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Beraprost sodium enhances neovascularization in ischemic myocardium by mobilizing bone marrow cells in rats

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Abstract

Beraprost sodium, an orally active prostacyclin analogue, has vasoprotective effects such as vasodilation and antiplatelet activities. We investigated the therapeutic potential of beraprost for myocardial ischemia. Immediately after coronary ligation of Sprague–Dawley rats, beraprost (200 μg/kg/day) or saline was subcutaneously administered for 28 days. Four weeks after coronary ligation, administration of beraprost increased capillary density in ischemic myocardium, decreased infarct size, and improved cardiac function in rats with myocardial infarction. Beraprost markedly increased the number of CD34-positive cells and c-kit-positive cells in plasma. Also, four weeks after coronary ligation of chimeric rats with GFP-expressing bone marrow, bone marrow-derived cells were incorporated into the infarcted region and its border zone. Treatment with beraprost increased the number of GFP/von Willebrand factor-double-positive cells in the ischemic myocardium. These results suggest that beraprost has beneficial effects on ischemic myocardium partly by its ability to enhance neovascularization in ischemic myocardium by mobilizing bone marrow cells.

Keywords: Prostacyclin analogue; Myocardial infarction; Neovascularization; Bone marrow mobilization

Interruption of myocardial blood flow leads to rapid death of cardiomyocytes and vascular structures, resulting in the development of heart failure [1]. Stem or progenitor cells are mobilized from bone marrow into the peripheral blood in response to tissue ischemia, migrate to sites of injured tissues, and differentiate into endothelial cells and cardiomyocytes [2–4]. However, the compensatory mechanisms are insufficient to heal infarcted myocardium. Earlier studies have shown that bone marrow cells artificially mobilized by cytokines repair the infarcted heart and improve cardiac function after acute myocardial infarction [5,6]. Therefore, enhancement of bone marrow cell mobilization leading to neovascularization following revascularization would be beneficial for the treatment of acute myocardial infarction.

Beraprost sodium (BPS) is a chemically stable prostacyclin analogue owing to its cyclo-pentabenzofuranyl structure [7]. It has been well established that BPS has vasoprotective effects such as vasodilation and antiplatelet activities [8–11]. Thus, BPS has been used in the treatment of peripheral arterial disease [12,13] and pulmonary arterial hypertension [14,15]. Although a limited number of studies suggest therapeutic potential of prostacyclin for the treatment of myocardial ischemia [16–18], the underlying mechanisms still remain unclear. In addition, little information is available regarding the therapeutic potential of prostacyclin analogues such as BPS for myocardial ischemia. A recent study has shown that BPS activates endothelial

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nitric oxide synthase (eNOS) through the c-AMP/protein kinase A pathway [19]. Activation of eNOS is known to contribute to bone marrow cell mobilization, leading to neovascularization [20]. These results raise the possibility that BPS may have beneficial effects on the ischemic myocardium through enhancement of bone marrow cell mobilization.

Thus, the purposes of this study were: (1) to examine the effect of BPS on mobilization and recruitment of bone marrow cells after acute myocardial infarction, (2) to investigate whether BPS induces neovascularization in the ischemic myocardium, and (3) to investigate whether treatment with BPS improves cardiac function in rats with myocardial infarction.

Methods

Model of myocardial infarction. We used male Sprague–Dawley rats (Japan SLC Inc., Hamamatsu, Japan) weighing 185–215 g. Myocardial infarction was produced by left coronary ligation, as described previously [21]. Briefly, after rats were anesthetized with sodium pentobarbital (30 mg/kg), they were artificially ventilated with a volume-regulated respirator. The heart was exposed via a left thoracotomy incision. Then, the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. Finally, the heart was restored to its normal position, and the chest was closed. Experimental protocols were performed in accordance with the "Guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute", which complies NIH Guidelines.

Administration of BPS. Immediately after coronary ligation, BPS (200 μg/kg/day, Astellas Pharma Inc., Tokyo, Japan) was subcutaneously administered to surviving rats using an osmotic mini-pump for 4 weeks (BPS group, $n = 12$). As a control, saline was similarly administered to rats receiving coronary ligation (Control group, $n = 12$).

Echocardiographic studies. Echocardiographic studies were performed 4 weeks after coronary ligation. M-mode tracings were obtained at the level of the papillary muscles using an echocardiographic system equipped with a 7.5-MHz phased-array transducer (HP SONOS 5500; Hewlett Packard Co., Andover, MA). Anterior and posterior end-diastolic and end-systolic wall thickness, LV end-diastolic and end-systolic dimensions, and LV fractional shortening were measured by the American Society for Echocardiography leading-edge method in three consecutive cardiac cycles. LV meridional wall stress was estimated as $0.344 \times \text{LV pressure} \times \{\text{LV dimension}/(1 + \text{PWT}/\text{LV dimension})\}$, where PWT is posterior wall thickness [22].

Hemodynamic studies. Hemodynamic studies were performed 4 weeks after coronary ligation, following echocardiography. After anesthesia with pentobarbital sodium, a 1.5F micromanometer-tipped catheter (Millar Instruments Inc., Houston, TX) was advanced into the LV through the right common carotid artery. Hemodynamic variables were measured with a pressure transducer connected to a polygraph. After completion of these measurements, the left and right ventricles and the lungs were excised and weighed. Infarct size was determined as a percentage of the entire LV area ($n = 5$ in each group), as reported previously [23]. Briefly, incisions were made in the posterior LV so that the tissue could be pressed flat. The circumference of the entire flat LV and of 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 infarct size and was expressed as a percentage of LV surface area.

Measurement of plasma ANP level. Blood samples were obtained 4 weeks after coronary ligation. Plasma atrial natriuretic peptide (ANP), a marker for heart failure, was measured by enzyme immunoassay (Peninsula Laboratories Inc., San Carlos, CA).

Mononuclear cell mobilization and FACS analysis. To investigate whether administration of BPS mobilizes bone marrow cells, an additional 12 rats were randomized to receive BPS (200 μg/kg/day, BPS group, $n = 6$) or saline (Control group, $n = 6$). On the third day of BPS or saline treatment, 4 ml of blood was drawn from the inferior vena cava of each rat. Peripheral blood was obtained at the end of infusion. After mononuclear cells were counted, they were incubated for 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz) and CD45 (clone OX-1), and FITC-conjugated rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz). Immunofluorescence-labeled cells were analyzed by quantitative flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Isotype-identical antibodies served as controls.

RT-PCR assay. To investigate whether bone marrow cells express the prostacyclin receptor (IP receptor), we analyzed expression of its mRNA by reverse transcription-polymerase chain reaction (RT-PCR). In brief, total RNA of bone marrow cells was extracted with guanidine isothiocyanate (RNeasy Mini Kit, Qiagen). Then, reverse-transcribed single-stranded cDNA was subjected to PCR (PCR Amplification Kit, Takara) using primer sets for the IP receptor (Hokkaido System Science Co., Ltd., Sapporo, Japan, forward, 5′-GGCACGAGAGGATGAAGTTTACC-3′; reverse, 5′-GTCAGAGGCACAGCAGTCAATGG-3′) and G3PDH (Clonetech Laboratories Inc., Mountain View, CA, forward, 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′; reverse, 5′-CATGTAGGCCATGAGGTCCACCAC-3′).

Creation of bone marrow-chimeric rats. To assess recruitment of bone marrow cells after BPS administration, bone marrow transplantation was performed by using male normal Sprague–Dawley rats as recipients and male Green fluorescent protein (GFP)-transgenic rats (SD-Tg [Act-EGFP] CZ-004Osb, Japan SLC Inc.) as donors, using a previously described method [24]. Briefly, bone marrow was harvested by flushing the cavity of femurs and tibias from GFP-transgenic rats with phosphate-buffered saline. Then, 3×10^7 GFP-positive bone marrow cells were individually administered to 12 lethally irradiated (900 Gray) rats via the tail vein. Four weeks after transplantation, flowcytometric analysis determined that 90% of peripheral blood mononuclear cells from both donors and 8 of 12 chimeric rats were GFP-positive, suggesting the establishment of stable chimerism. These chimeric rats were subjected to left coronary ligation, followed by administration of BPS (200 μg/kg/day, BPS group, $n = 4$) or saline (Control group, $n = 4$) using an osmotic mini-pump for 4 weeks.

Histological examination. To detect fibrosis in the cardiac muscle, the LV myocardium ($n = 5$, each group) was fixed in 10% formalin, cut transversely in three sections, embedded in paraffin, and stained with Masson's trichrome. To detect capillary endothelial cells in the peri-infarct area, we performed DAB staining (LSAB2 System HRP, Dako Cytomation Co., Denmark) using rabbit polyclonal anti-von Willebrand factor (vWF) antibody (Dako). A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted in the peri-infarct area using a light microscope at 200× magnification. Capillary density was expressed as the mean number of capillaries per square millimeter. Also, 4 weeks after coronary ligation in bone marrow-chimeric rats ($n = 4$ in each group), the LV myocardium was excised, embedded in OCT compound, snap-frozen in liquid nitrogen, and cut transversely into 6-μm-thick sections from base to apex. Immunofluorescent staining was performed using rabbit polyclonal anti-vWF antibody (Dako), mouse monoclonal anti-cardiac troponin T antibody (Neomarkers, Fremont, CA), and rabbit polyclonal Alexa 488-conjugated anti-GFP antibody (Molecular Probes Inc., Eugene, OR). The nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). We measured the number of GFP/vWF-double-positive cells incorporated into vascular structures in 10 randomly selected fields in the peri-infarct area per section in a blinded fashion using a fluorescence microscope.

Statistical analysis. Numerical values are expressed as means ± SEM. Comparisons of parameters between two groups were made by unpaired Student's t test. A value of $p < 0.05$ was considered significant.

Results

Cardiac structure

Body weight at 4 weeks after coronary ligation was significantly greater in the BPS group than in the Control group (Table 1). Right ventricular weight and lung weight in the BPS group were significantly smaller than those in the Control group, although LV weight did not differ between the two groups. Moderate to large infarcts were observed in the Control group (Fig. 1A). However, administration of BPS significantly decreased infarct size in rats with myocardial infarction (Fig. 1A and B). BPS significantly decreased LV end-diastolic dimension (LVDD) (Fig. 1C).

Table 1
Physiological profiles of experimental groups

	Control	BPS
Number	12	12
Body weight (g)		
Baseline	198 ± 3	204 ± 3
After treatment	319 ± 6	352 ± 9*
LV wt/body wt (g/kg)	2.28 ± 0.04	2.27 ± 0.04
RV wt/body wt (g/kg)	0.99 ± 0.05	0.61 ± 0.02**
Lung wt/body wt (g/kg)	6.55 ± 0.62	3.88 ± 0.1**
Plasma AND level (pg/ml)	798 ± 99	498 ± 57*

Control, infarct rats without treatment; BPS, infarct rats treated with BPS administration; AND, atrial natriuretic protein. Data are expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group.

