

## Endothelin A Receptor Blockade Influences Apoptosis and Cellular Proliferation in the Developing Rat Kidney

Endothelin systems are believed to play important roles in the emergence and maintenance of functions of various organs during perinatal development, including the kidney. The present study was designed to investigate the roles of endothelin systems on physiologic renal growth and development. Newborn rat pups were treated with either Bristol-Myers Squibb-182874 (30 mg/kg/day), a selective endothelin A receptor (ET<sub>A</sub>R) antagonist, or vehicle for 7 days. To identify cellular changes, kidneys were examined for apoptotic cells by terminal deoxynucleotide transferase-mediated nick-end labeling stain and proliferating cell nuclear antigen (PCNA) by immunohistochemical (IHC) stain. To clarify the molecular control of these processes, immunoblots and reverse transcriptase-polymerase chain reaction for Clusterin, Bcl-2, Bcl-X<sub>L</sub>, Bax, and p53 were performed. ETAR antagonist treatment resulted in reduced kidney weights, decreased PCNA-positive proliferating cells, and increased apoptotic cells. The protein expressions of renal Bcl-X<sub>L</sub> and Bax in the ETAR antagonist-treated group were significantly decreased, whereas the mRNA expressions of these genes were not changed. There were no significant differences in the expressions of Clusterin, Bcl-2, and p53. In conclusion, inhibition of endogenous endothelins impairs renal growth, in which decreased cellular proliferation, increased apoptosis and decreased expressions of renal Bcl-X<sub>L</sub> and Bax are possibly implicated.

**Key Words :** Endothelins; Growth and Development; Apoptosis; Cell Proliferation; Clusterin; Bcl-2; Bcl-X<sub>L</sub>; Bax; p53; Kidney

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## INTRODUCTION

The endothelin (ET) system is thought to play important roles in the emergence and maintenance of functions of various organs during perinatal development, including the kidney. It has been reported that expression of ET-1 increases in these organs toward the later stages of embryonic development (1, 2). The expression of ET receptors has also been shown to increase around the time of birth in animal models (3). The evidence suggests an influential role for the ET system during normal physiologic growth and development of the kidney, and evokes the possibility of an influence of ET-1 receptor antagonists during postnatal maturation and development of the kidney. However, it is still unknown whether endogenous activation of the paracrine ET systems plays a physiologic role in renal development.

An unexpected role of the ET system in embryonic and fetal development has been recently discovered and appears to be essential for cardiovascular and neuronal crest-derived cell differentiation (4, 5). Despite the definitive demonstrations of a crucial role of the ET system during early devel-

opment, little is known about the importance of the ET system during the perinatal period, including the late embryonic and neonatal stages.

It is now well-accepted that the growth impairment of developing organs is often the result of an imbalance between the rate of cell proliferation and that of apoptosis. Our previous study (6) showed that ET inhibition by selective endothelin A receptor (ET<sub>A</sub>R) blockade decreased apoptosis and the expression of its related genes in the developing rat heart. The ET<sub>A</sub>R antagonist also decreased cellular proliferation. The decreased cell turnover due to the ET<sub>A</sub>R antagonist in the developing rat heart may account for neonatal cardiac growth impairment. These findings indicate the likelihood of important functions for the ET<sub>A</sub>R antagonist in the development of the kidney, which is another hemodynamically important system. Thus, an imbalance between cell proliferation and apoptosis due to ET<sub>A</sub>R blockade might contribute to the normal growth and development of the kidney during the perinatal period.

In this study, we selected the developing kidney model of the neonate rat and investigated the roles of the ET system in

the renal growth and development. The experimental approach consisted of inhibiting endogenous ET by the chronic administration of Bristol-Myers Squibb (BMS)-182874, a competitive, selective, and orally active ET<sub>A</sub>R antagonist, to the neonatal rat pups. The effects of the ET<sub>A</sub>R antagonist on cellular proliferation and apoptosis in the neonatal rat kidney were investigated. In addition, to clarify the molecular control of these processes, the changes in expression of various regulatory genes at the protein and mRNA levels by the ET<sub>A</sub>R antagonist were determined.

## MATERIALS AND METHODS

### Animal preparation

Forty-two neonatal rat pups from seven pregnant Sprague-Dawley rats were breastfed by their own mother throughout the study. The newborn rats were divided into two groups and a dose of 30 mg/kg of BMS-182874 (BMS Korea, Seoul, Korea) (BMS group) or 10 cc/kg of normal saline (control group) were fed to the rat pups via an orogastric tube for 7 days, respectively. After 7 days, their kidneys were harvested and RNA analysis, protein assays, and immunohistochemical staining for cell proliferation and apoptosis were performed. The protocols for the animal experimentation and the handling of animals were approved by the Animal Care Committee of the Korea University Guro Hospital.

