Arterial Smooth Muscle Cell Phenotype in Stroke-Prone Spontaneously Hypertensive Rats

Francis Contard, Abdelkarim Sabri, Marina Glukhova, Saviero Sartore, Françoise Marotte, Jean Pierre Pomies, Pierre Schiavi, David Guez, Jane-Lyne Samuel, Lydie Rappaport

The aim of this study was to determine the phenotype of smooth muscle cells in the arteries of chronically hypertensive animals and to analyze the effects of treatments known to increase the survival of the animal without a clear effect on its hypertensive state. Stroke-prone spontaneously hypertensive rats (SHRSP) kept on a 1% sodium drinking solution were untreated or treated with one of two diuretics, indapamide (3 mg/kg per day) or hydrochlorothiazide (20 mg/kg per day), from 6 to 13 weeks of age. Phenotype was characterized by the immunolabeling of arteries with antibodies raised against a cellular form (EIIIA) of fibronectin, α-smooth muscle actin, and nonmuscle myosin. We demonstrated that phenotypes of smooth muscle cells of the SHRSP differ from those found in Wistar-Kyoto rats. The difference in phenotype is specific for the vessel type: ie, an increased expression of nonmuscle myosin in the aorta and of both EIIIA fibronectin and nonmuscle myosin in the coronary arteries. The two diuretics (1) had no effect on blood pressure, (2) prevented or did not prevent the increase in medial thickness, and (3) prevented changes in both smooth muscle cell phenotype and ischemic tissular lesions. Taken together, the results suggest that in SHRSP the changes in the phenotype of smooth muscle cells and the thickness of arteries are unrelated events. We propose that the maintenance of the contractile phenotype of the arterial smooth muscle cells could be an essential parameter involved in the prevention of the deleterious consequences characteristic of a severe hypertensive state. (Hypertension. 1993;22:665-676.)

**KEY WORDS** • muscle, smooth, vascular • fibronectin • myosin • actins • diuretics • indapamide • hydrochlorothiazide • antihypertensive agents

Hypertrophy of the heart, as well as hypertrophy and/or hyperplasia of medial smooth muscle cells, develops in salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP). Cardiac hypertrophy in hypertensive rats is correlated with a shift in the myosin heavy chain pattern of striated cardiac myocytes toward a fetal phenotype. Both quantitative and qualitative alterations may be normalized by antihypertensive treatment. Changes in the phenotype of smooth muscle cells occur in vivo during ontogenesis. A well-differentiated phenotype is predominant in the vessels when cells contract in response to chemical and mechanical stimuli and are involved in the control of blood pressure and flow. At this stage, α-smooth muscle actin (αSM-actin) and smooth muscle myosin heavy chain (SM2) are the major contractile protein isoforms expressed in human, rat, or rabbit smooth muscle cells. A phenotypic shift in the expression of the contractile proteins and of a protein of the extracellular matrix, fibronectin, toward the forms expressed in the fetus is observed in the aorta during the early phase of atherogenesis. Changes in the expression of fibronectin isoforms also occur in the coronary arteries and aorta as a result of pressure overload.

In vivo, the heterogeneity of the smooth muscle cell phenotype throughout the vascular system in normotensive conditions would suggest specific changes in the phenotype of the smooth muscle cells as a result of hypertension. Therefore, the present study was conducted to determine whether an increase in size of the aortic and coronary artery media in SHRSP is associated with changes in the phenotype of the smooth muscle cells. It has been shown in the salt-loaded SHRSP that several types of drugs, such as angiotensin converting enzyme inhibitors, β-blockers, and diuretics, prevent the occurrence of fibrinoid necrosis as well as the characteristic ischemic lesions in the brain, kidney, and heart and decrease stroke-related mortality without substantially lowering blood pressure. These reports suggest that factors other than high blood pressure may be involved in the development of tissular lesions in salt-loaded SHRSP. To investigate the relative importance of the smooth muscle phenotype in inducing lesions that develop in SHRSP, we analyzed the effect of two diuretic treatments, indapamide and hydrochlorothiazide, that have beneficial effects on left ventricular and vascular hypertrophy without a significant reduction in blood pressure in salt-loaded SHRSP. Thiazides are the largest diuretic class, and hydrochlorothiazide accounts for half of all diuretic prescriptions. Indapamide is a nonthiazide indol derivative of chlorosulfonamide; its identity is clearly distinct from that of hydrochlorothiazide. In the present study, treatment was started at 6 weeks of age, when there is...
no hypertension and no morphologically detectable alterations of the coronary vessels in SHRSP.