Cardiac function

Neither heart rate nor mean arterial pressure differed between the BPS and Control groups (Table 2). LV fractional shortening and LV maximum dP/dt in the BPS group were significantly greater than those in the Control group (Fig. 2A and B). LV end-diastolic pressure (LVEDP) in the BPS group was significantly lower than that in the Control group (Fig. 2C). LV minimum dP/dt was also improved by BPS (Fig. 2D). Treatment with BPS attenuated the increase in plasma ANP level after myocardial infarction (Table 1). BPS significantly increased anterior wall thickening, although it did not significantly alter posterior wall thickening (Table 2). Thickness of the anterior and posterior walls tended to be greater in the BPS group, but these changes did not reach statistical significance. LV diastolic wall stress in the BPS group was significantly lower than that in the Control group.

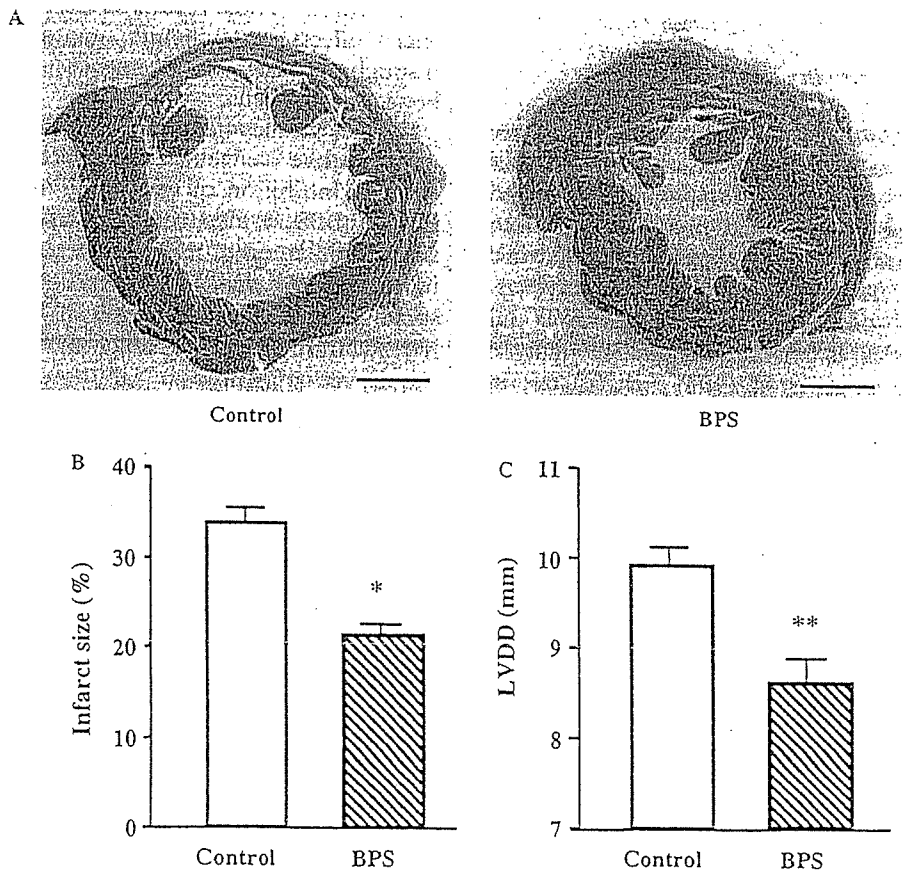

Fig. 1. (A) Representative examples of Masson's trichrome staining of transverse sections of LV myocardium 4 weeks after coronary ligation. Scale bars = 2 mm. (B,C) Quantitative analysis of infarct size and LV end-diastolic dimension (LVDD). Infarcted area and LVDD in the BPS group were significantly smaller than those in the Control group. Data are expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group.

Table 2
Echocardiographic and hemodynamic data

	Control	BPS
AWT diastole (mm)	0.62 ± 0.04	0.74 ± 0.05
AW thickening (%)	17 ± 3	34 ± 6*
PWT diastole (mm)	1.55 ± 0.07	1.70 ± 0.04
PW thickening (%)	43 ± 4	49 ± 3
Heart rate (bpm)	458 ± 7	471 ± 10
Mean arterial pressure (mmHg)	103 ± 5	115 ± 4
LV systolic pressure (mmHg)	113 ± 4	127 ± 5*
LV diastolic wall stress (kdyne/cm^2)	24 ± 4	5 ± 1**
LV systolic wall stress (kdyne/cm^2)	267 ± 18	225 ± 14

AWT, anterior wall thickness; AW, anterior wall; PWT, posterior wall thickness; PW, posterior wall. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

Mobilization of bone marrow cells

RT-PCR demonstrated that IP receptor mRNA was expressed in bone marrow cells (Fig. 3A), indicating a direct effect of BPS on these cells. Three-day administration of BPS significantly increased the number of peripheral blood mononuclear cells compared to saline administration (Fig. 3B). Administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells (Fig. 3C and D). BPS also increased the number of CD45-positive hematopoietic lineage cells (Fig. 3E).

BPS-induced neovascularization

Chimeric rats with GFP-expressing bone marrow were used to assess recruitment of bone marrow cells. Four weeks after coronary ligation, bone marrow-derived GFP-positive cells were incorporated predominantly into the infarcted region and its border zone (Fig. 4A), while these cells were rarely detected in the noninfarcted myocardium. Some of the GFP-positive cells stained for vWF and formed vascular structures. Semi-quantitative analysis demonstrated that the number of GFP-positive cells in the myocardium was significantly greater in the BPS group than in the Control group (Fig. 4B). The number of GFP-vWF double-positive cells (bone marrow-derived endothelial cells) in the ischemic myocardium was significantly greater in the BPS group than in the Control group (Fig. 4C). In addition, a small number of GFP-troponin T-double-positive cells were observed in the BPS group (Fig. 4D).

Capillary density

In the peri-infarct area, clustering of relatively small vessels was seen in BPS-treated hearts, which is indicative of recent endothelial regeneration (Fig. 5A). Semi-quantitative analysis also demonstrated that administration of BPS significantly increased the capillary density in the peri-infarct area compared to the Control group (Fig. 5B).

Discussion

In the present study, we demonstrated that treatment with BPS (1) decreased infarct size and improved cardiac structure and function in rats with acute myocardial infarction, (2) increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells in rats, and (3) increased the number of bone marrow-derived endothelial cells and the capillary density in the ischemic myocardium. These results suggest that BPS may have beneficial effects on ischemic myocardium at least in part through enhancement of neovascularization by mobilizing bone marrow cells.

Earlier studies have reported that prostacyclin has cardioprotective effects in ischemia–reperfusion injury through inhibition of neutrophil activation and migration [25,26]. BPS is also reported to inhibit chemotaxis and superoxide anion production of neutrophils which contribute to tissue damage by releasing tissue destructive lysosomal enzymes [27]. Infusion of BPS has been shown to reduce infarct size in the dog heart with left coronary occlusion by reducing myocardial oxygen demand and by inhibition of the migration of neutrophils [28]. However, these

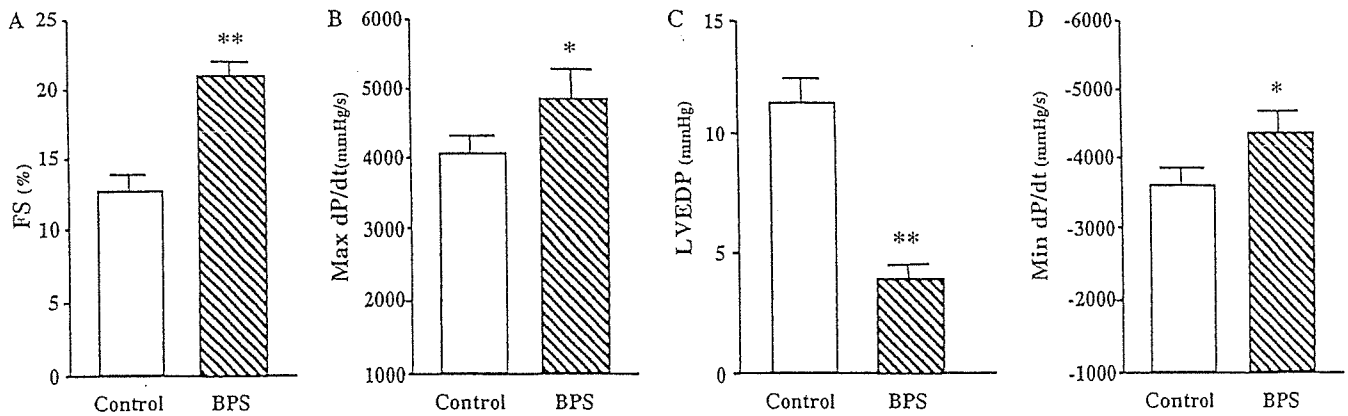

Fig. 2. Cardioprotective effects of BPS on echocardiographic and hemodynamic parameters. FS, fractional shortening; LVEDP, LV end-diastolic pressure; Max and Min dP/dt, maximum and minimum dP/dt. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

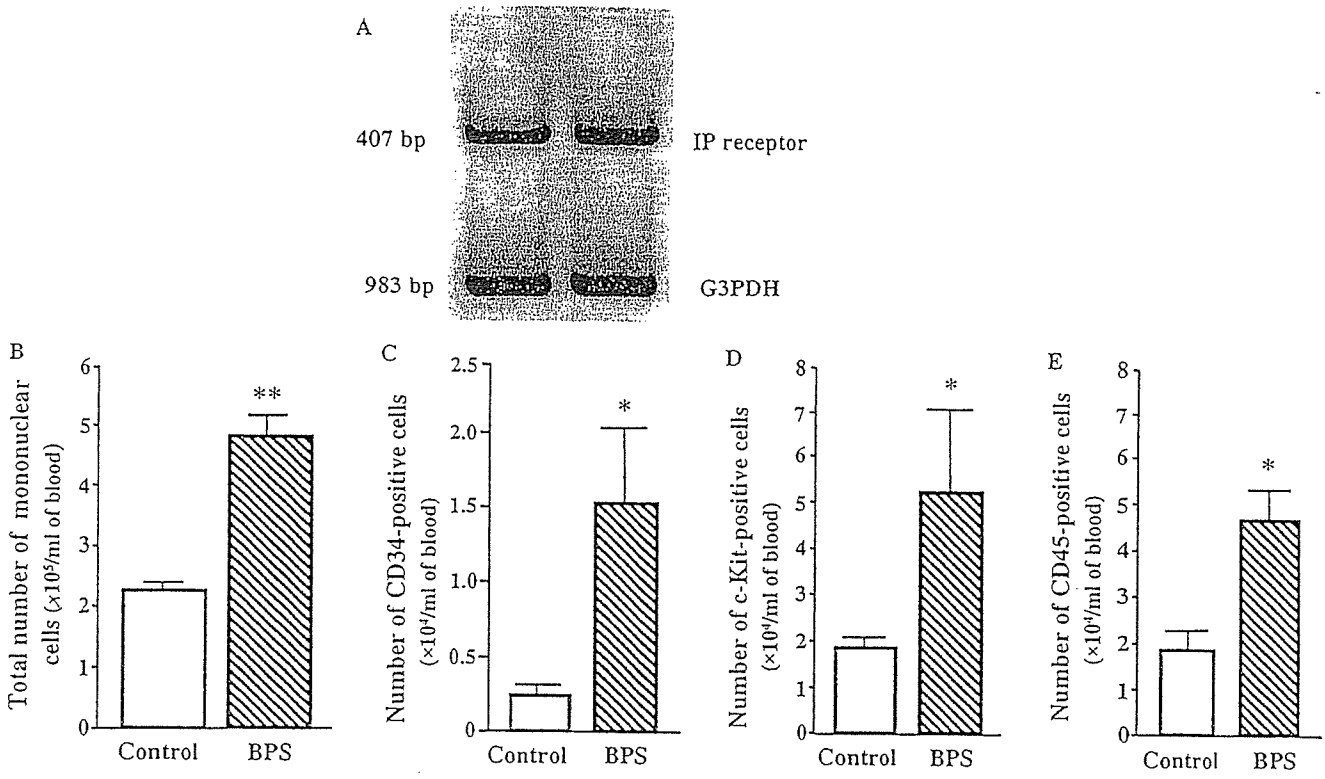

Fig. 3. BPS-induced mobilization of bone marrow cells. (A) Expression of prostacyclin receptor (IP receptor) on bone marrow cells. (B–E) Quantification of BPS-induced MNC mobilization by FACS analysis. Administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells. BPS also increased the number of CD45-positive hematopoietic lineage cells. Data are expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group.

biological activities of BPS appear to be insufficient to explain the decrease in infarct size as well as suppression of LV remodeling.

