Table 3. Quantitative Angiographic Data and Clinical Outcomes at 8 Months of Follow up

Target lesion (n)	SES group (n=53)	BMS group (n=125)	P value
Follow up			
Reference diameter (mm)	2.89±0.45	2.93±0.54	0.66
MLD (mm)	2.01±1.08	2.41±0.91	0.01
Diameter stenosis (%)	30.3±33.6	17.9±26.6	0.02
Angiographic re-restenosis, n (%)	16 (29)	15 (12)	<0.01
TLR, n (%)	11 (21)	10 (8)	0.02
TVR, n (%)	11 (21)	11 (9)	0.04
MACE, n (%)	11 (21)	11 (9)	0.04
Stent thrombosis, n (%)	2 (4)	1 (1)	0.16

Data are expressed as mean±SD when appropriate.
TLR, target lesion revascularization; TVR, target vessel revascularization; MACE, major adverse cardiac event. Other abbreviations see in Tables 1 and 2.

adverse cardiac events (MACE) including all-cause death, myocardial infarction (MI), target lesion revascularization (TLR) and target vessel revascularization (TVR). We defined MI as the presence of a new Q wave in ≥2 contiguous ECG leads and/or a creatine kinase-MB concentration elevated to >3-fold the upper normal limit. We defined TLR as repeated re-intervention to treat an ISR accompanied by symptoms or objective signs of ischemia (ischemia-driven TLR). We then defined TVR as any repeated revascularization procedure involving a target vessel with an ischemic sign (ischemic-driven TVR). Stent thrombosis was defined according to the designation established by the Academic Research Consortium.[11]

Statistical Analysis

Data are expressed as mean±SD. The differences between the 2 groups were analyzed using an unpaired Student's t-test or the Mann-Whitney U test when the variance was skewed. Categorical variables were compared by Fisher's exact probability test. We also assessed independent predictors of angiographic re-restenosis and MACE using multivariate logistic regression analysis with independent variables that were considered significant according to univariate analyses. All tests were 2-sided and a P value of <0.05 was considered statistically significant.

Results

We studied a total of 179 ISR lesions in 158 consecutive patients (53 SES restenotic lesions in 49 patients, and 126 BMS restenotic lesions in 109 patients) between August 2004 and June 2007. All patents were successfully implanted with SES and 178 patients (99%) were followed up by angiography, for which 1 patient refused to undergo.

Tables 1 and **2** show baseline demographic, clinical, and angiographic characteristics and procedural variables. Patients with post-SES restenosis had a higher prevalence of diabetes mellitus and a lower prevalence of MI history than those with post-BMS restenosis (**Table 1**). Patients with SES restenosis also had more frequent focal restenosis, lesions with a significantly smaller MLD, a higher rate of %diameter stenosis and shorter lesions than those with BMS restenosis (**Table 2**). No periprocedural MI, TLR or stent thrombosis or in-hospital deaths occurred.

Table 3 and **Figure** show the QCA data and clinical outcomes at 8 months of follow up. The MLD was significantly smaller and the rate of %diameter stenosis was more increased in ISR lesions originally treated with SES than with

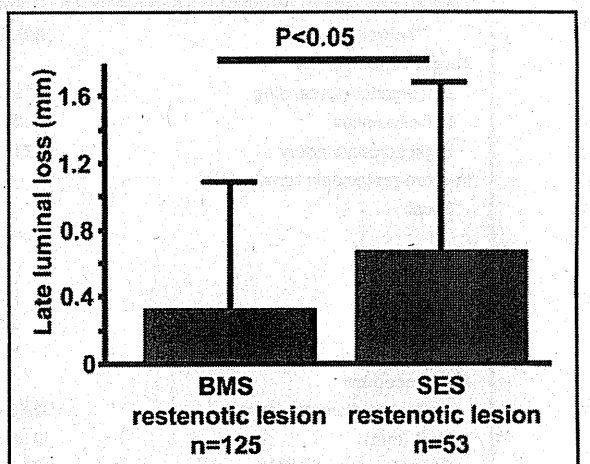

Figure. Late luminal loss in groups with post-BMS and post-SES restenosis. Data are expressed as means±SD. BMS, bare-metal stent; SES, sirolimus-eluting stent.

BMS. Interestingly, the rates of angiographic re-restenosis (29% vs 12%, P<0.01), TLR (21% vs 8%, P=0.02), TVR (21% vs 9%, P=0.04) and MACE (21% vs 9%, P=0.04) were significantly higher in the group with post-SES than post-BMS restenosis (**Table 3**). Moreover, late luminal loss was significantly greater in the SES than in the BMS group (0.68±1.01 vs 0.36±0.77 mm, P=0.04; **Figure**). The type of re-restenosis was generally focal in both groups (data not shown). No cardiac death and Q-wave MI occurred during follow up. Stent thrombosis resulting in unstable or stable angina pectoris developed in 1 (1%) and 2 (4%) patients in the BMS and SES groups, respectively, but the difference was not statistically significant.

Multivariate logistic regression analysis also showed that repeated SES implantation to treat restenosis of SES is an independent predictor of angiographic re-restenosis and MACE (odds ratio (OR) 3.4, 95% confidence interval (CI) [1.28–9.02], P=0.01 and OR 3.37, 95%CI [1.08–10.49], P=0.04, respectively) compared with BMS, after including all variables that were significant in the univariate analyses listed in **Tables 1** and **2** (**Tables 4,5**).

Also, the incidence of re-restenosis and MACE did not differ between patients with and without diabetes mellitus (19% vs 16%, P=0.7 and 14% vs 11%, P=0.78, respectively).

Table 4. Univariate and Multivariate Predictors of Re-Restenosis

	Univariate			Multivariate		
	OR	95%CI	P value	OR	95%CI	P value
SES group	3.2	1.44–7.1	<0.01	3.4	1.28–9.02	0.01
Diabetes mellitus	1.24	0.57–2.71	0.59	0.92	0.4–2.12	0.85
Previous MI	0.68	0.31–1.47	0.68	0.81	0.36–1.85	0.62
Pre-procedure						
MLD	0.52	0.19–1.45	0.21	0.48	0.08–2.91	0.42
Lesion length	0.96	0.91–1.02	0.18	0.97	0.92–1.03	0.36
% Diameter stenosis	1.01	0.99–1.04	0.34	0.99	0.95–1.04	0.72
Lesion type: Focal	1.28	0.57–2.84	0.55	0.59	0.22–1.56	0.29

Variables were P<0.05 in univariate analyses listed in Tables 1 and 2.
OR, odds ratio; CI, confidence interval. Other abbreviations see in Tables 1 and 2.