The vascular phenotype was analyzed by immunochemical methods using antibodies directed against (1) total fibronectin (plasmatic and cellular fibronectin), (2) EIHA fibronectin, a cellular form of fibronectin typical of the immature phenotype of smooth muscle cells, (3) αSM-actin, and (4) nonmuscle myosin heavy chain (NM-myosin). These proteins have been chosen because their expression characterizes different cell functions and is modulated during development. The differences between elastic (aorta) and muscular (coronary) vessels have been defined mainly according to morphological criteria, sensitivity to chemical and mechanical stimuli, or both. Thus, we studied phenotypes of both types of arteries in the SHRSP in parallel to assess whether changes in phenotype depend on the size and characteristics of the vessels.

We describe a shift in the well-differentiated phenotype of the medial smooth muscle cells toward a less mature state in the SHRSP. The phenotypic shift is vessel specific and is prevented by both diuretic treatments despite their poor effect on blood pressure.

Methods

Animals

All procedures were in accordance with institutional guidelines for animal experimentation. Male Wistar-Kyoto (WKY) rats and SHRSP were obtained from Ifa Credo, Lyon, France, at 4 weeks of age. Animals were placed on a normal chow diet and water supplemented with 1% NaCl ad libitum. After a 2-week acclimatization period, SHRSP were randomized into three groups (n=6 to 8 per group) and housed individually.

Medications and Treatment Follow-up

Diuretic treatments were started at 6 weeks of age and administered daily by oral gavage in the form of a suspension in 0.1% carboxymethylcellulose (vehicle) to hypertensive rats during 44 days: untreated SHRSP (vehicle only), SHRSP treated with 3 mg/kg per day indapamide (Servier, France), and SHRSP treated with 20 mg/kg per day hydrochlorothiazide (Sigma Chemical Co, St Louis, Mo). Doses were those reported in the literature. The experimental protocol has been published elsewhere. Briefly, body weights and blood pressures (systolic and diastolic) were measured in conscious animals at 4 weeks of age and then every other week by the tail-cuff method (Pressure Computer LE 5007, Letica). Twenty-four-hour urine samples were collected from each animal once before and once after the treatment period. Blood samples were collected after ether anesthesia at the level of the retro-orbital sinus before the treatment period and by puncture of the lower part of the descending aorta at the end of treatment.

Rats were examined daily for clinical signs of stroke and survival throughout the study. After 44 days on a 1% NaCl drinking solution, six of eight animals (75%) survived in the untreated SHRSP group and were subsequently analyzed. Survival was 100% in the other groups.

Urine output was significantly increased (P<.001) at the end of the study (30±3.7 versus 10±0.8 mL/d at day 1) in all SHRSP and WKY groups, with no statistical difference among the groups, as previously described. Potassium and sodium excretions were similar among the different groups and at any time of the study (not shown). Blood electrolyte determination demonstrated a higher kalemia in the indapamide- and hydrochlorothiazide-treated groups (4.6±0.3 and 4.2±0.2 mmol/L, respectively) than in the SHRSP untreated group (3.7±0.2 mmol/L), but this increase was significant only in the indapamide-treated group (P<.05).

Experimental Protocol

After ether anesthesia, all animals were killed by exsanguination, the chest was opened, and the heart and aorta were quickly removed. The hearts were trimmed of large vessels, weighed, and cut transversally at the equator of both ventricles. A segment (4 mm) of the ascending part of the aorta was also collected and adherent connective tissue removed. The upper parts of the heart and aorta were mounted and frozen in liquid nitrogen-cooled isopentane. Blocks were kept at −70°C until use for immunohistochemical study.

Autopsies were performed on rats for routine pathological examination of the brain and kidneys. These organs were fixed by immersion in 10% Formalin and SHRP and WKY groups, with no statistical difference among the groups, as previously described. Potassium and sodium excretions were similar among the different groups and at any time of the study (not shown). Blood electrolyte determination demonstrated a higher kalemia in the indapamide- and hydrochlorothiazide-treated groups (4.6±0.3 and 4.2±0.2 mmol/L, respectively) than in the SHRSP untreated group (3.7±0.2 mmol/L), but this increase was significant only in the indapamide-treated group (P<.05).

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Antibodies. Basal membrane protein immunolabeling was performed using polyclonal anti-laminin antibodies (gift from Dr H. Kleinman, National Institute for Dental Research, National Institutes of Health, Bethesda, Md) and monoclonal antibodies directed either against a domain common to all fibronectin isoforms (total fibronectin) (gift from Dr M. Cheruousov, Cardiology Research Center, Moscow, Russia) or against the EDA domain (EIIIA fibronectin, kindly provided by Dr L. Zardi, Instituto Nazionale per la Ricerca sul Cancro, Genoa, Italy). Both monoclonal antibodies were raised against human fibronectin, and their specificities toward rat fibronectin have been described elsewhere. Monoclonal antibodies directed against human smooth muscle actin (DAKO, Glostrup, Denmark) and NM-myosin (NM-G2 antibody) were also used. The NM-G2 antibody recognized an epitope present in nonmuscle cell myosin extracted from human platelets.

