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# Overexpression of P104L mutant caveolin-3 in mice develops hypertrophic cardiomyopathy with enhanced contractility in association with increased endothelial nitric oxide synthase activity

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The effect of endogenous nitric oxide synthase (NOS) on cardiac contractility and architecture has been a matter of debate. A role for NOS in cardiac hypertrophy has recently been demonstrated by studies which have shown hypertrophic cardiomyopathy (HCM) with altered contractility in constitutive NOS (cNOS) knockout mice. Caveolin-3, a strong inhibitor of all NOS isoforms, is expressed in sarcolemmal caveolae microdomains and binds to cNOS *in vivo*: endothelial nitric oxide synthase (eNOS) in cardiac myocytes and neuronal nitric oxide synthase (nNOS) in skeletal myocytes. The current study characterized the biochemical and cardiac parameters of P104L mutant caveolin-3 transgenic mice, a model of an autosomal dominant limb-girdle muscular dystrophy (LGMD1C). Transgenic mouse hearts demonstrated HCM, enhanced basal contractility, decreased left ventricular end diastolic diameter, and loss and cytoplasmic mislocalization of caveolin-3 protein. Surprisingly, cardiac muscle showed activation of eNOS catalytic activity without increased expression of all NOS isoforms. These data suggest that a moderate increase in eNOS activity associated with loss of caveolin-3 results in HCM.

## INTRODUCTION

Caveolae are 50–100 nm flask-shaped invaginations of the plasma membrane that are primarily composed of the 21–24 kDa integral membrane proteins, caveolin-1, -2 and -3 (1). These structures participate in vesicular trafficking events and signal transduction by acting as scaffold proteins for specific lipids and lipid-modified signaling molecules (e.g. cholesterol, G-proteins, G-protein coupled receptors, receptor tyrosine kinases and nitric oxide synthase) (2–4).

Caveolin-3 is a myocyte-specific isoform that assembles to ~350 kDa homo-oligomers in the sarcoplasmic reticulum (SR) (2). Caveolin-3 homo-oligomers are translocated to the plasma membrane via the trans-Golgi network (2,4) and inhibit all NOS isoforms *in vitro* and bind to constitutive NOS (cNOS)

*in vivo*: endothelial nitric oxide synthase (eNOS) in cardiac myocytes and neuronal nitric oxide synthase (nNOS) in skeletal myocytes (5–8).

Autosomal dominant limb-girdle muscular dystrophy 1C (LGMD1C) and autosomal dominant rippling muscle disease (AD-RMD) result from heterozygous mutations of the skeletal muscle caveolin-3 gene (*CAV3*) (9,10). We previously generated transgenic (Tg) mice (TgCAV3M1 mice) with severe myopathy secondary to overexpression of P104L mutant caveolin-3 as a model of LGMD1C (11). Further study of this transgenic animal model revealed increased sarcolemmal nNOS activity without alternations of nNOS expression at both mRNA and protein levels. Other reports demonstrated that caveolin-3 is down-regulated in the hypertrophic hearts of spontaneously hypertensive rats (12) and that circulatory NO

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(13) or eNOS mRNA in cardiac muscle (14) increases in human heart failure. These observations suggest that loss of caveolin-3 may modulate cardiac architecture and function via disinhibition of NOS activity. The present study investigated this hypothesis by characterizing the biochemical and cardiac parameters of TgCAV3M1 mice.

## RESULTS

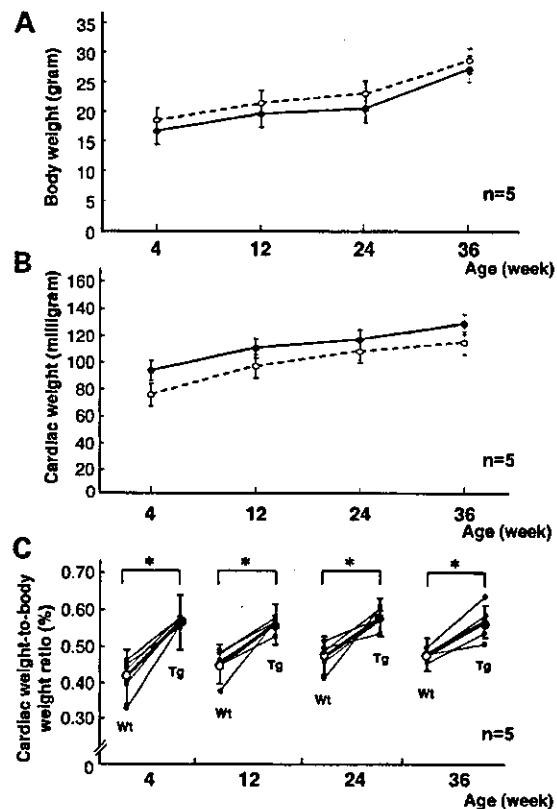
### TgCAV3M1 mice show hypertrophic cardiomyopathy with enhanced contractility

TgCAV3M1 mice were generated as described previously (11). Tg mice showed poor growth and were significantly smaller than their wild-type (Wt) littermates at 4, 12, 24 and 36 weeks of age (Fig. 1A) and demonstrated kyphosis of the spine and paralysis of the hindlimbs from 12 weeks of age. The Tg mice exhibited increased cardiac weight and statistically significant increases in the cardiac weight-to-body weight ratio at 4, 12, 24 and 36 weeks of age when compared with their Wt littermates (Fig. 1B and C). However, from the clinical standpoint, the Tg mice have so far not developed any significant symptoms of heart failure, nor have any significant differences in life span been observed between the Wt and Tg mice.

Gross appearance of the hearts from 6-week-old Wt and Tg mice at the lower ventricular level showed thickening of the interventricular septum and the posterior wall of the left ventricle, resulting in a smaller left ventricular chamber in the Tg mice (Fig. 2A). Hematoxylin and eosin staining of cardiac muscle sections showed hypertrophy of cardiac myofibers in the Tg mice (Fig. 2B). The diameters of 1000 cardiac muscle fibers from five Tg mice and five Wt mice, respectively, were measured. The frequency distribution (percentage) of the cardiac myofiber diameter in Tg mice showed a distribution skew to the right and marked size variability on both longitudinal and transverse sections when compared to Wt mice (Fig. 2C). The mean cardiac muscle fiber diameter and standard deviation were significantly larger ( $P < 0.05$ ) in Tg mice ( $8.53 \pm 1.43 \mu\text{m}$  on longitudinal section and  $9.35 \pm 1.17 \mu\text{m}$  on transverse sections) than in Wt mice ( $6.55 \pm 0.91$  and  $7.32 \pm 0.83 \mu\text{m}$ , respectively; Fig. 2C). Gene expression of cardiac myocyte hypertrophic markers, atrial natriuretic peptide (ANP) and brain-derived natriuretic peptide (BNP) was analyzed by northern blotting. Densitometry using BAS2000 Image Analyzer demonstrated that both the ANP and BNP transcripts in the Tg mouse hearts were up-regulated by 1.69- and 1.54-fold, respectively (Fig. 2D). Transthoracic echocardiogram performed in 24-week-old Tg mice ( $n = 7$ ) revealed unique pathophysiological characteristics for HCM; increased thickness of the interventricular septum and left ventricular posterior wall, hypercontractility (increased left ventricular fractional shortening) and diastolic dysfunction (decreased left ventricular end diastolic diameter; Table 1).