Table 5. Univariate and Multivariate Predictors of MACE

	Univariate			Multivariate		
	OR	95%CI	P value	OR	95%CI	P value
SES group	3.04	1.2–7.67	0.02	3.37	1.08–10.49	0.04
Diabetes mellitus	1.37	0.55–3.42	0.5	1.05	0.4–2.74	0.92
Previous MI	0.54	0.21–1.35	0.19	0.61	0.23–1.61	0.32
Pre-procedure						
MLD	0.83	0.26–2.59	0.74	0.82	0.1–6.79	0.86
Lesion length	0.97	0.91–1.04	0.43	0.99	0.92–1.05	0.65
% Diameter stenosis	1	0.98–1.03	0.8	0.99	0.94–1.04	0.75
Lesion type: Focal	1.22	0.48–3.12	0.68	0.61	0.2–1.88	0.39

Variables were P<0.05 in univariate analyses listed in Tables 1 and 2.
Abbreviations see in Tables 1–4.

Discussion

The main finding of the present study was that repeated SES implantation for post-SES restenosis although safe, was associated with relatively higher rates of recurrent restenosis and MACE than that for post-BMS restenosis at 8 months of follow up. We also discovered significantly greater late luminal loss in the SES group.

The implantation of SES has significantly decreased the incidence of restenosis and the need for TLR.[1–3] In the clinical environment, SES are increasingly being implanted into patients with various higher risk indications such as ISR, small vessels, chronic total occlusions coronary artery calcification, and diabetes.[3–7,12,13] These applications have led to an observed rate of DES restenosis of >5%.[3,6,7,12,13] More uses of SES in complex settings will probably increase the incidence of restenosis. Thus, SES restenosis will become an important issue even in the DES era. However, because of the relatively low incidence of SES restenosis, few studies have evaluated the effectiveness of repeated SES implantation for SES restenosis, and the clinical and angiographic outcomes of re-intervention with SES to treat restenosis of SES and BMS have not been determined. The present study addressed these issues. Several retrospective studies[6,10,14–17] have suggested higher restenosis and TLR rates after treating restenosis of various DES, including SES, than after treating de novo lesions. Furthermore, current therapies for DES ISR or DES thrombosis are associated with a high long-term rate of MACE (42.9%).[18] The present study prospectively discovered that repeated SES implantation to treat SES restenosis is associated with a higher incidence of late restenosis, ischemia-driven TLR and MACE than SES implantation to treat BMS restenosis. The rates of re-restenosis, TLR or MACE in the present study were relatively similar to the findings of several retrospective studies.[6,10,14–17] Our results support previous findings and indicate that although SES is an excellent approach to treating patients with BMS restenosis, the same cannot be said for treating those with SES restenosis.

Local features, angiographic findings of restenosis type and diabetes mellitus might play important roles in post-SES restenosis.[5–7,12] Lemos et al[16] reported that recurrent SES restenosis is particularly high among patients with hypercholesterolemia, previous angioplasty, failed brachytherapy and post-SES restenosis treated with balloon dilation. Several investigators then also suggested that repeated SES implantation to treat non-focal SES restenosis and smaller-diameter vessels is an independent predictor of re-restenosis.[10,17] However, multivariate logistic regression analysis showed that diabetes mellitus, type of restenosis on angiograms, lesion length and procedural MLD did not significantly influence the outcomes after repeated intervention in our series.

The mechanisms of the high rates of recurrent restenosis after repeated SES implantation for SES restenosis are poorly understood. Possible explanations include enhanced allergic or inflammatory responses to the polymer or the drug as well as a late catch-up phenomenon, excessive intimal hyperplastic responses that are inadequately blocked by the drug or the amount of drug delivered, stent fracture and stent underexpansion.[6,19–22] In the present study, IVUS-guided PCI was performed to exclude underexpansion at the second intervention. In contrast, although the drug-resistance (sirolimus-resistance) hypothesis seems attractive, no clinical studies have yet demonstrated a clear clinical benefit of implanting a DES that is different from the original.[6,15,17] The small

sample size of these retrospective non-randomized studies might have limited the statistical power to demonstrate differences or detect potential adverse effects. Further study is required to evaluate this issue.

The limitations of this study are as follows. First, although this is a prospective study with a high rate of angiographic follow up, the small sample size might not have been sufficient to reach a definitive conclusion. However, to recruit a large number of patients is complicated by low SES restenosis rates. Further investigations are required to confirm our observations. Second, this is a non-randomized analysis at a single center. Third, in this study, we did not compare the effect of repeated SES implantation with that of other possible therapies, including balloon angioplasty or implantation with a different DES. Recently, some investigators have reported the efficacy of repeated DES implantation for DES restenosis compared with conventional balloon angioplasty.[10,23] In the preliminary study, we also assessed the rate of re-restenosis of balloon angioplasty for SES restenosis. The re-restenosis rate of balloon angioplasty for SES restenosis tended to be higher than in that of repeated SES implantation, but the difference did not reach significance (data not shown).

In conclusion, the incidence of late restenosis and MACE is higher after repeated SES implantation to treat restenosis of SES, than of BMS. These results might affect the mid-term clinical outcomes of re-intervention with SES.

Disclosure

Conflict of interest: None declared.

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NEPHROLOGY

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Original Article

Effects of mineralocorticoid and angiotensin II receptor blockers on proteinuria and glomerular podocyte protein expression in a model of minimal change nephrotic syndrome

AKIHIRO FUKUDA,[1] SHOUICHI FUJIMOTO,[1] SHUJI IWATSUBO,[1] HIROSHI KAWACHI[2] and KAZUO KITAMURA[1]

[1]Division of Circulatory and Body Fluid Regulation, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, and [2]Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

KEY WORDS:
nephrotic syndrome, podocyte, proteinuria, rennin–angiotensin–aldos terone system, slit diaphragm.

Correspondence:
Dr Akihiro Fukuda, Division of Circulatory and Body Fluid Regulation, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Kihara 5200, Kiyotake, Miyazaki, 889-1692, Japan. Email: mrgiants@fc.miyazaki-u.ac.jp

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SUMMARY AT A GLANCE

This is an interesting paper describing the effects of mineralocorticoid and angiotensin II receptor blockers in a model of minimal change disease that shows preservation of podocytes with treatment.

