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Gene expression in uremic left ventricular hypertrophy: effects of hypertension and anemia

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Abbreviations: ACE, angiotensin converting enzyme; ANF, atrial natriuretic factors; Ang II, angiotensin II; DBP, diastolic blood pressure; EPO, erythropoietin; HW/BW, heart weight/body weight ratios; IEF, immediate early genes; LV/BW, left ventricular weight/body weight ratios; LVH, left ventricular hypertrophy; MAP, mean arterial pressures; TGF-β1, transforming growth factor-β1

Abstract

Hypertension and anemia may be causes of left ventricular hypertrophy (LVH) in uremia but the molecular mechanism is not known. Uremia was induced in male Sprague Dawley rats by 5/6 nephrectomy. The following groups of rats were studied for 6 weeks; uremic rats (U) fed *ad. lib.*, control rats (C) pair-fed with U, U rats given hydralazine (100 mg/kg/day) (UH), U rats given erythropoietin (48U/kg/week, i.p.) (UE). Both diastolic and mean arterial pressures are higher ($P < 0.01$) in U and UE compared with C whereas both pressures in UH were normalized. Hemoglobin in U was lower than in C, and was normalized in UE. U, UH and UE had higher heart weight/body weight ratios (HW/BW) as well as left ventricular weight/body weight ratios (LV/BW) compared with C ($P < 0.01$). Compared with U, UH has lower HW/BW and LV/BW ($P < 0.05$) and UE has normal HW/BW but lower LV/BW than U ($P < 0.05$). To see if the gene expression in uremic LVH is similar to that described in pressure overload LVH in which mRNA levels of angiotensin converting enzyme (ACE), transforming growth factor-β1 (TGF-β1), atrial natriuretic factors (ANF) and skeletal α-actin were increased, we measured these mRNA levels by Northern analysis. TGF-β1, ACE and α-actin mRNA levels were not changed in all 4 groups. ANF mRNA in U and UE was increased 3 fold over C, and normalized in UH. Treatment of anemia with erythropoietin improved uremic LVH but did not change ANF mRNA; whereas treatment of hypertension with hydralazine normalized ANF mRNA but did not completely correct uremic LVH. Thus, gene expression in uremic LVH is distinct from that in pressure-overload LVH, suggesting that other unidentified factor(s) might be involved in uremic LVH.

Keywords: anemia; cardiac hypertrophy; hypertension; left ventricular hypertrophy; uremia

Introduction

LVH is one of the commonest cardiac abnormalities on autopsy in uremic patients and a leading cause of death (Rostand *et al.,* 1991; Collins, 2003). Long standing arterial hypertension has been postulated as a major cause of LVH since LVH in patients with essential hypertension can be reversed by effective antihypertensive therapy (Frohlich *et al.,* 1992; Susic and Frohlich, 2000). The relationship, however, between hypertension and LVH is not so clear in uremia. Whereas some studies showed that prolonged control of hypertension in dialysis patients significantly decreased left ventricular mass (Cannella *et al.,* 1993; Klingbeil *et al.,* 2000), other studies showed that LVH did not change (London *et al.,* 1990; London *et al.,* 1994) or could even progress (Huting *et al.,* 1988; Parfrey *et al.,* 1990) in spite of adequate blood pressure control in uremic patients. Anemia is another common complication in uremia and may also contribute to the pathogenesis of LVH because it is associated with a high output state placing the left ventricle at risk for failure. Correction of anemia with erythropoietin has been reported to decrease left ventricular chamber and reduce cardiac indices (Smith *et al.,* 2003). On the other hand, it has been reported that correction of anemia or treatments with several anti-hypertensive agents did not prevent increase of

heart weight in rats with renal insufficiency (Rambausek *et al.,* 1985; Tanabe *et al.,* 2000).