Recent studies have shown that mobilization of bone marrow cells by cytokines promotes myocardial repair and regeneration after acute myocardial infarction [5,6]. In the present study, three-day administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells in rats. In addition, treatment with BPS enhanced recruitment of bone marrow cells to the ischemic myocardium and increased capillary density in the peri-infarct area. Earlier studies have shown that CD34-positive cells have angiogenic potential to treat ischemic heart [29–31]. Also, another stem cell fraction, c-kit-positive cells have ability to repair ischemic myocardium by differentiating into vascular endothelial cells [32,33]. These findings suggest that administered BPS induces neovascularization partly via enhancement of bone marrow cell mobilization. RT-PCR demonstrated that IP receptor mRNA was expressed in bone marrow cells, indicating a direct effect of BPS on these cells. A recent study has shown that BPS increases eNOS expression in cultured endothelial cells through activation of c-AMP/Protein kinase A signal transduction [19]. Also, earlier studies have shown that eNOS plays essential role in the recruitment of EPCs to the ischemic myocardium [20]. Taken together, administered BPS may act as a potent stimulator of cell mobilization from bone marrow, although further studies are necessary to examine the underlying mechanisms.

In the present study, treatment with BPS significantly attenuated infarct size after myocardial infarction. BPS improved cardiac function and attenuated the development of LV remodeling after acute myocardial infarction, as indicated by increases in LV fractional shortening and maximum dP/dt, and decreases in LVEDP and LVDD. Taken together, BPS may attenuate myocardial infarction through enhancement of neovascularization via modification of bone marrow kinetics. Interestingly, a small fraction of mobilized bone marrow cells expressed cardiac troponin T in the ischemic myocardium in the BPS group, suggesting that BPS may partially contribute to myocardial regeneration after acute myocardial infarction. Earlier studies have demonstrated that BPS has other beneficial effects for ischemic heart disease including anti-thrombotic activity [34], inhibition of reperfusion injury [35], and prevention of coronary spasm [36], and re-stenosis [37]. These findings suggest that administration of BPS may be a promising therapy for acute myocardial infarction.

Granulocyte colony stimulating factor (G-CSF) is currently used agent for mobilization of bone marrow. Infusion of G-CSF after myocardial infarction improves LV function increasing peripheral stem cell fraction [5,38]. A recent clinical trial, however, claimed the G-CSF therapy

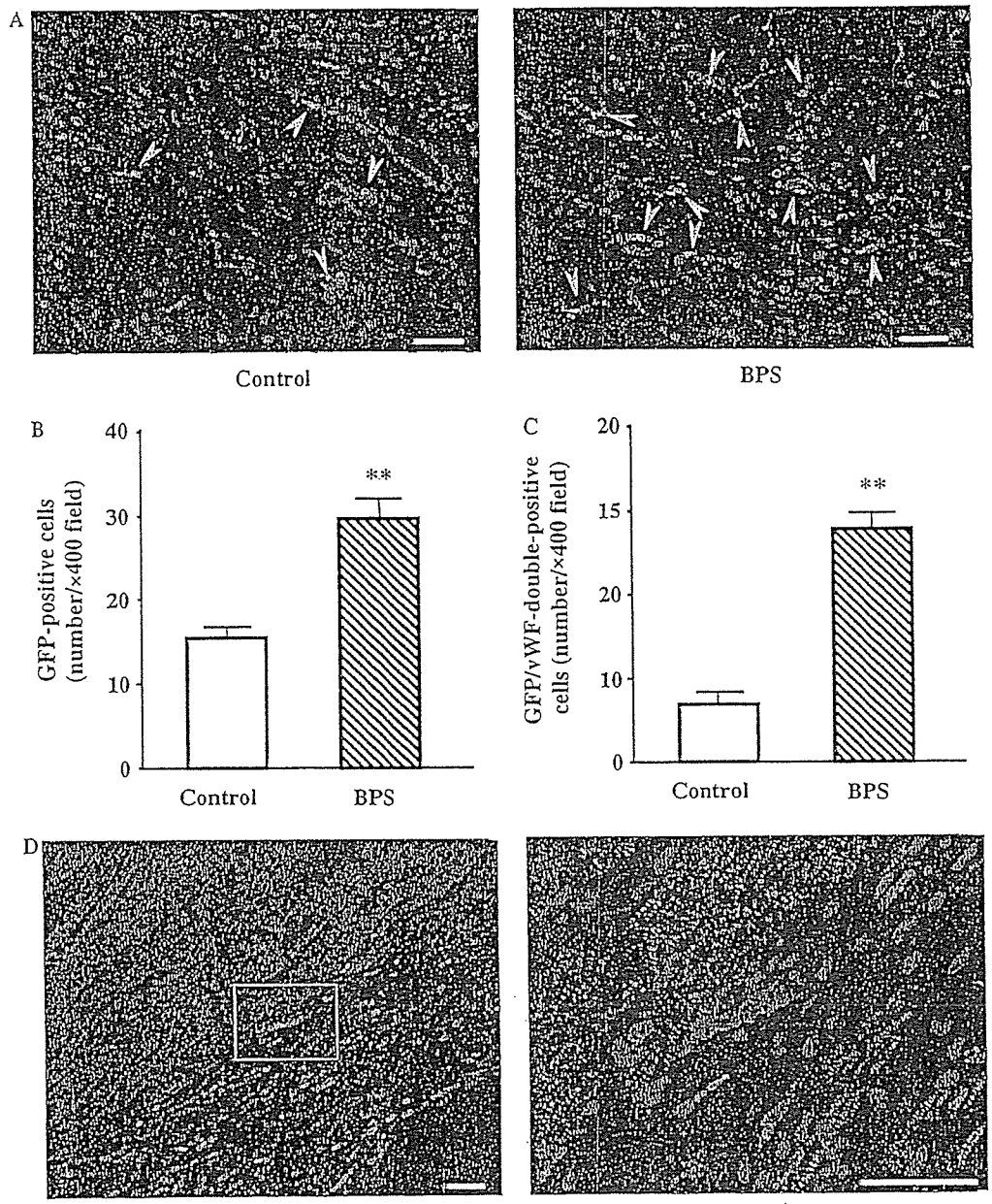

Fig. 4. BPS-induced neovascularization. (A) Representative immunofluorescent images stained with antibodies to von-Willbrand factor (vWF, red) and green fluorescent protein (GFP, green). Nuclei were counterstained with DAPI (blue). (B,C) Semi-quantitative analyses of numbers of GFP-positive cells and GFP-vWF double-positive cells in the peri-infarct area. (D) Representative immunofluorescent image of GFP-positive cells (green) expressing cardiac troponin T (red) observed in the BPS group. Scale bars = 50 μm. Data are expressed as means ± SEM. $^{**}p < 0.01$ vs. Control group.

has serious problem with re-stenosis after recanalization [39]. On the other hand, the safety of BPS has been identified in the treatment of peripheral arterial disease [12,13] and pulmonary arterial hypertension [14,15]. A randomized, controlled clinical trial failed to demonstrate therapeutic potential of prostacyclin for the treatment of severe congestive heart failure [40], which has long discouraged the pursuit of prostacyclin as a therapeutic option for the treatment of acute myocardial infarction. Interestingly, however, double-blinded, randomized, placebo-controlled, large-scale studies showed that treatment with BPS decreased vascular events in patients with peripheral arterial disease [41,42]. Thus, adequate use of BPS for only acute myocardial infarction may have beneficial effects on ischemic myocardium, although further preclinical trials are required to verify the safety and efficacy of BPS.

Conclusion

In summary, administration of BPS improved cardiac structure and function in rats with acute myocardial infarction. This beneficial effect of BPS may be mediated partly by its ability to enhance neovascularization in ischemic myocardium by mobilizing bone marrow cells.

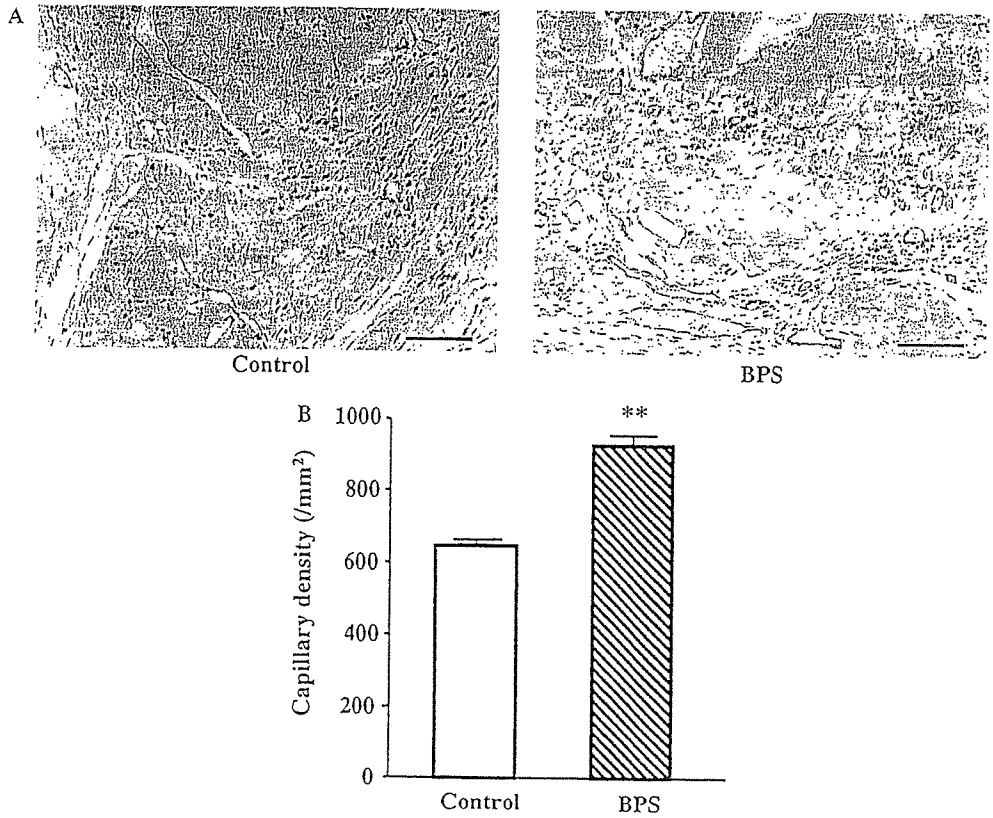

Fig. 5. (A) Representative samples stained with antibody to von Willebrand factor by bright-field DAB. (B) Quantitative analysis of capillary density in peri-infarct area. Administration of BPS increased capillary density by 37%. Scale bars = 50 μm. Data are expressed as means ± SEM. $^{**}p < 0.01$ vs. Control group.