Immunolabeling. For fibronectin/laminin immunolabeling, consecutive serial ventricular and aortic cryosections (5 μm) were labeled using a double immunolabeling technique. Sections were incubated overnight at 4°C with total fibronectin or EIIIA fibronectin antibodies at respective dilutions of 1:200 and 1:5 in phosphate-buffered saline (PBS) (mmol/L: NaCl, 150; KCl, 2.5; phosphate buffer, 10; pH 7.2) containing 2% rat serum. Sections were rinsed three times at room temperature in PBS and incubated for 30 minutes at room temperature with biotinylated anti-mouse IgG (1:200 dilution in PBS, Vector Laboratories, Biosys, Compiègne, France). Sections were then incubated after three washing steps for 30 minutes at room temperature with anti-laminin antibodies (1:200 dilution in PBS). After two washings, sections were incubated with a 30-fold dilution of anti-rabbit immunoglobulin antibodies conjugated to...
fluorescein isothiocyanate fluorochrome (Amersham, Les Ulis, France) to detect the anti-laminin antibodies. The biotinylated antibodies were revealed with a streptavidin-Texas Red complex (1:50 dilution in PBS, BioMérieux, Marcy l’Etoile, France). Fluorescein isothiocyanate fluorochrome (Amersham).

NM-myosin and actin immunolabeling was performed in parallel on serial nonconsecutive sections of the heart and aorta. Sections were incubated overnight at 4°C with anti–NM-actin or anti–NM-myosin antibodies at a dilution of 1:50 and 1:500, respectively, in 2% rat serum in PBS. Subsequent steps were identical to those described for other monoclonal antibodies.

Sections were mounted in aqueous medium (Medium-Prep, BioMérieux, Marcy l’Etoile, France). Fluorescence was visualized using a Leitz microscope equipped with epifluorescence optics (Leica, Reuil-Malmaison, France).

We verified that each immunolabeling pattern was identical among distant sections (10 to 50 μm) using trinocular assays.

**Morphology and Quantitative Analysis**

Morphology and quantitative analysis of the coronary arteries. Analysis of coronary artery labelings with antibodies against fibronectin or laminin was performed using a video imaging microscopy technique. Video images from a low-light level camera (C-2400, Hamamatsu, Pareil-Vielle-Poste, France) were transmitted to a microcomputer (Macintosh IIx, Bruno Rives, Paris, France) equipped with an image analyzer program (Optilab, Graphtek, Bruno Rives). This software permitted us to store real-time images in digitally calibrated formats for later processing. Coronary arteries in the heart and aorta were studied using two images from a low-light level camera (C-2400, Hamamatsu, Pareil-Vielle-Poste, France) equipped with an image analyzer program (Optilab, Graphtek, Bruno Rives). This software permitted us to store real-time images in digitally calibrated formats for later processing.

Coronary arteries were examined throughout the two ventricles, and only those found to have a transverse orientation were further analyzed. Arterial sections with a transverse orientation were further analyzed. The concentric distribution of the smooth muscle cells in the media, the elongated shape of the cells, and the homogeneous thickness of the media throughout the entire vessel section, as previously described. In each section, 17 to 30 and 2 to 4 vessels with a lumen size less than and more than 8×10^4 μm^2, respectively, were analyzed. The specific location of laminin in the media of coronary arteries permitted us to trace the internal and external medial perimeters and to calculate the delimited areas. The medial area was then obtained by the subtraction of the two measured areas. It also permitted us to pair the vessels corresponding to the same coronary artery in two consecutive serial sections and thus to compare the relative importance of total fibronectin and EIIIA fibronectin labelings and to analyze the ratio of EIIIA fibronectin to media. The tracing of these areas was directly performed on the image display (magnification, ×1100). The image analyzer software automatically evaluated the positive labelings using a threshold method, i.e., selecting pixels whose intensity level was greater than a threshold value (background).

**Morphology of the aortas.** The thickness of the aortic media was determined directly by planimetry on the magnified image (×32) of sections stained with hematoxylin and eosin in an optical microfilm reader (PC Printer 80, Canon, Paris, France). The internal and external perimeters were traced directly on the magnified image using a digital tablet (ASM Leitz apparatus, Leica). Subsequent perimeters were identical to those previously described.