In cardiac hypertrophy a return to fetal state of cardiocyte differentiation has been observed, suggesting the existence of common mechanism of transcriptional regulation between fetal heart and hypertrophic heart (Ghatpande *et al.,* 1999). Immediate early genes (IEG), such as *c-fos, c-myc,* and *c-jun,* were induced by mechanical stimuli (stretch) and these may up-regulate the transcription of fetal class of cardiac genes (Sadoshima and Izumo, 1993), including atrial natriuretic factor (ANF) (Chien *et al.,* 1991), transforming growth factor β1 (TGF-β1) (Patel *et al.,* 2000) and α-actin (Suurmeijer *et al.,* 2003). ANF and TGF-β have a Fos/Jun heterodimer complex (AP-1) binding site (TRE/AP-1) in the 5'-flaking region which may be a target of IEGs, while skeletal α-actin have no consensus AP-1 site in its promoter suggesting that there is other transcriptional activation mechanism. ANF and skeletal α-actin genes have been utilized as markers for the induction of the embryonic gene program during hypertrophy (Chien *et al.,* 1993). A number of growth factors including acidic and basic fibroblast growth factors (FGF), TGF-β, insulin-like growth factor I and II, myotrophin (Sen *et al.,* 1990), have been reported to exist in heart and some of them can induce cardiac hypertrophy and specific gene expression *in vitro*. Angiotensin converting enzyme (ACE) activity and mRNA expression was increased in pressure overload LVH (Makino *et al.,* 1997). Then later angiotensin II (Ang II) have been demonstrated as a potent hypertrophic stimulus which causes significant increase in TGF-β1 expression (Schneider, 2002; Schultz *et al.,* 2002). Despite extensive studies of gene expression in cardiac hypertrophy caused by mechanical stress the molecular mechanism studies of LVH in uremia remains largely unknown.

In this study, we induced uremia in rats by 5/6 nephrectomy and tested the effects on the size of left ventricles (LV) of correcting hypertension and anemia in the nephrectomized rat models by treatments with hydralazine and erythropoietin (EPO), respectively. Then we measured the mRNA levels of ANF, TGF-β1, α-actin and ACE in left ventricles (LV) to check if the same genetic markers were expressed and regulated in uremic hypertrophy.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing between 130 *g* and 160 *g* were housed in an environmentally controlled room with a 12 h light/dark cycle, where they had free access to standard rat chow. After one week of equilibration in the new environment, chronic uremia was induced in the animals by 5/6 nephrectomy in one stage procedure. Under anesthesia (intraperitoneal pentobarbital 50 mg/kg), approximately 70% of one kidney was removed. When hemostasis was achieved, the contralateral kidney was then removed. Control animals underwent the same anesthesia and sham operation. Kidneys were exposed and the capsules were stripped. Four groups were studied: U, Uremic rats fed ad libitum with regular rat chow containing 40% protein (casein); UH, Uremic rats given hydralazine (100 mg/kg/day) in their drinking water; UE, Uremic rats given intraperitoneally erythropoietin (48 U/kg/week); and C, sham-operated control rats. Groups UH, UE, and C were weight matched and pair-fed with Group U. Five weeks and 4 days after subtotal nephrectomy, the animals were anesthetized again with intraperitoneal pentobarbital. The left carotid artery was also exposed and a catheter was advanced through the carotid artery until its tip reached the aortic arch. The free end of the catheter was tunneled subcutaneously around the side to the back of the neck where it was exteriozed through a skin incision. At the end of the procedure, the catheter was filled with a viscous solution of polyvinylpyrollidone (PVP-10, 160 w/v% of saline containing 4,000 U heparin) to prevent refluxing of blood into the catheter lumen and capped with a piece of polyethylene tubing and sealed at one end (6). Three days later, (6 weeks after nephrectomy) arterial pressure was measured at about 8 am after an overnight fast by connecting the carotid artery catheter *via* a PE-50 tubing to a pressure transducer (Physiograph model MK-III-S, Narco Biosystems Inc., Houston, Texas).

During the measurements, the animals were allowed to move freely within the confines of a large cage. The animals usually slept or rested calmly. Under pentobarbital anesthesia, the heart was dissected out, weighed and further dissected into left (LV) and right ventricles (RV) and weighed again respectively. The liver was also dissected out and weighed. Serum creatinine was measured in all groups with a Beckman analyzer. Hemoglobin was measured in Groups U, UE, and C by usual methods. All data are expressed as mean ± standard deviation. Statistical analysis was performed using unpaired t tests and significance was accepted at the 5% level.

