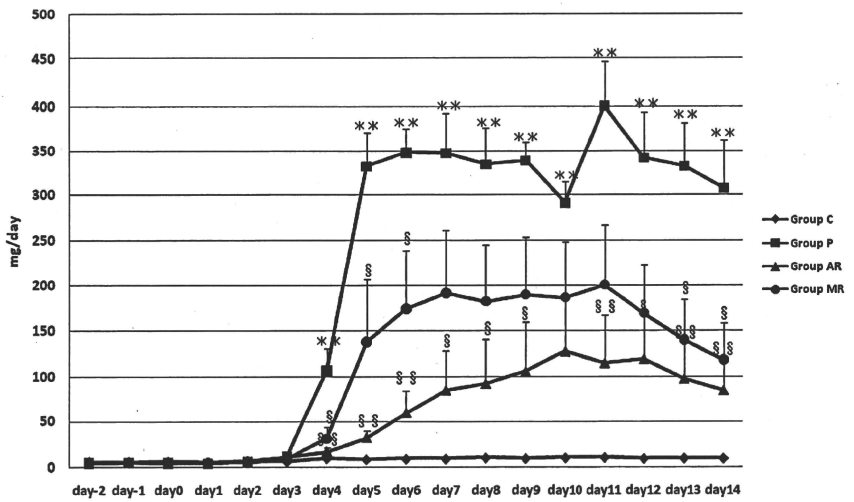

Fig. 1 Urinary protein excretion. $^{**}P < 0.01$ *vs* group C; $^{\S}P < 0.05$ *vs* group P; $^{\S\S}P < 0.01$ *vs* group P (tested by one-way ANOVA, followed by Scheffe's *F*-test).

Table 2 Systolic blood pressure among treatment groups

	Day 0	Day 7	Day 14
Control ($n = 5$) (mmHg)	105.2 ± 8.1	112.5 ± 6.8	98.2 ± 2.1
PAN ($n = 6$) (mmHg)	108.0 ± 3.5	124.3 ± 5.0	112.7 ± 4.3
MR ($n = 6$) (mmHg)	111.0 ± 2.7	108.6 ± 3.1	102.5 ± 2.3
AR ($n = 6$) (mmHg)	111.8 ± 3.8	112.5 ± 3.1	109.3 ± 5.6

PAN, puromycin aminonucleoside-treated rats; MR, PAN followed by eplerenone; AR, PAN followed by losartan. Values are mean ± standard error of the mean. Blood pressure levels in each group, and among groups on days 0, 7 and 14 after PAN administration did not statistically differ (tested by one-way ANOVA).

and plasma aldosterone levels did not differ among the groups and systolic BP did not significantly change throughout the study in any group (Table 2).

Proteinuria was significantly decreased in the groups MR and AR compared with group P (urinary protein excretion on day 14 after PAN administration in groups C, P, MR and AR was 9.4 ± 1.5, 308 ± 55, 119 ± 39 and 85 ± 35 mg/day, respectively; group P *vs* AR, was $P < 0.01$; group P *vs* MR, $P < 0.05$) (Fig. 1).

Gene expression of nephrin

Nephrin gene expression in glomeruli on day 14 after PAN administration was significantly decreased in group P compared with group C ($P < 0.05$, unpaired Student's *t*-test) (Fig. 2a). On the other hand, nephrin mRNA expression did not statistically differ among the groups P, MR and AR although the level tended to be higher in groups MR and AR compared with in group P (Fig. 2b).

Immunofluorescence for podocyte proteins

Glomerular immunostaining for nephrin, podocin and podocalyxin was less intense in group P than group C. Immunostaining for these proteins recovered in groups MR and AR (Fig. 3a). Its intensity was evaluated by semiquantitative analysis (score: 0, no; 1, weak; 2, intermediate; and 3, strong staining). Nephrin, podocin and podocalyxin expression was significantly decreased in group P compared with group C ($P < 0.01$). This reduction was recovered in groups MR and AR ($P < 0.01$) (Fig. 3b).

DISCUSSION

The present study showed that AR and MR blockers improved proteinuria in a model of acute nephrotic syndrome by protecting the slit diaphragm independently of BP. Previous studies showed that blockers of the RAAS are effective against proteinuria in models of chronic hypertensive and protein-induced renal damage,[3–5,9] and also in a PAN rat model,[10,11] but these effects have been considered the result of improvement

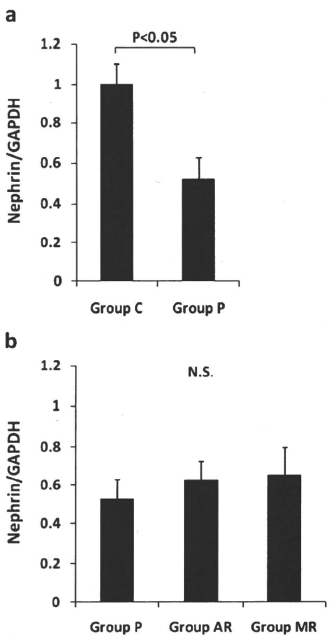

Fig. 2 Gene expression of nephrin in glomeruli on day 14 after puromycin aminonucleoside (PAN) administration. (a) Nephrin gene expression is significantly decreased in group P ($n = 6$) compared with group C ($n = 5$; $P = 0.017$, Student's unpaired *t*-test). (b) Nephrin mRNA did not statistically differ among groups P ($n = 6$), MR ($n = 6$) and AR ($n = 6$; $P = 0.730$, one-way ANOVA followed by Scheffe's *F*-test). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

of glomerular hypertension. Recently, a few studies show that type 1 receptor-mediated angiotensin II action reduced the expression of the slit diaphragm-associated molecules, and that type 1 receptor blockade ameliorated proteinuria by preventing the function of angiotensin II on the slit diaphragm.[3] On the other hand, the effect of MR blockers has not been examined in a model of acute nephrotic syndrome. So, we selected AR and MR blockers to determine the effects against proteinuria in this model through protecting the slit diaphragm independently of BP.

A dysfunctional slit diaphragm is involved in the development of proteinuria in several common diseases such as minimal change nephrotic syndrome and membranous nephropathy.[12] In fact, decreased mRNA expression and protein components of slit diaphragm are related to the degree of proteinuria in several models of kidney diseases.[12] Podocyte damage leads to proteinuria and initiates glomerulosclerosis, finally resulting in progressive loss of kidney function. As such, proteinuria must be decreased to preserve kidney function. Nephrin, a type 1 transmembrane protein, links molecules like podocin, CD2AP, Neph1 and others. In addition to functioning as a structural protein in the slit diaphragm, nephrin also transmits signals from the slit diaphragm into the interior of podocytes. Nephrin is phosphorylated by an Src family kinase[13] and a recent study has shown that decreased nephrin phosphorylation might lead to proteinuria in rat and human nephrosis.[14] Thus, nephrin is a key functional and structural component of the podocyte slit diaphragm.

We demonstrated that RAAS treatment recovered immunostaining for nephrin, podocin and podocalyxin and decreased proteinuria in PAN rats. The combination of AR and MR blockers tended to reduce proteinuria more effectively, and increase the degree of mRNA expression and immunostaining of slit diaphragm proteins in/on the glomeruli compared with either AR or MR alone, but these levels did not statistically differ (data not shown). A dual blockade of the renin–angiotensin system (RAS) and aldosterone might further prevent long-term renal disease progression compared with the inhibition of either system alone.[5,15,16] However, levels of BP differed between the groups in these studies, indicating that the effects are dependent upon BP. A recent large study[17] showed that the combination therapy with angiotensin-converting enzyme (ACE) inhibitor and AR blocker leads to a greater reduction in proteinuria than monotherapy with either drug, but dual RAS blockade worsens renal outcome. The reason may be related to the more reduction of BP, hyperkalaemia or aldosterone escape phenomenon, as described by Epstein.[18,19] Here, we used relatively low doses of AR and MR blockers; thus, levels of BP did not significantly differ among the RAAS treatment groups. The combination of AR and MR blockers also leads to reduced proteinuria without BP changes, but the levels of proteinuria did not significantly differ between the groups with combination therapy and monotherapy with either drug (data not shown). However, the possibility remains that the low-dose combination of AR and MR blockers may be the first treatment for podocyte injury disease, not only proteinuric nondiabetic disease but also diabetic disease. More studies are needed to clarify the superiority of combination of AR and MR blockers compared to an ACE inhibitor and/or an AR blocker.

