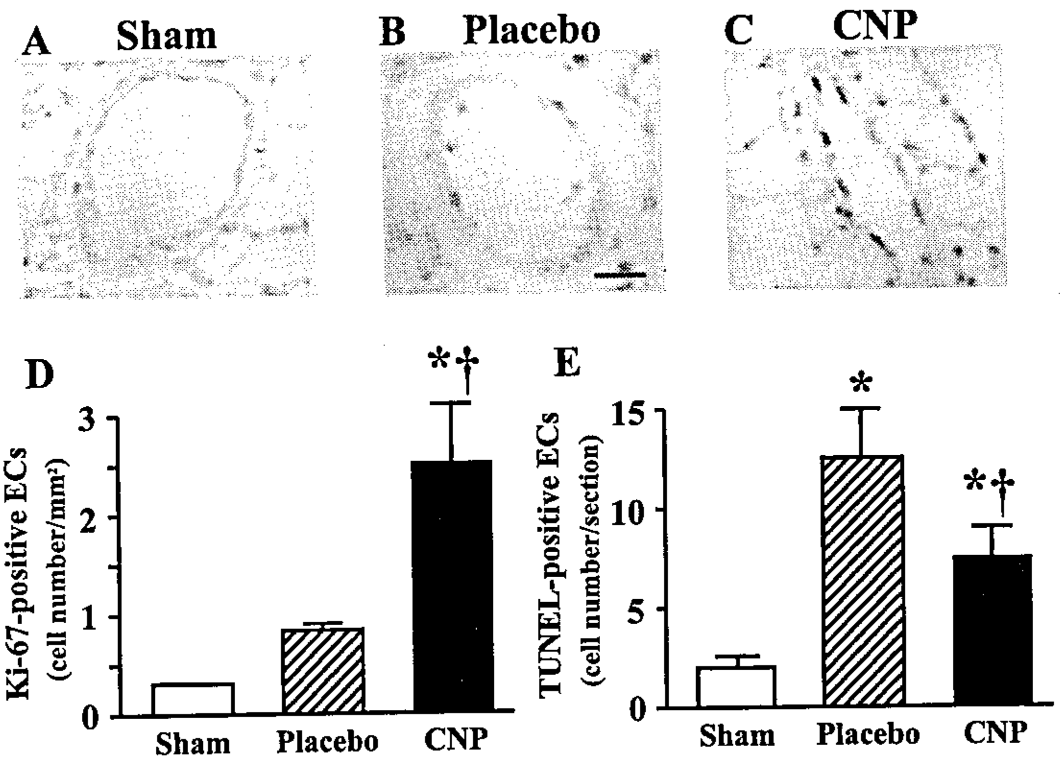

Figure 2. (*A–C*) Immunohistochemical demonstration of Ki-67 antigen, a marker for cell proliferation, in pulmonary endothelial cells (ECs) on Day 7. *Scale bars* = 20 μm. (*D*) Quantitative analysis of Ki-67–positive ECs in pulmonary vessels. The number of Ki-67–positive ECs in the CNP group was significantly increased compared with that in the placebo group. (*E*) Quantitative analysis of terminal dUTP nick-end labeling (TUNEL)-positive ECs in lungs on Day 7. Data are mean ± SEM. $^*p < 0.05$ versus sham; $^\dagger p < 0.05$ versus placebo.

onstrated that (*3*) these effects of CNP may be attributable to regeneration of pulmonary endothelial cells, inhibition of pulmonary endothelial cell apoptosis, and prevention of monocyte/macrophage infiltration and PAI-1 expression.

Endothelial cell injury caused by MCT activates platelets and vasoconstrictive factors, resulting in pulmonary hypertension and vascular remodeling (20). We demonstrated that CNP infusion significantly attenuated the increases in RV systolic pressure and the ratio of RV weight to body weight, suggesting that CNP infusion ameliorates MCT-induced pulmonary hypertension. CNP has been shown to be less expressed than atrial natriuretic peptide (21). Nevertheless, continuous infusion of CNP had benefi-

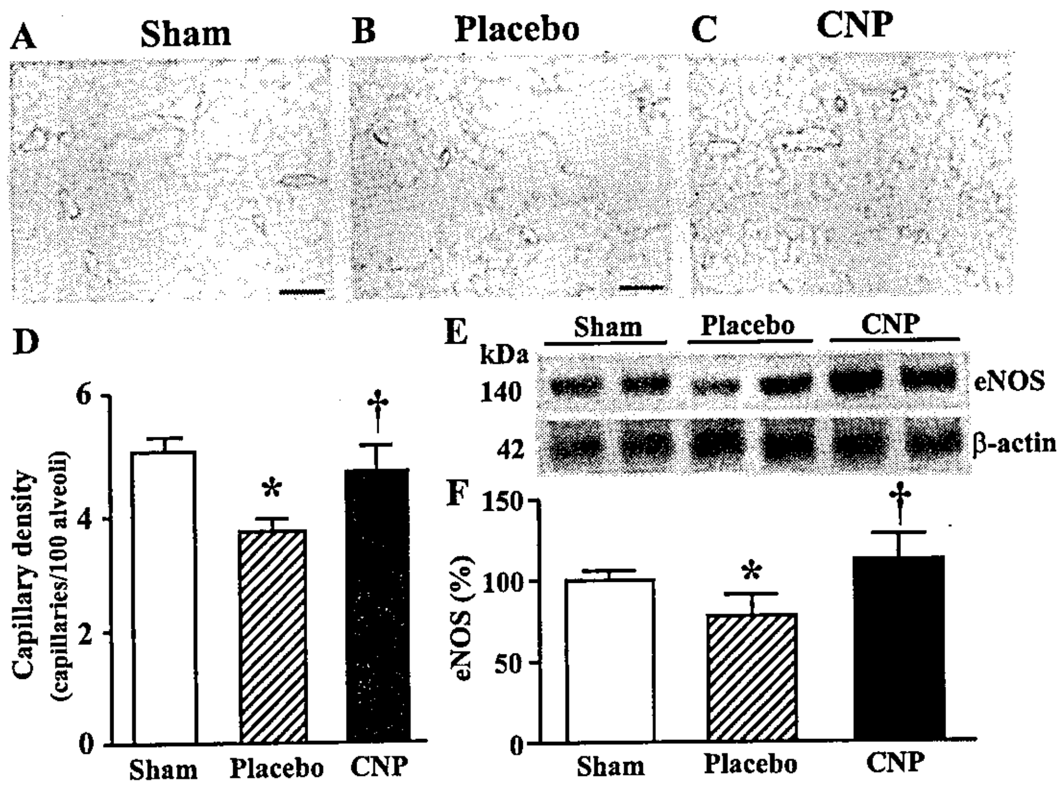

Figure 3. (*A–C*) Immunohistochemical demonstration of factor VIII antigen in lungs on Day 21. *Scale bars* = 100 μm. (*D*) Quantitative analysis of capillary density. (*E*) Representative Western blotting for endothelial nitric oxide synthase (eNOS) and β-actin (protein loading control) in lungs on Day 21. (*F*) Quantitative analysis of lung tissue content of eNOS. eNOS protein is shown as the percent of the level expressed in sham rats. CNP infusion significantly increased eNOS protein compared with placebo. Data are mean ± SEM. $^*p < 0.05$ versus sham; $^\dagger p < 0.05$ versus placebo.

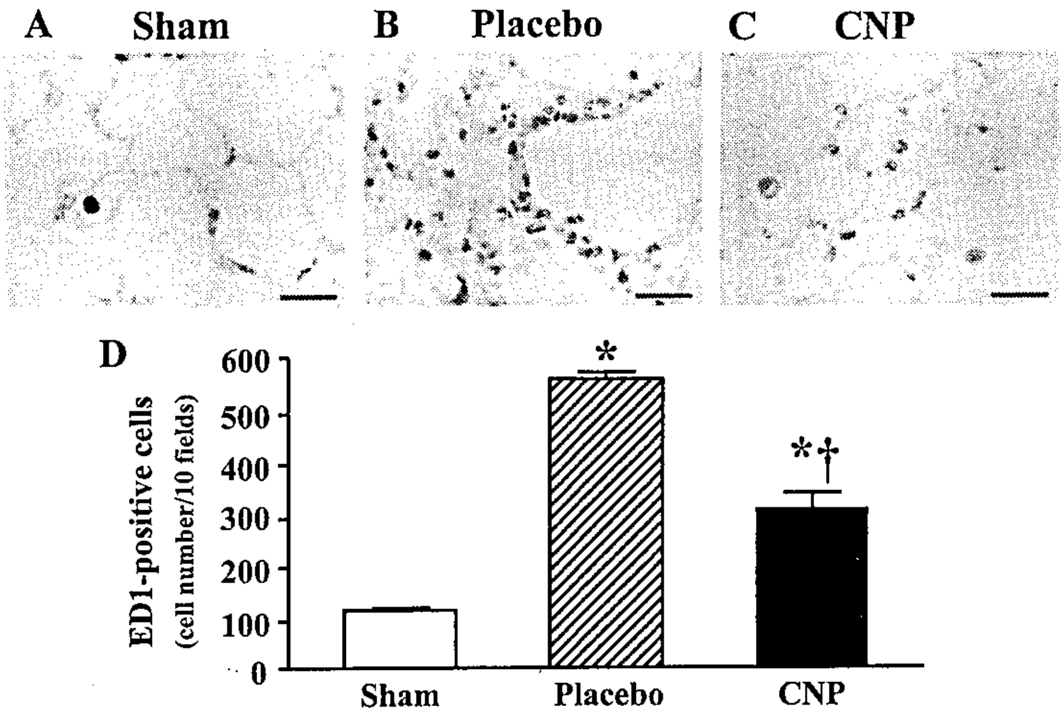

Figure 4. (*A–C*) Immunohistochemical demonstration of ED1 antigen, a marker for rat monocytes/macrophages, in lungs on Day 21. *Scale bars* = 50 μm. (*D*) Quantitative analysis of ED1-positive cells in lungs. The number of ED1-positive cells was significantly decreased in the CNP group compared with the placebo group. Data are mean ± SEM. *$p < 0.05$ versus sham; †$p < 0.05$ versus placebo.

cial effects in MCT rats, even if endogenous CNP had little physiologic significance under the condition of pulmonary hypertension. Earlier studies have shown that the vasodilator effect of CNP is much less potent than those of atrial natriuretic peptide (approximately 1:100) (9, 13, 21). In fact, unlike atrial natriuretic peptide, CNP infusion did not alter any hemodynamic parameters. These findings suggest that the pharmacologic effects of CNP are attributable to vasoprotective effects rather than to vasodilator activity.