RNA isolation and Northern Blot

Total RNA from LV and RV were isolated as described (Chomczynski and Sacchi, 1987; Eum *et al.,* 2003). The RNA (20 μg) was separated on 1% agarose/2.4 M formaldehyde gels in 1x MOPS buffer containing 0.7 mg/ml ethidium bromide. The RNA was transfered to Nitroplus 2,000 paper (Microseparations

Inc., Westborough, MA) by capillary blotting overnight. The paper was UV irradiated (Stratagene: auto cross link mode). The filters were prehybridized at 42°C for 2 h in 50% formamide, 6x SSC, 2x Denhardt's solution, 0.1% SDS and 0.1 mg/ml salmon sperm DNA and then hybridized with a ^{32}P-labeled cDNA probe (prepared by random priming method) in the same solution used for prehybridazation at 42°C for 16-24 h. The filters were washed twice with 1x SSC/0.1% SDS at room temperature for 15 min, twice with 0.25x SSC/0.1% SDS at 60°C for 15 min and then twice with 0.1x SSC/0.1% SDS at 60°C for 15 min. The filters were then air-dried and placed into sealed bags and exposed to x-ray film at -80°C. The bands were quantified using laser densitometer (The Discovery Series, PDI, NY). After obtaining appropriated film for quantification, the filters were stripped by incubating twice at 95°C in 0.1x SSC/0.1% SDS for 30 min and then hybridized with other probes as described above.

cDNA probes

The 1.4 kb fragment of chicken skeletal α-actin cDNA (a generous gift from Dr. Ordahl, Dept. of Antomy, UCSF) was purified after EcoRI and BamHI digestion. ANF cDNA (a generous gift form Dr. Chien, Dept. of Medicine, UCSD) probe was prepared by PstI and HindIII digestion (620 bp). Porcine TGF-β1 (PstI fragment, 0.8 kb) probe was obtained from Dr. Gibbons (Cardiovascular Medicine, Stanford University). Rat ACE cDNA (a generous gift from Dr. R. Pratt, Cardiovascular Medicine, Stanford University) probe (0.6 kb) was prepared by EcoRI-EcoRV digestion. Rat glyceraldehyde 3-dehydrogenase probe (1.0 kb) was synthesized by RT-PCR (primers were TCT TCT TGT GCA GTG CCA GC and CTC TCT TGC TCT CAG TAT CC, 13) using cDNA synthesized from rat liver RNA. All probes were labeled using random primer method in the presence of ^{32}P-α-dCTP.

Results

Experimental data in the 4 different groups are presented in Table 1. Each group consists of 4-6 animals. There was no difference in the initial weights in the four groups. The weight measured on the day of catheterization was considered as a final weight since the surgical procedure of placing arterial catheter caused weight loss in all four groups. The final weight in U was significantly lower than that of C and hydralazine and erythropoietin (EPO) treatments did not correct the growth failure of uremia. Both diastolic blood pressure (BP) and mean arterial pressures (MAP) were significantly ($P < 0.01$) higher (20-30%) in U and UE compared with C whereas both pressures in UH were normalized to the level of C. Hemoglobin level of U was 15% lower than that of C ($P < 0.01$), and EPO treatment corrects this to the C level. Serum creatinine levels of U, UH and UE were significantly higher than those of C ($P < 0.01$).

Organ weights of each group are presented in Table 2. Heart weight/body weight (HW/BW) and left ventricular weight/body weight (LV/BW) ratios in the different groups are presented in Figure 1A and 1B, respectively. Group U, UH and UE had 33%, 20% and 47% higher in HW/BW and 77%, 46%, and 38% higher in LV/BW compared with group C ($P < 0.01$), respectively. Compared with U, UH had lower HW/BW (10%) as well as lower LV/BW (18%) and UE had 22% lower LV/BW without a change in HW/BW compared with group U ($P < 0.05$). Thus all experimental groups have cardiac hypertrophy and LVH compared with controls and treatments of hypertension and anemia corrected LVH partly but not completely. Right ventricular weight/body weight (RV/BW) and weights of other organs (liver and spleen) in the 3 experimental groups are not different from those of controls. Remnant kidneys of experimental groups were hypertrophied to the original size which was in agreement of observation reported by others (Packham *et al.,* 1992).

Table 1. Effects of nephrectomy and hydralazine and erythropoeitin (EPO) treatments on blood pressures and chemistry.