### Tg CAV3M1 mouse hearts show loss and cytoplasmic mislocalization of caveolin-3

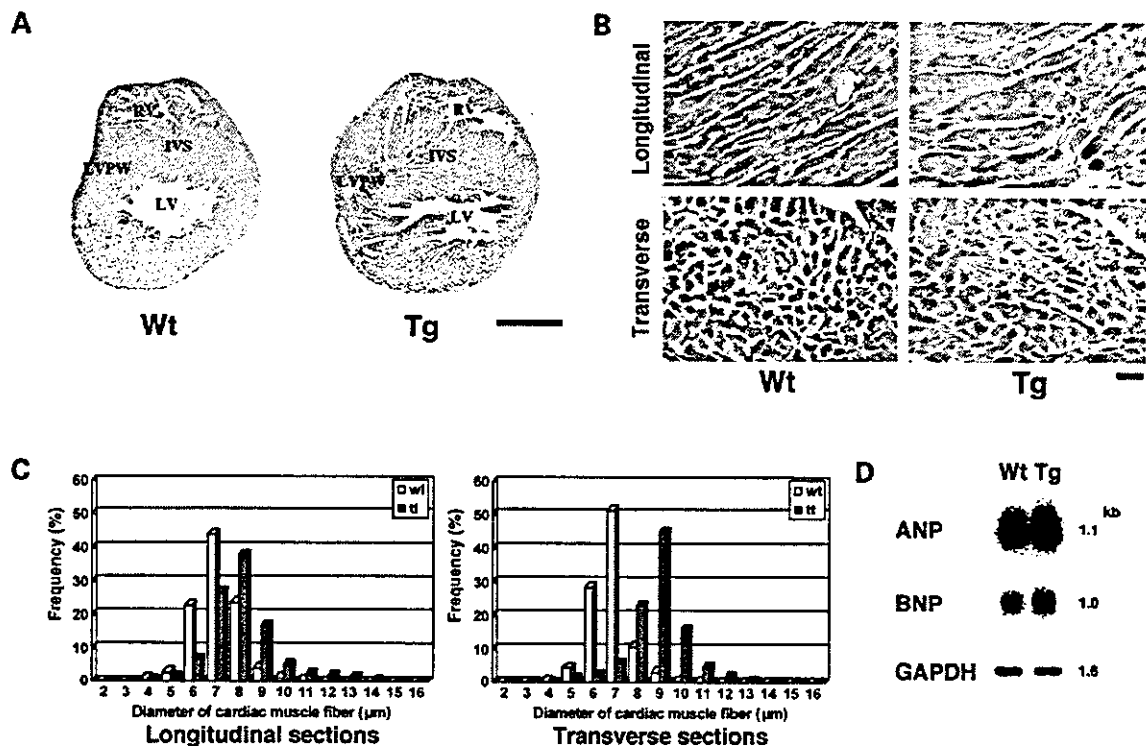
Northern blot analysis of Tg mouse cardiac muscle showed overexpression of smaller-sized ( $\sim 1.1$  kb) mutant caveolin-3



**Figure 1.** Comparison of body weight (A), cardiac weight (B) and the cardiac weight-to-body weight ratio (C) between Wt (open circle,  $n = 5$ ) and Tg (solid circle,  $n = 5$ ) mice at 4, 12, 24 and 36 weeks of age. Note that the smaller body weight and larger cardiac weight in the Tg mice results in statistically significant increases in the mean cardiac weight-to-body weight ratios. Error bars are  $\pm$ SD. \*Statistical significance was determined by Welch's *t*-test ( $P < 0.05$ ).

mRNA (Fig. 3A). Detection of endogenous caveolin-3 mRNA ( $\sim 1.4$  kb) in the Tg mice was interfered with by an excessive amount of the mutant caveolin-3 mRNA. Then, the differential expression of endogenous and mutant caveolin-3 mRNA was analyzed by RT-PCR using two distinct reverse primers as described previously (11). RT-PCR confirmed the expression of endogenous caveolin-3 mRNA in Tg mouse hearts as well as in Wt mouse hearts, as shown in Figure 3B.

In sharp contrast to overexpression of mutant caveolin-3 mRNA, immunoblot analysis showed a marked reduction ( $\sim 95\%$ ) of caveolin-3 protein in Tg mouse cardiac muscle (Fig. 3C). Immunohistochemical analysis demonstrated sarcolemmal localization of caveolin-3 protein in the Wt mice, while the majority of cardiac muscle fibers of the Tg mice showed only weak sarcolemmal immunoreactivity with small, cytoplasmic, dot-like immunoreactivity (Fig. 3D). There was no change in sarcolemmal dystrophin expression in the Tg mice (Fig. 3D). Caveolin-1 was expressed in endothelial cells and showed no compensatory overexpression in Tg mouse cardiac myocytes (Fig. 3D).  $\beta$ -Dystroglycan expression was similar in the Wt and Tg mice (Fig. 3C).



**Figure 2.** Histology of hearts from 6-week-old Wt and Tg mice. (A) Gross appearance of transverse lower ventricular sections from Wt and Tg mouse hearts. Note that apparent thickening of the interventricular septum and posterior wall of the left ventricle results in a tiny ventricular chamber in the Tg mouse heart (right). LV, left ventricle; IVS, interventricular septum; LVPW, left ventricular posterior wall; RV, right ventricle. Bar, 1 mm. (B) H&E staining of cardiac muscle sections from 6-week-old Wt or Tg mice. Note the myofiber hypertrophy in the Tg mouse hearts in both longitudinal (Upper panels) and transverse (lower panels) sections. Bar, 10  $\mu$ m. (C) Histogram of cardiac myofiber diameter from 6-week-old Wt or Tg mice on longitudinal sections (left) and on transverse sections (right). The diameters of 1000 cardiac muscle fibers from five Tg mice and five Wt mice were measured. Note that the frequency distribution (%) of the cardiac muscle fiber diameter in the Tg mice compared with that in the Wt mice shows a skewed distribution to the right and marked size variability, thus indicating cardiac myocyte hypertrophy. The diameter of each cardiac muscle fiber was measured by the IBAS 2000 image analysis system (Zeiss). (D) Northern blot analysis of cardiac myocyte hypertrophic markers; ANP and BNP. Radiointensities of ANP and BNP transcripts in the Tg mouse hearts were up-regulated by 1.69- and 1.54-fold, respectively.

**Table 1.** Transthoracic echocardiographic analysis of hearts from 24-week-old Wt and Tg mice

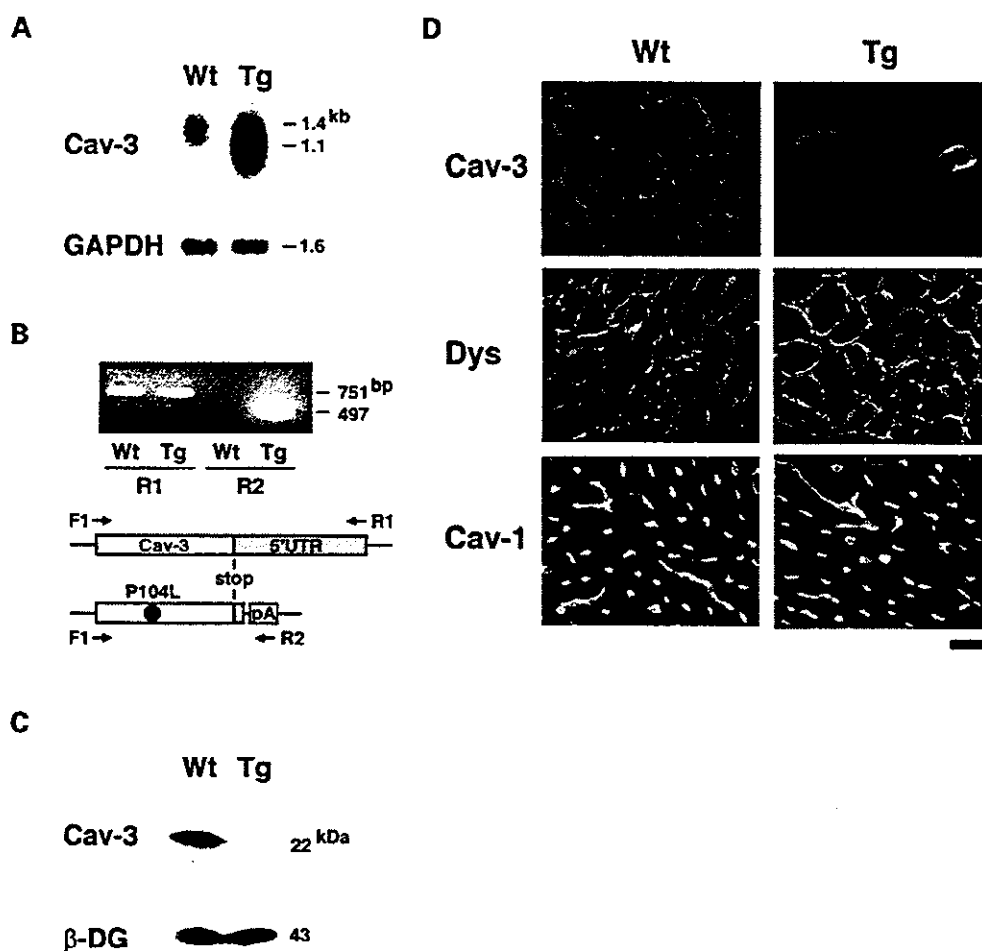
Group	Interventricular septal thickness (mm)	Left ventricular posterior wall thickness (mm)	Left ventricular end diastolic diameter (mm)	Left ventricular end systolic diameter (mm)	Left ventricular fractional shortening (%)
Wt	0.83 ± 0.11	0.78 ± 0.08	3.94 ± 0.28	2.14 ± 0.23	45.60 ± 4.00
Tg	1.04 ± 0.12*	1.07 ± 0.11*	3.00 ± 0.30*	1.15 ± 0.18*	61.20 ± 4.70*

\* $P < 0.01$ . Wt ( $n = 7$ ); Tg ( $n = 7$ ).