MCT induces pulmonary endothelial cell injury and decreases the number of pulmonary capillaries (20, 22), which contributes to the development of MCT-induced pulmonary hypertension. A recent study has demonstrated that transplantation of endothelial progenitor cells attenuates MCT-induced pulmonary hypertension (23), suggesting that endothelial regeneration has beneficial effects on pulmonary hemodynamics. CNP has been shown to induce endothelial regeneration in an ischemic hindlimb model through the cGMP/cGMP-dependent protein kinase pathway (17). In this study, CNP infusion enhanced the expression of Ki-67, a marker for cell proliferation, in pulmonary endothelial cells. In addition, CNP increased the number of pulmonary capillaries in MCT rats. Interestingly, we demonstrated that CNP infu-

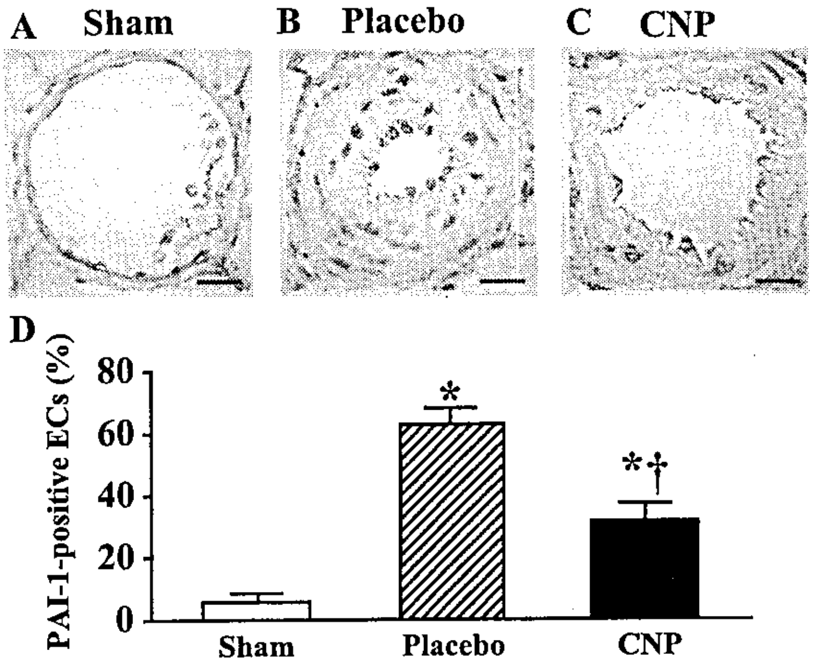

Figure 5. (*A–C*) Immunohistochemical demonstration of plasminogen activator inhibitor type 1 (PAI-1) expression in pulmonary ECs on Day 21. *Scale bars* = 20 μm. (*D*) Semiquantitative analysis of PAI-1–positive ECs. The percentage of PAI-1–positive ECs was calculated as (number of PAI-1–positive ECs/total number of ECs) × 100. Data are mean ± SEM. *$p < 0.05$ versus sham; †$p < 0.05$ versus placebo.

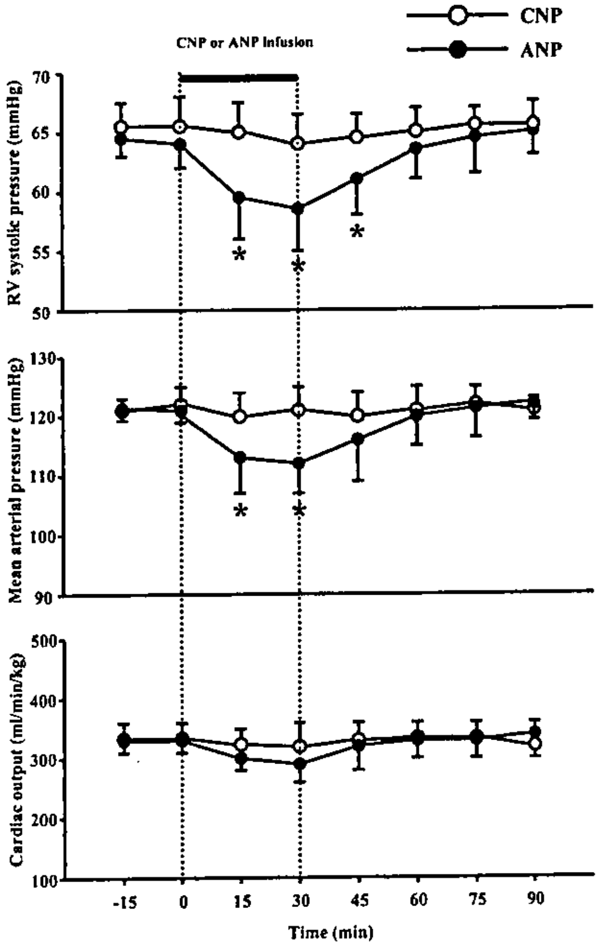

Figure 6. Acute hemodynamic responses to infusion of CNP (*open circles*) or arterial natriuretic peptide (ANP; *closed circles*) in MCT rats. Data are mean ± SEM. $^*p < 0.05$ versus time 0.

sion significantly augmented lung tissue content of endothelial nitric oxide synthase protein. Endothelial nitric oxide synthase is an enzyme that produces nitric oxide in vascular endothelial cells (24, 25), which has a pivotal role in the regulation of pulmonary vascular tone (26). In fact, Champion and colleagues have demonstrated that intratracheal gene transfer of endothelial nitric oxide synthase to the lung attenuates hypoxia-induced pulmonary hypertension in mice (27). Thus, the therapeutic effects of CNP on pulmonary hypertension may be mediated by regeneration of pulmonary endothelium and improvement in nitric oxide bioavailability in MCT rats.

MCT induces apoptosis of pulmonary endothelial cells *in vivo* and *in vitro* (28–30). In fact, in this study, MCT injection increased the number of apoptotic pulmonary endothelial cells. Recent studies have shown that inhibition of pulmonary endothelial apoptosis attenuates MCT-induced pulmonary hypertension (29, 30). Interestingly, CNP infusion decreased the number of apoptotic cells in the lung of MCT rats. Thus, not only an increase in cell proliferation but also a decrease in cell apoptosis may contribute to improvement in pulmonary hemodynamics by CNP therapy.

Inflammatory cells, including macrophages, neutrophilis, lymphocytes, and mast cells, are observed in pulmonary arteries under the condition of pulmonary hypertension in animals and humans (6–8, 31). Particularly, monocyte/macrophage infiltration has a pivotal role in the development of MCT-induced pulmonary hypertension in rats (32, 33). In this study, CNP infusion inhibited monocyte/macrophage infiltration in the lungs, as indicated by a marked decrease in ED1-positive cells in pulmonary arterioles. These findings suggest that inhibition of monocyte/macrophage infiltration by CNP contributes to the improvement in pulmonary hemodynamics.

PAI-1, the principle inhibitor of the plasminogen system, irreversibly inactivates both tissue and urokinase plasminogen activators (34). PAI-1 is secreted by endothelial cells (35), smooth muscle cells, and macrophages (36). Inhibition of plasminogen activation by PAI-1 impairs fibrinolysis and thereby promotes thrombosis (37). It has been reported that the fibrino-

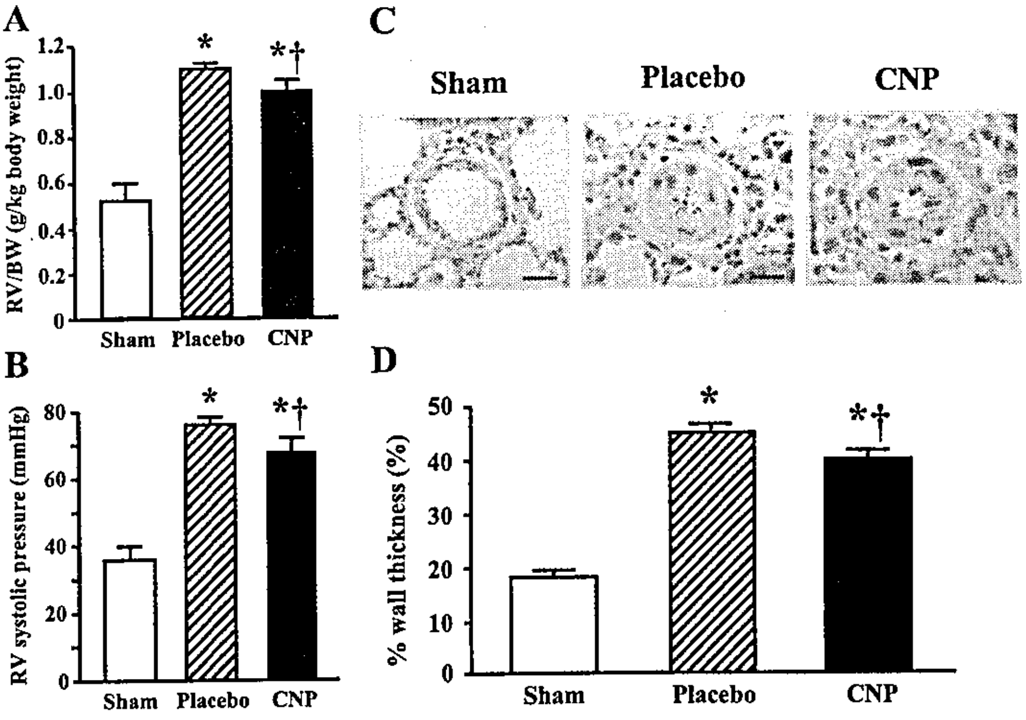

Figure 7. Effects of CNP infusion on established pulmonary hypertension. A continuous infusion of CNP was started 3 weeks after MCT injection. (*A*) RV/BW. (*B*) RV systolic pressure. (*C*) Representative photomicrographs of peripheral pulmonary arteries. *Scale bars* = 20 μm. (*D*) Quantitative analysis of percentage wall thickness in peripheral pulmonary arteries. Data are mean ± SEM. $^*p < 0.05$ versus sham; $^†p < 0.05$ versus placebo.

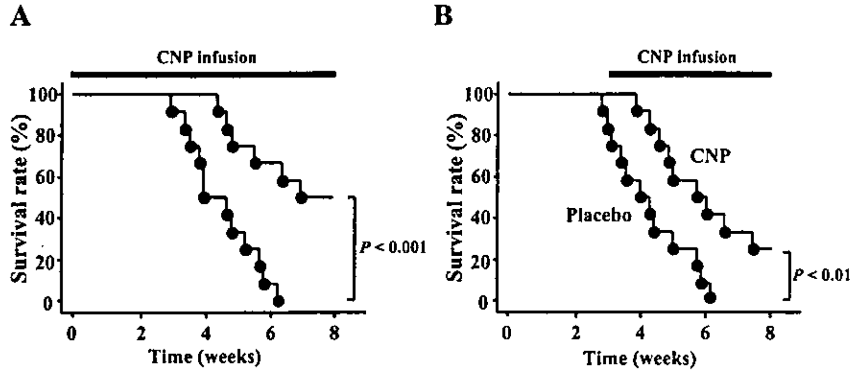

Figure 8. Kaplan-Meier survival curves. (*A*) Continuous infusion of CNP was initiated immediately after MCT injection. (*B*) CNP infusion was started 3 weeks after MCT injection.

lytic activity of lung tissue is decreased in MCT rats (38). These findings raise the possibility that PAI-1 may have a role in the development of MCT-induced pulmonary hypertension. In fact, immunohistologic examination demonstrated that MCT injection increased PAI-1 expression in pulmonary vessels. Recently, CNP has been shown to suppress PAI-1 expression in vascular smooth muscle cells and endothelial cells through an elevation of cGMP *in vitro* (39, 40). In this study, CNP infusion inhibited the MCT-induced increase in PAI-1 expression in pulmonary vessels. These findings suggest that CNP infusion ameliorates MCT-induced pulmonary hypertension at least in part through inhibition of fibrinolysis impairment.