Group ID	Initial Weight (g)	Final Weight[a] (g)	Weight Gain (g)	Diastolic BP (mmHg)	MAP (mmHg)	Serum Creatinine (mg/dl)	Hemoglobin (mg/dl)
Control (C)	136±4	322±7	186±6	116±1	126±2	0.36±0.08	15.86±0.52
Uremic (U)	136±4	285±9*	149±12*	141±6*	152±5*	1.34±0.24*	13.43±0.81*
U + Hydralazine(UH)	134±3	294±21*	160±19*	108±3	119±2	1.13±0.08*	ND
U + EPO (UE)	145±1	277±22*	133±23*	124±17*	138±12*	1.10±0.15*	15.48±1.29*

Data = Mean ± SD. *$P < 0.01$ vs. C. a: The weight measured on the day of catheterization (3 days before sacrifice). MAP, mean arterial pressure; ND, not determined.

Table 2. Effects of nephrectomy and hydralazine and erythropoeitin (EPO) treatments on organ weight.

Group ID	Heart weight (g)	LV weight (g)	RV weight (g)	Liver (g)	Spleen (g)	Renmant kidney (g)
Control (C)	0.97±0.05	0.43±0.05	0.19±0.02	9.22±0.82	0.70±0.01	1.10±0.09
Uremic (U)	1.15±0.08*	0.65±0.07*	0.18±0.02	8.11±0.67	0.71±0.08*	1.14±0.21
U+Hydralazine (UH)	1.05±0.1*	0.55±0.09*	0.15±0.02	8.19±0.65	0.89±0.13	1.36±0.10
U+EPO (UE)	1.23±0.18*	0.51±0.05*	0.17±0.07	7.51±1.31	ND	1.25±0.15

Data = Mean ± SD. *$P < 0.01$ vs. C. ND, not determined.

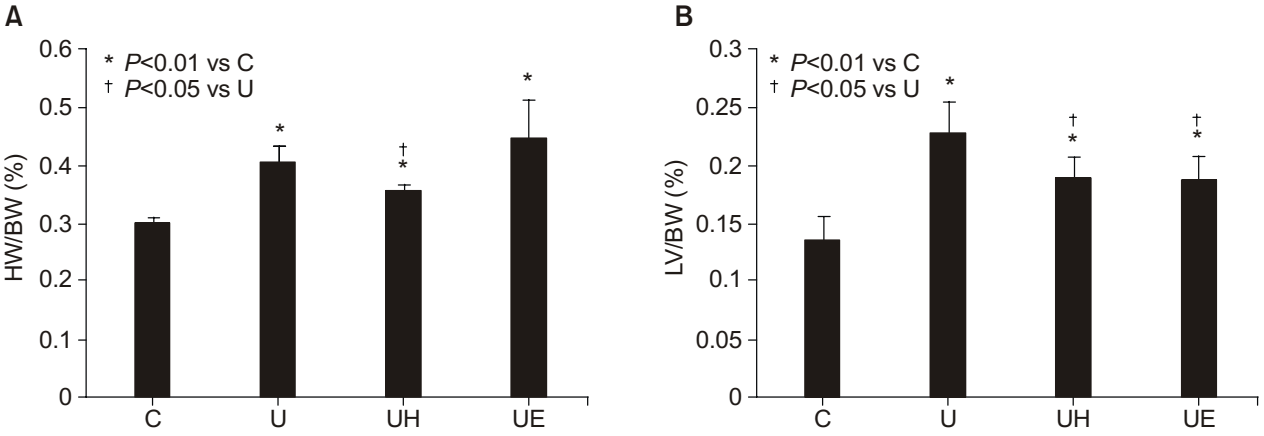

Figure 1. Effects of hydralazine or erythropoietin on weights of total heart and left ventricle of uremic rats. Chronic uremia was induced after 6 weeks after 5/6 nephrectomy of SD rats (U). The nephrectomized rats were administered with either hydralazine (UH, 100 mg/kg/day) in drinking water or erythropoietin (UE, 48 U/kg/week, i.p.) during induction of chronic uremia. Total heart and LV were weighed after sacrifice. Heart weight/body weight ratios (HW/BW) and left ventricular weight/body weight ratios (LV/BW) are presented. $n = 7$ for each group. *: $P < 0.01$ vs C and † : $P < 0.05$ vs U.

