The effect of metanephros transplantation on blood pressure in anephric rats with induced acute hypotension

Shinya Yokote1,2, Takashi Yokoo1,2, Kei Matsumoto1,2, Yasunori Utsunomiya1, Tetsuya Kawamura1 and Tatsuo Hosoya1

1Division of Nephrology and Hypertension, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan and 2Project Laboratory for Kidney Regeneration, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan

Correspondence and offprint requests to: Takashi Yokoo; E-mail: tyokoo@jikei.ac.jp

Abstract

Background. The kidney is an important organ for maintaining blood pressure. We have previously reported that transplanted metanephroi can reproduce some kidney functions. The aim of the present study was to determine the metabolic function of transplanted metanephroi with particular reference to maintaining blood pressure.

Methods. Male Wistar rats were transplanted with metanephroi (transplanted group, n = 28), following unilateral nephrectomy. For comparison, we performed unilateral nephrectomy without transplantation in 32 rats (non-transplanted group, n = 18; haeminephrectomy control group, n = 14). The remaining kidney was removed 2 weeks after the initial operation, while control rats had a sham operation. Hypotension was induced by intravenous infusion of diltiazem hydrochloride or rapid withdrawal of blood. Mean arterial blood pressure (MAP) was invasively monitored and plasma renin activity (PRA) was analysed at multiple time points. Renin expression by metanephroi was evaluated by real-time polymerase chain reaction and immunohistochemistry.

Results. Metanephroi in the transplanted group expressed renin messenger RNA. Metanephros transplantation significantly raised PRA and maintained MAP compared with the non-transplanted group. No significant differences between the transplanted and control groups were found with respect to PRA or MAP. PRA was positively correlated with metanephroi weight as well as MAP in the transplanted group.

Conclusion. The present study shows that transplantation of metanephroi produces PRA and contributes to raising MAP in a rat model of acute hypotension.

Keywords: diltiazem hydrochloride; hypotension; metanephros transplantation; nephrogenesis; renin

Introduction

End-stage renal disease (ESRD) is a major clinical health problem with high levels of morbidity and mortality [1]. Renal transplantation demonstrates a better 1-year survival rate than dialysis [2]. However, available organs for transplantation are so limited that dialysis is the most common form of renal replacement therapy. In Japan, over 296 000 people with ESRD are currently receiving dialysis at a cost of 1 trillion per annum (3% of Japan’s health budget) [3] and the number of people receiving dialysis is increasing by ~8000 annually, so placing a group of this size onto dialysis is likely to overburden the health care system.

One potential alternative approach is the transplantation of a fetal kidney rudiment, the metanephros. Recent studies have emphasized the use of renal primordia as an alternative to the transplantation of developed adult organs, including the transplantation of whole metanephroi into intra-peritoneal locations [4–6]. These studies provide important insights into the ontogenetic development of transplanted renal tissue in both allogeneic [4, 6] and xenogeneic models [5, 7]. In addition, we have successfully rebuilt part of the metanephros from human mesenchymal stem cells using a method of nephrogenesis in a growing xeno-embryo [8] and were able to establish mature organoids [9] that express erythropoietin and 1α-hydroxylase [10] in the rat omentum. Our ultimate goal is to establish an entire functional kidney in ESRD patients. We have researched the best site for metanephros transplantation and previously reported that transplantation into the para-aortic area is better for the establishment of renin-producing tissue [11]. However, any difference in the effect on blood pressure between different transplantation sites remains unknown. This is especially important as blood pressure variability is associated with cardiovascular events in elderly patients [12] and is an adverse prognostic risk factor in ESRD patients [13]. Furthermore, haemodialysis-associated hypotension is an independent risk factor in haemodialysis patients [14]. Therefore, controlling blood pressure variability is important in patients with chronic kidney disease.

Thus in the present study, we used two rat models of hypotension, induced by diltiazem or rapid withdrawal of blood, to investigate the effect of metanephros transplantation on blood pressure maintenance and whether the para-aortic area is the best site for metanephros transplantation with respect to blood pressure. In addition, we...
examined the metabolic function of the implanted meta-
nephroi in the host animal.

Materials and methods

Experimental protocol

All experiments were approved by the Animal Committee of the Jikei University School of Medicine. Adult male Wistar rats (10 or 12 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and were fed a standard CE-2 diet (Nihon CLEA, Tokyo, Japan). They were kept in cages in pairs and allowed free access to food and water. The rats were divided into four groups: haeminephrectomy control group (n = 14); para-aorta (PA)-transplanted group (n = 13); omentum/epididymis (OE)-transplanted group (n = 15) and non-transplanted group (n = 18; Supplementary Table 1). Two separate experiments (Experiments 1 and 2) were carried out using blood pressure monitoring. The experimental designs are summarized in Supplementary Figure 1.