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Unblinded Pilot Study of Autologous Transplantation of Bone Marrow Mononuclear Cells in Patients With Thromboangiitis Obliterans

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Background—The short-term clinical benefits of bone marrow mononuclear cell transplantation have been shown in patients with critical limb ischemia. The purpose of this study was to assess the long-term safety and efficacy of bone marrow mononuclear cell transplantation in patients with thromboangiitis obliterans.

Methods and Results—Eleven limbs (3 with rest pain and 8 with an ischemic ulcer) of 8 patients were treated by bone marrow mononuclear cell transplantation. The patients were followed up for clinical events for a mean of 684±549 days (range 103 to 1466 days). At 4 weeks, improvement in pain was observed in all 11 limbs, with complete relief in 4 (36%). Pain scale (visual analog scale) score decreased from 5.1±0.7 to 1.5±1.3. An improvement in skin ulcers was observed in all 8 limbs with an ischemic ulcer, with complete healing in 7 (88%). During the follow-up, however, clinical events occurred in 4 of the 8 patients. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with an incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient showed worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year.

Conclusions—In the present unblinded and uncontrolled pilot study, long-term adverse events, including death and unfavorable angiogenesis, were observed in half of the patients receiving bone marrow mononuclear cell transplantation. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving this treatment. (*Circulation.* **2006;114:2679-2684.**)

Key Words: angiogenesis ■ collateral circulation ■ endothelium ■ peripheral vascular diseases

The clinical consequences of severe peripheral arterial disease or critical limb ischemia include rest pain and the loss of tissue integrity in the distal limb.[1–3] Therapeutic options for such patients are limited. These conditions are often refractory to conservative measures and are typically unresponsive to drug therapy. When vascular obstruction involves a long segment or is widespread, percutaneous revascularization may not be feasible. Surgical therapy, consisting of arterial bypass or amputation, is complicated by variable morbidity and mortality, and its effectiveness depends on the short- and long-term patencies of the conduit employed. Therapeutic angiogenesis thus constitutes a potential alternative treatment strategy for such patients.[4,5]

Previous investigators have suggested that endothelial progenitor cells, originating from bone marrow, circulate in adult peripheral blood and participate in postnatal neovascularization.[6–8] Subsequent experiments have shown that bone marrow or bone marrow–derived cells have the potential to stimulate angiogenesis and thereby modulate the hemodynamic deficit in ischemic limbs in vivo.[9,10] The Therapeutic Angiogenesis by Cell Transplantation (TACT) study first demonstrated that the magnitude of angiogenesis stimulated by these cells is sufficient to constitute a therapeutic benefit in patients with critical limb ischemia.[11] In that study, the investigators injected bone marrow mononuclear cells (BM-MNCs) into the ischemic limb of patients and documented a significant improvement in the hemodynamic deficit as well as the relief of ischemic symptoms. Although the TACT

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study established the concept of using BM-MNCs for therapeutic angiogenesis, limited information is available about the long-term safety and efficacy of this strategy.

The purpose of the present study was to determine the long-term safety and clinical impact of BM-MNC transplantation for "no-option" patients with thromboangiitis obliterans.

Methods

Patients

Eight patients with thromboangiitis obliterans were treated with an autologous transplantation of BM-MNCs between March 2002 and September 2004. The diagnosis of thromboangiitis obliterans was based on the criteria proposed by Olin[12]: (1) onset before age 45; (2) current (recent) history of tobacco use; (3) the presence of distal-extremity ischemia (infrapopliteal or infrabrachial) indicated by claudication, rest pain, ischemic ulcers, or gangrene; (4) exclusion of autoimmune or connective tissue diseases, hypercoagulable states, and diabetes mellitus; (5) exclusion of a proximal source of emboli by echocardiography and arteriography; and (6) consistent arteriographic findings in the clinically involved and noninvolved limbs.

Patients qualified for cell transplantation if they had chronic limb ischemia, with rest pain or a nonhealing ischemic ulcer, present for a minimum of 4 weeks without evidence of improvement in response to conventional drug therapy; showed angiographic evidence of vasculopenia in the affected limb; and were not candidates for percutaneous or surgical revascularization. The exclusion criteria included severe concurrent illness, the presence of proliferative diabetic retinopathy, and a history or clinical evidence of a malignant disorder.

All the patients involved in the present study received continuous medical therapy for >2 months before BM-MNC transplantation to confirm that conventional measures would be insufficient to achieve improvement in rest pain or skin ulcer/gangrene. During this period, no surgical therapies such as bypass grafting, extensive debridement, skin grafting, or limb amputation were performed. In addition, the patients were admitted to the hospital for a minimum of 1 month before BM-MNC transplantation to exclude the likelihood of spontaneous improvement in ischemic symptoms resulting from an enrollment bias. It should be also pointed out that the patients remained in the hospital and received the same therapy for at least 1 month after BM-MNC transplantation to avoid changes in their treatment.

BM-MNC Transplantation

While the patients were under general anesthesia, marrow cells were aspirated from the ileum. BM-MNCs were sorted on an AS-104 blood-cell separator (Fresenius HemoCare, Redmond, Wash) and concentrated to a final volume of ≈50 mL. After bone marrow cells were sorted on the AS-104 blood-cell separator, a small fraction of the cells was used for BM-MNC counting; the concentration of BM-MNCs in the final product was determined by using a microscope counting chamber after May-Giemsa staining. By using another fraction of cells, the number of $CD34^+$ cells in the BM-MNCs was also determined by fluorescence-activated cell sorting (FACS SCAN flow cytometer; Becton Dickinson, San Jose, Calif). The cells were incubated with the FITC-conjugated mouse monoclonal antibody against human CD34 (clone 581; Becton Dickinson) according to manufacturer's instructions.

For each patient, ≈100 aliquots of BM-MNCs (0.5 mL per aliquot) were administered via a syringe with a 27-gauge needle. Injection was performed into 9 lower limbs in 7 of the patients and the bilateral hands in 1. Injection sites were arbitrarily selected according to angiographic findings (ie, the degree of vasculopenia) and included calf muscles such as the soleus and gastrocunemius muscles as well as the sole muscles of the foot. For the patient with hand ischemia, injection was performed in palm muscles.

Assessment of Short-Term Outcome

Ischemic pain was assessed with a visual analog pain scale (VAS) with 10 levels. Ischemic ulcers were documented by color photography. Resting ankle-brachial pressure index (ABI) was calculated as the quotient of absolute ankle pressure and brachial pressure (the patient who received BM-MNC transplantation in his hands was excluded from ABI analysis). Angiographic assessment was performed with magnetic resonance angiography, computed tomographic angiography, or digital subtraction angiography. Adverse events were defined as death, limb amputation, pathological angiogenesis, recurrence/worsening of ischemic symptoms (ie, rest pain, skin ulcer, gangrene), myocardial infarction, stroke, and malignant disease.

Assessment of Long-Term Outcome

The mean length of follow-up was 684±549 days (range 103 to 1466). Patients were followed up by history analysis, physical examination, routine blood testing, ABI, and angiography at prescribed intervals during the first year, after which they were contacted at an outpatient clinic or by telephone to track events.

Data Analysis

All data are presented as mean±SD (range) or frequencies (percentage).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Diagnosis

The diagnosis of thromboangiitis obliterans was made according to the criteria described above. Among the 8 patients, only patient 6 did not completely fulfill the criteria; ie, this patient had no history of tobacco use (Table 1). Laboratory screening excluded the possibility of other underlying diseases, however, including autoimmune and connective tissue diseases. It should be also pointed out that patient 6 had diabetes mellitus at the time of cell transplantation but not at the onset of thromboangiitis obliterans. With the typical characteristic angiographic findings of thromboangiitis obliterans, such as multiple segmental arterial involvement (skip lesions) and "cork-screw" collateral vessels, we diagnosed patient 6 as having thromboangiitis obliterans, even though the patient did not have a history of tobacco use.

Patient Characteristics

The demographic and clinical data of the 8 patients are shown in Table 1. The mean age of the patients enrolled was 46±14 years (range 28 to 63). Seven patients (88%) were male. One patient had undergone prior femoral-tibial artery bypass grafting, and 1 had undergone sympathetic ganglion block. These treatments were performed >1 year before BM-MNC transplantation. Seven patients (88%) had a history of smoking, all of whom stopped smoking at least 1 month before transplantation.

Short-Term Outcome

The total volume of cells aspirated from the ileum was 728±72 mL (range 600 to 800) per patient, and the total volume of injected BM-MNCs was 45±7 mL (range 30 to 50) per patient. Total number of injected BM-MNCs was $3.5\pm0.8\times10^9$ (range 2.0 to 4.7×10^9), and that of $CD34^+$ cells was $6.8\pm2.6\times10^7$ (range 2.4 to 9.7×10^7).

TABLE 1. Patient Characteristics

Patient	Age	Sex	Fontaine Stage	Previous Treatment	DM	HT	HLP	Smoking	BM-MNC ($\times 10^9$)	$CD34^+$ in BM-MNC ($\times 10^7$)	ABI, Baseline	ABI, 1 Month	VAS, Baseline	VAS, 1 Month
1	63	M	III(lt)	Bypass graft	−	−	+	+	3.0	6.6	0.34	0.55	5	0
2	31	M	IV(rt)	Medical	−	−	−	+	4.7	9.7	0.49	0.39	5	1
3	52	M	IV(lt)	Medical	−	−	−	+	4.1	9.0	0.65	0.67	7	2
4	28	M	IV(lt)	Sympathetic ganglion block	−	−	−	+	2.0	6.8	0.50	0.26	5	0
5	32	M	IV(rt)	Medical	−	−	−	+	3.8	2.4	−	−	5	2
			IV(lt)								−	−	5	2
6	55	F	IV(lt)	Medical	+	+	−	−	3.4	4.0	0.53	0.51	4	3
7	63	M	III(rt)	Medical	−	+	−	+	3.0	9.1	1.10	0.91	5	3
			IV(lt)								0.76	0.94	5	3
8	43	M	IV(rt)	Medical	−	+	−	+	3.6	6.8	1.00	1.04	5	0
			III(lt)								1.00	1.07	5	0

DM indicates diabetes mellitus; HT, hypertension; and HLP, hyperlipidemia.

