Blood sampling for these humoral factors and echocardiographic study were performed just before hemodialysis, because of the necessity to evaluate the relationship between humoral factors and cardiac function in concurrence with excessive blood volume in the present study.

Statistical analysis

Data are expressed as the mean ± SD. Statistical comparisons were made with the Student unpaired *t* test or chi-square analysis between the 2 groups. The significance of differences between more than 2 groups was evaluated by unpaired analysis of variance with a subsequent Fisher multiple comparison tests. Univariate and stepwise multivariate regression analyses were used to detect independent related factors of 2-dimensional, Doppler echocardiographic parameters, plasma CRP level, or removal fluid volume among mAM, log [ANP], log [BNP], and log [NE]. Event-free curves were estimated by the Kaplan-Meier product-limited method and compared with the Mantel (log-rank) test. The prognostic value of each humoral factor was tested by Cox multivariate proportional-hazards regression analysis. The median value of humoral factors was chosen as the cutoff value (mAM 4.55 pmol/L, ANP 230 pg/mL, BNP 442 pg/mL, NE 473 pg/mL). Differences were considered to be statistically significant when the *P* value was < 0.05.

RESULTS

Clinical characteristics of the study population and echocardiographic findings just before hemodialysis are listed in Table 1 and Table 2, respectively. Causes of renal failure included chronic glomerulonephritis ($N = 25$), diabetic nephropathy ($N = 19$), hypertensive nephrosclerosis ($N = 9$), polycystic kidney disease ($N = 4$), renal stone ($N = 2$), renal tuberculosis ($N = 1$), or unknown ($N = 7$). All patients had normal cardiac sinus rhythm, whereas all patients had cardiovascular diseases, which consisted of coronary artery disease ($N = 40$), peripheral arterial occlusive disease ($N = 22$), hypertensive heart disease ($N = 9$), cerebrovascular attack ($N = 7$), aortic aneurysm ($N = 6$), aortic valve stenosis ($N = 4$), ventricular tachycardia ($N = 3$), paroxysmal supraventricular tachycardia ($N = 1$), primary pulmonary hypertension ($N = 1$), and cardiac tumor ($N = 1$). Delta body weight (= excessive body fluid) was also presented in Table 1.

There was no significant difference in plasma mAM level between the different causes of renal failure (chronic glomerulonephritis 4.77 ± 1.80; diabetic nephropathy 4.72 ± 2.10; nephrosclerosis 4.62 ± 1.89; polycystic kidney disease 5.33 ± 1.72; others or unknown 4.92 ± 1.31 pmol/L, $P = 0.9735$). There was no significant difference in the distribution of causes of renal failure between the classifications by E/A ratio, S/D ratio, and the existence of coronary artery disease (E/A ratio $P = 0.5451$; S/D ratio $P = 0.7806$; coronary artery disease $P = 0.1628$).

Distribution of crude values of mAM, ANP, BNP, NE, and CRP in patients with cardiovascular disease was shown in Figure 1. Because plasma levels of BNP, ANP, and NE had much greater deviations than mAM or CRP, the clinical significance of plasma levels of BNP, ANP, and NE was evaluated using logarithmic values.

Table 1. Clinical characteristics of the study population

Age *years*	64.7 ± 10.4
Sex *Male/female*	48/19
Duration of hemodialysis *years*	6.6 ± 8.0
Cause of renal failure *number of patients*	
Chronic glomerulonephritis	25
Diabetic nephropathy	19
Nephrosclerosis	9
Polycystic kidney disease	4
Renal stone	2
Renal tuberculosis	1
Unknown	7
Cardiovascular disease *number of events*	
Coronary artery disease	40
Peripheral arterial occlusive disease	22
Hypertensive heart disease	9
Cerebrovascular attack	7
Aortic aneurysm	6
Aortic valve stenosis	4
Ventricular tachycardia	3
Paroxysmal supraventricular tachycardia	1
Primary pulmonary hypertension	1
Cardiac tumor	1
Heart rate *bpm*	71 ± 13
Systolic blood pressure *mm Hg*	145 ± 22
Diastolic blood pressure *mm Hg*	70 ± 11
Plasma mature adrenomedullin level *pmol/L*	4.8 ± 1.8
Delta body weight *kg*	1.92 ± 1.08
Plasma C-reactive protein level *mg/dL*	1.15 ± 1.84

Data are expressed as number or mean ± SD.

Table 2. Echocardiographic findings just before hemodialysis

A *cm/sec*	103.4 ± 25.5
E *cm/sec*	90.6 ± 25.6
DcT *msec*	254.6 ± 65.9
Ad *msec*	145.5 ± 21.0
S *cm/sec*	63.6 ± 16.7
D *cm/sec*	47.6 ± 20.6
PVa *cm/sec*	26.0 ± 6.2
PVad *msec*	122.0 ± 18.5
E/A ratio	0.88 ± 0.23
S/D ratio	1.51 ± 0.46
Ad-PVad *msec*	23.1 ± 27.4
LVEDVI *mL/m²*	58.0 ± 21.4
LVESVI *mL/m²*	25.7 ± 18.1
LVEF%	59.2 ± 13.8

Abbreviations are: A, peak velocity of atrial filling; E, peak velocity of early diastolic filling; DcT, deceleration time of early diastolic filling; Ad, duration of atrial filling; S, peak forward-flow velocity during ventricular systole; D, peak forward-flow velocity during ventricular diastole; PVa, peak reverse-flow velocity at atrial contraction; PVad, duration of the PVa wave; LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; LVEF, left ventricular ejection fraction. Data are expressed as mean ± SD.

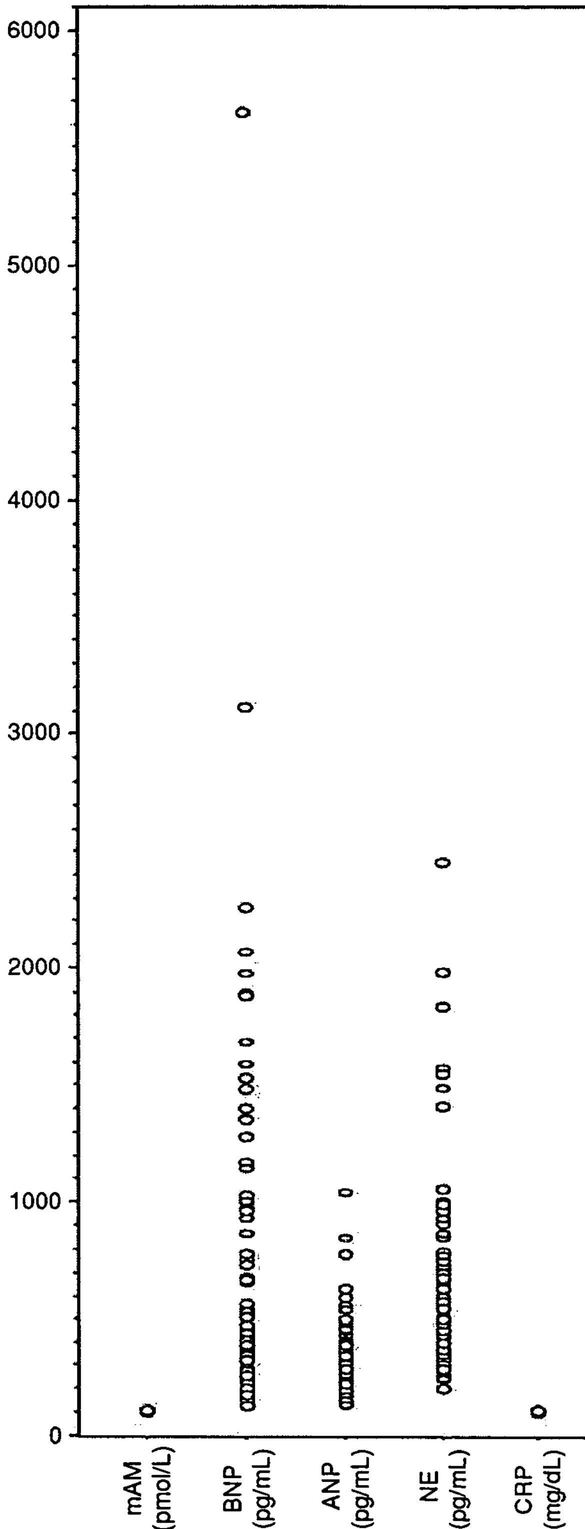

Fig. 1. Distribution of crude values of mAM, BNP, ANP, NE, and CRP in patients with cardiovascular disease.

Table 3. Univariate correlation between neurohumoral factors and echocardiographic findings in patients with hemodialysis

	Age		mAM		Log [BNP]	
	R	*P*	*R*	*P*	*R*	*P*
SBP *mm Hg*	0.153	0.2157	−0.366	0.0024	−0.020	0.8742
DBP *mm Hg*	−0.155	0.2241	−0.261	0.0386	−0.183	0.1507
A *cm/sec*	0.234	0.1092	−0.212	0.1479	0.150	0.3086
E *cm/sec*	−0.266	0.0593	0.270	0.0554	0.304	0.0303
DcT *msec*	0.350	0.0128	−0.412	0.0030	0.064	0.6605
Ad *msec*	0.463	0.0009	−0.253	0.082	0.024	0.8710
S *cm/sec*	0.069	0.6463	−0.208	0.1602	−0.510	0.0002
D *cm/sec*	−0.231	0.1104	0.345	0.0152	0.120	0.4116
PVa *cm/sec*	−0.030	0.8452	−0.126	0.4091	−0.466	0.0012
PVad *msec*	0.001	0.9934	−0.149	0.3275	−0.278	0.0646
E/A ratio	−0.560	<0.0001	0.288	0.0470	0.099	0.5023
S/D ratio	0.275	0.0613	−0.465	0.0010	−0.295	0.0443
Ad-PVad *msec*	0.377	0.0106	−0.099	0.5191	0.287	0.0564
LVEDVI *mL/m²*	−0.048	0.7512	0.375	0.0095	0.227	0.1241
LVESVI *mL/m²*	−0.061	0.6817	0.429	0.0026	0.305	0.0369
LVEF %	0.085	0.5691	−0.392	0.0065	−0.335	0.0215

	Log [ANP]		Log [NE]	
	R	*P*	*R*	*P*
SBP *mm Hg*	0.175	0.1569	−0.173	0.1619
DBP *mm Hg*	0.008	0.9482	−0.143	0.2644
A *cm/sec*	0.102	0.4890	0.243	0.0967
E *cm/sec*	0.219	0.1233	0.213	0.1326
DcT *msec*	0.004	0.9773	−0.028	0.8443
Ad *msec*	−0.037	0.8045	0.054	0.7161
S *cm/sec*	−0.338	0.0200	−0.011	0.9426
D *cm/sec*	0.214	0.1403	0.228	0.1150
PVa *cm/sec*	−0.555	<0.0001	0.111	0.4696
PVad *msec*	−0.0081	0.5956	−0.189	0.2147
E/A ratio	0.010	0.9460	−0.119	0.4218
S/D ratio	−0.288	0.0499	−0.131	0.3809
Ad-PVad *msec*	0.103	0.5013	0.182	0.2325
LVEDVI *mL/m²*	0.206	0.1653	0.137	0.3578
LVESVI *mL/m²*	0.239	0.1051	0.279	0.0579
LVEF %	−0.223	0.1321	−0.362	0.0124

Abbreviations are: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAM, mature adrenomedullin; BNP, B-type natriuretic peptide; ANP, atrial natriuretic peptide; NE, nor epinephrine. For others, see Table 2.

