End-organ damage in hypertensive transgenic Ren-2 rats: influence of early and late endothelin receptor blockade

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Abstract

The rat strain transgenic for the murine Ren-2 renin gene (TGR) is defined as a monogenic model of angiotensin II-dependent hypertension with endogenous activation of the renin-angiotensin system. Homozygous males TGR develop malignant hypertension with a strong salt sensitive component. These animals show severe hypertension, proteinuria and high mortality. Morphological changes of renal parenchyma corrrespond to chronic ischemic glomerular changes. Heterozygous TGR develop only mild hypertension and thus provide a more suitable model of hypertension regarding to clinical studies. Within the renal parenchyma, secondary focal segmental glomerulosclerosis (FSGS) predominates. High-salt diet in heterozygous animals induces transition from benign to malignant phase of hypertension. In this case, ischemic glomerular changes are superimposed on preexisting secondary FSGS. In the regression model of hypertesion (late-onset treatment) the effect of salt intake is attenuated.

In homozygous TGR, early selective ET_A receptor blockade decreased blood pressure and ameliorated end-organ damage. Late selective ET_A receptor blockade reduced podocyte injury despite final severe hypertension. Survival rate was markedly improved in both regimens with ETA selective blockade, while only partly with early non-selective blockade. Both bosentan and atrasentan decreased ET-1 levels in both regimens.

In heterozygous TGR, early and late ET_A treatment substantially while ET_A/AT_B partly improved survival rate. Significant effect on BP was found with early and late ET_A blockade, while ET_A/ET_B blockade had no effect. Bosentan and atrasentan similarly decreased ET-1 levels on both regimens.

In conclusion, selective ET_A receptor blockade is superior to nonselective ET_A/ET_B receptor blockade in attenuating hypertension and end-organ damage. Its effect is more pronounced when adapted early in the life.

Introduction

Although the treatment of hypertension has significantly advanced in the recent decades, chronic elevation of blood pressure still results in progressive renal damage as evidenced by escalating incidence of end-stage renal disease (ESRD). Hypertension as a disease category is the second leading cause of ESRD following diabetes.

The transgenic rat strain TGR(mRen2)27 (TGR) is the first successful transgenic hypertensive rat model, in which the development of hypertension is a result of insertion of a single gene, namely the mouse Ren-2 renin gene into the rat genome (Mullins *et al.* 1990). Thus, TGR represents a well defined monogenic model of angiotensin II-dependent hypertension with endogenous activation of the renin-angiotensin system (Jacinto *et al.* 1999).

Endothelin (ET) is known to be one of the most powerful vasoconstrictors (Yanagisawa *et al.* 1988). The action of ET is mediated by the activation of two G-protein-coupled receptor subtypes ET_A and ET_B. ET_A receptors localized on vascular smooth muscle cells cause vasoconstriction, while ET_B receptors on endothelial cells exert largely vasodilatation. Numerous studies have shown that ET system plays an important role in the pathogenesis of high blood pressure and associated end-organ damage in salt-sensitive models of hypertension (Roux *et al.* 1999). Animal models of angiotensin-dependent hypertension demonstrate further elevation in arterial blood pressure and increased risk for end-organ damage when fed a high-salt diet (Callahan *et al.* 1996, Floege *et al.* 1992).

The primary morphological lesions in hypertension are vascular, so that glomerular damage is the major pathological event leading to renal insufficiency. Hypertension causes mesangial cell growth and glomerulosclerosis. A variety of mechanisms have been suggested to explain glomerulosclerosis. Exuberant mesangial and/or interstitial cell proliferation with subsequent matrix deposition has been proposed as a central mechnisms

by a number of researchers (Floege *et al.* 1992, Kashgarian and Sterzel 1992). Later, podocyte injury and loss were established as crucial mechanism in the development of focal segmental sclerosis (FSGS), either primary or secondary (Pavenstädt *et al.* 2002). Adhesion of podocytes to Bowman's capsule is critically associated with podocyte damage. Characteristic changes of filtration barier and podocytes are best evaluated at the ultrastructural level using transmission electron microscopy or immunohistochemically using various podocyte markers.

The purpose of this review was to compare the efficacy of selective versus nonselective endothelin receptor blockade applied at various ages in the homozygous or heterozygous lines of Ren-2 TGR from the morphological point of view.

Rat models

Rats from all hypertensive models exhibit cardiac hypertrophy and demonstrate impaired endothelium-dependent relaxation of isolated arteries (Pinto *et al.* 1998). However, more severe forms of end-organ damage such as heart failure, stroke and kidney failure, occur only in subset of them. Rat hypertensive models can be devided into a low renin form, with salt and water retention (DOCA-salt rats (Gomez-Sanchez *et al.* 1996), Dahl salt-sensitive rats (Meneely and Ball 1958), Lyon hypertensive rats (Lantelme *et al.* 1997), Milan hypertensive rat (Ferrari *et al.* 1987) and high renin form (SHR (Okamoto 1963), two-kidney one-clip model (Koletsky *et al.* 1971), TGR (Mullins *et al.* 1990) which is amenable to RAS inhibition but responds poorly to salt and water restriction.