All qualitative and morphometric analyses were performed blinded and in parallel by two investigators.

**Statistical Analysis**

Data are expressed as mean±SEM except when otherwise noted. Qualitative analysis of the NM-myosin expression in aortas was compared among groups using the χ^2 test. Other statistical analyses were carried out by one-factor analysis of variance, and when F values were significant, group means were evaluated using the Scheffé F test. This test is considered to be the most appropriate for evaluating arbitrary combinations of groups against each other. A value of P≤.05 was considered statistically significant. Concerning coronary arteries, because of the homogeneity of results among hearts in the treated SHRSP groups, the data handling of four hearts was sufficient to give statistical significance.

### Table 1. Arterial Pressure and Characteristics of Aortic Media

<table>
<thead>
<tr>
<th>Group</th>
<th>SAP, mm Hg</th>
<th>DAP, mm Hg</th>
<th>Aortic Media Thickness, μm</th>
<th>No. of Rats With Aorta Exhibiting Homogeneous Expression of NM-Myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated SHRSP</td>
<td>265±22</td>
<td>195±18</td>
<td>198±2.9</td>
<td>6</td>
</tr>
<tr>
<td>HCTZ (n=6)</td>
<td>247±10</td>
<td>199±9</td>
<td>195±11.0</td>
<td>1*</td>
</tr>
<tr>
<td>HCTZ (n=6)</td>
<td>251±9</td>
<td>202±11</td>
<td>183±7.0</td>
<td>4</td>
</tr>
<tr>
<td>WKY (n=6)</td>
<td>142±9t</td>
<td>108±7†</td>
<td>143±4.9‡</td>
<td>1*</td>
</tr>
</tbody>
</table>

SAP indicates systolic arterial pressure 24 hours after gavage; DAP, diastolic arterial pressure 24 hours after gavage; NM-myosin, nonmuscle myosin; SHRSP, stroke-prone spontaneously hypertensive rats; IDP, indapamide-treated SHRSP; HCTZ, hydrochlorothiazide-treated SHRSP; and WKY, Wistar-Kyoto rats. Values are mean±SEM and frequency data. Because of the constitutive expression of NM-myosin in normal aorta, its intramural expression was qualitatively estimated as either restricted to the inner part of the aorta (see Fig 2B) or homogeneously distributed throughout the entire media (see Fig 2E).

*P<.01 vs untreated SHRSP, χ^2 test. †P<.05 vs untreated SHRSP, Scheffé F test. ‡P<.001 vs untreated SHRSP, Scheffé F test.
Results

Characteristics of the SHRSP Model and of Diuretic Treatments

The morphological and physiological characteristics of the diuretic-treated SHRSP have been previously described. Only those parameters necessary for the understanding of the present study are reported here. At the beginning of the treatment (6 weeks of age), arterial systolic pressure was similar in SHRSP and WKY rats (138±4 and 125±4 mm Hg, respectively). By the end of the experiments (13 weeks of age), blood pressure was unchanged in the WKY rats and dramatically increased in the untreated SHRSP group (Table 1). After 44 days of treatment with either indapamide or hydrochlorothiazide, the systolic and diastolic blood pressures were not significantly different in the treated groups compared with the untreated group (Table 1), but the ratios of heart weight to body weight were lowered (4.80±0.07 and 4.89±0.16, respectively, versus 5.67±0.20 in the SHRSP, P<.01).

Fig 1 shows that the brain and kidney ischemic lesions that developed in the untreated SHRSP were almost absent in all treated animals. No vascular lesions characteristic of fibrinoid necrosis were observed in the diuretic-treated groups. The same results were observed at the level of the heart tissue, as previously described.
Hypertension and Medial Phenotypes

Smooth Muscle Cell Phenotypes of the Aortic Wall

The aortic mediae were thickened in the hypertensive untreated group compared with the normotensive group (+38%, Table 1), as illustrated by immunolabeling of αSM-actin (Fig 2). αSM-actin was homogeneously distributed throughout the media, whereas both total fibronectin (not shown) and EIIIA fibronectin were mostly present in the intimal layer and sometimes in focalized areas of the media (Fig 2). The distribution and labeling intensity of the three proteins were similar in the WKY and SHRSP strains. The major difference in the phenotype of the aorta between the two strains concerned NM-myosin (Fig 2). Indeed, positive staining for NM-myosin was found only in the inner part of the media and was almost always absent from the midwall and outer part in 83.3% of the WKY aortas. In contrast, in SHRSP, the protein was homogeneously distributed throughout the whole media in all the animals (χ² test on the frequency distribution, P≤.01 versus WKY). In the untreated SHRSP, the NM-myosin distribution was identical to that of αSM-actin, indicating that NM-myosin was coexpressed with αSM-actin by aortic smooth muscle cells.