Experiment 1. All 12-week-old rats (n = 30) underwent a unilateral nephrectomy under isoflurane anaesthesia. At the same time, both transplanted groups were implanted with metanephros in the para-aortic area (PA-transplanted group, n = 6) or the omentum and epididymis (OE-transplanted group, n = 8). Two weeks after surgery (when the rats were 14 weeks old), rats in the OE- and PA-transplanted groups and the non-transplanted group (n = 10) had their remaining kidney removed to stop production of native renin and blood pressure was monitored under isoflurane anaesthesia. Rats in the control group (n = 6) underwent a sham operation and were monitored with their remaining kidney intact. Two hours after the second nephrectomy, diltiazem hydrochloride was administra-
ted intravenously at a dose rate of 0.025 mg/kg/min for 2 h, fol-
lowed by a dose rate of 0.05 mg/kg/min for another 2 h using a syringe pump (Model 100; BAS Inc., Tokyo, Japan). Blood samples for bio-
chemical analysis were obtained from the right femoral vein before ne-
phrectomy (0 min) and at 0, 1, 2, 3 and 4 h during diltiazem administra-
tion (120, 180, 240, 300 and 360 min). After blood pressure monitoring, implanted metanephros from the transplanted groups and native kidneys from the haeminephrectomy control group were removed and all rats were killed by abdominal aortic puncture under isoflurane anaesthesia.

Experiment 2. The effect of metanephros transplantation on a more rapid drop in blood pressure, without anti-hypertensive drugs, was exam-
ined. Ten-week-old rats (n = 30) underwent a unilateral nephrectomy under isoflurane anaesthesia. At the same time, both transplanted groups were implanted with metanephros in the para-aortic area (PA-transplanted group, n = 7) or the omentum and epididymis (OE-transplanted group, n = 7). Two weeks after surgery (when the rats were 12 weeks old), rats in the OE- and PA-transplanted groups and the non-transplanted group (n = 8) had their remaining kidney removed and blood pressure was moni-
tored under isoflurane anaesthesia. Rats in the haeminephrectomy control group (n = 8) underwent a sham operation and were monitored with their remaining kidney intact. Blood was then rapidly withdrawn from the inferior vena cava at a volume of 2% vol/wt body weight while blood pressure was continuously monitored under isoflurane anaesthesia. Two hours after blood withdrawal (120 min), the rats were killed and blood and kidney specimens were collected for further analysis.

Preparation of metanephros transplantation

Rat metanephros were dissected and transplanted into the adult rat hosts as described previously [4, 15, 16]. Briefly, metanephros were surgically resected from 15-day-old (E15) Wistar rat embryos. Metanephros were transplanted within 60 min both into the omentum (number of trans-
planted metanephros = 6.3 ± 0.1) and the fat around bilateral epididy-
mides (right, n = 6.4 ± 0.2; left, n = 6.4 ± 0.2) of the OE-transplanted group and into the para-aortic area (n = 19.6 ± 0.6) of the PA-trans-
planted group under isoflurane anaesthesia (Supplementary Table 2).

Blood pressure monitoring and diltiazem administration

The rats were anaesthetized with isoflurane and placed on a heated pad. A polyethylene catheter (Natsume Seisakusho, Tokyo, Japan) was in-
serted into the abdominal aorta through the left femoral artery for blood pressure recording. A second catheter was implanted into the left femoral vein for intravenous administration of diltiazem. Mean arterial blood pressure (MAP) was recorded using an amplifier (PA-001; Star Medical, Tokyo, Japan). Diltiazem hydrochloride (Sigma–Aldrich, St. Louis, MO) was dissolved in normal saline (5 mg/ml) and infused via the left femoral vein using a syringe pump.

Blood biochemistry

Blood samples for biochemical analysis were obtained from the right femoral vein or inferior vena cava while rats were under isoflurane anaesthesia. Plasma renin activity (PRA) was measured using a radioimmuno-
assay kit (TFB, Inc., Tokyo, Japan).