All strains mentioned above displayed the same morphological changes leading to glomerulosclerosis. Only the degree of morphological changes is markedly different between them. Glomerulosclerosis produced closely resembles the lesions of FSGS. Acute ischemic changes of glomeruli are only sporadically present in models with accelerated

hypertension (homozygous TGR and models with salt-sensitive component on high salt diet (heterozygous TGR, DOCA). It should be mentioned out that morphological descriptions of renal parenchyme is mostly restricted to glomerulosclerosis.

Homozygous TGR

Homozygous male TGR is generally accepted as an animal model of malignant hypertension (Langheinrich *et al.* 1996, Mullins *et al.* 1990). The affected rats have highly elevated blood pressure, typical renal morphological changes and die at 50-90 days of age. Malignant (accelerated) hypertension is mostly accompanied by organ damage including kidney and heart. Hematuria is present in part of animals, significant proteinuria and high serum creatinine levels are also found (Dvořák *et al.* 2004, Whitworth *et al.* 1995). General morphological glomerular changes may be acute or chronic. Acute changes are focal, while majority of glomeruli look unchanged. The most characteristic change is fibrinoid necrosis of capillary loops, usually segmental, accompanied often by mesangiolysis and followed by crescent formation. Glomeruli with thickening of capillary walls due to duplication of glomerular basement membrane (GBM) and subendothelial widening are occasionally seen. Uninvolved glomeruli show marked congestion and dilatation of the capillary lumina. Chronic glomerular changes are of two types. The classical lesions are ischemic with shrinkage of the glomerular tuft, narrowing of the capillaries with wrinkling of their walls and collapse with filling of Bowman's space with collagen, or with subendotelial widening of GBM accompanied by collagenization of Bowman's space. Glomerular changes in homozygous male TGR mostly correspond to the above described chronic ischemic changes represented by shrinkage of the tuft and collapse (Fig. 1A). Acute changes with fibrinoid necrosis and crescent formation were seen only sporadically. A part of glomeruli showed segmental sclerosis and represented probably benign hypertension

occuring before the onset of malignant phase (Dvořák *et al.* 2004, Vaněčková *et al.* 2005, Opočenský *et al.* 2006).

Ultrastructural studies of acute glomerular changes demonstrate dense amorphous material in the area of fibrinoid necrosis. The mesangium may be intact or show areas of loosening of the matrix, sometimes with finely granular material. The loss of attachment of the GBM to the matrix denudes areas of mesangiolysis. Endothelial cells may be swollen, in more severely affected areas they may be necrotic. The widened subendothelial space contains floculent material of variable density. The glomeruli with long-term changes show wrinkling of the GMB which becomes both thicker and denser. In the resting glomeruli podocyte changes dominate. Regressive changes of podocytes are characterized by microvillous transformation, fusion of their foot processes and vacuolization of cytoplasm with numerous resorption droplets (Fig. 1B) (Opočenský *et al.* 2006). Injured podocytes detache from the basement membrane and die.

Tubulointerstitial changes vary with the severity of renal involvement. Early in the course, cellular changes range from minimal alterations to severe cell swelling or to individual cell necrosis. Only in sudden onsets of accelerated hypertension patchy necrosis of tubules may be seen. The tubules are sometimes separated by markedly edematous interstitium. The tubules in either benign or malignant hypertension share the same morphological changes, they may be atrophic and sometimes contain hyaline casts. Majority of tubules is not affected. The interstitium is widened in areas with atrophic tubules. Moderate chronic inflammatory cells are dispersed in the areas of scaring. Often, there is the transdifferentiation of tubular cells, which may express vimentin or other mesenchymal markers (Vernerová *et al.* 2008). There is also some evidence that transdifferentiated tubular cells may contribute to fibrogenesis in later stages

(Vongwiwatana *et al.* 2005, Strutz *et al.* 1994). The smaller of the interlobular arteries may also show fibrinoid necrosis, which is usualy a segmental change, and onion skin lesions.

Heterozygous TGR

Heterozygous male TGR provide a suitable model of hypertension, since in contrast to homozygous animals, which develop severe malignant hypertension, their hypertension is milder, thus allowing long-term studies. Such experiments are much closer to a clinical situation. Heterozygotes showed target organ damage in hearts and kidneys but no other (functional or morphological) characteristics of accelerated hypertension were found (Opočenský *et al.* 2004). Blood pressure, proteinuria, serum creatinine level and cardiac hypertrophy were only moderately increased. The earliest morphological hypertensive changes are ischemic, represented by two distinct glomerular lesions, a classical one with a collapse of capillary loops and GBM wrinkling (Fig. 1C), or a more recently recognized type – focal segmental glomerulosclerosis, often associated with hyalinosis (Fig. 1D). The glomeruli are slightly enlarged, but with progression of the disease there is an augmentation of the mesangial matrix and the process of sclerosis typically begins adjacent to the hilus with adhesion between the capillaries and Bowman's capsule. The sclerosis, which is starts as focal one, finally involves the whole glomerulus. The same morphological picture was seen in our studies where FSGS predominated (Vaněčková *et al.* 2006, Vernerová *et al.* 2008).

Tubulointerstitial changes did not differ from morphological changes in homozygous animals, but tubular atrophy is milder and ischemic tubular necrosis is not present. Vascular changes differ with the size of the vessel involved and also differ among individual animals. Arcuate and larger arteries show the alteration typical for atherosclerosis manifested by fibrous intimal thickening that results in the reduction of the

vessel lumen. The internal elastic membrane may show splitting. Elastic reduplication is particularly prominent in the cases with a prolonged period of benign hypertension before the onset of malignant one.

