Effect of angiotensin II inhibition on the epithelial to mesenchymal transition in developing rat kidney

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= Abstract =

Purpose: To investigate the effects of angiotensin II inhibition on the epithelial to mesenchymal transition (EMT) in the developing kidney, we tested the expression of EMT markers and nestin in angiotensin converting enzyme (ACE) inhibitor-treated kidneys.

Methods: Newborn rat pups were treated with enalapril (30 mg/kg/d) or a vehicle for 7 days. Immunohistochemistry for the expression of α-smooth muscle actin (SMA), E-cadherin, vimentin, and nestin were performed. The number of positively-stained cells was determined under 100 magnification in 10 random fields.

Results: In the enalapril-treated group, αSMA-positive cells were strongly expressed in the dilated tubular epithelial cells. The number of αSMA-positive cells in the enalapril-treated group increased in both the renal cortex and medulla, compared to the control group (P<0.05). The expression of E-cadherin-positive cells was dramatically reduced in the cortical and medullary tubular epithelial cells in the enalapril-treated group (P<0.05). The number of vimentin- and nestin-positive cells in the cortex was not different in comparisons between the two groups; however, their expression increased in the medullary tubulointerstitial cells in the enalapril-treated group (P<0.05).

Conclusion: Our results show that ACE inhibition in the developing kidney increases the renal EMT by up-regulating αSMA and down-regulating E-cadherin. Enalapril treatment was associated with increased expression of vimentin and nestin in the renal medulla, suggesting that renal medullary changes during the EMT might be more prominent, and ACE inhibition might differentially modulate the expression of EMT markers in the developing rat kidney. (Korean J Pediatr 2009;52:944-952)

Key Words: Angiotensin II, Cell Transdifferentiation, Growth and Development, Kidney Diseases

Introduction

Dynamic cellular transitions, switching between the epithelial and mesenchymal states, are pivotal events during embryogenesis and organogenesis. The epithelial to mesenchymal transition (EMT) and the mesenchymal to epithelial transition (MET) occur at multiple occasions during development, tumor progression and organ fibrosis. Over the past several years, there has been substantial progress in demonstrating the significance of the EMT in chronic kidney disease. The enhanced conversion of the renal tubular epithelial cells to myofibroblasts/fibroblasts via the EMT leads to the disruption of polarized renal tubular epithelial layers and an increase in fibrotic scar formation.

Of note, is that the conversion of the metanephric mesenchyme into epithelium via the MET is a fundamental process during kidney development. Reciprocal inductive interactions between the metanephric mesenchyme and ureteric bud transform the ureteric bud into the renal collecting system; this occurs while the metanephric mesenchyme condenses and subsequently undergoes MET to give rise to nephrons. In this context, the renal EMT may be characterized as a process of reverse embryogenesis. There is a great deal of experimental data on the modulation of the EMT, as a novel therapeutic target, in the setting of chronic renal fibrosis.

A substantial number of EMT inducers and mediators have been studied and among them, αSMA, E-cadherin and vimentin...
have been identified as markers of both the EMT and the MET. During the EMT associated with renal fibrosis, tubular epithelial cells lose the epithelial cell marker E-cadherin and acquire mesenchymal features, characterized by αSMA and vimentin markers. Cadherin-mediated adhesion is a vital component for determining and maintaining the epithelial phenotype; the role that E-cadherin plays in modulating the epithelial phenotype and expression patterns during kidney development is of great interest.

Nestin is considered a marker of neurogenic and myogenic precursor cells; however, relatively little is known about its role in the kidney. In the developing kidney, nestin has been detected in immature glomerular podocytes and proximal tubular epithelial cells. Nestin has been shown to be associated with vimentin in vivo and in vitro studies, suggesting the possibility of nestin expression in vimentin-positive cells of injured kidneys. In the anti-Thy1 nephritis experimental model, most mesangial cells have been shown to express nestin associated with the activation marker αSMA and the proliferation marker.

The renin–angiotensin system (RAS) has been implicated as a potent mitogen for the regulation of genes involved in cell proliferation and growth of the developing kidney; in addition, it is a major factor involved in the development of renal disease. We and others have demonstrated that the neonatal interruption of angiotensin II induces renal growth impairment that is linked to renal papillary atrophy and tubulointerstitial damage; these abnormalities are characterized by tubular dilatation/atrophy and interstitial inflammation/fibrosis. Given the fact that abnormal renal development is linked to subsequent susceptibility to cardiovascular disease in adulthood, and RAS is a candidate for perinatal programming, the RAS–mediation of renal development is an important focus of study.