CNP infusion also attenuated the increase in medial wall thickness of peripheral pulmonary arteries. CNP has also been shown to suppress the growth of vascular smooth muscle cells through an elevation of cGMP *in vitro* and inhibit the development of vascular remodeling of injured arteries *in vivo* (14). Thus, direct inhibitory effects of CNP on smooth muscle cell proliferation may contribute to inhibition of vascular remodeling.

Finally, we examined the effect of CNP on established pulmonary hypertension. CNP administration was started 3 weeks after MCT injection. CNP slightly but significantly attenuated the development of MCT-induced pulmonary hypertension. Importantly, CNP infusion improved survival not only in developing pulmonary hypertension but also in established pulmonary hypertension. Thus, continuous infusion of CNP may be a promising treatment for severe pulmonary hypertension.

This study includes some study limitations. First, the histology shown in the MCT model involves only smooth muscle hypertrophy. In contrast, the histopathology of pulmonary arterial hypertension in humans includes endothelial proliferation and fibrosis in addition to smooth muscle hypertrophy (2, 3). Thus, the results obtained from the MCT model may not be predictive of response to therapy in humans. Therefore, the initial success of CNP therapy reported here should be confirmed by further studies using other animal models of pulmonary hypertension before clinical trials. Second, the effects of CNP on pulmonary hemodynamics were modest. Unlike other vasodilators, however, CNP did not decrease systemic arterial pressure, which may be appropriate in the management of pulmonary hypertension. The improvement in pulmonary hypertension by CNP was mediated by multiple vasoprotective effects rather than by vasodilator activities. Thus, addition of CNP to conventional vasodilator therapy may be beneficial effects in patients with pulmonary hypertension. Finally, because the pathophysiologic role of CNP in cardiovascular disease is less well understood in humans, further studies are necessary to confirm the therapeutic potential of CNP in patients with pulmonary hypertension.

In conclusion, continuous infusion of CNP ameliorated MCT-induced pulmonary hypertension and improved survival in rats. These beneficial effects may be mediated by regeneration of pulmonary endothelium, inhibition of endothelial cell apoptosis, and prevention of monocyte/macrophage infiltration and fibrinolysis impairment after MCT injection.

Conflict of Interest Statement: T.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; N.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.I.-U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; C.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Review

Adrenomedullin in the treatment of pulmonary hypertension

Noritoshi Nagaya[a,*], Kenji Kangawa[b]

[a] *Department of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujisjirodai, Suita, Osaka 565-8565, Japan*
[b] *Department of Biochemistry, National Cardiovascular Center Research Institute, 5-7-1 Fujisjirodai, Suita, Osaka 565-8565, Japan*

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Abstract

Adrenomedullin (AM) is a potent, long-lasting pulmonary vasodilator peptide. Plasma AM level is elevated in patients with primary pulmonary hypertension (PPH), and circulating AM is partially metabolized in the lungs. These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. We have demonstrated the effects of three types of AM delivery systems: intravenous administration, inhalation, and cell-based gene transfer. Despite endogenous production of AM, intravenously administered AM at a pharmacologic level decreased pulmonary vascular resistance in patients with PPH. Inhalation of AM improved hemodynamics with pulmonary selectivity and exercise capacity in patients with PPH. Cell-based AM gene transfer ameliorated pulmonary hypertension rats. These results suggest that additional administration of AM may be effective in patients with pulmonary hypertension. AM may be a promising endogenous peptide for the treatment of pulmonary hypertension.

Keywords: Adrenomedullin; Pulmonary hypertension; Inhalation; Gene therapy

Contents

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* Corresponding author. Tel.: +81 6 6833 5012; fax: +81 6 6833 9865.
E-mail address: nagayann@hsp.ncvc.go.jp (N. Nagaya).

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1. Introduction

Primary pulmonary hypertension (PPH) is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure, and death [42,43]. Median survival is considered to be 2.8 years from the time of diagnosis. Because the presence of endothelial injury in the pulmonary vascular bed develops pulmonary vasoconstriction, smooth muscle cell proliferation, and in situ thrombosis [1], a variety of vasodilators, anti-proliferative agents, and anticoagulants have been proposed as therapeutic agents of PPH [3,10,23,45]. Despite therapeutic medical advances including prostacyclin therapy [3,23,45], some patients ultimately require heart–lung or lung transplantation [38,41]. Thus, a novel therapeutic strategy is desirable for the treatment of pulmonary hypertension including PPH.

Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma [19]. The peptide consists of 52 amino acids with an intramolecular disulfide bond, sharing slight homology with calcitonin gene-related peptide and amylin. Immunoreactive AM has subsequently been detected in plasma and a variety of tissues including blood vessels and lungs [13,47]. AM is metabolized by neutral endopeptidase protein in the kidney and by receptor binding in a variety of tissues. The half-life of AM is approximately 15 min. Earlier studies have shown that plasma AM level is increased in patients with hypertension [14] or heart failure [34]. Taking together its potent vasodilatory effect [19] and diuretic and natriuretic effects [21], AM may be involved in the regulation of the body fluid and thus in the cardiovascular homeostasis. We have shown that plasma AM level is elevated in patients with PPH, and that the plasma AM level increases in proportion to the severity of pulmonary hypertension [16]. It has been reported that there are abundant binding sites for AM in the lungs [37]. In fact, circulating AM is partially metabolized in the lungs [52]. These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. Earlier studies have shown that AM has a variety of biological effects, which are necessary for the treatment of pulmonary hypertension (Table 1). These actions of AM are mediated by calcitonin receptor-like receptor (CRLR) which functions as a selective AM receptor depending on the expression of the subtypes 2 and 3 of a family of receptor-activity-modifying proteins (RAMPs) [22]. AM acts through some signaling pathways: the cyclic adenosine 3′, 5′-monophosphate (cAMP), cyclic guanosine 3′, 5′-monophosphate (cGMP), phosphatidylinositol 3-kinase (PI3K)/Akt, and etc. These actions are induced by 0.01~0.1 ug/(kg min) in vivo and by 10^{-10} to 10^{-7} M in vitro. This article will summarize the therapeutic potential of AM for the treatment of pulmonary hypertension.

2. Intravenous administration of AM

In vivo studies have shown that intravenously administered AM causes vasodilation, diuresis, and a positive inotropic effect in an experimental model of heart failure [40]. In humans, intravenous administration of AM decreases systemic and pulmonary vascular resistance and increases cardiac output in patients with congestive heart failure, together with slight increases in urine volume and urinary sodium excretion [31]. Endogenous AM production is enhanced in a variety of cardiovascular diseases through a compensatory mechanism [29]. Nonetheless, additional supplementation of AM has beneficial effects in these diseases [27]. These results suggest that endogenous AM level is not sufficient enough to improve deteriorated conditions in spite of the increased AM production.

Experimental studies have shown that intralobar arterial infusion of AM causes dose-related decreases in pulmonary vascular resistance under conditions of high pulmonary vascular tone [9,20,36]. The vasodilatory effect is mediated by cAMP-dependent and nitric oxide-dependent mechanisms [15,32]. Thus, AM is known to be one of the most potent endogenous vasodilators in the pulmonary vascular bed. However, little information is available regarding the hemodynamic effects of intravenously administered AM in patients with pulmonary hypertension. Accordingly, we examined the hemodynamic and hormonal responses to intravenous infusion of AM (0.05 μg/kg/min) or placebo, were examined in 13 patients with pulmonary arterial hypertension including PPH [28]. Because AM-induced hypotension

Table 1
Beneficial effects of adrenomedullin for the treatment of pulmonary hypertension

Biological activity	Second messenger or signal
1. Potent pulmonary vasodilation	cAMP, NO/cGMP, PI3K/Akt
2. Inhibition of endothelial cell apoptosis	PI3K/Akt
3. Inhibition of smooth muscle cell proliferation and migration	cAMP, Ca^{2+}
4. Positive inotropic effect	cAMP, protein kinase C, Ca^{2+} release or influx
5. Diuresis and natriuresis	NO/cGMP, cAMP
6. Suppression of aldosterone production	Ca^{2+}
7. Induction of angiogenesis	PI3K/Akt, MEK/ERK
8. Anti-inflammation	cAMP

cAMP: cyclic adenosine 3′, 5′-monophosphate, cGMP: cyclic guanosine 3′, 5′-monophosphate, PI3K: phosphatidylinositol 3-kinase, NO: nitric oxide, ERK: extracellular signal-regulated kinase, MEK: mitogen-activated protein ERK kinase.

may cause adverse effects in patients with pulmonary hypertension, we used a relatively low dose of AM. Intravenous infusion of AM increased plasma AM level in patients with pulmonary hypertension (15 ± 1 to 48 ± 8 fmol/ml, cf. 10 ± 1 fmol/ml in healthy subjects). Infusion of AM significantly decreased pulmonary vascular resistance by 32%. In addition, AM decreased systemic vascular resistance without inducing a marked hypotension. The hemodynamic effects of AM lasted at least 15 min after the end of infusion. These results suggest that AM has potent, relatively long-lasting pulmonary vasodilator activity in patients with pulmonary hypertension. We have shown that administered AM increases plasma cAMP, but not cGMP, in patients with pulmonary hypertension, in association with its hemodynamic effects. The increase in cAMP in smooth muscle cells by AM activates protein kinase A, resulting in the decrease in calcium content in smooth muscle cells. It is therefore possible that AM may relax vascular smooth muscle through a cAMP/protein kinase A-dependent mechanism. On the other hand, Nossaman et al. [36] have shown that AM regulates pulmonary vascular tone in rats through an endothelium-derived nitric oxide-dependent mechanism. Nishimatsu et al. [35] have shown that AM induces Akt activation in the endothelium via the Ca^{2+}/calmodulin-dependent pathway and that this is implicated in the production of nitric oxide, which in turn induced endothelium-dependent vasodilation. Because the vascular effects of AM are known to vary with species and vascular regions, further studies are necessary to elucidate the mechanisms responsible for pulmonary vasodilator activity of AM in humans.

Intravenous infusion of AM markedly increased cardiac index in patients with pulmonary hypertension [28], consistent with our previous results from left sided heart failure [31]. Considering the strong vasodilator activity of AM in the systemic and pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for increased cardiac index with AM. On the other hand, a previous binding study has shown abundant, specific binding sites for AM in ventricular myocardium [37]. AM has been shown to increase cardiac cAMP [33], which is known to mediate the positive inotropic action of beta-adrenergic stimulants. Alternatively, AM has been shown to produce a positive inotropic action through cAMP-independent mechanisms [49]. These findings suggest that the increase in cardiac index may be attributable not only to a fall in cardiac afterload but also to the direct positive inotropic action of AM.

