DEVELOPMENT OF SUBSTRAINS
IN SPONTANEOUSLY HYPERTENSIVE RATS:
GENEALOGY, ISOZYMES
AND EFFECT OF HYPERCHOLESTEROLEMIC DIET

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Genealogical study and experimental fat-cholesterol and salt loadings showed that the present strain (F26–27) of spontaneously hypertensive rats consisted of several substrains with no difference in the level of blood pressure but with a marked difference in the incidence of cardiovascular lesions. Biochemical specificities of these substrains were demonstrated by ALPase and esterase isozymes in the liver and serum. Different responses in serum cholesterol level to the hypercholesterolemic diet served as a further differentiation of some lines among these substrains and were seemingly related to their vulnerability to cardiovascular lesions under these experimental conditions.

HYPERTENSION seems to play some role in the cause of cardiovascular lesions such as cerebrovascular complications and ischemic heart disease as shown by epidemiological studies in man1–3 and also by the effectiveness of blood pressure control in the reduction of these complications.4–7 On the other hand, there are abundant reports about the acceleration of atherosclerosis in the animals with renal hypertension fed on a hypercholesterolemic diet.8–12 Effect of hypercholesterolemic diet on cardiovascular lesions was also studied in spontaneously hypertensive rats (SHR) [Okamoto and Aoki]13,14 which have attracted more attention nowadays as the best animal model for the studies on essential hypertension,15 and high incidence of cardiovascular lesions was obviously noted in SHR fed on hypercholesterolemic diet.6 These epidemiological, clinical and experimental studies provide us with undeniable evidences about the dominant role of hypertension in the cardiovascular complications.

However, in the course of the experimental feeding of SHR on a high fat-cholesterol and salt diet, we observed a rather marked difference in the incidence of cerebrovascular and other cardiovascular lesions among the substrains of SHR.17–19 As there was no noticeable difference in the level of high blood pressure among these substrains, not only hypertension itself but also some genetic differences seemed to contribute to a different susceptibility to cardiovascular lesions. Therefore, genealogy and different characteristics of these substrains were intensively

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studied to clarify what genetico-biochemical profiles were related to the vulnerability to cardiovascular lesions in SHR. Such profiling in SHR may throw a new light on the differentiation of benign and malignant conditions of hypertensive disease as recently pointed out in essential hypertension by Laragh.20

MATERIALS AND METHODS

Spontaneously hypertensive rats used in this experiment were mainly from F24 to F26 generations which had been established as an inbred strain in Oct., 1969 after 20 generations of sib-breeding15,17. Control normotensive rats (CR) were the age-matched rats either of Wistar-Kyoto, a closed colony at Animal Center Laboratory of Kyoto University, from which the ancestors of SHR were separated, or of the inbred strain of Wistar-Mishima (F60–61), which were introduced from the National Institute of Genetics, Mishima, Japan and kept in our laboratory.

Rats were fed on commercial stock chow diet CA-1 (Clea Co., Tokyo, Japan) and tap water for drinking. Selected groups of male animals were fed on high fat cholesterol diet16–19 containing 20% of “tempura” oil (Japanese fry oil), 5% of cholesterol, 2% of bile powder (crude sodium cholate) and 73% of the powdered diet of CA-1 and were also loaded with 1% salt water for drinking. This regimen of high fat-cholesterol and salt diet or hypercholesterolemic diet (FCS-diet) was started at the age of 45 to 60 days and continued for about 8 weeks.

All rats were housed in an air-conditioned room with constant temperature (22–25°C) and humidity (50 to 60 per cent).

Blood pressure was measured indirectly by applying a cuff around the tail of anesthetised rats (water-plethysmographic method)13 at weekly intervals and also before sacrifice.

One group of rats were sacrificed at the age of 50 to 60 days and 90 to 120 days, corresponding to the incipient and early stage of hypertension in SHR, respectively. After decapitation, blood was collected and organs were extirpated, weighed and kept frozen at −20°C for zymographic analysis. Preparation of the organ homogenate and detailed technique for starch gel electrophoresis were reported previously.21 Nonspecific (α-naphthyl acetate) esterase21 acid and alkaline phosphatase isozymes22,23 were routinely examined by the zymographic technique, and L-leucyl-2-naphthylamidase22 lactate and glucose-6-phosphate dehydrogenase22 were also checked to probe the difference among the substrains. As the gel and electrode buffers, borate buffers (pH 8.5), 0.03 and 0.3 M, respectively, were used, but 0.005 M histidine and 0.41 M sodium citrate buffers (pH 8.0) were used especially for the clear separation of alkaline phosphatase isozymes of the serum. The discrete zymogram of serum alkaline phosphatase was obtained by incubating the gels at 37°C in the mixture of the following contents: 0.5 mg of sodium α-naphthyl phosphate, 1 mg of Fast Violet B salt, 5 mg of polyvinylpyrrolidon per ml of 0.1 M tris buffer (pH 8.5) containing 0.0005 M MgCl2 and 0.3 M NaCl.