ABSTRACT:

Aim: **Several proteins constituting the slit diaphragm are considered important for maintaining capillary wall permselectivity. Early intervention with blockers of angiotensin II receptors (AR) and mineralocorticoid receptors (MR) is effective against proteinuria in models of chronic hypertensive and protein-induced renal damage. However, the effects of AR and/or MR blockers in a model of acute nephrotic syndrome remain unknown. The effects of AR and MR blockers were examined in puromycin aminonucleoside (PAN)-treated rats.**

Methods: **Six week old male Sprague–Dawley (SD) rats were injected with PAN or vehicle and assigned to groups as follows: vehicle (group C); PAN (group P); PAN followed 3 days later by administration of the MR blocker, eplerenone (group MR), and by the AR blocker, losartan (group AR). Blood pressure and urinary protein excretion were measured and all rats were killed for immunohistochemical investigation on day 14 after PAN administration.**

Results: **Blood pressure did not change throughout the study period. Proteinuria was decreased in groups MR and AR compared with group P (on day 14 after PAN administration, respectively; group P *vs* AR, $P < 0.01$; group P *vs* MR, $P < 0.05$). Nephrin, podocin and podocalyxin staining was preserved in the glomeruli of groups MR and AR compared with group P.**

Conclusion: **The MR and AR blockers decreased proteinuria in the acute model of nephrotic syndrome with preserved expression of glomerular podocyte protein independently of blood pressure.**

The filtration barrier of the kidney glomerulus that prevents plasma proteins from leaking into primary urine comprises endothelial cells, a glomerular basement membrane and visceral epithelial cells (podocytes). That the slit diaphragm located between the adjacent foot processes of podocytes functions as the final barrier of the glomerular capillary wall is becoming accepted.[1] Several proteins that constitute the slit diaphragm, such as nephrin and podocin, are considered important for maintaining capillary wall permselectivity, because mutations in the genes of these proteins cause congenital nephrotic syndrome and focal segmental glomerulosclerosis (FSGS).[2]

A dysfunction of the slit diaphragm is involved in the development of proteinuria. Recent studies show that early intervention with blockers of the renin–angiotensin–aldosterone system (RAAS) is effective against proteinuria in models of chronic hypertensive and protein-induced renal damage.[3–5] This mechanism is not well understood, but angiotensin II receptors (AR) and mineralocorticoid receptors (MR) are located in podocytes, and angiotensin II or aldosterone infusion induces proteinuria.[6,7] Therefore, the inhibition of AR or MR signalling in podocytes would improve impaired capillary wall permselectivity. However, the effects of AR and/or MR blockers in models of acute nephrotic syndrome remain unknown. To clarify the role of the RAAS on proteinuria and the components of slit diaphragm in minimal change nephrotic syndrome, we examined the effects of AR and MR blockers in puromycin aminonucleoside (PAN) rats.

METHODS

Animals

Six week old male Sprague–Dawley (SD) rats (Charles River Japan, Atsugi, Japan) weighing 180–220 g were maintained in individual metabolic cages in a temperature-controlled room with free access to water and normal salt diet. All experiments proceeded according to the regulations of the Animal Research Committee of the University of Miyazaki. This investigation also conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

Experimental protocols

Puromycin aminonucleoside (Sigma Chemical, St Louis, MO, USA) was induced by a single dorsal i.v. injection at a dose of 10 mg per 100 g bodyweight in each group. Animals injected with vehicle only served as controls. Rats were assigned to the following groups: vehicle (group C, $n = 5$); PAN (group P, $n = 6$); PAN followed 3 days later by the MR blocker, eplerenone, 100 mg/kg per day (provided by Pfizer, Tokyo, Japan) (group MR, $n = 6$), and by the AR blocker, losartan, 30 mg/kg per day (provided by Merck, Whitehouse Station, NJ, USA) (group AR, $n = 6$). MR/AR blockers were given p.o. by using sonde. Blood pressure (BP), urinary protein excretion and serum markers were measured and then all rats were killed for immunohistochemical investigation on day 14 after PAN administration. Urine was collected for 24 h using metabolic cages. Systolic BP in conscious rats was measured by tail-cuff plethysmography after the animals were maintained in an incubator at 37°C for 15 min to ensure vasodilatation.

Real-time quantitative polymerase chain reaction (PCR)

The kidneys were fully perfused *in situ* with ice-cold saline and removed, and the cortex was dissected into small pieces. Glomeruli were isolated by sequentially pressing the kidney tissue through 125, 105 and 53 μm sieves. Each sieve was thoroughly washed with ice-cold saline. Tissue samples containing glomeruli were finally collected from the 53 μm sieve. Total RNA (2 μg) extracted from glomeruli (Total RNA Isolation Reagent; Invitrogen, San Diego, CA, USA) was reverse-transcribed using Superscript reverse transcriptase (Invitrogen) to yield the respective cDNA. Nephrin mRNA expression was analyzed by real-time quantitative PCR (7300 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA, USA). The PCR products were used as standards and the mRNA levels were compared after normalization relative to those of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. The forward and reverse primers for nephrin were 5′-CAACAATCCAAGCTGC CAGT-3′ and 5′-AAACGGGTGTTGTGAAGCGT-3′, and GAPDH were 5′-TCCTGCACCACCAACTGCTTAG-3′ and 5′-CACAGCCTTGGCAG CACCAGT-3′, respectively. The probe specific to nephrin was FAM-5′-CCAGACCCCTCCCTCATCTGGTTTAAGG-TAMRA-3′, and GAPDH was FAM-5′-TGACCACAGTCCATGCCATCACTGCCACTC-TAMRA-3′ in which FAM is 6 carboxyfluorescein and TAMRA (quencher) is 6-carboxy-tetramethylrhodamine.

Immunofluorescence microscopy

Immunofluorescence studies proceeded essentially as described[8] using the primary antibodies, rabbit antirat nephrin antibody,[8] rabbit antihuman podocin antibody (29040; Immuno-Biological Laboratories, Gunma, Japan), rabbit antirat podocalyxin antibody (KR064; Trans Genic, Kobe, Japan) and the secondary antibody, fluorescein isothiocyanate-conjugated swine antirabbit immunoglobulin (F234; DAKO, Tokyo, Japan).

Scoring of podocyte markers

For the evaluation of nephrin, podocin and podocalyxin, a semi-quantitative grading system was used (0, no; 1, weak; 2, intermediate; and 3, strong staining) and the average staining score was calculated.

Statistical analyses

All data are presented as mean ± standard errors of the mean. Parametric data between two groups were compared using an unpaired Student's *t*-test and among three or more groups using one-way ANOVA. The significance of individual difference was evaluated using Scheffe's *F*-test if the ANOVA findings were significant. $P < 0.05$ was considered statistically significant for all calculations.