### Immunohistochemical stain

Immunostaining was performed on paraffin sections as described previously (7, 8). The proliferating cell nuclear antigen (PCNA) cells and terminal deoxynucleotide transferase-mediated nick-end labeling (TUNEL)-positive apoptotic cells were detected using the avidin-biotin immunoperoxidase method (Vectastain ABC kit, Burlingame, CA, U.S.A.) and the TACS TM 2 TdT In Situ Apoptosis Detection Kit

(Trevigen, Gaithersburg, MD, U.S.A.), respectively. We compared three slides of each kidney in both groups using a light microscope (400×). The number of PCNA-positive cells and TUNEL-positive apoptotic cells were measured by counting 15 areas (250×250 μm) and obtaining the average. The PCNA-positive cells were counted if they had a central ovoid nucleus with a clear zone and abundant eosinophilic cytoplasm. Only morphologically distinct nuclei that stained positively by the TUNEL assay were counted as apoptotic.

### Isolation of kidney RNA and protein

Each five left kidneys of the control and the BMS group were selected for RNA analysis and protein assays. The TRI-REAGENT (Molecular Research Center, Cincinnati, OH, U.S.A.) method was used for RNA and protein isolation, according to the recommendations of the manufacturer, and prepared as previously described (8).

### cDNA synthesis by reverse transcription (RT) and polymerase chain reaction (PCR)

Oligo dT primed 1st strand cDNA was synthesized from the template RNA (1 μg) by using a cDNA Synthesis Kit (Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.). The primers used in the PCR reaction are presented in Table 1. The PCR was carried out with a different mode of time and temperature for each reaction period by using a Perkin Elmer Cetus DNA Thermal Cycler (Model 2400, Foster City, CA, U.S.A.). These amplified PCR products were visible as fluorescent bands at regular intervals under ultraviolet light after performing electrophoresis in 2% agarose gel and ethidium bromide staining. The Polaroid photographs were scanned using an Epson GT-9500 (Seiko Corp., Nagano, Japan) and the pictures were quantified by a densitometer (Image PC alpha 9, N.I.H., Bethesda, MD, U.S.A.), and the values were revised glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. The primer sequences and the expected product sizes for RT-PCR analysis

Genes	Primers	Sequence	PCR product
GAPDH	Forward	5'-AATGCATCCTGCACCACCA-3'	515 bp
	Reverse	5'-GTAGCCATATTCATTGCATA-3'	
Clusterin	Forward	5'-CGGGTACCGACAATGAGCTCCA-3'	310 bp
	Reverse	5'-GGCCGCGGCCACTTCTGCAGAC-3'	
Bcl-2	Forward	5'-TGGGATACTGGATGAAGA-3'	478 bp
	Reverse	5'-AGACAGCCAGGAGAAATCAAA-3'	
Bcl-X <sub>L</sub>	Forward	5'-GGTGGTTGACTTTCTCTCCTAC-3'	491 bp
	Reverse	5'-GAAAAGTATCCAGCCGCC-3'	
Bax	Forward	5'-GACAVVTGAGCTGACRRGG-3'	310 bp
	Reverse	5'-GAGGAAGTCCAGTGCCAGC-3'	
p53	Forward	5'-CTGAGGTCGGCTCCGACTATACCACTATCC-3'	360 bp
	Reverse	5'-CTGATTCAGCTCTCGAACATCTCGAAGCG-3'	

RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### Immunoblot analysis

Immunoblots were performed as in previous studies (6-8). Briefly, equal amounts of 5-15  $\mu\text{g}$  of proteins were subjected to 10% SDS-polyacrylamide gels and then transferred onto