For the determination of serum cholesterol level in the other group of male rats blood samples were collected from incised tail tips with capillary hematocrit tubes before and at various time intervals after the beginning of the hypercholesterolemic diet-loading and serum was separated by centrifugation (11,000 rpm for 5 min). 50 μl of the serum was assayed for cholesterol by Zurkowski’s method24.

All numerical data were statistically analyzed by Student’s small sample t-test.

RESULTS

1. Genealogical Study

SHR kept in our Department at present consist of 3 main substrains, tentatively named A, B and C. As shown in the outline of the pedigree (Fig.1), substrain A had been kept only by successive sib-breeding for 26 generations up to the present. They are subdivided into at least 3 lines, A1, A2 and A3. The former two were separated at F22 generation and the last was started at F21 generation. Among these lines of A substrain, comparatively high incidence of spontaneous cerebral softening and/or bleeding was observed in some lines, and A1-sb was started from the litter which showed the comparatively high incidence at F24 generation and the original male parent of F24 itself died of spontaneous cerebral softening after mating at the age of 220 days.

B and C substrains were produced by nonsib-breeding in the course of establishing inbred SHR in order to avoid the risk of continuing the strain only by sib-breeding for many generations. The B substrain was separated from the original strain at the F12 generation by nonsib-breeding and was kept by sib-breeding thereafter as two lines, B1 and B2. In the B2 line spontaneous cerebral softening and/or bleeding was not ob-

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TABLE 1  BLOOD PRESSURE, BODY WEIGHT AND ORGAN WEIGHT IN MALE A, B, AND C SUBSTRAINS OF SHR, WISTAR-KYOTO (WK) AND WISTAR-MISHIMA (WM) AT 4 MONTHS OF AGE

<table>
<thead>
<tr>
<th></th>
<th>SHR</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (6)</td>
<td>B (6)</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>186 ± 6**</td>
<td>188 ± 6**</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>322 ± 16</td>
<td>328 ± 20</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>1.45 ± 0.06**</td>
<td>1.47 ± 0.04**</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>2.56 ± 0.14</td>
<td>2.68 ± 0.30</td>
</tr>
<tr>
<td>Hypophysis (mg)</td>
<td>11 ± 8</td>
<td>11 ± 8</td>
</tr>
<tr>
<td>Thyroid (mg)</td>
<td>26 ± 3.6</td>
<td>25 ± 3.2</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>50 ± 3.7</td>
<td>47 ± 3.4</td>
</tr>
</tbody>
</table>

M ± S.D.

( ): number of rats examined

*: **: statistically significant differences from the value of WK, ** (p<0.01), * (0.01<p<0.05)

served and there were some adult rats of the B₂ line with heavier body weights than the weights of ordinary SHR (male and female adults, under 400 and 250 g, respectively, under the present feeding condition). The body weights of these slightly obese males and females were 400–580 and 260–360 g, respectively. These rats with a tendency to become obese (B₂-ob) were selected at F₂₁ generation from a male (430 g) and a female (260 g) in the same litter and sib-breeding was continued thereafter.

D substrain, which exist no longer, was produced at F₁₆ generation by nonsib-breeding between a male from A and a female from B

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substrain. The present C substrain was started at F20 generation by the mating between a female from D and a male from B substrain.

2. Blood Pressure and Organ Weight

Both young and adult SHR showed a significantly higher blood pressure and heavier heart weight than young and adult CR, respectively. However, no significant difference in blood pressure and organ weight was noted among A, B and C substrains of SHR. Blood pressure, body weight and organ weight of the young male rats sacrificed at the age of 4 months are summarized in Table I.