The neonatal rat is a useful model for kidney developmental studies. Rats are born with immature kidneys and they undergo considerable postnatal renal development. The developing rat kidney is dependent on an intact RAS from gestational day 15 to postnatal day 13. All components of the RAS are crucially expressed in the developing kidney in a spatial and temporal pattern. In the present study, we investigated the effects of angiotensin (Ang) II inhibition on the EMT in the developing rat kidney. We examined the expression of EMT markers and nestin in angiotensin converting enzyme (ACE) inhibitor–treated rat kidneys.

### Materials and Methods

Neonatal rat pups from pregnant Sprague Dawley rats were breastfed by their mothers throughout the study. They were treated with 30 mg/kg of enalapril (the enalapril–treated group, n=22) or vehicle (the control group, n=16) via an orogastric tube daily from birth. This dose of enalapril is known to block the effects of Ang II. The rats were sacrificed at eight days of age (N8), and their kidneys were processed for the study. All rats were used in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals and by guidelines established by the Animal Care Committee of the Korea University Guro Hospital.

1. Immunohistochemical staining

For assessing expression, five kidneys in each group were selected for representative immunohistochemical staining of αSMA, E-cadherin, vimentin and nestin using an avidin–biotin immunoperoxidase method (Vectastain ABC kit, Burlingame, CA, USA). The harvested kidneys were treated in 10% formalin solution (Sigma Chemical Co., St. Louis, U.S.A.) and embedded in paraffin. The samples were then cut into 4-μm sections and dried and placed onto siliconized slides (Muto–Glass, Japan). The paraffin sections were deparaffinized with xylene, followed by rehydration in a descending series of ethanols. Then, the endogenous peroxidase activity was quenched in 0.6% hydrogen peroxide for 15 min. Antigen retrieval was performed with 0.1% citric acid (DAKO Co., CA, USA). The following staining and immunohistochemical techniques were used.

2. Identification of EMT markers and nestin

After quenching and antigen retrieval, the sections were incubated with primary antibodies against αSMA (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:800), E-cadherin (Santa Cruz Biotechnology; dilution 1:400), vimentin (Santa Cruz Biotechnology; dilution 1:400) and nestin (Santa Cruz Biotechnology; dilution 1:200). As negative controls, the primary antibody was substituted with PBS. The incubation time was overnight at 4°C. After incubation, the sections were washed twice in PBS for 5 min., and incubated for 30 min with secondary antibodies [peroxidase–conjugated anti-rabbit IgG (Vectastain ABC kit, Burlingame, CA, USA; dilution 1:200)]. Then, the slides were washed in PBS, and incubated for 50 min with the Vectastain ABC reagent. The immuno-
reaction products were developed using 3, 3-diaminobenzidine as the chromogen, at the standard development times. The sections were counterstained in 0.5% methyl green solution (Trevigen, Gaithersburg, MD, USA) for 5 min, dehydrated, and evaluated using light microscopy (×400). The number of positively-stained cells was determined under 100 magnification in 10 random fields.

3. Statistical analysis

Data are presented as the mean±SEM. Differences between the groups were analyzed by the Student t-test. Statistical significance was defined as a P<0.05. The SigmaStat version 2.0 for Windows was used for the analysis.

Results

1. Expression of αSMA

For the immunohistochemical staining, αSMA was more intensely expressed in the medullary and cortical dilated tubular cells in the enalapril-treated group, compared to the controls (Fig. 1A-F). In the control group, αSMA was weakly detected only in the vascular smooth muscle cells and in some interstitial cells; there was scant expression of αSMA in the tubular epithelial cells and glomeruli (Fig. 1A, C and E). In the enalapril-treated group, αSMA was observed to be highly expressed in the dilated tubular epithelial cells (Fig. 1B, D and F). The number of αSMA-positive cells significantly increased in both the renal cortex and medulla in the enalapril-treated group (P<0.05) (Fig. 1G and H).