Infusion of AM significantly decreased plasma aldosterone, although there was no significant change in plasma renin activity. In vitro, AM has been shown to inhibit Ang II-induced secretion of aldosterone from dispersed rat adrenal zona glomerulosa cells [51]. Therefore, the inhibition of plasma aldosterone by AM was probably due to a direct effect on adrenal gland, as is the case for atrial natriuretic peptide [46].

It appears that a number of similarities in pharmacologic actions, i.e. vasodilatation, cardiac effect, and cAMP production, exist between AM and prostacyclin that is used for reducing pulmonary resistance in PPH. Unlike prostacyclin, however, AM has diuretic and natriuretic activities. AM inhibits inflammation and aldosterone production [7,51]. These biological effects may be the advantages of AM over prostacyclin in respect of therapeutic effectiveness. Exogenously administered AM at a pharmacologic level increased plasma cAMP in association with hemodynamic effects. Thus, additional administration of AM may be effective in patients with pulmonary hypertension.

3. Inhalation of AM

The goal of vasodilator therapy for patients with PPH is to reduce pulmonary vascular resistance without producing systemic hypotension, and improve quality of life and survival. We have shown that intravenous administration of AM markedly decreases pulmonary vascular resistance in patients with PPH [28]. Nevertheless, systemically administered AM decreases systemic arterial pressure, which may be harmful in treating patients with PPH. Recently, inhalation of aerosolized prostacyclin and its analogue, iloprost, has been shown to cause pulmonary vasodilation without systemic hypotension in patients with PPH [11,53]. In addition, inhalant application of vasodilators does not impair gas exchange because the ventilation-matched deposition of drug in the alveoli causes pulmonary vasodilation matched to ventilated areas. In clinical settings, inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. Thus, the purpose of this study was to investigate the effects of AM inhalation on hemodynamics and exercise capacity in patients with PPH.

Interestingly, Champion et al. [5] have shown that intratracheal gene transfer of calcitonin gene-related peptide (CGRP), a member of the same peptide family as AM, to bronchial epithelial cells attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. These results raise the possibility that intratracheal delivery of a vasodilator peptide may be sufficient to alter pulmonary vascular function. In fact, inhalation of AM significantly decreased pulmonary vascular resistance in patients with pulmonary hypertension, whereas it did not alter systemic arterial pressure or systemic vascular resistance [26]. The ratio of pulmonary vascular resistance to systemic vascular resistance was significantly reduced by AM inhalation. These results suggest that inhaled AM improves hemodynamics with pulmonary selectivity. This is consistent with earlier findings that inhaled prostacyclin or its analogue, iloprost, acts transepithelially with pulmonary selectivity and improves pulmonary hypertension.

We examined the long-term effects of inhaled AM in monocrotaline (MCT)-induced pulmonary hypertension rats [30]. AM or saline was inhaled as an aerosol using an ultrasonic nebulizer, for 30 min, four times a day. Repeated inhalation of AM for three weeks markedly decreased mean pulmonary arterial pressure and pulmonary vascular resistance in MCT rats without systemic hypotension. The potent, long-lasting pulmonary vasodilator effect of inhaled AM may contribute to the strong inhibition of the development of pulmonary hypertension. In addition, considering intermittent delivery of AM to the lungs, the chronic effects of inhaled AM appear to go beyond acute pulmonary vasodilation. Inhalation of AM inhibited an increase in the medial wall thickness of peripheral pulmonary arteries of MCT rats. In vitro studies have shown that AM inhibits the migration and proliferation of vascular smooth muscle cells [12,17]. Given the known potent vasoprotective effects of AM such as vasodilation and inhibition of smooth muscle cell migration and proliferation, it is interesting to speculate that AM trapped in the bronchial epithelium or alveoli leaks to the pulmonary arteries to maintain pulmonary vascular integrity in MCT rats. Importantly, Kaplan–Meier analysis demonstrated that the 6-week survival rate for MCT rats treated with aerosolized AM was significantly high (70%) as compared with 10% in those given saline [30]. Thus, treatment with aerosolized AM may be an alternative approach for severe pulmonary hypertension that is refractory to conventional therapy.

We have demonstrated that inhalation of AM has beneficial hemodynamic effects in animals and humans [26,30]. Recently, pulmonary delivery of a dry-powder insulin has been shown to improve glycemic control without adverse pulmonary effects [48]. Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary AM delivery, combining AM inhalation therapy with other modalities that have a different mode of action may have beneficial effects in patients with PPH.

4. Cell-based AM gene transfer

The pulmonary endothelium plays an important role in the regulation of pulmonary vascular tone through the release of vasoactive substances such as nitric oxide and prostacyclin [6]. Dysfunction of the endothelium may play a role in the pathogenesis of pulmonary hypertension including PPH [4]. Thus, pulmonary endothelial cell may be a therapeutic target for the treatment of pulmonary hypertension. Recently, endothelial progenitor cells have been discovered in adult peripheral blood [2]. EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ [8,18,50]. These findings raise the possibility that transplanted EPCs may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. Thus, we investigated whether cell (EPCs)-based AM gene transfer ameliorates MCT-induced pulmonary hypertension in rats.

We obtained EPCs from cultured human umbilical cord blood mononuclear cells and constructed AM plasmid DNA. We used cationic gelatin to produce ionically linked DNA–gelatin complexes. Interestingly, EPCs phagocytosed plasmid DNA–gelatin complexes, which allowed nonviral, highly efficient gene transfer into EPCs [24]. Recently, intravenously administered hematopoietic cells have been shown to be attracted to sites of cerebral injury [39]. Intravenously injected EPCs accumulate in ischemic myocardium after acute myocardial infarction [18]. These findings suggest that progenitor cells have the capability to sense injured tissues. In fact, intravenously administered gene-modified EPCs were incorporated into pulmonary arterioles and capillaries in MCT rats and differentiated mature endothelial cells [25]. MCT injures endothelial cells of small arteries and capillaries in the lungs, resulting in pulmonary hypertension [44]. Taking these findings together, transplanted EPCs may circulate in the blood and attach to injured pulmonary endothelia in MCT rats. Thus, EPCs may serve not only as a vehicle for gene delivery to injured pulmonary endothelia, but also as a tissue-engineering tool in restoring intact pulmonary endothelium. Transplantation of EPCs without gene modification slightly, but significantly decreased pulmonary vascular resistance in MCT rats [25]. EPCs have been shown to express endothelial nitric oxide synthase and produce nitric oxide [24]. We showed that EPCs produce AM even when its gene is not transduced. These results suggest that vasodilator substances secreted from EPCs contribute to improvement in pulmonary hypertension. We also investigated whether transplantation of gene-modified EPCs causes further improvement in pulmonary hemodynamics and survival in MCT rats [25]. Interestingly, EPCs cultured with AM DNA–gelatin complexes markedly secreted AM protein for more than 2 weeks. These results suggest relatively long-lasting AM secretion from EPCs. The consequence of this synthesis in MCT rats was a marked decrease in mean pulmonary arterial pressure and pulmonary vascular resistance. Histological examination revealed that transplantation of AM-expressing EPCs inhibited an increase in medial wall thickness of pulmonary arteries. Expectedly, transplantation of AM-expressing EPCs caused significantly greater improvement in pulmonary hypertension and vascular remodeling than transplantation of EPCs alone. Given the known potent vasoprotective effects of AM such as vasodilation and inhibition of smooth muscle cell proliferation [12,17], it is interesting to speculate that AM secreted from EPCs may act not only as a circulating factor but also as a autocrine/paracrine factor in the regulation of pulmonary vascular tone and vascular remodeling in MCT rats. Importantly, a single transplantation of AM-expressed EPCs improved survival in MCT rats as compared with administration of EPCs alone or culture medium. These results suggest that ex vivo gene transfer into EPCs greatly enhances therapeutic effects of EPCs transplantation. Further studies are necessary to examine whether repeated administration of EPCs produces an even greater effect than single transplantation.

5. Summary

This article described the therapeutic potential of AM for the treatment of pulmonary hypertension. Baseline plasma AM is significantly higher in patients with pulmonary arterial hypertension. Nevertheless, exogenously administered AM at a pharmacologic level induces hemodynamic improvement. This suggests that an additional administration of AM may be effective in patients with pulmonary hypertension. We have demonstrated the effects of three types of AM delivery systems: intravenous administration of AM peptide, inhalation of AM peptide, and cell-based AM gene transfer. Further studies are necessary to examine which delivery system is the best in clinical settings. AM induces potent pulmonary vasodilation and has vasoprotective effects beyond vasodilation. Thus, AM is a promising endogenous peptide for the treatment of pulmonary arterial hypertension.

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Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats

Takafumi Fujii,[1] Noritoshi Nagaya,[2,3] Takashi Iwase,[2] Shinsuke Murakami,[2] Yoshinori Miyahara,[1] Kazuhiro Nishigami,[3] Hatsue Ishibashi-Ueda,[5] Mikiyasu Shirai,[1] Takefumi Itoh,[2] Kozo Ishino,[6] Shunji Sano,[6] Kenji Kangawa,[4] and Hidezo Mori[1]

Departments of [1]Cardiac Physiology, [2]Regenerative Medicine and Tissue Engineering, [3]Internal Medicine, [4]Biochemistry, and [5]Pathology, National Cardiovascular Center, Osaka; and [6]Department of Cardiovascular Surgery, Okayama University Medical School, Okayama, Japan

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Fujii, Takafumi, Noritoshi Nagaya, Takashi Iwase, Shinsuke Murakami, Yoshinori Miyahara, Kazuhiro Nishigami, Hatsue Ishibashi-Ueda, Mikiyasu Shirai, Takefumi Itoh, Kozo Ishino, Shunji Sano, Kenji Kangawa, and Hidezo Mori. Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* 288: H1444–H1450, 2005. First published November 11, 2004; doi: 10.1152/ajpheart.00266.2004.—Adrenomedullin (AM), a potent vasodilator, induces angiogenesis and inhibits cell apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. Transplantation of bone marrow-derived mononuclear cells (MNC) induces angiogenesis. We investigated whether infusion of AM enhances the therapeutic potency of MNC transplantation in a rat model of myocardial infarction. Immediately after coronary ligation, bone marrow-derived MNC (5×10^6 cells) were injected into the ischemic myocardium, followed by subcutaneous administration of 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ AM (AM-MNC group) or saline (MNC group) for 3 days. Another two groups of rats received subcutaneous administration of AM alone (AM group) or saline (control group). Hemodynamic and histological analyses were performed 4 wk after treatment. Cardiac infarct size was significantly smaller in the MNC and AM groups than in the control group. A combination of AM infusion and MNC transplantation demonstrated a further decrease in infarct size. Left ventricular (LV) maximum change in pressure over time and LV fractional shortening were significantly improved only in the AM-MNC group. AM significantly increased capillary density in ischemic myocardium, suggesting the angiogenic potency of AM. AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Although MNC apoptosis was frequently observed 72 h after transplantation, AM markedly decreased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells among the transplanted MNC. In conclusion, AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC.

angiogenesis; apoptosis; mononuclear cell

DESPITE THE RECENT REMARKABLE progress in medical and surgical treatment for ischemic heart disease, this disease remains a major cause of death worldwide (5). Bone marrow-derived mononuclear cells (MNC) contain various kinds of cell lineages and numerous cytokines that contribute to neovascularization (1, 15). In fact, autologous transplantation of bone marrow cells has been shown to enhance angiogenesis and improve cardiac function in an animal model of cardiac ischemia (6, 9, 10). Recent human studies have demonstrated beneficial effects of transplanted MNC in patients with ischemic heart disease (23, 25). However, some patients fail to respond to this cell therapy. Thus a novel therapeutic strategy to enhance the angiogenic property of MNC is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (8). We have shown that infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure (17). On the other hand, AM has been shown to activate the phosphatidylinositol 3-kinase (PI3-kinase)/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis including endothelial cell proliferation, migration, and capillary-like formation (14, 22). In fact, we have shown that AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia via activation of Akt (24). These findings suggest that AM may play an important role in the regulation of vascular regeneration. In addition, AM has been shown to exert an antiapoptotic effect on a variety of cells including vascular endothelial cells (7, 20). Taking these findings together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of ischemic heart disease.