Angiographic assessment at 4 weeks after transplantation revealed an apparent increase in limb vascularity in 3 of the 8 (38%) patients (4 of the 11 limbs) (Figure 1). Hemodynamic assessment also failed to document evidence of improved collateral development. Specifically, an increase in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs), whereas a decrease in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs). As a result, mean ABI measured at 4 weeks (0.71±0.30) did not differ from that at the baseline (0.70±0.27). Because 2 patients had sites of arterial occlusion distal to the ankle, they showed normal ABIs before treatment. Even after the exclusion of these 2 patients, ABI showed no changes between before (0.55±0.15) and after transplantation (0.55±0.24).

In contrast to the angiographic and hemodynamic results, improvement in limb status was observed in all 8 patients (100%). Improvement in VAS was observed in all 11 limbs, with a decrease from a mean of 5.1±0.7 to 1.5±1.3. Furthermore, complete pain relief was achieved in 4 of the 11 limbs (36%). Improvement in skin ulcers was also observed in all 8 limbs (100%), with complete healing in 7 (88%). Although surgical amputations of the distal limb were performed in 2 patients at 1 month, these operations were intentionally scheduled to be performed after transplantation with the expectation of sufficiently improving the limb perfusion to distally advance the site of amputation (Table 2; Figure 2A and 2B).

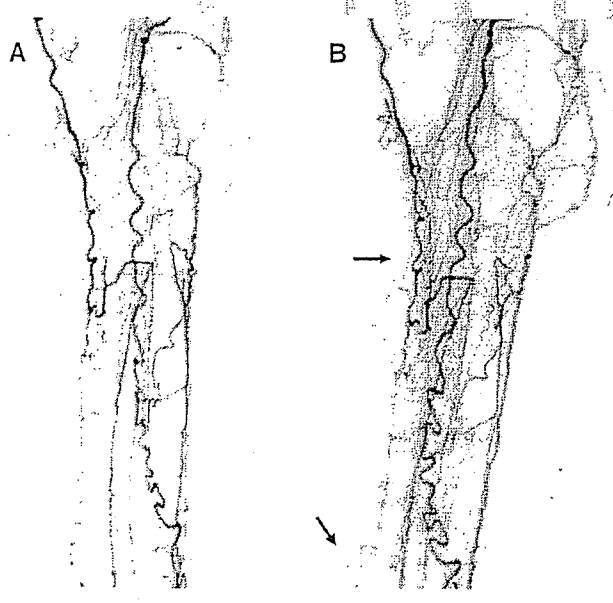

Figure 1. Digital subtraction angiography at (A) baseline and (B) 1 month after cell transplantation. Arrows indicate newly visible collateral vessels at the calf level.

Long-Term Outcome

The mean follow-up period was 684±549 days (range 103 to 1466). At the final follow-up, VAS score remained unchanged from that observed at 1 month after transplantation in 5 of the 8 patients (63%). The mean VAS score at follow-up also remained low (2.3±1.9) compared with that observed at baseline (5.1±0.7).

In contrast to the pain scale results, adverse events were observed in as many as 4 patients (50%) (Table 2). At age 30

TABLE 2. Adverse Outcomes After Autologous Transplantation of BM-MNCs in Patients With Thromboangiitis Obliterans

Adverse Outcomes	30 Days	Final Follow-Up
Death	0	1 (13)
Major amputation	0	0
Minor amputation	2 (25)*	0
Unexpected angiogenesis	0	1 (13)
Recurrence/worsening of skin ulcer/gangrene	0	2 (25)†
Recurrence/worsening of pain	0	1 (13)
Cardiovascular event	0	0
Cerebrovascular event	0	0
Malignancy	0	0

Values are expressed as n (%).

*Amputation was intentionally scheduled to be performed at 1 month after transplantation.

†One patient was the same one who developed unexpected angiogenesis.

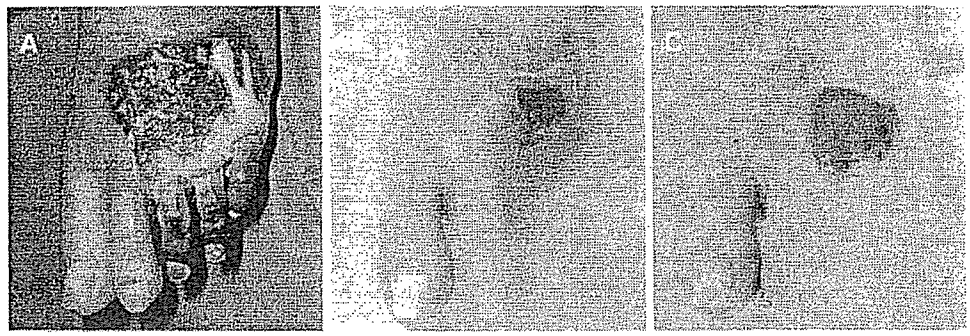

Figure 2. Skin ulcer at (A) 1 month, (B) 2 months, and (C) 4 months after cell transplantation. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient received a prescheduled amputation of the distal limb at 1 month, and the skin ulcer continued to improve thereafter. At 4 months, however, the skin lesion began to enlarge.

years, patient 4 suddenly died of an unknown cause at 20 months after transplantation. This patient had previously been a smoker, but had stopped smoking before cell transplantation. He had no history of diabetes, hypertension, or hyperlipidemia. Furthermore, ^{201}thallium myocardial scan performed before BM-MNC transplantation showed no signs of myocardial ischemia. After cell transplantation, his limb pain disappeared within 1 week and his skin ulcer resolved by 1 month. Thereafter, he was completely free of limb symptoms. Twenty months after cell transplantation, however, he was found dead at his home. He had never experienced chest pain up to the time of his death. Because no autopsy was performed, the cause of his death remains unknown.

Patient 6 showed worsening of an ischemic ulcer at 4 months. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient underwent a prescheduled amputation of the distal limb at 1 month (see Short-Term Outcome) (Figure 2A), and the skin ulcer continued to improve thereafter (Figure 2B). At 4 months, however, the skin lesion began to increase in size (Figure 2C). The patient subsequently received a second round of cell therapy.

In patient 7, despite complete healing of the skin ulcer, rest pain did not completely resolve after transplantation, with a VAS score of 3 at 1 month. At 8 months, the patient experienced worsening of rest pain (VAS score=4). After a combination of exercise training and maximal drug therapy, the pain improved and became well tolerated.

Patient 8 experienced swelling and recurrence of the skin ulcer in his foot at 7 months. Computed tomographic angiography documented an early venous return of contrast material in his right limb (Figure 3B) that was not observed at the baseline (Figure 3A). Ultrasound examination disclosed an arterialized waveform in the dorsal vein at the base of his third toe, suggesting the presence of an arteriovenous shunt. By 1 year, the swelling and skin ulcer had spontaneously regressed. The systolic pulsatile component in the venous waveform was found to be diminished on ultrasound examination, and early venous filling had disappeared on computed tomographic angiography (Figure 3C).

Discussion

In the present unblinded and uncontrolled pilot study, we documented that the transplantation of BM-MNCs was associated with an improvement in ischemic symptoms for up to 4 years. Indeed, VAS scores improved from 5.1±0.7 to 2.3±1.9 at follow-up. Furthermore, skin ulcers remained completely healed in 6 of 7 patients. In this regard, the present findings extend previous observations[11] by establishing the potential long-term benefit of BM-MNC transplantation for the treatment of arterial insufficiency.

It should be noted, however, that half of the patients suffered adverse events during follow-up. Such a high rate of adverse events cannot be explained by the natural course of the disease itself. In general, the prognosis of patients with thromboangiitis obliterans is directly related to tobacco

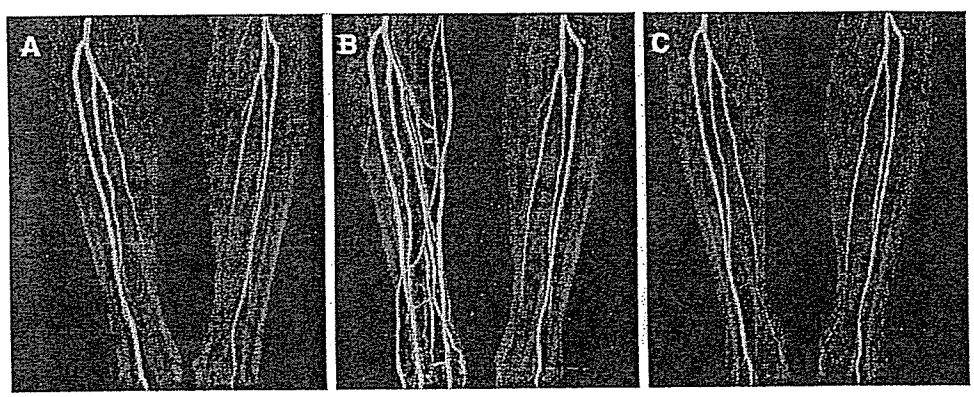

Figure 3. Computed tomographic angiography at (A) baseline, (B) 7 months, and (C) 1 year after cell transplantation. Early venous return of contrast material observed at 7 months spontaneously regressed by 1 year.

use.[12,13] Patients who are able to stop smoking avoid the recurrence of the disease and amputation.[14] In addition, unlike those with atherosclerosis of the extremities, these patients rarely show involvement of visceral vessels and do not appear to be at an increased risk of stroke or myocardial infarction. The mortality rates are thus not higher than those of age- and sex-matched populations.[15,16] It is entirely possible that the high rate of adverse events observed in our patients may have been directly related to BM-MNC transplantation rather than to the progression of the disease itself.

Patient 4 suddenly died at 20 months after transplantation at the age of 30 years. The deaths of patients receiving BM-MNC transplantation were previously reported in the TACT study, in which 2 of 25 patients died of acute myocardial infarction within 24 weeks after transplantation.[11] The patients' backgrounds in the TACT study, in terms of age and comorbidity, may have been totally different from those of our study, in which only patients with thromboangiitis obliterans were recruited. As mentioned above, in patients with thromboangiitis obliterans, coronary involvement is rare, and they usually do very well as long as they discontinue smoking.[12–16] Patient 4 had no risk factors for atherosclerosis and stopped smoking before BM-MNC transplantation. Furthermore, ^{201}thallium scintigraphy performed before transplantation documented no sign of myocardial ischemia. Considering the patient's background and the natural course of the disease,[12–16] the possibility that his death was related to BM-MNC transplantation cannot be excluded. In this regard, several studies have suggested the possible role of BM-MNCs in atherogenesis. A recent report by Silvestre et al,[17] for example, demonstrated that the transplantation of BM-MNCs into ischemic limbs of apolipoprotein E–knockout mice led to a significant increase in atherosclerotic plaque size at a distant site. More recently, George et al[18] have also shown that an intravenous injection of bone marrow cells into apolipoprotein E–knockout mice results in an increase in atherosclerotic lesion size, whereas an injection of endothelial progenitor cells influences plaque stability. These reports indicate that attempts to enhance neovascularization by using BM-MNCs could also enhance unwanted plaque growth and instability, thus suggesting the possibility that our young patient died of an acute coronary event due to accelerated atherogenesis after BM-MNC transplantation.