Histopathological examination

For histological analysis, metanephros from each animal in the trans-
planted groups were placed in a 10% formalin solution in phosphate buffer. The metanephros were paraffin-embedded, cut into 3 μm sections and mounted on silanized/charged slides. Slides were stained with haematoxylin–eosin and also examined for the expression of renin (sc-22752; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) by immunohis-
tochemistry. For immunohistochemical staining of endothelial cells, rabbit anti-CD31 polyclonal antibody (Thermo Scientific, Rockford, IL) was used.

Reverse transcription–polymerase chain reaction and real-time polymerase chain reaction

RNA was extracted from each tissue using TRZol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Genomic DNA was removed using DNase I (Takara Bio, Otsu, Japan) and cDNA was synthesized from the total RNA using the Superscript reverse tran-
scription–polymerase chain reaction (PCR) system. The RNA was then digested with RNase H (Invitrogen). The primer sets are depicted in Sup-
plementary Table 3. Renin messenger RNA (mRNA) levels were semi-
quantified using real-time PCR and were normalized against the expression of rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as the internal control. In brief, for renin qPCR, we used TaqMan Gene Expression Assays (Rn00561847_m1; Applied Bio-
systems, Foster City, CA) and TaqMan Rodent GAPDH Control Reagents (No. 4308313; Applied Biosystems). PCR conditions were as follows: an initial denaturation at 95°C for 2 min, 95°C for 10 min and then 40 cycles of amplification (denaturation: 95°C, 15 s; annealing: 60° C, 60 s).

Statistical analysis

Data are presented as the means ± SEM. Statistical analyses were per-
fomed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Differences between all experimental groups for MAP and PRA over time were examined by repeated measures analysis of variance (ANOVA). Differences for baseline characteristics and mRNA levels were tested by one-way ANOVA, in combination with Bonferroni correction when multiple comparisons were made. A Bartlett test was used to compare the variances of the groups. If the variances were unequal, stat-
istical significance was determined by the Kruskal–Wallis test. The Pear-
son’s Correlation Coefficient was used to evaluate correlations between the weight of metanephros, PRA and MAP. A value of P < 0.05 was con-
sidered significant.

Results

Baseline characteristics

The baseline characteristics of the study groups before monitoring blood pressure are outlined in Supplementary Table 2. There were no differences in body weight between the four groups of rats.
Metanephros transplantation maintains mean arterial pressure in diltiazem-treated rats

The effects of metanephros transplantation on blood pressure were evaluated in Experiment 1 using diltiazem-treated anephric rats.

Figure 1A illustrates the time course of the blood pressure change induced by diltiazem in Experiment 1. There was no significant difference in MAP at 0 min between all groups. Diltiazem administration and regular blood sampling caused a pronounced fall in MAP in all groups. However, MAP in the control and transplanted groups was higher than in the non-transplanted group (P < 0.05 and P < 0.001, respectively). MAP in the OE-transplanted group was the same as the PA-transplanted group (data not shown). There was no significant difference in MAP at sacrifice between the control and transplanted groups. Figure 1B illustrates the time course of PRA change in Experiment 1. There was no significant difference in PRA at 0 min between all groups. Compared with values in the non-transplanted group, PRA at sacrifice was significantly higher in the control and both transplanted groups (P < 0.001 and P < 0.0001, respectively; Figure 1B). There were no significant differences in PRA between the control, PA- and OE-transplanted groups during the course of Experiment 1.

Metanephros transplantation raises mean arterial pressure in rats post-rapid blood withdrawal

The effects of metanephros transplantation on a more rapid drop in blood pressure were analysed in Experiment 2 using a rapid blood removal.

Figure 2A illustrates the change in the blood pressure induced by blood withdrawal, which caused a rapid fall in MAP in all groups in Experiment 2. There was no significant difference in MAP at 0 min between all groups. However, MAP at sacrifice in both the OE- and PA-transplanted groups was higher than in the non-transplanted group (both P < 0.0001). There was no significant difference in MAP between both the transplanted groups and the control group during the course of Experiment 2. Compared with the non-transplanted group, PRA was significantly increased in both transplanted groups at sacrifice (Figure 2B). There were also no significant differences in PRA between both the transplanted groups and the control group.

Fig. 1. (A) MAP in control, non-transplanted and OE- and PA-transplanted rats in Experiment 1. Data are presented as means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 for PA-transplanted compared with corresponding non-transplanted group; ††P < 0.01 for OE-transplanted compared with corresponding non-transplanted group; ‡‡P < 0.05 for control compared with corresponding non-transplanted group; n.s., not significant. (B) PRA in control, non-transplanted and OE- and PA-transplanted rats in Experiment 1. Data are presented as means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with corresponding non-transplanted group; n.s., not significant.

