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The Tsukuba hypertensive mouse (transgenic mouse carrying human genes for both renin and angiotensinogen) as a model of human malignant hypertension: development of lesions and morphometric analysis

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Abstract The renin-angiotensin system has a pivotal role in hypertension. The Tsukuba hypertensive mouse (THM; a transgenic mouse carrying human genes for both renin and angiotensinogen) was generated to allow further examination of the renin-angiotensin system in a variety of pathologic conditions. We evaluated the development of renal lesions in these mice and in controls by morphometric, immunohistochemical and ultrastructural methods. Blood pressure was significantly higher in THM than in control mice; 1 year after birth, it was approximately 40 mmHg higher. The kidney-to-body weight ratio was also higher in THM than in control. Morphometrical analysis revealed that the glomerular sclerosis index was significantly elevated in THM with 10% of the glomeruli sclerotic at 18 months. The grade of vascular lesion and the frequency of fibronoid arteritis of the kidney exhibited the same tendency as the glomerular sclerosis index. Murine renin was located exclusively in the juxtaglomerular apparatus, whereas human renin was expressed not only in the juxtaglomerular apparatus, but also in periarteriolar smooth muscle cells and in mesangial and epithelial cells of the glomeruli. Light and electron microscopy revealed significant fibrinoid arteritis of the kidney in THM and also "onion skinning", both pathognomonic for malignant nephrosclerosis. THM may be an excellent model of human malignant hypertension.

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Introduction

Hypertension is associated with a variety of aetiological factors. It is inherited as a multifactorial trait, and several different genes seem to be involved in the regulation of blood pressure and the pathogenesis of hypertension. The difficulties that have been encountered in attempts to identify these genes in humans include problems related to definition of phenotype, identification of informative families for genetic studies, and the probable heterogeneity of the disease [12, 15, 29]. Both genetic and environmental factors, either singly or in combination, appear to have important roles in the aetiology of hypertension [15, 22, 30]. Among the numerous risk factors identified, the renin-angiotensin system (RAS) has received considerable attention. As the rate-limiting factor in the renin angiotensin system is the initial step between renin and angiotensinogen [30], this reaction has long been considered to be of key importance in the regulation of blood pressure and balance of the fluid volume, together with other biological responses.

Production of transgenic animals has provided some excellent animal models for analysis of the control of gene expression and the mechanisms of certain human diseases [5–7, 27]. We have constructed a chimeric RAS comprising both human renin and human angiotensinogen genes through cross-moting separate lines of transgenic mice carrying either the human renin gene or the human angiotensinogen gene against a mouse genetic background possessing endogenous renin activity and murine angiotensinogen II receptors. This newly established transgenic mouse line is called the Tsukuba hypertensive mouse (THM) [7]. In THM and in other transgenic mouse and rat models, various aspects of baseline physiological regulation have been examined, such as the response to sodium depletion, inhibition of angiotensin-

converting enzyme, and human renin mRNA expression and release of the protein [7, 8, 26], but extensive histopathological examination of transgenic animals has not been performed, in particular with emphasis on these animals as models for human disease.

We analysed the morphology and development of lesions on this animal, and in particular we examined the blood vessels of the kidney and the glomeruli, in addition to other organs.

Methods

The mice used in this study were progeny of those initially described by Fukamizu et al. [7]. Transgenic mice carrying either or both of the 15-kb human renin and 14-kb human angiotensinogen genes (THM) were produced and bred under standard conditions of the Laboratory Animal Research Centre, University of Tsukuba. They had free access to food and autoclaved water. A total of 59 mice, ranging in age from 1 to 18 months, were used. B6 mice of corresponding ages (37 mice) were used as controls. Male and females were included and identified in all groups. Systolic blood pressure (BP) was measured with a programmable sphygmomanometer (BP-98, Softron, Japan) by the tail-cuff method. Unanaesthetized mice were introduced into a small holder mounted on a thermostatically controlled warming plate and maintained at 37° C during measurements. Under intraoperitoneal pentobarbital anaesthesia (100 mg/kg), THM were killed at 3 (n=15), 6 (n=10), 9 (n=10), 12 (n=15) and 18 months (n=4) of age. The numbers of control mice examined were 8 each at 3, 6, 9, and 12 months and 5 at 18 months. The heart and vascular trees, lungs, and kidneys were dissected out for examination.