2. Expression of E-cadherin

In the control group, E-cadherin was intensely expressed in the developing medullary and cortical regions and in the maturing tubular epithelial cells, compared to the enalapril-treated group (Fig. 2A–F). The immunohistochemical staining showed that E-cadherin expression was easily

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Fig. 1. Immunohistological examination of αSMA in the rat newborn control kidneys (A, C, E) and in the enalapril–treated kidneys (B, D, F). Low magnification of the kidney shows that αSMA is more intensely detected in the medullary and cortical cells in the enalapril–treated group, compared to the controls (A, B). αSMA is faintly observed only in the vascular smooth muscle cells and some interstitial cells in the control kidneys (C, E) (arrows); however, αSMA was more strongly stained in the renal cortex and medulla, especially in the dilated tubular epithelial cells in the enalapril–treated group (D, F) (arrows). The number of αSMA–positive cells was increased in both the renal cortex and medulla of the enalapril–treated group (G, H) (P<0.05) (black bar: control, white bar: enalapril–treated group) (A, B original magnification ×40; C, D ×200; E, F ×400).
detected within glomeruli and tubular epithelial cells throughout the cortex and medulla in the control N8 kidneys (Fig. 2A, C and E). In the enalapril–treated group, E-cadherin was weakly observed in some dilated tubular epithelial cells (Fig. 2B, D and F). The expression of E-cadherin-positive cells was dramatically reduced in the cortex and medulla in the enalapril–treated group ($P<0.05$) (Fig. 2G and H).

3. Expression of vimentin

In the enalapril–treated group, vimentin expression in the renal medulla was more prominent than in the control group, and almost all medullary interstitial cells showed strong positive vimentin staining (Fig. 3A–F). In the control group, vimentin was detected in the vascular endothelial cells, glomeruli and medullary interstitial cells (Fig. 3A, C and E). The expression of vimentin was stronger in almost all of the medullary interstitial cells in the enalapril–treated group, compared to the controls (Fig. 3B, D and F). The number of vimentin–positive cells was not different in the renal cortex between the two groups; however, the expression increased in the medullary tubulointerstitial cells in the enalapril–treated group ($P<0.05$) (Fig. 3G and H).

4. Expression of nestin

Nestin–positive cells were more strongly expressed in the medullary tubulointerstitial cells in the enalapril–treated group, compared to the controls (Fig. 4A–F). The expression of the nestin–positive cells in the renal cortex was observed in the glomeruli, endothelial cells of arterioles, and a few tubular cells; it was not much different from that of the control group (Fig. 4C and D). Nestin was more abundantly detected in the dilated medullary tubular epithelial cells in the enalapril–treated group (Fig. 4E and F). The number of nestin–positive cells was not different in the renal cortex of the two groups; however, the expression of nestin increased in medullary tubulointerstitial cells of the enalapril–treated group ($P<0.05$) (Fig. 4G and H).
Fig. 3. Immunohistological examination of vimentin in the rat newborn control kidneys (A, C, E) and in the enalapril−treated kidneys (B, D, F). Low magnification of the kidney shows vimentin expression in the renal medulla was detected significantly more in the enalapril−treated group, compared to the controls (A, B). In the cortex of both groups, vimentin was detectable within the glomeruli, vascular endothelial cells and some interstitial cells (C, D) (arrows). In the renal medulla of enalapril−treated kidneys, almost all interstitial cells had strong positive vimentin staining and more vividly expressed than in the control group (E, F) (arrows). The number of vimentin−positive cells in the renal cortex was not different between the two groups; however, their expression in the medulla was increased in the enalapril−treated group (G, H) (\( P<0.05 \)) (black bar: control, white bar: enalapril−treated group) (A, B original magnification \( ×40 \); C, D, E, F \( ×400 \)).

Discussion

The main finding of the present study was that ACE inhibition, in the developing rat kidney, increased the renal EMT. We demonstrated that ACE blockade, in the newborn rat kidney, up−regulated the dilated tubular expression of αSMA, a representative mesenchymal marker, and down−regulated the expression of the most common epithelial cell marker, E−cadherin. Enalapril treatment was associated with increased expression of vimentin and nestin in the renal medulla; however, there were no significant changes noted in the renal cortex. These results suggest that the RAS is important for modulating the renal EMT of the developing kidney, and that medullary tubulogenesis via renal MET or EMT might be affected by neonatal ACE inhibition.