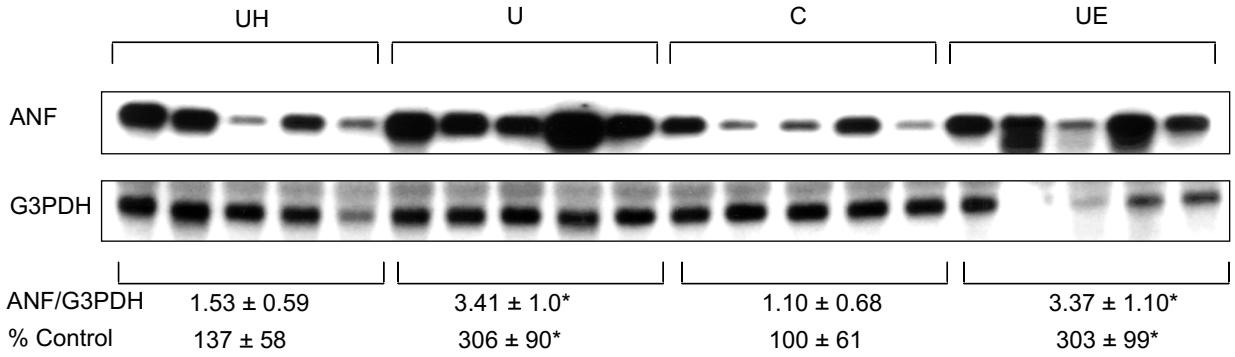

Figure 2. Northern blot analysis of ANF. Left ventricles (LV) were dissected from uremic rats (U), U treated with hydralazine (UH) or erythropoietin (UE) and sham-operated control (C) rats. Total RNA (20 μg) were separated on 1% formaldehyde-agarose gel and transferred onto nylon membrane. The membrane was probed with ^{32}P-labeled cDNA fragment (620 bp) of ANF or G3PDH (1.0 kb). The band intensities of ANF on X-ray film was quantified by densitometry and normalized by G3PDH. Mean ± SD of ANF/G3PDH ratios and % control are presented. *: $P < 0.05$ vs C.

To see if the gene expression in uremic LVH is similar to that described in pressure overload LVH, mRNA levels of atrial natriuretic factors (ANF), angiotensin converting enzyme (ACE), transforming growth factor-β1 (TGF-β1), and skeletal α-actin in LV were measured by Northern analysis. As shown in

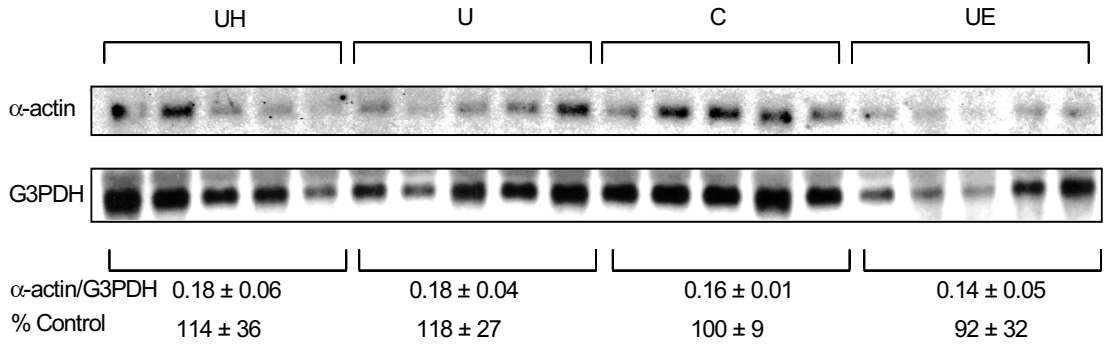

Figure 3. Expressions of α-actin in LV were analyzed by Northern blot. Total RNA (20 μg) from LVs of U, UH, UE, and C groups were separated on 1% formaldehyde-agarose gel and transferred onto nylon membrane. The membrane was probed with ^{32}P-labeled cDNA fragment (1.4 kb) of α-actin or G3PDH (1.0 kb). The band intensities of α-actin on X-ray film was quantified by densitometry and normalized by G3PDH. Mean ± SD of α-actin/G3PDH ratios and % control are presented.