We also encountered the development of an arteriovenous shunt, which could be a potential consequence of BM-MNC transplantation. Indeed, concerns have been raised about the potential adverse effects of cell transplantation, ie, unregulated differentiation and proliferation. Wakitani et al[19] reported that teratoma formation could occur after embryonic stem cell transplantation. Yoon et al[20] documented intramyocardial calcification after the transplantation of bone marrow cells in rats. Our observations may provide another cautionary example of unregulated differentiation and proliferation. Although the arteriovenous shunt in our case was self-limited, it may represent unwanted angiogenesis; thus, careful monitoring is warranted for future patients who receive BM-MNC transplantation.

Worsening or recurrence of ischemic symptoms was observed in 3 patients. The short-term outcome at 1 month was poor in these patients except in the patient with an arteriovenous shunt. The improvement in rest pain was not substantial in 1 patient. Healing of the skin ulcer was incomplete in another. It is anticipated that a poor response 1 month after BM-MNC transplantation could result in a poor long-term outcome. It is important to note that, in the latter patient with incomplete healing of the skin ulcer, the angiographic improvement of the collateral network at 1 month remained unchanged at 5 months when worsening of the skin ulcer was observed. It is suggested that the temporal sequence of improvement in ischemic limb status does not necessarily parallel the temporal evolution of collateral development.

Conclusions

In this unblinded and uncontrolled pilot study, long-term adverse events after BM-MNC transplantation, including death and unfavorable angiogenesis, were observed in half of the patients with thromboangiitis obliterans. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving BM-MNC transplantation.

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Disclosures

None.

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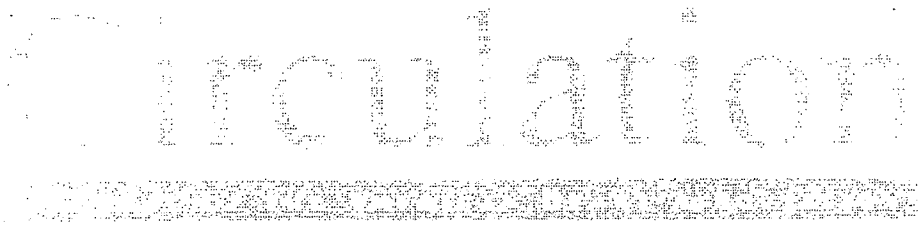
CLINICAL PERSPECTIVE

The favorable short-term outcome of bone marrow mononuclear cell transplantation (BM-MNC) transplantation has been established in patients with critical limb ischemia. However, the long-term outcome of this treatment strategy has not been determined yet. In our case series, we documented that long-term adverse events, including death and unfavorable angiogenesis, were observed in 4 of 8 patients receiving BM-MNC transplantation. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient had worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year. Given the current incomplete knowledge on the safety and efficacy of this strategy, it is suggested that careful long-term monitoring is required in patients receiving BM-MNC transplantation. To our knowledge, this is the first report on the long-term outcome of transplantation of BM-MNCs for critical limb ischemia, and the first that documents the development of unfavorable angiogenesis and sudden death after therapeutic angiogenesis.



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Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice

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Murakami, Shinsuke, Noritoshi Nagaya, Takefumi Itoh, Masaharu Kataoka, Takashi Iwase, Takeshi Horio, Yoshinori Miyahara, Yoshiki Sakai, Kenji Kangawa, and Hiroshi Kimura. Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 290: L59–L65, 2006. First published September 9, 2005; doi:10.1152/ajplung.00042.2005.—The balance between prostacyclin and thromboxane A_2 (TXA_2) plays an important role in pulmonary homeostasis. However, little information is available regarding the therapeutic potency of these prostanoids for pulmonary fibrosis. We have recently developed ONO-1301, a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity. Thus we investigated whether repeated administration of ONO-1301 attenuates bleomycin-induced pulmonary fibrosis in mice. After intratracheal injection of bleomycin or saline, mice were randomized to receive repeated subcutaneous administration of ONO-1301 or vehicle. Bronchoalveolar lavage (BAL) and histological analyses were performed at 3, 7, and 14 days after bleomycin injection. In vitro studies using mouse lung fibroblasts were also performed. ONO-1301 significantly attenuated the development of bleomycin-induced pulmonary fibrosis, as indicated by significant decreases in Ashcroft score and lung hydroxyproline content. ONO-1301 significantly reduced total cell count, neutrophil count, and total protein level in BAL fluid in association with a marked reduction of TXB_2. A single administration of ONO-1301 significantly increased plasma cAMP level for >2 h. In vitro, ONO-1301 and a cAMP analog dose-dependently reduced cell proliferation in mouse lung fibroblasts. The reduction in cell proliferation by ONO-1301 was attenuated by a protein kinase A (PKA) inhibitor. Furthermore, bleomycin mice treated with ONO-1301 had a significantly higher survival rate than those given vehicle. These results suggest that repeated administration of ONO-1301 attenuates the development of bleomycin-induced pulmonary fibrosis and improves survival in bleomycin mice, at least in part by inhibition of TXA_2 synthesis and activation of the cAMP/PKA pathway.

adenosine 3′,5′-cyclic monophosphate; fibroblast; neutrophil; survival

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a life-threatening disease characterized by progressive dyspnea and worsening of pulmonary function (6, 22). The pathological features of IPF are fibroblast proliferation with increased amounts of extracellular matrix and varying degrees of persistent inflammation of the alveolar septa (22). Thus a novel therapeutic strategy against these abnormalities may be effective for the treatment of IPF.

Prostanoids, which are metabolites of arachidonic acid, are important regulators of pulmonary homeostasis. Prostacyclin inhibits migration, proliferation, and collagen synthesis in fibroblasts (14, 29). Conversely, thromboxane A_2 (TXA_2) promotes fibroblast proliferation, increases pulmonary vascular permeability, and induces lung inflammation (18, 21, 24). Interestingly, a recent study has demonstrated that the decreased ratio of prostacyclin synthesis to thromboxane synthesis is associated with the development of pulmonary fibrosis (4). Thus we hypothesized that compounds that regulate the balance between prostacyclin and TXA_2 may have beneficial effects in IPF.

Recently, we have developed a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase, named ONO-1301. Unlike prostacyclin, ONO-1301 does not possess a five-membered ring and allylic alcohol in its molecule, which contributes to the biological and chemical stability of this compound. As a result, this compound can be given by subcutaneous administration twice a day. Its inhibitory effect on thromboxane synthesis is mediated by binding of thromboxane synthase to 3-pyridine moiety and a carboxylic acid group in ONO-1301 (30).

Thus the purpose of this study was to investigate whether modulation of prostacyclin/TXA_2 balance by ONO-1301 attenuates bleomycin-induced pulmonary fibrosis and improves survival in bleomycin-injected mice.

METHODS

Animals. We used specific pathogen-free female C57BL/6 mice weighing 18–20 g. The mice were randomly given intratracheal injection of either bleomycin (Nippon Kayaku, Tokyo, Japan) or saline, and assigned to receive repeated administration of ONO-1301 or vehicle. This protocol resulted in the creation of three groups: sham mice given vehicle (Sham group, $n = 34$), bleomycin mice given vehicle (Vehicle group, $n = 34$), and bleomycin mice treated with ONO-1301 (ONO-1301 group, $n = 34$). Twenty-four additional mice were studied to evaluate the effect of ONO-1301 administration on survival in bleomycin mice. Twenty-five mice were studied to examine the effect of ONO-1301 on plasma cAMP. Finally, 15 mice were

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used to examine the effects of ONO-1301 on established pulmonary fibrosis. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute.

ONO-1301. We have recently developed a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase, {7,8-dihydro-5-[(E)-2-[(α-(3-pyridyl)benzylidene)amino-oxy]ethyl]-1-naphtyl-oxy} acetic acid, named ONO-1301 (Fig. 1). This compound has two interesting structural features. First, as stated above, unlike prostacyclin, ONO-1301 does not possess a five-membered ring and allylic alcohol in its molecule. This structural feature contributes to the biological and chemical stability of this compound. Second, this compound possesses a 3-pyridine moiety at one end of the molecule and a carboxylic acid group at the other. The inhibitory effect of ONO-1301 on thromboxane synthesis is mediated by binding of thromboxane synthase to 3-pyridine moiety and a carboxylic acid group in ONO-1301.

In vivo experimental protocol. After the mice were anesthetized by intraperitoneal injection of pentobarbital sodium, they were given intratracheal injection of either bleomycin (0.02 or 0.03 units/mouse) dissolved in 50 μl of saline or saline alone. Then, ONO-1301 (6 $mg \cdot kg^{-1} \cdot day^{-1}$) dissolved in 100 μl of saline, or saline was administered by subcutaneous injection twice a day throughout the experiment. This dose was determined to obtain maximum effects, based on dose-response experiments. Systolic blood pressure in the conscious state was measured by the indirect tail-cuff method with a blood pressure monitor (MK-2000; Muromachi Kikai, Tokyo, Japan) 0, 30, 60, 120, and 360 min after administration. ONO-1301 at a dose of 6 $mg \cdot kg^{-1} \cdot day^{-1}$ did not influence blood pressure. The mice were maintained under standard conditions with free access to food and water.

The effects of ONO-1301 on pulmonary fibrosis were analyzed by the following parameters: *1*) the severity of pulmonary fibrosis such as histological examination using the Ashcroft score and lung hydroxyproline content on *day 14* (0.02 units/mouse), 2) the severity of acute lung injury such as that reflected in total cell count, differential cell count, and total protein level in bronchoalveolar lavage (BAL) fluid on *day 3* and *day 7* (0.02 units/mouse), *3*) TXB_2 and active transforming growth factor-β1 (TGF-β1) levels in BAL fluid on *day 3* and *day 7* (0.02 units/mouse), *4*) intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in whole lung tissue on *days 3* and *7* (0.02 units/mouse), and *5*) the survival rate on *day 21* (0.03 units/mouse).

Finally, to investigate the effects of ONO-1301 on established pulmonary fibrosis, 15 mice were randomly given an intratracheal injection of either bleomycin or saline. ONO-1301 or saline was administered from *day 14* to *28* (Sham, Vehicle, and ONO-1301 groups, $n = 5$ each). These mice were evaluated on *day 28*.

BAL analysis. Total and differential cell counts in BAL fluid were determined as described previously ($n = 5$ each) (20). The supernatant of BAL fluid was used for the measurement of total protein, TXB_2, which is a stable metabolite of TXA_2, and active TGF-β1 levels. The total protein level was measured by Bradford assay (Bio-Rad, Tokyo, Japan). The TXB_2 level was measured with an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The active TGF-β1 level was measured with a mouse TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN).

Histological examination. The right lung was fixed by inflation with 4% paraformaldehyde and embedded in paraffin ($n = 5$ each). Sections 4 μm thick were stained with hematoxylin-eosin. The Ashcroft score was used for semiquantitative assessment of fibrotic changes (1). The severity of fibrotic changes in each histological section of the lung was assessed as the mean score of severity from observed microscopic fields. Thirty fields in each section were analyzed. Grading was performed in a blinded fashion by three observers, and the mean was taken as the fibrosis score.