Thus the purposes of this study were *1*) to investigate whether infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of myocardial infarction, and 2) to investigate the effects of AM on survival and differentiation of the transplanted MNC to examine the underlying mechanisms of the effects induced by AM.

MATERIALS AND METHODS

Animal model. Myocardial infarction was produced in male Lewis rats weighing 200–220 g by left coronary ligation. In brief, after rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt), they were ventilated artificially. The heart was exposed via left thoracotomy, and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium using a 6-0 proline suture. Finally, the heart was restored to its normal position, and the chest was closed. The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

Address for reprint requests and other correspondence: N. Nagaya, Dept. of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan (E-mail: nnagaya@ri.ncvc.go.jp).

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Preparation of MNC. After Lewis rats were killed, bone marrow from the femur and tibia was collected and put in PBS. Marrow cells were loaded on a 1.077 gradient of Ficoll (Lymphoprep; Nycomed Pharma, Oslo, Norway) and centrifuged at 1,500 rpm for 20 min. The cells were then washed with 10 ml PBS to remove the Ficoll and centrifuged at 2,000 rpm for 10 min. The cells were finally suspended in PBS at a concentration of 5×10^6 cells in 50 μl PBS for transplantation. Fluorescence-activated cell sorting analysis demonstrated that 22 ± 1% of MNC were positive for lectin from ulex europaeus (UEA)-1 lectin (Sigma, St. Louis, MO).

MNC transplantation and AM infusion. Transplantation of bone marrow-derived MNC and/or 3-day infusion of AM was performed immediately after coronary ligation. MNC (5×10^6 cells in 50 μl PBS) were injected into the myocardium at five points in the border zone surrounding the infarct by using a 27-gauge needle. Recombinant human AM (0.05 μg·kg^{-1}·min^{-1}) was subcutaneously administered by using an osmotic minipump (model 2004; Alza, Palo Alto, CA) for 3 days. The pump was positioned in a pocket constructed in the subcutaneous tissue just below the subscapular region. For control, 5% glucose was infused in a similar manner in the rats receiving coronary ligation. This protocol resulted in the creation of four groups: *1*) AM infusion plus MNC transplantation (AM-MNC group, $n = 15$), *2*) vehicle infusion plus MNC transplantation (MNC group, $n = 14$), *3*) AM infusion plus PBS injection (AM group, $n = 14$), and *4*) vehicle infusion plus PBS injection (control group, $n = 13$).

Echocardiographic studies. Echocardiographic studies were performed 4 wk after surgery using a 7.5-MHz phased-array transducer (model HP SONOS 5500; Hewlett-Packard, Andover, MA). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt) as a supplement to maintain mild anesthesia. M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular (LV) end-diastolic and end-systolic dimension, and LV fractional shortening were measured from three consecutive cardiac cycles by the American Society for Echocardiology leading-edge method (21).

Cardiac catheterization. Cardiac catheterization was performed 4 wk after surgery. Rats were anesthetized with intraperitoneal pentobarbital and placed on a heating pad to maintain body temperature at 37–38°C throughout the study. A 1.5 Fr micronanometer-tipped catheter was inserted in the right carotid artery for measurement of heart rate and mean arterial pressure. The catheter was then advanced into the LV for measurement of LV end-diastolic pressure and then replaced with a thermomicroprobe for measurements of cardiac output. These hemodynamic variables were measured with a pressure transducer (UFI, Morro Bay, CA) connected to a polygraph and recorded with a thermal recorder (model 7758 B system; Hewlett-Packard).

Infarct size measurement. After completion of hemodynamic measurements, the heart was arrested by an injection of 2 mmol KCl through the carotid artery, and the cardiac ventricles were excised. The size of myocardial infarction was determined by a previously described method (2). In brief, incisions were made in the LV so that the tissue could be pressed flat. The circumference of the entire flat LV and the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarction size and was expressed as a percentage of LV surface area.

Histological analysis of microvessel density. LV myocardium was fixed in 10% formalin. Three cross sections of the LV, cut from apex to base, were obtained from individual rats for comparison among four groups ($n = 5$ each). They were embedded in paraffin and stained with Masson's trichrome for measurement of interstitial fibrosis. In other rats ($n = 5$ each), LV myocardium was embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen, and cut into 5-μm-thick sections. Tissue sections were stained for alkaline phosphatase with an indoxyltetrazolium method to detect capillary endothelial cells ($n = 5$ in each group). The number of capillary vessels was counted in the peri-infarct area (a 1.0-mm band next to the scar) excluding scar region using a light microscope at a magnification of ×200. The numbers in five high-power fields in each rat were averaged and expressed as the number of capillary vessels. These morphometric studies were performed by two examiners who were blinded to treatment.

Detection of MNC apoptosis. To examine the antiapoptotic effect of AM on transplanted MNC, red fluorescence-labeled MNC were transplanted into ischemic myocardium in rats with ($n = 5$) and without ($n = 5$) AM infusion. Before implantation into the ischemic heart, suspended MNC were labeled with fluorescent dyes with a PKH26 (Red Fluorescent Cell Linker Kit; Sigma), as reported previously (13). AM was subcutaneously administered by using a minipump for 3 days. Rats were killed 72 h after MNC transplantation. The LV was enucleated, and muscle samples were embedded in OCT compound and snap frozen in liquid nitrogen for the detection of apoptosis. Serial sections of the heart were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) for apoptosis using an in situ apoptosis detection kit (model S7111 Apoptag Fluorescein Kit; Intergen). Apoptosis of transplanted MNC was also evaluated by the detection of cleaved caspase-3-positive cells. In brief, the frozen tissue sections were incubated with anticleaved caspase-3 antibody (Cell Signaling), followed by incubation with FITC-conjugated IgG antibody (BD Pharmingen, San Diego, CA). The number of TUNEL/PKH26 double-positive cells and caspase-3/PKH26 double-positive cells was counted in 10 fields of each rat using a confocal microscopy (Fluoview model 500; Olympus, Tokyo, Japan).

The antiapoptotic effect of AM on MNC was also evaluated by in vitro TUNEL assay. MNC were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 h with control buffer, AM (1×10^{-7} M), or AM plus wortmannin, a PI3-kinase inhibitor (50 nM). Randomly selected microscopic fields ($n = 10$) were evaluated for calculating the ratio of TUNEL-positive cells to total cells.

Monitoring of implanted MNC in ischemic heart. Additional rats were used to examine whether transplanted MNC differentiate into endothelial cells, cardiomyocytes, vascular smooth muscle cells, or macrophages in the ischemic heart. PKH26 (red fluorescence)-labeled MNC were injected into the ischemic heart in rats with ($n = 8$) and without ($n = 8$) AM infusion. These subgroups of rats were killed 4 wk after coronary ligation. To identify vascular endothelial cells in vivo, FITC-labeled UEA-1 lectin was intravenously administered 30 min before the rats were killed ($n = 5$ in each group). The LV was enucleated, and muscle samples were then embedded in OCT compound, snap frozen in liquid nitrogen, and cut into sections. Sections were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) to detect nuclei. The number of DAPI/PKH26 double-positive cells and lectin-positive cells in the peri-infarct area was counted in 10 fields of each rat using a confocal microscopy. Frozen sections from other rats ($n = 3$ in each group) were incubated with mouse anticardiac troponin T (Novocastra, Newcastle, UK), anti-α-smooth muscle actin antibody (Dako, Copenhagen, Denmark), and anti-ED1 antibody (Serotec, Oxford, UK), followed by incubation with FITC-conjugated IgG antibody. In other rats (MNC group, $n = 5$; AM-MNC group, $n = 5$), the cardiac muscle from base to apex was transversely cut into 6-μm slices to calculate the number of transplanted MNC present within the heart 4 wk after transplantation. These morphometric studies were performed by two examiners who were blinded to treatment.

Statistical analysis. Numerical values were expressed as means ± SE. Comparisons of parameters among the four groups were performed by one-way ANOVA, followed by Newman-Keuls test for unpaired data. Comparisons of parameters between two groups were made by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

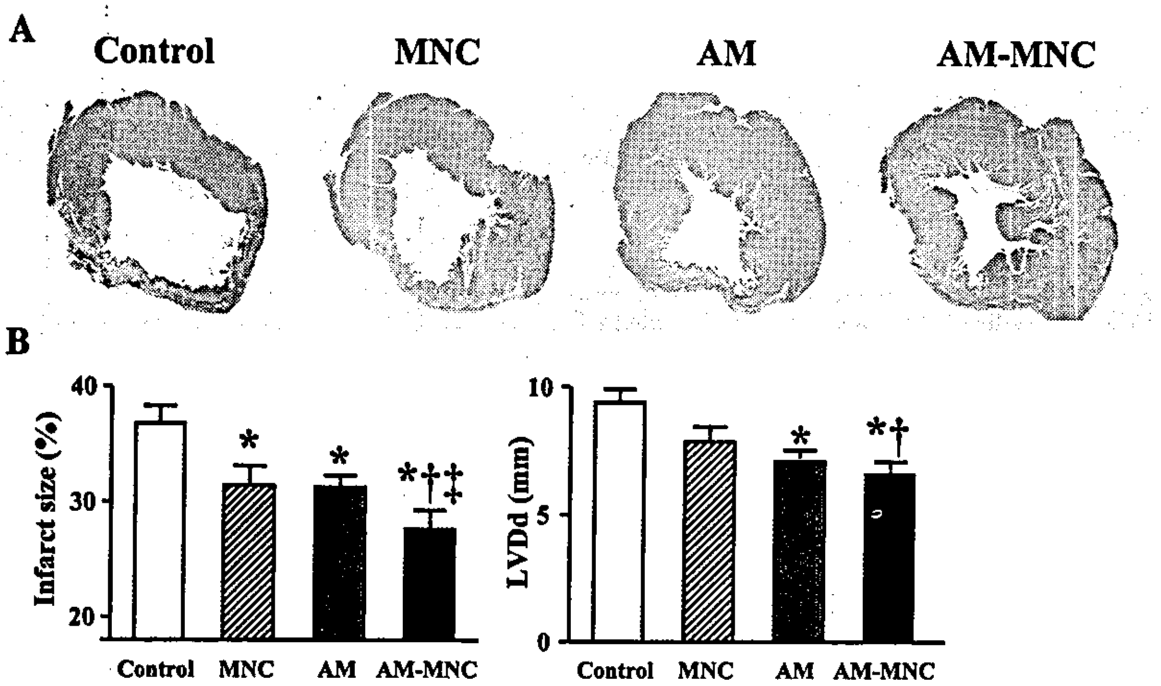

Fig. 1. *A*: representative examples of Masson trichrome-staining of transverse sections of left ventricular (LV) myocardium 4 wk after coronary ligation. *B*: quantitative analysis of infarct size and LV chamber size. Infarcted area and LV end-diastolic diameter (LVDd) of the adrenomedullin-mononuclear cell (AM-MNC) group were significantly smaller than those of the other groups. Values are means ± SE. $*P < 0.05$ vs. control; $†P < 0.05$ vs. MNC; $‡P < 0.05$ vs. AM.

