Contribution of Genetic Factors to Renal Lesions in the Stroke-Prone Spontaneously Hypertensive Rat

Bruna Gigante, Speranza Rubattu, Rosita Stanzione, Alessia Lombardi, Alfonso Baldi, Feliciano Baldi, Massimo Volpe

Abstract—Stroke-prone spontaneously hypertensive rats (SHRSP) develop renal lesions more frequently than the closely related control strain, the stroke-resistant SHR. The aim of this study was to investigate the contribution of genetic factors to the enhanced susceptibility to renal damage of SHRSP in an SHRSP/SHR F2 intercross by means of a genotype/phenotype cosegregation analysis. For this purpose, 154 6-week-old F2-SHRSP/SHR rats (79 male, 75 female) were fed a stroke-permissive Japanese diet for 4 weeks. Systolic blood pressure (SBP) was recorded at the end of the dietary period. Renal damage was scored from 0 to 3, and 274 genetic markers polymorphic between SHR and SHRSP were genotyped. Linkage of genotype markers to the degree of renal disease was determined by \( \chi^2 \) test. Experimental threshold level to declare linkage was calculated by QTL cartographer. SBP was not correlated to renal damage (\( \rho \) coefficient, 0.201; \( P=\text{NS} \)). Grade 2 and grade 3 lesions were more frequent in male than in female rats (\( P=0.01 \)). Two loci, D1Rat238, on chromosome 1 and the IGF receptor-binding protein 4 (Rbp4g) on chromosome 10, were significantly linked to the degree of renal damage, with SHRSP allele being aggressive at D1Rat238 locus and protective at Rbp4g locus. In male rats only, the SHRSP allele at one locus on chromosome 16, D16Mit2, was associated with a more severe degree of renal disease. Our results demonstrate that in this intercross, susceptibility to renal damage is influenced by several genetic loci acting independently from high blood pressure levels and also shows a sexual dimorphism. (Hypertension. 2003;42[part 2]:702-706.)

Key Words: genetics ■ rats, stroke-prone SHR ■ renal disease ■ hypertension, genetic

The stroke-prone spontaneously hypertensive rat (SHRSP) strain develops a severe form of hypertension and shows a high incidence of injuries in different vascular beds. In particular, histological lesions in renal vasculature and parenchyma are commonly observed in this animal model, whereas they are milder or even absent in its closely related rat strain, the stroke-resistant spontaneously hypertensive rat (SHR),\(^1,2\) from which the SHRSP has been derived.\(^3\)

Indeed, severe renal failure is not constantly observed in the SHRSP, probably because during the establishment of this strain, this additional trait would have resulted in an intolerable reduction of fitness.\(^3\) On the other hand, previous studies indicate that development of renal lesions precede cerebrovascular lesions and suggest that they are involved in the pathogenesis of stroke in this model.\(^2,4\) In this latter regard, high blood pressure levels, abnormalities in the renin-angiotensin system, and an elevated salt intake have been recognized as important factors in the pathogenesis of cerebral as well as of renal vascular lesions in this strain.\(^2,5\) More recently, however, elegant experiments of kidney transplantation have pointed out a central role of genetic factors in the occurrence of renal damage in the SHRSP.\(^6\)

Experiments performed by our group have formerly investigated the role of genetic factors in the pathogenesis of stroke\(^7\) and of endothelial dysfunction\(^8\) in the SHRSP through a genotype/phenotype cosegregation analysis of these 2 traits in an SHRSP/SHR F2 intercross. In this intercross, elevated blood pressure levels do not segregate, whereas latency to stroke\(^7,8\) and the impairment of endothelial function\(^8,9\) do segregate, along with the susceptibility to a high salt, low potassium diet.

The aim of the current study was to investigate the potential role of genetic factors in the susceptibility to renal histological lesions in the SHRSP/SHR F2 intercross by using a large panel of polymorphic markers between the 2 parental strains.\(^10\)

Methods

Animals
A cohort of 154 F2-hybrid rats (79 male, 75 female), derived from the original SHR and SHRSP colonies established in Japan in 1974,\(^9\) were studied, according to the Guidelines for Animal Research of our Institution.

Rats were kept at constant temperature with a 12-hour day-night cycle, with free access to regular chow and water. At 6 weeks of age, rats were shifted to a stroke-permissive diet (Japanese-style diet)

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(Laboratori Dottori Piccioni), with an altered sodium/potassium ratio, low protein content, and NaCl 1% supplement in the drinking water for 4 weeks. At this time, systolic blood pressure (SBP) was measured noninvasively in conscious restrained rats by means of a tail-cuff sphygmomanometer (PE-3000, Narco Biosystem Inc). To minimize the variability of this technique, multiple (n=5) measurements were obtained for each rat after they became accustomed to the device. To avoid the confounding effect of the predictable onset of stroke and death that may occur after several weeks of exposure to the Japanese diet, rats were killed at this stage.