High-salt diet (HSD) augmented blood pressure and allowed the onset of the malignant phase of hypertension in heterozygous male TGR (Dvořák *et al.* 2004). The cause of the switch from benign to malignant hypertension is not yet fully understood. Many authors have suggested that the renin-angiotensin system is important for this, namely that inappropriate activation of RAS contribute to hypertension. HSD increases mortality, proteinuria, serum creatinine level and deteriorates cardiac and renal injury. Morphological picture of the renal tissue corresponds to accelerated hypertension with preceding period of benign hypertension characterized by secondary FSGS. Chronic ischemic changes in accelerated phase are augmented and the number of glomeruli with advanced sclerosis is increased (Vaněčková *et al.* 2006). Acute changes were not present. Tubular changes also progressed.

ET blockade in homozygous TGR

Prevention study (Fig 2, left panel) („**early treatment**" started at the age of 29 days, ended at the age of 90 days) in homozygous male Ren-2 transgenic rats fed a high-salt diet has demonstrated that the pharmacological blockade either with non-selective ET_A/ET_B or with selective ET_A receptor improved mortality (52% in untreated, 21% in bosentan-treated and 8% in atrasentan-treated TGR) (Fig.2A) and renal and left ventricular structure. However, only ET_A receptor blockade had antihypertensive effects (systolic BP 236 mmHg in untreated, 228 mmHg in bosentan-treated and 190 mmHg in atrasentan-treated TGR) (Fig.2B). Both nonselective and selective ET_A/ET_B receptor blockers substantially reduced ET levels in the left ventricle (Fig. 2C) (Vaněčková *et al.* 2005).

When the treatment was begun after substantial hypertension had already developed (**regression study)** (Fig 2-right panel), (**„late treatment“** started at the age of 51 days, ended at the age of 90 days), untreated homozygous animals showed a high mortality, podocyte injury, thickening of GBM and proteinuria but not yet glomerulosclerosis (Opočenský *et al.* 2006). Although there was a temporary drop of blood pressure in ET_A antagonist-treated rats, there was no difference in the hypertension between different treatment groups of animals at the end of experiment. Surprisingly, only selective blockade of the ET_A receptor but not combined ET_A/ET_B receptor blockade had striking effect on survival and elevated cardiac ET levels. Moreover, ET_A receptor blocker (atrasentan) almost normalized glomerular barrier and prevented or even reversed podocyte injury and proteinuria (Opočenský *et al.* 2006).

ET blockade in heterozygous TGR

Prevention study in heterozygous male TGR (Fig 3, left panel) (Vaněčková *et al.* 2006) showed similar results as with homozygous animals. We confirmed that the treatment with either non-selective ET_A/ET_B or selective ET_A receptor blocker improved survival, cardiac hypertrophy, glomerulosclerosis and left ventricular ET-1 tissue concentration, whereas only ET_A receptor blockade reduced proteinuria and hypertension.

Regression study in heterozygous TGR (Fig 3, right panel) also showed similar effect of ET receptor blockade. Similarly, in this experiment ET_A blockade was superior to ET_A/ET_B blockade. ET_A blockade improved not only biochemical parameters, blood pressure and proteinuia, but also almost restored podocyte structure and reversed podocyte phenotype changes represented by the expression of CD 10, desmin and vimentin (Vernerová *et al.* 2008).

It is difficult to explain small efficacy of ET_A/ET_B receptor blocker (bosentan) in heterozygous animals. We did not confirm our previous results (Opočenský *et al.* 2004) where bosentan markedly improved survival, proteinuria, glomerulosclerosis and cardiac hypertrophy, except for the fact that our last studies (Vaněčková *et al.* 2006, Vernerová *et al.* 2008) lasted only half the time of the previous one (90 vs 180 days of age).

Role of salt

Dietary sodium plays an important role in the pathogenesis of hypertension not only in humans (Weinberger 1996) but also in salt-sensitive models of hypertension (Opočenský *et al.* 2004, Dahl *et al.* 1968). Ren-2 transgenic rats represent a model with a strong salt-dependent component. In our previous study (Vaněčková *et al.* 2006) HSD applied to young animals significantly accelerated the development of hypertension in heterozygous rats and worsened all measured parameters except for the blood pressure at the end of experiment. We supppose that one reason for this could be that sudden onset of hypertension in animals on high-salt diet may cause severe organ damage in this time and this could lead to the worsening of renal parameters. Animals on normal salt diet, in which the BP rise is gradual, can probably better adapt to hypertension, which results in milder organ damage. Interestingly, we found great similarities (Vernerová *et al.* 2008, Vaněčková *et al.* 2006) when comparing our data from regression protocol of heterozygous TGR on high-salt diet with those obtained from TGR on normal salt diet. These results are in agreement with those of Chuang et al (Chuang *et al.* 1993), who also used old animals with established hypertension (3-5 months old). We suppose that the effect of salt component in regression model is attenuated. One explanation for this could be the fact that young animals are more susceptible to hypertensive injury (Zícha and Kuneš 1999). On the other hand, it should also be mentioned that in this type of experiment the increase

in blood pressure is gradual and hypertensive acceleration due to high salt diet is attenuated.