For microscopy, both kidneys were cut on the frontal plane, and specimens fixed with 4% neutral buffered formaldehyde were further processes for standard LM. They were stained with haematoxylin-eosin (H&E), Masson- trichrome, elastica-van Gieson (EVG), periodic acid – Schiff (PAS), and PAS methenamine silver (PASM). Tissues other than kidneys were usually stained with H&E, but were stained by other methods when appropriate. Tissues for TEM were prepared by previously described techniques [1]. In brief, tissues that had been perfusion fixed with 2.5% glutaraldehyde in cacodylate buffer were postfixed in 1% phosphate-buffered osmium. After osmium fixation, they were dehydrated in ethanol and embedded in Epon. Thick sections (1 μm) were cut from each block, stained with toluidine blue, and evaluated for lesions by LM. The sections with lesions were cut further with a diamond knife and stained with uranyl acetate and lead citrate for evaluation by TEM. Thin sections were viewed with a transmission electron microscope (JEOL 100CS; Tokyo, Japan) operated at 100 kV.

For immunohistochemical analysis, the monoclonal antibodies specific for human renin and those specific for mouse submandibular gland renin were used as specific markers for each renin [5]. For immunostaining, endogenous peroxidase activity was blocked by incubating each section in 0.3% H_2O_2 with 1% NaN_3. The primary antibodies, appropriately diluted (approximately 1:800), were applied to frozen-cut, acetone-fixed sections in a humid atmosphere at room temperature for 2 h. The specimens were washed in PBS and incubated with the secondary antibody. After washing with PBS, they were treated with horseradish-peroxidase-conjugated streptavidin. The peroxidase reaction was visualized by incubation with 0.02% diaminobenzidine and 0.001% H_2O_2 for 5 min. Normal mouse IgG was used for negative controls. Sections were counterstained with methyl green.

To evaluate glomerular and vascular lesions in the kidneys, an index and a grading system were introduced [1, 21]. For an index of glomerular sclerosis (GSI), glomerular lesions were graded from 0 to 4+ on the basis of the extent of the glomerulus with evidence of involvement: 0, no evidence of lesions; 1+, up to 25% of tuft affected by sclerosis; 2+, tuft involvement of 25–50%; 3+, tuft involvement of 50–75%; 4+, almost all the tuft involved. For each kidney, the sum of the results for 100 glomeruli, taken from the maximum frontal section of the kidney, was taken as the GSI.

The grade of vascular lesions (GVL) was determined as follows. Extreme changes in blood vessels, sites of fibrinoid arteritis and arteriolitis were easily recognized by standard histological examination. Each mouse examined was graded as follows: 0, no fibrinoid arteritis on the entire plane of the frontal renal section; 1, 1–3 sites of fibrinoid arteritis were found; 2, 4–10 sites of fibrinoid arteritis were found, 3, 11 or more sites of fibrinoid arteritis were found.

Data are expressed as means±SD unless otherwise indicated. Differences between means for the two groups were tested for statistical significance by Student's t-test.

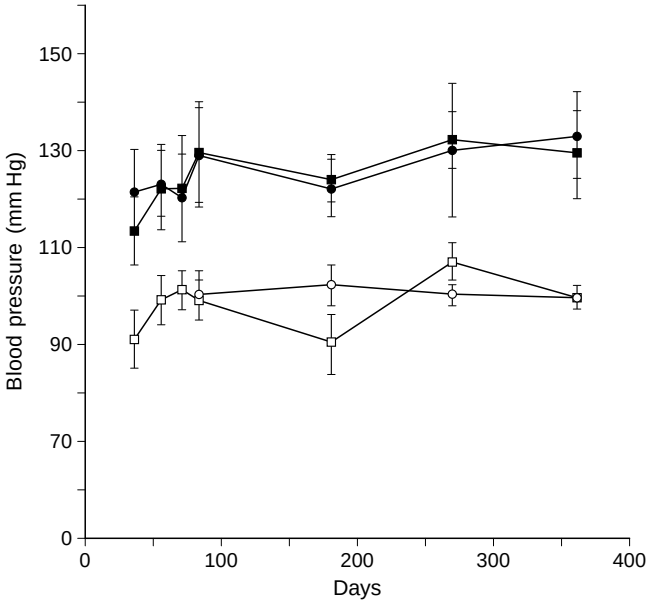

Fig. 1 Blood pressure of THM and control B6 mice, measured by the tail-cuff method. Throughout the experiment, the blood pressure of THM was higher by 20–40 mmHg, regardless of sex (■ THM male, ● THM female, □ B6 male, ○ B6 female)