In univariate linear regression analysis, age correlated positively with DcT, Ad, and Ad-PVad, and negatively with E/A ratio, whereas mAM correlated negatively with systolic and diastolic blood pressure, DcT, S/D ratio, and LVEF, and positively with D, E/A ratio, LVEDVI, and LVESVI (Table 3). Log [BNP] correlated negatively with S, PVa, S/D ratio, and LVEF, and positively with E and LVESVI (Table 3). Log [ANP] also correlated negatively with the S, PVa, and S/D ratio, but not left ventricular volume indexes or LVEF (Table 3). Log [NE] correlated negatively with only LVEF (Table 3).

We further examined the independent relationship between echocardiographic findings and humoral factors by using stepwise multiple regression analysis. Independent variables were selected as related factors from the results of univariate linear regression analysis. Because mAM was the only related factor for LVEDVI from the results of the univariate linear regression analysis, a stepwise multiple regression analysis was not performed for

Table 4. Stepwise regression analyses of the DcT, E/A ratio, S/D ratio, LVESVI, and LVEF

Independent variables	Beta-coefficient	*P*
DcT		
Age	0.274	0.0409
mAM	−0.353	0.0094
E/A ratio		
Age	−0.560	<0.0001
mAM		NS
S/D ratio		
mAM	−0.465	0.0010
Log [BNP]		NS
Log [ANP]		NS
LVESVI		
mAM	0.429	0.0026
Log [BNP]		NS
LVEF		
mAM	−0.347	0.0118
Log [BNP]		NS
Log [NE]	−0.312	0.0224

For abbreviations, see Tables 2 and 3. NS, not significant.

Table 5. Univariate linear regression and stepwise multivariate regression analyses between neurohumoral factors and C-reactive protein levels and removal fluid volume in patients with hemodialysis

	CRP			
	Univariate		Multivariate	
	r	*P*	Beta-coefficient	*P*
mAM	0.328	0.0067	0.328	0.0067
Log [BNP]	0.224	0.0685		NS
Log [ANP]	0.189	0.1253		NS
Log [NE]	0.225	0.0671		NS
	Removal fluid volume			
	Univariate		Multivariate	
	r	*P*	Beta-coefficient	*P*
mAM	0.329	0.0066	0.329	0.0066
Log [BNP]	0.251	0.0403		NS
Log [ANP]	0.263	0.0312		NS
Log [NE]	0.016	0.8998		NS

Abbreviations are: CRP, C-reactive protein. For others, see Table 3.

LVEDVI. As listed in Table 4, mAM was independently associated with the DcT, S/D ratio, LVESVI, and LVEF, while NE was independently associated with LVEF.

Furthermore, in univariate and stepwise multivariate regression analyses, plasma mAM level correlated independently with plasma CRP level before hemodialysis or removal fluid volume during hemodialysis better than cardiac natriuretic peptides and NE (Table 5).

To evaluate the possible effects of LV diastolic dysfunction on the plasma mAM level, we compared the plasma mAM level between the patients with E/A >1 and E/A ≦1, and between the patients with S/D <1, 1≦S/D<2, and S/D≧2. In patients with E/A >1, the plasma mAM level was significantly higher than those with E/A ≦1. Furthermore, in patients with S/D <1, the plasma mAM level was significantly higher than the other 2 groups (Fig. 2).

To clarify the influence of coronary artery disease (CAD) on the plasma levels of humoral factors, we subdivided the patients according to the presence or absence of concomitant CAD and compared the plasma levels of humoral factors and echocardiographic findings between the 2 groups. The plasma levels of mAM and NE were comparable between the 2 groups, whereas the ANP and BNP levels were significantly higher in hemodialysis patients with CAD than those without (Fig. 3). There was no significant difference in S/D ratio, LVEDVI, and LVESVI between the 2 groups (Fig. 4). We also compared plasma levels of ANP and BNP between patients with CAD before coronary intervention ($N = 14$) and after coronary intervention ($N = 12$) to clarify influences of intervention therapy. There were no significant differences in plasma ANP or BNP levels between the 2 groups (ANP 304 ± 177 vs. 278 ± 162 pg/mL; BNP 803 ± 656 vs. 743 ± 540 pg/mL, respectively).

During the 1-year follow-up period, 7 patients died and 8 had additional cardiovascular events requiring hospitalization (unstable angina pectoris $N = 3$, peripheral arterial occlusive disease $N = 3$, stroke $N = 2$). Event-free Kaplan-Meier curves according to the mAM cutoff value are shown in Figure 5. The median value of mAM (4.55 pmol/L) was used as a cutoff value in the present study. Twelve of 33 patients with a mAM level of 4.55 pmol/L or greater, and 3 of 34 subjects with a level less than 4.55 pmol/L experienced an episode of death and additional cardiovascular events that led up to hospitalization during the follow-up periods. Patients with greater mAM levels had a markedly greater rate of episodes than those with lower mAM levels (log-rank test, $P = 0.0056$). By multivariate Cox proportional hazards regression analysis, mAM was shown to be related to the occurrence of the episodes during the follow-up periods, whereas ANP, BNP, and NE were not (Table 6).

There was no difference in the frequency of usage concerning Ca-antagonist, angiotensin-converting enzyme inhibitor, or angiotensin II receptor blocker between the 2 groups classified by the level of plasma mAM, BNP, ANP, or NE (Table 7). However, the usage of beta-blockers was more frequent in patients with higher plasma BNP or ANP levels than the patients with lower levels of these natriuretic peptides (Table 7).

DISCUSSION

In the present study, we showed that: (*1*) plasma mAM level was negatively related to blood pressure, DcT, S/D, and LVEF, and positively to E/A, LVEDVI, and LVESVI in hemodialysis patients with cardiovascular disease; (*2*) plasma mAM reflects LV diastolic and systolic dysfunction and LV enlargement, which were defined by decreases in DcT, S/D, and LVEF and increases in E/A, LVEDVI, and LVESVI, better than natriuretic peptides and NE; (*3*) plasma mAM reflects systemic inflammatory status and/or removal fluid volume by ultrafiltration

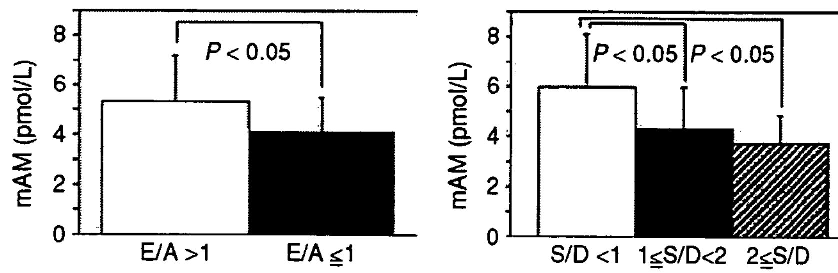

Fig. 2. Comparison of mAM levels between the E/A >1 group ($N = 11$) and E/A≦1 group ($N = 37$), or between the S/D<1 group ($N = 6$), 1≦S/D<2 group ($N = 33$), and S/D≧2 group ($N = 8$).

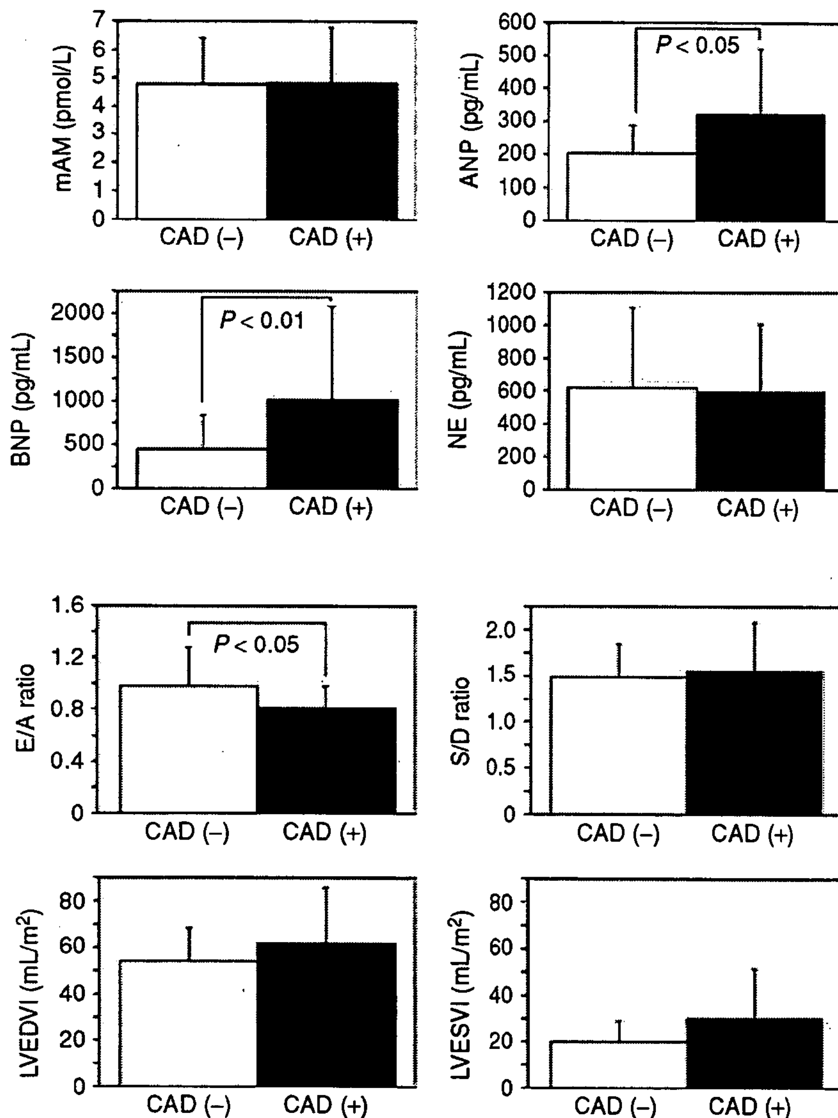

Fig. 3. Comparison of plasma levels of the mAM, ANP, BNP, and NE between hemodialysis patients with CAD and those without.

Fig. 4. Comparison of echocardiographic findings between hemodialysis patients with CAD and those without.

during hemodialysis better than natriuretic peptides and NE; (*4*) patients with high mAM had higher mortality and additional cardiovascular morbidity than those with low mAM; (*5*) plasma ANP and BNP levels were significantly increased in patients with CAD compared to patients without CAD, whereas there were no significant differences in plasma mAM levels between the 2 groups.

Plasma levels of ANP and BNP have recently been reported to reflect LV mass and LV systolic dysfunction and predict mortality in hemodialysis patients without clinical evidence of heart failure [25–27]. Plasma NE levels have also been reported to be associated to concentric LV hypertrophy and predict mortality and cardiovascular event morbidity in end-stage renal disease without heart failure

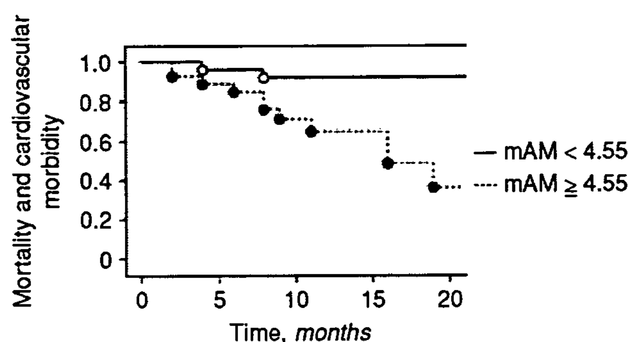

Fig. 5. Event-free curves obtained using the Kaplan-Meier method in hemodialysis patients classified according to plasma mAM level [<4.55 pmol/L ($N = 34$; 3 events) or ≥4.55 pmol/L ($N = 33$; 12 events)].