**Table 2.** Characteristics and sources of clusterin, Bcl-2, Bcl-X<sub>i</sub>, Bax, and p53 antibodies

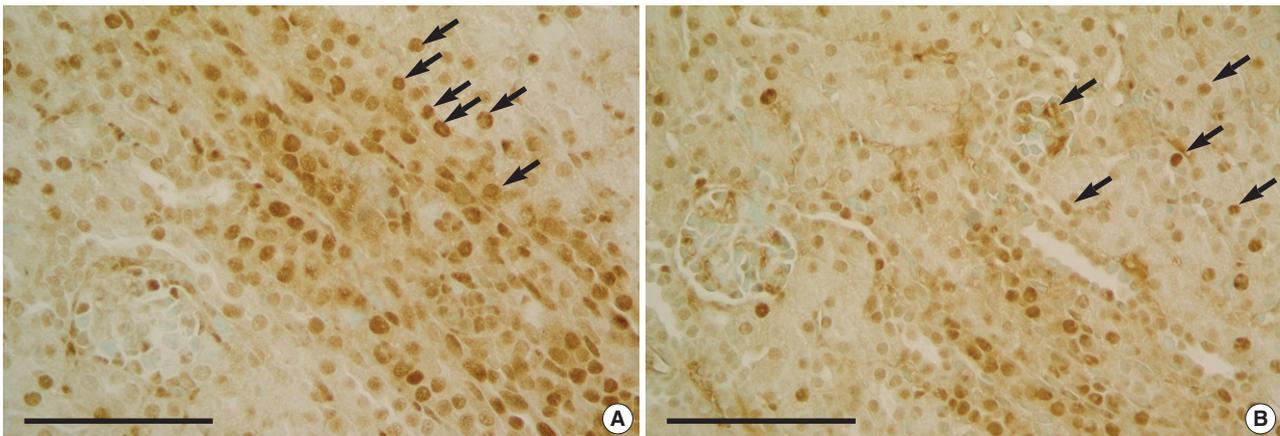
	Type	Host	Dilution	Source
Clusterin	Monoclonal	Rabbit	1:400	Upstate
Bcl-2	Monoclonal	Mouse	1:500	R&D
Bcl-X <sub>i</sub>	Polyclonal	Rabbit	1:1,000	Calbiochem
Bax	Polyclonal	Mouse	1:500	BD
p53	Polyclonal	Mouse	1:1,000	Santacruz
Tubulin	Monoclonal	Mouse	1:1,000	Oncogen

Upstate Biotechnology (Lake Placid, NY, U.S.A.); R&D systems, Inc. (Minneapolis, MN, U.S.A.); Calbiochem (La Jolla, CA, U.S.A.); BD Biosciences (Los Angeles, CA, U.S.A.); Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); Oncogene Biotechnology (Boston, MA, U.S.A.).

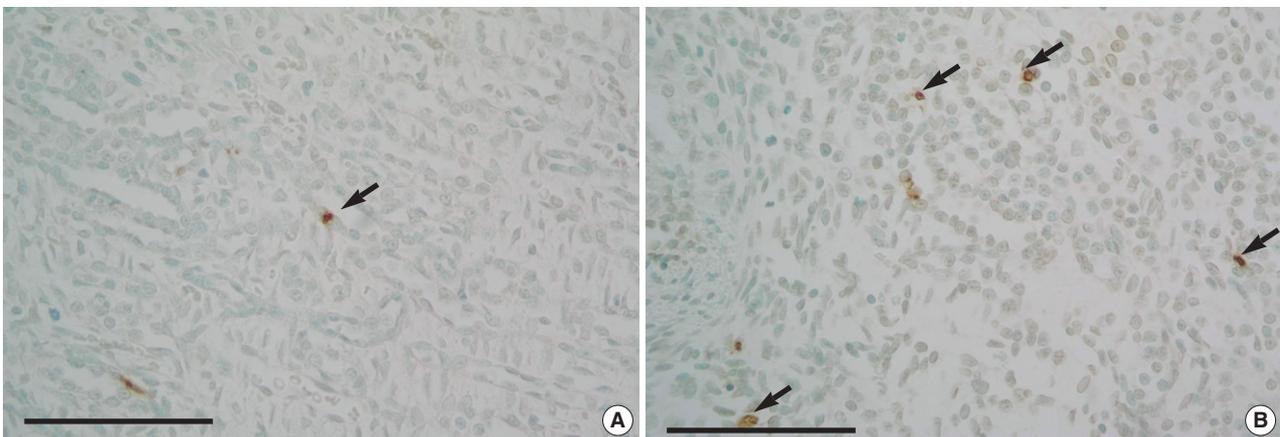
nitrocellulose membranes (Amersham Life Science, Buckinghamshire, U.K.). The membrane was first blotted for the antibodies directed against clusterin, Bcl-2, Bcl-X<sub>i</sub>, Bax, and p53. Then, it was reblotted for  $\alpha$ -tubulin (1:1,000 dilution; Oncogene, Boston, MA, U.S.A.) to control equal loading. The characteristics of the antibodies directed against clusterin, Bcl-2, Bcl-X<sub>i</sub>, Bax, and p53 are listed in Table 2. The relative densities of these antibodies versus  $\alpha$ -tubulin bands were measured by a computerized densitometer (Image PC alpha 9).

### Statistical analysis

The results are expressed as the means  $\pm$  SEM. Differences between groups were tested for their significance by using the Student t-test and the Mann-Whitney rank sum test, and a value of  $p < 0.05$  was considered statistically significant.