#### NOS activity is increased in TgCAV3M1 mouse hearts

Expression of all NOS isoforms was similar when Wt and Tg mice cardiac muscles were compared by northern blot and immunoblot analyses (Fig. 4A and B). In addition, subsarcolemmal and vascular endothelial localization of eNOS was similar in Tg and Wt mouse hearts (Fig. 4C). NOS activity was quantified in crude extracts from freshly isolated hearts from six-week-old Wt and Tg mice ( $n = 8$ ). Total NOS

and eNOS activities in the Tg mouse hearts were significantly higher ( $P < 0.05$ ) than those in the Wt mouse hearts: total NOS activity (pmol/mg protein/min)—Tg mouse,  $5.10 \pm 0.32$ , Wt mouse,  $3.38 \pm 0.32$ ; eNOS activity (pmol/mg protein/min)—Tg mouse,  $4.03 \pm 0.32$ , Wt mouse,  $2.31 \pm 0.24$ . No significant differences were observed in either nNOS activity (pmol/mg protein/min)—Tg mouse,  $0.77 \pm 0.07$ , Wt mouse,  $0.75 \pm 0.06$ —or iNOS activity (pmol/mg protein/min)—Tg mouse,  $0.30 \pm 0.02$ , Wt mouse,  $0.31 \pm 0.03$  (Fig. 4D).

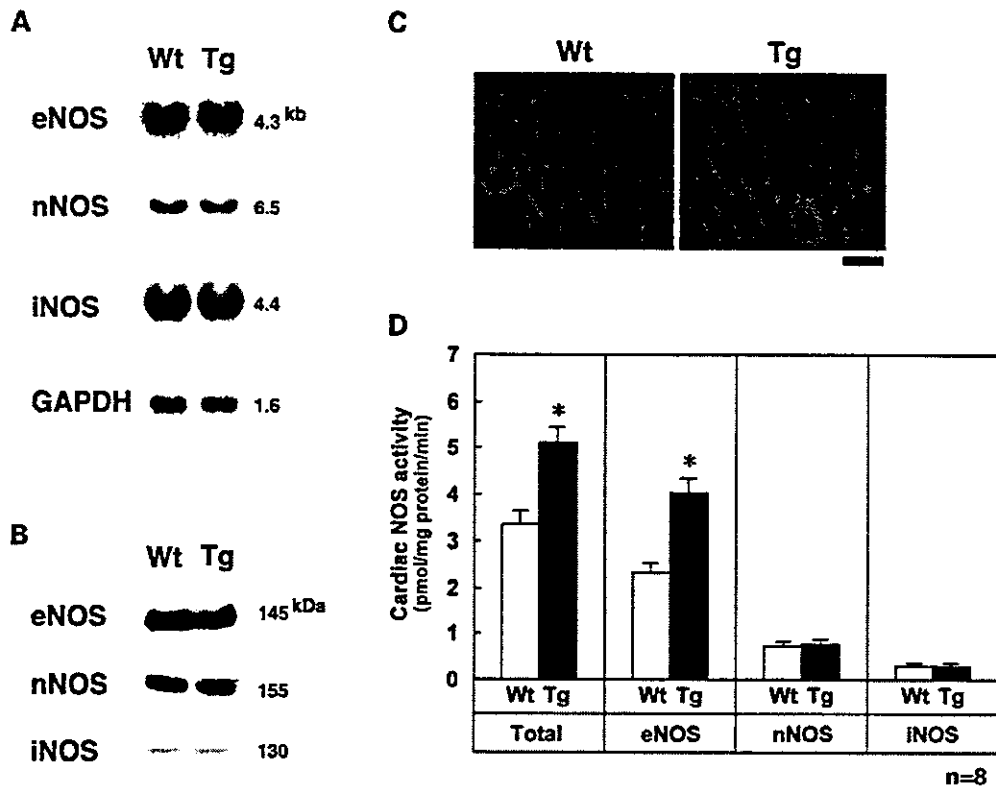


**Figure 3.** (A) Northern blot analysis of caveolin-3 in cardiac muscle from 6-week-old Wt and Tg mice. The smaller-sized (~1.1 kb) mutant caveolin-3 mRNA was expressed excessively in the Tg mice compared with the Wt mice. (B) RT-PCR analysis of endogenous or mutant caveolin-3 in cardiac muscle from 6-week-old Wt and Tg mice. The endogenous caveolin-3 was detectable in Tg mice as well as Wt mice using primers of F1/R1 (upper panel, left). The mutant caveolin-3 mRNA was detectable only in Tg mice using primers of F1/R2 (upper panel, right). R1 corresponds to the 5'-untranslated region (5'-UTR) of the caveolin-3 transcript, whereas R2 corresponds to the SV40 polyadenylation site (pA) of the transgene transcript (lower panel). (C) Immunoblot analysis of caveolin-3 in total protein extracts from 6-week-old Wt and Tg mouse cardiac muscles. Caveolin-3 expression was markedly reduced in the Tg mice. In contrast,  $\beta$ -DG expression was similar in the Wt and Tg mice. (D) Immunohistochemical analysis of cardiac muscle from 6-week-old Wt and Tg mice. Lower ventricular cryosections from the Wt and Tg mice were stained with antibodies against caveolin-3 (Cav-3), dystrophin (Dys) and caveolin-1 (Cav-1). Caveolin-3 localized to the sarcolemma in the Wt mice, but the Tg mice generally lacked caveolin-3 except for a few fibers that showed weak cytoplasmic localization of caveolin-3. The expression of dystrophin and caveolin-1 was similar in the Wt and Tg mice. Bar, 10  $\mu$ m.

## DISCUSSION

Caveolin-3 is a strong physiological inhibitor of NOS and expressed specifically in cardiac and skeletal myocytes (5–8). The present study demonstrated the occurrence of hypertrophic cardiomyopathy with enhanced contractility in association with increased eNOS activity in caveolin-3-deficient transgenic mice without changes in NOS expression. Therefore, loss of NOS inhibition secondary to deficient caveolin-3 may contribute to the pathogenesis of hypertrophic cardiomyopathy.

NOS activity may modulate cardiac contractility and architecture (15–18). eNOS is localized in caveolae (5,8) and plays a role in inhibition of  $\beta$ -adrenergic-induced contractility (19,20). NO generated by eNOS may also interact with the SR ryanodine receptor (21). In contrast, nNOS, located in the SR (15,22), increases SR  $Ca^{2+}$  release and enhances cardiac contractility (15,23). eNOS knockout mice show enhanced contractility (15), while overexpression of eNOS results in reduced cardiac size and contractility (24). Unexpectedly, our Tg mouse hearts with moderately increased eNOS activity showed enhanced



**Figure 4.** Northern blot analysis (A) and immunoblot analysis (B) of NOS isoforms in cardiac muscle from 6-week-old Wt and Tg mice. Note the comparable expression of all NOS isoforms in the Wt and Tg mice. (C) Immunohistochemical analysis of eNOS in cardiac muscle from 6-week-old Wt and Tg mice. The expression pattern of eNOS was similar in the Wt and Tg mice. Bar, 10 μm. (D) NOS activities in crude extracts from cardiac muscle from 6-week-old Wt and Tg mice. Note that significant increases in total NOS and eNOS activity were observed in the Tg mice, compared to the Wt mice ( $n=8$ ). In contrast no significant difference was observed in either nNOS or iNOS activity between the Tg and Wt mice. Data are expressed as mean  $\pm$  SD. \*Statistical significance was determined using Welch's *t*-test ( $P < 0.05$ ).

contractility and hypertrophic cardiomyopathy. It is noteworthy that exogenous NO, derived from pharmacological NO donors, produces a biphasic contractile response in cardiac tissue with augmentation at low NO levels and depression at high NO levels (25–27). Based on these exogenous NO donor experiments, we postulate that a moderate activation of eNOS activity, as seen in the mutant caveolin-3 Tg mouse hearts enhances contractility and leads to hypertrophic cardiomyopathy, whereas an extremely high activation of eNOS activity, as seen in mouse hearts in which eNOS is overexpressed, reduces contractility and cardiac size.