Angiotensin type I receptor (AT1R), angiotensin type II receptor (AT2R) and MR are expressed in podocytes,[1,6,20] and AT1R overexpression and activated MR signalling induce proteinuria, nephron loss and the development of glomerulosclerosis in rats.[21,22] This explains why AR and MR blockers were effective against proteinuria in the present study. Activation of a local tissue angiotensin system leads to an increase in podocyte apoptosis, mainly in an AT1R-mediated fashion.[23] Others have shown that MR activation is important in the pathogenesis of cardiovascular and renal damage in both high-aldosterone states and in low-aldosterone

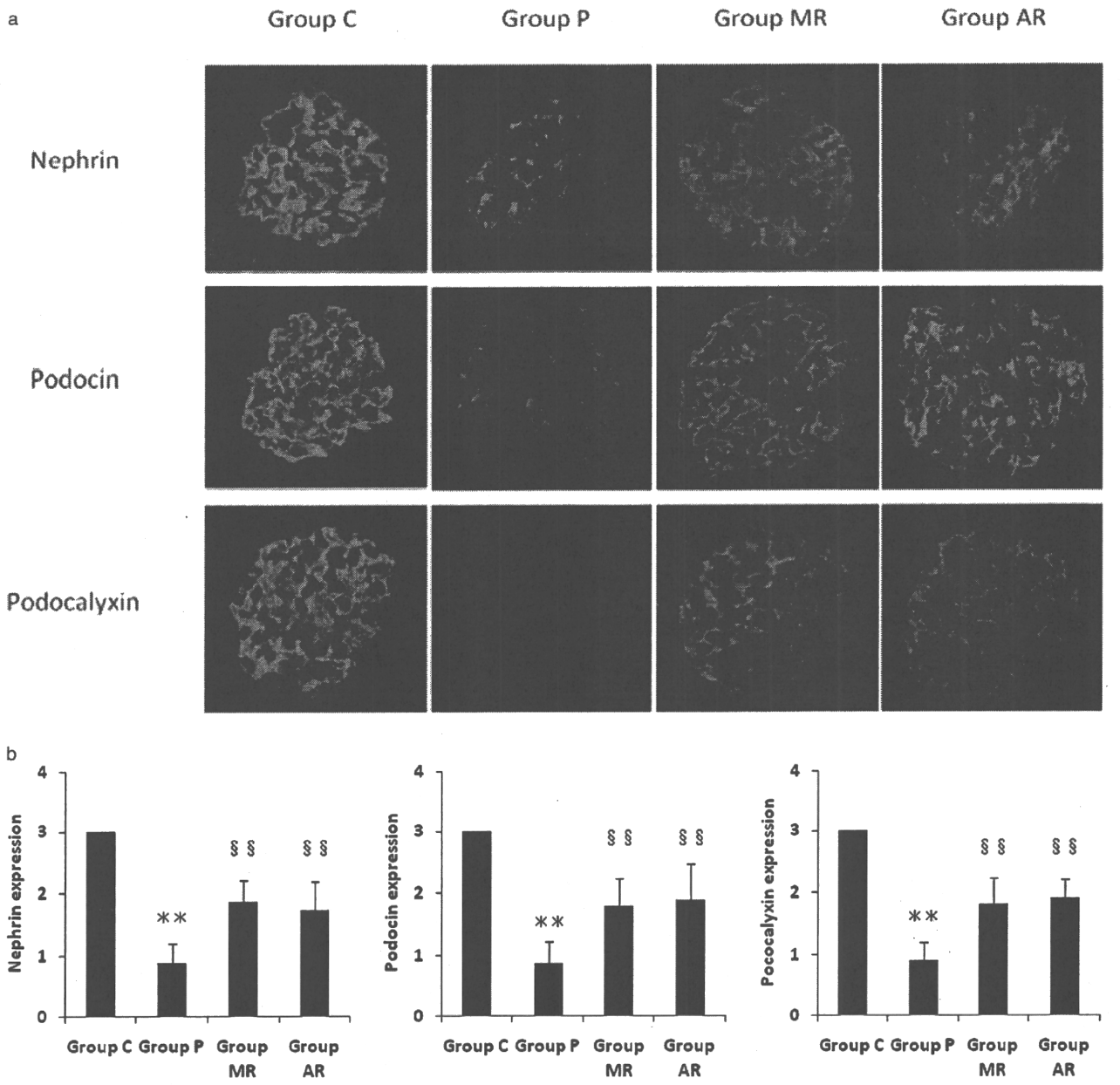

Fig. 3 Effects of eplerenone and losartan on nephrin, podocin, podocalyxin expression in puromycin aminonucleoside (PAN)-induced rat model. (a) Representative micrographs of immunostaining of nephrin, podocin and podocalyxin in glomeruli from controls, PAN-induced rats and from PAN-induced rats given eplerenone or losartan. (b) Semiquantitative immunofluorescence scoring of nephrin, podocin, podocalyxin in each rat group (group C, $n = 5$; group P, $n = 6$; group MR, $n = 6$; group AR, $n = 6$; $^{**}P < 0.01$ *vs* group C; $^{\S\S}P < 0.01$ *vs* group P (tested by one-way ANOVA, followed by Scheffe's *F*-test).

hypertension.[4] Salt-induced renal injury is accompanied by activated MR signalling in the kidney, without elevation of serum aldosterone.[22] We found here that MR blocker decreased proteinuria, while the plasma aldosterone concentrations did not differ between groups C and P.

As the cause of experimental nephrotic syndrome in this study, oxidative stress initiation might be associated with PAN-induced podocyte impairment.[24] Immunological, metabolic, toxic, infectious, haemodynamic and other mechanisms can damage podocytes.[25] Angiotensin II induces oxidative stress and leads to podocyte damage and decreases the integrity of the filtration barrier.[26] Angiotensin II might induce proteinuria through increasing glomerular capillary pressure or through direct cellular effects independently of

haemodynamics, that is, direct podocyte damage.[7] Aldosterone also causes podocyte damage through oxidative stress.[27] Nagase *et al.* demonstrated that aldosterone causes podocyte damage with proteinuria through a mechanism that is independent of BP, that is, possibly through the induction of oxidative stress.[6,28] Local angiotensin II, renal sympathetic nerve activation, oxidative stress or unidentified MR modulators are currently thought to increase MR activation.[23] Because the AR and MR blockers used in the present study have antioxidant effects,[28,29] we postulate that a blockade of angiotensin II activation and MR signalling improves podocyte damage through an antioxidative effect in PAN nephrosis. This notion seems to concur with previous findings showing the effect of other antioxidants on podocyte damage in PAN rats.[30]

In summary, our findings suggest that AR and MR blockers equally improve proteinuria independently of BP in a model of acute nephrotic syndrome with preserved expression of glomerular podocyte protein. Further studies are required to determine whether MR blockers are effective against minimal change nephrotic syndrome in humans.

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REVIEW

Stromal Cell Biology
– A Way to Understand the Evolution of Cardiovascular Diseases –

Toshihiro Tsuruda, MD; Takuroh Imamura, MD; Kinta Hatakeyama, MD*; Yujiro Asada, MD*; Kazuo Kitamura, MD

Stromal cells, composed of fibroblasts, microvascular endothelial cells, immune cells and inflammatory cells, are critical determinants of the mechanical properties and function of the heart and vasculature, and the mechanisms whereby these types of cells are activated are important to understand the progression of cardiovascular diseases. Emerging studies have suggested that the activation of autocrine and paracrine signaling pathways by stromal cell-derived growth factors, cytokines and bioactive molecules contributes to disease progression. Disruption of the stromal network will result in alterations in the geometry and function in these organs. Interventions targeting the stromal cells (eg, myofibroblasts, microvascular endothelial cells, inflammatory cells) by pharmacological agents or direct gene delivery/small interfering RNA would be potential novel therapeutic strategies to prevent/attenuate the progression of cardiovascular disorders. (*Circ J* 2010; **74**: 1042–1050)

Key Words: Adventitia; Angiogenesis; Fibroblast; Matrix metalloproteinase

Extracellular matrix (ECM) serves as a physical scaffold (structural support) for cells but also provides specific molecular and spatial information that influences cell proliferation, differentiation and apoptosis.[1] Stromal cells are defined as connective tissue cells of organs found in loose connective tissue, which is composed of fibroblasts, immune cells, pericytes, microvascular endothelial cells and inflammatory cells (**Table 1**). Emerging studies have suggested that the activation of autocrine and paracrine signaling pathways by stromal cell-derived growth factors, cytokines and bioactive molecules contributes to the pathogenesis of cardiovascular diseases. Hemodynamic overload of the heart not only activates hypertrophy in cardiac myocytes, but also induces hyperplasia among fibroblasts and microvessels accompanied by inflammatory responses in the interstitium.[2–5] The elastin and collagen network provides skeletal support for hemodynamics in the vasculature, while the proportion of collagen to elastin increases in the aneurysmal abdominal aorta.[6] The acute reaction of stromal composition to hemodynamic, inflammatory and growth alteration appears to be "adaptive", but chronic (prolonged) stimuli of stromal cells can become "maladaptive", evoking inadequate ECM turnover, resulting in the pathological manifestation, such as inward (eg, stiffness of heart and vasculature) or outward remodeling (eg, dilatation of the heart and aneurysmal formation). This review highlights the multiple roles of stromal cells in the pathogenesis of cardiovascular diseases, such as heart failure, post-myocardial infarction (MI), atherosclerosis and abdominal aortic aneurysm.

