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Changes in Extracellular Matrix in Subcutaneous Small Resistance Arteries of Patients with Primary Aldosteronism

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Context and Objective: It has been previously demonstrated that aldosterone may possess a strong profibrotic action *in vitro* and in animal models of genetic or experimental hypertension. Our aim was to evaluate whether such a profibrotic action is present also in the human microcirculation.

Design and Patients: We investigated 13 patients with primary aldosteronism, seven patients with essential hypertension, and 10 normotensive controls. All subjects were submitted to a biopsy of gluteal sc fat tissue. Small resistance arteries were dissected and mounted on an isometric myograph, and the tunica media to internal lumen ratio was measured.

Main Outcome Measures: The total collagen content within the tunica media was detected (Sirius red staining and image analysis), and collagen subtypes were evaluated using polarized light microscopy; under this condition thicker type I collagen fibers appear orange or red, whereas thinner type III collagen fibers are yellow or green.

Results: Tunica media to internal lumen ratio was significantly increased in primary aldosteronism and in essential hypertension compared with normotensive controls. Clinic blood pressure values were similar in primary aldosteronism and in essential hypertension, and greater than in normotensive controls. Normotensive controls had less total and type III collagen (3.23 ± 0.58 and 1.60 ± 0.22%, respectively) in respect to the two hypertensive groups ($P < 0.001$). Total collagen and type III vascular collagen were significantly greater in primary aldosteronism (total collagen, 8.17 ± 1.38%; type III collagen, 6.06 ± 0.74%; $P < 0.05$) than in essential hypertension (total collagen, 6.84 ± 1.15%; type III collagen, 5.25 ± 0.80%).

Conclusions: Our results indicate that, in small resistance arteries of patients with primary aldosteronism, a pronounced fibrosis may be detected, even more evident than in blood-pressure-matched patients with essential hypertension. **(*J Clin Endocrinol Metab* 91: 2638–2642, 2006)**

THE RENIN ANGIOTENSIN-ALDOSTERONE system may play a relevant role in the pathophysiology of arterial hypertension, contributing to the development of vascular damage both in the macrocirculation and in the microcirculation (1). In particular, aldosterone is able to induce *in vitro* vascular smooth muscle cell hypertrophy or hyperplasia (2), both directly and through an increase in the effects of angiotensin II (3). Aldosterone stimulates cell growth and vascular remodeling also *in vivo*, as demonstrated by the observation of an increased left ventricular mass (4) and an increased tunica media to lumen ratio in sc small resistance arteries (5) in patients with elevated circulating levels of aldosterone, such as those with primary aldosteronism. However, aldosterone also induces profound changes in the extracellular matrix, leading to collagen deposition, and, subsequently, to fibrosis (6). In animal models, alterations in the composition of small resistance artery wall induced by angiotensin II infusion (remodeling, fibrosis) (7) or sodium loading (8) may be completely prevented by the coadministration of an aldosterone receptor antagonist, such as eplerenone. In a subgroup of the Randomized Aldactone Evaluation Study, levels of serum markers of collagen turnover (in particular amino-terminal peptide of type III procollagen, PIIINP), which, in this study, were demonstrated to have prognostic significance, were reduced by spironolactone therapy (9).

It is possible that oxidative stress may be involved in the profibrotic effect of aldosterone, at the microcirculatory level (10). In fact, an increased activity of the renin-angiotensin system is associated with an increase in vascular oxidative stress, partly through an activation of NAD(P)H oxidase, which may be prevented, again, by spironolactone (7, 11). However, no data about the effects of high circulating levels of aldosterone on extracellular matrix of human small resistance arteries are presently available.

Therefore, the aim of the present study was to evaluate whether a profibrotic action of aldosterone is present also in the human microcirculation. In particular, we investigated extracellular matrix in sc small resistance arteries of patients with primary aldosteronism, compared with patients with essential hypertension as well as with normotensive control subjects.

