Immunosuppressant HR-325 Attenuates Progression of Malignant Arteritis in the Kidney of Dahl Salt-Sensitive Rats

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We investigated the effects of the immunosuppressant HR-325 on arterial lesions in Dahl rats with salt-induced hypertension. Forty-eight 6-wk-old Dahl salt-sensitive (DS) rats were divided into 1) a low-salt (0.3% NaCl) group, 2) a high-salt (4% NaCl) group, 3) a high-salt and low-dose (1 mg/kg) HR-325 group, and 4) a high-salt and high-dose (30 mg/kg) HR-325 group. The rats were treated for 8 wk. Various variables of renal function and morphological alterations in the kidney were assessed. Blood pressure was measured by the tail-cuff method. HR-325 significantly decreased systolic blood pressure in a dose-dependent manner throughout the study. HR-325 tended to decrease plasma creatinine level and increase creatinine clearance rate. Morphological studies revealed that HR-325 treatment strikingly resolved infiltration of immune-related cells in perivascular and intraluminal lesions, thereby decreasing the total arterial injury score by 32%. High-dose HR-325 also attenuated glomerulosclerosis and tubular injury by 35% and 34%, respectively, as compared with untreated high-salt Dahl S rats. Reduced levels of immune-related cells resulted in a decrease in urinary nitrite excretion. These data indicate that long-term treatment with the immunosuppressant HR-325 decreases systolic blood pressure in Dahl salt-sensitive rats, and that this decrease is associated particularly with resolution of infiltration of immune-related cells in arterial lesions. Hyperimmune state is responsible in part for the susceptibility of Dahl S rats to hypertensive organ damage. (Hypertens Res 1997; 20: 91-97)

Key Words: Dahl salt-sensitive rats, immunosuppressant, nitric oxide, kidney injury, cytokine

Evidence has accumulated for participation of immune dysfunction in the pathogenesis of hypertension (1, 2). White and Grollman reported that autoimmunity plays an important role in the development of hypertension after renal infarction (3). Recent studies have demonstrated that spontaneously hypertensive rats (SHR) have impaired suppressor T cell function and that hyper-immunoglobulinemia or autoantibodies contribute to vascular and renal damage following hypertension (4). Morphological investigations have revealed that arteries in the kidney or mesentery show infiltration of immune-related cells in SHR, and these lesions are attenuated by immunosuppressants (5-7). Arterial injury observed in salt-induced hypertension is not reproduced in nude mice (8). In addition, some forms of cytokines, humoral immunomediators produced by immunocompetent cells, exhibit antihypertensive effects when administered to hypertensive animals (9-11).

On the other hand, a considerable amount of evidence indicates that Dahl salt-sensitive (Dahl S) rats are susceptible to arterial injury in hypertension, as compared with spontaneously hypertensive rats with similar blood pressure levels (12-14). The arterial lesions in the kidney have been classified into three types of injuries: medial necrosis, immune-related cell infiltration in periartrial regions, and thrombus formation. Particularly during the malignant phase of hypertension in Dahl S rats with salt-induced hypertension as well as in stroke-prone SHR, lesions in the cerebral, mesenteric, and kidney arteries are associated with periarteritis nodosa, strongly suggesting participation of a hyperimmune state in the genesis of hypertension and arterial injuries in Dahl S rats. Despite such a possibility, to our knowledge there have been few studies investigating whether or how immunosuppressants affect the progression of arterial lesions in this rat model.

To test the hypothesis that immune dysfunction contributes to the development of malignant arterial injuries in Dahl S rats, we treated Dahl S rats with the novel immunosuppressant HR-325 and investigated the beneficial effects of this drug on renal function and the morphological lesions in the kidney.