Perivascular and Interstitial Space of Heart

The heart is composed of parenchyma (cardiac myocytes) and stroma (connective tissue). Cardiac myocytes (cardiocytes) comprise 75% of volume and 30–40% of number, but the remaining 60–70% of the cell population is composed of non-myocytes: endothelial cells, smooth muscle cells, fibroblasts, macrophages and mast cells.[7,8] These stromal cells form a continuum between different cell types within the myocardium and provide a structural supporting network to maintain myocardial geometry during the cardiac cycle.[9] Thickening of the adventitia surrounding intramyocardial coronary arteries in response to systemic hypertension has been thought to reduce oxygen and nutrient supply to the myocardium, re-

Table 1. Stromal Cell Composition

Fibroblast
Immune cell
Pericyte
Microvascular endothelial cell
Inflammatory cell

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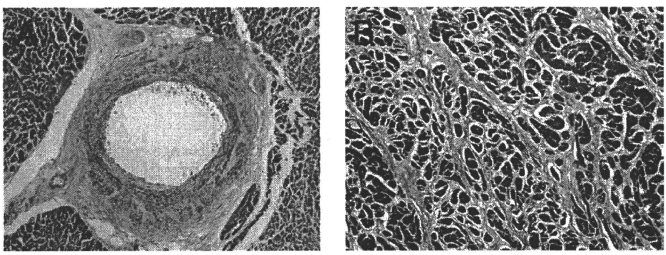

Figure 1. Autopsy of an 87-year-old male patient who suffered from uncontrolled hypertension and died suddenly. Slides were stained with Azan-Mallory. Gross fibrosis was observed in the (**A**) intra-myocardial coronary artery and (**B**) interstitial space of the hypertrophied left ventricle. Original magnification ×100.

sulting in the deterioration of ventricular function.[10] **Figure 1** illustrates the left ventricle of an 87-year-old male patient who suffered from uncontrolled hypertension and died suddenly.

Vascular Wall

The arterial wall is composed of 3 layers (intima, media and adventitia). The media is the main structural component supported by ECM elements, such as elastin, collagen and fibronectin.[11] The adventitia is populated by terminal nerve fibers, vasa vasorum and surrounding connective tissue, which contains a few resident fibroblasts and inflammatory cells. The vasa vasorum penetrates the outer media and adventitia of large arteries and veins, providing an entire microvascular bed within the wall of host blood vessels. Recent studies have shown that the "adventitial layer" is an important modulator of arterial remodeling through its interactions with the media and intima.[12,13]

Myofibroblast Differentiation

Myofibroblasts are specialized fibroblast-like cells that show induced expression of α-smooth muscle actin in response to injury.[14,15] These cells have ultrastructural features between a fibroblast and a smooth muscle cell. Stimulated myofibroblasts proliferate and increase the production of ECM proteins, including collagen I and III, fibronectin and laminin. Although myofibroblasts are essential for wound healing by generating contractile force, overproduction of matrix proteins from this type of cell produces a pathological manifestation. Myofibroblasts have been identified at the site of the healing stage of MI,[16] in the hypertrophied heart,[4,17] and the injured arteries following angioplasty.[15] Transforming growth factor (TGF)-β1, angiotensin II (Ang II), aldosterone and endothelin are recognized to play an important role in promoting the transition from fibroblasts to myofibroblasts.[14] The expression of TGF-β1 and smooth muscle α-actin was most abundant in the adventitia after balloon injury in the rat carotid artery, which was followed by the accumulation of collagenous ECM, suggesting that the adventitial layer plays a central role in inward remodeling.[18]

Inflammation

The roles of inflammatory cells in the progression of atherosclerosis have been reviewed.[19] We will focus on the roles of macrophages and mast cells in the evolution of cardiovascular diseases in this review.

Macrophages

The heterogeneity of macrophages is well recognized, and is determined by specific tissue- and immune-related stimuli.[20] At least two subsets of macrophages are distributed in the infarcted heart and atherosclerotic aorta. One subset (M1) is conventional foam cells in atheromatous plaque, promoting inflammation and ECM destruction. The other (M2) is monocyte-like cells seen in atheromatous plaque with hemorrhage, that is, extravasated erythrocytes[21] and in the outer media and adventitia.[22] The latter uniquely expresses CD163, a glycoprotein belonging to the scavenger receptor cysteine-rich superfamily.[23] Interestingly, it functions as a scavenger for hemoglobin by binding and clearing haptoglobin–hemoglobin complexes,[24] and as a tumor necrosis factor-like inducer of apoptosis.[25] In addition, this receptor has been reported to play important roles in the regulation of immune responses at atherosclerotic plaques.[21,26] Macrophage accumulation in the perivascular area of intramyocardial coronary arterioles precedes fibrous formation in the rodent model of pressure overload,[4] which is mediated by the transient upregulation of intracellular adhesion molecule-1 on capillary endothelial cells.[27] Okamoto et al showed that the infiltration of macrophages was greater in the adventitia than in the intima after balloon angioplasty of porcine coronary arteries.[28] They demonstrated that the recruitment of macrophages to the adventitia was accompanied by the expression of cell adhesion molecules in the endothelium of the vasa vasorum. Gong et al also showed that matrix metalloproteinase (MMP)-9 activation was required for macrophage migration to the adventitia in an experimental model of abdominal aortic aneurysm.[29]

Mast Cells

Mast cells develop from progenitor cells that in turn arise from uncommitted hematopoietic stem cells in the bone marrow.[30] Stem cell factor binds to the receptor tyrosine kinase c-kit on mast cells, and subsequent signaling, such as mito-

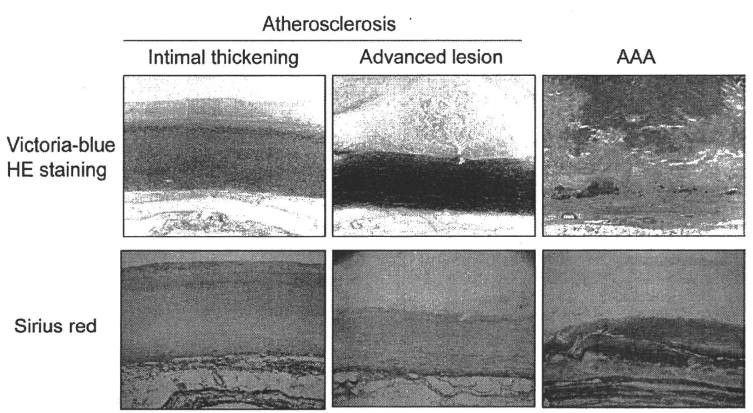

Figure 2. Elastin and collagen formation in the intimal thickening, advanced atherosclerosis and aneurysmal abdominal aorta. Distribution of elastin and collagen was determined by Victoria blue and Sirius red, respectively. Collagen deposition was observed to increase in the outer media and adventitia of abdominal aortic aneurysm (AAA) as a consequence of elastin degradation. Original magnification ×25.

gen-activated protein kinase cascade and phosphoinositide-3 kinase, is necessary for mast cells to differentiate, home, prolong viability and enhance mediator production.[31] Fibroblasts contribute to the further differentiation and maturation of mast cells.[32] Mediators secreted by mast cells can be subdivided into preformed (secretory granule-associated) and newly synthesized after cell activation.[33] Preformed mediators include histamine, proteoglycan, tryptase and chymase, and newly generated products include prostaglandin D_2, leukotriene, inflammatory cytokines and chemokines. The majority of mast cells contain both tryptase and chymase in the adventitia of atherosclerotic aorta and aneurysm,[22] which activate MMPs,[34,35] and induce the apoptosis of smooth muscle cells.[36] Mast cells are uniquely positioned around capillary vessels, suggesting their potential contribution to angiogenesis.[37] These cells are reported to be involved in the pathogenesis of various cardiovascular diseases, such as atherosclerosis,[38] hypertensive heart disease,[39] myocarditis,[40] heart failure[41] and abdominal aortic aneurysm[22] in experimental animal models and humans.

Metabolism of ECM

The regulation of ECM turnover is defined by the balance between its synthesis and degradation. The normal rate of ECM turnover varies on species, and is particularly very slow with a half-life of approximately 100 days (collagens) in the heart[42,43] and at age 40–70 years (elastin) in the arterial wall in humans;[44] but substantial collagen synthesis (deposition) is accelerated in the interstitium of the hypertrophied heart,[45] failing heart with hypertension,[46] and during worsening heart failure,[47] probably due to the compensatory mechanism to prevent further dilatation. Collagen deposition was also observed to increase in the outer media and adventitia of an abdominal aortic aneurysm, as a consequence of elastin degradation (**Figure 2**).[6]

MMPs are enzymes that degrade different components of the ECM, but their functions expand beyond matrix degradation.[9] The more than 20 different members are classified into five groups: collagenases (such as MMP-1 and MMP-13), stromelysin (such as MMP-3), gelatinases (such as MMP-2 and MMP-9), membrane type (such as MMP-14) and others (such as metalloelastase, matrilysin). MMPs and their endogenous inhibitors, named tissue inhibitors of metalloproteinases (TIMPs)-1–4, not only inhibit the action of MMPs, but are also essential components of myocardial and vascular structures and function by modulating cell–cell and cell–matrix interactions.[48] Under physiological conditions, the activities of MMPs are strictly regulated by the transcriptional activation of pro-MMP precursor zymogens and TIMPs, and collagen I and the collagenase MMP-1 are important biological determinants of cardiac performance.[42] Shear stress influences various vascular function,[49] including MMP activities in vascular endothelial cells and smooth muscle cells.[50] Activated MMPs are thought to optimize shear stress and wall tension in the vasculature and to preserve the lumen size,[51] delaying the development of flow-limiting stenosis; but ECM metabolism becomes dysregulated in the failing heart[9] and aneurysmal aorta.[52] In particular, gelatinases MMP-2 and MMP-9 activities have been reported to rise in atherosclerosis,[52] after MI[53] and in heart failure,[9] facilitating structural alteration in the heart and vasculature. Moreover, MMP-2 works as an interstitial collagenase,[54] and MMP-2 and MMP-9 are capable of degrading elastin,[55] and are thereby specifically involved in aneurysmal formation.[56] Thus, it is suggested that the degradation of ECM over its synthesis may eventually promote dilatation of the heart and aorta.