Patients and Methods

We have investigated 13 patients with primary aldosteronism (six aldosterone-producing adenomas and seven bilateral adrenal hyper-

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plasia), seven patients with essential hypertension, and 10 normotensive controls. Demographic characteristics of our population are summarized in Table 1.

The control subjects were considered hypertensive if their clinic blood pressure (average of three different sphygmomanometric measurements each performed on 3 separate days, after a wash-out period of at least 2 wk if previously treated with antihypertensive drugs) was greater than 140/90 mm Hg. The subjects were considered normotensive if their systolic blood pressure was lower than 140 mm Hg, and their diastolic blood pressure was lower than 90 mm Hg. The presence of primary aldosteronism was assessed with standard laboratory tests or imaging approaches, as previously described (1). In particular, a radionuclide scintigraphy with 131-I-iodocholesterol, or adrenal veins blood sampling with evaluation of local aldosterone concentration were performed. In the six patients with aldosterone-producing adenoma, the diagnosis was confirmed by pathology. Patients previously treated with angiotensin-converting enzyme inhibitors, spironolactone, and angiotensin receptor blockers were excluded. Characteristics of previous antihypertensive therapy were similar in the two hypertensive groups. Venous blood samples were taken from all subjects in the supine position, after a wash-out period of 2 wk when appropriate, for measurement of plasma renin activity (RIA, Renctk; Sorin Biomedica, Turin, Italy), plasma, and urinary aldosterone (RIA, Aldoctk; Sorin Biomedica).

Micromyography

All subjects underwent a biopsy of sc fat from the gluteal region (3 cm long, 0.5 cm wide, 1.5 cm deep) (12, 13). Small arteries (about 100–280 μm of average diameter in relaxed conditions, 2 mm long) were dissected from the sc fat of the biopsies and mounted as a ring preparation on an isometric myograph (Danish Myo Technology, Aarhus, Denmark), by threading onto two stainless steel wires (40 μm diameter). The following morphological parameters were evaluated: media thickness, normalized internal diameter, media to lumen ratio, media cross-sectional area. Details about the micromyographic technique of evaluation of small artery morphology were previously reported (14–16). The average values obtained from two vessels in each experiment were considered. The protocol of the study was approved by the ethics committee of our institution (Medical School, University of Brescia), and informed consent was obtained from each participant. The procedures followed were in accordance with institutional guidelines.

Determination of the composition of small artery walls

Human arteries obtained from sc biopsy were isolated and fixed in paraformaldehyde 4% for 24 h. Two vessels for each patient were washed in 0.12 M phosphate buffer for 24 h, dehydrated in a series of alcohol, embedded in paraffin, and cut on a microtome at 5 μm thickness section (five sections for each vessel). After deparaffinization and hydration, the sections were stained for 5 min in 1% acid phosphomonolibdic aqueous solution and then stained for 3 min in 6% Sirius red in an aqueous solution. After dehydration in alcohol series and clarification with xylene, the slides were mounted in mounting medium (DPX; Fluka, Buchs, Switzerland). All sections were then analyzed using a light microscope under normal and polarized light. Tissue sections were examined at a magnification of ×40 and the quantitative evaluation of 10 fields per each vessel was performed under both normal and polarized light. The percentage of total collagen occupying the media layer of vessels was evaluated under standard light, where the percentage of the area red stained by Sirius red is easily quantifiable using an image analyzer (ImageproPlus; Immagini e Computer, Milan, Italy). The determination of the different types of collagen content with Sirius red staining was made using polarized light microscopy (17), which allows visualization of collagen fibers of different thickness with different colors. Type I collagen fibers are orange to red, whereas the thinner type III collagen fibers appear yellow to green (18, 19). The percentage of the different types of collagen occupying the media layer was evaluated with the same automated image analyzer. Intraassay variation coefficients ranged from 11–15% (total collagen and collagen subtypes), whereas interassay variation coefficients were 13–19%, respectively. Quantification of collagen content was performed by one of the coauthors who was blinded as to the source of tissue.