Table 6. Multivariate Cox proportional hazard models for mortality and additional cardiovascular morbidity based on humoral factors

Parameter	Hazard Ratio	95% CI	*P*
mAM ≥4.55 pmol/L	4.55	1.23–16.80	0.0230
BNP ≥442 pg/mL	1.18	0.30–4.60	0.8106
ANP ≥230 pg/mL	1.41	0.36–5.56	0.6218
NE ≥473 pg/mL	1.10	0.36–3.35	0.8655

For abbreviations, see Table 3.

Table 7. The evaluation of influences of pharmacotherapies on plasma humoral factors

	Ca-antagonist	ACEI	ARB	Beta-blocker
Number%	41	11	18	36
mAM ≥4.55 pmol/L	17 (41)	5 (45)	7 (39)	16 (44)
mAM <4.55 pmol/L	24 (59)	6 (55)	11 (61)	20 (56)
BNP ≥442 pg/mL	21 (51)	5 (45)	7 (39)	23 (64)[a]
BNP <442 pg/mL	20 (49)	6 (55)	11 (61)	13 (36)
ANP ≥230 pg/mL	23 (56)	6 (55)	7 (39)	25 (69)[a]
ANP <230 pg/mL	18 (44)	5 (45)	11 (61)	11 (31)
NE ≥473 pg/mL	20 (49)	5 (45)	8 (44)	20 (56)
NE <473 pg/mL	21 (51)	6 (55)	10 (56)	16 (44)

Abbreviations are: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker. For others, see Table 3.
[a]$P < 0.05$ vs. another group.

[28–30]. However, there are few reports of a similar evaluation in hemodialysis patients with apparent concomitant cardiovascular disease. In hemodialysis patients with CAD, plasma levels of ANP and BNP have been reported to correlate with pulmonary artery pressure, pulmonary artery wedge pressure, LV end-diastolic pressure, LVEF, LVEDVI, and LVESVI [34]. The plasma AM level has been reported to be increased in patients with heart failure in association with the severity of cardiac dysfunction [8–11], suggesting that AM might also be a useful biochemical marker for the severity of heart failure. However, there are few reports concerning the significance of the plasma AM level in hemodialysis patients with cardiovascular disease. Thus, we evaluated the relationship between plasma levels of these humoral factors and cardiac conditions in hemodialysis patients with cardiovascular disease. Interestingly, the plasma mAM level significantly correlated with cardiac dysfunction and LV size better than the other humoral factors, and mAM could be a new predictive factor of mortality and additional cardiovascular morbidity in those patients. Osajima et al [22] reported that mAM level is less useful than natriuretic peptide levels as a marker of cardiac function in hemodialysis patients with coronary artery disease. Although the exact reason for the existence of discrepancy between the previous report and our present results is unclear, one possibility might be the discrepancy of the distribution of complicated cardiovascular disease. The previous report evaluated plasma peptide levels and intracardiac pressures in hemodialysis patients with coronary artery disease. In contrast, our patients had 54 events of cardiovascular disease apart from 40 coronary artery disease, as shown in Table 1. Another possibility might be the difference of the methods of data analysis. The previous report evaluated the clinical significance of plasma natriuretic peptide levels using crude values of these levels. In contrast, in the present study, the significance of natriuretic peptide was evaluated using logarithmic values.

Numerous previous studies demonstrated that the plasma AM levels were significantly higher in patients with end-stage renal disease than those in controls [15–21]. AM has been reported to be involved in the regulation of systemic blood pressure in hemodialysis patients because the plasma AM level negatively correlated with systemic blood pressure before hemodialysis [16, 17, 19], as in the present results. Furthermore, there are reports that mAM is a marker to evaluate circulating blood volume in hemodialysis patients without cardiovascular disease [15, 21]. Although excessive blood volume causes increase of blood pressure and increased AM level in hemodialysis patients reflects excessive systemic blood volume, plasma AM level was negatively correlated with systolic and diastolic pressure in the present study. Taken together with the fact that AM has potent vasodilatory action, our results supported the previous reports that AM might be involved in the suppressive regulation of blood pressure in hemodialysis patients. In the present study, plasma mAM level was a useful biochemical marker for cardiac dysfunction and LV size better than BNP in hemodialysis patients with cardiovascular disease. BNP is considered to be a biochemical marker for evaluating the severity of LV dysfunction and remodeling [35, 36], and the effect of specific therapy against underlying heart disease [37]. In hemodialysis patients, BNP has also been reported to be a marker for evaluating cardiac dysfunction and predicting overall and cardiovascular mortality [25, 26]. However, concomitant CAD with end-stage renal disease causes an increase in plasma BNP levels [19]. The increases in BNP in patients with CAD also existed in a group with normal LV function and

were comparable between patients who had CAD with proximal lesions and those with distal lesions [38]. Furthermore, in the present study, there were no significant differences in plasma BNP levels between the hemodialysis patients who had undergone a procedure to improve the blood supply to the heart and those who had not. Thus, these results suggested the possibility that the hemodialysis patients with CAD might have higher plasma BNP levels than those without CAD, despite having similar LV function. In fact, the plasma BNP levels were significantly higher in patients with CAD compared with the patients without CAD, whereas there were no significant differences in plasma mAM level, S/D, LVEDVI, or LVESVI between the 2 groups in the present study. One possible reason for the close relationship between mAM and LV dysfunction and LV size may be that ischemic heart disease does not affect the plasma mAM level in hemodialysis patients.

Plasma mAM level was correlated negatively with DcT and positively with E/A ratio in the present study. An increase in E/A ratio with a decrease in DcT had been reported to show a restrictive filling (or psuedonormalized) pattern [31, 32]. Our results suggested that the increased mAM level in hemodialysis patients with cardiovascular disease may reflect the progression of LV diastolic dysfunction. Because volume overload leads to LV dysfunction through an increase in blood pressure, LV hypertrophy, and LV dilatation, sustained volume overload worsens prognosis in hemodialysis patients [3]. Increasing level of CRP is associated with increased risk of death in patients undergoing long-term hemodialysis [4, 5]. Thus, the present results that plasma mAM level reflects not only cardiac dysfunction but also systemic blood volume and inflammatory status better than cardiac natriuretic peptides might be the possible mechanism how plasma mAM level could become a more reliable predictor of cardiovascular morbidity and mortality than cardiac natriuretic peptides.

Which organ or cell is the source of increased plasma AM in hemodialysis patients? Previous reports demonstrated that shear stress induces up-regulation of AM mRNA expression in cultured human umbilical vein endothelial cells [39], and volume overload induced by aortocaval shunt in experimental rats increases biventricular AM mRNA expression and AM peptide level [40]. In addition, tumor necrosis factor alpha, which is one of the most common inflammation-related cytokines, had been reported to increase basal secretion of AM in cultured monocyte/macrophage cell line [41]. Taken together with these reports, our results that plasma AM level reflects excessive fluid volume, LV dysfunction, and systemic inflammatory status in hemodialysis patients with cardiovascular disease suggest that the source of increased plasma AM level might be cardiac ventricle, vascular endothelial cells, and circulatory monocytes/macrophages.

Study limitations

We performed echocardiographic studies to evaluate the condition of LV diastolic and systolic function and LV size noninvasively, instead of left-sided and right-sided catheterizations, which are more precise methods for these evaluations. Concomitant cardiovascular disease was disproportionately distributed in the present study. CAD was more frequent than the other cardiovascular diseases. Although there was no significant difference in plasma mAM level between the different causes of renal failure, including diabetic nephropathy, we could not deny an influence on our results exerted by the different causes of renal failure. Furthermore, although high frequency of beta-blocker usage in patients with higher plasma natriuretic peptide levels might be due to specific pharmacotherapy against heart failure, we could not deny an effect of the drug on the plasma natriuretic peptide levels. Therefore, additional investigations should be conducted, taking these factors into consideration.

CONCLUSION

The present results suggest that plasma mAM levels may reflect cardiac dysfunction, excessive blood volume, and systemic inflammation better than ANP, BNP, and NE, resulting in a possible predictor of mortality and additional cardiovascular morbidity in hemodialysis patients with concomitant cardiovascular disease. Further studies are necessary to clarify the pathophysiologic role of mAM in hemodialysis patients complicated with various cardiovascular diseases.

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Adrenomedullin Enhances Angiogenic Potency of Bone Marrow Transplantation in a Rat Model of Hindlimb Ischemia

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Background—Previous studies have shown that adrenomedullin (AM) inhibits vascular endothelial cell apoptosis and induces angiogenesis. We investigated whether AM enhances bone marrow cell–induced angiogenesis.

Methods and Results—Immediately after hindlimb ischemia was created, rats were randomized to receive AM infusion plus bone marrow–derived mononuclear cell (MNC) transplantation (AM+MNC group), AM infusion alone (AM group), MNC transplantation alone (MNC group), or vehicle infusion (control group). The laser Doppler perfusion index was significantly higher in the AM and MNC groups than in the control group (0.74±0.11 and 0.69±0.07 versus 0.59±0.07, respectively, $P<0.01$), which suggests the angiogenic potency of AM and MNC. Importantly, improvement in blood perfusion was marked in the AM+MNC group (0.84±0.08). Capillary density was highest in the AM+MNC group, followed by the AM and MNC groups. In vitro, AM inhibited MNC apoptosis, promoted MNC adhesiveness to a human umbilical vein endothelial cell monolayer, and increased the number of MNC-derived endothelial progenitor cells. In vivo, AM administration not only enhanced the differentiation of MNC into endothelial cells but also produced mature vessels that included smooth muscle cells.

Conclusions—A combination of AM infusion and MNC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation alone. This effect may be mediated in part by the angiogenic potency of AM itself and the beneficial effects of AM on the survival, adhesion, and differentiation of transplanted MNCs. (***Circulation*. 2005;111:356-362.**)

Key Words: peptides ■ angiogenesis ■ peripheral vascular disease

Peripheral vascular disease is a crucial health issue that affects an estimated 27 million people.[1] Despite recent advances in medical intervention, the symptoms of some patients with critical limb ischemia fail to be controlled. Bone marrow–derived mononuclear cells (MNCs) include a variety of stem and progenitor cells, such as endothelial progenitor cells (EPCs), and contribute to pathological neovascularization.[2] MNC transplantation induces therapeutic angiogenesis in ischemic limb[3,4]; however, some patients fail to respond to this cell therapy. Thus, a novel therapeutic strategy to enhance the angiogenic property of MNCs is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[5] Previous studies have reported that abnormalities of vascular structure are present in homozygous AM knockout mice.[6,7] A recent study has demonstrated that blood flow recovery in ischemic limb and tumor angiogenesis are substantially impaired in heterozygous AM knockout mice.[8] Furthermore, AM has been shown to inhibit vascular endothelial cell apoptosis and induce angiogenesis through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[9,10] These results suggest that AM is indispensable for modulating angiogenesis and vasculogenesis. When these findings are taken together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of severe peripheral vascular disease. Thus, the purposes of the present study were (1) to investigate whether local infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

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Methods

Animal Model of Hindlimb Ischemia

Male Lewis rats (weight 250 to 275 g; Japan SLC Inc, Hamamatsu, Japan) were used in the present study. The left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg). The distal portion of the saphenous artery and all side branches and veins were dissected free and excised. The right hindlimb was kept intact and used as the nonischemic limb. Transplantation of bone marrow–derived MNCs and infusion of AM were performed in 40 rats immediately after hindlimb ischemia was created. This protocol resulted in the creation of 4 groups: (1) AM infusion plus MNC transplantation (AM+MNC group, n=10), (2) AM infusion plus PBS injection (AM group, n=10), (3) vehicle infusion plus MNC transplantation (MNC group, n=10), and (4) vehicle infusion plus PBS injection (control group, n=10). The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

MNC Transplantation and AM Infusion

Bone marrow was harvested from the femur and tibia in other male Lewis rats, and MNCs were isolated by Ficoll density gradient centrifugation (Lymphoprep, Nycomed). MNCs (5×10^6 cells per animal) or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at 5 different points. Human recombinant AM (0.01 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or vehicle was administered for 7 days with a mini-osmotic pump (ALZET, Palo Alto) implanted in the left inguinal region.