Fig. 2. (A) Change in MAP in control, non-transplanted and OE- and PA-transplanted rats in Experiment 2. Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 for PA-transplanted compared with corresponding non-transplanted group; ‡‡‡P < 0.001 and ‡‡‡‡P < 0.0001 for OE-transplanted compared with corresponding non-transplanted group; ††P < 0.01, ‡‡‡P < 0.001 and ‡‡‡‡‡P < 0.0001 for control compared with corresponding non-transplanted group; n.s., not significant. (B) PRA at sacrifice in control, non-transplanted and OE- and PA-transplanted rats in Experiment 2. Data are presented as means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with corresponding non-transplanted group; n.s., not significant.
PRA produced by metanephroi has a significant correlation with mean arterial blood pressure

PRA generated in transplanted animals was comparable with that in control rats. However, the mass of metanephric tissue in the transplanted animals was only \( \sim 275 \) mg compared with kidneys of \( > 1 \) g in the controls, which implied that the metanephroi were releasing far more renin per mass than the control animals with a native kidney. So renin expression in the engrafted metanephroi and the mechanism underlying the observed blood pressure maintenance effect seen with metanephros transplantation were examined in greater detail. Fourteen days after transplantation, implanted metanephroi had engrafted successfully (Supplementary Figure 2), as described previously [11, 16]. Immunohistochemical examination of renin showed that juxtaglomerular (JG) cells expressed renin in the native kidney of the control group (Figure 3A and B). On the other hand, the renin expression pattern in the engrafted metanephroi was different to the native

Fig. 3. Appearance of the metanephroi 2 weeks after transplantation. Expression of renin by the native kidney of the control group (A, B) and the metanephroi of the transplanted group (C, D, E, F) were detected by immunohistochemistry. Arrowheads indicate renin-expressing cells. Original magnification: \( \times 40 \) (A, C); \( \times 400 \) (B, D, E, F). Bars indicate 500 \( \mu \)m (A, C) and 100 \( \mu \)m (B, D, E, F). * Indicates vascular invasion from the host rat.
kidney. The number of renin-expressing cells in the metanephros was higher than in the control native kidney. In addition, the renin-producing cells were seen near the vascular invasion of the cortex and co-expressed the endothelial marker, CD31 (Supplementary Figure 3), suggesting that they were in various areas—interlobular arteries, afferent arterioles and near the JG area (Figure 3C–F) as previously reported [11]. Furthermore, real-time PCR revealed that renin expression in the transplanted metanephroi was ∼10-fold higher than in the native kidneys of the control rats (Figure 4).

Therefore, we analysed the correlations between the number of transplanted metanephroi per rat and the total weight of developed metanephroi, PRA and MAP in both transplanted groups at sacrifice (Figure 5). The number of transplanted metanephroi per rat was significantly correlated with the weight of the developed metanephroi (Figure 5A). PRA had a significant correlation with the weight of the developed metanephroi (Figure 5B) as well as with MAP (Figure 5C). These data reflected that the blood pressure maintenance effect seen in the transplanted group is due to renin produced by the transplanted metanephroi.

**Fig. 4.** (A) Reverse transcription-polymerase chain reaction for renin in the metanephroi of the OE-transplanted (MN1) and PA-transplanted (MN2) groups and the native kidneys (NK) from the control group. Lane 1 (m), maker (φ×174/HaeIII); n.c., no template cDNA. (B) Renin mRNA expression, normalized against GAPDH, in metanephroi from the OE- and PA-transplanted groups and native kidney from the control group in Experiment 1 and (C) in Experiment 2. Data are presented as means ± SEM. *P < 0.05 compared with native kidney from the control group.

**Discussion**

In the present study, we examined the effect of metanephros transplantation on blood pressure in the host animal and found that transplanted metanephroi contribute to raising blood pressure in anephric rats with induced acute hypotension, suggesting that metanephros transplantation can contribute to the regulation of sudden falls in blood pressure.