RESULTS

Infarct size and ventricular weight. Moderate-to-large infarcts were observed in the control group after coronary ligation (Fig. 1). However, infarct size was smaller in the MNC, AM, and AM-MNC groups than in the control group. In particular, it was very small in the AM-MNC group. Quantitative analysis also demonstrated that cardiac infarct size in the AM-MNC group was smallest among the four groups. Right ventricular weight was significantly lower in the AM and AM-MNC groups than that in the control group (Table 1). LV weight did not significantly differ among the four groups.

Echocardiographic findings. LV diastolic dimension was smallest in the AM-MNC group, followed by the AM, MNC, and control groups (Fig. 1). LV fractional shortening in the AM-MNC group was also higher than that in the control, MNC, and AM groups (Table 2). Diastolic thickness of the anterior wall was significantly attenuated in the MNC, AM, and AM-MNC groups compared with the control group.

Table 1. *Physiological profiles of four experimental groups*

	Control	MNC	AM	AM-MNC
Number	13	14	14	15
Body weight, g	274±3	285±5	287±3	305±4*
Heart rate, bpm	410±24	404±30	398±33	387±36
MAP, mmHg	101±11	104±13	103±9	116±14*
LV wt/body wt, g/kg	2.4±0.2	2.5±0.2	2.6±0.1	2.5±0.2
RV wt/body wt, g/kg	1.1±0.1	0.9±0.1	0.8±0.1*	0.7±0.1*

Values are means ± SE; number is number of rats in each group. Control group, myocardial infarction rats given vehicle; MNC group, those given mononuclear cells; AM, those given adrenomedullin; AM-MNC, those given AM and MNC; MAP, mean arterial pressure; LV, left ventricle; RV, right ventricle. $*P < 0.05$ vs. control.

Hemodynamics. Cardiac output in the AM-MNC group was significantly higher than that in the control, MNC, and AM groups (Fig. 2). LV end-diastolic pressure in the MNC, AM, and AM-MNC groups was significantly lower than that in the control group. LV maximum change in pressure over time (dP/d*t*) in the MNC and AM-MNC group were significantly higher than that in the control group. Similarly, LV minimum dP/d*t* was significantly decreased only in the AM-MNC group.

Capillary density. Alkaline phosphatase staining of ischemic myocardium showed marked augmentation of neovascularization in the MNC, AM, and AM-MNC groups compared with the control group (Fig. 3*A*). Quantitative analysis demonstrated that capillary density was significantly higher in the AM-MNC group than in the MNC and AM groups (Fig. 3*B*). Cartilage, bone, or fat was not observed in the transplanted area. No tumor-like cells were seen.

Antiapoptotic effect of AM on MNC. Red fluorescence-labeled MNC were detected in each recipient heart 72 h after transplantation (Fig. 4). TUNEL-positive cells were frequently observed in the MNC group. In contrast, these apoptotic cells

Table 2. *Echocardiographic findings*

	Control	MNC	AM	AM-MNC
LVDd, mm	9.9±0.2	8.3±0.3	7.3±0.2*	6.9±0.3*†
LVDs, mm	8.4±0.3	6.6±0.4	5.8±0.2*	5.1±0.2*
%FS, %	14±1	22±1*	21±1*	26±1*†‡
AWT diastole, mm	1.0±0.2	1.3±0.3*	1.3±0.3*	1.4±0.4*
PWT diastole, mm	1.5±0.5	2.2±0.4	2.1±0.4	2.2±0.4

Values are means ± SE. LVDd, LV diastolic dimension; LVDs, LV systolic dimension; %FS, LV fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. $*P < 0.05$ vs. control; $†P < 0.05$ vs. MNC; $‡P < 0.05$ vs. AM.

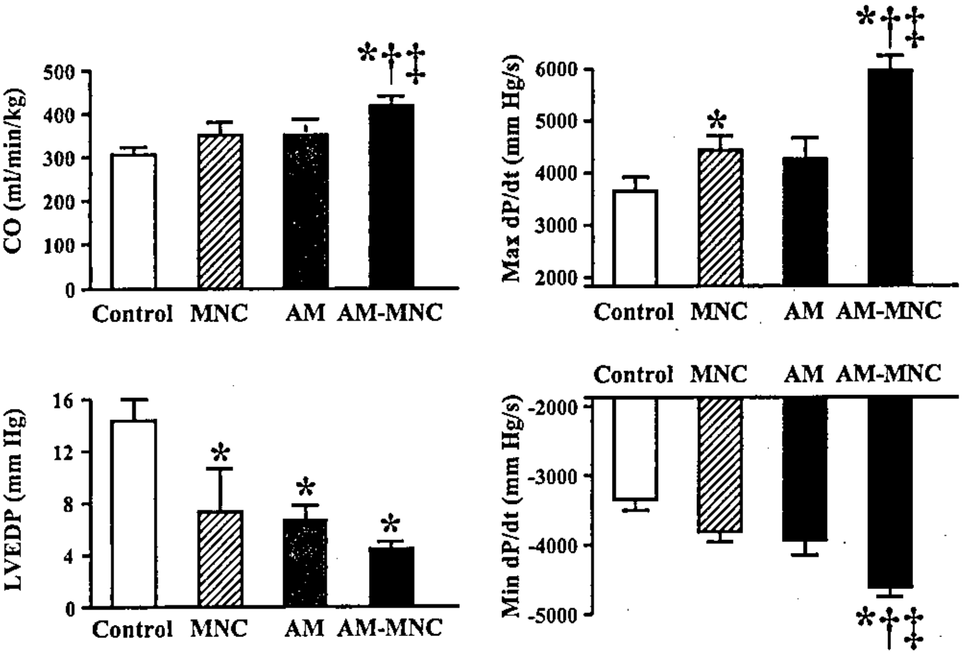

Fig. 2. Effects of AM infusion and MNC transplantation on hemodynamic parameters. CO, cardiac output; LVEDP, LV end-diastolic pressure; Max dP/d*t*, LV maximum change in pressure over time; Min dP/d*t*, LV minimum dP/d*t*. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

were hardly detected in the AM-MNC group. Semiquantitative analysis demonstrated that the number of TUNEL-positive MNC was significantly lower in the AM-MNC group than in the MNC group. Similarly, the number of caspase-3-positive MNC was significantly lower in the AM-MNC group than in the MNC group. These results suggest that infusion of AM inhibits apoptosis of transplanted MNC.

In vitro, serum starvation induced MNC apoptosis. When incubated in the presence of AM (1×10^{-7} M), the percentage of TUNEL-positive cells decreased significantly (19 ± 1 to 9 ± 1%, $P < 0.05$). However, pretreatment with wortmannin, a PI3-kinase inhibitor, diminished the antiapoptotic effect of AM (17 ± 1%).

Differentiation of MNC into endothelial lineage. Four weeks after transplantation, fluorescence-labeled transplanted cells

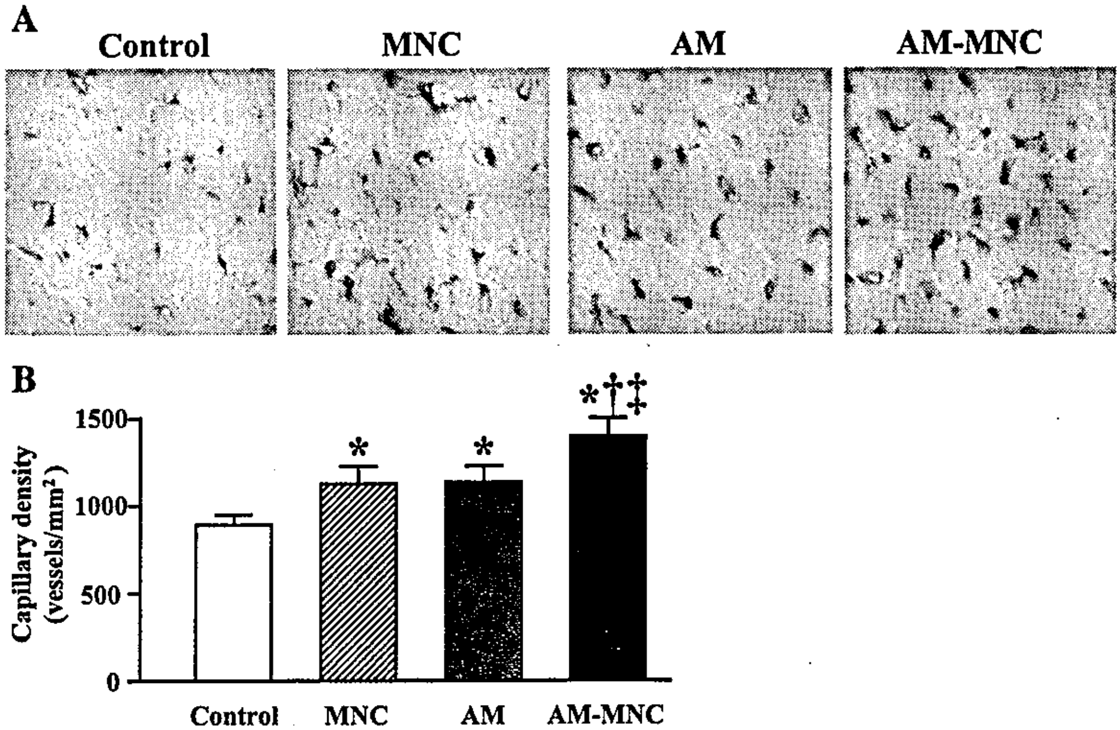

Fig. 3. *A*: representative examples of alkaline phosphatase staining in peri-infarct area. A combination of AM infusion and MNC transplantation markedly induced myocardial neovascularization. Magnification, ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Capillary density in the AM-MNC group was significantly higher than that in the MNC and AM groups. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