3. Zymogram analyses

a) Strain specificity: SHR had some strain specific isozymes as reported previously. The SHR-specificity of α-naphthyl acetate esterase zymogram patterns of the kidney and liver were constantly observed in all three substrains, A, B and C, compared with two normotensive controls, Wistar-Kyoto and Wistar-Mishima. Moreover, SHR specificity of the esterase isozymes detected in arterial tissue homogenate was

<table>
<thead>
<tr>
<th>TABLE II CHARACTERISTIC ZYMOGAMS OF A, B, AND C SUBSTRAINS IN SHR, COMPARED WITH WISTAR-KYOTO</th>
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</thead>
<tbody>
<tr>
<td>SHR</td>
</tr>
<tr>
<td>A   B   C</td>
</tr>
<tr>
<td>Alkaline phosphatase of liver</td>
</tr>
<tr>
<td>Alkaline phosphatase of serum</td>
</tr>
<tr>
<td>Esterase of serum*</td>
</tr>
<tr>
<td>Esterase of kidney,** liver, artery</td>
</tr>
<tr>
<td>Acid phosphatase of liver*</td>
</tr>
<tr>
<td>Acid phosphatase of thyroid gl.</td>
</tr>
</tbody>
</table>

S or D: Zymograms are same or different from those of Wistar-Kyoto.
* : Zymogram is also different in Wistar-Mishima.
B, H : Borate or histidine buffer at pH ( ).
observed commonly in these substrains.

Acid phosphatase of the liver in these substrains showed a common zymogram pattern different from that of Wistar-Kyoto but similar to that of Wistar-Mishima. Acid phosphatase of the thyroid gland in three substrains commonly showed a zymogram with intense enzyme activity (Table II) but with no difference in electrophoretic movability from that in CR, and confirmed the previous quantitative data of the enzyme activity.

b) Substrain specificity: Three isozyme bands of serum alkaline phosphatase, clearly demonstrated in histidine buffer system, migrated faster to the anode in B and C substrains than A substrain and CR as shown in Fig.2. A similar acceleration in the electrophoretic migration of alkaline phosphatase of the liver was noted in B and C substrains compared to A substrain and CR, although the liver had the only one main band of the enzyme.

On the other hand, serum esterase zymogram
### Table III: Serum Cholesterol of A, B, and C Substrains in SHR and Control Rats, Fed on Fat-Cholesterol and Salt Diet.

<table>
<thead>
<tr>
<th>Feeding period in weeks</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>ca 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₁-sb (a)</td>
<td>72 ± 8 (14)</td>
<td>214 ± 39 (10)</td>
<td>c' e f g h i</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₁ (b)</td>
<td>72 ± 5 (16)</td>
<td>174 ± 33 (6)</td>
<td>f' g i</td>
<td>242 ± 45 (8)</td>
<td>323 ± 57 (10)</td>
<td>463 ± 251 (12)</td>
<td>494 ± 237 (15)</td>
</tr>
<tr>
<td>A₂ (c)</td>
<td>73 ± 10 (19)</td>
<td>176 ± 36 (13)</td>
<td>a' e' f g i</td>
<td>e' β i</td>
<td>e β i</td>
<td>β h' i</td>
<td>β h i</td>
</tr>
<tr>
<td>A₃ (d)</td>
<td>73 ± 6 (10)</td>
<td>191 ± 44 (6)</td>
<td>e' f g h' i</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SHR</strong></td>
<td></td>
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</tr>
<tr>
<td>B₁ (e)</td>
<td>72 ± 7 (17)</td>
<td>150 ± 30 (17)</td>
<td>a c' d' g i</td>
<td>182 ± 47 (7)</td>
<td>215 ± 30 (7)</td>
<td>274 ± 48 (7)</td>
<td>288 ± 42 (6)</td>
</tr>
<tr>
<td>B₂ (f)</td>
<td>77 ± 20 (12)</td>
<td>132 ± 27 (8)</td>
<td>a b' c d h' i</td>
<td>142 ± 18 (8)</td>
<td>163 ± 17 (8)</td>
<td>215 ± 31 (11)</td>
<td>207 ± 27 (10)</td>
</tr>
<tr>
<td>B₂-ob (g)</td>
<td>74 ± 13 (14)</td>
<td>112 ± 16 (11)</td>
<td>a b c d e h' i</td>
<td>α e' h</td>
<td>α e h</td>
<td>α e h' i</td>
<td>α e h i</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(h)</td>
<td>74 ± 10 (22)</td>
<td>156 ± 28 (22)</td>
<td>a d' f' g</td>
<td>243 ± 39 (9)</td>
<td>269 ± 42 (8)</td>
<td>265 ± 60 (10)</td>
<td>266 ± 45 (10)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Wistar-Kyoto) (i)</td>
<td>78 ± 11 (28)</td>
<td>111 ± 9 (19)</td>
<td>a b c d e f h</td>
<td>145 ± 15 (11)</td>
<td>169 ± 35 (21)</td>
<td>166 ± 23 (19)</td>
<td>189 ± 29 (16)</td>
</tr>
</tbody>
</table>

M ± S.D.: cholesterol level (mg/dl), determined by Zarkowski's method.