Assessment of Blood Perfusion

To measure serial blood flow for 3 weeks, we used a laser Doppler perfusion image (LDPI) analyzer (Moor Instrument). After blood flow was scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion.[11]

Histological Assessment

Three weeks after MNC transplantation and/or AM infusion, 4 pieces of ischemic tissue from the adductor and semimembranous muscles were obtained and snap-frozen in liquid nitrogen. Frozen tissue sections were stained with alkaline phosphatase by an indoxyl tetrazolium method to detect capillary endothelial cells.[3,11] Five fields were randomly selected to count the number of capillaries. The capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the 4 groups.[3]

Monitoring of Transplanted MNCs in Ischemic Hindlimb Muscle

To examine differentiation of transplanted MNCs, 5×10^6 MNCs labeled with red fluorescent dye (PKH26-GL, Sigma Chemical Co) were transplanted into the ischemic thigh muscle in rats with (n=3) and without (n=3) AM infusion. Three weeks after transplantation, frozen tissue sections from ischemic muscle were incubated with anti-von Willebrand factor antibody (vWF, DAKO), anti-CD31 antibody (BD Pharmingen), and anti-α-smooth muscle actin antibody (α-SMA, DAKO), followed by incubation with Alexa Fluor 633 IgG antibody (Molecular Probes) and FITC-conjugated IgG antibody (BD Pharmingen), respectively. Five high-power fields (40×) of each section were randomly selected to count the number of transplanted MNCs, vWF-positive cells, and α-SMA–positive cells.

In Situ Detection of MNC Apoptosis

PKH26-labeled MNCs (5×10^6 cells per animal) were transplanted into the ischemic muscle in rats with (n=2) and without (n=2) AM infusion. Twenty-four hours after transplantation, apoptosis of transplanted MNCs in ischemic tissue was evaluated by terminal dUTP nick-end labeling (TUNEL) assay (ApopTag Fluorescein kit, Serological Corporation), as reported previously.[12]

In Vitro Apoptosis Assay

The antiapoptotic effect of AM on MNCs was evaluated by TUNEL assay. Human MNCs, isolated from peripheral blood, were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 hours with control buffer, AM, or AM plus wortmannin, a PI3K inhibitor (50 nmol/L). TUNEL for detection of apoptotic nuclei was performed according to the manufacturer's instructions. MNCs were then mounted in medium that contained 4',6-diamidino-2-phenylindole (DAPI). Randomly selected microscopic fields (n=10) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

Adhesion Assay

We evaluated whether AM enhances MNC adhesiveness according to a previously reported method.[13] In brief, human umbilical vein endothelial cells (HUVECs) were cultured to confluence on 6-well plates with or without pretreatment with tumor necrosis factor-α (1 ng/mL). In the absence or presence of AM (10^{-7} mol/L), 1×10^6 MNCs labeled with PKH26 were incubated on an HUVEC monolayer for 24 hours. Nonadherent MNCs were removed, and the number of PKH26-positive cells in each well was counted.

Cell ELISA

Expression of adhesion molecules in HUVECs was measured by cell ELISA, as reported previously.[14] In brief, confluent HUVECs on 96-well plates were treated with AM (10^{-7} mol/L) or control buffer for 4 hours. HUVECs were then incubated with monoclonal mouse antibodies against intercellular adhesion molecule-1 (ICAM-1, R&D Systems) and vascular adhesion molecule-1 (VCAM-1, R&D Systems). A protein detector ELISA kit (KPL) was used to detect bound monoclonal antibodies.

EPC Culture Assay

Culture of EPCs was performed as described previously.[11,15,16] In brief, 2×10^6 MNCs were plated in Medium-199 supplemented with 20% FCS, heparin, and antibiotics on fibronectin-coated 6-well plates. AM (10^{-7} mol/L), human recombinant vascular endothelial growth factor (VEGF; 20 ng/mL), or control buffer was added to each plate. After 7 days of culture, nonadherent cells were removed, and adherent cells were incubated with acetylated LDL labeled with DiI (DiI-acLDL, Biomedical Technologies) and FITC-labeled lectin from ulex europaeus (Sigma). Double-positive cells for DiI-acLDL and FITC-labeled lectin were identified as EPCs.[16] Randomly selected microscopic fields (n=10) were evaluated to count the number of EPCs.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting was performed to identify characteristics of adherent cells after 7 days of culture.[16] Cells were incubated for 30 minutes at 4°C with anti-human CD31 antibodies (clone L133.1, Becton Dickinson), anti-human KDR antibodies (clone KDR-1, Sigma), and anti-human VE-cadherin antibodies (clone BV6, Chemicon). Isotype-identical antibodies served as controls. Fluorescence-activated cell sorting analyses were performed with a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences).

Real-Time Polymerase Chain Reaction

Expression of calcitonin receptor-like receptor (CRLR), a receptor for AM, was examined by real-time polymerase chain reaction (PCR). Total RNA was extracted from MNCs, EPCs, and HUVECs with an RNA extraction kit (RNeasy Mini Kit, Qiagen) and converted to cDNA by reverse transcription. Real-time PCR was performed with SYBR green dye (QuantiTect SYBR Green PCR kit, Qiagen) and a Prism 7700 sequence detection system (Applied Biosystems). The PCR primers for CRLR were as follows: sense primer 5'-CATTCAACAAGCAGAAGGCG-3' and antisense primer 5'-AGCCATCCATCCCAGGTTC-3'. For GAPDH, the primers were as follows: sense primer 5'-CAATGCCTCCTGCACCACCAA-3' and antisense primer 5'-GAGGCAGGGATGATGTTCTGGA-3'. Levels of CRLR mRNA were normalized to that of

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GAPDH mRNA. PCR-amplified products were also electrophoresed on 2% agarose gels to confirm that single bands were amplified.

In Vitro Matrigel Assay

HUVECs (1×10^5 cells) were seeded onto 24-well plates coated with Matrigel (Becton Dickinson) in the presence of the combination of control buffer, AM (10^{-7} mol/L), VEGF (10 ng/mL), or neutralizing antibodies against KDR (2 μg/mL, R&D Systems). After incubation for 18 hours, tube formation area was measured as described previously.[17] The control was defined as 100% tube formation, and the percent increase was calculated for each sample.

Measurements of Cytokines

A total of 1×10^6 MNCs or HUVECs were plated in serum-free medium with or without AM (10^{-7} mol/L) on 12-well plates. After 24-hour incubation, the conditioned medium was collected, and VEGF, basic fibroblast growth factor, and hepatocyte growth factor were measured with enzyme immunoassay kits (R&D Systems).

Migration Assay

Migration assay of smooth muscle cells (SMCs) was performed with Transwell (Coster) 24-well plates composed of a collagen-coated membrane with 8-μm pores. Human aortic SMCs, preincubated with serum-free medium for 24 hours to maintain quiescence, were seeded on the upper chamber at a concentration of 1×10^6 cells/mL. Serum-free medium containing control buffer, AM (10^{-7} mol/L), or AM plus wortmannin (50 nmol/L) was placed in the lower chamber. After incubation for 12 hours, the number of migrated cells was counted in the randomly selected fields (n=5).

Statistical Analysis

All values are expressed as mean±SEM. Student's unpaired t test was used to compare differences between 2 groups. Comparisons of parameters among 3 or 4 groups were made by 1-way ANOVA, followed by Scheffé multiple comparison test. Comparisons of the time course of the LDPI index were made by 2-way ANOVA for repeated measures, followed by Scheffé multiple comparison tests. A probability value <0.05 was considered statistically significant.

Results

Blood Perfusion and Capillary Density

Blood perfusion of the ischemic hindlimb increased modestly but gradually in the AM and MNC groups after treatment (Figure 1A). Interestingly, blood perfusion in the AM+MNC group markedly improved within 2 weeks after treatment and showed further improvement thereafter. The LDPI index was significantly higher in the AM, MNC, and AM+MNC groups than in the control group 3 weeks after surgery (Figure 1B). Importantly, the LDPI index was highest in the AM+MNC group among the 4 groups.

Alkaline phosphatase staining of ischemic muscle showed significant augmentation of neovascularization in the AM, MNC, and AM+MNC groups (Figure 2A). The capillary/muscle fiber ratio of ischemic muscle was highest in the AM+MNC group, followed by the MNC group, AM group, and control group (Figure 2B).

Differentiation of Transplanted MNCs

Three weeks after MNC transplantation, PKH26-labeled MNCs were frequently observed in the AM+MNC group, and these transplanted cells were positive for vWF (Figure 3A). Most of these cells were also stained by CD31 (data not shown). The number of PKH26/vWF double-positive cells was significantly higher in the AM+MNC group than in the MNC group (Figure 3B). Although PKH26/α-SMA double-positive cells were not detected in ischemic muscle of each group, newly formed vascular structures in the AM+MNC group included α-SMA–positive cells (Figure 3C). The number of α-SMA–positive cells in the MNC-derived vascular structures was significantly higher in the AM+MNC group than in the MNC group (Figure 3D).

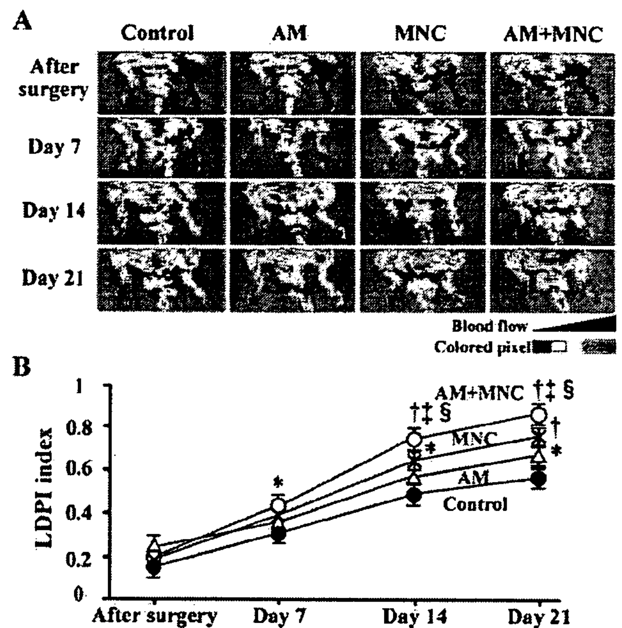

Figure 1. A, Representative examples of serial laser Doppler perfusion images. Blood perfusion of ischemic hindlimb increased notably in AM+MNC group (red to yellow). B, Quantitative analysis of hindlimb blood perfusion with LDPI index, ratio of ischemic to nonischemic hindlimb blood perfusion. Data are mean±SEM. *P<0.05 and †P<0.01 vs control; ‡P<0.01 vs AM; §P<0.05 vs MNC.