Woodman *et al.* (28) reported that caveolin-3 null mice showed cardiomyopathy and suppressed contractility, possibly secondary to alterations in the p42/44 MAPK pathway, but they did not characterize changes in NOS isoform profiles. Based on previous studies and the present data, we would predict greater NOS activity in caveolin-3 null mouse hearts than in the hearts of mutant caveolin-3 transgenic mice. This higher NOS activity in caveolin-3 null mouse hearts may result in suppression of cardiac contractility.

To date hypertrophic cardiomyopathy has not been reported in LGMD1C patients. However, in only one German pedigree of autosomal dominant rippling muscle disease (AD-RMD) (29),

which was proven to carry a caveolin-3 missense mutation (A45V) (10), two patients died suddenly of possible cardiac arrhythmia. An autopsy study of one patient in this pedigree disclosed non-obstructive cardiomyopathy, which was poorly described (29). Cardiac involvement, cardiomyopathy and/or arrhythmia may be a rare but possible feature of CAV-3 missense mutations. Further extensive clinical examination of cardiac function in LGMD1C patients is necessary to test this possibility. Furthermore, it is plausible that the higher copy number of the mutant caveolin-3 gene in TgCAV3M1 mice promotes a disease process leading to HCM.

In conclusion, we propose that moderate eNOS activation caused by loss of caveolin-3 may be involved in a caveolin-3-mediated hypertrophic signal pathway of cardiac myocytes.

## MATERIALS AND METHODS

### RT-PCR

cDNA templates were reverse-transcribed from 1 μg of total RNA from mouse cardiac muscle, primed with an oligo(dT)<sub>12-18</sub>

primer and then subjected to PCR. For amplification of the endogenous caveolin-3 gene, we used F1 (5'-CCCAGCCTCACAAATGATGACCGAAG-3') and R1 (5'-CATGTGAACGCAAAGCC TTGC-3'). For amplification of the transgene, we used F1 and R2 (5'-GCATTCTAGTTGGTTT-3'). As illustrated in Figure 3B, R1 and R2 correspond to the 5'-untranslated region of caveolin-3 cDNA and the SV40 polyadenylation site, respectively, as described previously (11).

#### Northern blot analysis

RT-PCR product of endogenous caveolin-3 was subcloned into TA vector pCR2.1 (Invitrogen) and then digested by *EcoRI*. The digested insert was extracted from agarose gel using Concert Kit (GibcoBRL). This cDNA was then labeled as a probe with [ $\alpha$ -<sup>32</sup>P]dCTP using MegaPrime DNA labeling system (Amersham Pharmacia Biotech). Ten micrograms of total RNA from 6-week-old Wt and Tg cardiac muscle were separated on 0.7% agarose gels containing 7% formaldehyde and blotted onto Hybond-N+ (Amersham Pharmacia Biotech). Hybridization was performed at 42°C for 12–24 h and autoradiography was performed on a Fuji imaging plate (Fuji Film). Signal intensity of transcripts was measured by BAS2000 Image Analyzer (Fuji Film). Using the same method, we hybridized northern blots with RT-PCR generated and labeled fragments of ANP cDNA, BNP cDNA, eNOS cDNA, nNOS cDNA, iNOS cDNA and internal control GAPDH cDNA.

#### Immunoblot analysis

Six-week-old Wt or Tg mouse cardiac muscle was homogenized in 10 vols (w/v) of a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM  $\beta$ -mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM benzamide. These crude extracts were denatured and size fractionated by SDS-PAGE (3–12%) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked using 5% milk in phosphate-buffered saline (PBS) and incubated with rabbit polyclonal antibody against caveolin-3 (Transduction Laboratories, C38330), raised against synthetic peptide corresponding to the amino terminus of caveolin-3 of rat and mouse origin, a monoclonal antibody against  $\beta$ -dystroglycan (Novo Castra), a rabbit polyclonal antibody against eNOS (Santa Cruz Biotechnology), a rabbit polyclonal antibody against nNOS (Santa Cruz Biotechnology) and a rabbit polyclonal antibody against iNOS (Transduction Laboratories) overnight at room temperature. After washing with 5% milk in PBS, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Amersham Pharmacia Biotech). Immunoreactive bands were visualized with ECL (Amersham Pharmacia Biotech).

#### Immunohistochemical analysis

Unfixed cardiac muscle samples were snap frozen in liquid nitrogen-cooled isopentane, sectioned on a cryostat (10  $\mu$ m), and melted directly onto glass slides. Sections were then post-fixed in 4% freshly depolymerized paraformaldehyde in PBS for 15–30 min at 4°C. After blocking with 3% BSA in PBS,

sections were immunostained with a goat polyclonal antibody against caveolin-3 (Santa Cruz Biotechnology, sc-7665), recognizing the epitope mapped at the amino terminus of caveolin-3 of mouse origin, a rabbit polyclonal antibody against caveolin-1 (Santa Cruz Biotechnology), a goat polyclonal antibody against dystrophin (Santa Cruz Biotechnology), and a rabbit polyclonal antibody against eNOS (Santa Cruz Biotechnology) for 1 h at room temperature. After extensive washing with PBS, sections were incubated with FITC-conjugated or Cy3-conjugated secondary antibody. Rabbit and goat polyclonal antibodies against caveolin-3 used in immunoblot and immunohistochemical analyses can recognize both mutant and wild type forms of caveolin-3.

#### Transthoracic echocardiogram

Transthoracic echocardiography was performed on 24-week-old Tg mice and Wt littermates ( $n = 7$ ) under light anesthesia using intraperitoneal pentobarbital as described previously (30).

#### NOS assay

Total NOS activity was measured by monitoring the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline, as previously described (18,31–33). Briefly, freshly prepared cardiac muscle from 6-week-old Tg mice and Wt littermates ( $n = 8$ ) was homogenized in 10 vols (w/v) of a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 1  $\mu$ M leupeptin. Aliquots from crude homogenates were quickly assayed in 100  $\mu$ l reactions containing 100 000 cpm (40 Ci/mmol) of L-[<sup>3</sup>H]arginine, 1 mM NADPH, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1.2 mM CaCl<sub>2</sub>, 10  $\mu$ g/ml calmodulin, 1 mM DTT and 10  $\mu$ M each of tetrahydrobiopterin, FAD and FMN. After an incubation of 10 min at 37°C, assays were terminated with 900  $\mu$ l of ice cold H<sub>2</sub>O. After brief sonication, samples were applied to 2 ml of Dowex AG 50W-X8 (Na<sup>+</sup> form) column. L-[<sup>3</sup>H]citrulline was quantified using 1 ml flow-through by liquid scintillation spectroscopy. The combined activity of eNOS plus iNOS was also measured in the same reaction buffer containing specific inhibitor of nNOS (0.1  $\mu$ M *N*<sup>ω</sup>-propyl-L-arginine) (TOCRIS) (34,35). iNOS activity was also measured in the presence of 1.5 mM EDTA and 1.5 mM EGTA, which replaced Ca<sup>2+</sup> ion in the reaction buffer. The above three assays were performed simultaneously. Then, nNOS activity was calculated by subtraction of the combined activity of eNOS and iNOS from total NOS activity. eNOS activity was calculated by subtraction of iNOS activity from the combined activity of eNOS and iNOS. Data are expressed as mean  $\pm$  SD. Statistical significance was determined using Welch's *t*-test ( $P < 0.05$ ).