**Fig. 1.** PCNA stains for proliferating cells in the control group (A) and the BMS group (B). PCNA-positive cells were rarely found in the BMS group as compared with the control group. Cortical tubular epithelial and interstitial cell proliferations were decreased following treatment with the ETA receptor antagonist as compared with the control group. Bar+0.1  $\mu\text{m}$ . Magnification:  $\times 400$ .



**Fig. 2.** TUNEL stains for apoptotic cells in the control group (A) and the BMS group (B). In the BMS group, TUNEL-positive apoptotic cells were more frequently found in the dilated tubular epithelial cells, as compared with the control group. Bar+0.1  $\mu\text{m}$ . Magnification:  $\times 400$ .

## RESULTS

### Kidney weight

On harvest, the average kidney weights were significantly reduced in the BMS group ( $124 \pm 18$  mg) as compared with the control group ( $160 \pm 29$  mg;  $p < 0.05$ ).

### PCNA

The PCNA-positive cells were found in both the tubules and the interstitium of both groups. A small number of proliferating glomerular cells and vascular cells were also observed. However, the number of PCNA-positive cells was decreased in the BMS group compared with the control group

(Fig. 1). The number of PCNA-positive cells was  $19.5 \pm 0.3$  in the control group and  $12.9 \pm 1.1$  in the BMS group in each  $250 \times 250 \mu\text{m}$  field. There was a significant 33.8% reduction in the PCNA-positive cells in the BMS group ( $p < 0.05$ ).

### Apoptosis

TUNEL-positive apoptotic cells were rarely found in the renal cortices of both groups. In the BMS group, apoptotic cells were occasionally observed in the dilated tubular epithelial cells and they were more frequently found as compared with the control group (Fig. 2). The number of TUNEL-positive apoptotic cells was  $2.46 \pm 0.09$  in the control group and  $2.79 \pm 0.12$  in the BMS group in each field of  $250 \times 250 \mu\text{m}$ , an increment of 13.4% ( $p < 0.05$ ).

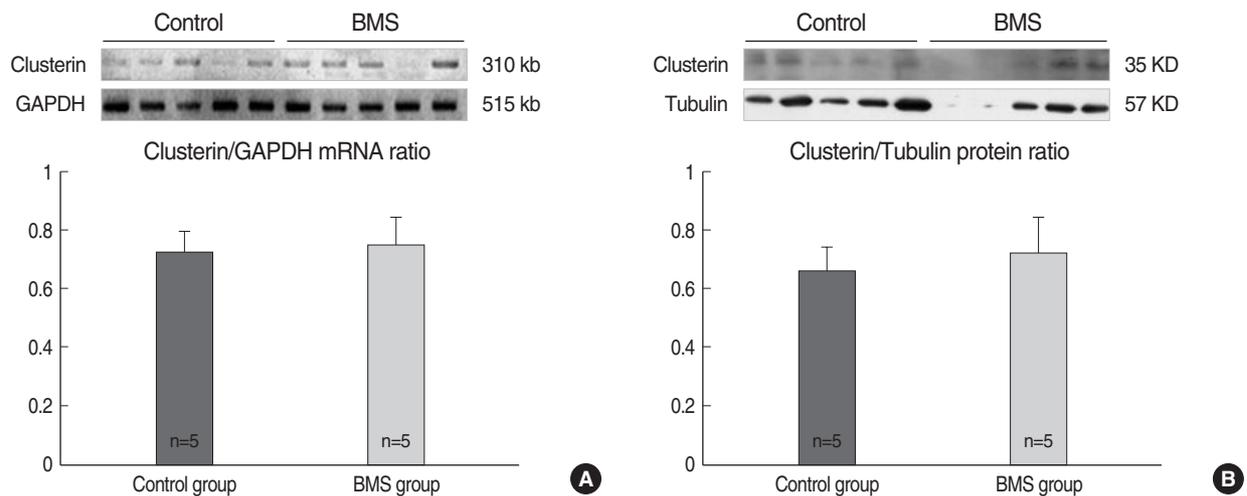


Fig. 3. Representative expressions of clusterin mRNA and protein. ■, control group; □, BMS group. (A) On the semi-quantitative RT-PCR, the clusterin/GAPDH mRNA expressions were not different between the two groups. (B) On the immunoblot analysis, the clusterin/tubulin protein expressions were not changed in the BMS group as compared with the control group.