Measurement of hydroxyproline content. To quantify lung collagen content as an indicator of pulmonary fibrosis, the hydroxyproline content in the lung was measured in each group according to the previously described method ($n = 5$ each) (20). The left lung was quick-frozen and kept at −80°C until the assay. After the lung was homogenized, the suspension was hydrolyzed with 0.5 ml of 12 N hydrochloric acid for 20 h at 100°C. After neutralization, a 0.1 ml aliquot of supernatant was mixed in 1.5 ml of 0.3 N lithium hydroxide solution. The hydroxyproline content was analyzed by high-performance chromatography.

Quantification of ICAM-1 and VCAM-1 expression by ELISA. We investigated the effect of ONO-1301 on ICAM-1 and VCAM-1, which are key molecules in leukocyte migration into lung tissues, expression in the bleomycin-treated lung. The treated lungs were quick-frozen and kept at −80°C until the assay. The lungs were homogenized in 1.5 ml of saline. The homogenates were centrifuged at 2,000 *g* for 10 min at 4°C, and the supernatants were assayed for ICAM-1 and VCAM-1 concentrations by ELISA kits (R&D Systems).

Measurement of cAMP level. To evaluate the effect of ONO-1301 on plasma cAMP, normal mice were assigned to receive a single administration of ONO-1301 (3 mg/kg). Blood samples were obtained 0, 30, 60, 120, and 360 min after administration and were immediately transferred into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) and centrifuged immediately at 4°C

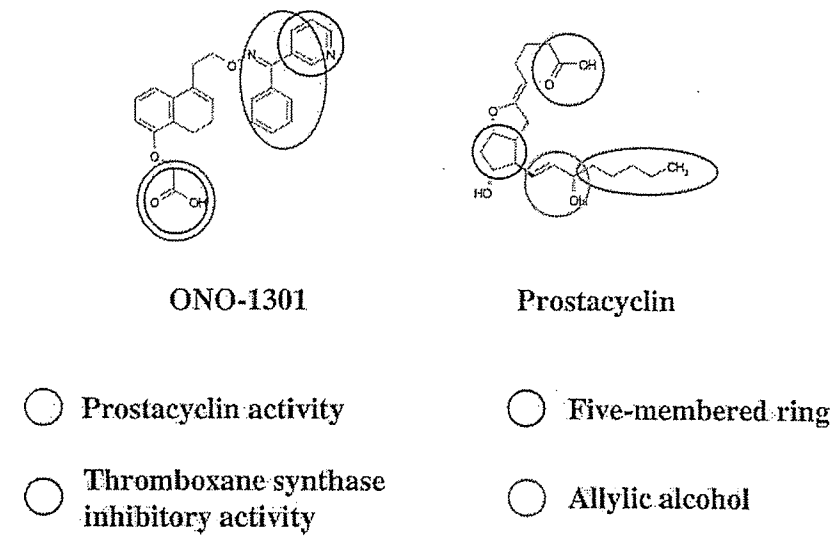

Fig. 1. Molecular structures of ONO-1301 and prostacyclin. Unlike prostacyclin, ONO-1301 does not possess a 5-membered ring and allylic alcohol, which contributes to the biological and chemical stability of this compound. ONO-1301 possesses a 3-pyridine moiety at 1 end of the molecule and a carboxylic acid group at the other, which contribute to inhibition of thromboxane synthesis. Green circle indicates prostacyclin activity; red circle indicates thromboxane synthase inhibitory activity; blue circle indicates 5-membered ring; orange circle indicates allylic alcohol.

Table 1. *Physiological profiles of three experimental groups*

	Sham	Vehicle	ONO-1301
n	8	8	8
Body weight, g	22.1±0.2	19.3±0.6*	21.8±0.4†
Lung weight/body weight, mg/g	5.8±0.1	14.8±1.7*	9.4±0.5*†

Data are means ± SE. These measurements were performed at 14 days after bleomycin injection. Sham, sham mice given vehicle; Vehicle, bleomycin mice given vehicle; ONO-1301, bleomycin mice treated with ONO-1301; *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. Vehicle group.

(n = 5 each). The plasma cAMP level was measured with a radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan) as described previously (13).

Survival analysis. To evaluate the effect of ONO-1301 on survival in bleomycin-injected mice, 24 mice received repeated administration of ONO-1301 (n = 12) or vehicle (n = 12) for 21 days. Survival was estimated from the date of bleomycin injection to the death of the mouse or 21 days after injection.

In vitro study. Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion with collagenase type III (10 mg/lung; Worthington Biochemical, Lakewood, NJ) for 80 min at 37°C, as reported previously (11). Cell suspension was filtered through 70-μm filters (BD Biosciences, Mountain View, CA). Then, these cells were centrifuged, washed, and cultured in complete medium composed of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Fibroblasts were used after the first cell passage. To evaluate the effect of ONO-1301 on intracellular cAMP, mouse lung fibroblasts grown in 24-well plates were incubated with various concentrations of ONO-1301 in the presence of 5×10^{-4} M 3-isobutyl-1-methylxanthine (Nacalai Tesque, Kyoto, Japan), a phosphodiesterase inhibitor (n = 8 each), for 10 min. The intracellular cAMP level was measured with a radioimmunoassay kit as described previously (10). The effects of ONO-1301 and 8-bromo cAMP (Sigma, St. Louis, MO), a cAMP analog, on cell proliferation were assessed using a CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's directions (n = 8 each). Cells were treated for 48 h with fresh medium containing 2.5% FCS along with various concentrations of ONO-1301 or 8-bromo cAMP. The effect of ONO-1301 (10^{-6} M) on cell proliferation in the presence of a myristoylated protein kinase A (PKA) inhibitor (10^{-6} M) (Protein Kinase A Inhibitor 14–22 Amide, Cell-permeable, Myristoylated; Calbiochem, Cambridge, MA) was also evaluated. Finally, to investigate the underlying mechanism responsible for regulation of cell proliferation, mouse lung-derived fibroblasts were treated with various concentrations of imidazole (Wako Pure Chemical Industries, Osaka, Japan), a thromboxane synthesis inhibitor, or beraprost sodium (Cayman Chemical), a stable prostacyclin analog.

Statistical analysis. All data are expressed as means ± SE unless otherwise indicated. Comparisons were made by one-way ANOVA followed by Newman-Keuls test. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Physiological profiles. The physiological profiles of the three experimental groups are shown in Table 1. Body weight was significantly lower in bleomycin mice given vehicle (Vehicle group) than in normal mice given vehicle (Sham group). However, a significant decrease in body weight was not observed in bleomycin mice treated with ONO-1301 (ONO-1301 group). Bleomycin injection significantly increased wet lung weight to body weight ratio. However, the increase was significantly attenuated by treatment with ONO-1301.

Inhibition of pulmonary fibrosis. The normal alveolar structure was maintained in the Sham group (Fig. 2*A*). Fourteen days after bleomycin injection, the alveolar walls were thickened and the air spaces were collapsed in the Vehicle group. In contrast to the findings in mice treated with bleomycin alone, pulmonary fibrosis was less severe in the ONO-1301 group. Semiquantitative assessment by the Ashcroft score demonstrated that the degree of pulmonary fibrosis in the ONO-1301 group was significantly lower than that in the Vehicle group (Fig. 2*B*). The hydroxyproline content in the lung was significantly increased after bleomycin injection (Fig. 2*C*). However,

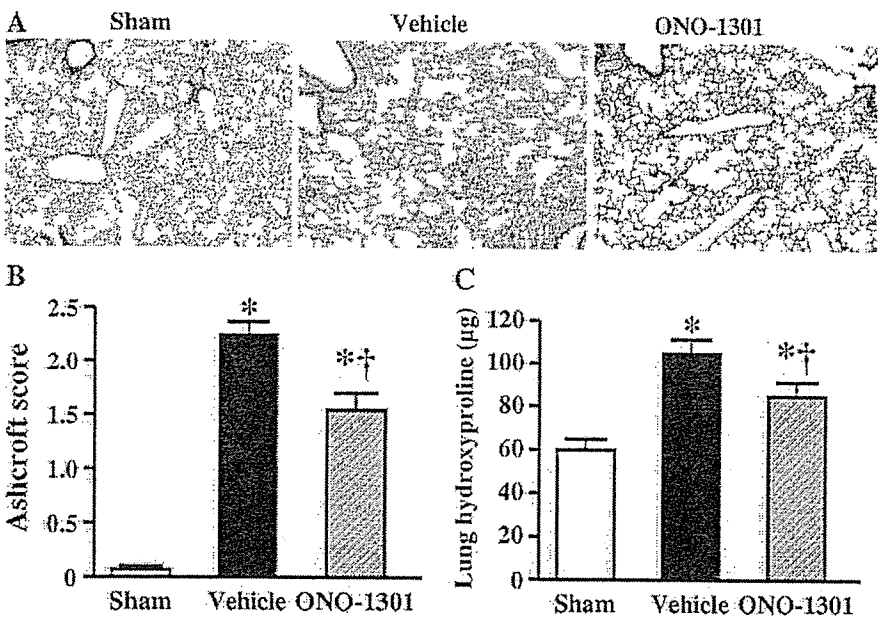

Fig. 2. *A*: representative photomicrographs of lung tissue at 14 days after bleomycin injection. Bleomycin-induced pulmonary fibrosis was attenuated by treatment with ONO-1301. Hematoxylin-eosin stain; magnification ×100. *B*: semiquantitative analyses of lung tissue using the Ashcroft score, a marker for pulmonary fibrosis. *C*: effect of ONO-1301 administration on hydroxyproline content in left lung of bleomycin-injected mice. Data are means ± SE. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. Vehicle group.