Neither indapamide nor hydrochlorothiazide treatments prevented thickening of the aortic media in SHRSP (Table 1). However, after indapamide treatment, only 16.7% of the aortas exhibited the homogeneous accumulation of NM-myosin throughout the entire media. This frequency was identical to that observed in the WKY group. After hydrochlorothiazide treatment, the positive and uniform pattern of NM-myosin was still observed in 66.7% of the aortas. The two diuretic treatments had no effect on the expression of αSM-actin and the fibronectins (not shown).

Smooth Muscle Cell Phenotypes of the Coronary Walls

In the SHRSP strain, throughout each ventricular section at least two to three myocardial arterioles exhibited typical fibrinoid necrosis (Fig 3). In these vessels, both the media and intima were labeled with antibodies directed against laminin. Both EIIIA fibronectin and total fibronectin were highly expressed and similarly distributed throughout all the vascular layers, including the adventitia. αSM-actin was detectable in the media, whereas the NM-myosin was almost undetectable throughout the vessels. No arterioles with

![Fig 2. Photomicrographs show distribution of EIIIA fibronectin, nonmuscle myosin, and α-smooth muscle actin within aortic wall of Wistar-Kyoto rat (A, B, C) and untreated stroke-prone spontaneously hypertensive rat (SHRSP) (D, E, F). Serial aorta cryosections were incubated with antibodies directed against EIIIA fibronectin (A, D), nonmuscle myosin (B, E), and α-smooth muscle actin (C, F). Note that nonmuscle myosin was present in the inner part of the aortic wall of Wistar-Kyoto rats, whereas it was expressed throughout the entire wall of untreated SHRSP. Lumen of the aorta is on the top. Bar=10 μm.](http://hyper.ahajournals.org/)}
fibrinoid lesions were found in the WKY animals or in the SHRSP treated with diuretics.

In the coronary arteries of the two strains, αSM-actin staining was intense and homogeneous in the whole media; it revealed, as seen with laminin labeling, a larger media cross-sectional area in the SHRSP strain (Fig 4). NM-myosin staining, almost absent in the WKY arteries, appeared as scattered dots visible only in a limited number of medial cells in the SHRSP heart sections. Total fibronectin was detected in all the layers of the coronary arteries, whatever the strain, but expanded dramatically throughout the media of SHRSP. The pattern of EIIIA fibronectin in the coronary arteries appeared strain specific; the WKY strain was characterized by either a positive EIIIA fibronectin staining in the adventitia (Fig 4) or an absence of staining throughout the vessels, whereas an accumulation of the EIIIA fibronectin in the media was more typical of the SHRSP.

Analyses of both the media cross-sectional area and the frequency of NM-myosin–positive and fibronectin–positive coronary media in the large (≥8×10³ µm² lumen) and small (<8×10³ µm²) arteries were performed. The frequency of positive vessels was always very high in the SHRSP, but the percentage of immunoreactive vessels clearly differed depending on both the proteins tested and the size of the vessels (Tables 2 and 3).

In the large coronary arteries, the media cross-sectional area in the SHRSP did not differ from that of WKY rats (Table 2). The proportion of large coronary arteries positive for EIIIA fibronectin was dramatically increased in the SHRSP (90%) compared with the WKY rats (12%); no difference was found for NM-myosin–stained media because the percentage of positive vessels was already high in the WKY strain. The two treatments were without a marked effect on the percentage of media labeled with either EIIIA fibronectin or NM-myosin.

In the small coronary arteries, larger media cross-sectional area (+47%) was observed in the hypertensive untreated group (Table 3) and was prevented by hydrochlorothiazide treatment only (Table 3). The percentage of vessels staining for either NM-myosin or fibronectin was also significantly increased in the SHRSP group (Table 3, Fig 5), and both indapamide and
hydrochlorothiazide treatments were able to prevent the accumulation of these two proteins in the media of small coronary vessels, even though the percentage of positive vessels remained elevated compared with the WKY animals (Table 3). As described above, EIIIA fibronectin was present in the adventitia of WKY rats but not SHRSP (Fig 4); 31.5% of the coronary arteries exhibited positive EIIIA fibronectin labeling in the adventitia in the WKY rats, and only 9.6% did so in the SHRSP (P<.05). Diuretic treatments did not normalize the pattern of EIIIA fibronectin in the adventitia.