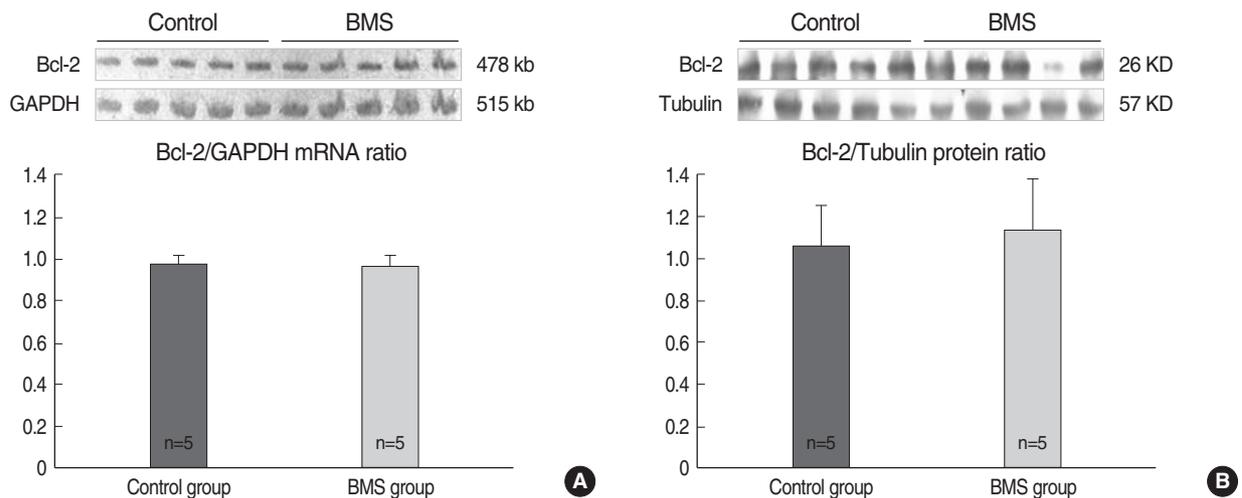


Fig. 4. Representative expressions of Bcl-2 mRNA and protein. ■, control group; □, BMS group. (A) On the semi-quantitative RT-PCR, the Bcl-2/GAPDH mRNA expression was not different between the two groups. (B) On the immunoblot analysis, the Bcl-2/tubulin protein expression were not changed in the BMS group as compared with the control group.

### Expression of clusterin mRNA and protein

The clusterin/GAPDH mRNA expressions were  $0.73 \pm 0.04$  in the control group and  $0.75 \pm 0.05$  in the BMS group. The clusterin/tubulin protein expression was not changed in the BMS group ( $0.72 \pm 0.06$ ) compared with the control group ( $0.66 \pm 0.06$ ; Fig. 3).

### Expression of Bcl-2 mRNA and protein

The Bcl-2/GAPDH mRNA expression was  $0.97 \pm 0.03$  in the BMS group and  $0.96 \pm 0.03$  in the control group. The Bcl-2/tubulin protein expression was not changed in the BMS group ( $1.12 \pm 0.27$ ) compared with the control group ( $1.05 \pm 0.18$ ; Fig. 4).

### Expression of Bcl-X<sub>L</sub> mRNA and protein

The Bcl-X<sub>L</sub>/GAPDH mRNA expression was  $1.05 \pm 0.12$  in the control group and  $1.14 \pm 0.08$  in the BMS group. The Bcl-X<sub>L</sub>/tubulin protein expression was decreased significantly in the BMS group ( $0.70 \pm 0.24$ ) compared with the control group ( $1.04 \pm 0.09$ ,  $p < 0.05$ ; Fig. 5).

### Expression of Bax mRNA and protein

The Bax/GAPDH mRNA expression was  $0.98 \pm 0.13$  in the control group and  $0.87 \pm 0.09$  in the BMS group. In addition, the Bax/tubulin protein expression was decreased significantly in the BMS group ( $0.78 \pm 0.08$ ) compared with the control group ( $1.39 \pm 0.03$ ,  $p < 0.01$ ; Fig. 6).