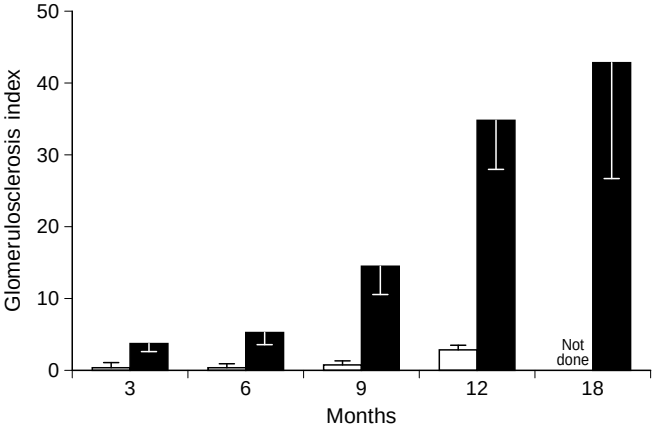

Fig. 2 Time-course of the change in glomerulosclerosis index (GSI). The glomerular lesion was graded 0 to 4+ on the basis of the extent of the glomerulus that showed evidence of involvement: 0 no evidence of lesions, *1+* up to 25% of tuft showing sclerosis, *2+* 25–50% of tuft involvement, *3+* 50–75% involvement, *4+* most of the tuft involved. For each kidney, the sum of 100 glomerular indices, taken from the maximum frontal section of the kidney, was expressed for GSI (□ B6 mice, ■ THM)