RESULTS

Animal data

Table 1 shows serum markers in the groups C, P, MR and AR on day 14 after PAN administration. The serum albumin level in group P was significantly decreased compared with group C ($P < 0.05$), but did not differ from those in groups MR and AR. The serum total cholesterol level in group P was significantly increased compared with groups C, MR and AR ($P < 0.01$). Level of blood urea nitrogen (BUN) and potassium in group P were significantly increased compared with group C (BUN, $P < 0.05$; potassium, $P < 0.01$), but did not differ from those in groups MR and AR. Serum creatinine

Table 1 Biological parameters in groups C, P, MR and AR at 14 days after puromycin aminonucleoside administration

Group	Group C ($n = 5$)	Group P ($n = 6$)	Group MR ($n = 6$)	Group AR ($n = 6$)
Alb, g/dL	2.12 ± 0.06	1.85 ± 0.07*	1.97 ± 0.05	2.03 ± 0.03
T-cho, mg/dL	49.8 ± 3.68	151.8 ± 18.7**	81.0 ± 10.5‡	70.3 ± 7.90‡
BUN, mg/dL	17.8 ± 0.65	24.5 ± 1.96*	21.0 ± 1.03	21.9 ± 1.19
Cre, mg/dL	0.25 ± 0.01	0.31 ± 0.02	0.26 ± 0.02	0.27 ± 0.02
K, mEq/L	3.84 ± 0.10	4.93 ± 0.18**	4.30 ± 0.13	4.57 ± 0.25
Aldosterone, pg/mL	31.4 ± 7.87	36.0 ± 6.46	29.1 ± 6.94	27.0 ± 11.5

Values are means ± standard error of the mean. *$P < 0.05$ *vs* group C; **$P < 0.01$ *vs* group C; ‡$P < 0.01$ *vs* group P (tested by one-way ANOVA, followed by Scheffe's *F*-test). Alb, serum albumin; T-cho, serum total cholesterol; BUN, blood urea nitrogen; Cre, serum creatinine; K, serum potassium.

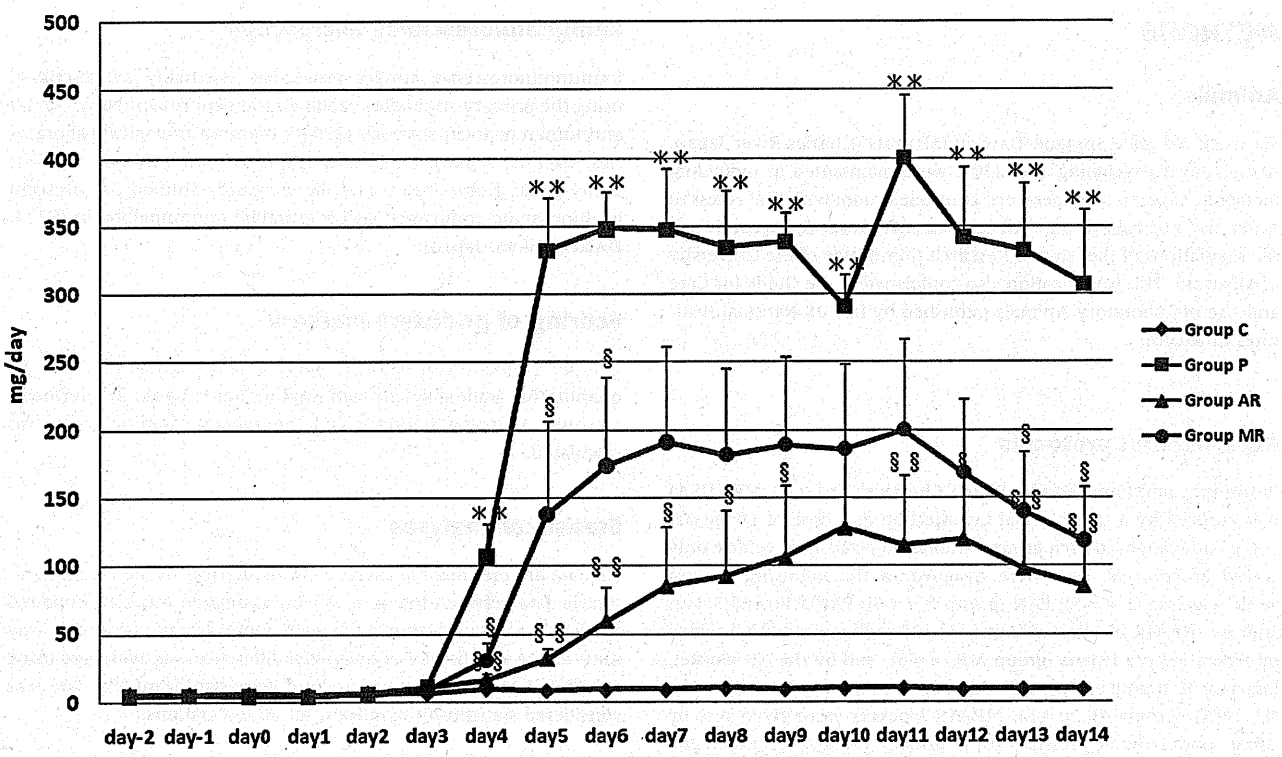

Fig. 1 Urinary protein excretion. $^{**}P < 0.01$ *vs* group C; $^{\S}P < 0.05$ *vs* group P; $^{\S\S}P < 0.01$ *vs* group P (tested by one-way ANOVA, followed by Scheffe's *F*-test).