Ang II, the main peptide of the RAS, has been implicated as a potent mitogenic regulator of genes involved in cell proliferation and growth of the developing kidney, as well as in the progression of renal damage. This peptide activates tubuloeipithelial cells, interstitial fibroblasts, and glomerular cells, that regulate cell growth and extracellular matrix synthesis. Accumulating evidence suggests that Ang II is the key modulator of renal fibrosis and EMT. In cultured tubuloeipithelial cells, Ang II induces αSMA expression and mesenchymal features. Infusion of Ang II into rats causes tubular injury associated with the neoexpression of αSMA and vimentin in the renal interstitial cells; therefore, it is reasonable to speculate that Ang II might be involved in the EMT of the kidney.

The results of the present study indicated, for the first time, that enalapril treatment promotes renal EMT and hinders renal MET in the developing rat kidney. In accordance with tubular dilatation and interstitial damage caused by ACE inhibition, the expression of mesenchymal markers (αSMA and vimentin)
and nestin increased, and the expression of the epithelial marker (E-cadherin) decreased. These changes were more evident in the renal medulla than in the cortex of the enalapril–treated rats. Consistent with our findings, prior reports have indicated that the interruption of Ang II signaling during nephrogenesis in neonatal rats perturbs renal tubular development. Lasaitiene et al.\(^{24}\) showed that neonatal losartan treatment caused significant changes in the phenotype of the developing medullary and cortical regions as well as a thick ascending limb of Henle. ACE inhibition in the neonatal rat has a detrimental effect on the tubular mitochondrial structure and function during renal morphogenesis\(^{25}\). Enalapril treatment also caused a reduced expression of E-cadherin in dilated medullary collecting tubules, suggesting that suppressed E-cadherin levels could be involved in the development of tubular dilatation and that RAS is important in mediating normal medullary tubulogenesis\(^{25}\).

The expression of αSMA has been used as a marker for myofibroblasts, a subset of fibroblasts that are a major source of collagen synthesis\(^{27}\). Indeed, increasing numbers of αSMA–positive myofibroblasts has been associated with progressive renal dysfunction in many experimental studies\(^{28}\). αSMA may provide a structural foundation not only for defining the morphology of transformed cells, but also for their migration, invasion, and their capacity for contractility. αSMA–positive tubular epithelial cells are a stable, terminally--differentiated, irreversible, transformed cell type\(^{29}\). In the present study, positive staining for αSMA was detected exclusively in the vascular smooth muscle cells of the control group. De novo αSMA expression might be detected primarily in the dilated cortical tubular epithelial cells and medullary tubulointerstitial cells of enalapril–treated neonatal rats.

E-cadherin is an epithelial cell specific intracellular adhesion molecule that can induce MET, when overexpressed in cells of mesenchymal lineage\(^{30}\). The suppression of E-cadherin expression is regarded as a key step that precedes other

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**Fig. 4.** Immunohistological examination of nestin in the rat newborn control kidneys (A, C, E) and in the enalapril–treated kidneys (B, D, F). Low magnification of the kidney shows nestin expression in the renal medulla was more strongly stained in the enalapril–treated group, compared to the controls (A, B). In the cortex of both groups, nestin was detectable within the glomerular, vascular endothelial cells and a few tubular cells; the immunoactivities did not differ between the two groups (C, D) (arrows). In the renal medulla of the enalapril–treated kidneys, nestin was more highly detected at the dilated tubular epithelial cells, compared to the controls (E, F) (arrows). The number of nestin–positive cells in the renal cortex was not different between the two groups; however, their expression in the medulla was increased in the enalapril–treated group (G, H) (\( \* P < 0.05 \)) (black bar: control, white bar: enalapril–treated group) (A, B original magnification \( \times 100 \); C, D, E, F \( \times 400 \)).
major events during renal EMT. The importance of E-cadherin in the development of normal epithelium has been established in gene knockout mice. We found that the expression of E-cadherin was significantly decreased in the cortical and medullary dilated tubules of the enalapril-treated group. Under normal conditions, E-cadherin expression was easily detected within the glomeruli and tubular epithelial cells throughout the cortex and medulla. The de novo expression of αSMA and the loss of E-cadherin imply that the mesenchymal programmed genes are turned on while the epithelial programmed genes have been turned off.