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Materials and Methods

Protocol of Study
Dahl-Iwai salt-sensitive (Dahl S) rats were obtained from the Tsukuba Research Laboratories of Eisai Co., Ltd., Tokyo, Japan. These rats originated from Brookhaven National Laboratories (Upton, New York, USA). They were maintained on a low-salt (0.3% NaCl) diet for 6 weeks after weaning. Thirty-six 6-wk-old Dahl S rats were then assigned randomly to the following three groups: 1) 12 rats fed a high-salt (4% NaCl) diet alone (high-salt control), 2) 12 rats fed a high-salt diet and given orally low-dose (1 mg/kg body weight per d) HR-325 (low-salt control), 3) 12 rats fed a high-salt diet and given high-dose (5 mg/kg body weight per d) HR-325 (high-salt control). These rats were treated for 8 wk. Another 12 Dahl S rats aged 6 wk were fed a low-salt diet for 8 wk (low-salt control). Blood pressure was measured at weekly intervals by the tail-cuff method of Freedman and Freed (15).

On the last three days of the study, 24-h urine specimens were collected. Blood samples and organs of interest were obtained under pentobarbital anesthesia (25 mg/kg body weight). The organs of interest were weighed and studied morphologically.

Renal Function
Creatinine concentrations in urine and plasma were measured with the use of a Dainabot model TDX creatinine analyzer system (Dainabot Co., Ltd., Tokyo, Japan). Urinary protein concentration was determined by an assay using sodium metabisulfite and o-phenylenediamine (13, 14). N-acetyl-β-D-glucosaminidase activity (NAG) was measured by the sulfosalicylic acid method (13, 14). N-acetyl-β-D-glucosaminidase activity (NAG) was measured by the sulfosalicylic acid method (13, 14).

Nitric oxide (NO) in urine was determined as the total amount of nitrite and nitrate in urine (17). Briefly, nitrate was converted to nitrite with 0.5 M NaI solution. The reduced urine was mixed with an equal volume of Griess reagent containing one part 1% sulfanilamide (Wako Pure Chemical Co., Ltd., Tokyo, Japan) in water and one part 0.1% naphthylethylenediamine (Wako Pure Chemical) in 5% phosphoric acid solution. The reaction mixture was incubated at 24°C for 10 min, and the specific optical absorption (OD540) of the diazo-coupling structure was determined with a Nihon-Koden Model V-540 spectrophotometer (Nihon-Koden Co., Ltd., Tokyo, Japan).

Histologic Investigation
Renal tissue was processed for light microscopy as described previously (13, 14). Briefly, half of each kidney was fixed in 3.5% formalin, and sagittal slices were cut and embedded in paraffin. Then 2-μm sections were cut and stained with hematoxylin and eosin, periodic acid-methenamine silver, and periodic acid-Schiff (PAS) stains.

The types of arterial injury were classified as follows: 0, no lesions (type 0); 1+, medial necrosis and/or increased medial thickness with luminal narrowing (type I); 2+, infiltration of immune-related cells in the periarterial or intraluminal space in the artery (type II); and 3+, medial necrosis and/or increased medial thickness accompanied by thrombus formation and periarterial fibrotic changes (type III) (Fig. 2) (18). Each kidney section included arteries with different grades of injury. We estimated the percentage of arteries with each grade of injury. To obtain the overall arterial injury score, the severity score (0 to 3+) was multiplied by the percentage of arteries displaying the same degree of injury, and these figures were summed up.

A minimum of 100 glomeruli in each specimen was examined, and the severity of lesions was graded from 0 to 4+ according to the percentage of the glomerulus involved: 0, no lesions; 1+, 1-25%; 2+, 25-50%; 3+, 50-75%; and 4+, 75-100% (19). To obtain the overall glomerulosclerosis score, the severity score (0 to 4+) was multiplied by the percentage of glomeruli displaying the same degree of injury, and these figures were summed up.

Tubular damage was scored according to a modification of the method of Rosen et al. (20). Renal morphological alterations in the inner stripes were semiquantitatively evaluated and graded from 0 to 4+ as follows: 0, no lesions; 1+, mild focal tubular dilation; 2+, an increased number of dilated tubules associated with interstitial widening; 3+, fairly extensive dilation of tubules with cyst formation and interstitial widening; and 4+, complete atrophy of tubules. To obtain the overall tubular injury score, the severity score (0 to 4+) was multiplied by the percentage of glomeruli displaying the same degree of injury, and these figures were summed up.