Neovascularization

As capillary vessels supply oxygen and nutrients in response to demand, angiogenesis would be beneficial for maintaining structure and function in the heart and vasculature.[3,57,58] Tissue hypoxia is an important component to trigger the activation of hypoxia-inducible factor (HIF)-1α, a key transcriptional factor to regulate angiogenesis, stimulating the expression of vascular endothelial growth factor (VEGF).[59] Perivascular fibroblasts are important for blood vessel formation by secreting growth factors, cytokines and proteolytic enzymes, specifically VEGF and MMPs, which allow for endothelial cells to proliferate, migrate, and grow, cooperating to stimulate angiogenesis.[60] In addition, adventitial fibroblast activation induces basic fibroblast growth factor, which is associated with the development of collateral vessels, followed by chronic occlusion of the coronary artery.[61]

Accumulating evidence suggests that the vasa vasorum might contribute to the pathogenesis of cardiovascular disorders.[62–64] Because inflammatory cells are recruited from circulating blood, it is not clear whether the angiogenic pathway due to the upregulation of HIF-1α and VEGF expression is related to inflammation and adverse ventricular remodeling.[65–68] Newly formed intimal vessels mainly originate from the adventitial vasa vasorum of human atheromatous coronary arteries,[69] and a ruptured and/or leaky vasa vasorum conceivably functions as a conduit for the entry of macrophages and inflammatory factors that may potentially promote disease.[70,71] In contrast, Barker et al have reported that removal of the adventitial vasa vasorum in the carotid artery induced intimal hyperplasia in rabbits, suggesting that impaired arterial wall oxygenation is related to atherogenesis.[72] Their hypothesis was further proven by showing that intimal hyperplasia already established was regressed by the generation of highly vascular neo-adventitia with the polyvinyl chloride tubing surrounding the carotid artery. Thus, it appears case dependent as to whether angiogenesis is beneficial or detrimental in the progression of cardiovascular diseases.

Bioactive Molecules

Fibroblasts are target cells for bioactive molecules, such as norepinephrine,[73] Ang II,[74] endothelin-1,[75] aldosterone,[74,76] natriuretic peptides[77–80] and adrenomedullin (AM)[81,82] in the turnover of ECM. **Table 2** lists the pro-fibrotic and anti-fibrotic bioactive molecules involved in the regulation of ECM metabolism.

Table 2. Pro-Fibrotic and Anti-Fibrotic Bioactive Molecules in the Regulation of ECM Metabolism

Bioactive molecules	Putative receptor	Reference
Pro-fibrotic		
Norepinephrine	β1-adrenergic	73
Ang II	AT_1	74
Endothelin-1	ET_A	75
Aldosterone	MR	74, 76
Anti-fibrotic		
ANP, BNP	NPR-A	77–79
CNP	NPR-B	80
AM	CRLR/RAMP	81, 82

ECM, extracellular matrix; Ang II, angiotensin II; AT_1, angiotensin II type 1 receptor; ET_A, endothelin type A receptor; MR, mineralocorticoid receptor; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; NPR, natriuretic peptide receptors; CNP, C-type natriuretic peptide; AM, adrenomedullin; CRLR/RAMP, calcitonine receptor-like receptor/receptor activity-modifying protein.

Specifically, the renin–angiotensin–aldosterone system (RAAS) is relevant in the pathogenesis of atherosclerosis and cardiac hypertrophy and fibrosis.[2,83,84] Adipose tissue, macrophages and mast cells in the adventitia of atherosclerotic and/or aneurysmal aorta,[85] and fibroblasts in the interstitium of the heart[86,87] have been shown to contribute to Ang II generation. Immunoreactivity of angiotensin-converting enzyme (ACE) was increased and distributed in the perivascular and interstitial fibroblasts of the pressure-overloaded rat left ventricle (**Figure 3**).[86,88] Various stimuli have been shown to stimulate ACE synthesis in the cardiovascular system,[89,90] in which activity was increased during myofibroblast transformation in cultured cardiac fibroblasts.[91] Activated macrophages also produce ACE,[92] but chymase in mast cells appears to be more responsible for generating Ang II formation in humans.[87,93] Locally synthesized in the

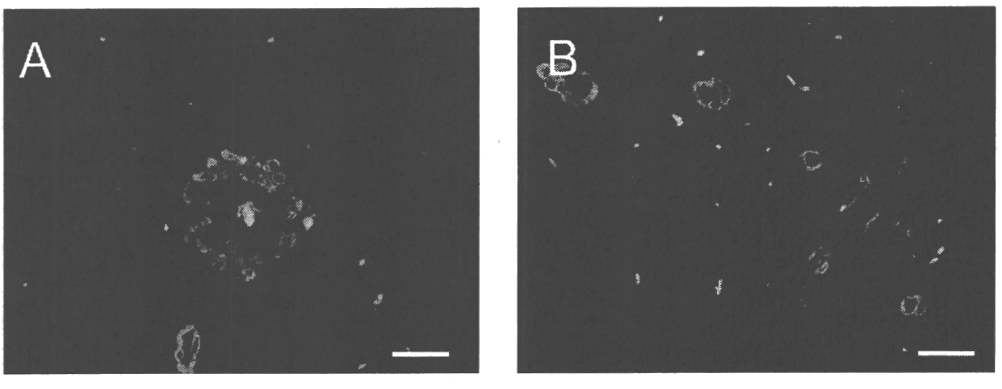

Figure 3. Distribution of immunoreactive angiotensin-converting enzyme (ACE, red) and α-smooth muscle actin (green) in the (**A**) intra-myocardial coronary artery and (**B**) interstitial space of the left ventricle with aortic constriction in adult rats. ACE immunoreactivity increased and was distributed not only in endothelial cells of intramyocardial arteries but also in perivascular and interstitial fibroblasts in the pressure-overloaded heart. Bar, 200 μm.

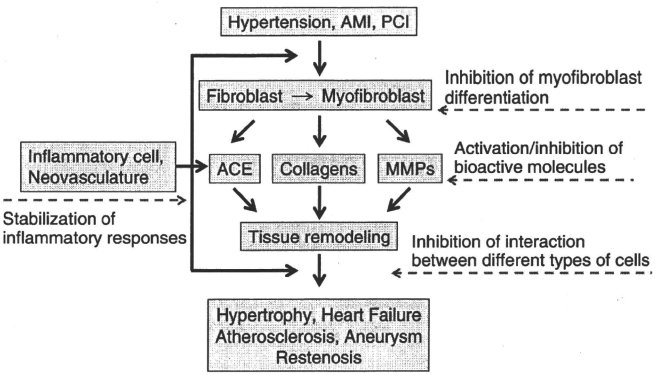

Figure 4. Contribution of stromal cells in the pathogenesis of cardiovascular diseases. Fibroblasts differentiate into myofibroblasts in hypertension, following acute myocardial infarction (AMI) or percutaneous coronary intervention (PCI). Myofibroblasts synthesize angiotensin-converting enzyme (ACE) and matrix proteins, such as collagen I and III, as well as producing matrix metalloproteinases (MMPs). Infiltrated inflammatory cells and neovasculature further amplify tissue remodeling, altering the architecture and function, resulting in stimulation of pathogenesis in the heart and vasculature. Several steps in therapeutic approaches, which include (1) inhibition of myofibroblast differentiation, (2) activation/inhibition of bioactive molecules, (3) stabilization of inflammatory responses and (4) interaction between the different types of cells, are thought to prevent/attenuate the progression of cardiovascular diseases.

cardiac tissue and vascular wall, Ang II can induce potent inflammatory responses by stimulating the release of growth factors (such as platelet-derived growth factor, TGF-β, insulin-like growth factor-1), cytokines (such as interleukin-1, -6) and chemokines (such as monocyte chemotactic protein-1 and interleukin-8), which in turn activates T-lymphocytes and induces monocyte migration and fibroblast differentiation into myofibroblasts.