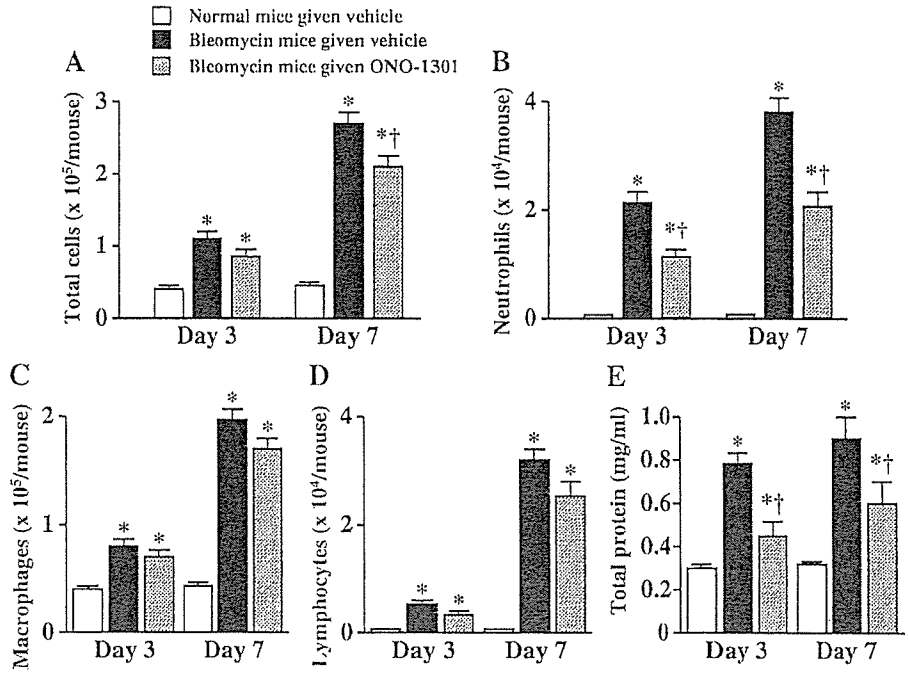

Fig. 3. Effects of ONO-1301 administration on total and differential cell counts (macrophages, *C*; lymphocytes, *D*) in bronchoalveolar lavage (BAL) fluid at 3 and 7 days after bleomycin injection. ONO-1301 significantly inhibited the increases in total cell count at 7 days (*A*) and neutrophil count at 3 and 7 days (*B*) after bleomycin injection. *E*: effect of ONO-1301 administration on total protein level in BAL fluid at 3 and 7 days after bleomycin injection. ONO-1301 significantly inhibited the increase in total protein level in BAL fluid. Data are means ± SE. $*P < 0.05$ vs. Sham group, $\dagger P < 0.05$ vs. Vehicle group.

subcutaneous administration of ONO-1301 significantly decreased the hydroxyproline content in bleomycin-injected mice.

Attenuation of lung inflammation. The recovery rate of BAL fluid was >85% in all groups. Total and differential cell counts were increased at 3 and 7 days after bleomycin injection (Fig. 3, *A–D*). However, subcutaneous administration of ONO-1301 significantly attenuated the increases in total cell count at 7 days and neutrophil count at 3 and 7 days after bleomycin injection. ONO-1301 administration tended to attenuate the increases in macrophage and lymphocyte counts, although these changes did not reach statistical significance. The total protein level was significantly increased at 3 and 7 days after bleomycin injection (Fig. 3*E*). However, the increase was significantly inhibited by ONO-1301.

Inhibition of thromboxane synthesis. The TXB_2 level in BAL fluid was significantly increased at 3 and 7 days after bleomycin injection (Fig. 4*A*), suggesting a pathological role of thromboxane in bleomycin-injected mice. Subcutaneous administration of ONO-1301 markedly inhibited the increase in TXB_2 level. The active TGF-β1 level in BAL fluid was significantly increased at 7 days after bleomycin injection (Sham group, 148 ± 6; Vehicle group, 251 ± 8; ONO-1301 group, 238 ± 9 pg/ml). ONO-1301 did not significantly alter the active TGF-β1 level in BAL fluid.

Inhibitory effect of ONO-1301 on ICAM-1 and VCAM-1 expression. The lung ICAM-1 and VCAM-1 levels were significantly increased at 3 and 7 days after bleomycin injection (Fig. 4, *B* and *C*). ONO-1301 administration sig-

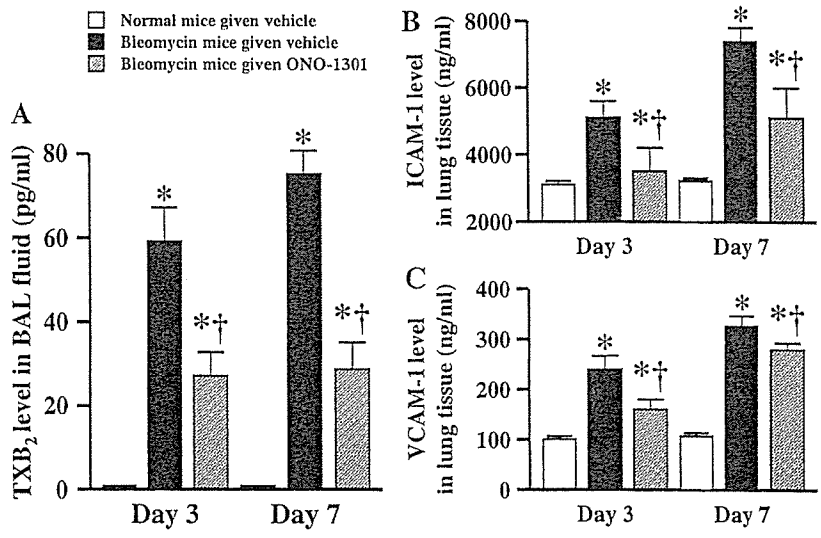

Fig. 4. *A*: effect of ONO-1301 administration on thromboxane B_2 (TXB_2) level in BAL fluid at 3 and 7 days after bleomycin injection. ONO-1301 markedly inhibited the increase in TXB_2 level in BAL fluid. Data are means ± SE. Effects of ONO-1301 administration on ICAM-1 (*B*) and VCAM-1 (*C*) levels in lung tissue at 3 and 7 days after bleomycin injection. $*P < 0.05$ vs. Sham group, $\dagger P < 0.05$ vs. Vehicle group.

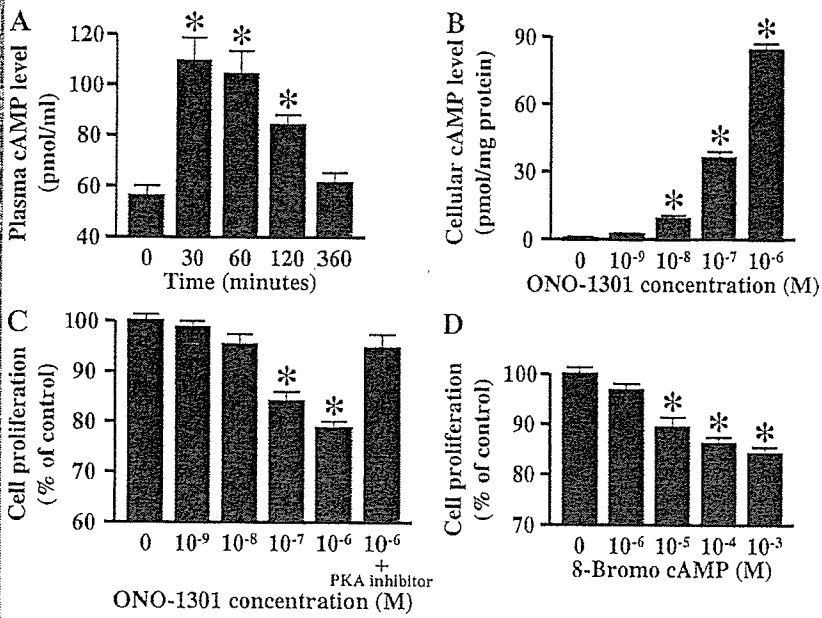

Fig. 5. *A*: time course of plasma cAMP level after a single subcutaneous administration of ONO-1301. *B*: dose-dependent effects of ONO-1301 on intracellular cAMP level. *C*: effect of ONO-1301 on fibroblast proliferation. ONO-1301 significantly reduced cell proliferation at concentrations of 10^{-7} M or greater, and this inhibitory effect was attenuated by a protein kinase A (PKA) inhibitor. *D*: effect of 8-bromo cAMP on fibroblast proliferation. Data are means ± SE. $*P < 0.05$ vs. Control.

nificantly attenuated the increases in ICAM-1 and VCAM-1 levels.

Activation of the cAMP/PKA pathway. A single subcutaneous administration of ONO-1301 significantly increased plasma cAMP level (Fig. 5*A*). The increase lasted longer than 2 h. In vitro, ONO-1301 dose-dependently increased intracellular cAMP level in mouse lung fibroblasts (Fig. 5*B*). ONO-1301 significantly reduced proliferation of mouse lung fibroblasts at concentrations of 10^{-7} M or greater, and this inhibitory effect was attenuated by a PKA inhibitor (Fig. 5*C*). The reduction in cell proliferation by ONO-1301 was reproduced by 8-bromo cAMP (Fig. 5*D*). Beraprost sodium (10^{-7} M) significantly reduced fibroblast proliferation (86% of control). However, imidazole at different concentrations (10^{-6}-10^{-9} M) did not significantly regulate cell proliferation.

Survival analysis. Kaplan-Meier survival curves demonstrated that bleomycin mice treated with ONO-1301 had a significantly higher survival rate than those given vehicle (75 vs. 33% 21-day survival, log-rank test, $P < 0.05$, Fig. 6).

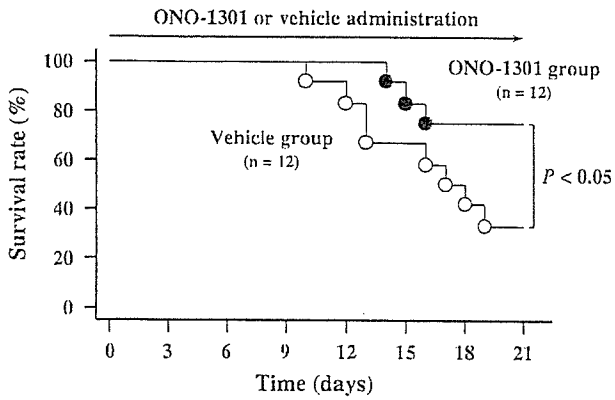

Fig. 6. Kaplan-Meier survival curves. Bleomycin mice treated with ONO-1301 (●) had a significantly higher survival rate than those given vehicle (○) (log-rank test, $P < 0.05$).

Delayed therapy. There were no significant differences in Ashcroft score and lung hydroxyproline content between the Vehicle group and ONO-1301 group at 28 days after bleomycin injection (data not shown).

DISCUSSION

In the present study, we demonstrated that *1*) repeated subcutaneous administration of ONO-1301 attenuated the development of bleomycin-induced pulmonary fibrosis, as indicated by decreases in Ashcroft score and lung hydroxyproline content, *2*) ONO-1301 attenuated the increases in total cell count, neutrophil count, and total protein level in BAL fluid, and *3*) ONO-1301 increased the survival rate in bleomycin-injected mice. We also demonstrated that *4*) ONO-1301 decreased the TXB_2 level in BAL fluid and inhibited ICAM-1 and VCAM-1 expression in the bleomycin-treated lung, and *5*) ONO-1301 inhibited lung fibroblast proliferation through activation of the cAMP/PKA pathway.

The balance between prostacyclin and TXA_2 plays an important role in pulmonary homeostasis. However, little information is available regarding the therapeutic potency of these prostanoids for pulmonary fibrosis. Recently, we have developed ONO-1301, a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase. Thus this compound was administered by subcutaneous injection twice a day.

Bleomycin induces lung inflammation followed by fibrosis when intratracheally injected in experimental animals (27). In the present study, subcutaneous administration of ONO-1301 significantly attenuated bleomycin-induced increases in total cell counts, neutrophil counts, and total protein level in BAL fluid. Earlier studies have shown that TXA_2 acts as a proinflammatory mediator via enhancement of pulmonary vascular permeability (18, 24) and neutrophil adhesion (33). In the present study, TXB_2, a stable metabolite of TXA_2, was significantly increased after bleomycin injection, which is consistent with previous studies (2, 8). However, ONO-1301 markedly