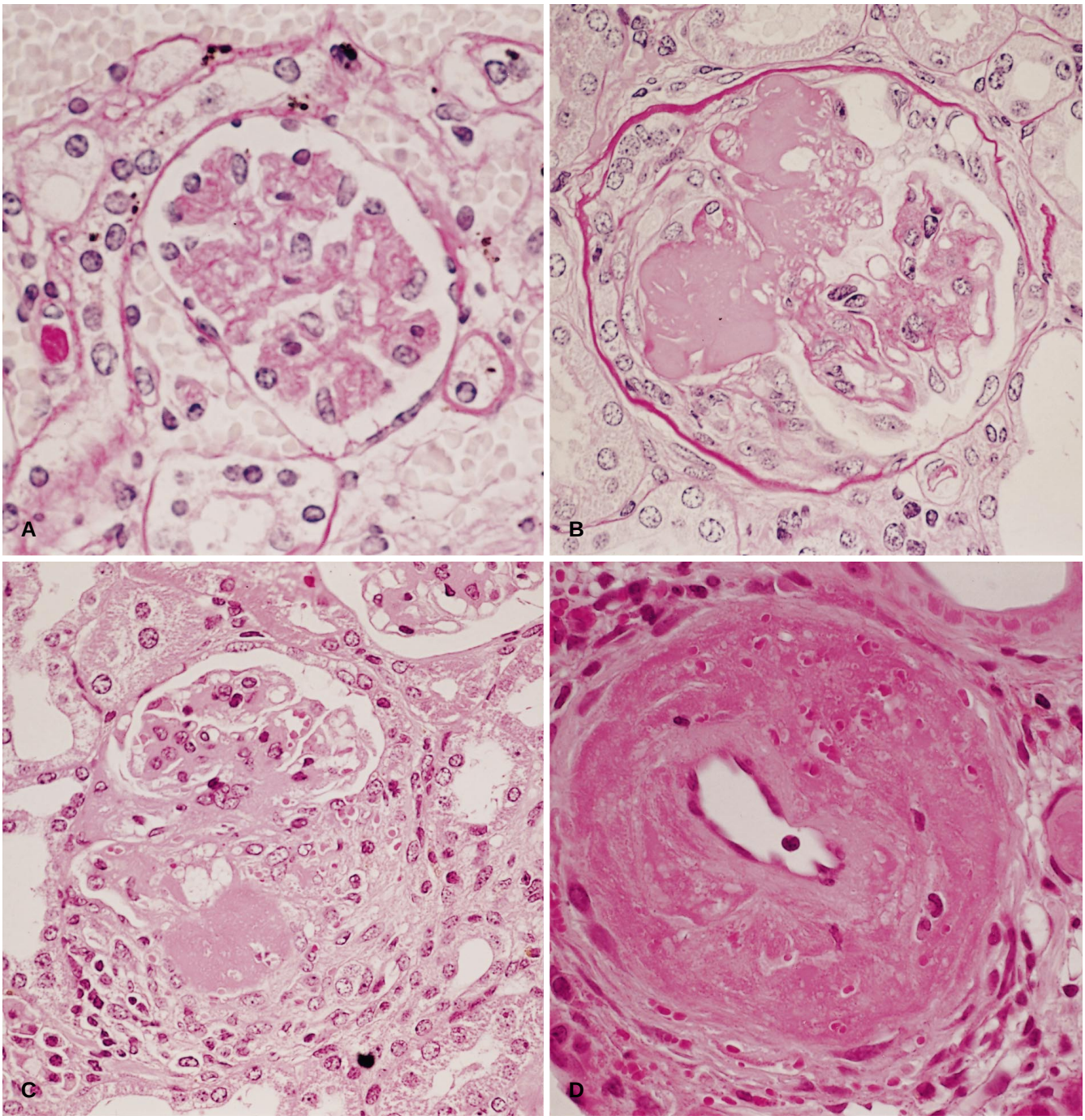

Fig. 3A–D Light micrographs of representative glomerular and vascular lesions in THM at 12 months of age. **A** Ischaemic glomerular collapse. The glomerulus has shrunken, with thickening of the basement membrane. PAS, ×800. **B** Focal segmental lesion. The left half of the glomerular tuft is enlarged as a result of accumulation of PAS-positive homogeneous materials associated with formation of a cellular crescent. The right half of the glomerulus remains unaffected. PAS, ×510. **C** Formation of hilar granuloma. In the hilar area, focal and nodular accumulation of mononuclear inflammatory cells is evident. H&E, ×410. **D** Fibrinoid arteritis of the kidney. The arteriolar wall is thickened as a result of the accumulation of eosinophilic homogeneous materials. Extravasated erythrocytes are scattered both in the thickened wall and the adventitia. H&E, ×540

Results

To document changes in BP over time, we measured BP at various times by the tail-cuff method. The results are shown in Fig. 1. Each point represents the mean value for the mice examined, and the values for males and females are given at each time-point. As shown in Fig. 1, BP was dramatically elevated in THM, regardless of sex. One month after birth, BP of THM was roughly 20–30 mmHg higher than that of normal controls. Thereafter, BP of THM was consistently elevated, being 30–40 mmHg higher than that of normal controls throughout the later life of the animals.

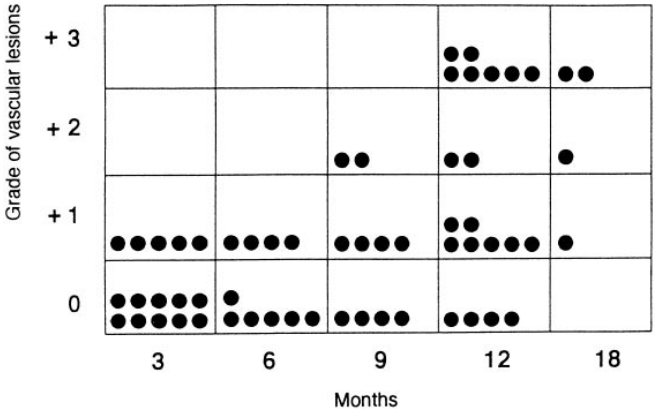

Fig. 4 Distribution of animals according to the severity of vascular lesions (GVL) in THM. Each mouse is represented by ●, and each was graded as follows: *0* no sites of fibrinoid arteritis on the entire plane of the frontal renal section, *+1* one to three sites of fibrinoid arteritis, *+2* four to ten sites, *+3* 11 or more sites. The GVL of control mice is not shown, because it was 0 throughout the experiment