Reagents
All reagents were of analytical grade. HR-325 was supplied by Nippon Roussel Co., Ltd., Tokyo, Japan.

Fig. 1. Chemical structure of HR-325. The chemical structure of HR-325, 2-cyano-3-cyclopropyl-3-hydroxy-3'-methyl-4'-(trifluoromethyl)-acrylanilide (MW 310.28), is shown in the figure. This compound is a white powder, stable at 40°C for 6 months.
Statistical Analysis
All results are expressed as the means ± SE. Differences were analyzed by Student's t-test, Dunnet test, one-way analysis of variance, two-way analysis of variance, or the chi-square test for independence.

Results

Hemodynamic Effects
All rats in the untreated high-salt control groups survived the experiment. One rat in the low-salt control group, 3 in the HR-low group and 4 in the HR-high group died during the study. These rats were in good general condition and died suddenly. Macroscopic and microscopic studies performed immediately after death disclosed no significant organ changes, e.g., kidney damage or bleeding from the cerebrovascular or gastrointestinal systems, which are common causes of death in Dahl S rats with hypertension. In a preliminary study, the 50% lethal dose of HR-325 was more than 300 mg/kg body weight/d, and no rat died at 10 mg/kg body weight/d (unpublished data). Although the exact cause of death was uncertain, the animals apparently did not die of insufficient protective effects or toxic effects of the immunosuppressant.

There were no differences in body weight among the four study groups. Changes in systolic blood pressure during the study are shown in Fig. 3. The

![Fig. 2. Micrographs of various types of arterial injury in Dahl S rats. Arcuate arteries in the kidney were examined microscopically. The micrograph (a) shows an artery with normal appearance. An artery with medial necrosis (arrows) is shown in graph (b). Immune-related cells infiltrate into the periarterial lesions (left arrow) or intraluminal areas (right arrow). Some immune-related cells adhere to endothelial cells (c). Graph (d) shows an artery with thrombus formation (arrows). Pass stain. ×200.](image)

![Fig. 3. Alterations of systolic blood pressure in Dahl S rats. Blood pressure time-dependently increased in untreated Dahl S rats fed a high-salt diet. There was no difference in blood pressure between the untreated and low-dose HR groups. In contrast, in the high-dose HR group the blood pressure levels were significantly lower than in the high-salt control rats. Differences in blood pressure reduction during the experiment were analyzed by two-way analysis of variance with repetition. High-dose HR-325 significantly lowered the systolic blood pressure, as compared with high-salt, untreated Dahl S rats (p < 0.02). There was no difference between low-dose HR-325 and the high-salt control rats. *p < 0.05 vs. the value in untreated Dahl S rats fed a high-salt diet (Dunnet test).](image)
blood pressure time-dependently increased in Dahl S rats fed a high-salt diet. The elevation of blood pressure was gradually attenuated during the experiment in the rats treated with high-dose HR-325, as compared with the untreated, high-salt rats. At the end of experiment, the blood pressure reduction in the high-dose HR-325 group was significantly greater than that in the untreated high-salt control group. There were no differences in organ weights, except for that of the spleen, among the three high-salt groups. Spleen weight was slightly, but not significantly, lower in the high-dose HR-325 group than in the other two high-salt groups (Table 1).

**Renal Function**
Variables of renal function are shown in Table 2. Dahl S rats fed a high-salt diet tended to have a higher plasma creatinine level and a lower creatinine clearance rate than did the two other high-salt groups, suggesting deterioration of renal function with the development of hypertension. In addition, urinary protein excretion and urinary NAG excretion were significantly higher in high-salt control Dahl S rats than in low-salt normotensive Dahl S rats. High-dose HR-325 treatment was associated with a slightly lower plasma creatinine level and a slightly higher creatinine clearance rate, as compared with untreated high-salt rats. HR-325 treatment affected neither the urinary protein nor the urinary NAG excretion. In addition, we determined nitrite excretion in urine. HR-325 treatment tended to decrease urinary nitrite excretion in a dose-dependent manner; however, the difference was not statistically significant.