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

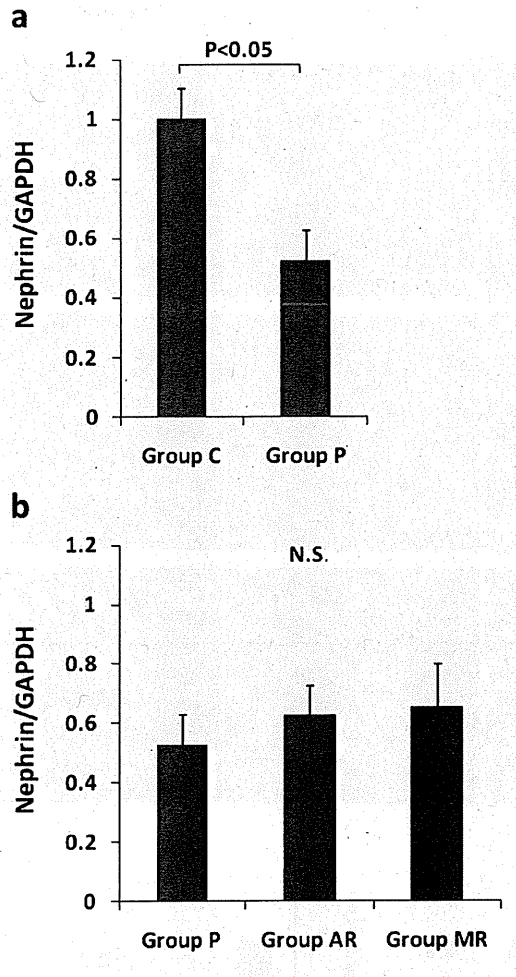

Fig. 2 Gene expression of nephrin in glomeruli on day 14 after puromycin aminonucleoside (PAN) administration. (a) Nephrin gene expression is significantly decreased in group P ($n = 6$) compared with group C ($n = 5$; $P = 0.017$, Student's unpaired *t*-test). (b) Nephrin mRNA did not statistically 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 non-diabetic 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

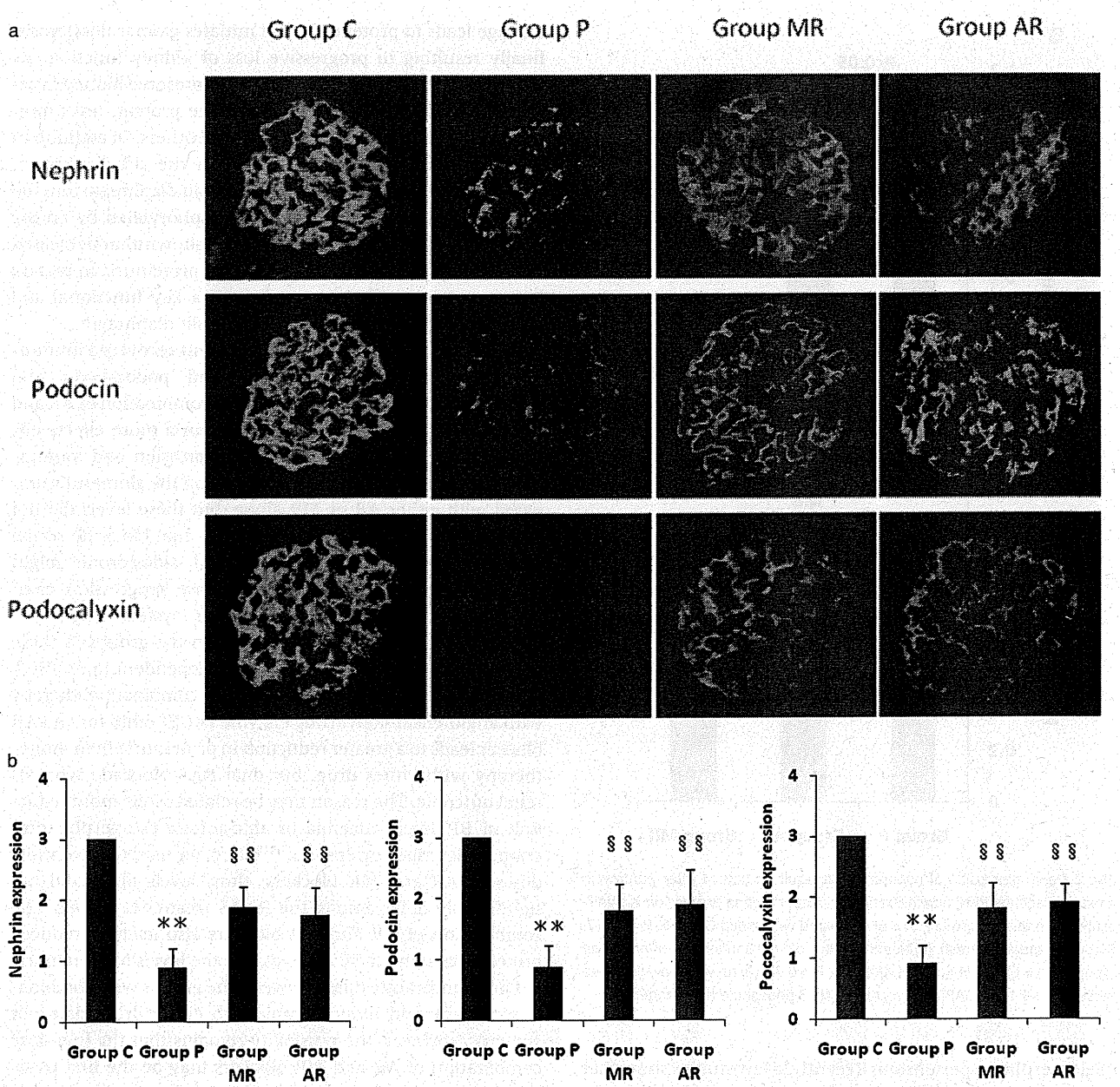

Fig. 3 Effects of eplerenone and losartan on nephrin, podocin, podocalyxin expression in puromycin aminonucleoside (PAN)-induced rat model. (a) Representative micrographs of immunostaining of nephrin, podocin and podocalyxin in glomeruli from controls, PAN-induced rats and from PAN-induced rats given 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; $^{§§}P < 0.01$ *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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Department of Internal Medicine, Circulatory and Body Fluid Regulation, *Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
Mailing address: Toshihiro Tsuruda, MD, Department of Internal Medicine, Circulatory and Body Fluid Regulation, University of Miyazaki, 5200 Kihara Kiyotake, Miyazaki 889-1692, Japan. E-mail: ttsuruda@med.miyazaki-u.ac.jp
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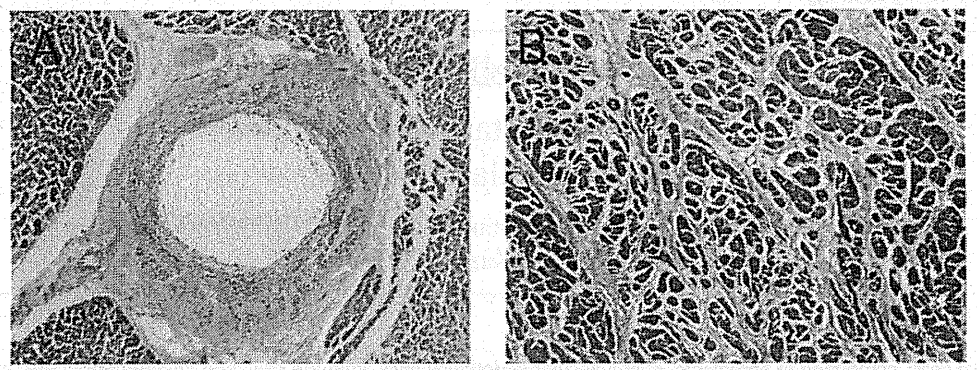