Statistical analysis

All data are expressed as mean ± SD, unless otherwise stated. One-way ANOVA was used to evaluate differences between groups. Because this was a hypothesis-generating study, no correction for multiple comparison was performed. The relation between continuous variables was evaluated by linear regression (Pearson's or Spearman-Kendall's correlation coefficients, according to the presence of a normal or a not normal distribution). All the statistical tests were two-tailed. $P < 0.05$ was considered statistically significant. All analyses were carried out with the BMDP statistical package (BMDP Statistical Software Inc., Los Angeles, CA).

Results

Normotensive subjects, patients with essential hypertension, and patients with primary aldosteronism were well matched for demographic characteristics (Table 1), whereas

TABLE 1. Demographic and clinical data in the different groups

	Normotensive controls (n = 10)	Essential hypertension (n = 7)	Primary aldosteronism (n = 13)
Age (yr)	50 ± 10	48 ± 16	53 ± 14
Gender (M, male/F, female)	5 M, 5 F	3 M, 4 F	6 M, 7 F
Weight (kg)	64 ± 12	66 ± 16	65 ± 13
Height (cm)	165 ± 9	164 ± 10	163 ± 8
Body mass index (kg/m^2)	23.5 ± 3.3	24.5 ± 3.9	24.5 ± 3.5
Known duration of hypertension (yr)		4.0 ± 2.5	2.9 ± 2.3
Known duration of previous hypertensive treatment (yr)		3.3 ± 2.1	2.1 ± 1.8
Systolic blood pressure (mm Hg)	124 ± 11	161 ± 8[a]	164 ± 10[a]
Diastolic blood pressure (mm Hg)	80 ± 6	93 ± 10[a]	92 ± 7[a]
Blood pressure values before therapy withdrawal (mm Hg)		138/86 ± 8/5	142/89 ± 9/6
Plasma renin activity (ng/ml·h)	1.2 ± 1.1	1.5 ± 1.3	0.2 ± 0.2[b,c]
Plasma aldosterone (nmol/ml)	0.30 ± 0.29	0.44 ± 0.31	1.97 ± 1.72[c,d]
Urinary aldosterone (nmol/24 h)	30.0 ± 24.7	37.1 ± 28.5	106 ± 73.2[c,d]
Previous antihypertensive therapy			
None		2/7	1/7
Diuretics		3/7	2/7
Calcium antagonists		3/7	4/7
β-blockers		1/7	1/7

[a] $P < 0.001$ *vs.* normotensive controls.
[b] $P < 0.05$ *vs.* normotensive controls.
[c] $P < 0.05$ *vs.* essential hypertension.
[d] $P < 0.05$ *vs.* normotensive controls.

the two hypertensive groups showed similar blood pressure values (Table 1). The duration of hypertension and the duration of previous antihypertensive treatment were similar in the two groups of hypertensive patients (Table 2).

Morphological characteristics of sc small resistance arteries are reported in Table 2. Media to lumen ratio and media thickness of small arteries were significantly greater in patients with primary aldosteronism and in those with essential hypertension, compared with normotensive controls, whereas internal diameter was significantly smaller in patients with primary aldosteronism. Media cross-sectional area was similar in all groups, thus suggesting the presence of mainly eutrophic remodeling in patients with primary aldosteronism and essential hypertension. These data confirm those previously reported (1, 5).

Total collagen and type III vascular collagen were significantly greater in patients with primary aldosteronism and with essential hypertension compared with normotensive controls. However, patients with primary aldosteronism had more total and type III collagen also compared with patients with essential hypertension (Table 2 and Figs. 1–3), despite similar blood pressure values. Type I collagen was slightly, not significantly, less in patients with primary aldosteronism than in normotensive controls. No significant correlation was observed between media to lumen ratio and total, type I, or type III vascular collagen. In addition, no significant correlation was observed between vascular collagen content and duration of hyperaldosteronism, or aldosterone serum levels.