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## **Cytokine therapy prevents left ventricular remodeling and dysfunction after myocardial infarction through neovascularization**

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### **ABSTRACT**

Pretreatment with a combination of granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) has been reported to attenuate left ventricular (LV) remodeling after acute myocardial infarction (MI). We here examined whether the cytokine treatment started after MI has also beneficial effects. Anterior MI was created in the recipient mice whose bone marrow had been replaced with that of transgenic mice expressing enhanced green fluorescent protein (GFP). We categorized mice into five groups according to the following treatment: 1) saline; 2) administration of G-CSF and SCF from 5 days before MI through 3 days after; 3) administration of G-CSF and SCF for 5 days after MI; 4) administration of G-CSF alone for 5 days after MI; 5) administration of SCF alone for 5 days after MI. All the three treatment groups with G-CSF showed less LV remodeling and improved cardiac function and survival rate after MI. The number of capillaries, which express GFP, was increased and the number of apoptotic cells was decreased in the border area of all the treatment groups with G-CSF. Even if the cytokine treatment is started after MI, it could prevent LV remodeling and dysfunction after MI—at least in part—through an increase in neovascularization and a decrease in apoptosis in the border area.

**Key words:** apoptosis • bone marrow • G-CSF

**I**t is important to prevent left ventricular (LV) remodeling after acute myocardial infarction (MI) because it causes heart failure and poor prognosis (1). After MI, many cardiomyocytes undergo cell death by the mechanisms of necrosis and apoptosis in the infarcted area, which is then replaced by fibrotic tissue. The infarcted area is gradually extended by the subsequent death of cardiomyocytes in the border area and expanded by abnormal wall tension (2). Myocardial ischemia plays a critical role in the cardiomyocyte death in the border area after MI and thus greatly affects LV remodeling (2). Recently, Orlic et al. have reported that a subset of bone marrow stem cells (BMSCs) differentiate into cardiomyocytes when injected into peri-infarcted area, which results in regeneration of the infarcted heart (3). They have also reported that a pretreatment with granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), which strongly induce mobilization of BMSCs from bone marrow (BM), attenuates LV remodeling after MI (4–6). Although these results suggest that the cytokine treatment is beneficial

to prevent LV remodeling, the cytokine treatment was started before MI and thus this protocol cannot be applied to humans. Furthermore, the molecular mechanism of how the cytokine treatment repairs the infarcted heart is not fully understood.

In the present study, we examined three points: 1) whether the cytokine treatment started after MI is as effective as the pretreatment; 2) whether the combination treatment with G-CSF and SCF is more effective than the single treatment with G-CSF alone or SCF alone; and 3) how G-CSF prevents LV remodeling and dysfunction after MI. We used mice that were replaced by BM cells of enhanced green fluorescent protein (GFP)-expressing mice to elucidate the role of BM cells in the reduction of infarct size.

## MATERIALS AND METHODS

### Murine MI model

C57BL/6 male mice at 12-weeks-old were used in this study. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University. Mice were anesthetized with pentobarbital and artificially ventilated with a respirator, and MI was produced by permanent ligation of the left coronary artery with a 10-0 nylon surgical suture under a dissecting microscope as previously described (7). Sham operation was performed by cutting pericardium.

### Cytokine treatments

Mice were divided into the following five groups: 1) administration of vehicle (control,  $n=22$ ); 2) administration of G-CSF (100  $\mu\text{g}/\text{kg}/\text{day}$ , Kyowa Hakko Kogyo Co., LTD. Tokyo, Japan) and SCF (200  $\mu\text{g}/\text{kg}/\text{day}$ , Kirin Brewery Co., LTD. Tokyo, Japan) from 5 days before MI through 3 days after (Pre-GS,  $n=16$ ); 3) administration of G-CSF (100  $\mu\text{g}/\text{kg}/\text{day}$ ) and SCF (200  $\mu\text{g}/\text{kg}/\text{day}$ ) for 5 days after MI (Post-GS,  $n=20$ ); 4) administration of G-CSF (100  $\mu\text{g}/\text{kg}/\text{day}$ ) alone for 5 days after MI (Post-G,  $n=20$ ); 5) administration of SCF (200  $\mu\text{g}/\text{kg}/\text{day}$ ) alone for 5 days after MI (Post-S,  $n=21$ ). We gave first injection of vehicle, G-CSF, or SCF at 2 h after MI subcutaneously (Fig. 1A). In addition, we added a sham-operated group ( $n=5$ ) to clarify whether cytokine treatments act completely or partly on MI heart.

### Physiological analysis

For physiological analysis, we examined hemodynamic parameters of the survived mice (Sham,  $n=5$ ; Control,  $n=5$ ; Pre-GS,  $n=10$ ; Post-GS,  $n=16$ ; Post-G,  $n=14$ ; Post-S,  $n=9$ ) at 2 weeks after MI by cardiac catheterization. Mice were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine (Sigma Chemical Co., St. Louis, MO) and 5 mg/kg xylazine (Sigma). The right carotid artery was cannulated by Millar Mikro-Tip transducer (model SPR-612, Millar Instruments, Houston, TX) as described previously (8). Pressure signals were recorded by using MacLab 3.6/s data acquisition system (AD Instruments, Milford, MA) with sampling rate 2000 Hz. LV systolic pressure (LVP), LV end-diastolic pressure (LVEDP), and positive and negative first derivatives for maximal rates of LV pressure development ( $dP/dt$  and  $-dP/dt$ ) were measured.

### **Histological analysis**

We excised hearts (Sham,  $n=5$ ; Control,  $n=5$ ; Pre-GS,  $n=10$ ; Post-GS,  $n=16$ ; Post-G,  $n=14$ ; Post-S,  $n=9$ ) for histological analysis after catheterization. LV was fixed with 10% formalin overnight and dehydrated methanol and was embedded in paraffin. Serial sections at 4  $\mu\text{m}$  were stained with hematoxylin-eosin and Azan-Mallory. The scar area was evaluated by tracing the blue area in Azan-Mallory staining. We evaluated LV remodeling as described previously (9).

### **Capillary density, inflammatory cells, and apoptotic cell death**

We analyzed inflammatory cells, capillary density, and apoptotic cells by another series of experiment (Control,  $n=5$ ; Pre-GS,  $n=5$ ; Post-GS,  $n=5$ ; Post-G,  $n=5$ ). The capillaries in the border area of MI hearts were identified by staining endothelial cells with antibody against platelet/endothelial cell adhesion molecule-1 (PECAM-1; Santa Cruz Biotechnology, Santa Cruz, CA; 10). The number of capillaries per square millimeter was counted in the border area of MI hearts at 4 days after MI. Twenty fields of 200  $\mu\text{m} \times 200 \mu\text{m}$  for the border area were analyzed for each mouse at a magnification of  $\times 400$ /high-power field (HPF). The inflammatory cells in the border area of MI hearts were identified with anti-Ly6G antibody (granulocytes), anti-Mac3 antibody (macrophages), and anti-CD3 antibody (T-lymphocytes; BD Pharmingen, San Diego, CA) at 4 day after MI. Furthermore, we performed an additional experiment to analyze apoptotic cells by TUNEL method at Days 1, 4, and 7 after MI (Control,  $n=15$ ; Post-G,  $n=15$ ). TUNEL assay was performed with an apoptosis detection kit (Takara Syuzo, Kyoto, Japan) as described previously (11). Furthermore, we examined double-immunohistochemical analysis with anti-von Willebrand factor antibody (DAKO, A/S, Denmark) to identify apoptotic endothelial cells.

### **Bone marrow transplantation**

BM cells of GFP transgenic mice (12) were transplanted into C57BL/6 male mice as described previously (13). Flow cytometry analysis revealed that the BM chimerism was more than 70%. BM cells were identified by immunostaining with anti-GFP antibody (Medical and Biological Laboratories, CO., Nagoya, Japan) as described previously (14).

### **Statistics**

All data are presented as mean  $\pm$  SD. Multiple group comparison was performed by one-way ANOVA followed by the Bonferroni procedure for comparison of means. Survival rates of mice were analyzed by Kaplan-Meier method. Probability values less than 0.05 were considered to be statistically significant.

## **RESULTS**

### **Mortality**

The survival rates of mice at 14 days after MI were significantly higher in the three cytokine treatment groups with G-CSF (pre-GS, 63%; post-GS, 80%; and post-G, 70%) than in the control group (23%,  $P<0.05$ ) and the post-S group (43%,  $P<0.05$ ) in Kaplan-Meier method (Fig. 1B). There was no significant difference in the survival rates between pre- and post-treatment with

cytokines and between the combination treatment with G-CSF and SCF, and the treatment with G-CSF alone.