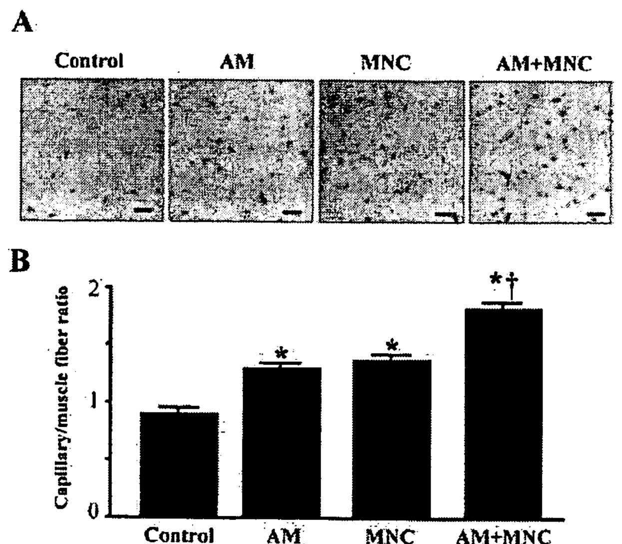

Figure 2. A, Representative photographs of alkaline phosphatase staining in ischemic hindlimb muscles. Capillary density in AM+MNC group was markedly higher than that in other groups. B, Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean±SEM. *P<0.01 vs control; †P<0.01 vs AM and MNC. Scale bars: 50 μm.

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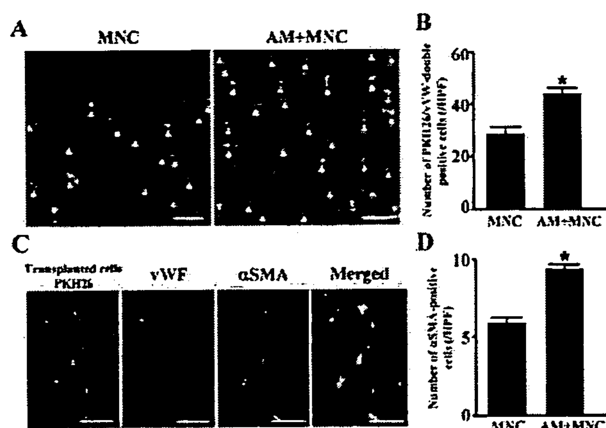

Figure 3. In vivo differentiation of transplanted MNCs. A, Representative photographs of MNC-derived vascular structures in MNC and AM+MNC groups. Red fluorescence (PKH26)-labeled MNCs were transplanted into ischemic thigh muscle. PKH26 (red)/vWF (blue) double-positive cells (pink, arrows) were frequently observed in AM+MNC group. B, Number of PKH26/vWF double-positive cells (MNC-derived endothelial cells) was significantly higher in AM+MNC group than in MNC group. C, Representative photographs of newly formed mature vessels in AM+MNC group. MNC-derived vascular structures often included α-SMA–positive cells (green). D, Number of α-SMA–positive cells in MNC-derived vessels was significantly higher in AM+MNC group than in MNC group. Data are mean±SEM. *$P<0.01$ vs MNC. Bars: 50 μm. HPF indicates high-power field.

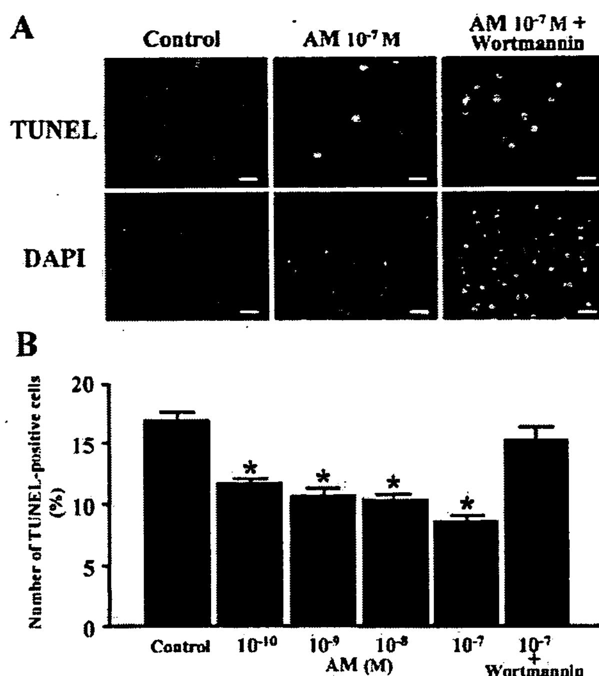

Figure 4. Apoptosis assay. A, Apoptosis of MNC was detected by TUNEL assay (green). Nuclei of MNC were stained with DAPI (blue). AM inhibited MNC apoptosis in serum-free medium. B, Quantitative analysis. AM decreased percentage of TUNEL-positive cells in dose-dependent manner. Pretreatment with wortmannin, a PI3K inhibitor, diminished antiapoptotic effect of AM. Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm.

Antiapoptotic Effect of AM on MNCs

In vitro, serum starvation induced MNC apoptosis, as indicated by detection of TUNEL-positive cells (Figure 4A). When incubated in the presence of AM, the percentage of TUNEL-positive cells markedly decreased in a dose-dependent manner (Figure 4B). However, pretreatment with wortmannin, a PI3K inhibitor, diminished the antiapoptotic effect of AM. Similarly, in vivo, local administration of AM decreased TUNEL-positive MNC 24 hours after transplantation (data not shown).

Effect of AM on MNC Adhesiveness

The number of adherent MNCs on an HUVEC monolayer increased significantly in the presence of AM (10^{-7} mol/L) compared with control (Figures 5A and 5B). With pretreatment using tumor necrosis factor-α, AM also enhanced the adhesiveness of MNCs to HUVECs. AM significantly enhanced expression of ICAM-1 and VCAM-1 in HUVECs (Figure 5C).

Effect of AM on EPC Expansion

After 7-day culture of human MNCs, spindle-shaped or cobblestone-like adherent cells were observed (Figure 6A). Most of the adherent cells were double stained with DiI-acLDL and FITC-labeled lectin. These adherent cells expressed endothelial cell–specific markers: KDR, VE cadherin, and CD31 (Figure 6B). Thus, we identified the major population of the adherent cells as EPCs. Culture of MNCs with AM significantly increased the number of EPCs (Figure 6C). The effect of AM was equivalent to that of VEGF. Real-time PCR revealed that MNCs, EPCs, and HUVECs expressed mRNA of CRLR (Figure 6D). Expression of CRLR mRNA was highest in HUVECs, followed by EPCs and MNCs.

Effects of AM on Tube Formation and SMC Migration

Like VEGF, AM induced tube formation in HUVECs in vitro (Figure 7A). Blocking antibodies against KDR significantly inhibited VEGF-induced tube formation, whereas they did not suppress AM-induced tube formation (Figure 7B). AM did not significantly alter VEGF, basic fibroblast growth factor, or hepatocyte growth factor levels in conditioned medium of cultured MNCs or HUVECs (data not shown). AM significantly increased the number of migrated SMCs compared with control (Figures 7C and 7D). Pretreatment with wortmannin diminished the effect of AM on SMC migration.

Discussion

In the present study, we demonstrated in vivo that AM infusion or MNC transplantation alone induced angiogenesis in a rat model of hindlimb ischemia, the combination of AM infusion and MNC transplantation enhanced MNC-induced angiogenesis, and AM increased the number of MNC-derived vWF-positive cells and generated α-SMA–positive vascular structures. We also demonstrated in vitro that AM inhibited serum starvation–induced MNC apoptosis, promoted MNC adhesiveness to an HUVEC monolayer, increased the number of MNC-derived EPCs, and stimulated SMC migration.

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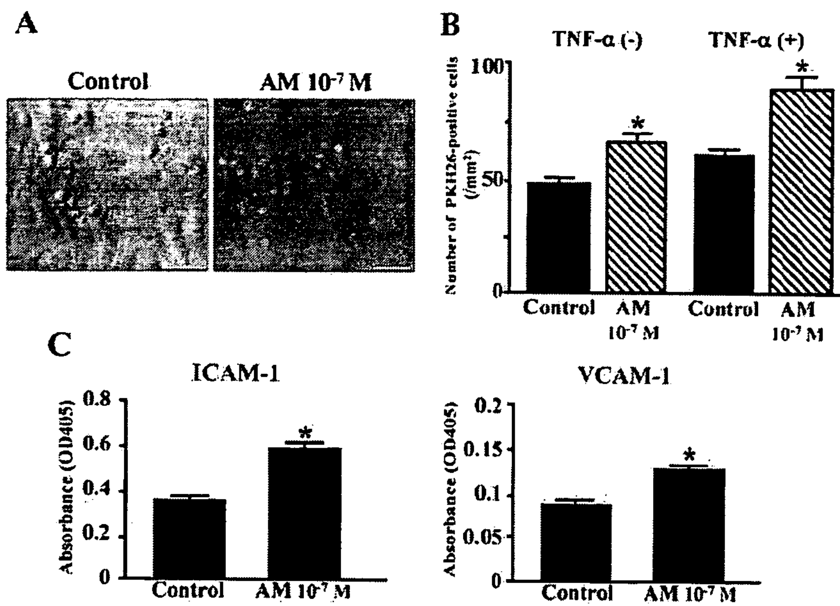

Figure 5. A and B, Adhesion assay. Representative photographs of red fluorescence-labeled MNC adhesion to HUVEC monolayer with and without AM (A). Quantitative analysis of MNC adhesion (B). Bars: 50 μm. C, Surface expression of ICAM-1 and VCAM-1 in HUVECs with or without AM. Data are mean±SEM. TNF indicates tumor necrosis factor. *$P<0.01$ vs control.