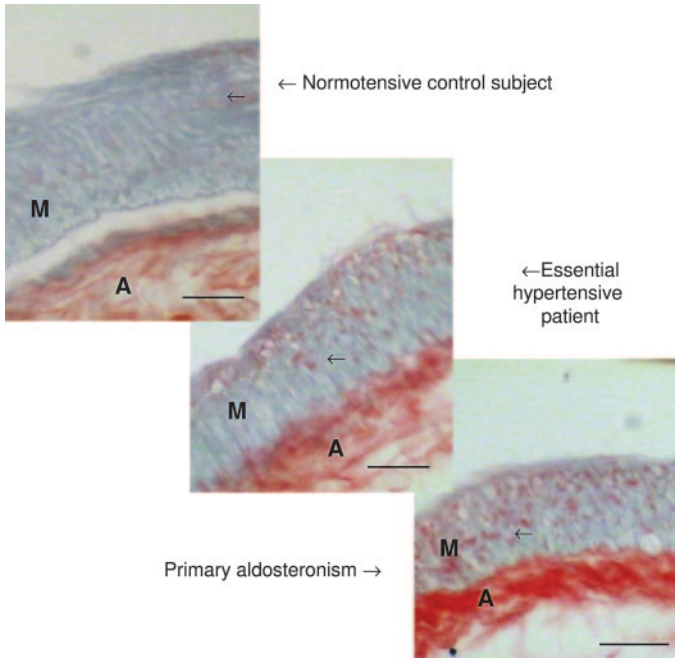

FIG. 1. Sirius red staining (*arrows*) of collagen content in the media (M) of sc small resistance arteries. *Bar*, 50 μm. Only medial collagen was quantified. The tunica adventitia was labeled with A.

Discussion

In the present study, for the first time, it was demonstrated that, in patients with primary aldosteronism, presenting elevated circulating and, probably, tissue levels of aldosterone associated with suppression of angiotensin II production, a relevant fibrosis of sc small resistance arteries (more pronounced than in blood-pressure-matched essential hypertensive patients) may be detected. Therefore, the strong profibrotic action of aldosterone, previously demonstrated to be present only *in vitro*, is present also in humans, at least when its circulating levels are very high.

It is well established that changes in extracellular matrix proteins, namely collagen and elastin content, may play a relevant role in the development of cardiovascular damage in hypertension (20–23). In particular, cardiac fibrosis is frequently present in human essential hypertension (20). Also, in small resistance arteries, an increase in collagen and a decreased elastin content within the tunica media was previously observed (24, 25). Surprisingly, these changes in the extracellular matrix components are not usually associated with concomitant changes in the mechanical properties of the vessels. In fact, no changes (26–29) or modest changes (24) in the wall stress-incremental elastic modulus relationship were found in the different studies when hypertensive patients and normotensive controls (24, 26, 27–29) or patients with renovascular hypertension and primary aldosteronism were considered (28). However, changes in the extracellular matrix may profoundly influence microvascular structure (21–23), and therefore, may have a relevant pathophysiological meaning, although there are, at present, no data supporting a prognostic role of microvascular fibrosis in hypertension.

As previously mentioned, aldosterone may have a relevant role in the development of cardiovascular fibrosis. The

TABLE 2. Morphological characteristics of sc small resistance arteries in the different groups

	Normotensive controls (n = 10)	Essential hypertension (n = 7)	Primary aldosteronism (n = 13)
Media thickness (μm)	15.1 ± 2.81	20.5 ± 4.89[a]	22.1 ± 5.18[b]
Internal diameter (μm)	298 ± 63	255 ± 57	242 ± 70[c]
Media to lumen ratio	0.051 ± 0.012	0.081 ± 0.018[a]	0.091 ± 0.032[c]
Media cross-sectional area (μm^2)	15,829 ± 8,181	160,128 ± 7,922	18,015 ± 8,478
Total collagen (%)	3.23 ± 0.58	6.84 ± 1.15[b]	8.17 ± 1.38[b,d]
Type I collagen (%)	2.51 ± 0.55	2.31 ± 0.40	2.22 ± 0.43
Type III collagen (%)	1.60 ± 0.22	5.25 ± 0.80[b]	6.06 ± 0.74[b,d]