The relative accumulation of fibronectin isoforms in arteries was quantitated by measuring the surface area staining for the two fibronectin forms. Measurements included the entire coronary wall because total fibronectin was present in both adventitia and media, whereas EIIIA fibronectin was detected either in the media or adventitia. A dramatic increase in the surface area staining for both total fibronectin (1.7-fold) and EIIIA fibronectin (4.6-fold) was found in the untreated SHRSP (Table 4). After the two diuretic treatments, although lesser amounts of both total fibronectin (±40% versus SHRSP) and EIIIA fibronectin (±62% versus SHRSP) were found in the small coronary arteries of the treated animals, only the total fibronectin–labeled surface area remained similar to values observed in the normotensive WKY rats (Table 4). However, when EIIIA fibronectin quantitation was restricted to the media, excluding the WKY vessels, in which only low-frequency positive media was present, it was found that both indapamide and hydrochlorothiazide significantly prevented the accumulation of EIIIA fibronectin in the coronary media (Table 4). Consequently, the proportion of the medial surface containing EIIIA fibronectin decreased from 36±4% in the untreated group to 16±2% and 13±2% in the indapamide and hydrochlorothiazide groups, respectively (P≤.001).

Discussion

The results of the present study demonstrate that phenotypes of smooth muscle cells in both the aorta and coronary arteries of hypertensive SHRSP differ from those in WKY rats. The difference in phenotype is not identical in elastic and muscular vessels. However, in both types of vessels these changes are always indicative of a less mature phenotype, ie, the only expression of NM-myosin in the aorta and of both EIIIA fibronectin and NM-myosin in the coronary arteries. To our knowledge this is the first demonstration of a smooth muscle phenotypic shift in SHRSP. The two diuretic treatments, indapamide and hydrochlorothiazide, partially prevent the phenotypic shift independently of their effect on the hypertrophy of the media and despite the absence of a consistent lowering in blood pressure.

The increase in vascular wall mass secondary to arterial hypertension results from two different mechanisms, hyperplasia and hypertrophy of smooth muscle cells. Cellular hyperplasia occurs in various resistance arteries and arterioles early during the development of hypertension, whereas cellular hypertrophy is observed at a later stage and is more prominent in the large elastic arteries (eg, aorta). In the present study we show that the changes in gene expression clearly differ in the aorta and coronary vessels. In the aorta, NM-myosin is expressed by smooth muscle cells at the early stage of ontogeny or during the development of atherosclerosis. In the adult WKY rat, there remains a cell subpopulation with an immature phenotype within the aortic wall. Moreover, smooth muscle cells expressing either NM-myosin or EIIIA fibronectin seem to belong to two different cell populations, except in the inner part of the media. In the SHRSP, the...
accumulation of NM-myosin in all the aortic smooth muscle cells shown here indicates that the majority of the medial cells rather than a cellular subpopulation are implicated during development of malignant hypertension. However, the absence of changes in αSM-actin pattern and the great similarity between the distribution of constitutive αSM-actin and NM-myosin throughout the media suggest that cells expressing NM-myosin conserve muscular characteristics even if there is a shift toward a less-mature phenotype. As for myosin, a change in the pattern of the expression of the actin isoforms (β, γ) cannot be excluded. A previous biochemical analysis showed no major changes in the two smooth muscle myosin isoforms (SM1 and SM2) in the aortas of SHR compared with WKY rats. Thus, changes in myosin expression, as found in the present study, may involve only the NM-myosin isoforms. The absence of expression of EIIIA fibronectin in these cells contrasts with previous findings showing an increased accumulation of EIIIA fibronectin mRNA in the rat aorta during the course of experimental (deoxycorticosterone acetate–salt, angiotensin II) or genetic (SHR) hypertension. However, the biosynthetic rate of fibronectin by aortic rings in vitro is unchanged in 40-week-old SHR compared with WKY rats. Therefore, the level of accumulation of cellular fibronectin as well as NM-myosin within the aorta could depend on the hypertensive etiology and/or the age of the animal. Taken together, the absence of changes in both αSM-actin and EIIIA fibronectin expression supports the hypothesis of restricted and specific changes in the phenotype of aortic smooth muscle cells in response to malignant hypertension.

In the SHRSP coronary arteries, the major alteration in the phenotype is reflected by the higher number of coronary vessels expressing EIIIA fibronectin and the relative accumulation of the protein in the media. An accumulation of EIIIA fibronectin mRNA or its encoded protein (this report) in the media of the coronary wall occurs in conditions of long-lasting and severe hypertension (this report) as well as in response to a sudden and severe pressure overload. Because EIIIA fibronectin expression is barely detected in the media of adult normotensive animals and is not present in plasma, the protein must be synthesized locally by smooth muscle cells in response to increased transmural pressure. The present study does not permit us to propose a mechanism responsible for the expression of the protein nor to distinguish between cellular hyperplasia and hypertrophy. The limited number of cells expressing NM-myosin in these vessels together with the dramatic increase in cellular fibronectin suggest a limited change in the phenotypic expression of smooth muscle cells in coronary vessels, as already proposed for aortic smooth muscle cells. It must be pointed out that unlike the aorta the coronary media of WKY rats is almost devoid of smooth muscle cells exhibiting an immature phenotype. The difference between the two types of arteries (this report) and previous observations in the Wistar aorta and mesenteric artery may represent a feature characteristic of each artery type (elastic or muscular).