MNC transplantation causes therapeutic angiogenesis by supplying EPCs and multiple angiogenic cytokines such as VEGF.[3,4] The present study showed that local infusion of AM significantly increased blood perfusion and capillary density in ischemic hindlimb muscle. Furthermore, a combination of AM infusion and MNC transplantation significantly increased blood perfusion and capillary density of the ischemic hindlimb compared with MNC transplantation alone. AM has been shown to induce angiogenesis in vitro and in vivo through the PI3K/Akt pathway.[10,18] In the present study, AM-induced tube formation was not blocked by neutralizing antibodies against KDR. In addition, AM did not enhance VEGF secretion from MNCs and HUVECs. Thus, beneficial effects of combination therapy

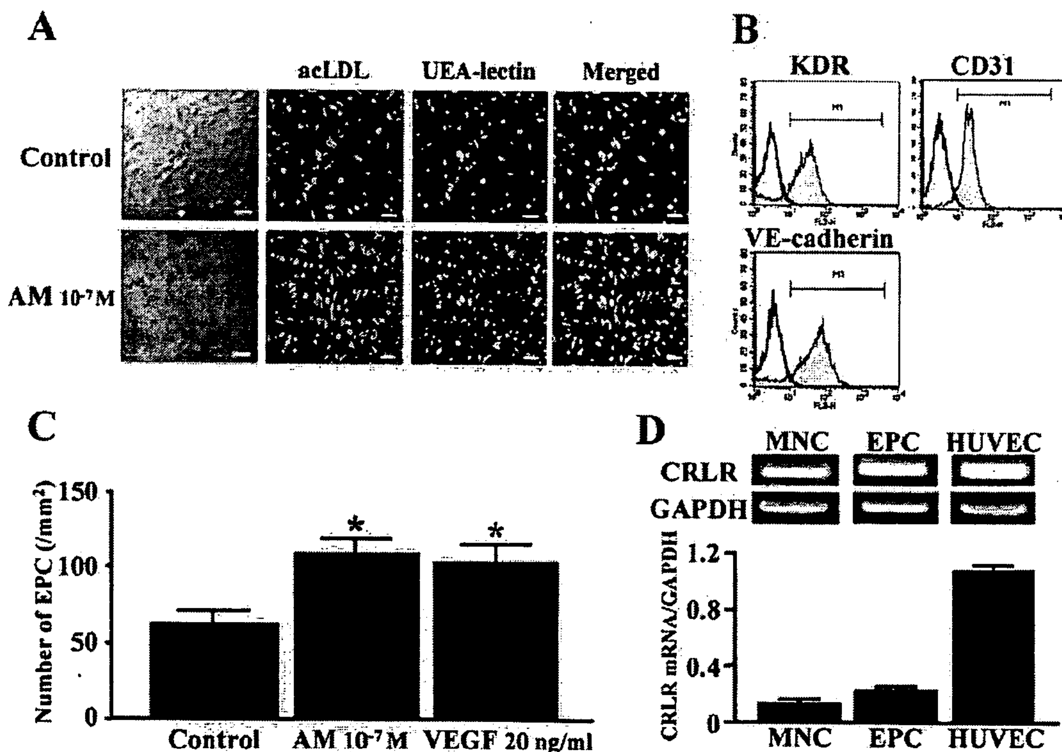

Figure 6. A through C, EPC culture assay. Cultured adherent cells took up DiI-acLDL (red) and FITC-labeled lectin (green) in same fields (A). Fluorescence-activated cell sorting analyses revealed that most adherent cells expressed KDR, VE cadherin, and CD31 (B). Culture of MNCs with AM significantly increased number of EPCs. Effect of AM was equivalent to that of VEGF (C). Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm. D, Quantitative analysis of AM receptor (CRLR) mRNA expression in MNCs, EPCs, and HUVECs. UEA indicates ulex europaeus.

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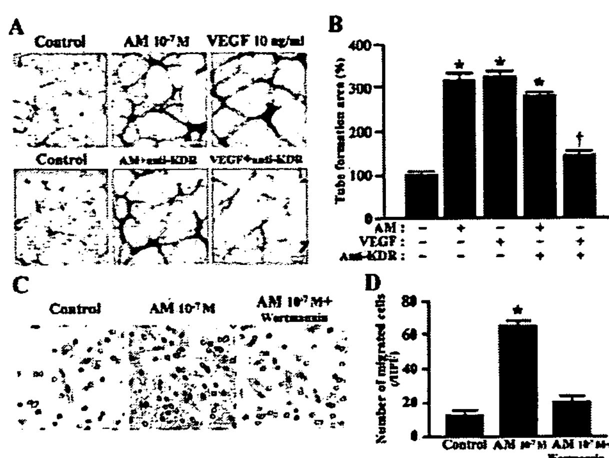

Figure 7. A and B, Matrigel assay. Representative photographs of tube formation (A). Quantitative analysis of tube formation area (B). Data are mean±SEM. *$P<0.01$ vs control; †$P<0.01$ vs VEGF. Bars: 20 μm. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses of SMC migration (D). Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm.

with AM and MNCs may be attributable in part to the angiogenic properties of AM itself.

An earlier study has shown that transplanted MNCs disappear from ischemic muscle 7 days after transplantation.[19] We demonstrated that apoptosis of MNCs occurred in ischemic muscle 24 hours after MNC transplantation. These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. In the present study, AM inhibited apoptosis of MNCs in vitro and in vivo, and the antiapoptotic effect of AM was suppressed by wortmannin, a PI3K inhibitor. These findings suggest that AM prolongs MNC survival through the PI3K/Akt pathway and thereby enhances neovascularization in ischemic tissue.

In the present study, AM promoted adhesiveness of MNCs to an HUVEC monolayer. AM significantly enhanced expression of ICAM-1 and VCAM-1 in HUVECs, both of which facilitate adhesion of MNCs to endothelial cells.[20] These findings suggest that AM increases MNC adhesiveness to endothelial cells via activation of adhesion molecules. A recent study has shown that MNC adhesiveness to endothelial cells is indispensable for MNC differentiation into endothelial lineage.[21] Thus, it is possible that AM infusion enhances the angiogenic potency of MNCs at least in part through promotion of adhesion of MNC to host vascular endothelial cells.

VEGF has been shown to increase the number of EPCs in vitro and in vivo, resulting in angiogenesis and vasculogenesis.[13,22] The present study showed that MNCs and EPCs expressed CRLR, a receptor of AM. In vitro, AM increased the number of MNC-derived EPCs that expressed VE cadherin, KDR, and CD31. The effect of AM on EPC expansion was equivalent to that of VEGF. In vivo, AM infusion increased the number of MNC-derived vWF-positive cells, although incorporation of these cells in the capillaries may be due in part to incorporation of hematopoietic cells. These findings suggest that AM may accelerate MNC differentiation into endothelial lineage.

SMC is essential for the generation of functional and mature blood vessels.[23] We demonstrated in vivo that local infusion of AM increased the number of α-SMA–positive cells (SMCs) in MNC-derived vascular structures. In vitro, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is indispensable for vascular morphogenesis.[6,7] When these findings are taken together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via the PI3K/Akt-dependent pathway.

Currently, a new therapeutic approach to augment the efficacy of MNC transplantation is awaited for the treatment of severe peripheral vascular disease. The present study demonstrated that local infusion of AM enhanced the angiogenic potency of MNC transplantation. In the present study, AM inhibited MNC apoptosis and increased the total number of engrafted cells in ischemic tissue, although this study did not show the effect of AM on specific cell populations of MNCs. In addition, AM promoted cell proliferation, migration, and differentiation. We have already demonstrated the safety of AM infusion in patients with congestive heart failure.[24] Thus, combination therapy with AM infusion and MNC transplantation may be a novel and promising therapeutic strategy for the treatment of severe peripheral vascular disease.

Conclusions

A combination of AM infusion and MNC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation alone. This effect may be mediated in part by the angiogenic potency of AM itself and the beneficial effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

Acknowledgments

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CNP infusion attenuates cardiac dysfunction and inflammation in myocarditis

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Abstract

Myocarditis is an acute inflammatory disease of the myocardium for which there is currently no specific therapy. We investigated the therapeutic potential of C-type natriuretic peptide (CNP) in acute experimental autoimmune myocarditis. One week after injection of porcine myosin into male Lewis rats, CNP (0.05 μg/kg/min) was continuously administered for 2 weeks. CNP infusion significantly increased maximum dP/dt, decreased left ventricular end-diastolic pressure, and improved fractional shortening compared with vehicle administration. In vehicle-treated hearts, severe necrosis and marked infiltration of CD68-positive inflammatory cells were observed. Myocardial and serum levels of monocyte chemoattractant protein-1 were elevated in myocarditis. However, these changes were attenuated by CNP infusion. In addition, treatment with CNP significantly increased myocardial capillary density. Guanylyl cyclase-B, a receptor for CNP, was expressed in myocarditic heart, and cyclic guanosine monophosphate was elevated by CNP infusion. In conclusion, CNP infusion attenuated cardiac function in acute myocarditis through anti-inflammatory and angiogenic effects.
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Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation. Acute myocarditis is associated with rapidly progressive heart failure, arrhythmias, and sudden death [1]. Immunomodulatory therapies such as immunoglobulin and interferon are regarded as promising for myocarditis [2,3]; however, the efficacy of those treatments still remains controversial [3,4]. Other treatment options are restricted to supportive care for heart failure or arrhythmias. The lack of specific treatment and the potential severity of the illness underlie the importance of novel and effective therapeutic strategies for myocarditis.

There are three main natriuretic peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), all of which signal through natriuretic receptors and cyclic guanosine monophosphate (cGMP) signaling pathways. ANP and BNP are predominantly secreted from cardiac myocytes. They have anti-hypertrophic effects on cardiac myocytes in an autocrine manner and also have inhibitory effects on collagen synthesis of cardiac fibroblasts in a paracrine manner, and thus have suppressive effects on cardiac remodeling. Cardioprotective effects of ANP and BNP have already been demonstrated, and they are used clinically for the treatment of heart failure. On the other hand, CNP, originally identified in the porcine brain [5], is predominantly

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expressed in vascular endothelial cells and plays a role in the local regulation of vascular tone and remodeling [6]. However, the potential therapeutic effects of CNP in heart disease are not well understood.

Recently, it has been shown that CNP is also synthesized in cardiac fibroblasts and inhibits collagen synthesis of cardiac fibroblasts more potently than ANP and BNP [7]. In addition, CNP proved to have more potent anti-hypertrophic effects than ANP in cultured cardiac myocytes [8]. More recently, infusion of CNP has been shown to improve cardiac function after myocardial infarction through anti-fibrotic and anti-hypertrophic effects [9]. These findings indicate the therapeutic potential of CNP in heart disease. However, it remains unknown whether CNP infusion improves acute myocarditis leading to severe heart failure. In the present study, cardiac myosin purified from pig hearts was injected into rats, and autoimmune myocarditis was induced [10].

Thus, the purposes of this study were (1) to investigate whether infusion of CNP improves cardiac function in a rat model of acute myocarditis, and (2) to investigate the mechanisms responsible for the effect of CNP on the myocarditic heart.

Materials and methods

Model of acute myocarditis. We produced a rat model of acute myocarditis by injecting pig cardiac myosin. In brief, purified myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [11]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl, mixed with an equal volume of complete Freund's adjuvant containing 11 mg/ml of Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg) and 0.2 ml of the antigen-adjuvant emulsion was injected into the foot pads.

CNP preparation and treatment. CNP was diluted in PBS with 5% glucose and administered via an ALZET mini-osmotic pump (DURECT Corporation, Cupertino, CA, USA) inserted subcutaneously, which discharged CNP at a rate of 0.05 μg/kg/min for the duration of 14 days beginning 1 week after myosin injection.

Experimental groups. Rats with sham operation or those with acute myocarditis were treated with vehicle or CNP. Fifty-four male 10-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were randomly placed into four groups and received the following treatments: (1) sham rats given vehicle ($n = 12$), (2) sham rats given CNP ($n = 12$), (3) myocarditis rats given vehicle ($n = 15$), and (4) myocarditis rats given CNP ($n = 15$).

Echocardiography. Echocardiography was performed at day 21 post-myosin injection. Rats were anesthetized with sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). Left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), anterior wall thickness (AWT), and posterior wall thickness (PWT) were measured, and taken as an average of three beats. Fractional shortening (%FS) was calculated as follows;

$$\%FS = (LVDd - LVDs)/LVDd \times 100$$

Hemodynamic study. Hemodynamic measurements were taken at day 21 post-myosin injection. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery (Millar Instruments, Houston, TX, USA). Heart rate was also monitored with electrocardiogram. As indexes of hemodynamics, heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dP/dt, and minimum dP/dt were used. Anesthesia was maintained with sodium pentobarbital, and the above mentioned indexes were recorded simultaneously during spontaneous ventilation after an equilibration period of a minimum of 20 min.