Role of endothelin blockade

We found similar changes in male heterozygous as in male homozygous TGR with early-onset and late-onset ET_A receptor blocker treatment, i.e. decline in blood pressure seen as early as one week after the beginning of the treatment, an almost identical survival and similar changes in proteinuria. On the other hand, cardiac and kidney hypertrophy were slightly greater together with higher tissue ET-1 concentrations in our study with the early-onset ET receptor blockade (Vaněčková *et al.* 2006), probably reflecting high dietary sodium exposure of these rats earlier in life, which causes marked acceleration of hypertension (Zícha and Kuneš, 1999). We have repeatedly confirmed the efficacy of selective ET_A receptor blockade over non-selective blockade in Ren-2 rats (Vaněčková *et al.* 2006, Vaněčková *et al.* 2005), which is probably due to the fact that non-selective receptor blockers inhibit concomitantly not only vasocontrictor ET_A receptors but also ET_B receptors mediating vasodilatation and natriuresis. The blood pressure lowering effect of ET_A receptor blockers was shown in several other rat strains and was usually accompanied by antiproteinuric actions, which has been explained by the effect on preserving the integrity of podocytes. The exact mechanism(s) leading to the reduction of proteinuria by ET_A receptor blocade are not known yet.

Podocyte damage

Podocytes are terminally differentiated epithelial cells which serve several function including regulation of glomerular permselectivity (Tryggvason and Wartiowaara 2001), structural support for glomerular capillaries together with mesangial cells, remodeling of

glomerular basement membrane (GBM) in cooperation with endothelial and mesangial cells (St John and Abrahamson 2001) and endocytosis of filtred proteins (Ina *et al.* 2002). Podocyte is a primary target of injury in many types of proteinuric glomerular diseases including diabetic nephropathy, membranous nephropathy, minimal change disease and FSGS. Podocytes are probably primarily or secondarily injured by mechanical forcesresulting from glomerular capillary hypertension. Most of the morphological patterns of renal injury do not correspond to a single specific disease entity, i.e. different etiology might cause the same histological picture. Durvasula and Shankland (Durvasula and Shankland 2005) showed that the resulting mechanical stress experienced by podocytes initiates a series of maladaptive response including impaired function, apoptosis and detachement. We found first podocyte impairment immuno-histochemically even before the onset of ultrastructural changes (Vernerová *et al.* 2008). We confirmed results of Nagase (Nagase *et al.* 2006), who reported first immunological changes of podocyte in Dahl sensitive rats at the time, when proteinuria was only moderate. Ultrastructural changes followed three weeks later. Pugliese et al (Pugliese *et al.* 2007) showed the same results in Milan hypertensive strain where synaptopodin and nephrin expression were reduced without morphological or ultrastructural podocyte changes. We suppose that pathologic changes of podocytes and of GBM occur early in the course of some diseases connected with nephrotic syndrome (not only of diabetic nephropathy but also of hypertension) and may play an important role for albuminuria development.

Conclusion

Structural changes in men vary depending on the form and duration of hypertension. Accelerated (malignant) hypertension is usually preceded by a benign form, although it may arise de novo. In accelerated phase of hypertension, glomerular changes

are more severe in men. Usually, at least one-third of glomeruli disclose fibrinoid necrosis of their capillaries, mesangium and associated afferent arteriole. Chronic changes in malignant hypertension found in Ren-2 TGR model are mostly those seen in benign hypertension, in that there is a collapse of the capillary tuft with wrinkling of the GBM. Second type of the collapse (almost acellular, collagenized glomeruli) was found only sporadically.

Two basic types of glomerulosclerosis are seen in benign hypertension, the first is ischemic, classical lesion associated with the collapse of capillary loops. The second type of glomerular lesion corresponds to FSGS. It is similar to the primary FSGS associated with nephrotic syndrome, as well as multiple secondary forms. These lesions are similar in both species.

Emerging data from human biopsy samples and animal models suggest that podocyte injury following various insults (irrespective whether primary or secondary) causes similar morphologic features. Summarizing our results in Ren-2 TGR animals we propose the following sequence of morphological changes of podocytes:

1. *Adaptive changes of podocytes* with normal glomerular pattern in the light microscope, focal segmental foot processes fusion ultrastructurally. Only histochemical changes are present due to the impairment of proteins of filtation barrier and cytoskeleton.

2. *Phenotypic changes of podocytes:* the injury is reflected in altered cell morphology due to the diffuse fusion of foot processes and regressive changes of cytoplasm. These changes are obvious on the ultrastructural level.

3. *Depletion of podocytes*: injured podocytes detach from the underlying GBM and die.

4. *Podocyte death* is followed by synechia formation and accumulation of extracellular matrix, which corresponds to focal segmetal glomerulosclerosis.

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Figure legend:

Fig. 1A. Glomerulus with wrinkling of glomerular basement membrane, upper right (PAS, x 400). **1B**. Electron micrograph showing regressive changes of podocytes. Podocytes with foot process effacement and a lot of droplets within the cytoplasm. (x 7 100). **1C.** Glomerulus with collapsing capillary loops and wrinkling of the glomerular basement membrane (PAS, x 400). **1D.** Glomerulus with focal segmental sclerosis of capillary loops. (PAS, x 400).