### Table 2. Smooth Muscle Cell Phenotype of Large Coronary Arteries

<table>
<thead>
<tr>
<th>Group</th>
<th>Media Cross-sectional Area, μm² x 10⁻³</th>
<th>Percent of Coronary Media Expressing</th>
<th>Total FN</th>
<th>EIIIA FN</th>
<th>NM-Myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated SHRSP</td>
<td>23.0±4.2</td>
<td>90±12</td>
<td>75.6±8.2</td>
<td>70.6±7.2</td>
<td>73±5</td>
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<tr>
<td>IDP (n=4)</td>
<td>26.8±2.4</td>
<td>60±12</td>
<td>30.0±6.6†</td>
<td>19.0±7.9*</td>
<td>37±6‡</td>
</tr>
<tr>
<td>HCTZ (n=4)</td>
<td>18.8±4.3</td>
<td>67±14</td>
<td>12.0±0.9§</td>
<td>13.7±1.1§</td>
<td>33±7§</td>
</tr>
<tr>
<td>WKY (n=6)</td>
<td>18.5±2.3</td>
<td>12±12</td>
<td>46.9±8.3‡</td>
<td>4.9±3.1§</td>
<td>17±4*</td>
</tr>
</tbody>
</table>

FN indicates fibronectin; NM-myosin, nonmuscle myosin; SHRSP, stroke-prone spontaneously hypertensive rats; IDP, indapamide-treated SHRSP; HCTZ, hydrochlorothiazide-treated SHRSP; and WKY, Wistar-Kyoto rats. Values are mean±SEM. The two immunolabelings were qualitatively estimated as positive or negative. Values in columns 2 through 4 correspond to percent of positively labeled coronary media.

*P<.01 vs untreated SHRSP, Scheffé F test.
†P<.05 vs untreated SHRSP, Scheffé F test.
‡P<.05, HCTZ vs IDP, Scheffé F test.
§P<.001 vs untreated SHRSP, Scheffé F test.
Fig 5. Photomicrographs show distribution of total fibronectin, EIIIA fibronectin, and nonmuscle myosin in small coronary arteries in untreated stroke-prone spontaneously hypertensive rats (SHRSP) (A, D, G) and in either indapamide-treated (B, E, H) or hydrochlorothiazide-treated (C, F, I) SHRSP. Serial heart sections were incubated with antibodies directed against total fibronectin (A, B, C), EIIIA fibronectin (D, E, F), and nonmuscle myosin (G, H, I). Note that total or EIIIA fibronectin staining was decreased in the treated group arteries. Bar=15 μm.
Nevertheless, the expression and accumulation of either NM-myosin or EIIIA fibronectin in the media of the aorta and coronary arteries of the SHRSP indicate some degree of smooth muscle cell "dedifferentiation"14,24 and may be associated with a partial loss of contractile properties by the cells. Fibronectin itself may contribute locally to the phenotypic changes of the smooth muscle cells.29 Indeed, in vitro the interaction between smooth muscle cells and fibronectin leads to a decrease in aSM-actin mRNA level even though the encoded protein is still detected.29 Consequently, aSM-actin may not be considered a definite marker of the smooth muscle differentiated state.29 This observation concurs with our finding, because we did not observe significant aSM-actin alterations in smooth muscle cells expressing either EIIIA fibronectin or NM-myosin. On the other hand, the expression of contractile proteins in arterial smooth muscle cells has been correlated with the overall differentiated state of the cells, during both normal development and atherogenesis. Therefore, the continued expression of the aSM-actin would indicate that smooth muscle cells from SHRSP do conserve some features of the contractile phenotype.