Natriuretic peptides (atrial natriuretic peptide, ANP; brain natriuretic peptide, BNP and C-type natriuretic peptide, CNP) exert anti-fibrotic actions.[77-80,94] They are not only synthesized in cardiomyocytes or vascular endothelial cells but are also secreted from fibroblasts; ANP in myofibroblasts in the infarct region of myocardium in sheep;[95] BNP in cultured adult canine ventricular myofibroblasts;[79] and CNP in cultured adult rat cardiac fibroblasts.[80] The anti-fibrotic action of natriuretic peptides mediates the cyclic guanosine monophosphate (cGMP), inhibiting fibroblast proliferation and collagen synthesis, and stimulating the production of matrix-degrading enzymes.[79] Using complementary DNA microarray analysis, Kapoun et al showed that BNP treatment of human cardiac fibroblasts resulted in the marked reduction of TGF-β effects on genes related to fibrosis, myofibroblast conversion, proliferation and inflammation.[78]

AM was discovered from human pheochromocytoma,[96] and has been recognized to be widely distributed in tissues and organs, including the heart and vasculature, in humans and rodents. AM exerts biological actions via calcitonin receptor-like receptor/receptor activity-modifying protein complex, stimulating the cyclic adenosine monophosphate (cAMP).[97] AM is synthesized from cultured fibroblasts obtained from the neonatal heart[81] and the adventitia of aorta of rats.[82] We have shown that synthetic AM inhibited cardiac fibroblast proliferation and collagen synthesis through cAMP elevation.[81] In addition, a subdepressor dose of AM administration into Ang II-induced hypertensive rats significantly attenuated collagen deposition in the perivascular area of intramyocardial arteries accompanied by the inhibition of myofibroblast differentiation.[17] Furthermore, AM was found to be synthesized in mast cells distributed in the outer media and adventitia of an abdominal aortic aneurysm, a possible role of mast cell-derived AM in pathogenesis.[98]

The importance of bioactive molecules in the regulation of MMP/TIMP activity in the myocardium has been previously reviewed.[99] Ang II and endothelin-1 have been shown to reduce MMP (collagenase) activities,[74,75] whereas BNP and AM appear to oppose pro-fibrotic bioactive molecules, stimulating MMP activities in culture;[79,82] but the biological actions of MMPs modulated by bioactive molecules seem to be complicated in vivo.[99]

Interaction of Stroma on Structure and Function in Cardiovascular System

As described in this review, stromal cells play important roles in cardiovascular structure and function; for example, endothelin-1 synthesized in fibroblasts is necessary to induce cardiomyocyte hypertrophy.[100] In addition, cultured ventricular cardiomyocytes have been shown to induce depolarization-induced automaticity when co-cultured with myofibroblasts, a possible contribution to arrhythmogenesis in the hypertrophied or infarcted heart.[101,102] In contrast, cardiomyocytes serve to secrete active TGF-β in Ang II-mediated collagen synthesis in fibroblasts.[103] In the vasculature, Moreno

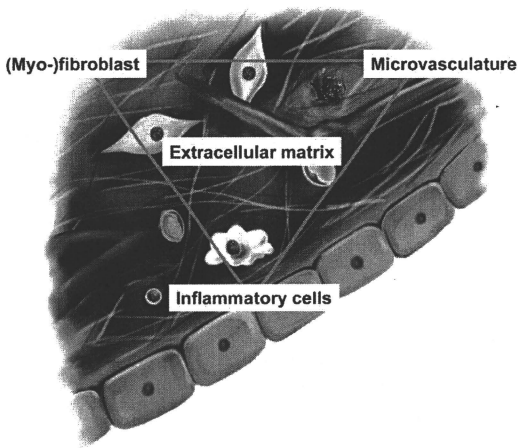

Figure 5. Stromal network in the heart and vasculature. Stromal cells are critical for maintaining myocardial and vascular geometry and function, whereas disruption of the stromal network will result in the development of cardiovascular diseases.

et al showed that vulnerable plaque is associated with an increased incidence of adventitial inflammation.[104] Although the relative contribution of adventitial fibroblasts in neointimal growth remains controversial,[105] Shi et al showed that neointimal hyperplasia is preceded by the activation and proliferation of adventitial fibroblasts, which migrate to the neointima from adventitia.[106]

Future Therapeutic Perspective

Dysregulation of matrix synthesis and degradation might result in the alteration of architecture and function in the heart and vasculature. Further understanding of the mechanisms, and development of effective pharmacological or biological agents targeting the stromal cells and ECM metabolism would be important advances for overcoming cardiovascular diseases. **Figure 4** summarizes the contribution of stromal cells to amplifying structural alteration in the heart and vasculature. Potential therapeutic approaches targeting these types of cells are also shown briefly. The first, several growth factors, which include the circulating and tissue components of RAAS and TGF-β, are potent activators of collagen synthesis in (myo)fibroblasts; therefore, inhibition of these factors could be an attractive therapeutic target to inhibit fibrosis.[4] Of importance, AT$_1$ receptor antagonism is reported to be effective not only for preventing the progression of fibrosis, but also for reversing fibrosis by stimulating collagen degradation in spontaneously hypertensive rats[107] and hypertensive/diabetic human subjects.[108,109] The second, targeting the inhibition of fibroblasts to myofibroblast transition, might theoretically be effective to attenuate fibrosis. Pharmacological abrogation of Ang II activity with ACE inhibition and AT$_1$ receptor blockade,[110,111] inhibition of aldosterone activity with mineralocorticoid receptor blocker,[112] direct stimulation of cGMP[113] or cAMP signaling[17] are potential therapies to attenuate fibrosis after MI and in the hypertensive heart by antagonizing myofibroblast transformation. The third, based upon the significance of adventitia in determining the direction of vascular remodeling, gene transfer of NAD(P)H oxidase inhibitor[114] or L-arginine administration[115] to the adventitial layer would be beneficial to prevent re-stenosis following balloon angioplasty. The inhibition of vasa vasorum formation with statins (3-hydroxy-3-methylglutryl-CoA (HMG-CoA) reductase inhibitors) has been shown to attenuate the progression of atherosclerosis, independent of the lipid-lowering effect.[63,116] The fourth, pharmacological intervention for attenuating the inflammatory cell infiltration,[117] switching the phenotype of macrophages[118] and stabilization of mast cells[22,41] might reduce disease progression. Last, considering the hypothesis that many signals for developing cardiovascular diseases are shared with signaling pathways in cancer biology,[119] it might be considered whether targeting stromal–cancer cell interactions with small interfering RNAs could be applied to novel treatment modalities in cardiovascular disorders.[120,121]

Conclusion

Figure 5 illustrates the schema of the stromal network in the heart or vasculature. Disruption of the stromal network will result in alterations of myocardial and vascular geometry and function. Further understanding of the function and regulation of stromal cells is needed in order to develop effective therapeutic methods for preventing/attenuating the evolution of these disorders.

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Plasma and tissue levels of proangiotensin-12 and components of the renin–angiotensin system (RAS) following low- or high-salt feeding in rats

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ABSTRACT

The renin–angiotensin system (RAS) is an essential regulator of the blood pressure and body fluid balance, but the processing cascade or role of the tissue RAS remains obscure. Proangiotensin-12 (proang-12), a novel angiotensin peptide recently discovered in rat tissues, is assumed to function as a factor of the tissue RAS. To investigate the tissue production of proang-12, we measured the circulating and tissue components of the RAS including proang-12 following low-, normal-, or high-salt feeding in rats. Twelve-week-old male Wistar rats were fed a low-salt 0.3% NaCl or high-salt 8% NaCl diet for 7 days and compared with those fed a normal-salt diet of 0.7% NaCl. Low-salt feeding elevated the plasma renin activity and aldosterone concentration, resulting in significant increases in Ang I and Ang II levels in the plasma or kidney tissue, as compared with the normal- or high-salt group. Despite the increases in plasma renin activity, Ang I, and Ang II, the proang-12 levels in plasma and various tissues including the kidneys, small intestine, cardiac ventricles, and brain remained unchanged following low-salt feeding. These results suggest that peptide levels of proang-12 in rat plasma and tissues are regulated in a manner independent of the circulating RAS.

1. Introduction

The renin–angiotensin system (RAS) plays an important role in regulating the blood pressure and fluid balance. The protease enzyme renin cleaves angiotensinogen circulating in the blood to produce angiotensin I (Ang I), which, in the presence of angiotensin-converting enzyme (ACE), is converted to Ang II, a potent pressor peptide mediating the major actions of the RAS as a circulating hormone [4,18]. A number of studies have been conducted on the tissue RAS, the function of which is assumed to be regulated independently of the circulating RAS [1,7,16,19]. There are, however, many unanswered questions regarding the tissue RAS, such as the processing cascade of angiotensin peptides or roles in regulating the blood pressure or fluid balance. Proangiotensin-12 (proang-12) is a C-terminal extended form of Ang I (Ang I-Leu-Tyr) recently isolated and identified in the rat small intestine [17]. When injected intravenously into rats, proang-12 exerts vasoconstrictor and pressor effects, which are abolished by an ACE inhibitor or an Ang II type I receptor blocker, suggesting the role of this novel peptide as a precursor of Ang II. As the levels of proang-12 in various tissues including the heart, kidneys, and small intestine are much higher than in the blood, proang-12 is assumed to be a factor of the tissue RAS in rats [10,17,22]. However, there are also a number of unresolved issues concerning the production cascade and function of this novel peptide. In the present study, we measured the plasma and tissue levels of proang-12, Ang I, and Ang II in rats under the condition of low-salt feeding, where the systemic RAS is activated, in an effort to answer these important questions.