**Histology and Phenotype Definition**

Histopathological studies were performed in all F2 hybrids rats, in 27 parental SHRSP (F0), and in 27 parental SHR (F0). After the rats were killed, kidneys were removed, cleaned of fat, and fixed in 10% phosphate-buffered formalin cut in 2 halves on a frontal plane and embedded in paraffin blocks, following standard procedures. For assessment of renal vascular and parenchymal damage, standard transverse 5-μm sections of each individual kidney, including the apex of the pyramidal, the corticomedullary junction, and the cortex, were examined. For light microscopic analysis, sections were stained with eosin for the nuclei, eosin van Gieson trichrome for connective tissue, Gomori for precollagen reticule, and PAS Alcian blue to detect mucopolysaccharides. Renal lesions were graded according to the degree of vascular and parenchymal damage, following classification criteria formerly described as absence of renal lesions grade 0, presence of mild lesions as grade 0.5, presence of moderate lesions as grade 1, and finally, presence of severe lesions as grades 2 and 3. In particular, grade 0 corresponds to the absence of vascular and parenchymal damage in all the sections examined (Figure 1A). Grade 0.5 refers to the mildest lesions: initial alterations of the vascular wall, ie, disorganization of smooth muscle cells (SMCs), and a lumen reduction are constantly observed in the culprit vessels. At this stage, only arterioles with a diameter <20 μm are involved, and this lesion is observed in ~20% of the arterioles examined. No parenchymal lesions are observed. Lesions defined as grade 1 (Figure 1B) are characterized by a rarefaction of SMCs within the vascular wall and their substitution with an extra cellular matrix proteic in origin (hyalinosis) that represents the prominent component of vascular wall at this stage. Signs of early necrosis of vascular wall can already be observed. These abnormalities involved vessels with a diameter <40 μm and were observed in ~40% of all the arterioles examined. Parenchymal lesions are still absent at this stage. Grade 2 lesions (Figure 1C) are represented by hyalinosis and moderate arterial necrosis of vessels with a diameter ≥40 μm, involving 60% of the arterioles observed. At this stage, presence of parenchymal damage is documented by the observation of focal tubular necrosis and regeneration in <50% of cortical and medullary parenchyma. Parenchymal lesions (Figure 1D) were observed in few animals and were characterized by extensive and severe necrosis of vascular wall of arteries ≥40 μm involving ~80% of the vessels studied, accompanied by regenerative tubular changes and focal infarcts of glomeruli. These lesions were present in >50% of the cortical and medullary parenchyma. Vascular and parenchymal lesions are summarized in Table 1.

**Genotyping and Linkage Analysis**

A random marker genome screening was performed by using a panel of 274 SSLPs, shown to be polymorphic between the 2 parental strains.10 SSLPs were obtained from different sources: markers identified as DxMghy, DxMity, and DxRaty were from Research Genetics Inc; those defined as DxWoxy were from the Wellcome Institute for Human Genetics; and markers developed from published sequences were reported with their GenBank locus name (http://www.ncbi.nlm.nih.gov).

All markers were assayed by PCR and resolved on 7% polyacrylamide gels, following published protocols.7 A linkage genetic map was developed with MAPMAKER/EXP3.0 computer package.11

**TABLE 1. Different Degrees of Vascular and Parenchymal Lesions Observed in F2 Cohort Kidneys**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
<th>Arterioles Diameter, μm</th>
<th>Parenchymal Lesions</th>
<th>n ( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>81 (52.5)</td>
</tr>
<tr>
<td>0.5</td>
<td>Disorganization of smooth muscle cells within the vascular wall; lumen reduction</td>
<td>&lt;20</td>
<td>...</td>
<td>28 (18.2)</td>
</tr>
<tr>
<td>1</td>
<td>Rarefaction of smooth muscle cells and hyalinosis of vascular wall</td>
<td>&lt;40</td>
<td>...</td>
<td>22 (14.2)</td>
</tr>
<tr>
<td>2</td>
<td>Hyalinosis and moderate degree of necrosis of vascular wall</td>
<td>&gt;40</td>
<td>Focal tubular necrosis and regeneration involving less than 50% of cortical and medullary parenchyma</td>
<td>15 (9.7)</td>
</tr>
<tr>
<td>3</td>
<td>Severe necrosis of vascular wall</td>
<td>&gt;40</td>
<td>Focal infarcts of glomeruli and regenerative tubular changes present in more than 50% of the cortical and medullary parenchyma</td>
<td>8 (5.2)</td>
</tr>
</tbody>
</table>

n indicates number of animals.
Linkage of genotype markers to the degree of renal disease was determined, grouping together animals with a severe score, grade 2 and 3, of histological lesions, by a 4X3 contingency table and $\chi^2$ test with 6 df. Experiment-wise threshold level to declare linkage was calculated by QTL cartographer from 1000 permutations of each genotype marker against the phenotype in the population as a whole and in male and female separately. Linkage was reported as significant if the $\chi^2$ statistic for a marker was greater than the critical value at 0.05 and suggestive if greater than the critical value at 0.10.