( ): No. of cases; male rats at 45–60 days of age were fed on fat-cholesterol-salt diet.

Statistical analysis: a (a'), b (b'), c (c'), d (d'), e (e'), f (f'), g (g'), h (h'), i (i'), α (α'), and β (β') show statistically significant differences; p<0.01 and (0.01< p<0.05), from the values of A₁-sb, A₁, A₂, A₃, B₁, B₂, B₂-ob, C control, combined A and combined B₂ substrains, respectively.
in A showed a weak reaction at the first band in the anodal direction and was different in intensity from those in B, C substrains, especially in B, and Wistar-Kyoto as shown in Fig.3.

As summarized in Table II, these zymographic findings of alkaline phosphatase of liver and serum, or of serum esterase enabled us to distinguish A from B and C substrains, but differentiation between B and C substrains, which are genealogically close to each other, was impossible by various zymogram analyses so far as examined.

4. Effect of Hypercholesterolemic Diet

As reported in the previous articles, the experimental feeding of high fat-cholesterol and salt (FCS) diet showed a rather marked difference among A, B2 and C substrains in the incidence of cardiovascular lesions, especially cerebrovascular lesions such as cerebral softening and/or bleeding. The incidence of cerebrovascular lesions in A, B2 and C substrains were 79, 34 and 58 per cent, respectively. Therefore, response in serum cholesterol level to the FCS diet was scrutinized by determining the serum cholesterol level repeatedly in the course of the experimental FCS loading and the difference in the response among these substrains was demonstrated. As shown in Table III, a rapid increase in serum cholesterol level was noted in the A substrate, while the B2 substrate showed a slow development of hypercholesterolemia, not much different from that in Wistar-Kyoto during the first 3 weeks of the experimental FCS loading. The difference in the response between A and B2 substrate was detected even one week after the initiation of the experiment and became obvious in the second week of the experiment. The C substrate showed an intermediate response between A and B2. Consequently, the hypercholesterolemic responses among A, B2 and C substrains appeared to be parallel to the aforementioned incidence of the cardiovascular lesions during the FCS loading.

Recent observations showed a comparatively high incidence of spontaneous cerebral softening and/or bleeding in the male rats of A1 lines (A1-sb) and conversely, no incidence of these cerebral lesions in B2 line, from which a line of slightly obese SHR (B2-ob) was separated as shown in the aforementioned genealogical study. Even a one-week FCS loading showed an obvious difference in the hypercholesterolemic response among these subdivided lines of A and B substrains: A1-sb showed the greatest response, while B2-ob showed the least response similar to that in Wistar-Kyoto. A highly significant difference in the serum cholesterol level was proved between A1-sb (214 ± 39 mg/dl) and B2-ob (112 ± 16 mg/dl). The response in B1 line was close to that in C and the responses of the A1, A2 and A3 lines were roughly intermediate between those in A1-sb and B1. These different responses to the FCS diet showed a possible genetic differentiation of several lines both in A and B substrains.

Discussion

Genealogical study showed the developmental course of different substrains in SHR, and zymogram analyses confirmed the establishment of the substrains by detecting the biochemical differences among these substrains, A, B2 and C with different susceptibilities to cardiovascular lesions as reported previously.

The different responses in serum cholesterol level to FCS diet showed a possibility of further differentiation of these substrains as to cholesterol and/or salt metabolism. These genetic metabolic differences seemed to be related to the different susceptibility to cardiovascular lesions under the FCS loading, for the present study suggested that there was a parallelism between the hypercholesterolemic response and the incidence of the cardiovascular lesions among these substrains.

Augmented responses in serum and liver cholesterol levels and in the lipid infiltration of heart and aortic tissue to hypercholesterolemic diet were observed in senescent rats and age-dependent change of cholesterol metabolism was recently characterized as a general delay in the absorption, biosynthesis, degradation and excretion of cholesterol. Although further elucidation of the detailed mechanism of hypercholesterolemic response observed in A, B1 and C substrains is necessary, their high vulnerability to vascular lesions may be related causally to the premature decay as to cholesterol metabolism or may depend on other basic metabolic alteration similar to that in the aging process which can be detected as the hypercholesterolemia response in one side. Whatever the cause is, this kind of hypercholesterolemic response may serve for the differentiation of vascular vulnerability.