Figure 1. Autopsy of an 87-year-old male patient who suffered from uncontrolled hypertension and died suddenly. Slides were stained with Azan-Mallory. Gross fibrosis was observed in the (A) intra-myocardial coronary artery and (B) interstitial space of the hypertrophied left ventricle. Original magnification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

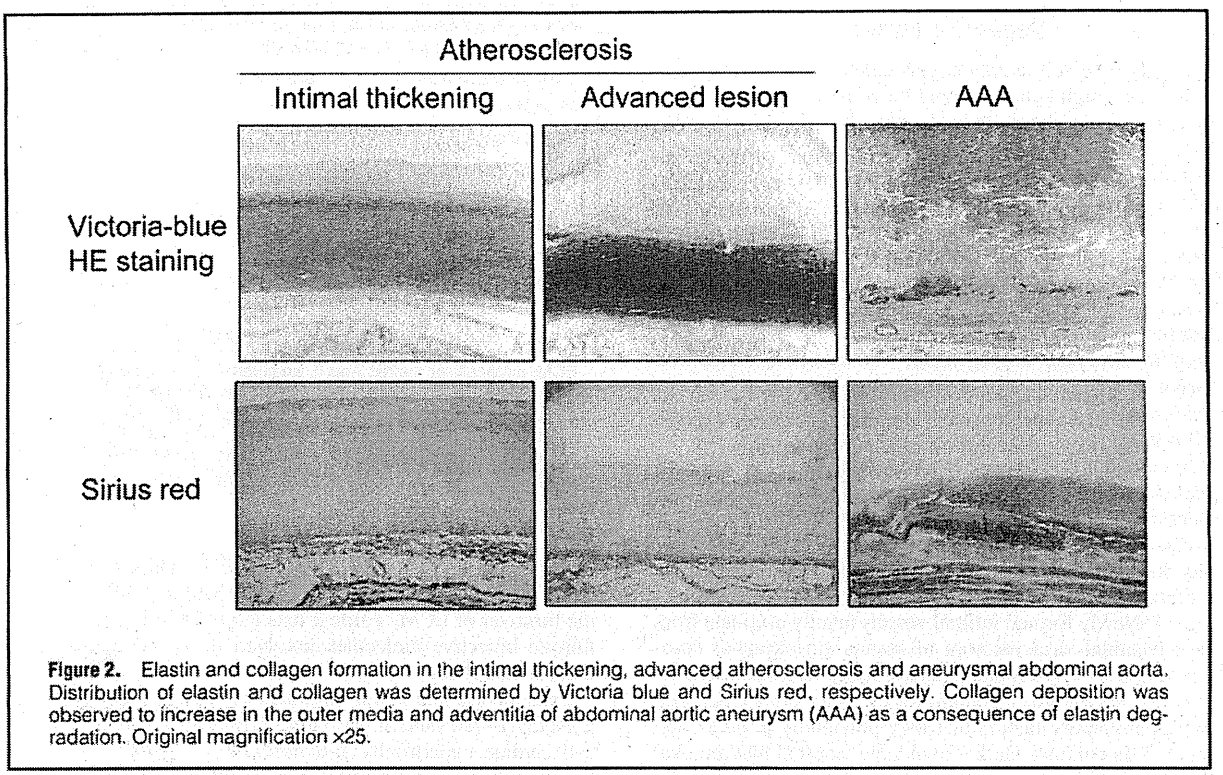

Figure 2. Elastin and collagen formation in the intimal thickening, advanced atherosclerosis and aneurysmal abdominal aorta. Distribution of elastin and collagen was determined by Victoria blue and Sirius red, respectively. Collagen deposition was observed to increase in the outer media and adventitia 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

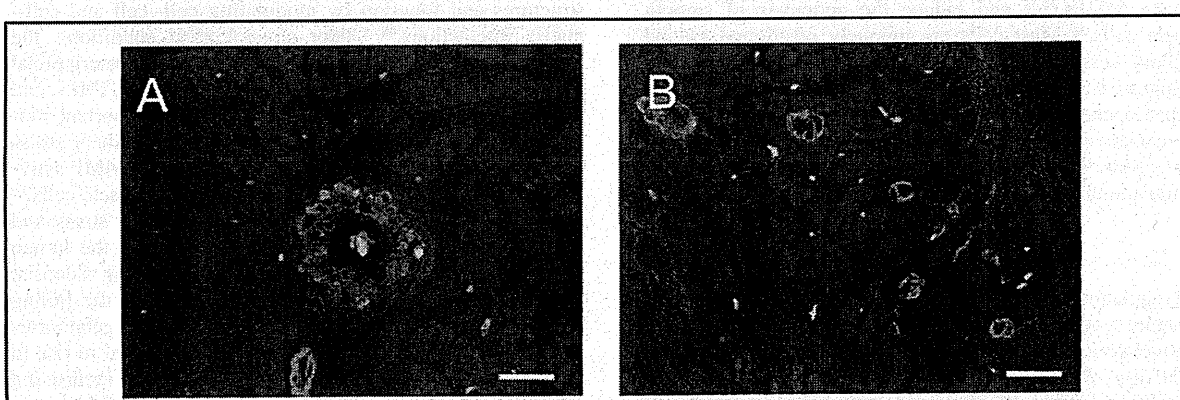

Figure 3. Distribution of immunoreactive angiotensin-converting enzyme (ACE, red) and α-smooth muscle actin (green) in the (**A**) intra-myocardial coronary artery and (**B**) interstitial space of the left ventricle with aortic constriction in adult rats. ACE immunoreactivity increased and was distributed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