During the experiment, we weighed kidneys and bodies at four time points and calculated the ratio of kidney to body weight. The ratio was slightly higher in THM than in normal controls at the age of 6 months, regardless of sex. When mice were 9 and 12 months old, the ratio was significantly higher in THM (approximately 1.5%) than in normal controls (approximately 1.0%).

Tissue sections from normal (total 37) and THM (total 54) male and female mice from 3 to 18 months of age were examined by LM to document the development of lesions. We focused our attention on glomerular and vascular renal lesions, since the pathological changes putatively associated with malignant hypertension are most conspicuous in the kidney, even though they also occur in other organs.

The values for GSI in THM and control mice are shown in Fig. 2. The GSI in the control mice was consistently below 3, whereas that of THM increased dramatically with age: at 18 months of age, GSI was more than 40. Thus, approximately 10% of the glomeruli examined were globally sclerotic. Figure 3A and B shows representative glomerular lesions, namely ischaemic glomerular collapse or glomerular simplification (Fig. 3A), and focal segmental lesions (Fig. 3B). In the former case, the capillary walls had collapsed, with wrinkling and thickening of the basement membrane. In the latter, the affected capillary tufts were widened and contained homogeneous plasma-like material that occluded capillary lumina. Semi-circumferential formation of crescents was associated with such lesions in this case. Approximately one third of the glomerulus was intact. Fibrinoid arteritis was occasionally noted in hilar regions, where granulomatous lesions had formed (so-called hilar granuloma; Fig. 3C). In later life the juxtaglomerular epithelioid cells in rather intact glomeruli were invariably hypertrophied and contained elevated numbers of secretory granules, while this part of the juxtaglomerular apparatus was acutely disrupted because of the extensive fibrinoid necrosis of the hilar arterioles, as indicated in Fig. 3.

With respect to development of vascular lesions, we graded each mouse as described above, searching sites of fibrinoid arteritis on the whole plain of the frontal section of each kidney (Fig. 4). In THM, the GVL remained at 0 or 1+ until mice reached 6 months of age. At 9 months, mice with GVL 2+ were observed (2/10). Beyond this age mice were graded as 3+ in increasing num-