2. Materials and methods

2.1. Animals and experimental protocol

Male Wistar rats at 10 weeks of age were purchased from CHARLES RIVER LABORATORIES (Kanagawa, Japan) and maintained under a 12-h light/12-h dark cycle in specific pathogen-free conditions with a normal diet containing 0.7% NaCl for 14 days. Thereafter, the rats were randomly divided into three groups fed a low-, normal-, or high-salt diet containing NaCl of 0.3, 0.7, and 8% (ORIENTAL YEAST Co., Ltd., Tokyo, Japan), respectively ($n = 7$–8 in each group). Blood pressure levels were measured before and after the low- or high-salt feeding by means of the tail-cuff method (model BP-98A; Softron Co., Ltd., Tokyo, Japan). After the 7 days of feeding, blood samples were collected following decapitation into a tube containing 10 mg/ml EDTA and 500 KIU/ml aprotinin and immediately centrifuged at 3000 × *g* for 10 min at 4 °C to obtain the plasma.

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The present study was performed in accordance with the Animal Welfare Act and with the approval of the University of Miyazaki Institutional Animal Care and Use Committee (2008-501-2).

2.2. Sample preparation for radioimmunoassay (RIA)

Tissues were carefully resected after decapitation and boiled for 10 min in 10 volumes of distilled H_2O. After boiling, acetic acid was added to the samples to a final concentration of 1.0 mol/l, as previously described [11,15]. The samples were then homogenized using a Polytron mixer and immediately centrifuged at 12 000 rpm for 20 min at 4 °C. Both the plasma and the tissue samples were applied to a Sep-Pak C18 cartridge and eluted with 60% acetonitrile in 0.1% trifluoroacetic acid. Eluted samples were lyophilized and store at −20 °C until RIA.

2.3. Measurement of proang-12 and other components of the RAS

To specifically detect proang-12 in tissues and plasma, we developed a RIA, as previously described, with antiserum raised against the C-terminal portion of the peptide [17]. The Ang I and Ang II levels in tissues and plasma were similarly determined using RIAs with the anti-C-terminal of Ang I and Ang II antisera purchased from Miles and CORTEX BIOCHEM, INC. (San Leandro, USA), respectively [8,13]. The angiotensinogen levels in plasma were determined by employing an ELISA purchased from Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan). Plasma renin activity and aldosterone levels were measured using RIAs, as previously described [12].

2.4. Statistical analysis

Comparisons of all data were made employing analysis of variance (ANOVA) followed by Scheffe's tests. Values are presented as means ± SE, and significance was set at $P < 0.05$.

3. Results

No significant differences were noted in systolic and diastolic blood pressures (SBP and DBP, respectively) between the low-, normal-, or high-salt groups (SBP, 121 ± 5, 135 ± 3, 129 ± 4; DBP, 94 ± 3, 101 ± 3, 96 ± 4 mmHg). Fig. 1 shows the plasma renin activity as well as plasma aldosterone and angiotensinogen concentrations in the three groups of rats. Compared with the normal-salt group, a 117% increase was observed in the plasma renin activity in the low-salt group, and, accordingly, the plasma aldosterone concentration was increased by 120% (Fig. 1A and B). In contrast, both the plasma renin activity and the aldosterone concentration were reduced in the high-salt group, though the reductions were non-significant. The angiotensinogen level of plasma was slightly elevated by low-salt feeding, but the difference was non-significant (Fig. 1C).

Fig. 2 shows the plasma levels of proang-12, Ang I, and Ang II in the three groups. When compared with the normal-salt group, low-salt feeding resulted in a slight increase of plasma Ang I levels, and the Ang II level was elevated by 989% (Fig. 2B and C). Despite the increases in Ang I and Ang II, there was no significant difference in the plasma levels of proang-12 among the three groups. Similar changes were observed in tissue proang-12 levels in the kidneys (Fig. 3). In comparison with the normal-salt group, the Ang I and Ang II levels in the low-salt group were elevated by 85 and 57%, respectively; however, the proang-12 level remained unchanged. As shown in Fig. 4, we measured proang-12 levels in various tissues, but the differences were non-significant between the three groups except for those of the liver.

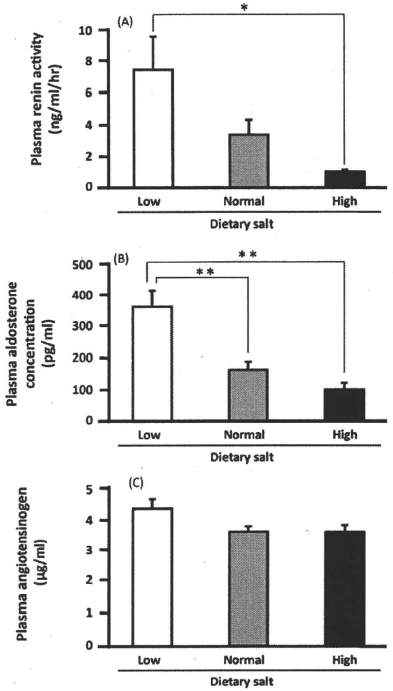

Fig. 1. Plasma renin activity (A), aldosterone (B), and angiotensinogen (C) levels in rats fed a low-, normal-, or high-salt diet. The results are shown as the means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

Proang-12, a novel angiotensin peptide first identified in the rat small intestine, is thought to be produced from angiotensinogen and converted into Ang I or Ang II [17,21]. In the present study, we measured the plasma and tissue levels of proang-12 following low-salt feeding, which activated the systemic RAS, as evidenced by augmentations of plasma renin activity, aldosterone, and Ang I and II concentrations. In contrast to Ang I or Ang II in the plasma or tissues, the levels of proang-12 remained unchanged in rats fed the low-salt diet. On comparison of the plasma and tissue levels of peptides, proang-12 levels in various rat tissues were much higher than in plasma, suggesting a role for this peptide as a component of the tissue RAS. However, almost the opposite was the case for Ang I, and this was further evident in the plasma and kidneys of rats placed under low-salt conditions in the present study.

An important question is whether or not proang-12 is produced and processed by the action of renin in the systemic circulation or local tissues. To investigate the role of systemic renin, we previously measured tissue proang-12 levels following bilateral nephrectomy in rats. In that experiment, despite a marked reduction in the plasma levels of Ang I and Ang II, the tissue levels of

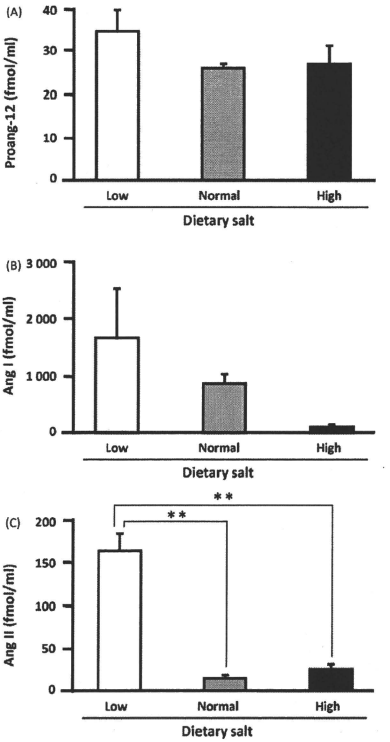

Fig. 2. Immunoreactive proangiotensin-12 (A), Ang I (B), and Ang II (C) levels in the plasma of rats fed a low-, normal-, or high-salt diet. The results are shown as the means ± SE. Proang-12 levels in the low-, normal-, and high-salt groups were 34 ± 5, 25 ± 1, and 26 ± 4 fmol/ml, respectively. $^{**}P < 0.01$.

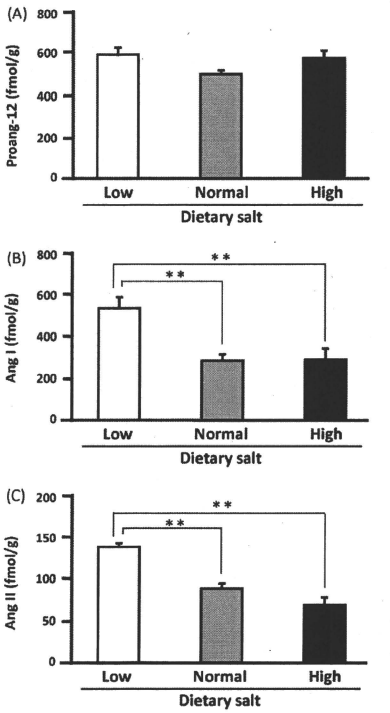

Fig. 3. Immunoreactive proangiotensin-12 (A), Ang I (B), and Ang II (C) levels in the kidney tissues of rats fed a low-, normal-, or high-salt diet. The results are shown as the means ± SE. $^{**}P < 0.01$.

three angiotensin peptides were found to be elevated in the cardiac ventricles [6]. In the conversion of proang-12 to Ang I or other angiotensin peptides, a renin inhibitor failed to inhibit Ang I production from proang-12 in perfused rat hearts *ex vivo* [21]. Prosser et al. reported that chymase inhibition resulted in the attenuation of proang-12-induced cardiac damage following ischemia-perfusion in rat hearts *ex vivo*, suggesting that chymase is involved in the conversion of proang-12 to Ang I or Ang II [20]. In the present study, despite the increase in plasma renin activity induced by low-salt feeding, the tissue levels of proang-12 remained unchanged in a number of organs including the kidneys, heart, and small intestine. Collectively, it is unlikely that renin is involved in the production or processing of proang-12 in rats, and proang-12 levels in the plasma and tissues appear to be regulated independently of the systemic RAS.