Statistical Analysis
SBP is expressed as mean±SEM. Correlation between SBP and degree of renal pathology was assessed by calculating the coefficient by Spearman test. Difference in gender distribution of renal disease in the F2 cohort was calculated by $\chi^2$ test with 2 df.

Results
At the end of the dietary treatment, SBP averaged 188±2.3 mm Hg in the rats of the F2 cohort (189±3.6 in male rats; 187±3.8 in female rats). Analysis of correlation between SBP levels and the degree of renal damage in this intercross did not achieve the level of significance (coefficient, 0.201; P=NS). When male and female rats were analyzed separately, no correlation was found in either group (coefficient, 0.154; P=0.302, for male rats; coefficient=0.222; P=0.134 for female rats).

Frequencies of renal lesions in the overall F2 population are shown in Table 1. Grade 2 and 3 lesions were more frequently observed in male than in female rats (19.7% versus 9.3%; $\chi^2=7.95, P=0.01$).

A random-marker genome screening was performed by using a panel of 274 genetic markers polymorphic between the 2 parental strains. A genetic linkage map with an average intermarker distance of 7 kcM was generated for the present intercross. As shown in Figure 2, 2 areas centered at marker D1Rat238 on chromosome 1 ($\chi^2=13.9$) and at a microsatellite marker within the insulin-like growth factor receptor binding protein 4 (Rbp4g) gene on chromosome 10 ($\chi^2=20.9$) were significantly linked to the degree of renal damage (Table 2) in the population as a whole, with SHR and SHRSP alleles exerting a protective effect at the D1Rat238 locus and at the Rbp4g locus, respectively (experiment-wise threshold level, $\chi^2=12.1$, for $\alpha=0.05$). Two additional loci, namely kallikrein ($\chi^2=10.5$) on chromosome 1 and the anonymous marker D4Mgh7 ($\chi^2=11$) on chromosome 4, showed a suggestive linkage to renal disease, with the SHR allele exerting a protective effect (experiment-wise threshold level, $\chi^2=10$ for $\alpha=0.10$).

Linkage analysis performed on male and female rats separately identified one locus on chromosome 16, D16Mit2, where the SHRSP allele was significantly associated with a more severe degree of renal disease ($\chi^2=13.8$; experiment-wise threshold level for male $\chi^2=12.2$, for $\alpha=0.05$).

Histopathological evaluation of SHR and SHRSP parental strains showed a highly significant difference in the development of renal lesions during exposure to the stroke-permissive diet. In detail, in SHR, no detectable vascular or parenchymal lesions on kidney sections were found after 4 weeks or a prolonged exposure (>12 weeks) to the Japanese style diet. SHRSP showed progressive and marked renal histopathological lesions during exposure to the Japanese style diet. After 4 weeks of diet, SHRSP displayed minimal to moderate lesions (grade 0.5 to 1), whereas after 8 to 12 weeks, more severe lesions were observed (grades 1 to 3).
Kidneys16 and showed an inhibitory effect on the growth model of chronic uremia, Rbp4g expression was increased in potentially reducing its biological effects. In an experimental factor with higher affinity than the receptor itself, thus as one of the potential mediators of growth retardation in are elevated in chronic renal failure and have been proposed development of severe renal lesions in male rats only.

We identified an additional locus on chromosome 16, D16Mit2, linkage analyses were performed on male rats and female rats, segregation study in a hybrid SHRSP/SHR F2 cohort. Our results demonstrate that at least 2 areas centered at an anonymous marker, D1Rat238, on chromosome 1 and at the insulin-like growth factor receptor binding protein 4 (Rbp4g) on chromosome 10, show a significant association and an opposite effect on the severity of renal damage. In particular, the SHR allele at the D1Rat238 marker and the SHRSP allele at the Rbp4g locus show a protective effect on the development of severe vascular and parenchymal renal damage. In our experimental model, moderate to severe renal lesions were more frequently detected in male than in female rats. When separate linkage analyses were performed on male rats and female rats, we identified an additional locus on chromosome 16, D16Mit2, where the SHR allele exerted a protective effect on the development of severe renal lesions in male rats only.