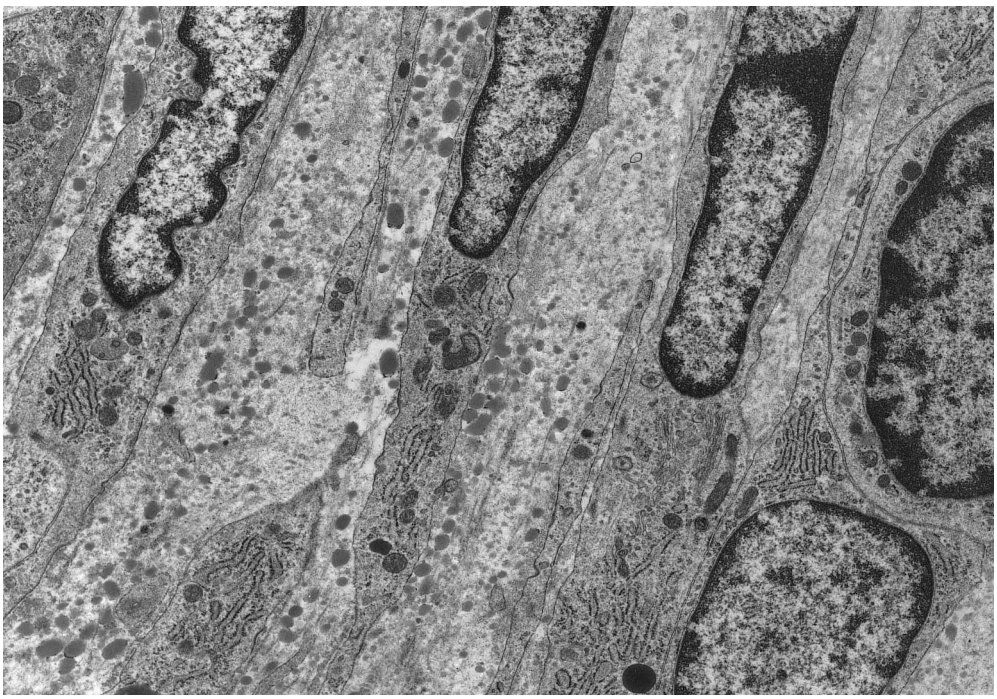

Fig. 5 Electron micrograph of the adventitial site of fibrinoid arteritis. In and around the smooth muscle cells, a number of electron-dense granules are visible. ×9600

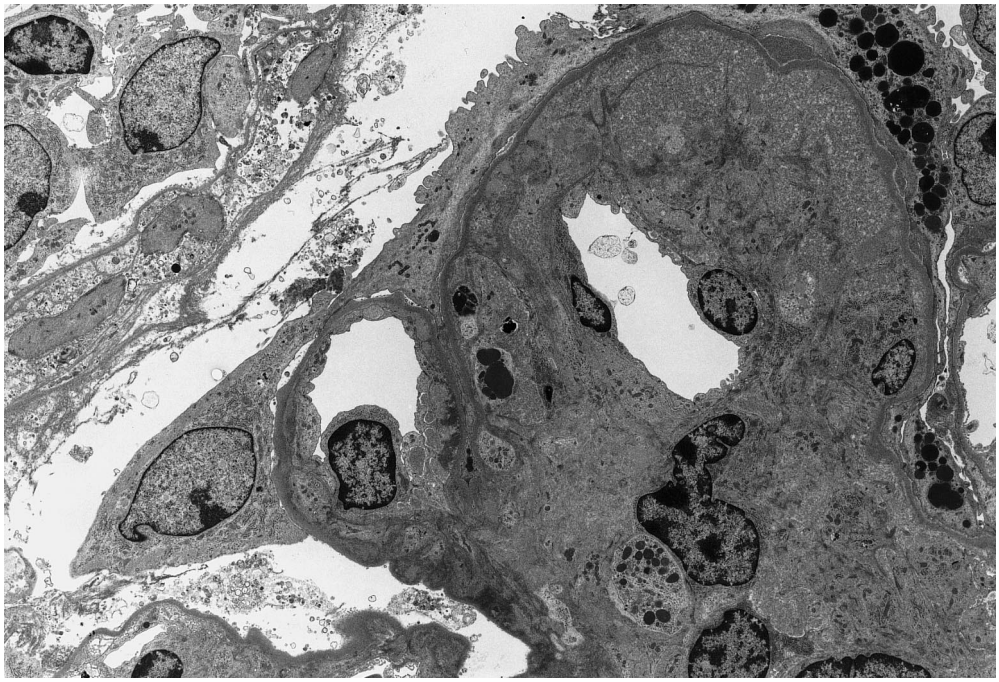

Fig. 6 Electron micrograph of an affected glomerulus. Accumulation of fine granular materials is visible in the subendothelial region. Electron-dense granules are visible in the mesangial cells and epithelial cells. ×3000

bers. Among those aged 18 months, half the mice (2/4) were graded as 3+. Thus, it was easy to find sites of fibrinoid arteritis after THM reached 9 months of age.

A typical example of fibrinoid arteritis is shown in Fig. 3D. The lumen was often narrowed by the lesion, which occupies the entire layer. The media was replaced by intensely eosinophilic homogeneous fibrinoid materials. Insudated erythrocytes and the fragmented debris of such cells were scattered within the fibrinoid materials and in the adventitia. Fibrinoid materials, mostly occupying the media, were stained red with Azan staining. Endothelial cells were swollen, but no denudation was apparent. Spindle-shaped smooth muscle cells and mononuclear inflammatory infiltrates encircled the lesion. Arterioles with concentric fibrosis and elastic duplication, so-called onion skinning, were also observed in the kidney.