The mechanism responsible for the maintenance of blood pressure seems to be the production of renin by the implanted metanephroi. Renin is a regulator of systemic blood pressure and its expression is regulated by a sensitive monitoring system in the kidney [17]. In fact, renin-producing cells develop along with newly formed vessels during kidney development [18, 19]. Renin is first found in the arcuate arteries at day E14 and renin expression shifts from the proximal to distal parts of the arcuate arterial tree. Five days after birth, renin is found only in mature afferent arterioles [19]. Many of the renin-secreting cells in the metanephroi were found outside the JG area in this study, suggesting that because of the slower development of engrafted metanephroi compared to the native kidney, the pattern of renin distribution in the metanephroi was more immature than in the native kidney. While the volume of renin-expressing cells in the native kidney is only ∼0.025% of the total native kidney volume, that in the developing kidney (post-natal day 1) is ∼0.3% of the total developing kidney volume [19]. So metanephros transplantation could increase PRA even though only a small mass of metanephroi was engrafted in the rat. However, it was not known whether renin produced by the metanephroi could influence the host animal’s blood pressure. In this study, we have demonstrated for the first time that metanephros transplantation raises blood pressure during acute hypotension in the host animal.

We used diltiazem hydrochloride to induce hypotension in Experiment 1 of this study. However, diltiazem is an L-type calcium channel blocker and L-type calcium channels appear to be the pathway for calcium entry in response to angiotensin II-induced renin inhibition, and inhibition of the L-type channels stimulates renin release [20, 21]. Because diltiazem administration may have influenced renin production from the metanephroi, we also analysed another rat model of hypotension using rapid blood withdrawal without calcium channel blockers in Experiment 2. Renin secretion is also controlled through a complex interaction of different classical regulatory pathways including the renal baroreceptor, renal nerves, the macula densa, etc. [22]. Renin release has been shown to depend on decreased perfusion pressure [23] and is inhibited by high renal perfusion pressure through the renal baroreceptor mechanism [24]. Because rapid blood withdrawal reduced the perfusion pressure in the implanted metanephroi, renin production by the metanephroi was increased and PRA levels were raised in the transplanted rats.

In addition to the circulating renin activity, other factors affect blood pressure, including other components of the renin–angiotensin–aldosterone system such as prorenin,
angiotensin, aldosterone and focal renin activity, and the sympathetic nervous system. We cannot specify whether the blood pressure maintenance effect seen in this study was due to circulating PRA only or a combination with these other factors. However, MAP measured in all groups of our study were associated with PRA at sacrifice (Supplementary Figure 4) and there were significant correlations between PRA and the weight of metanephroi and MAP in the transplanted groups (Figure 5B and C), suggesting that the action to maintain blood pressure in the transplanted groups is due in part to renin produced by the transplanted metanephroi.

We previously reported that transplantation into the para-aortic area is better for the establishment of renin-producing tissue [11]. However, there were no significant differences in renin mRNA of metanephroi and PRA between the PA- and OE-transplanted groups at sacrifice in this study. This discrepancy might have resulted from differences in the experimental protocol. Firstly, in this study, we transplanted metanephroi into not only the omentum but also the epididymis in the OE-transplanted group. Secondly, the number of metanephroi transplanted into each rat in this study was higher, and the weight of the developed metanephroi in the rat was heavier, than in previous studies. Thirdly, time to sacrifice after blood removal was different; in our previous study [11], rats were sacrificed 90 min after blood withdrawal. Because of the longer time from blood withdrawal to sacrifice, renin mRNA in the OE-transplanted group might have increased more in this study. On the other hand, although not significantly different, average PRA levels in the PA-transplanted group tended to be higher than in the OE-transplanted group at 120 min in Experiment 1 (Figure 1B) and at sacrifice in Experiment 2 (Figure 2B). Recovery of MAP in the PA-transplanted group also seemed to occur earlier than in the OE-transplanted group. So the para-aortic area could be the best site for metanephros transplantation with respect to renin reactivity to blood withdrawal. In addition, the average weight of engrafted metanephroi in the PA-transplanted group tended to be heavier than in the OE-transplanted group (Supplementary Table 2). Since PRA had a significant correlation with the weight of the developed metanephroi (Figure 5B), this also suggests that the para-aortic area might be the best site for the growth of transplanted metanephroi and renin production. This point requires further consideration in the future.

In summary, we examined the effect of metanephros transplantation on blood pressure. Our findings indicate that metanephros transplantation can contribute to raising blood pressure in acutely hypotensive rats and is dependent on PRA produced by the implanted metanephroi.
Acknowledgements. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant No. 21591042).

Conflict of interest statement. None declared.

References
2. Sonoda T, Takahara S, Takahashi K et al. Outcome of 3 years of immunosuppression with tacrolimus in more than 1,000 renal transplant recipients in Japan. *Transplantation* 2003; 75: 199–204.

Received for publication: 27.9.2011; Accepted in revised form: 2.1.2012