### **Physiological analysis**

In the present study, we examined cardiac function by echocardiogram a few days before grouping. The mice with normal cardiac function were used in this study. Therefore, the degree of baseline LV function in mouse was equal among the groups before entry for the study. At 2 weeks after MI, there was no significant difference in the heart rate among all the groups (data not shown). LVP was higher in the three cytokine treatment groups with G-CSF than in the control group and the post-S group (Table 1). LVEDP was elevated in the control group and the post-S group but not the three treatment groups with G-CSF (Table 1). Furthermore, dP/dt and -dP/dt were larger in the three treatment groups with G-CSF than in control group and post-S group (Table 1). There was no significant difference in these parameters among the three cytokine treatment groups with G-CSF. These results suggest that all the three cytokine treatments with G-CSF improve systolic and diastolic functions of LV after MI.

### **Histological analysis**

After MI, LV free wall was very thin and LV cavity was markedly expanded in control group and post-S group but LV wall thickness and LV cavity were preserved normal in the three treatment groups with G-CSF (Fig. 2A). Although the percentage of fibrotic area to the whole LV area was similar among cytokine treatment groups and the control group (control,  $38.9 \pm 9.7\%$ ; pre-GS,  $35.2 \pm 3.4\%$ ; post-GS,  $33.1 \pm 11.0\%$ ; post-G,  $32.0 \pm 5.4\%$ ; post-S,  $32.8 \pm 4.2\%$ ; Fig. 2B), the wall thickness was greater in the three cytokine treatment groups with G-CSF (sham,  $1.14 \pm 0.06$  mm; pre-GS,  $0.52 \pm 0.14$  mm; post-GS,  $0.57 \pm 0.20$  mm; post-G,  $0.53 \pm 0.16$  mm) than in control group ( $0.23 \pm 0.07$  mm) and post-S group ( $0.31 \pm 0.08$  mm; Fig. 2C). There was no significant difference in the wall thickness among all the three treatment groups with G-CSF.

### **Infiltration of white blood cells**

G-CSF has been reported to increase the number of peripheral granulocytes (15). In this study, the number of granulocytes at 4 days after MI was higher in the three treatment groups (pre-GS,  $12886 \pm 773/\text{mm}^3$ ; post-GS,  $13912 \pm 1447/\text{mm}^3$ ; and post-G,  $14318 \pm 2736/\text{mm}^3$ ) than in the control group ( $3056 \pm 959/\text{mm}^3$ ,  $P < 0.05$ ), but there was no histological evidence suggesting immunoreactions in all organs examined in our animal model. We next examined whether the number of infiltrated cells into the heart was also increased and, if so, what kinds of cells infiltrated into the heart. Immunohistochemical analysis using specific antibodies revealed that there was infiltration of granulocytes, macrophages, and T lymphocytes in the hearts of 4 days after MI. More granulocytes were observed in the hearts of the three treatment groups with G-CSF (pre-GS,  $56.8 \pm 15.0/10^3$  cells; post-GS,  $62.2 \pm 14.0/10^3$  cells; post-G,  $58.7 \pm 15.0/10^3$  cells) than in control group ( $32.1 \pm 5.0/10^3$  cells,  $P < 0.05$ ) at 4 days after MI (Fig. 3A, B). There was no significant difference in the number of macrophages (Fig. 3C, D) and T lymphocytes (Fig. 3E and F) among the three treatment groups with G-CSF and control group.

### **Mobilization of BM cells**

We next examined whether BM cells were infiltrated into the heart by using the mice whose BM was replaced by that of GFP-expressing mice. Because it was difficult to discriminate

GFP-expressing BM cells from other types of cells because of autofluorescence of MI hearts (Fig. 4A), we identified BM-derived cells by using anti-GFP antibody. Many GFP-positive cells were recognized in the border area of all the three treatment groups but not the control group (Fig. 4B). Most of GFP-positive cells were infiltrated blood cells (arrows in Fig. 4B), and some GFP-positive cells were observed at capillary walls (Fig. 4C). There were few GFP-positive cardiomyocytes (less than 0.01%) in the border area as well as in the infarcted area and the remote area. Therefore, we next compared the number of vessels in the border area. The number of capillaries in the border area after MI was much greater in all the three treatment groups (pre-GS,  $8.0 \pm 2.3/\text{HPF}$ ; post-GS,  $7.8 \pm 1.7/\text{HPF}$ ; post-G,  $6.2 \pm 1.9/\text{HPF}$ ) than in the control group ( $2.0 \pm 0.82/\text{HPF}$ ,  $P < 0.05$ ; Fig. 4D and E).

### Apoptotic cells in border area

We examined apoptotic cell death in the border area after MI. The number of TUNEL-positive cells in the border area of infarcted heart at Day 4 after MI was significantly smaller in the three treatment groups (pre-GS,  $13.3 \pm 3.2/10^3$  cells; post-GS,  $10.8 \pm 1.5/10^3$  cells; post-G,  $12.8 \pm 1.0/10^3$  cells, not significant among three groups) than in control group ( $30.7 \pm 3.7/10^3$  cells,  $P < 0.05$ ; Fig. 4F and G). At Days 1 and 4, the number of TUNEL-positive cells was significantly smaller in Post-G group (Day 1,  $73.8 \pm 11.4/10^3$  cells; Day 4,  $12.8 \pm 1.0/10^3$  cells; Day 7,  $4.7 \pm 2.0/10^3$  cells) than in control group (Day 1,  $116.0 \pm 17.1/10^3$  cells; Day 4,  $30.7 \pm 3.7/10^3$  cells; Day 7,  $4.2 \pm 1.9/10^3$  cells,  $P < 0.05$ ; Fig. 5A). Furthermore, we examined double-immunohistochemical analysis to identify the cell type of apoptotic cells. We observed very few apoptotic cardiomyocytes at all the time points. The percentage of von Willebrand factor-positive cells in TUNEL-positive cells was significantly smaller in the Post-G group (Day 1,  $0.8 \pm 0.5\%$ ) than in control group (Day 1,  $5.5 \pm 1.5\%$ ,  $P < 0.05$ ; Fig. 5B).

## DISCUSSION

Because LV remodeling after MI determines subsequent cardiac function and prognosis, the inhibition of LV remodeling is clinically very important (1). After MI, many cardiomyocytes undergo cell death by the mechanisms of necrosis and apoptosis in the infarcted area, which is then replaced by fibrous tissue. The infarcted area is gradually extended by the subsequent death of cardiomyocytes and vascular cells in the border area and expanded by abnormal wall tension (2). Myocardial ischemia plays a critical role in the cardiomyocyte death in the border area after MI and thus greatly affects LV remodeling. It has been reported that a subset of BMSCs differentiates into cardiomyocytes when injected into peri-infarcted area, which results in regeneration of infarcted heart (3). Moreover, it has been reported that pretreatment with G-CSF and SCF attenuates LV remodeling after MI (4). Although these results suggest that the cytokine treatment is beneficial to prevent LV remodeling, the cytokine treatment was started before MI and thus this protocol cannot be applied to humans. Furthermore, the molecular mechanism of how the cytokine treatment repairs the infarcted heart is not fully understood. In the present study, 1) cytokine treatments started after MI were as effective as the pretreatment; 2) there was no significant difference in all parameters, including fibrotic area, cardiac function, and survival rate between the treatment with G-CSF alone and the combination treatment with G-CSF and SCF; 3) more capillaries were observed in the border area of the treatment groups with G-CSF compared with control group and SCF alone group, and the number of apoptotic cells was smaller in the three cytokine treatment groups with G-CSF than in control group.

It is very important to determine whether the combination therapy of G-CSF and SCF has additive effects on LV remodeling and cardiac function. Although it was reported that the combined treatment with G-CSF and SCF synergistically increased mobilization of BMSCs, it remains unknown whether the combined treatment also has synergistic effects on MI heart. Therefore, we compared the effects of G-CSF alone or SCF alone with those of combination treatment on LV remodeling and cardiac function after MI. Although the treatment with G-CSF alone showed effects similar to the combination treatment, the treatment with SCF alone could neither prevent LV remodeling nor improve survival rate after MI. It was previously reported that continuous treatment with SCF alone failed to induce any detectable change in the number of WBC throughout first 5 days and that a transient increase in WBC was increased at Day 7 after treatment in normal mice (16). Treatment with G-CSF alone increased the number of WBC at Day 4 after treatment. The different ability of BMSC mobilization between G-CSF and SCF may cause the different effects on LV remodeling after MI. These results suggest that G-CSF treatment started after MI is as beneficial as the pretreatment or the combination treatment with G-CSF and SCF to prevention of LV remodeling.