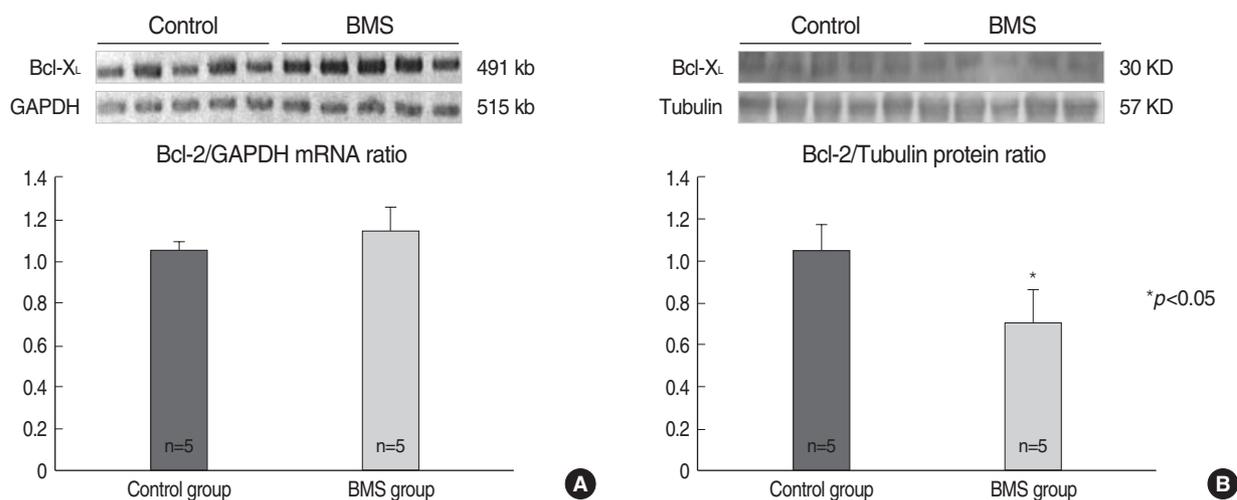


Fig. 5. Representative expression of Bcl-X<sub>L</sub> mRNA and protein. ■, control group; □, BMS group. (A) On the semi-quantitative RT-PCR, the Bcl-X<sub>L</sub>/GAPDH mRNA expression were not different between the two groups. (B) On the immunoblot analysis, the Bcl-X<sub>L</sub>/tubulin protein expression was decreased significantly in the BMS group as compared with the control group.

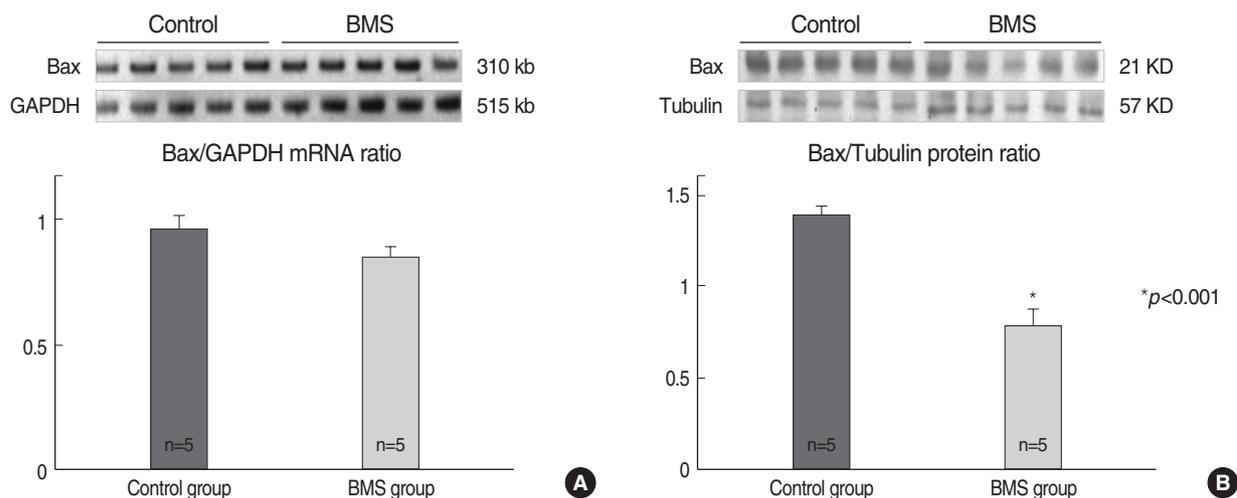


Fig. 6. Representative expression of Bax mRNA and protein. ■, control group; □, BMS group. (A) On the semi-quantitative RT-PCR, the Bax/GAPDH mRNA expression were not different between the two groups. (B) On the immunoblot analysis, the Bax/tubulin protein expression was decreased significantly in the BMS group as compared with the control group.