Serum insulin-like growth factor receptor binding proteins are elevated in chronic renal failure and have been proposed as one of the potential mediators of growth retardation in affected children. Indeed, they bind the insulin-like growth factor with higher affinity than the receptor itself, thus potentially reducing its biological effects. In an experimental model of chronic uremia, Rbp4g expression was increased in kidneys and showed an inhibitory effect on the growth cartilage. In our experimental model, the SHRSP allele at the Rbp4g locus exerts a protective effect on the development of severe renal lesions. Specific studies of the biological activity of the mutant gene, as well as the establishment of congenic lines are required to validate the hypothesis that Rbp4g might represent a candidate gene for renal damage in this experimental model.

A genetic predisposition to development of renal lesions has been described in different rat models. In the Fawn-Hooded Hypertensive Rat, a rat strain in which severe renal damage develops with mild hypertension, a QTL on chromosome 1 has been reported to determine the degree of renal damage independent from blood pressure, and a high susceptibility to renal lesions has been observed in SHR carrying a segment of chromosome 1 from Brown Norway rats after the induction of a controlled degree of deoxycorticosterone acetate salt hypertension. More recently, 3 QTLs were found to explain increased urinary albumin excretion in the Munich Wistar Fromter rat, a model of chronic nephropathy and mild hypertension.

None of these QTLs overlaps with the area on chromosome 1, linked to the degree of renal damage identified in the current study. On the other hand, these discrepancies can be explained by differences in the genetic background among the strains used for investigation and/or the density of the linkage genetic maps obtained for each experimental model and, finally, by the definition of the target phenotype. Of note, in the same experimental model, we have identified on chromosome 1 one QTL, STR1, linked to latency to stroke, and one locus, D1Wox4, potentially involved in the modulation of endothelium-dependent vasorelaxation. Taken together, these observations suggest that several regions of rat chromosome 1 might be involved in determining the susceptibility to vascular injury in different vascular beds in the SHRSP animal model.

The current investigation explored the genetic basis of renal injury in SHRSP by using as a target phenotype vascular and related parenchymal lesions in the kidney. Score of renal damage was previously obtained in SHR and SHRSP male rats and replicated in the current study. Although SHR rats do not have renal lesions after 4 weeks of diet and are unlikely to have renal lesions even after a prolonged exposure to a Japanese-style diet, SHRSP showed a minimal degree of renal lesions after 4 weeks of diet and progressive increase of severe grade 2 and 3 lesions after 8 and 12 weeks of diet, often associated with the onset of stroke. In the current study, F2 rats were studied after only 4 weeks of Japanese diet, when the incidence of stroke and stroke-related death is 1.8% in the F2 population. These findings might explain the low percentage of severe grade 2 and 3 renal lesions observed.

Although elevated levels of SBP as well as a high salt intake are essential for the development of cerebrovascular...
and renal lesions in the SHRSP,1,2,5,21 a genetic predisposition to development of renal damage has been recognized.6 In fact, SHRSP kidney transplanted in SHR recipients, kept on a high salt intake, had a more severe degree of renal damage than did the native SHR kidney, indicating that when exposed to the same blood pressure level and dietary regimen, the SHRSP kidney is intrinsically highly susceptible to renal injury. Consistent with these observations, we did not find any correlation between SBP levels and extent of renal damage in the present intercross. Indeed, SBP, a major confounding factor in vascular pathophysiology, does not segregate in the SHRSP/SHR F2 intercross.7–9 We cannot exclude, however, that diastolic blood pressure and/or mean arterial pressure values that were not evaluated in the current study may be correlated to the degree of renal damage.

In conclusion, the current results demonstrate that several genetic loci contribute to the development of renal damage in our experimental model. The study of genetic factors underlying intermediate disease phenotypes, such as renal disease in the SHRSP, might be helpful to dissect genetic factors underlying susceptibility to vascular injury and to fully explain the remaining genetic variance in latency to stroke in this experimental model.7 Given the discrete nature of the target phenotype analyzed in the current study, no conclusions regarding the overall contribution of genetic factors could be drawn.22

Perspectives
Renal lesions precede the onset of stroke in the SHRSP, and it has been suggested that they might be involved in the pathogenesis of stroke, thus representing a potential intermediate phenotype. Intermediate phenotypes have been proposed as complementary phenotypes to decrease the distance between genes and complex traits. In this context, dissection of the genetic basis of renal damage in this rat strain might represent one of the experimental approaches to shed light on the complexity of the vascular pathology in this experimental model as well as the first step to investigate the specific gene contribution in congenic strains carrying selective SHR or SHRSP chromosomal regions.

On a more general perspective, dissection of genetic factors involved in the determination of target organ damage during hypertension might help to gain further insights into the natural history and prevention of cardiovascular diseases and to achieve a deeper knowledge of the mechanisms involved in cardiovascular homeostasis.

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