Histopathology. The heart was excised above the origin of the great vessels, and the heart and body weights were recorded. A midventricular portion of the heart was fixed with formalin and embedded in paraffin, and 4-μm sections were cut and stained with either hematoxylin and eosin (H&E) or Masson's trichrome stain, or subjected to immunohistochemical staining. H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) for the characterization of myocardial injury and inflammation, without knowledge of the experimental groups, on the following sc ale: (0) no or questionable presence, (1) limited focal distribution, (2 and 3) intermediate severity, and (4) coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue.

Immunohistochemistry. Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Sections were incubated with DakoCytomation protein block, then with anti-von Willebrand factor (vWF) (DakoCytomation, Glostrup, Denmark), CD68 (DakoCytomation), or monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences, San Jose, CA, USA) antibodies, followed by sequential incubations with HRP-linked rabbit anti-mouse IgG (DakoCytomation). The reaction products were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide. Sections were counterstained with hematoxylin. The numbers of vWF-stained capillaries and CD68-stained cells were determined in ten randomly selected fields (vWF; 400×, CD68; 200×).

Enzyme-linked immunosorbent assay (ELISA). Serum MCP-1 level on day 21 post-myosin injection was measured using a Rat MCP-1 ELISA Kit (Biosource International, Camarillo, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of guanylyl cyclase-B (GC-B) mRNA, a receptor for CNP, was examined by RT-PCR. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and myocarditis rats given vehicle ($n = 5$ in each group). Total RNA was extracted from heart with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed (PCR Amplification Kit, Takara, Shiga, Japan). The complementary DNA was amplified by the PCR using specific primers for GC-B or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers for GC-B were as follows [12]: sense primer 5′-AACGGGCG CATTGTGTATATCTGCGGC-3′ and antisense primer 5′-TTATCA CAGGATGGGTCGTCCAAGTCA-3′. For GAPDH, the primers were as follows: sense primer 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and antisense primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

Radioimmunoassay. To investigate whether subcutaneous administration of CNP has a biological activity in heart, we measured myocardial level of cGMP. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and those given CNP ($n = 10$ in each groups). Myocardial level of cGMP was measured with a radioimmunoassay kit (cGMP assay kit; YAMASA Co., Chiba, Japan).

Statistical analysis. Data were presented as means ± SEM. Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $P < 0.05$.

Results

Improvement in cardiac function by CNP treatment

Myocarditis rats given vehicle had two deaths 19 and 21 days after myosin injection, respectively, whereas those treated with CNP showed no mortality. At 3 weeks post-myosin injection, Myocarditis rats given vehicle showed decreased maximum dP/dt and minimum dP/dt, and

increased LVEDP compared with the sham rats (Fig. 1A–C), indicating the presence of acute heart failure in this model. Such parameters subsequently returned to baseline with CNP treatment. On echocardiography, rats with myocarditis showed an increase in LVDd and a significant reduction in %FS (Fig. 1D–F). CNP infusion significantly improved %FS in myocarditis rats. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was reduced by CNP treatment (Table 1). MAP was significantly decreased in myocarditis rats, and the decrease was significantly attenuated by CNP treatment. CNP did not significantly influence cardiac function in sham rats.

Attenuation of inflammatory cell infiltration by CNP treatment

Histological examination showed that myocardial necrosis and tissue granulation as well as inflammation and edema were markedly increased in our model of acute myocarditis (Fig. 2A and B). CNP administration significantly attenuated necrotic changes observed in myocarditis rats. CNP-treated hearts exhibited a consistent tendency for a reduction of tissue granulation, inflammation and edema, on blinded histological grading by a cardiovascular pathologist (H.I-U.) as compared to vehicle-treated hearts. Although, CNP is known to have potent anti-fibrotic activ-

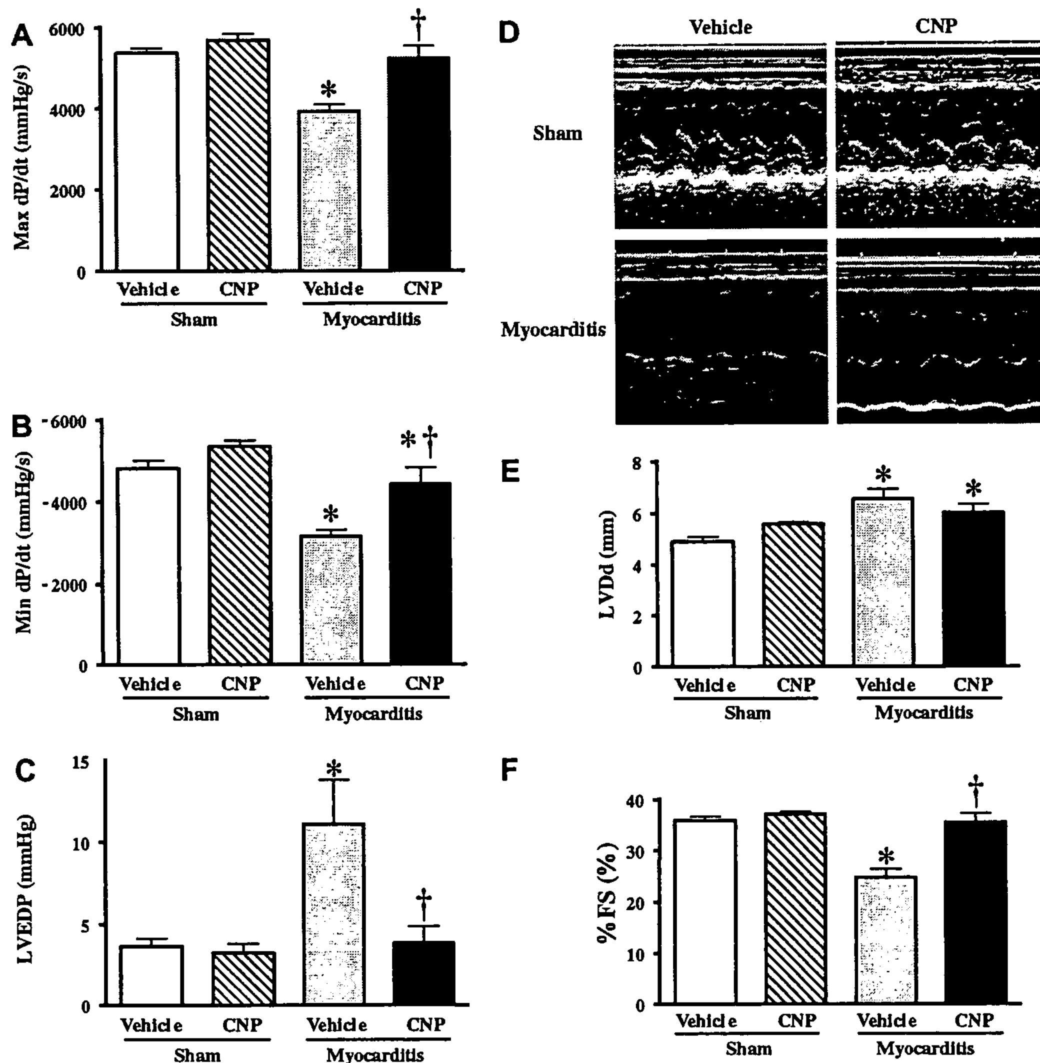

Fig. 1. Effects of CNP administration on hemodynamic parameters in acute myocarditis. (A) Maximum dP/dt (Max dP/dt), (B) minimum dP/dt (Min dP/dt), and (C) left ventricular end-diastolic pressure (LVEDP) were measured in sham rats given vehicle, sham rats given CNP, myocarditis rats given vehicle, and myocarditis rats given CNP. (D) Representative echocardiographic images showing wall thickening and poor myocardial movement in rats with myocarditis and improved cardiac contractility in those treated with CNP. (E,F) CNP administration in myocarditis tended to attenuate the increase in left ventricular diastolic dimension (LVDd) and significantly improved fractional shortening (%FS). Values are means ± SEM. *$P < 0.05$ *vs*. Sham-Vehicle, †$P < 0.05$ *vs*. Myocarditis-Vehicle.

Table 1
Physiological and catheter-based parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 12$)	CNP ($n = 13$)
BW (g)	282 ± 2	282 ± 3	208 ± 4*	224 ± 3*,†
HW/BW (g/kg)	2.86 ± 0.04	2.81 ± 0.03	6.33 ± 0.25*	5.29 ± 0.20*,†
HR (bpm)	428 ± 7	422 ± 5	367 ± 13*	431 ± 13*,†
MAP (mmHg)	111 ± 4	103 ± 4	87 ± 3*	105 ± 5†
LVSP (mm Hg)	124 ± 5	125 ± 4	104 ± 4*	123 ± 6†

BW, body weight; HW/BW, heart weight to body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure. Data are means ± SEM.
* $P < 0.05$ *vs*. Sham-Vehicle.
† $P < 0.05$ *vs*. Myocarditis-Vehicle.