**Morphologic Changes**
We analyzed the effects of HR-325 on arterial lesions in hypertensive Dahl S rats (Fig. 4). We determined the number of arteries with various types of arterial damage. In normotensive rats, the arteries had an almost normal appearance (type 0). A high-salt diet increased the number of arteries having medial necrosis (type I), perivascular or intraluminal infiltration of immune-related cells (type II), or thrombus formation with medial necrosis (type II).
High-dose HR-325 treatment significantly decreased the number of arteries with perivascular or intraluminal infiltration of immune-related cells (type II). HR-325 treatment slightly, but not significantly, decreased the number of arteries with medial necrosis (type I). HR-325 treatment did not affect thrombus formation (type III). In type III arterial lesions, however, infiltration of immune-related cells was almost completely eliminated by HR-325 treatment.

Overall injury scores were estimated as described in the methods section. A high-salt diet significantly increased the score, as compared with low-salt normotensive rats (Table 3). This arterial injury score was significantly reduced by 32% by high-dose HR-325 treatment, as compared with untreated high-salt rats. Similarly, a high-salt diet increased the glomerulosclerosis and tubular injury scores, and high-dose HR-325 significantly decreased these scores by 35% and 34%, respectively, as compared with untreated high-salt Dahl S rats.

Multivariate Analyses
To investigate predictors of urinary nitrite excretion, we analyzed the relationship between urinary nitrite excretion and morphological indices in the three high-salt groups (Table 4). The urinary nitrite excretion closely correlated with the percentages of arteries with type-II arterial injury, but not with total arterial injury score. Factor analysis revealed that two independent factors were involved; one factor was related to urinary nitrite excretion and type-II arterial injury, and the other factor was related to glomerulosclerosis and tubular injury. In addition, multiple regression analysis disclosed that type-II arterial injury was an independent predictor of urinary nitrite excretion.

Discussion
HR-325 has been developed as a drug to treat patients with rheumatoid arthritis. This drug, an inhibitor of pyrimidine synthesis, is known to potently inhibit the mitogenesis of T and B immune cells and thereby suppresses the function of both humoral and cellular immune systems (unpublished data). In Dahl S rats, many immune-related cells infiltrate into the lumen and periarterial regions (13, 14, 18). It seems quite possible that the arterial injury in Dahl S rats with hypertension is influenced by immune-mediated events; however, few studies have investigated the role of the immune system in the progression of organ injury in Dahl S rats. We therefore used HR-325, a potent immunosuppressant, to investigate the mechanistic relationship between the immune system and the development of arterial injury in Dahl S rats.

Our study clearly demonstrated that the immunosuppressant HR-325 significantly attenuated the elevation of blood pressure in Dahl S rats fed a high-salt diet. However, more than 4 wk of HR-325 treatment were required to reduce blood pressure. This time lag suggested that the reduction in blood pressure was not due to direct vasorelaxation but to attenuation of renal injuries and subsequent improvement in renal function. HR-325 did not change heart rate or urinary sodium excretion (unpublished data). This indicates that sodium handling in the kidney did not play a part in the blood pressure reduction. In this context, we demonstrated that HR-325 treatment decreased the number of arteries showing infiltration of immune-related cells in vascular lesions. This treatment lessened
glomerulosclerotic and tubular lesions in hypertensive Dahl S rats. Despite this, since glomerulosclerosis and tubular injury are likely to be influenced by hemodynamic changes, we could not neglect beneficial effects of blood pressure reduction on the kidney, particularly after high-dose HR-325 treatment.