Fig. 2. Survival rate (A), systolic blood pressure (B) and ET-1 concentration in the left ventricle (C) in **homozygous** Ren-2 transgenic rats.

Fig. 3. Survival rate (A), systolic blood pressure (B) and ET-1 concentration in the left ventricle (C) in **heterozygous** Ren-2 transgenic rats.

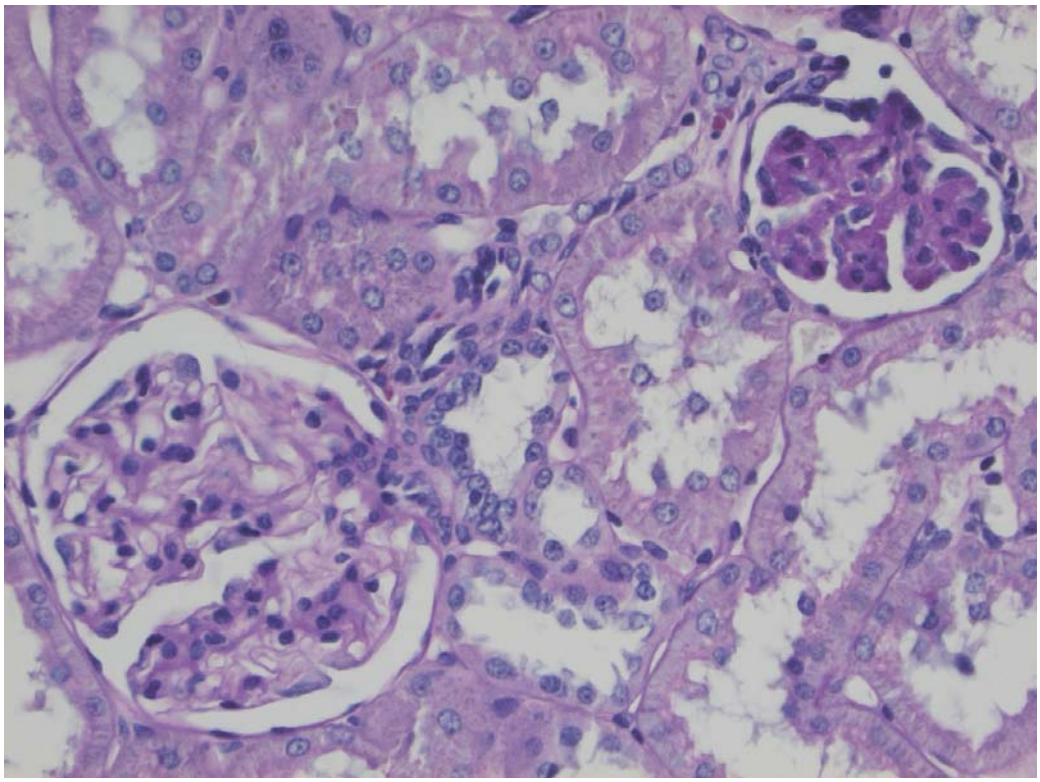

Fig. 1A

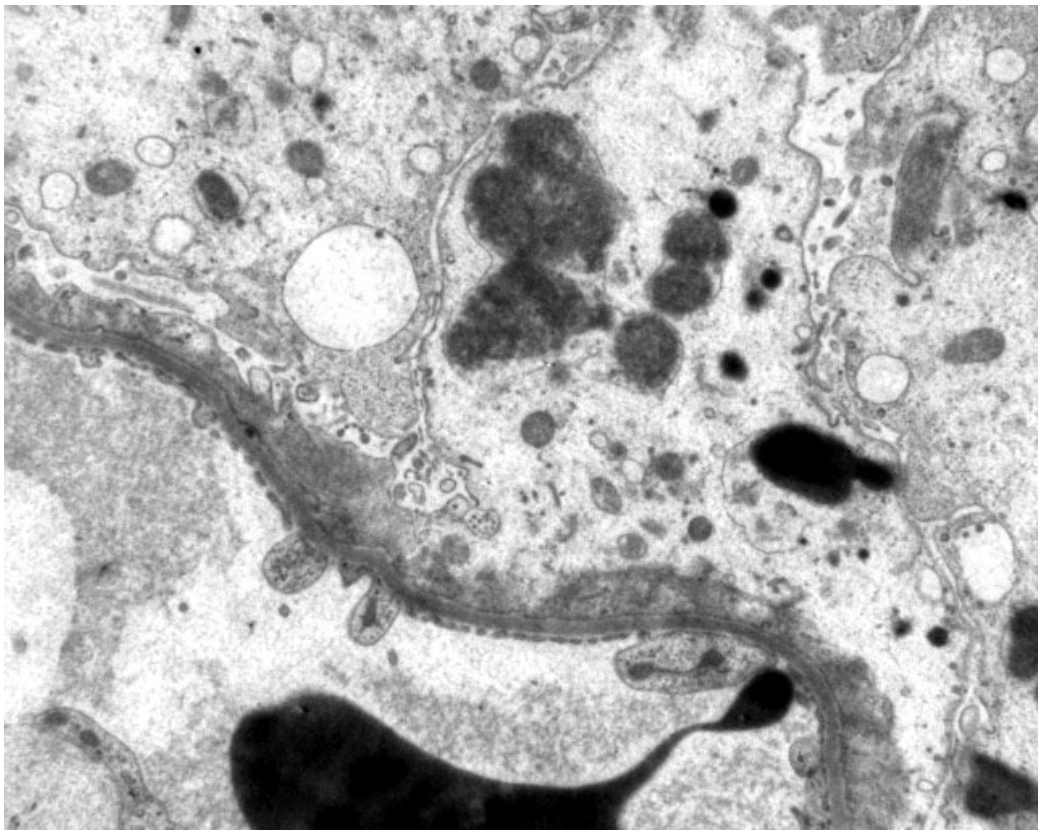

Fig. 1B

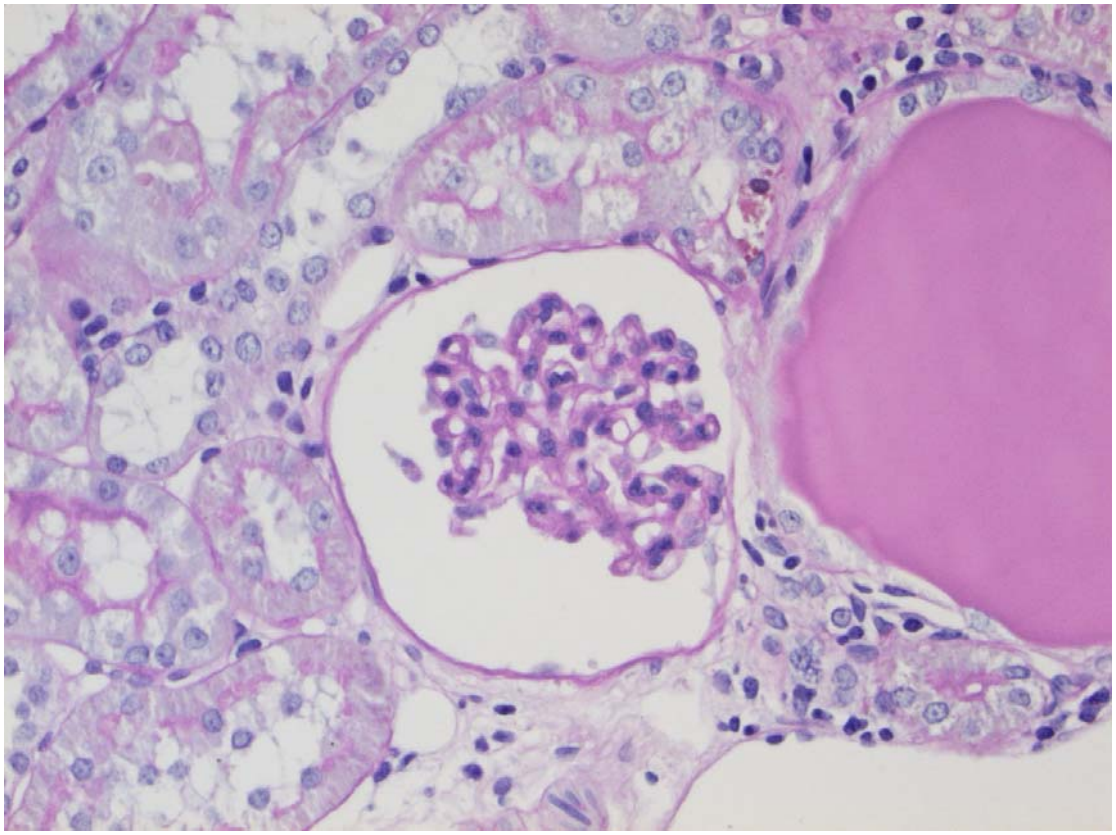

Fig. 1C

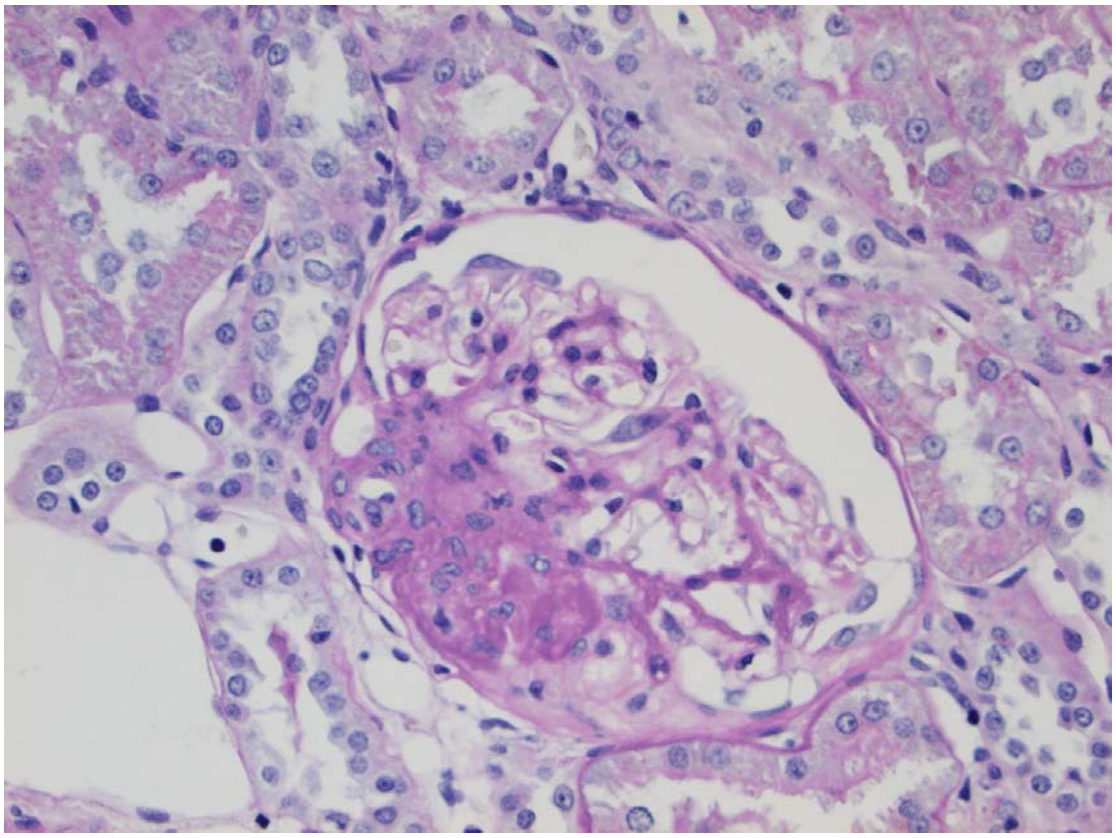

Fig. 1D

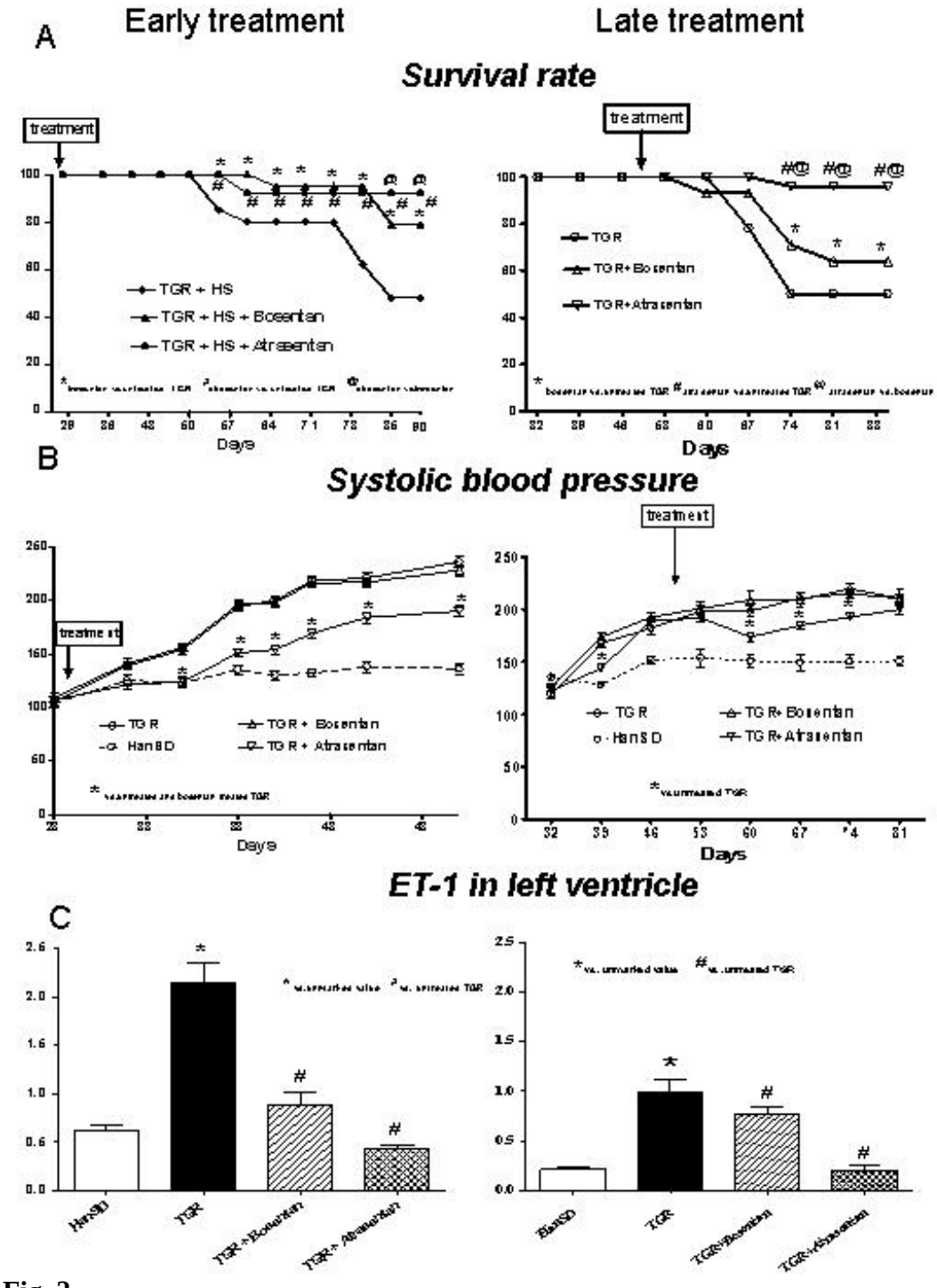
A
Early treatment
Late treatment
Survival rate
treatment
TGR + HS
TGR + HS + Bosentan
TGR + HS + Atrasentan
TGR
TGR+Bosentan
TGR+Atrasentan
Days
B
Systolic blood pressure
TGR
TGR + Bosentan
Han SD
TGR + Atrasentan
TGR+Bosentan
TGR+Atrasentan
Days
C
ET-1 in left ventricle
Han SD
TGR
TGR+Bosentan
TGR+Atrasentan