There is a substantial amount of evidence suggesting the active roles of the local RAS in tissues including the kidneys, cardiac ventricles, and brain [2,3,5,17]. In the present study, both the Ang I and the Ang II levels in the kidney were elevated by low-salt feeding, a

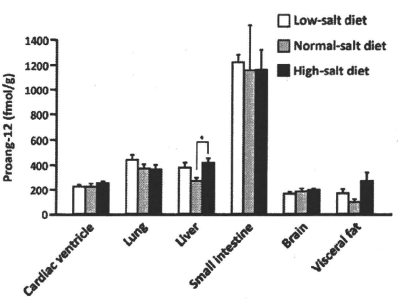

Fig. 4. Immunoreactive proangiotensin-12 levels in various tissues of rats fed a low-, normal-, or high-salt diet. The results are shown as the means ± SE. $^{*}P < 0.05$.

finding consistent with the view that angiotensins intrarenally produced by renin play an active role in retaining sodium in the renal tubules [14]. Despite the augmentations of Ang I and Ang II in kidneys, we observed no increase in these peptide levels in the cardiac ventricles following low-salt feeding (data not shown). The brain is also an organ where the tissue RAS is assumed to play an active role because components of the RAS are expressed at a significant level [7,17]. Recently, Isa et al. reported that the neutralization of brain proang-12 by a specific antibody resulted in blood pressure reduction in mRen2 transgenic rats, suggesting a role of proang-12 in centrally modulating blood pressure in the rat brain [9]. In the present study, the blood pressure remained unchanged, and we observed no significant changes in tissue proang-12 levels of the kidneys, cardiac ventricles, or brain in the three groups of rats. However, those findings do not necessarily exclude the possibility that proang-12 participates in the regulation of electrolytes and water balance under low-salt conditions. An example is provided by the plasma angiotensinogen level as shown in Fig. 1, where no clear differences were noted between the three groups. Further studies need to be aimed to further specify the roles of this novel peptide in disorders of blood pressure or fluid balance, in which the tissue RAS is involved.

In summary, the proang-12 levels in tissues and plasma remained unchanged following low-salt feeding despite the activation of the systemic RAS in rats. The present findings support the notion that proang-12 is produced independently of renin in the systemic circulation, suggesting that the proang-12 levels in tissues and plasma are differentially modulated in rats.

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ORIGINAL ARTICLE

Aldosterone antisecretagogue and antihypertensive actions of adrenomedullin in patients with primary aldosteronism

Toshihiro Kita, Mariko Tokashiki and Kazuo Kitamura

Adrenomedullin (AM) is located in the zona glomerulosa of the adrenal cortex and is considered to suppress aldosterone release. To determine the effect of AM in primary aldosteronism (PA), we infused AM (2.5 pmol kg^{-1} min^{-1}) for 27 h, followed by a 15-h recovery period, in a control group (essential hypertensives with plasma aldosterone levels ⩽100 pg ml^{-1}, n=7) and in a PA group (n=5). The control group was also infused with vehicle. Hemodynamic, hormonal, oxidative and inflammatory responses were studied. AM infusion caused similar and steady decreases in blood pressure and several markers for arteriosclerosis (for example, pulse wave velocity) in both groups. Interestingly, AM infusion suppressed aldosterone release to values within the normal range in the PA group (300.0 ± 58.4 to 111.6 ± 13.5 pg ml^{-1}, P<0.01). In the control group, aldosterone release suppression was significant but limited (81.7 ± 9.1 to 47.9 ± 9.9 pg ml^{-1}, P<0.01). The adrenocorticotropic hormone–cortisol system was not changed by AM infusion. Brain natriuretic peptide was cumulatively increased by prolonged AM infusion in both groups, probably because of cardiac overload. AM did not affect oxidative markers. In addition, a mild but significant increase in C-reactive protein (CRP) mediated by interleukin-6 was observed during AM infusion in every participant, without exception. This pathway might participate in CRP elevation in cardiovascular disease. In summary, AM seems to have an essential role in the suppression of aldosterone release in PA. AM may be an important modulator in PA, and intermediate-term (3 h) AM infusion could be used as an alternative renin-stimulating/aldosterone-suppressing test for PA detection.
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Keywords: adrenomedullin; aldosterone; C-reactive protein; humans; primary aldosteronism

INTRODUCTION

Adrenomedullin (AM) is a potent hypotensive peptide found ubiquitously in tissues and organs, especially in cardiovascular tissues, the kidneys, lungs and endocrine glands. AM has multiple functions in a wide range of tissues and acts mainly as a vasodilatory and proliferation-inhibitory factor.[1] AM was initially identified in the adrenal medulla, but similar densities of AM were detected in the zona glomerulosa of the adrenal cortex, where AM suppresses aldosterone release.[2,3] In addition, expression of AM and its receptor was detected in Conn's adenoma cells, and AM exerted aldosterone antisecretagogue action and proliferative effects on cultured Conn's adenoma cells.[4] These findings suggest that endogenous AM may be an important modulator of aldosterone release in normal and pathogenic hyperaldosteronism. In addition, AM antagonized aldosterone-induced vascular or cardiac remodeling[5,6] and suppressed aldosterone-induced oxidative stress in a malignant hypertensive model.[7] The characteristics that define this compound, from its release to alterations in target organs, suggest that AM may be an endogenous anti-aldosterone factor, especially in the cardiovascular system.

The effects of exogenous AM administration on aldosterone release were varied in experimental animal and human studies.[8] Short-term AM administration to healthy volunteers,[9] hypertensive patients[10] and patients with renal insufficiency[11] caused vigorous stimulation of renin release, but did not change aldosterone levels; consequently, the aldosterone/renin ratio decreased. AM suppressed aldosterone levels in secondary hyperaldosteronism, such as experimental heart failure in sheep[8] or congestive heart failure in patients.[12] These data suggest that AM may be a functional antagonist of aldosterone release, but further evidence is required. We investigated the effects of AM in patients with primary aldosteronism (PA) to confirm the antagonistic effect of AM against aldosterone.

METHODS

Study subjects

Initially, essential hypertensive patients were recruited for baseline data collection for future translational research on the clinical applications of AM. After a comprehensive screening, seven hypertensive subjects who showed normal aldosterone release (plasma aldosterone ⩽100 pg ml^{-1}; control group) were

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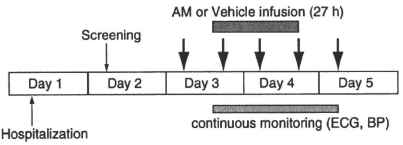

Figure 1 Experimental protocol. After a comprehensive examination of all participants, AM (2.5 pmol min^{-1} kg^{-1}) or vehicle was intravenously administered for 27 h, followed by a 15-h post-infusion period. Arrows indicate hemodynamic and blood sample assessments.

administered AM. Five patients with PA, owing to an aldosterone-producing adenoma (PA group), were then recruited to receive AM. The control group was administered vehicle after an interval of at least 1 month. All participants were admitted to our hospital and subjected to 2 days of comprehensive examinations, including urine and blood tests, chest X-ray, ECG, echocardiography, echo scan of the carotid artery and a brain MRI. Patients with narrowing or obstruction of extra- and/or intracranial major arteries, renal failure (serum creatinine >1.2 mg per 100ml), heart failure (left ventricular ejection fraction <50%), coronary heart disease, peripheral artery diseases, collagen diseases or active infections were excluded. All patients with PA had cleared the diagnostic criteria for PA, as proposed by the Japan Endocrine Society (http://square.umin.ac.jp/endocrine/rinsho_juyo/index.html). Potential adrenal adenomas were confirmed by CT and/or MRI. This study was approved by the ethics committee of the institute. All participants gave their written informed consent.

Preparation of human AM

Chemically synthesized human AM was purchased from the Peptide Institute (Osaka, Japan). The homogeneity of human AM was confirmed by reverse-phase high-performance liquid chromatography and amino acid analysis. AM was dissolved in distilled water with 3.75% D-mannitol and 0.05% aminoacetic acid and then sterilized by passing through a 0.22-μm filter (Millipore, Bedford, MA, USA). The chemical nature and level of human AM in vials were verified by reverse-phase high-performance liquid chromatography. No measurable endotoxin was detected (<0.01563 EU ml^{-1}), and the material was determined to be pyrogen-free by the Japan Food Research Laboratories (Tokyo, Japan).