Fibrinoid necrosis was confirmed by light and electron microscopy. On electron microscopy, the endothelial cells of the affected arterioles appeared taller than usual, with somewhat corrugated luminal contours. Nonetheless, no denudation was detected. Granular deposits beneath the endothelium replaced most of the widened media. The granular materials revealed by electron microscopy correspond to the fibrinoid necrosis observed under the light microscope. In the outer portion of the arterioles, concentric layers of spindle-shaped or oval cells were noted (Fig. 5). These periarteriolar cells had electron-dense granules within the cytoplasm and around the extracellular spaces. The granules were considered to be mature renin granules. In some glomeruli, the subendothelial zone was considerably widened owing to the deposition of fine granular materials, with resultant thickening of the glomerular tufts (Fig. 6). In the same glomeruli, we noted some mesangial cells and epithelial cells of the glomeruli that contained electron-dense renin granules of various size. They were mostly large and amorphous, and we considered them to be a mature type of renin granules. Smaller protogranules, often seen in the cisternae of the Golgi regions, were scarcely observed.

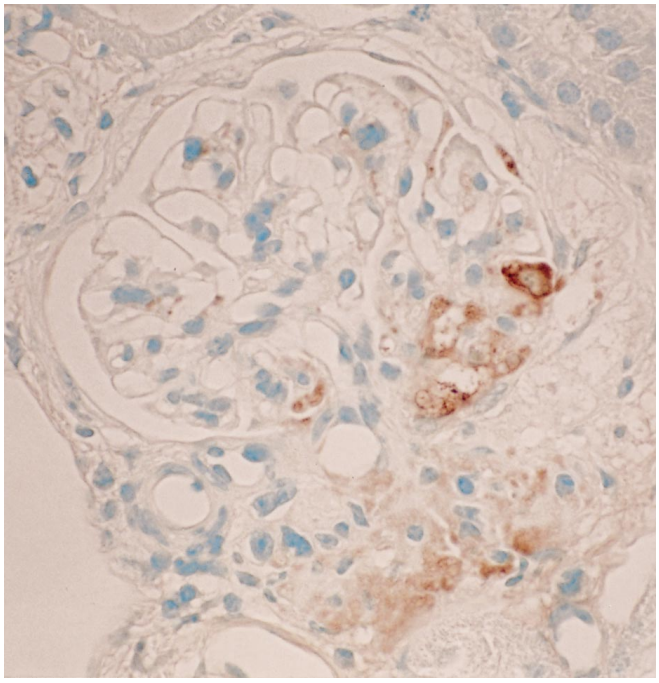

Fig. 7 Localization of human renin with immunostaining. Human renin is immunostained not only in the enlarged juxtaglomerular apparatus, but also in the mesangial cells and in the epithelial cells of the glomerulus ×560

Immunohistochemical staining with the avidin-biotin system revealed that murine renin was limited exclusively to the juxtaglomerular apparatus throughout the experiment. Figure 7, recorded in a 1-year-old THM, shows that the epithelioid cells of the juxtaglomerular apparatus were swollen and contained increased numbers of renin granules within their cytoplasm. Furthermore, human renin was localized not only in the juxtaglomerular apparatus but also in the cells around the arterioles, in mesangial cells and in epithelial cells. Taken together with the results of the ultrastructural study, these results suggest that cells other than the cells of the juxtaglomerular apparatus, such as the adventitial cells of the arterioles, have the ability to produce renin with the progression of hypertension.

The effects of hypertension were most prominent on the hearts of THM: left ventricular hypertrophy was conspicuous, and the heart-to-body weight ratio was 0.7–0.8% in THM, compared with 0.4–0.5% in control mice throughout the experiment (data not shown). In addition, we found several sites of fibrinoid arteritis, with or without rupture and haemorrhage, in THM hearts. In some hearts, fresh or organized thrombi were packed in the left atrium.