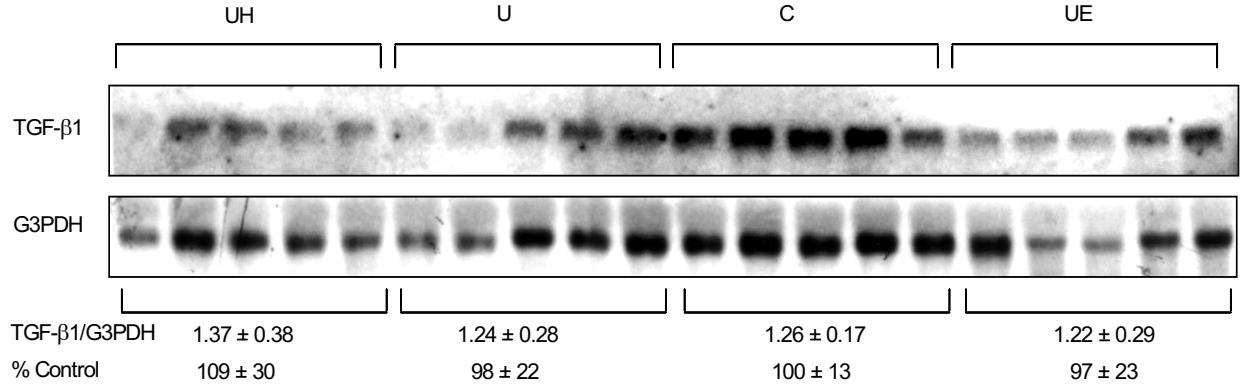

Figure 4. Northern blot analysis of TGF-β1. Total RNA (20 mg) from LVs of U, UH, UE, and C groups were separated on 1% formaldehyde-agarose gel and transferred onto nylon membrane. The membrane was probed with ^{32}P-labeled cDNA fragment (0.8 kb) of TGF-β1 or G3PDH (1.0 kb). The band intensities of TGF-β1 on X-ray film was quantified by densitometry and normalized by G3PDH. Mean ± SD of TGF-β1/G3PDH ratios and % control are presented.

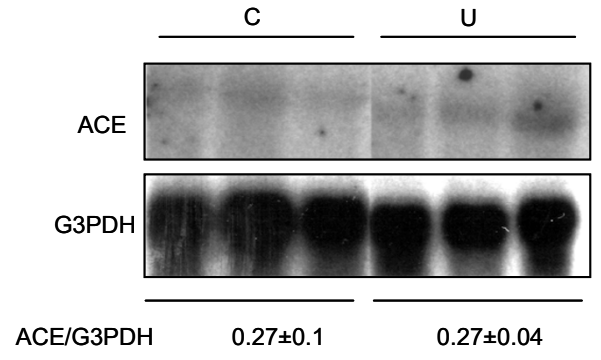

Figure 5. Northern blot analysis of ACE. Left ventricles were dissected from uremic rats (U) or sham-operated control (C) rats. Total RNA (20 μg) were separated on 1% formaldehyde-agarose gel and transferred onto nylon membrane. The membrane was probed with ^{32}P-labeled cDNA fragment (0.6 kb) of ACE or G3PDH (1.0 kb). The band intensities of ACE on X-ray film was quantified by densitometry and normalized by G3PDH. Mean ± SD of ACE/G3PDH ratios and % control are presented.

Figure 2, ANF mRNA in group U and UE were increased 3 fold over controls ($P < 0.01$), and normalized in UH. Thus, the treatment of anemia with EPO did not change the steady state ANF mRNA level despite it significantly decreased LV mass. On the other hand, the treatment of hypertension by hydralazine normalized ANF mRNA but did not totally correct uremic LVH. Skeletal α-actin mRNA was not changed in all 4 groups (Figure 3). In other words LVH was observed in all uremic groups (U, UH, UE) without an increase of skeletal α-actin gene expression. Furthermore, the levels of TGF-β1 and ACE mRNA in uremic LV were not different from the control (Figure 4 and 5). Therefore we concluded the gene expressions in uremic LVH were distinct from that in pressure-overload LVH and other unidentified factor(s) might be involved in uremic LVH.

Discussion

Ventricular myocytes lose the ability to undergo cell division soon after birth and further growth takes place by enlargement or hypertrophy of existing cells. The enlargement of individual myocytes in LV has been considered an adaptation to increased blood pressure. However, non-hemodynamic factors may also be involved in the pathogenesis of LVH since it can be found in patients with only mild hypertension (Martinez *et al.,* 2003). Cardiac hypertrophy has been studied using 3 representative models: aortic coarctation, monolayer cells cultured on stretchable mesh, and cardiac myocyte culture stimulated with different trophic factors. Even though many factors and genes have been identified using these models, the precise function of the factors and genes in hypertrophy is yet understood. It is not clear whether the increases of these factors and mRNAs are the cause of LVH or the simple response of stimuli which are not related with hypertrophy.

