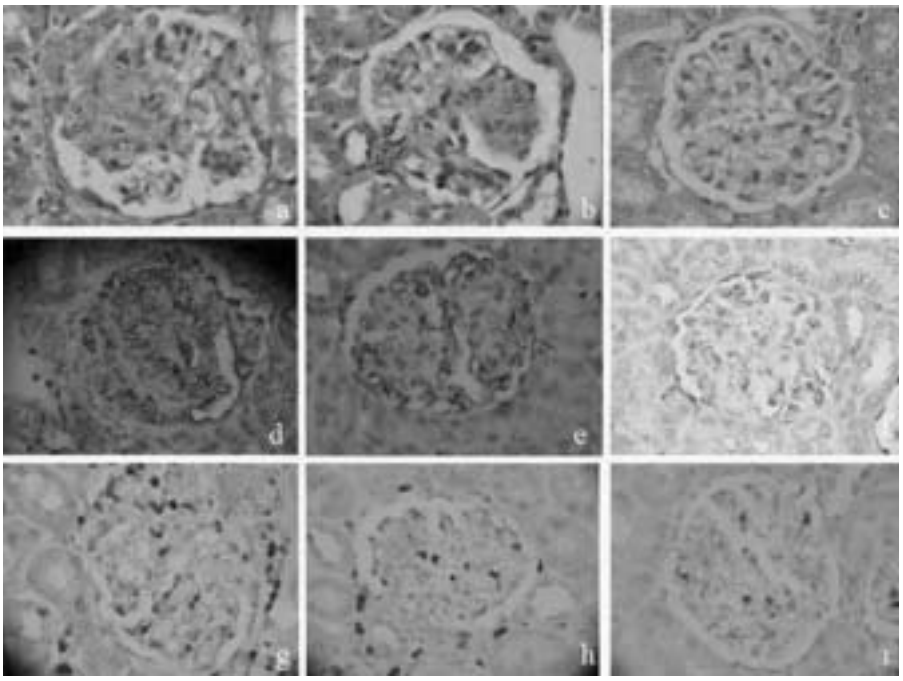

Figure 1. TGF β, Ki-67, and Hematoxylin-eosin glomerular immunostaining: a) group control hematoxylin-eosin staining, b) group valsartan hematoxylin-eosin staining, c) group spironolactone hematoxylin-eosin staining, d) group control TGF staining, e) group valsartan TGF staining, f) group spironolactone TGF staining, g) group control Ki-67 staining, h) group valsartan Ki-67 staining, i) group spironolactone Ki-67 staining.

RESULTS

There were no differences between the body weights of the rats before the experiment. Mesangioproliferative glomerulonephritis was formed by anti-thy 1.1 in all rats. The TGF β levels of the control group was significantly more than the other two groups ($p<0.01$). Ki-67 levels were significantly lower in both the spironolactone and valsartan groups than the control group ($p<0.01$). The TGF staining areas calculated by a computer-based image analysis system was significantly less than the control group. The artery and glomerular injury score evaluated between groups were found to be significantly decreased compared to control group ($p<0.05$). However, there were no significant differences in tubular injury scores ($p>0.05$). On the other hand, renal injury scores of both groups were significantly decreased compared to controls. When spironolactone and valsartan groups were compared, Ki-67 levels were significantly decreased in favor of spironolactone. Although TGF β levels were also decreased in the spironolactone group, the difference did not reach significance. There were no differences between renal injury scores. Aldosterone increased significantly in spironolactone–treated rats as compared with valsartan and control groups. Spironolactone and valsartan groups prevented the development of proteinuria compared with the control (see Table I).

When potassium levels were evaluated, there were mild increases in both groups compared to the control group; however, this difference was not significant. There were no differences between groups regarding creatinine levels.

DISCUSSION

Recent studies show that besides its classical effects, such as electrolyte transport, aldosterone also has a role in different phases of renal vascular remodeling.[20] The authors evaluated TGF-β, a marker of fibrosis, and Ki-67, a marker of proliferation, in this study to investigate the effect of aldosterone on fibrosis and proliferation. TGF-β is a cytokine that has roles in the initiation of fibroblast differentiation and proliferation, the up-regulation of synthesis and storage of collagen, and the matrix metalloprotein synthesis and down-regulation. It has an important place among cytokines that have roles in fibrogenesis. TGF-β release increases within the vascular smooth cells with the stimulation of angiotensin II, hypoxia, high glucose levels, autoantibodies, platelet derive growth factor β (PDGF-β), and basic fibroblast growth factor (bFGF).[21] Sun et al. showed that in rats with one kidney removed, aldosterone infusion increased ACE and AT II receptor density and TGF β expression, and renal cortical fibrosis developed in which collagen accumulation was seen.[22] Especially in the group that was infused aldosterone, having these changes despite the angiotensin receptor blockage shows that aldosterone has an effect on fibrosis independent from the renin-angiotensin system-related TGF β release.