Discussion

THM is a newly established hypertensive transgenic mouse that is the first animal model of human high-renin hypertension [7]. Several kinds of animal models of human hypertension are presently available. The spontaneously hypertensive rat (SHR) is widely used as a low-renin model of human essential hypertension because of its normal or even decreased level of plasma renin activity [20]. In the Dahl rat, the onset of the elevated BP is reported to be associated with the functioning of the RAS [22], but its implications have not been elucidated. Despite the usefulness of the SHR and other models as experimental tools, the genetic basis governing the development of the hypertension in these strains remains unclear. In stroke-prone SHR (SHRSP), two to four major genes are thought to influence BP, but details remain to be fully characterized [29]. In contrast to the cited animal models, the onset of high BP has been fully clarified in THM [5–7, 27]. The findings obtained to date are consistent with hypothesis that the marked elevation of BP is due solely to the physiological interaction between the products of the two human transgenes introduced. Furthermore, administration of captoril and other ACE inhibitors causes a significant decrease in BP in THM [7] . Thus, this system not only facilitates the examination of the biological effectiveness of drugs in vivo, but also allows long-term histopathological monitoring of the hypotensive effcts of drugs, which have been difficult to evaluate to date.

The representative disease state of human high-renin hypertension is well known under the name of malignant hypertension [13, 14]. This condition is defined by the pathological finding of blood vessels damaged by markedly elevated BP. The pathological changes associated with malignant hypertension are most conspicuous in the kidney, but they also occur in many other organs. From a histopathological perspective, the renal involvement has often been termed malignant nephrosclerosis [3]. The present study indicates that THM develops renal lesions that resemble those of human malignant nephrosclerosis.

In fulminant cases in humans, the characteristic pattern consists of enlarged swollen kidneys with a coloured surface [3, 13, 14, 16, 17]. In keeping with these observations, kidney-to-body weight ratio of THM increases significantly with age. The vessels that are most affected are the small arteries of the size of renal interlobular arteries, afferent arterioles and glomerular capillaries [13, 14, 16]. As each lesion progresses, the kidney develops typical vasculitic lesions, with fibrinoid necrosis characterized by the deposition of homogeneous, strongly eosinophilic and PAS-positive material that also stains for fibrin [3, 10, 16, 17]. The findings now presented are consistent with those seen in human malignant nephrosclerosis. However, in human cases, fibrinoid necrosis of the arterioles is not an obligatory finding. Inasmuch as samples at renal biopsy are limited to a few arterioles and glomeruli, such characteristic necrotic lesions are rather uncommon in human cases. Our findings contrast with the findings in human biopsy studies. In the latter case, lesions are more proliferative and insudative, with fibrinoid materials not seen so often [16]. The explanation for the frequent vasculitic lesions in THM remains to be discovered.

In the mouse, it is clear that the human RAS introduced affected the vascular microenvironment, and alterations in the activity of the RAS are the basic mechanism for the pathogenesis of this mouse model [7]. We detected renin at the perimeter of the vascular system, in mesangial cells and epithelial cells of the glomeruli and we confirmed the localization of electron-dense granules by electron microscopy; these are considered to be renin granules [11, 25]. It seems, therefore, that secretion of renin by juxtaglomerular cells is not controlled by the usual arteriolar or tubular mechanisms in THM. Our immunohistochemical and ultrastructural studies demonstrated that these cells contained renin, lending credence to the hypothesis that the mechanism responsible for hypertension in THM might be attributable to the hypersecretion of renin by the affected tissue.

The major sources of renin are the JGA cells [11, 13, 25], with a minority of renin-synthesizing cells in the adrenal cortex and anterior pituitary [8]. In the ischaemic areas of partially infarcted kidneys, renin-containing granules were found in cells of the juxtaglomerular apparatus and interlobar arteries [2, 23]. Moreover, there is a body of evidence that supports the notion that local renin-angiotensin systems exist [4, 19, 28]. Activity and synthesis of renin have been reported in cultured arterial smooth muscle cells and cultured endothelial cells, and angiotensin-converting enzyme has also been localized in the endothelium and smooth muscle [18,

24]. To date, there is no information on the renin production of glomerular epithelial cells. It is evident that more definitive findings require elucidation of the dynamics of the RAS in the diseased state and in vascular microenvironments.

In conclusion, THM was confirmed as an animal model of human malignant hypertension or malignant nephrosclerosis. The findings presented in this paper may lead to a better understanding of human malignant hypertension, as well as to better methods of therapeutic intervention in patients with hypertension.

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