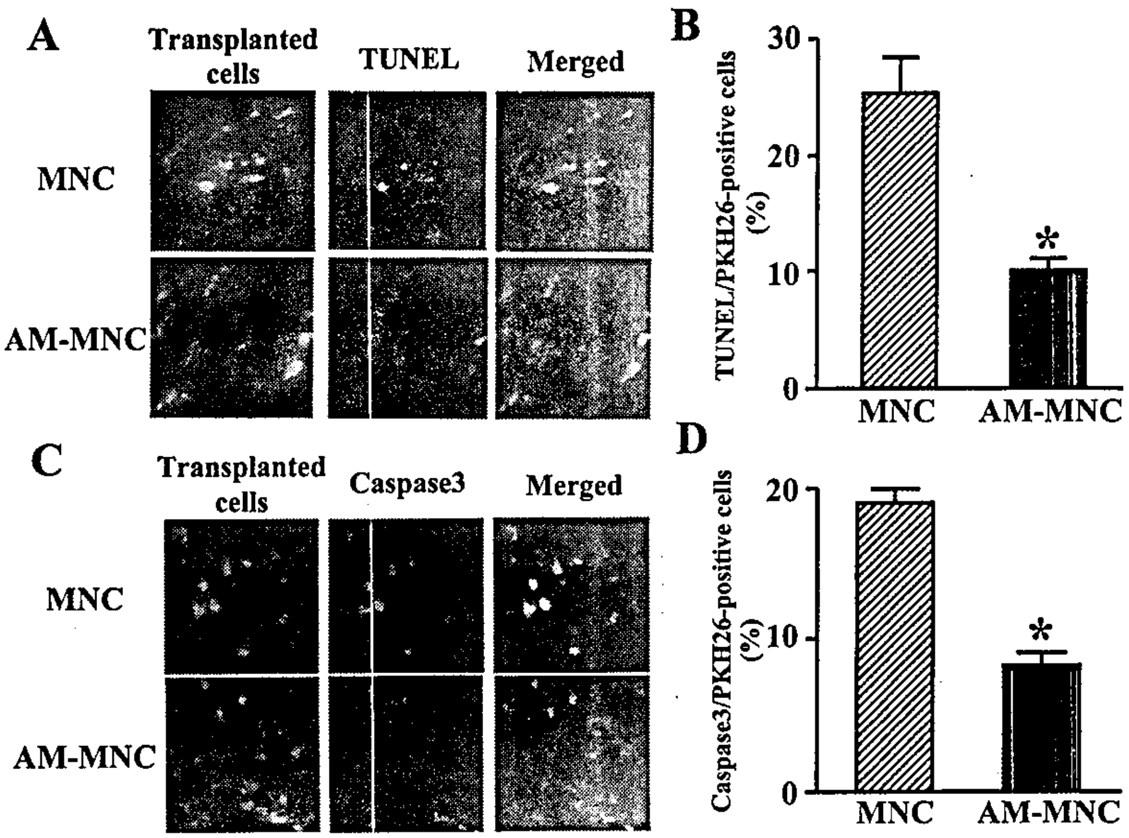

Fig. 4. Detection of transplanted cell apoptosis. *A*: representative photographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates TUNEL-positive cells. TUNEL-positive cells were frequently observed in the MNC group, whereas they were hardly detected in the AM-MNC group. Magnification, ×400. *B*: semiquantitative analysis of TUNEL-positive cells in the PKH26-positive (transplanted) cells. *C*: representative photographs of caspase-3 staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates caspase-3-positive cells. *D*: semiquantitative analysis of caspase-3-positive cells in the PKH26-positive cells. Values are means ± SE. *$P < 0.05$ vs. control.

were more frequently observed in the AM-MNC group than in the MNC group (6.4 ± 0.4 to 3.1 ± 0.2%, $P < 0.05$). Moreover, some of the transplanted cells were positive for UEA-1 lectin in the AM-MNC group (Fig. 5*A*), suggesting differentiation of MNC into vascular endothelial cells. Semiquantitative analysis demonstrated that the number of DAPI/PKH26 double-positive cells (viable transplanted cells) was significantly higher in the AM-MNC group than in the MNC group (Fig. 5*B*). Moreover, the ratio of lectin-positive cells to DAPI/PKH26 double-positive cells was significantly higher in the AM-MNC group than in the MNC group. The ratio of DAPI/PKH26 double-positive cells to lectin-positive cells was small, but significantly higher in the AM-MNC group than in the MNC group (23.9 ± 0.9 to 17.2 ± 0.6%, $P < 0.01$). Transplanted MNC were negative for troponin T or α-smooth muscle actin-positive cells. Some of the transplanted MNC were positive for ED1, a marker of macrophage (data not shown).

DISCUSSION

In the present study, we demonstrated that *1*) infusion of AM enhanced the angiogenic potency of MNC in a rat model of acute myocardial infarction, resulting in decreased infarct size and improved cardiac function. We also demonstrated that *2*) AM induced angiogenesis and inhibited apoptosis of the transplanted MNC. Thus a combination of AM and MNC may have beneficial effects in rats with myocardial infarction, partly through the angiogenic potency of AM itself and through its antiapoptotic effect on MNC.

Bone marrow-derived MNC include a variety of stem and progenitor cells (1, 15, 19), some of which can differentiate into endothelial cells and secrete numerous cytokines and chemokines (6, 9, 10). Earlier studies (6, 9, 10, 23, 25) have shown that autologous bone marrow transplantation induces angiogenesis and improves LV function in animals and humans. However, some patients are refractory to this cell therapy. Thus an approach to augment the angiogenic potency of MNC transplantation is required.

The present study showed that MNC transplantation or AM infusion alone reduced infarct size. A combination of AM infusion and MNC transplantation resulted in further decreases in infarct size and LV chamber size. MNC transplantation or AM administration modestly improved LV function. On the other hand, a combination of MNC and AM significantly improved cardiac performance compared with MNC or AM alone, as indicated by increases in cardiac output, fractional shortening, and LV maximum dP/d*t*. Earlier studies (6, 9, 10) have reported that MNC transplantation induces therapeutic angiogenesis and preserves LV function through inhibition of cardiomyocyte apoptosis in animal models of myocardial infarction. We have shown that AM infusion during the acute phase of ischemia-reperfusion inhibits apoptosis of cardiomyocytes and produces hemodynamic improvement in an animal

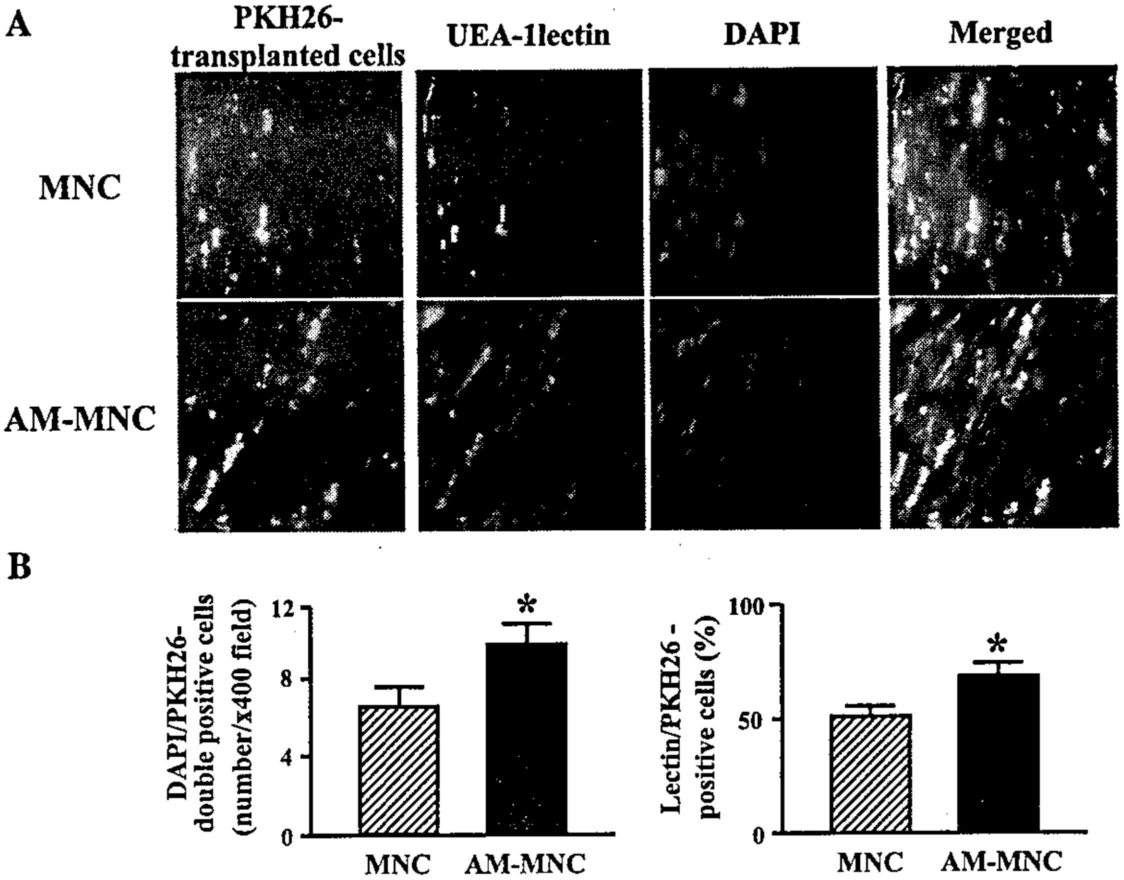

Fig. 5. *A*: representative examples of MNC differentiation into endothelial lineage. Red fluorescence (PKH26) marks transplanted cells; green fluorescence indicates ulex europaeus (UEA)-I lectin, a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial cells in the AM-MNC group. Magnification, ×400. *B*: quantitative analysis of living transplanted cells and endothelial differentiation. The number of living cells after transplantation was significantly higher in the AM-MNC group than in the MNC group. The ratio of lectin-positive cells to living transplanted cells was significantly higher in the AM-MNC group than in the MNC group. Values are means ± SE. $*P < 0.05$ vs. control. DAPI, 4′,6′-diamidino-2-phenylindole.

study (18). These findings suggest that the reduction of infarct size induced by this combination therapy may be attributable to additive cardioprotective effects of MNC and AM.

The present study showed that AM infusion significantly increased capillary density in ischemic myocardium. Furthermore, AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Contribution of transplanted MNC to neovascularization (the ratio of DAPI/PKH26 double-positive cells to lectin-positive cells) was significantly greater in the AM-MNC group than in the MNC group. A recent study (14) has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells and enhances angiogenesis in a murine gel plug assay through the PI3-kinase/Akt pathway. We have also shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a rabbit model of chronic hindlimb ischemia via activation of Akt (24). These findings suggest that the beneficial effects of combination therapy using AM and MNC may be attributable, in part, to the angiogenic properties of AM itself. Thus it is possible that AM infusion and MNC transplantation induce additive effects on myocardial damage after myocardial infarction. However, it still remains unknown whether AM infusion plus MNC transplantation induces synergetic effects.