A preliminary study showed that HR-325 strongly suppresses mitogenesis of immunocompetent cells, including T and B cells (unpublished data). This is accompanied by decreased secretion of immunoglobulins. Immunological suppression was also reflected in the decrease in spleen weight with HR-325 treatment. It seems quite interesting that HR-325 was more likely to improve arteries with infiltration of immune-mediating cells (type II arterial injury) than those with medial necrosis or thrombus formation. This strongly suggests that HR-325 lessened arterial injury in Dahl S rats by suppressing immune processes underlying the progression of such injury. At this time, we cannot determine whether this beneficial effect is specific to the immunosuppressant HR-325, or if drugs with similar properties, e.g., cyclophosphamide or azathioprine, have similar effects on kidney injury. However, recent studies have demonstrated that the immunosuppressant cyclosporin produces low-renin hypertension and may cause renal dysfunction (21-25). In contrast, FK-506, an analogue of cyclosporin, reportedly is not associated with hypertension and is less likely to produce renal injury (26, 27). These data suggest that effects on blood pressure and the kidney differ among immunosuppressants. Accordingly, further investigation of the mechanism by which HR-325 affects arterial injuries in hypertension may enhance our understanding of progression of organ injury in hypertension.

The results of the present study were consistent with those of previous studies reporting the role of immune processes in the progression of vascular injury in hypertension. Recent studies demonstrated that immunocompetent cells release various cytokines, such as interleukin-1, interleukin-6 or tumor necrotizing factors. Such active substances initiate expression of inducible nitric oxide synthase (iNOS) and increase generation of nitrite (28-30). This mechanism may aggravate vascular and organ injuries associated with hypertension, hyperlipidemia, and other factors that promote atherosclerosis. In this context, we demonstrated that HR-325 slightly decreases urinary nitrite excretion. HR-325 does not affect directly cytokine release from immunocompetent cells (unpublished data). More interestingly, multivariate analyses clearly demonstrated that type-II arterial lesions are an independent predictor of urinary nitrite excretion. In addition, urinary nitrite excretion tended to decrease with HR-325 treatment. Altered nitrite formation in the kidney is presumably related to the improvement in kidney function. In fact, Sharkey and Butcher have reported that the immunosup-

### Table 4. Multivariate Analyses of Urinary Nitrite Excretion

<table>
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<tr>
<th>Correlation Coefficients</th>
<th>UNO</th>
<th>Type-II AI</th>
<th>AI score</th>
<th>GS score</th>
<th>TI score</th>
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<tr>
<td>UNO</td>
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<td>0.4805*</td>
<td>0.0666</td>
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<tr>
<td>Type-II AI</td>
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<td>0.6522*</td>
<td>0.4815*</td>
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<tr>
<td>AI score</td>
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<td>0.6652*</td>
<td>0.201</td>
</tr>
<tr>
<td>GS score</td>
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<td></td>
<td></td>
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<td>0.5948*</td>
</tr>
<tr>
<td>TI score</td>
<td></td>
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*: *r* values more than 0.3739 indicate statistical significance.

### Factor Analysis

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<td>UNO</td>
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<tr>
<td>Type-II AI</td>
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<tr>
<td>GS score</td>
<td>0.8340*</td>
</tr>
<tr>
<td>TI score</td>
<td>0.9156*</td>
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Principal components were extracted by factor analysis and Varimax raw rotation method. *Marked loadings were > 0.7000.

### Multiple Regression Analysis

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<td>GS score</td>
<td>-0.0838</td>
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<tr>
<td>TI score</td>
<td>0.1631</td>
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</tbody>
</table>

Dependent variable is UNO, and independent variables are Type-II AI, GS score, and TI score. UNO, urinary excretion of nitrite; Type-II AI, arterial injury type-II; AI score, arterial injury score; GS score, glomerulosclerosis score; TI score, tubular injury score.
pressant FK506 prevents cerebral stroke by reducing inducible nitric oxide formation, suggesting that cytokine-induced nitric oxide generation is injurious to vascular structure (31). These data suggest that the reduction in urinary nitrite excretion is probably secondary to the decrease in infiltration of immunocompetent cells in arterial lesions after long-term HR-325 treatment and that this mechanism participates in attenuation of renal injuries in Dahl S rats. Immunosuppression may therefore be a new strategy to prevent progression of vascular injury in hypertension. Finally, our study demonstrated that immunosuppression lowers blood pressure and lessens vascular and kidney injury in Dahl salt-sensitive rats with hypertension. This strongly suggests that immune mechanisms participate in the progression of organ damage in salt-induced hypertension. Modulation of these mechanisms may provide a new strategy to treat patients with organ injury due to hypertension.

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References