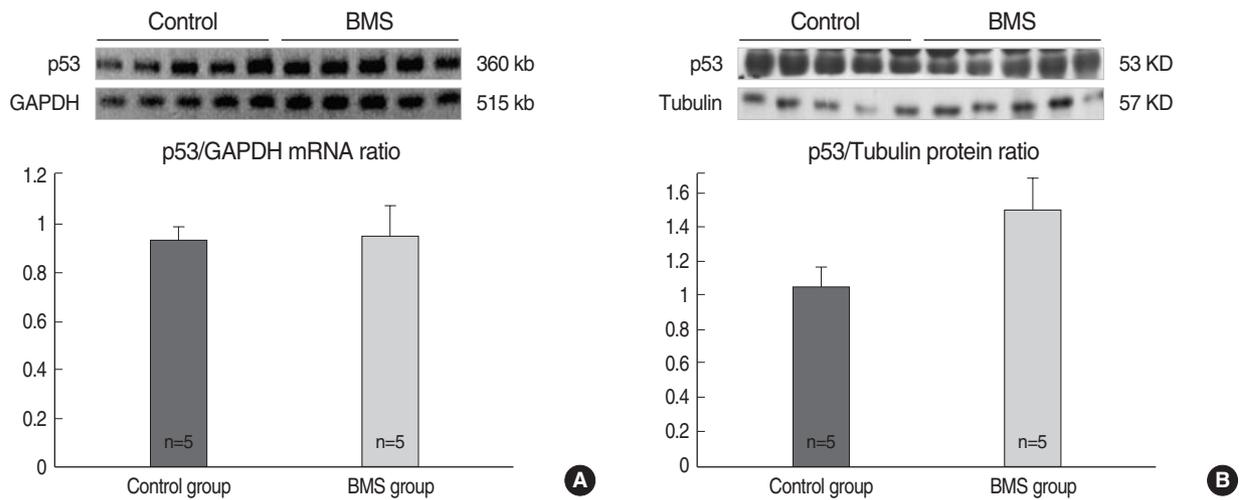


Fig. 7. Representative expression of p53 mRNA and protein. ■, control group; □, BMS group. (A) On the semi-quantitative RT-PCR, the p53/GAPDH mRNA expression was not different between the two groups. (B) On the immunoblot analysis, the p53/tubulin protein expression was not changed in the BMS group as compared with the control group.

### Expression of p53 mRNA and protein

The p53/GAPDH mRNA expression was  $0.92 \pm 0.07$  in the control group and  $0.94 \pm 0.03$  in the BMS group. The p53/tubulin protein expression was not changed significantly in the BMS group ( $1.49 \pm 0.18$ ) compared with the control group ( $1.05 \pm 0.11$ ,  $p=0.073$ ; Fig. 7).

## DISCUSSION

The renin-angiotensin system (RAS) and the ET system have features of the most potent vasopressor mechanisms identified to date. These neurohormones are regulated by the same G protein-coupled receptors. It has been hypothesized that angiotensin II (Ang II), the active peptide of the RAS, affects the synthesis of ET-1, which in turn can influence the RAS by acting at different steps. In keeping with this hypothesis, the identification of the consensus sequences for jun in the regulatory regions of the preproendothelin-1 gene has raised the possibility of its transcriptional regulation by Ang II (9). Ang II has been reported to augment pre-/pro-ET-1 expression not only in vascular endothelial cells, but also in non-endothelial cells, including vascular smooth muscle cells, myocardial cells, renal epithelial cells, and glomerular mesangial cells (10, 11). With this in mind, Ang II may have its cellular effects through the induction of ET. Therefore, it could be speculated that Ang II is directly involved in the development of tissue hypertrophy through the activation of the Ang II type 1 receptor and furthermore, Ang II is indirectly involved through the activation of the ET system. As a consequence, it is likely that under pathophysiologic conditions caused by the activation of the RAS, ET receptor antagonists may be effective for ameliorating the hypertensive

phenotypes.

In our previous study, we demonstrated that Ang II modulates growth in a variety of tissues, including the kidney and the heart (7, 8, 12, 13). Ang II inhibition by angiotensin converting enzyme (ACE) inhibitor in the developing rat kidney decreases the cellular proliferation and increases apoptosis, and this may account for neonatal renal growth impairment (7, 8, 12-15). These results suggest that the RAS is necessary for normal renal growth and development. So, ET-1, like Ang II, is implicated in postnatal renal maturation and development.

The present in vivo animal study has demonstrated that ET systems may play a role in renal growth impairment via ET<sub>A</sub>R inhibition. In particular, the chronic administration of ET<sub>A</sub>R antagonists diminished the number of PCNA-positive proliferating cells by 33.8% in cortical tubular epithelial cells. Our previous studies demonstrated that ACE inhibition by enalapril decreased cellular proliferation in the developing rat kidney. The PCNA-positive proliferating cells were also decreased significantly in the same cortical tubular epithelial cells (12, 15).

In the present study, we have shown here that TUNEL-positive apoptotic cells were increased in the BMS-treated group, and this was similar to the previous enalapril-treated group. The apoptotic cells primarily occurred in the dilated cortical tubules and the number was increased in the BMS-treated group. Diminished kidney size was associated with the lowered cellular proliferation rates, as well as with increased apoptosis. The long-term ET inhibition may have an effect on the renal hemodynamics, as a result of the hypotension, the microcirculation or the local ischemia seen in an in vivo model. None of these effects were seen when using hydralazine at the doses previously established to have equivalent effects on blood pressure (7, 16). These experiments suggest

that the effects of ET<sub>A</sub>R antagonists on renal growth are mediated through direct cell signaling rather than through the secondary hemodynamic effects.