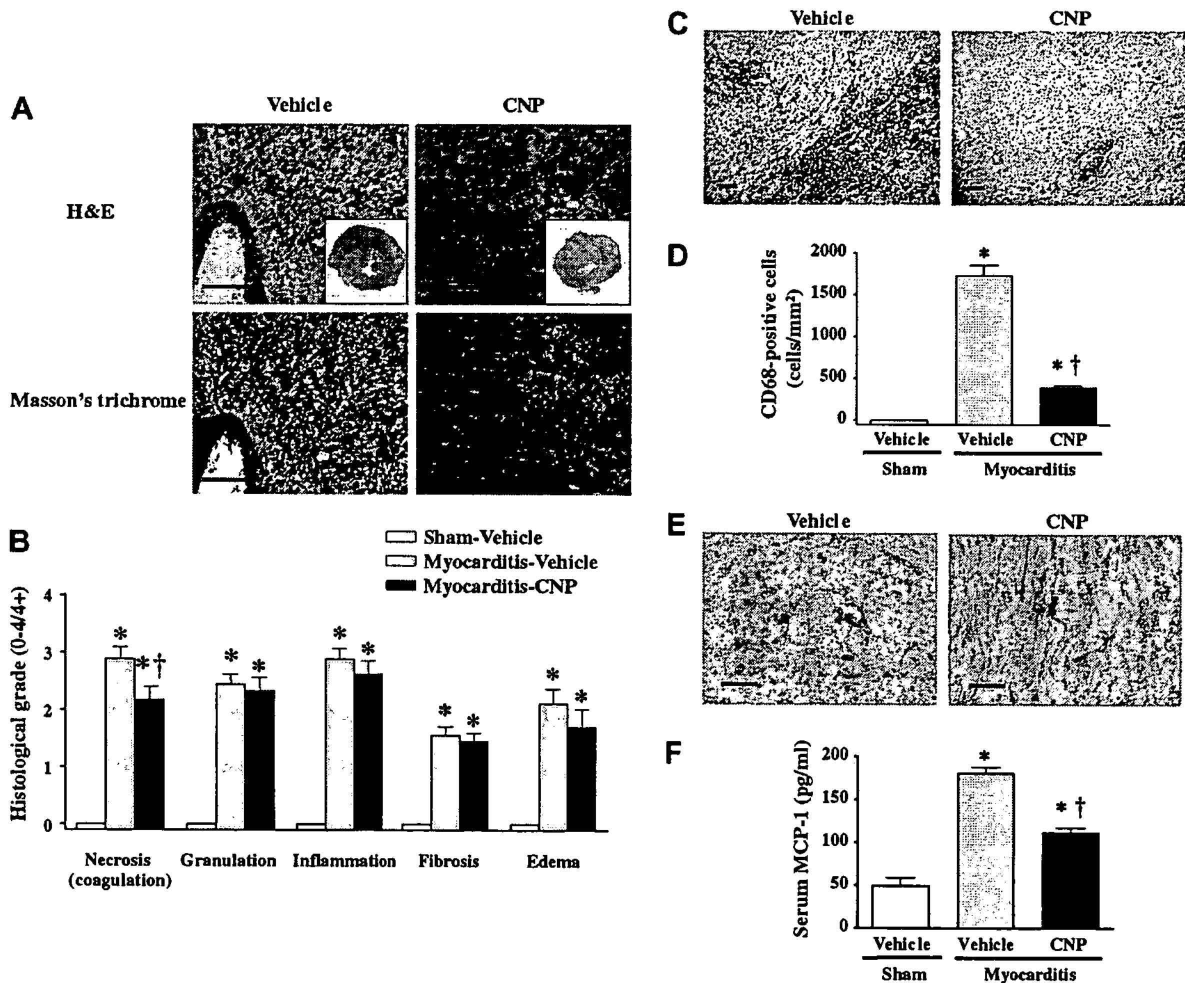

Fig. 2. Histological analysis of the myocardium. (A) Representative myocardial sections showed markedly decreased inflammation and tissue necrosis (H&E) and a comparable degree of early fibrosis (Masson's trichrome) in CNP-treated hearts as compared to myocarditic hearts. Insets are transverse section of the left ventricular section (H&E). (B) Semiquantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were lower in myocarditis rats treated with CNP as compared to untreated rats. Sham rats exhibited no measurable pathological change. Scale bar is 100 μm. (C) Representative myocardial sections immunohistochemically-stained for CD68 demonstrated a marked decrease in CD68-positive cells, including giant cells, in CNP-treated hearts as compared to vehicle-treated hearts. Scale bar is 100 μm. (D) Semi-quantitative counts of CD68-positive cells demonstrate a significant reduction in CNP-treated hearts. (E) Representative MCP-1-stained myocardial sections from rats with acute myocarditis. Scale bar is 100 μm. (F) Serum level of MCP-1 measured by ELISA. Values are means ± SEM. *$P < 0.05$ *vs*. Sham-Vehicle, †$P < 0.05$ *vs*. Myocarditis-Vehicle.

ity [9], myocardial fibrosis was not significantly attenuated by CNP infusion (Fig. 2B), probably due to the acute nature of this experiment (Table 2).

Notably, marked histiocytic infiltration was demonstrated by the presence of CD68-positive cells, including multinucleated giant cells, in rats with myocarditis, and this

Table 2
Echocardiographic parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 9$)	CNP ($n = 11$)
LVDd (mm)	5.6 ± 0.1	5.6 ± 0.1	6.5 ± 0.4*	6.0 ± 0.3
LVDs (mm)	3.6 ± 0.1	3.5 ± 0.1	4.9 ± 0.4*	3.9 ± 0.2†
%FS (%)	36 ± 1	37 ± 1	25 ± 2*	36 ± 2†
AWT diastole (mm)	1.9 ± 0.1	1.9 ± 0.1	3.1 ± 0.2*	2.8 ± 0.2*
PWT diastole (mm)	1.9 ± 0.1	1.8 ± 0.1	3.5 ± 0.3*	3.6 ± 0.4*

LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; %FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

was significantly attenuated by CNP treatment (Fig. 2C and D). In myocarditis, there was an increase in MCP-1 expression localized to the vascular endothelium and also in myocytes surrounding and adjacent to areas of inflammatory infiltration (Fig. 2E). The hearts in myocarditis rats treated with CNP showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in myocarditis rats, whereas it was significantly decreased in those treated with CNP (Fig. 2F).

Effect of CNP on angiogenesis

To determine the angiogenic effect of CNP treatment in the myocardium, immunohistochemical analysis of vWF was performed. Capillary density in the heart was increased in myocarditis, particularly in areas directly adjacent to tissue necrosis (Fig. 3). Notably, capillary density was increased over that in acute myocarditis alone. The clustering of relatively small vessels seen in CNP-treated myocarditic hearts was indicative of recent endothelial regeneration or angiogenesis. On the other hand, CNP did not significantly influence the capillary density in the sham rats.

Expression of GC-B and cGMP in myocardium

RT-PCR demonstrated that GC-B mRNA was expressed in myocarditic heart (Fig. 4A). Myocardial level

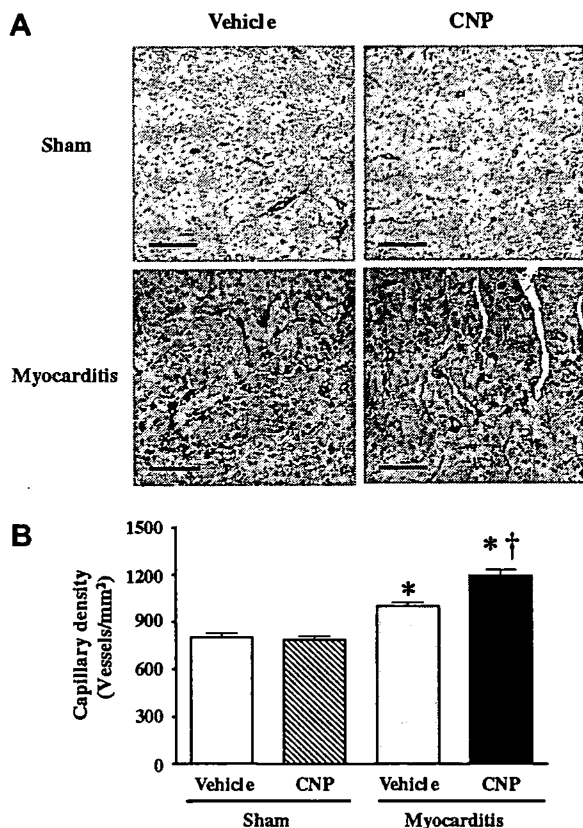

Fig. 3. Angiogenic potential of CNP in acute myocarditis. (A) Representative myocardial sections immunohistochemically-stained for vWF exhibit increased microvasculature in control myocarditic hearts, which was more marked in CNP-treated hearts. (B) Capillary density measured in 10 random representative high powered fields showed a significant increase in rats with acute myocarditis and a further increase in those treated with CNP. Scale bar is 100 μm. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

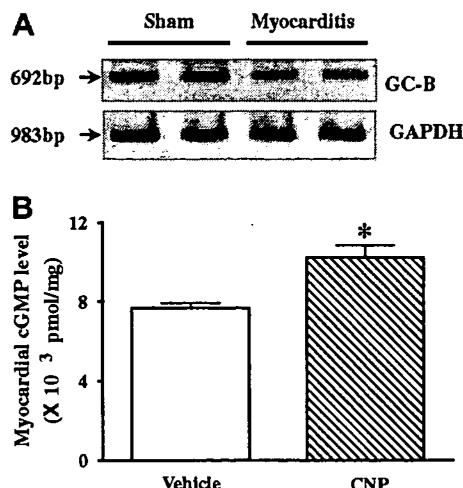

Fig. 4. Expression of GC-B and cGMP in the myocardium. (A) RT-PCR analysis of GC-B mRNA expression in myocarditic heart. (B) Myocardial level of cGMP measured by radioimmunoassay. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle.

of cGMP was significantly elevated by the subcutaneous infusion of CNP (Fig. 4B). These results suggest that subcutaneous infusion of CNP (0.05 μg/kg/min) has biological effects on myocaditic heart.

Discussion

In this study, we focused on the therapeutic potential of CNP in the acute phase of autoimmune myocarditis. We showed that CNP treatment 1 week following myosin injection but prior to the development of myocarditis (1) preserved cardiac function after acute myocarditis, (2) significantly decreased tissue necrosis, inflammatory cell infiltration and MCP-1 expression in the heart and serum, and led to a tendency for reduced overall inflammation, granulation and edema, and (3) stimulated angiogenesis in myocarditic hearts beyond the baseline increase seen in myocarditis.

The rat model of myosin-induced experimental autoimmune myocarditis closely resembles human giant cell myocarditis [11]. This disease model is triphasic, consisting of antigen priming phase from days 0–14, an autoimmune response phase from days 14–21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [13]. In our experiments, CNP was administered 1 week following myosin injection, corresponding to an early time point in the disease process. In the present study, CNP treatment significantly improved cardiac function as determined by increased maximum dP/dt and %FS as well as decreased LVEDP in rats with acute myocarditis. Importantly, earlier studies have shown that the vasodilator effect of CNP is much less potent than that of ANP [5,9,14,15]. ANP and BNP cause vasodilatation and hypotension, thus limiting their use as treatment for patients with severe heart failure. Because the effects of CNP on blood pressure and HR were very small, CNP treatment is considered as a safer alternative for the treatment of those patients [16]. Indeed, administration of CNP did not decrease arterial pressure, but sustained its biological activity.

Our data showed a significant decrease in inflammatory cell infiltration and a consistent tendency for decreased overall inflammation and edema by CNP treatment. In addition, CNP infusion decreased MCP-1 expression in the heart and serum. A previous study has demonstrated that CNP reduces macrophage infiltration by inhibition of MCP-1 expression [17]. These findings suggest that attenuation of inflammatory cell infiltration by CNP may be regulated, at least in part, by suppression of MCP-1 expression.

Recently, it was shown that CNP has anti-fibrotic properties in pulmonary fibrosis and myocardial infarction, through a cGMP-dependent pathway [9,18]. However, since the present experiments were carried out in the acute phase of myocarditis, the anti-fibrotic effect of CNP in the myocarditic heart was not clear. Further studies are necessary to examine the anti-fibrotic effects of CNP in the chronic phase of myocarditis.

We demonstrated that CNP induces endothelial regeneration beyond the increase seen in myocarditis. In rabbit balloon injury, infectious vein graft disease and hindlimb ischemia models, CNP overexpression stimulated reendothelialization via a cGMP-dependent pathway [19]. Endothelial dysfunction including microvascular constriction and microaneurysm formation has previously been reported in myocarditis [20], as well as chronic impairment of endothelial-dependent vasorelaxation of coronary resistance vessels in myocarditis [21]. Thus, the endothelial regenerative effects of CNP are likely to be beneficial in preventing myocardial injury and dysfunction in acute myocarditis. In this study, capillary density in normal heart was not increased by CNP infusion. In inflammatory tissue, it is speculated that CNP does not have an effect on initiation of angiogenesis, but promote angiogenesis at the phase of forming mature blood vessels. However, a further examination is necessary to elucidate the mechanisms of angiogenic effects.

Considering the importance of natriuretic peptides, such as ANP and BNP, in the diagnosis and treatment of cardiovascular diseases, there is currently much interest in the role of CNP. Since, CNP has marked cardioprotective effects including anti-inflammatory and angiogenic effects, and has less vasodilator effects, which enable the use of this peptide in patients with hypotension, this molecule may have great potential for the treatment of patients with acute myocarditis.

In summary, administration of CNP ameliorated cardiac dysfunction in a rat model of acute myocarditis. The beneficial effects may be due, at least in part, to anti-inflammatory and angiogenic effects. This work expands the beneficial effects of CNP to acute myocarditis, and increases our understanding of the role of natriuretic peptides in severe heart failure.

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