Study protocol

Fixed time points (0800 and 1700 hours) were assigned for comprehensive hemodynamic examination and blood sampling. Vehicle or AM administration was started at 1400 hours, as indicated in Figure 1. The comprehensive hemodynamic examination included blood pressure, heart rate (by ECG) and arteriosclerosis-related markers, such as pulse wave velocity (form PWV/ABI, BP-203RPE; Omron Colin, Komaki, Japan), augmentation index (HEM9010AI tonometer; Omron Healthcare, Kyoto, Japan) and elastic property of the carotid artery.[13] A 22-gauge cannula was inserted into the forearm vein for infusion of AM (2.5 pmol min^{-1} kg^{-1}) or vehicle diluted by 0.9% saline. Saline was administered at the rate of 5 ml h^{-1} for 27 h, followed by 15 h of recovery time. Continuous ECG monitoring and blood pressure measurements at 60-min intervals were performed throughout the experiment (Figure 1). The first urine sample was collected at around 0700 hours, before, during and after AM or vehicle administration. Plasma total and mature AM were measured using a specific immunoradiometric assay kit (Shionogi, Osaka, Japan). Plasma concentrations of other hormones, high-sensitivity C-reactive protein (CRP) and cytokines were measured using a commercially available laboratory testing service (SRL, Hachioji, Japan). Urinary concentrations of 8-isoprostane and 8-hydroxydeoxyguanosine were also measured by the laboratory testing service provided by SRL and normalized by the concentration of urinary creatinine.

Statistical analysis

All data were expressed as means ± s.e.m. Comparisons between the two groups (control *vs.* PA) were performed using the unpaired Student's *t*-test. The significance of differences was evaluated by one-factor analysis of variance with repeated measures on a time course of variables, followed by Bonferroni–Dunn *post hoc* comparison tests. A value of $P<0.05$ was the criterion for statistical significance.

Table 1 Basal characteristics

	Control	*PA*	*P-value*
Age (years)	65.3 ± 3.1	63.6 ± 2.5	NS
Sex (M/F)	7/0	3/2	
SBP (mm Hg)	154.4 ± 7.0	155.4 ± 5.8	NS
DBP (mm Hg)	93.7 ± 3.6	93.2 ± 4.5	NS
Heart rate (b.p.m.)	63.3 ± 2.8	63.6 ± 3.8	NS
PWV (cm s^{-1})	1943 ± 117	1939 ± 221	NS
Augmentation index (%)	83.7 ± 6.2	97.2 ± 4.4	NS
Elastic property (kPa)	109.7 ± 11.0	93.1 ± 7.9	NS
Aldosterone (pg ml^{-1})	81.7 ± 9.1	300 ± 58.4	0.001
Renin activity (ng ml^{-1} h^{-1})	0.90 ± 0.40	0.16 ± 0.04	NS
Adrenomedullin (fmol ml^{-1})	11.8 ± 0.6	19.2 ± 1.7	0.001
ANP (pg ml^{-1})	15.6 ± 2.8	23.4 ± 9.8	NS
BNP (pg ml^{-1})	16.6 ± 3.3	55.8 ± 36.2	NS
Noradrenaline (pg ml^{-1})	297 ± 31	384 ± 81	NS
High-sensitivity CRP (mg l^{-1})	0.78 ± 0.18	1.80 ± 0.83	0.06

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CRP, C-reactive protein; DBP, diastolic blood pressure; NS, non-significant; PA, primary aldosteronism; PWV, pulse wave velocity; SBP, systolic blood pressure.

RESULTS

To avoid extreme rises in blood pressure, the minimal amount of Ca^{2+}-channel blocker (amlodipine 5–10 mg day^{-1}) was administered to three of seven patients in the control group and to all five patients with PA. The baseline characteristics of the control group and PA group were fairly matched, particularly with regard to age and blood pressure (Table 1). The plasma concentration of aldosterone and, interestingly, the plasma concentration of AM were increased in the PA group (Table 1).

Prolonged AM administration caused a strong and steady decrease in blood pressure; this effect was quite similar in both control and PA groups (Figure 2). Heart rate was increased by almost the same magnitude in both groups during AM administration (approximately +26% in control and +31% in PA). In addition, similar decreases in arteriosclerotic markers, such as pulse wave velocity, augmentation index and elastic property of the carotid artery, were accompanied by reductions in blood pressure in both groups (Figure 2). These effects returned to baseline after a 15-h interval. Harmful symptoms or reactions were not observed in any of the participants.

As indicated in Figure 3, AM administration caused significant increases in total AM (approximately 3.1-fold in the control group and 2.1-fold in the PA group). Peak concentrations of AM were comparable in both groups. Plasma concentration of mature AM also increased: approximately 6.7-fold in both groups (Table 2). Most interestingly, AM administration caused strong and significant suppression of aldosterone release in the PA group; levels reached the normal range (111.6 ± 13.5 pg ml^{-1}) at the end of AM infusion (Figure 3). Significant but moderate suppression of aldosterone release was also observed in the control group during AM administration. AM infusion stimulated renin release in the control group, but this change was not significant. A very small but significant increase in renin was observed in the PA group (Figure 3). Standard renin-secretion stimulating tests (captopril loading and furosemide loading+walking) did not increase renin levels in the PA group (data not shown).

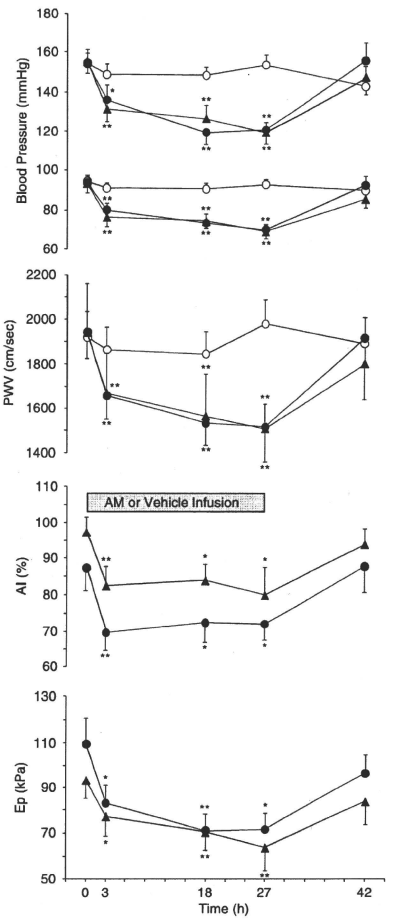

Figure 2 Changes in blood pressure, pulse wave velocity (PWV), augmentation index (AI) and elastic property (Ep) of the carotid artery during infusion of adrenomedullin (●: control group, ▲: PA group) or vehicle (○: control group). Data are means ± s.e.m. $*P<0.05$, $**P<0.01$, each *vs.* baseline.

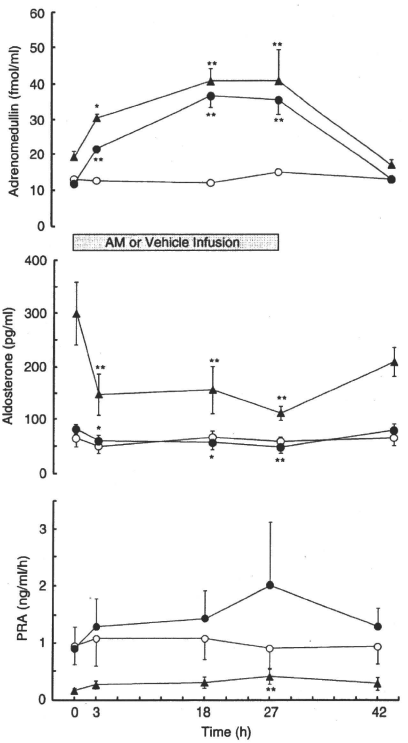

Figure 3 Changes in the plasma concentration of adrenomedullin, aldosterone and plasma renin activity (PRA) during infusion of adrenomedullin (●: control group, ▲: PA group) or vehicle (○: control group). Data are means ± s.e.m. $*P<0.05$, $**P<0.01$, each *vs.* baseline.

Other hormonal changes are summarized in Table 2. Levels of cAMP, the second messenger of AM, were unchanged in both groups. An increase in atrial natriuretic peptide (ANP) level, accompanied by a cGMP increase, was only observed in the PA group during AM administration. Brain natriuretic peptide (BNP) level was increased in both groups in a late phase of the experiment, but this alteration was not associated with the cGMP increase. It is noteworthy that AM did not affect the adrenocorticotropic hormone–cortisol system in either group.

To evaluate the acute effects of AM on oxidative stress and the immune system, we assessed oxidative stress markers, cytokines and high-sensitivity CRP. Basal levels of CRP were below the normal range ($<0.3\,mg\,dl^{-1}$) in all participants, and the average value was $0.12 \pm 0.04\,mg\,dl^{-1}$. AM administration did not affect the oxidative stress markers (8-isoprostane and 8-hydroxydeoxyguanosine) in either group (Table 2). Surprisingly, AM administration induced expression of interleukin-6 (IL-6), which was followed by an increase in CRP. This reaction was confirmed in every participant, without exception. All results are summarized in Figure 4. (The unit for CRP is $mg\,l^{-1}$.)