Fig. 2

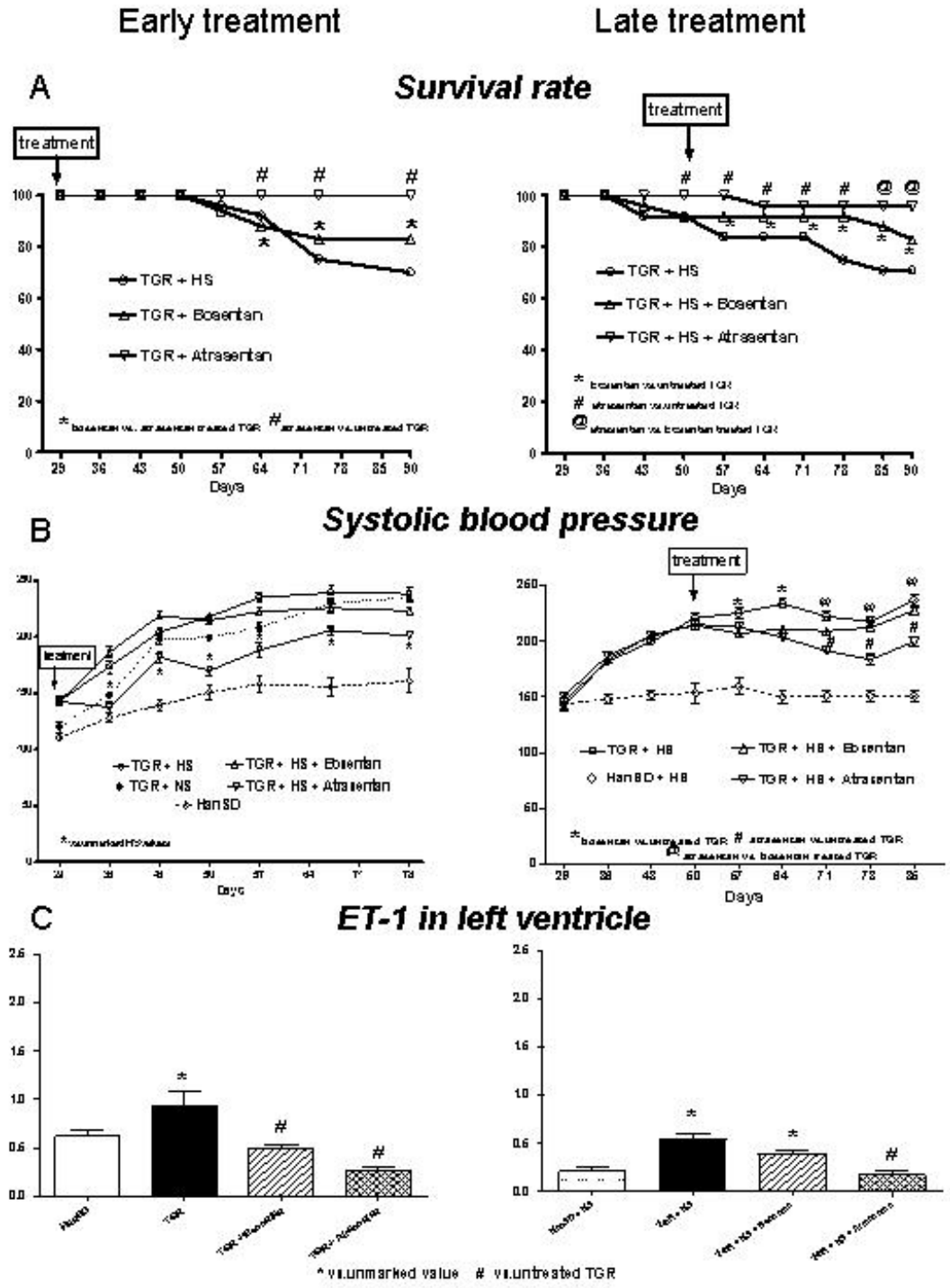
Early treatment
Late treatment
A
Survival rate
treatment
TGR + HS
TGR + Bosentan
TGR + Atrasentan
TGR + HS + Bosentan
TGR + HS + Atrasentan
Days
B
Systolic blood pressure
TGR + HS
TGR + HS + Bosentan
TGR + NS
TGR + HS + Atrasentan
Han SD
TGR + HB
TGR + HB + Bosentan
Han SD + HB
TGR + HB + Atrasentan
C
ET-1 in left ventricle
* vs.unmarked value # vs.untreated TGR

Fig. 3