[a] $P < 0.01$ *vs.* normotensive controls.
[b] $P < 0.001$ *vs.* normotensive controls.
[c] $P < 0.05$ *vs.* normotensive controls.
[d] $P < 0.05$ *vs.* essential hypertension.

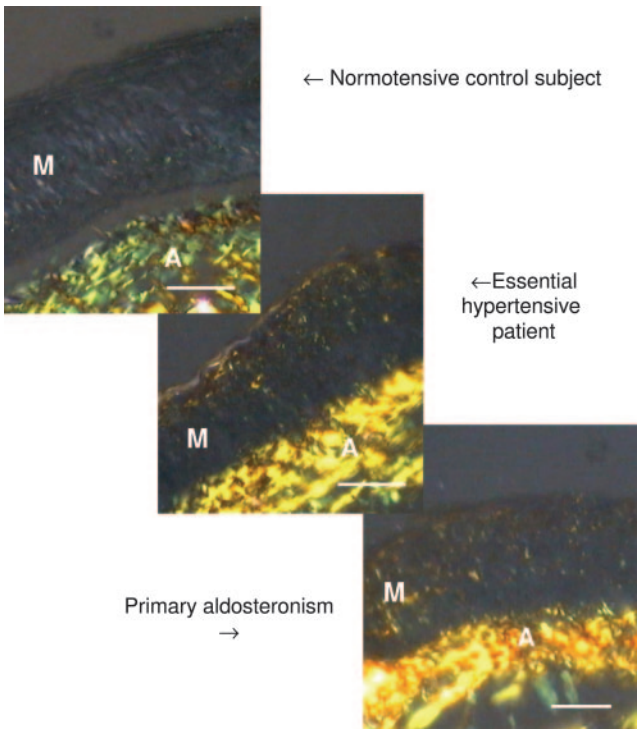

FIG. 2. Polarized observation of Sirius red stained type I (*orange/red*) and type III (*yellow/green*) collagen in the tunica media (M) of sc small resistance arteries. *Bar*, 50 μm. Only medial collagen was quantified. The tunica adventitia was labeled with A.

mechanisms underlying the profibrotic actions of aldosterone are only partially clear. An intracardiac aldosterone system has been described (30). This cardiac-generated aldosterone has possibly autocrine or paracrine actions. Normal cardiac tissue contains mineralocorticoid receptors (30). Mineralocorticoid receptors are not specific for aldosterone but they also bind glucocorticoids. Cardiac fibroblasts, however, contain the enzyme 11β-hydroxy-steroid dehydrogenase II, which converts these glucocorticoids to inactive metabolites. Discordant findings on the *in vitro* effect of aldosterone on the collagen synthesis in cardiac fibroblasts are reported and can be attributed at least partly to the

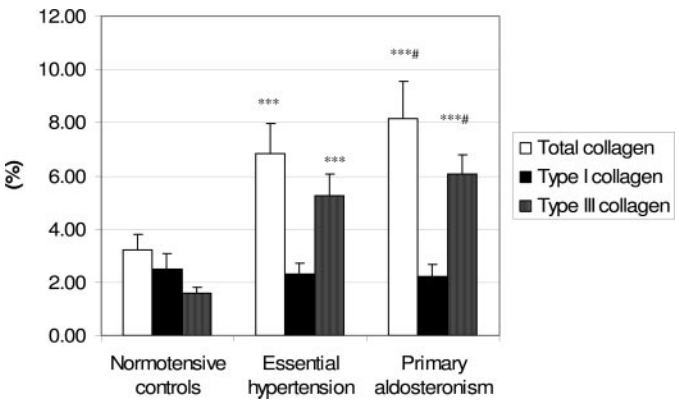

FIG. 3. Percent total collagen, type I collagen, and type III collagen content within the tunica media of sc small resistance arteries. ***, $P < 0.001$ *vs.* normotensive controls; #, $P < 0.05$ *vs.* essential hypertension.

presence of various fibroblast phenotypes. During the continuous infusion of aldosterone in the rat, the appearance of fibrosis was delayed and starts 4 wk after the beginning of the infusion, which argues against a direct effect of aldosterone (30).