It has been reported that G-CSF induces the mobilization of BMSCs from BM into the peripheral blood circulation (17). There were more capillaries in the border area of all the cytokine treatment groups with G-CSF than in control group after MI, and a part of the cells constituting the capillary wall were derived from BM cells. There were few GFP-positive cardiomyocytes in the hearts in spite of the cytokine therapy. We think two possible mechanisms by which the cytokine therapy has beneficial effects on MI heart. One mechanism is that the cytokine therapy mobilizes BMSCs into MI heart and induces angiogenesis. Several lines of evidence suggest that BMSCs injected into myocardium induce angiogenesis (18, 19). Another mechanism is that the number of apoptotic endothelial cells is decreased by the cytokine therapy. The number of both von Willebrand factor and TUNEL-positive cells was significantly smaller in the Post-G group than in the control group. These mechanisms may result in an increase in capillary density leading to a decrease in apoptosis of cardiac myocytes in the border area. Recently, we observed that G-CSF induced activation of Akt, which has been reported to play an important role in cell survival and angiogenesis (20), in the border area after MI in swine (unpublished data). These results suggest that an increase in Akt activity by G-CSF may be also associated with an increase in the number of capillary vessels and a decrease in the number of apoptotic cells in the border area, resulting in better myocardial perfusion and less remodeling in the border area. Moreover, we recently examined whether G-CSF influences expression level of an angiogenic growth factor, vascular endothelial growth factor (VEGF; 21), after MI in swine. The expression of VEGF protein in the border area was increased in G-CSF-treated group than in the control group after MI (unpublished data). Further studies are needed to clarify what kinds of growth factors and cytokines are involved in angiogenic effects of the cytokine therapy in mouse MI model, and it also remains to be determined which cells (e.g., hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells) become vascular cells and whether neovascularization is a major mechanism for G-CSF-induced prevention of LV remodeling after MI.

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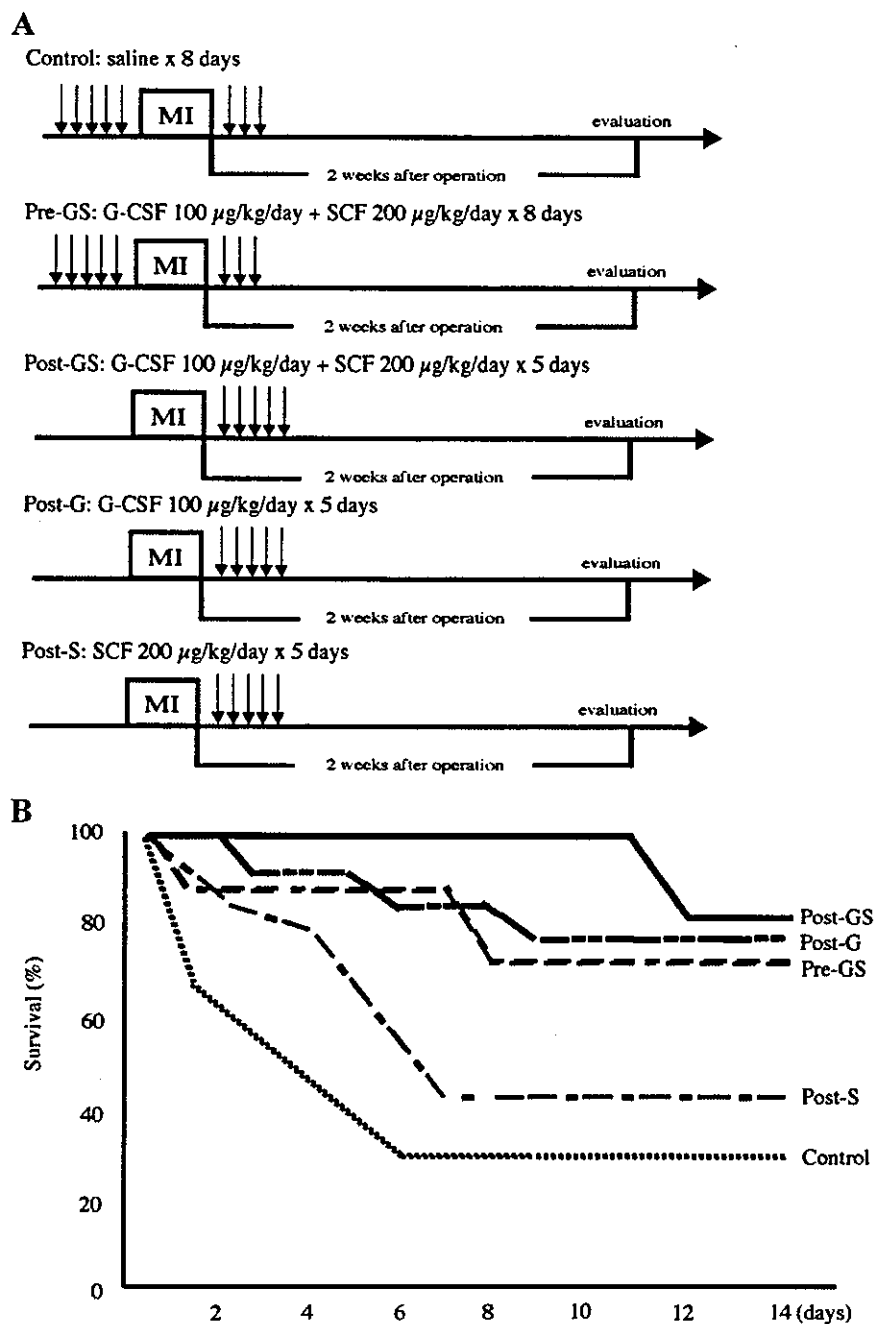


**Table 1****Hemodynamic parameter after MI**

	Sham (n=5)	Control (n=5)	Pre-GS (n=10)	Post-GS (n=16)	Post-G (n=14)	Post-S (n=9)
LVP (mmHg)	103.1±13.9	61.8±11.7	87.3±16.1*	89.9±17.5*	85.7±6.7*	63.3±5.5
LVEDP (mmHg)	2.0±0.8	13.3±6.8	4.1±2.5*	3.3±3.3*	4.2±2.6*	12.4±2.5
dP/dt (mmHg/s)	6034±302	2048±558	2860±567*	3241±710*	2558±373*	2106±466
-dP/dt (mmHg/s)	5805±364	2018±459	2778±567*	3169±703*	2598±371*	2011±479