The deposition of cholesterol in the vasculature may be due to the impairment of cholesterol esterification, for serum cholesterol esterifying enzyme seems to be important in promoting the
removal of free cholesterol from the arterial wall. Although α-naphthyl acetate esterase demonstrated zymographically in this study is nonspecific arylerase and its detailed physiological role is unknown, the possible role of serum arylerase is speculated as transesterification. The attenuation of one of the serum esterase isozenes in the A substrain may be involved in the impaired lipid metabolism detected as hypercholesterolemic response. However, such speculation of the relationship between the two is beyond our present state of knowledge.

Alkaline phosphatase zymogram of the liver and serum in the A substrain susceptible to cardiovascular lesion was the same as that in Wistar-Kyoto, and B and C sub strains with rather low susceptibility to cardiovascular lesion showed a different zymogram pattern from the original inbred A substrain separated only by successive sib-breeding from normotensive Wistar-Kyoto strain. It may follow as a natural consequence that the selection of the rats for hypertension resulted in the development of hypertensive rats able to live in spite of hypertension. Specific isozyme alterations detected in B and C strains might partly correspond to the mutational development of their resistant feature to cardiovascular lesions under the hypertensive state. The possible development of similar compensatory mechanisms to hypertension itself in SHR was reported in the low activity of renin-angiotensin system, decreased norepinephrine turnover of the heart with a decrease in cardiac output according to the aging process, hyporeactivity of aortic strips, dilatation of thoracic aorta and low tyrosine hydroxylase activity in the mesenteric vasculature.

As a different incidence of the cardiovascular lesions was also proved among these sub strains simply under salt loading, genetic deviation of salt metabolism rather than of cholesterol metabolism may be an indispensable factor for the vulnerability to cardiovascular lesions. Hypercholesterolemic response and vascular vulnerability in the A substrain might be influenced by a deviation in salt metabolism, for experimental study indicated that salt aggravated vascular lesions in the rats fed on atherogenic diet. Further metabolic studies and analyses of influential factors such as adrenal and thyroid hormones are necessary for elucidating the genetic difference of the vascular vulnerability among the sub strains. As Laragh indicated a possibility to differentiate benign and malignant conditions of essential hypertension by the level of plasma renin activity, the detection of such biochemical features in vasculessitive and -resistant substrains of SHR may provide a clue for the classification of the benign and malignant hypertensive states from genetic aspects. Even if the clue is not so closely related to the genetic lesions essential for vascular vulnerability as in the case of hypercholesterolemic responses, biochemical features related in any way to the genetic lesions such as the substrate specific isozymes proved in this study may serve as an index for the differentiation. Such a clue for differentiating the conditions of hypertension will be useful to the preventive medicine for complications of hypertension, the direct cause of death in hypertensive diseases, merely by the reduction of the blood pressure level or hopefully by the redressment of the genetic metabolic deviation in the subgroup of hypertensive subjects with high vulnerability to vascular lesions.

The present study also indicated that SHR could be further divided into several sub strains not only by the vascular vulnerability but also by some other genetic characteristics, which might serve for the elucidation of hormonal or metabolic disorder from genetic aspects. The obese SHR, recently reported by Koletsky is one of the extreme examples of such metabolic disorder among SHR.

**SUMMARY**

The A, B and C sub strains of spontaneously hypertensive rats (SHR) with high, low and intermediate vulnerability to cardiovascular lesions under high fat-cholesterol and salt (FCS) loading were studied genealogically, zymographically and as to their hypercholesterolemic response, and the following conclusions were obtained.

1) A, B and C substrains were genealogically different from each other. The A substrain was established only by sib-breeding, and B and C substrains were obtained by nonsib-breeding in the course of establishing inbred A substrain.

2) The A, B and C sub strains commonly had the SHR-specific isozymes of α-naphthyl acetate esterase in the kidney, liver and artery, and of acid phosphatase in the liver. The A substrain was differentiated from the B and C sub strains by the difference in alkaline phosphatase isozyme of the liver and serum and also in serum α-naphthyl acetate esterase isozyme.
Substrains in SHR

3) Hypercholesterolemic responses to FCS diet were great, least and intermediate in the A, B and C substrains, respectively. The difference in the initial response to FCS loading even for 1 to 3 weeks was likely to be parallel to the difference in the vulnerability to vascular lesions among these substrains.

4) These studies showed that SHR were not only the animal model for hypertension research but their substrains with different vulnerability to vascular lesions served for the elucidation of genitico-biochemical mechanisms pathogenetically related to the complications of hypertension, and further indicated that some substrains with different characteristic metabolic disorders could possibly be established among SHR.

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REFERENCES


28. YAMORI, Y., OOSHIMA, A., & OKAMOTO, K.: Genetic factors involved in spontaneous hyperten-