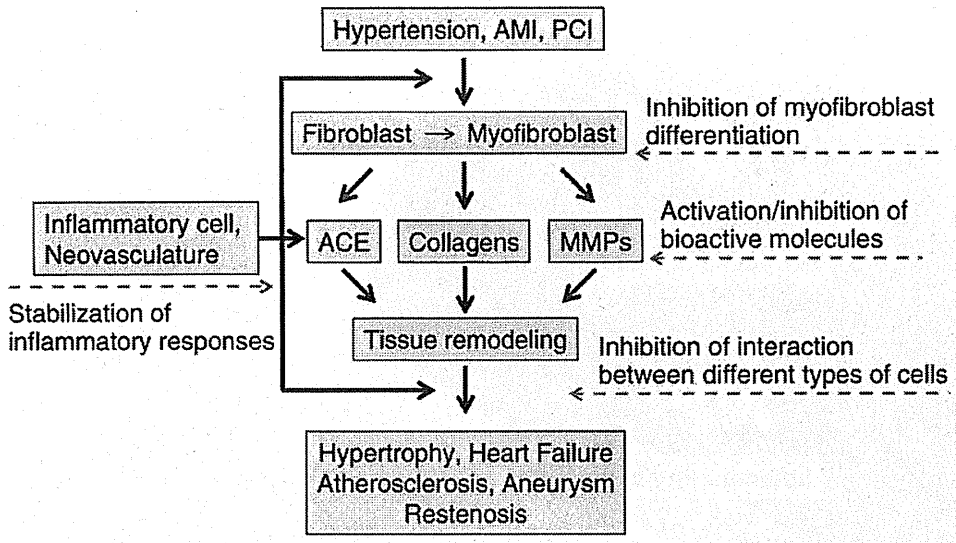

Figure 4. Contribution of stromal cells in the pathogenesis of cardiovascular diseases. Fibroblasts differentiate into myofibroblasts in hypertension, following acute myocardial infarction (AMI) or percutaneous coronary intervention (PCI). Myofibroblasts synthesize angiotensin-converting enzyme (ACE) 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

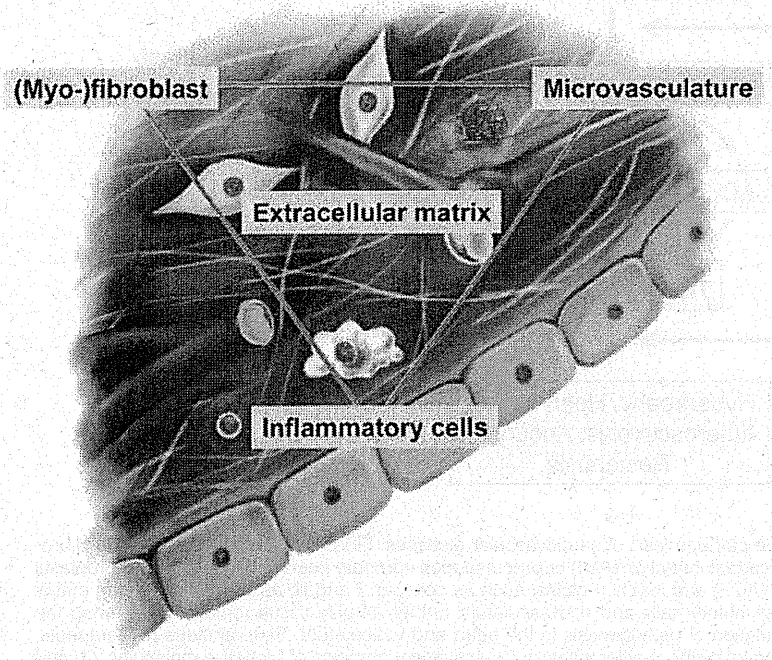

Figure 5. Stromal network in the heart and vasculature. Stromal cells are critical for maintaining myocardial and vascular geometry and function, whereas disruption of the stromal network will result in the development of cardiovascular diseases.

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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ORIGINAL ARTICLE

Disturbed blood flow induces erosive injury to smooth muscle cell-rich neointima and promotes thrombus formation in rabbit femoral arteries

T. SUMI*, A. YAMASHITA†, S. MATSUDA*, S. GOTO§, K. NISHIHIRA*, E. FURUKOJI‡, H. SUGIMURA‡, H. KAWAHARA¶, T. IMAMURA*, K. KITAMURA*, S. TAMURA‡ and Y. ASADA†

**Department of Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki; †Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki; ‡Department of Radiology, Faculty of Medicine, University of Miyazaki, Miyazaki; §Department of Medicine, Tokai University School of Medicine, Isehara, Kanagawa; and ¶Medical Research Institute, Tokai University School of Medicine, Isehara, Kanagawa, Japan*

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Summary. *Background:* Plaque erosion is a cause of atherothrombosis that preferentially occurs on smooth muscle cell (SMC)- and proteoglycan-rich rather than lipid-rich plaques. However, its underlying mechanisms remain unknown. *Objective:* To determine whether disturbed blood flow induces erosive injury and thrombus formation on SMC-rich neointima. *Methods:* Three weeks after balloon injury, SMC-rich neointima with increased tissue factor (TF) activity developed in rabbit femoral arteries that were narrowed with a vascular occluder to disturb blood flow after stenosis. Neointimal injury and thrombus formation were assessed at 15, 30, and 180 min after the vascular narrowing. *Results:* Endothelial detachment, platelet adhesion and neointimal cell apoptosis became evident at the post-stenotic regions of all femoral arteries ($n = 5$) within 15 min of narrowing. Mural thrombi composed of platelet and fibrin developed after 30 min, and then occlusive thrombi were generated in three out of five vessels after 180 min. The identical vascular narrowing of normal femoral arteries also induced endothelial detachment with small platelet thrombi at post-stenotic regions, but fibrin and occlusive thrombi did not develop. Computational simulation analysis indicated that oscillatory shear stress contributes to the development of erosive damage to the neointima. *Conclusions:* These results suggest that disturbed post-stenotic blood flow can induce erosive injury in SMC-rich plaques and promote thrombus formation that results in vascular events.

Correspondence to: Yujiro Asada, Department of Pathology, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan.
Tel.: + 81 985 85 2810, fax: + 81 985 85 7614.
E-mail: yasada@fc.miyazaki-u.ac.jp

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Introduction

Disruption of coronary atherosclerotic plaques is recognized as a trigger of coronary thrombosis that results in acute coronary syndrome. The two major morphological features of plaque disruption are rupture and erosion [1]. Precursor lesions to plaque rupture usually comprise a relatively large lipid core that is encapsulated by a thin fibrous cap and heavily infiltrated by inflammatory cells; disruption of the fibrous cap results in rupture [1,2]. Considerable evidence supports the notion that inflammation plays a crucial role in these processes. On the other hand, plaque erosion is characterized by superficial plaque injury. Eroded plaques are rich in smooth muscle cells (SMCs) and proteoglycan, rather than having a large lipid core, and the prevalence of inflammation is considerably lower than that in ruptured plaques. Plaque erosion is the cause of 20%–40% of coronary sudden deaths, and it is particularly common among patients younger than 50 years of age and smokers, with the highest frequency in women [3–6]. Although these histologic and clinical findings suggest that the underlying mechanisms of plaque erosion and rupture differ, details of the mechanisms leading to the development of plaque erosion are poorly understood.