It was proposed recently that aldosterone may induce cardiovascular fibrosis also through an increase in oxidative stress (10). Chronic infusion of aldosterone induced fibrosis in the heart, kidney, and aorta of Sprague Dawley rats (10). This effect was prevented by cotreatment with both an angiotensin II receptor blocker, losartan, and with an antioxidant, tempol (10). The protection from cardiovascular fibrosis was paralleled by a reduction in serum 8-isoprostane levels, superoxide tissue content, and NADPH oxidase activity (10). However, it should be mentioned that angiotensin receptor blockade may be effective only partially in blocking the deleterious effects of aldosterone, because *in vivo* an aldosterone escape may occur, whereby aldosterone levels return to or exceed baseline levels with consequent adverse cardiovascular effects (31, 32).

It is also possible that an interaction between aldosterone and endothelin system may be present; in fact, blockade of endothelin-1 receptors may reduce aldosterone-induced oxidative stress (33). Aldosterone may increase tissue concentrations of vascular endothelin-1; moreover, collagen deposition in cardiovascular tissues may be prevented by administration of an endothelin-1 type A receptor antagonist (34). Finally, aldosterone may also induce vascular fibrosis through its regulatory effects on plasminogen activator inhibitor-1 production, which, in turn, may modulate the collagen-degrading effects of metalloproteinases (35), or by inducing hypokalemia (36). Therefore, aldosterone, and the renin-angiotensin-aldosterone system activation in general, may induce vascular fibrosis through a variety of mechanisms, including oxidation, inflammation, and an intercellular and intracellular cascade of events that are, at least in part, independent from the hemodynamic load. Unfortunately, despite several attempts with different techniques (Western-Blot of nitrosylated proteins, confocal microscopy, *etc.*), due to methodological problems, we were unable to assess the level of oxidative stress in our small vessels; therefore, we cannot confirm, at present, the relevance of oxidative stress in the profibrotic action of aldosterone in our patients. Patients with primary aldosteronism and essential hypertension show the presence of an increased media to lumen ratio of sc small arteries, compared with normotensive controls. Media cross-sectional area was not statistically different between groups, thus suggesting the presence of mainly eutrophic remodeling in patients with primary aldosteronism or with essential hypertension. However, we have previously demonstrated that about 15% of the increase in media to lumen ratio in primary aldosteronism may be ascribed to hypertrophic remodeling (1, 5). No particular interference of the previous therapy should be expected because our patients were treated mainly with diuretics (thiazides) and calcium channels blockers, for a relatively short time. In general, their blood pressure was well controlled until shortly before the treatment was stopped.

In conclusion, our results indicate that, in small resistance arteries of patients with primary aldosteronism, a pro-

nounced fibrosis may be detected. The severity of fibrosis is greater that in blood-pressure-matched patients with essential hypertension. Therefore, the profibrotic effects of high circulating levels of aldosterone are present also in humans. Despite the fact that no demonstration of a prognostic role of microvascular fibrosis is presently available, it is conceivable that protection from the development of fibrosis in small arteries may contribute to the beneficial effects of drugs blocking aldosterone receptors (in addition to prevention or regression of cardiac and large artery fibrosis), observed in some pathological conditions, including heart failure. However, there are very few data available about effects of antihypertensive drugs on microvascular fibrosis (37), although drugs that block the renin-angiotensin system may have some advantage in this regard.

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