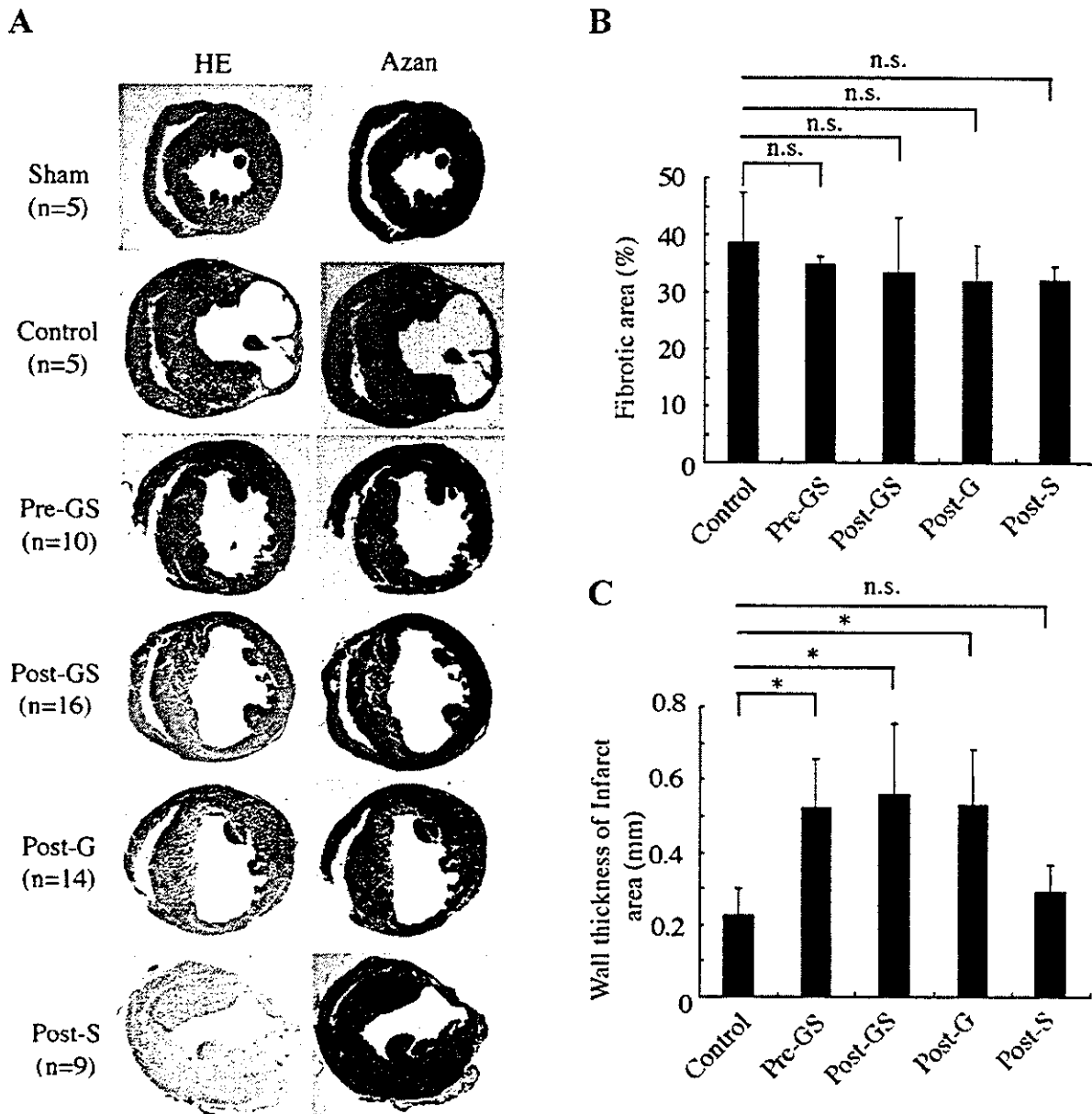
LVP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt and -dP/dt, positive and negative first derivatives for maximal rates of left ventricular pressure development. Values are mean ± SD. \*P < 0.05 vs. Control group.

**Fig. 1**



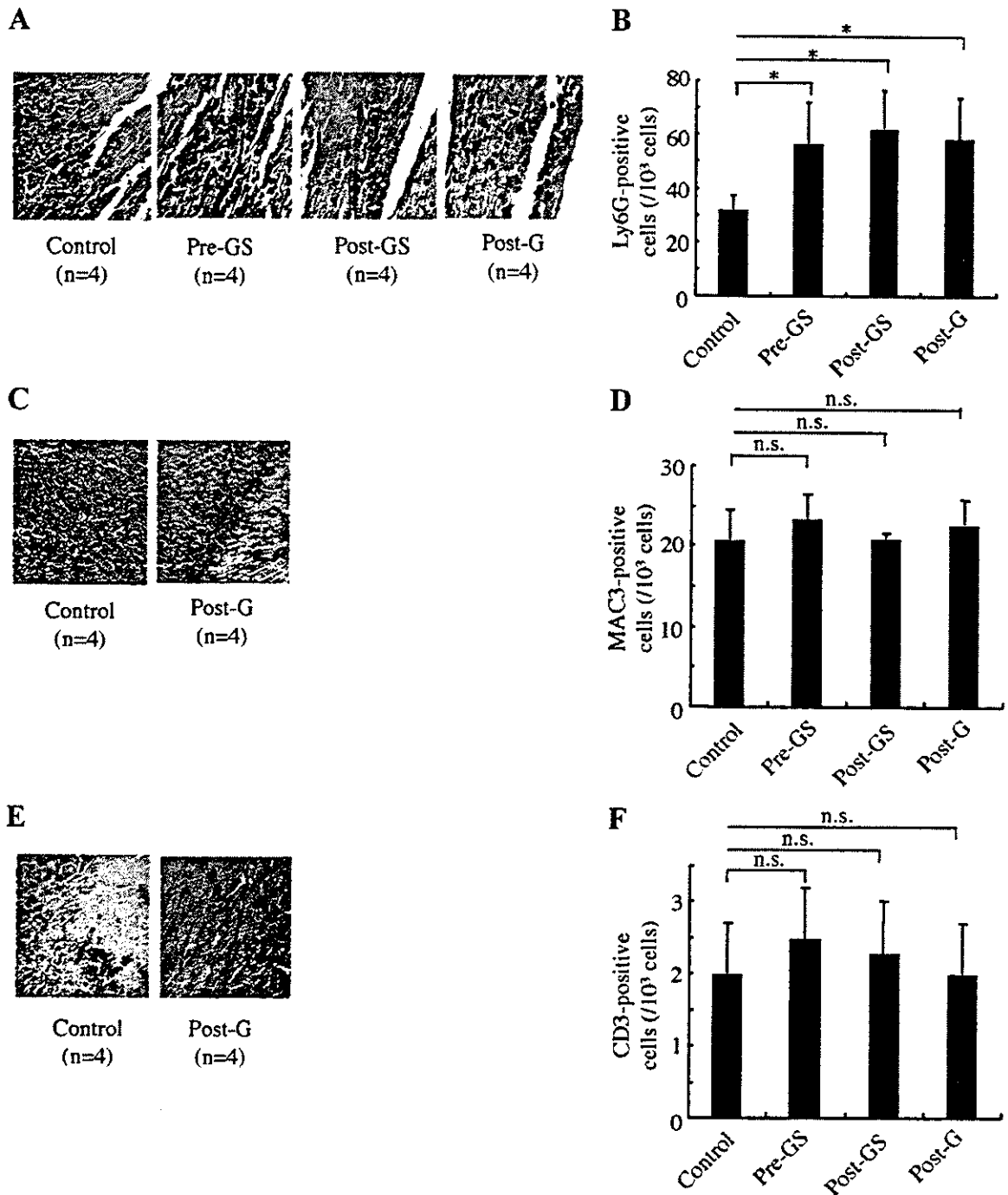
**Figure 1. Protocol of the cytokine treatment and survival rate. A)** Mice in pretreatment group (Pre-GS) were injected subcutaneously (s.c.) with SCF (200  $\mu$ g/kg/day) and G-CSF (100  $\mu$ g/kg/day) from 5 days before MI until 3 days after MI ( $n=16$ ). Mice in post-treatment groups were injected s.c. with G-CSF alone (Post-G;  $n=20$ ), SCF alone (Post-S;  $n=21$ ), or SCF and G-CSF once a day for 5 days after MI (Post-GS;  $n=20$ ). Mice in control group (Control) were injected with saline for 8 days before and after MI ( $n=22$ ). **B)** Survival rates of mice at 14 days were higher in 3 treatment groups with G-CSF than in control group in Kaplan-Meier method.

**Fig. 2**



**Figure 2. Morphological analysis.** We examined hemodynamic parameters of the survived mice (Sham,  $n=5$ ; Control,  $n=5$ ; Pre-GS,  $n=10$ ; Post-GS,  $n=16$ ; Post-G,  $n=14$ ; Post-S,  $n=9$ ) at 2 weeks after MI by cardiac catheterization and subsequently performed histological analysis. **A**) Light microscopic analysis in hematoxylin-eosin (HE) staining and Azan-Mallory staining. LV free wall was thinner in heart of control group and Post-S group than in heart of Post-G, Post-GS, and Pre-GS groups (HE: left panel). Infarct area was replaced completely by fibrotic tissue in control group, while remained myocardium was observed in the hearts of all the treatment groups with G-CSF (Azan: right panel). **B**) Fibrotic area in the whole LV area. There was no significant difference in the percentages of fibrotic area among the treatment groups and control group (n.s., not significant.). **C**) Wall thickness of infarct area was thinner in hearts of control group and Post-S group than in hearts of Post-G, Post-GS, and Pre-GS groups ( $*P<0.05$ ).

Fig. 3



**Figure 3. Inflammatory cells in the heart.** *A, B*) The number of Ly6G-positive cells (granulocytes) at the border area of infarcted heart was greater in the three treatment groups than in control group ( $*P < 0.05$ ) at 4 days after MI. There was no significant difference in the number of MAC3-positive cells (macrophages; *C, D*) and CD3-positive cells (T-lymphocytes; *E, F*) among the three treatment groups and control group.