The immunohistochemical evaluation of cell proliferation depends on the measurement of the amounts and distribution of several cellular proteins, such as Ki-67, DNA polymerase delta, and P125/65, in the cell cycle, proliferative cell nuclear antigen (PCNA). Ki-67, developed by Gerds et al., is a monoclonal antibody found only in proliferating cells and reacts with an unknown epitope on nuclear

Table 1
Biochemical and immunohistochemical values of groups

	Group 1 (CON)	Group 2 (SPRO)	Group 3 (VAL)
Cre(mg/dL)	0.42	0.45	0.42
K (mg/dL)	3.9±0.6	4.2±0.3	4.5±0.3
Ki-67 (positive nuclei/1000cell)	258.1±18.7	167.1±12.0*,‡	198.8±19.3*
TGF scores	2.6±0.5	1.3±0.5*	1.8±0.4*
TGF staining areas %	36.6±2.5	27.9±5.5†	26.6±6.3†
Glomerular injury scores	2.3±0.5	1.0±0.8†	1.5±0.5†
Arteriolar injury scores	1.3±0.5	0.3±0.5†	0.6±0.5†
Tubulointerstitial injury scores	1.6±0.5	1.1±0.7	1.3±0.8
Total renal damage scores	5.3±1.0	2.5±2.0†	3.5±1.3†
Proteinuria (mg/d)	110±13.7	55.6±16.1*	62.5±7.5*
Plasma aldosterone(ng/dL)	23.3±16.1§	81.8±23.3	38.6±25.1§

*$p<0.01$ versus control rats.
†$p<0.05$ versus control rats.
‡$p<0.05$ versus valsartan groups.
§$p<0.05$ versus spironolactone groups.

antigens.[23] As it is found in all active phases of the cell cycle, it is an ideal marker to show the growth fraction for a cell population. Although it can be ubiquitously found in all cell types during proliferation, its functional role in cell proliferation is still unknown.[24] It has been shown that cellular proliferation is determined by Ki-67 in routine renal biopsies. Clinically, Ki-67 is shown to be increased with tubular proliferation, as a marker of subclinical tubular damage, without the presence of renal function disorder.[25]

Angiotensin II also shows effects that are independent from its hemodynamic ones, such as an increase in renal cell proliferation, which triggers hypertrophy in tubular cells, the accumulation of extracellular matrix, the activation of macrophages, and the stimulation of phagocytosis. Both clinical and preclinical studies indicate the inhibition of RAAS in hypertensive cases regress renal damage. ACE inhibitors and AT receptor blockers are shown to slow the progress of glomerulosclerosis and decrease proteinuria in patients with or without diabetes by randomized clinical trials.[26–29] AASK, HOPE, RENAAL, and IRMA-IDNT studies clearly showed ACE inhibitors and AT receptor blockers slow down the renal progression.[30–33] The blockade of the RAAS with ACE inhibition or angiotensin receptor blockade often does not confer optimal protection from the effects of mineralocorticoids on small- and medium-sized blood vessels. Recent preliminary data from clinical studies indicate that aldosterone blockade protects the kidneys, sharply decreases proteinuria beyond the activities of ACE inhibition or angiotensin receptor blockade and independent of beneficial blood pressure effects, and can protect patients from vascular injury associated with diabetes mellitus and hypertension.[21] The evidence from many different laboratory animal models shows that aldosterone has a major role in the initiation of fibrogenesis and proliferation, persistence of hypertension, and renal damage. Horiuchi et al. found that while aldosterone levels in ten-week old normotensive rats were normal, they were significantly high in spontaneously hypertensive rats.[34] Similar to animals with chronic renal failure, aldosterone levels were found to be high in patients with renal failure. It has been shown in the group with a 50% decrease in creatinine clearance that aldosterone levels were significantly high regardless of renin and potassium levels. In a subsequent study, Rocha et al. studied the effects of chronic aldosterone infusion on vascular lesions in animals treated with captopril. Animals were divided into four groups—vehicle alone, captopril alone, aldosterone alone, and combined dosing with captopril plus aldosterone at two different dose levels. All animals had significantly elevated blood pressure. Animals in the control and aldosterone-infusion groups experienced marked proteinuria and similar degrees of renal injury. Conversely, captopril treatment reduced endogenous aldosterone levels and prevented the development of proteinuria and glomerular and renal vascular lesions. However, subsequent aldosterone infusion reversed the ability of captopril to confer this protection. The aldosterone-infused captopril-treated rats showed proteinuria and renal vascular and glomerular lesions despite ACE inhibitor treatment. Systolic blood pressure in captopril-treated SHSRPs administered aldosterone infusion was not significantly different than in SHSRPs treated with captopril alone. In this model, there is a clear role for aldosterone in the pathogenesis of vascular injury, and aldosterone-related injury is independent of the effects on blood pressure.[14] Clinical studies also have similar results. Aldosterone blockage diminished proteinuria both in diabetic nephropathy and chronic renal failure.[35]

Similar to previous studies, it was found that in anti-thy1.1 mesangioproliferative glomerulonephritis, aldosterone blockage affected the proliferation and immunohistopathological changes of fibrosis. There was a significant decrease in fibroproliferation in the valsartan group compared to the control group. Interestingly, although not statistically significant, fibrosis and proliferation were less in the spironolactone group than valsartan group. There was no difference between groups regarding creatinine and potassium levels. Similar to other preclinical and clinical evidence, this study also showed that aldosterone is a strong and independent mediator of renal vascular remodeling. Moreover, in addition to aldosterone's antagonism protector effect on fibrosis, the authors also showed that it can also contribute to renoprotection by inhibiting proliferation as much as AT II receptor blockers. If the aldosterone escape during chronic ACE inhibitors or AT receptor blocker treatments is considered, in order to have renoprotection, aldosterone blockage combination with these treatments may be considered in the future.

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