Blood flow is a key modulator of the development of atherosclerosis and thrombosis. Atherosclerosis probably develops in areas of disturbed flow or low shear stress, whereas regions under steady laminar flow and physiologically high shear stress are resistant to atherogenesis [7]. Endothelial cells preferentially undergo apoptosis at downstream areas of atherosclerotic plaques where blood flow is disturbed and shear stress is lower than at upstream areas [8]. The transcription of thrombogenic or anti-thrombotic genes is also regulated by shear stress [9,10]. Experimental aortic stenosis can induce

acute endothelial change or damage of the normal aorta [11,12]. Therefore, hemodynamic force, particularly disturbed blood flow induced by stenosis or vasoconstriction, could be a crucial factor in generating surface vascular damage and thrombosis. Although direct clinical evidence has not yet supported the notion that coronary artery vasospasm plays a role in plaque erosion, the morphological characteristics of plaques prone to vasospasm are similar to those of eroded plaques [3,4,6], and platelet and blood coagulation in coronary circulation are activated after vasospastic angina [13,14]. These findings suggest that an acute-onset disturbance of blood flow due to vasoconstriction could trigger plaque erosion. We therefore investigated whether post-stenotic disturbed blood flow could induce erosive injuries and thrombus formation in rabbit SMC-rich neointima.

Material and methods

Generation of SMC-rich neointima and stenosis in rabbit femoral arteries

The Animal Care Committee of University of Miyazaki approved the research protocols, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Aseptic surgery proceeded under general anesthesia induced by an intravenous injection of pentobarbital (25 mg kg^{-1}, body weight).

To develop SMC-rich neointima, the left femoral arteries of male Japanese white rabbits (Kyudo Corp, Kumamoto, Japan) weighing 2.5–3.0 kg were damaged by inserting a 2.5 (diameter) × 9 (length) mm angioplasty balloon catheter (Boston Scientific, Galway, Ireland) into the femoral artery via the carotid artery. Briefly, the right common carotid artery was cannulated, and then an angioplasty wire followed by an angioplasty balloon was fluoroscopically guided into the femoral artery. The catheter was then inflated at 1.5 atm and then pulled backwards by 5 cm, three times [15]. Blood flow (mL min^{-1}) in the femoral artery was continuously measured 3 weeks later using a Doppler probe placed over the femoral artery and a T106 transit time blood flow meter (Transonic Systems Inc., Ithaca, NY, USA). Data were analyzed using a PowerLab digital recording system (ADInstruments Pty. Ltd., Castle Hill, Australia) at a sampling rate of 1000 per s. The bilateral femoral arteries were constricted using a vascular occluder (Fine Science Tools Inc., North Vancouver, BC, Canada) to reduce the flow volume to 75%. The blood flow velocity and color Doppler mosaic area before and after the vascular narrowing were analyzed by ultrasonography (Logic7 with an M12L linear scan probe; GE Healthcare UK Ltd., Buckinghamshire, UK). The rabbits were intravenously injected with heparin (500 U kg^{-1}) 15, 30, and 180 min thereafter, and then euthanized with an overdose of pentobarbital (60 mg kg^{-1}, intravenous) to evaluate erosive damage to intima/neointima and thrombus formation.

The animals were perfused with 50 mL of phosphate-buffered saline (PBS) (0.01 mol L^{-1}) and then perfusion fixed with 100 mL of 4% paraformaldehyde for immunohistochemical evaluation or with 4% neutralized formaldehyde and 1% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer for scanning electron microscopy. The femoral arteries were dissected out for subsequent studies.

Tissue factor activities in undamaged and damaged femoral arteries

To evaluate tissue factor (TF) activities in the vascular walls, rabbit plasma clotting time initiated by vessel homogenate was measured using a Thrombotrack coagulation timer (AXIS-SHIELD; PoC AS, Oslo, Norway)[16]. The intima or neointima with media of the bilateral femoral arteries from 3 weeks after balloon injury without perfusion fixation were carefully separated from the adventitia. The intima/media was homogenized in Tris-buffered saline (pH 7.4) containing 5 mmol L^{-1} $CaCl_2$ and 0.1% Triton X-100 (Nakalai Tesque Inc., Kyoto, Japan) using a Polytron PT3000 (Kinematica, Littau, Switzerland). After centrifugation at 2500 × *g* for 10 min, the supernatant (vessel sample; 100 μL, 100 μg protein) was incubated for 1 min with rabbit plasma (100 μL), with or without 250 ng mL^{-1} of recombinant human TF pathway inhibitor (rTFPI; The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), and then clotting assays were started by adding 0.02 mol L^{-1} $CaCl_2$ (100 μL). Protein concentrations were determined using bicinchoninic acid (BCA) protein assay kits (Pierce, Rockford, IL, USA).

Light microscopy and immunohistochemistry

The femoral arteries were fixed in 4% paraformaldehyde for 24 h at 4 °C and longitudinally embedded in paraffin. Sections (3 μm thick) were stained with hematoxylin and eosin, and immunohistochemically examined using antibodies against the endothelial marker VWF (The Binding Site, Birmingham, UK), muscle actin (HHF35; Dako A/S, Glostrup, Denmark), rabbit macrophages (RAM11; Dako), glycoprotein (GP) IIb-IIIa (Affinity Biologicals Inc., Hamilton, CA, USA), rabbit fibrin (Takeda Chemical Industries Ltd., Osaka, Japan) and TF (The Chemo-Sero-Therapeutic Research Institute). The sections were stained with Envision (Dako) or donkey anti-sheep or guinea pig IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Horseradish peroxidase activity was visualized using 3, 3'-diaminobenzidine tetrahydrochloride, and the sections were faintly counterstained with Meyer's hematoxylin. Immunostaining controls included non-immune mouse IgG or non-immune sheep or guinea pig serum instead of the primary antibodies. Loss of endothelial covering (length of erosive injury) was assessed in VWF-stained sections, and measured using an image analyzing system (Axio Version 4.6; Carl Zeiss, Munchen, Germany). Areas of positive immunostaining for GPIIb-IIIa and fibrin were analyzed using color imaging morphometry (Win Roof; Mitani, Fukui, Japan) [16]. Thrombi were semi-quantified at the post-stenotic region of each artery under a ×20 objective lens. Two investigators