Our findings showed that up-regulation of vimentin and nestin expression was observed only in the renal medulla of enalapril-treated rats. The medullary EMT may be more susceptible to neonatal ACE inhibition than the cortex, or vimentin and nestin expression may play different roles during EMT. Vimentin and nestin are intermediate filament (IF) proteins that are typically found in mesenchyme and in neuroepithelial stem cells, respectively. Among the known cytoskeletal proteins, IF proteins are the most characteristic of podocytes. IFs contribute to the mesenchymal integrity of cells and tissues, and play a key role in a variety of cellular functions that range from the determination of cell shape and motility to cell cycle control and signal transduction. Alteration of IF gene expression, is often accompanied by tissue injury; this includes its effects on the kidneys.

Vimentin was not found in the tubular epithelial cells at any developmental stage. However, the cells of the collecting ducts showed transient expression of vimentin in the fetal kidneys. Interstitial staining of vimentin in diseased kidney tissues has shown increased fibrosis. Expression of vimentin by tubular cells has been found in acute tubular necrosis during which transient high levels of vimentin expression have been seen in proliferating tubules during the repair/regenerative phase; however, this is reversible and is not associated with progressive tubulointerstitial fibrosis. In the present study, vimentin was easily detected in the glomeruli and in some of the medullary collecting ducts of the neonatal control kidneys. Enalapril treatment increased the medullary interstitial expression of vimentin, suggesting some role in renal medullary EMT of neonatal rats.

Nestin is another IF protein and a marker for neuroepithelial and non-neural stem cells. Its presence in cells may identify multi-potentiality and regenerative potential. Sakairi et al. showed that the degree of nestin expression was correlated with the degree of tubulointerstitial fibrosis in the adult rat kidney following unilateral ureteral obstruction. The nestin-positive tubular cells expressed vimentin, indicating that these cells reverted to a mesenchymal phenotype. After experimental mesangial cell injury in vivo, glomerular nestin was also transiently increased during the repopulation phase. Nestin promoted mesangial cell proliferation in vitro, supporting a role for nestin during repair reactions. In this study, we found that nestin was expressed in glomeruli, endothelial cells of arterioles, and a few tubular cells involved in the control of the developing kidneys, consistent with the findings of others. In ACE inhibited neonatal rats, nestin was re-expressed and increased significantly in the medullary dilated tubular and interstitial cells, suggesting that nestin plays an important role in renal medullary EMT. However, the results of the present study do not provide enough data to confirm this. Further investigations involving the precise mechanism of vimentin–or nestin–associated renal EMT in the developing kidney are now needed.

The results of this study showed for the first time that Ang II inhibition was involved in renal EMT, in developing rat kidneys, through enhanced expression of αSMA, vimentin and nestin, and suppressed expression of E-cadherin. There were no significant cortical changes associated with vimentin and nestin expression after enalapril treatment. The interaction between RAS and renal MET/EMT in the developing kidney might provide a clue for improved understanding of the novel mechanisms involved in renal growth and development. To further clarify the RAS–associated MET/EMT changes, we now need to investigate the signal cascades involved in the developing kidneys.
자 및 nestin의 발현 양상을 조사하였다.

방 법 : 7일 동안 신생 백서에게 enalapril (30 mg/kg/d) 또는 vehicle을 투여하였으며, α-smooth muscle actin (SMA), E-cadherin, vimentin 및 nestin에 대한 면역 조직 화합 염색을 시행하였다.

결 과 : enalapril 투여군에서 대조군에 비해 신 피질 및 수질 모두에서 α-SMA 발현이 증가하였으며, 이는 확장된 세뇨관 상피 세포에서 투여하였다 (P<0.05). E-cadherin 발현은 enalapril 투여군의 신 피질 및 수질의 세뇨관 상피 세포에서 화학적 감소하였다 (P<0.05), vimentin 및 nestin 발현은 신 피질에서는 양군간의 차이가 없었으나, 신 수질에서는 enalapril 투여군에서 세뇨관 간질 세포에서 발현이 의미있게 증가하였다 (P<0.05).

결 론 : 신생 백서 신장에서 Ang II 억제는 α-SMA 발현을 증가시키고, E-cadherin 발현을 감소시킴으로써 발달하는 신장의 EMT를 증가시켰다. Enalapril 투여는 또한 신 수질에서 vimentin과 nestin의 발현을 증가시켰으며, 이는 신생 백서 신장에서의 Ang II 억제로 인한 EMT 과정 중 신 수질의 변화가 더욱 뚜렷한 것을 시사하며, Ang II 억제가 EMT 표지자들의 발현을 다르게 변화시킨 것으로 사료된다.

References