An earlier study has demonstrated that ischemia and mechanical stress induce apoptosis of transplanted cells in the early stage after MNC transplantation (9). These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. Kim et al. (7) have demonstrated that AM inhibits apoptosis of endothelial cells through the PI3-kinase/Akt pathway in vitro. Activation of the PI3-kinase/Akt pathway has been shown to inhibit apoptosis of endothelial progenitor cells and enhance neovascularization (11). In the present study, AM infusion significantly inhibited MNC apoptosis in ischemic tissue. In vitro, we showed that the antiapoptotic effect of AM on MNC was mediated by activation of the PI3-kinase/Akt pathway. Thus AM may enhance the therapeutic potency of MNC transplantation through a direct action of AM on MNC survival. Moreover, immunohistological examination demonstrated that infusion of AM increased the number of lectin-positive (endothelial) cells in transplanted MNC. These findings raise the possibility that AM may enhance differentiation of MNC into the endothelial lineage. Thus AM may directly act on transplanted MNC, which may result in synergetic effects on the ischemic myocardium.

This study includes some study limitations. Although the labeling efficacy of PKH26 has been shown to persist for >8 wk without cell toxicity (3, 4), the used vital marker PKH26 may have some cell toxic effects and cell or membrane fusion can lead to labeling of neighboring cells in the target tissue. Second, the present study demonstrated that AM prolongs MNC survival through the PI3-kinase/Akt pathway and enhances neovascularization in a peri-infarcted area. However, further studies are necessary to examine the effect of AM on MNC differentiation into endothelial cells.

Autologous cell transplantation may be an alternative treatment for ischemic heart disease in the clinical setting. Because their use does not require immunosuppression, the clinical use of MNC for cellular cardiomyoplasty appears to be most advantageous. Administration of AM peptide is simple and relatively noninvasive. We and others (12, 16, 17) have reported the safety of AM infusion in humans. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

In conclusion, infusion of AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

GRANTS

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SCIENTIFIC LETTER

Green tea reverses endothelial dysfunction in healthy smokers

N Nagaya, H Yamamoto, M Uematsu, T Itoh, K Nakagawa, T Miyazawa, K Kangawa, K Miyatake

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Green tea is popular in Asia, including Japan, although it is rarely consumed by western people. It contains large amounts of catechins which are potent free radical scavengers and therefore has an antioxidant effect.[1] Oxidative stress participates in the pathogenesis of many cardiovascular diseases. Recently, black tea consumption has been shown to reverse endothelial dysfunction in patients with coronary artery disease through its antioxidant effects.[2] However, the number of catechins in non-fermented tea (green tea) is higher than that in fermented tea (black tea).[1] These findings raise the possibility that green tea acts as a potent antioxidant.

Smoking is a well recognised risk factor for cardiovascular diseases. Free radicals in cigarette smoke are responsible for endothelial dysfunction, leading to atherosclerosis.[3] Earlier studies have shown that the flavonoid components of red wine and purple grape juice, which are powerful antioxidants, reverse endothelial dysfunction and reduce cardiovascular risk. However, any beneficial effect of green tea on vascular function in smokers remains unknown. Accordingly, the purpose of this study was to investigate whether green tea consumption reverses endothelial dysfunction in association with improvement of oxidative stress in healthy smokers.

METHODS

We studied 20 healthy male smokers (mean (SEM) age 33 (1) years, body mass index 22.9 (0.9) kg/m^2, smoking 15.5 (2.1) packs/year). They were randomised to consume green tea or hot water in a crossover design. All subjects gave written informed consent and the study was approved by the ethics committee of the National Cardiovascular Center. Subjects were studied on two separate days, at least one week apart, at 8:30 in the morning after they had fasted for at least 12 hours. Subjects did not smoke on the morning of the study. At baseline and two hours after consumption of 400 ml green tea or hot water, we measured the response of forearm blood flow (FBF) to reactive hyperaemia, an index of endothelium dependent vasodilatation, and to sublingual administration of glyceryl trinitrate (GTN), an index of endothelium independent vasodilatation. To induce reactive hyperaemia, FBF was occluded by inflating a cuff around the left upper arm to a pressure of 280 mm ?Hg for five minutes. After release of the ischaemic cuff occlusion, FBF was measured for three minutes. After FBF returned to the baseline in a 15 minute recovery period, 0.3 mg GTN was administered sublingually and FBF was measured for five minutes. Seven healthy volunteers (mean age 32 (2) years), who had not smoked for more than one year, served as age matched non-smoking controls.

FBF was measured using a mercury filled Silastic strain-gauge plethysmograph (EC-5R, DE Hokanson Inc, Washington, USA), as described previously.[1] FBF was expressed in ml/min/100 ml of forearm tissue volume. Blood and urine samples were repeatedly obtained for measurement of plasma catechin and urine 8-iso-prostaglandin-F2α (8-iso-PGF2α), an index of oxidative stress.[4] Numerical values were expressed as mean (SEM). Comparisons of the time course of parameters between the two groups were made by two way analysis of variance (ANOVA) for repeated measures, followed by Scheffe's multiple comparison test.

RESULTS

Green tea contained very large amounts of catechins (173.1 mg/dl), including epigallocatechin gallate (61.8 mg/dl), which have potent antioxidant effects. The content of epigallocatechin gallate in green tea was 10-fold higher than that in black tea. Unlike black tea, green tea also contained ascorbic acid (3.0 mg/dl). Plasma epigallocatechin gallate concentration was notably increased two hours after a single consumption of green tea (3 (1) pmol/ml to 694 (81) pmol/ml, $p < 0.001$). Green tea consumption significantly decreased the urinary concentration of 8-iso-PGF2α, an index of oxidative stress (541 (188) pg/mg creatinine to 396 (136) pg/mg creatinine, $p < 0.05$). Green tea had no significant effect on systolic and diastolic blood pressure, heart rate, fasting plasma lipid, or glucose concentration. These parameters remained unchanged after hot water consumption.

During reactive hyperaemia in healthy smokers, FBF was significantly less than that in age matched non-smoking controls (maximum FBF, 16.9 (1.1) ml/min/100 ml tissue *v* 29.3 (2.1) ml/min/100 ml tissue, $p < 0.01$), suggesting the presence of endothelial dysfunction in healthy smokers. Time course curves of FBF during reactive hyperaemia were similar before consumption of green tea and hot water. When the results of subjects randomised to receive green tea first and hot water first were combined, green tea consumption significantly increased FBF during reactive hyperaemia (maximum FBF, 16.9 (1.1) ml/min/100 ml tissue to 21.3 (1.3) ml/min/100 ml tissue, $p < 0.001$, fig 1).

These results suggest that green tea consumption improved endothelium dependent vasodilatation in smokers. In contrast, green tea had no effect on the increase in FBF after sublingual administration of GTN, an index of endothelium independent vasodilatation (maximum FBF, 16.9 (1.1) ml/min/100 ml tissue to 21.3 (1.3) ml/min/100 ml tissue, p = NS). These parameters remained unchanged after hot water consumption.

DISCUSSION

The present study demonstrated that, compared with black tea, green tea contained extremely large amounts of catechins including epigallocatechin gallate. We also showed that, unlike black tea, green tea contained ascorbic acid, which also has antioxidant effects. These results suggest that green tea may act as a potent antioxidant.

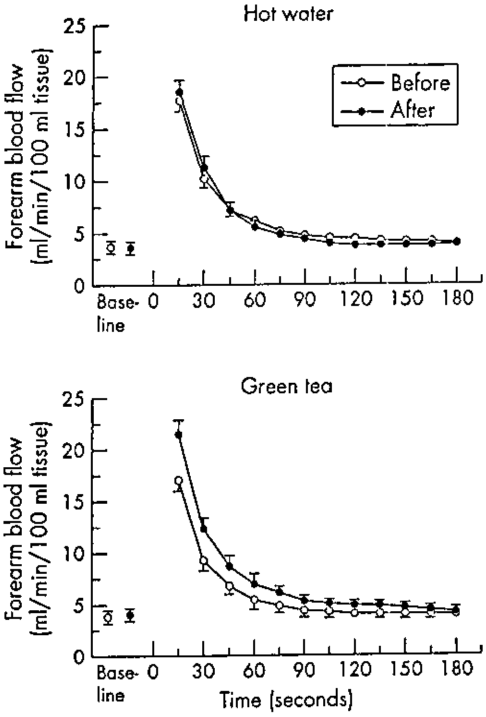

Figure 1 Forearm blood flow (FBF) at rest and during reactive hyperaemia before (open circles) and after (solid circles) consumption of hot water (upper panel) or green tea (lower panel). Green tea consumption augmented FBF during reactive hyperaemia ($p < 0.001$), whereas hot water consumption did not significantly alter it.

Cigarette smoke has been reported to contain nicotine and large amounts of free radicals, such as superoxide anion and hydroxyradicals.[3] Free radicals in cigarette smoke may not only degrade nitric oxide released from the endothelium, but also produce highly reactive intermediates, resulting in endothelial injury. In the present study, a single consumption of green tea notably increased plasma epigallocatechin gallate concentration and significantly decreased urinary 8-iso-PGF2α, a specific and stable product of lipid peroxidation, in healthy smokers. Another study has shown that green tea decreases oxidative DNA damage (measured through 8-OHdG (8-hydroxy-2′-deoxyguanosine) in smokers.[5] These results suggest that green tea consumption attenuates oxidative stress in healthy smokers, at least in part, through the antioxidant effects of catechins. Ascorbic acid included in green tea may also play a role in the regulation of oxidative stress. As expected, endothelium dependent vasodilatation was impaired in healthy smokers, consistent with many previous studies.

In the present study, a single consumption of green tea significantly increased FBF during reactive hyperaemia in smokers. In contrast, green tea had no effect on FBF at rest or GTN induced vasodilatation. These results suggest that green tea consumption reverses endothelial dysfunction in healthy smokers, possibly through its antioxidant effect. Earlier studies have demonstrated that endothelial dysfunction is important in the pathogenesis and clinical manifestation of cardiovascular disease. Thus, green tea consumption may be beneficial for the prevention and treatment of atherosclerotic vascular disease. Further prospective, randomised studies of green tea consumption are necessary to examine whether green tea consumption reduces the risk of cardiovascular events and mortality.

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Authors' affiliations

N Nagaya, H Yamamoto, T Itoh, K Miyatake, Department of Internal Medicine, National Cardiovascular Center, Osaka, Japan
M Uematsu, Cardiovascular Division, Kansai Rosai Hospital, Hyogo, Japan
K Nakagawa, T Miyazawa, Laboratory of Biodynamic Chemistry, Tohoku University Graduate School of Life Science and Agriculture, Sendai, Japan
K Kangawa, Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan

Correspondence to: Dr Noritoshi Nagaya, Department of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan; nagayann@hsp.ncvc.go.jp

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