The selective alterations of the smooth muscle cell phenotype within the arterial wall of the hypertensive animals shown in the present study together with the accumulation of other extracellular matrix components, such as elastin and collagen,30-32 could lead to vascular stiffening, which is one of the detrimental features characteristic of the hypertensive state. Besides, alterations in the phenotype of the arterioles, associated with a loss of contractile proteins, could lead to a decreased capacity of the arterial wall to withstand high systolic blood pressure and in turn favor the fibroinoid necrotic process, resulting in ischemia of the vascularized tissues33 and stroke, which characterize the rats developing malignant hypertension. The relative importance of the smooth muscle phenotype in inducing these characteristic lesions was analyzed using two diuretic treatments that prevent the formation of tissular lesions and partially vessel hypertrophy without a consistent effect on blood pressure (see Reference 2 and this report), as previously described with other antihypertensive drugs.1,19 The present study clearly demonstrates that the two diuretic treatments prevent the smooth muscle cell phenotype modification that occurred in the SHRSP; indapamide had no effect on the hypertrophy of the small coronary arteries and aortas, and hydrochlorothiazide prevented medial hypertrophy in small coronary vessels only, indicating that the changes in phenotype of smooth muscle cells and in the medial hypertrophy are unrelated events. Thus, the use of diuretics has permitted us to demonstrate that changes in the smooth muscle cell phenotype and the development of high blood pressure are independent processes and probably regulated by different mechanisms. If the occurrence of tissular lesions results from a partial loss in the contractile properties of the arterioles, the maintenance of the contractile phenotype as a result of the two drug treatments could explain the prevention of ischemic lesions despite the lack of a consistent decrease in blood pressure. However, it cannot be excluded that the respective effects of the drugs on the shift in smooth muscle phenotype and the formation of tissular lesions are independent events. Further studies are necessary to differentiate between these possibilities.

Multiple factors may be responsible for the phenotype alterations of smooth muscle cells associated with the development of malignant hypertension. Growth factors synthesized by the smooth muscle cells themselves34-35 or by the endothelium36 have been implicated. In vitro transforming growth factor-ß is known to induce connective tissue synthesis,37 although it sometimes stimulates and sometimes inhibits smooth muscle cell growth.38-40 A direct effect of transforming growth factor-ß on fibronectin synthesis by smooth muscle cells is unlikely, because fibronectin levels remained essentially unchanged in smooth muscle cells treated with transforming growth factor-ß.38,41 Angiotensin II, another growth factor, is known to induce aortic smooth muscle cell hypertrophy and EIIIA fibronectin expression.26,27 Interestingly, paradoxical elevated plasma renin activity was reported in SHRSP on a high-salt intake,42,43 and previous studies suggest an involvement of angiotensin II in the development of tissular lesions.1 Although the effect of diuretic therapy on systemic angiotensin II is not known, the beneficial effects of the diuretics could be obtained via the tissular renin-angiotensin system without involvement of systemic angiotensin II,44 as suggested for angiotensin converting enzyme inhibitors.1 It is also possible that induced hyperkalemia45,46 or the antioxidant properties of indapamide23 may play a role in preventing the formation of tissular lesions. Whatever the mechanism of action, it remains that the phenotype of smooth muscle cells from SHRSP exposed

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**TABLE 4. Quantitation of Total Fibronectin and EIIIA Fibronectin Surface Areas Within the Wall of Fibronectin-Positive Small Coronary Arteries**

<table>
<thead>
<tr>
<th>Group</th>
<th>Entire Wall</th>
<th>Media EIIIA FN Area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated SHRSP (n=6)</td>
<td>5556±712</td>
<td>670±251†</td>
</tr>
<tr>
<td>HCTZ (n=4)</td>
<td>3362±495*</td>
<td>660±158†</td>
</tr>
<tr>
<td>WKY (n=6)</td>
<td>3298±543†</td>
<td>ND</td>
</tr>
<tr>
<td>IDP (n=4)</td>
<td>2648±494†</td>
<td>680±246†</td>
</tr>
</tbody>
</table>
| HCTZ, hydrochlorothiazide-treated SHRSP; WKY, Wistar-Kyoto rats; and ND, not determined. Values are mean±SEM.

*P<0.05, †P<0.01, ‡P<0.001 vs untreated SHRSP, Scheffé F test.
to indapamide or hydrochlorothiazide tends to be normalized despite the lack of effect on hypertension. The regulatory mechanisms by which indapamide and hydrochlorothiazide act remain to be elucidated.

We demonstrate here that the phenotype of arterial smooth muscle cells in the salt-loaded SHRSP does not depend on blood pressure. Our results support the concept that the beneficial effect of various types of drugs in SHRSP may be associated with the prevention of a shift in the phenotype of smooth muscle cells from a well-differentiated to a less-mature state. Whether diuretic treatment would be effective in reverting changes in the phenotype after they have occurred has not been investigated, however. The maintenance of the contractile phenotype of arterial smooth muscle cells could be an essential parameter involved in the prevention of deleterious consequences characteristic of a severe hypertensive state.

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