Rambausek *et al.* reported that heart weight was increased in uremia despite correction of hypertension with different hypertensive agents or anemia by blood transfusion (Rambausek *et al.,* 1985). Increase of heart weight in uremia was due to neither the increased water or lipid content of heart nor an increase of interstitial space. All myocardial fiber components were qualitatively (not quantitatively) increased in uremic heart compared with contractile proteins when examined by transmission electromicroscopy. Fast-migrating V1 isomyosin was increased in uremia. In end-stage-renal disease the elevated serum hepatocyte growth factor (Malatino *et al.,* 2003) or calcium homeostasis (Kennedy *et al.,* 2003) seemed to be associated with LVH. However, the mechanism which this occurs and the major players in the pathogenesis are still unclear. Our study demonstrated that treatments of arterial hypertension and anemia partially ameliorated but did not completely correct cardiac and left ventricular hypertrophy in uremic rats, which was in an agreement with the results presented by others (Rambausek *et al.,* 1985; Kennedy *et al.,* 2003).

The mRNA expression of ANF, ACE, and skeletal α-actin are increased in other models, thus these genes have been cited as hypertrophic genetic markers (Komuro and Yazaki, 1993; Patel *et al.,* 2000; Schultz *et al.,* 2002; Suurmeijer *et al.,* 2003). We found a different pattern of gene expression in uremic LVH. In LV from 5/6 nephrectomized rats, ANF mRNAs were markedly increased over controls and almost normalized after hydralazine treatment. However, treatment of anemia with erythropoietin did not normalize ANF mRNA although in erythropoeitin improved LVH to the same extent as hydralazine did. Furthermore, skeletal α-actin mRNA levels in uremia were not different from controls. Skeletal α-actin mRNA was reported to be accumulated in hypertrophied rat heart 4 days after aortic coarctation, then slowly declined to normal level in 1 month even though heart was hypertrophied (Schwartz *et al.,* 1986). Since the uremic model used in this study was studied 6 weeks after nephrectomy, it was possible we simply missed the peak point of accumulation. It was also showed, however, that skeletal α-actin was persistently expressed more than the controls up to 30 days after coarctation (Black *et al.,* 1991) and it was unlikely we missed the time point of accumulation of skeletal α-actin mRNA. Therefore, we concluded the gene expression patterns in uremic LVH were quiet different from other LVH models, especially pressure-overload one. Another piece of evidence to support the conclusion is that ACE and TGF-β1 mRNA level is not distinguishably changed in uremia. ACE activity and mRNA levels were significantly higher than those of controls 8 weeks after aortic stenosis (Schunkert *et al.,* 1990). Ang II, which is produced by ACE, induced TGF-β1 activity and gene expression, then modulated vascular smooth muscle cell hypertrophy or hyperplasia (Gibbons *et al.,* 1992). Ang II induced cardiac hypertrophy and hyperplasia, many immediate-early genes (*c-fos, c-jun, jun B, Egr-1,* and *c-myc*), and "late" markers for cardiac hypertrophy, skeletal α-actin and ANF, and TGF-β1 expression (Sadoshima and Izumo, 1993). Experiments using TGF-β1-deficient mice demonstrated that TGF-β1 was required for Ang II-induced LVH and ANF expression (Schultz *et al.,* 2002).

We assumed at first if uremic hypertrophy followed the same mechanism as the pressure-overload hypertrophy, their gene expression patterns in LV should be similar to each other. Surprisingly, the expressions of marker genes for pressure-induced hypertrophy were different. Especially, TGF-β1, ACE and skeletal a-actin in LV were not altered at all. ANF was increased in uremic LV, but its expression level was out of accordance with LV size change. We concluded that the mechanism of uremic hypertrophy is different from that of hemodynamic one and the known genetic markers for pressure overload LVH are not applicable to uremic LVH. Furthermore, unidentified uremic factor(s) would be necessary to develop uremic hypertrophy, opening a further study.

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