Apoptosis is a crucial event in various physiologic processes, such as embryogenesis, organ development, and cell proliferation, as well as in pathologic processes, including autoimmune disease and cancer development. These phenomena are regulated by several genes. Of these genes, the clusterin gene has important roles in various pathophysiologic processes, including tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis. Clusterin has been shown to be one of the most potent inhibitors of apoptosis that was induced by a wide variety of stimuli. Our previous study (14) has indicated that the inhibition of Ang II by an ACE inhibitor on the developing rat kidney decreased clusterin protein expression. Thus, clusterin mRNA expression was increased as a secondary response to increased apoptosis and decreased its protein expression. However, in the present study, the ET<sub>A</sub>R inhibition did not change the protein and mRNA expressions of clusterin.

One of the major multigene families involved in the molecular control of cell survival or death is the Bcl-2 gene family. Members of the Bcl-2 gene family can form homo- or hetero-dimers, and the relative proportions of the anti-apoptotic members, such as Bcl-2 or Bcl-X<sub>L</sub> and the pro-apoptotic members, such as Bax are now thought to govern whether the cell lives or dies after damage (17).

During nephrogenesis, apoptosis tends to be inversely correlated with Bcl-2 expression. Bcl-2 is highly expressed early in the embryonic kidney and its level decreases significantly at later times such that its expression is normally low in the postnatal kidney (18). Our previous study (14) has indicated that the inhibition of Ang II by ACE inhibitor in the developing rat kidney decreased Bcl-2 protein expression. Bcl-2 mRNA expression was increased as a secondary response to the increased apoptosis and this decreased its protein expression. However, in the present study, ET<sub>A</sub>R inhibition did not change the Bcl-2 protein and mRNA expression.

Expression studies have demonstrated that Bcl-X<sub>L</sub> was predominantly expressed postnatally. Bcl-X<sub>L</sub> is five- to six-fold more abundant in the adult kidney, brain, and thymus than Bcl-2. Therefore, it also appears that Bcl-X<sub>L</sub> may play an important role in the regulation of cell death during development and tissue homeostasis. In the BMS-treated group, we found that Bcl-X<sub>L</sub> protein expressions were significantly decreased, as compared with the control group. The renal apoptosis induced by ET<sub>A</sub>R inhibition in the developing kidney was related with the decreased anti-apoptotic Bcl-X<sub>L</sub> protein expression, but the Bcl-X<sub>L</sub> mRNA expression was not changed. Unfortunately, as compared with the control group, the expression of proapoptotic Bax protein was decreased in the BMS-treated group. This deregulated expression of Bax and Bcl-X<sub>L</sub> was also found to impair the normal renal developmental processes (19, 20). Together these data can be ex-

plained in two ways. First, the Bcl-X<sub>L</sub> gene plays a more important role than the Bax gene in the regulation of cell death during the renal development. Alternatively, the increased Bax protein degradation can be a conceivable possibility without the change of mRNA expression (21).

p53 is a transcriptional factor that has effects on the cell cycle arrest or apoptosis in response to a variety of stresses. p53 is also a transcriptional modulator of the Bax gene that promotes apoptosis. Additionally, a p53-negative response element has been identified in the Bcl-2 gene, which protects from apoptosis. p53 is one of the representative apoptosis-promoting factors and, as has already been mentioned in the results, the levels of expression of this gene appears to be unaffected during the course of the apoptotic effect of the ET<sub>A</sub>R antagonist in the developing kidney models.

In summary, our results show an influential role of the ET system for the normal physiologic growth and development of the rat kidney, and this evokes the possibility of the influence of the ET-1 receptor antagonists on the postnatal maturation and development of the kidney. Inhibition of the endogenous ET production by chronic administration of an ET-1 receptor antagonist to neonatal rat seems to impair the renal growth, and in this process, the decrease of cellular proliferation and the increase of apoptosis may play a role in renal growth impairment. Especially, the Bcl-x<sub>L</sub> gene functions as a dominant regulator of apoptotic cell death in the developing rat kidney. Further studies are needed to look into the renal growth factor systems that are involved in cellular proliferation, which in turn control renal growth and development by the ET system.

Although this experiment did not evaluate the short-term outcomes, the exogenous ET-1 receptor antagonists are likely to contribute to the normal growth and development of kidney during the perinatal period. In the future, clinical trials using ET-1 receptor antagonists in newborn infants, investigators should pay special attention to the growth and developmental impairment of various vital organs. Further study is required to evaluate these agents for chronic pulmonary hypertension and vascular remodeling in neonatal animal models, and to establish their safety and appropriate dose. Subsequent clinical trials can then be performed in carefully selected preterm and full term infants with chronic lung disease and persistent pulmonary hypertension of newborn.

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