Early Combined Treatment with Cilostazol and Bone Marrow-Derived Endothelial Progenitor Cells Markedly Attenuates Pulmonary Arterial Hypertension in Rats

Cheuk-Kwan Sun, Fan-Yen Lee, Jiunn-Jye Sheu, Chun-Man Yuen, Sarah Chua, Sheng-Ying Chung, Han-Tan Chai, Yen-Ta Chen, Ying-Hsien Kao, Li-Teh Chang, and Hon-Kan Yip

Divisions of General Surgery (C.-K.S.), Thoracic and Cardiovascular Surgery (F.-Y.L., J.-J.S.), Neurosurgery (C.-M.Y.), and Urology (Y.-T.C.), Department of Surgery and Division of Cardiology, Department of Internal Medicine (S.C., S.-Y.C., H.-T.C., H.-K.Y.) and Department of Anesthesiology (Y.-H.K.), Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, Republic of China; and Basic Science, Department of Nursing, Meiho Institute of Technology, Pingtung, Taiwan, Republic of China (L.-T.C.)

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ABSTRACT

We investigated whether early combined cilostazol and bone marrow-derived endothelial progenitor cell (BMDEPC) treatment offers synergistic benefit in ameliorating monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) in rats. Male Sprague-Dawley rats (n = 10/group) were randomized to receive saline injection only (group 1), MCT (70 mg/kg) (group 2), MCT plus cilostazol (20 mg/kg/day) (group 3), MCT plus BMDEPCs (2.0 × 10^5 cells) (group 4), and MCT plus combined cilostazol/BMDEPCs (group 5). Intravenous BMDEPCs and oral cilostazol were given on day 3 after MCT administration. By day 42, connexin43 protein expression in right ventricle (RV) was reduced in group 2 compared with the other groups (all p < 0.05). In addition, mRNA expressions of matrix metalloproteinase-9, tumor necrosis factor-α, and caspase-3 were higher, whereas Bcl-2 and endothelial nitric-oxide synthase were lower in lung and RV in group 2 compared with the other groups (all p < 0.05). The number of alveolar sacs and lung arterioles was lower in group 2 than in other groups and lower in groups 3 and 4 than in group 5 (all p < 0.05). RV systolic pressure (RVSP) and weight were increased in group 2 compared with the other groups (all p < 0.001). Moreover, RVSP and RV-to-left ventricle plus septum weight ratio were higher in groups 3 and 4 than in groups 1 and 5 (p < 0.01) but showed no difference between groups 1 and 5. In conclusion, early combined autologous BMDEPC/cilostazol treatment is superior to BMDEPC or cilostazol only for preventing MCT-induced PAH.

Pulmonary artery hypertension (PAH) is a devastating disease (Pousset et al., 1997; Gaine and Rubin, 1998; Galiè et al., 2002; Runo and Loyd, 2003). Although severe PAH is uncommon, it drastically limits physical capacity and seriously reduces life expectancy (Gaine and Rubin, 1998; Runo and Loyd, 2003). Prognosis is poor, and detection is critical (D’Alonzo et al., 1991; Koh et al., 1996; Gaine and Rubin, 1998; Sanchez et al., 1999; Runo and Loyd, 2003). Without treatment, median life expectancy is only 2.8 years after diagnosis (D’Alonzo et al., 1991; Koh et al., 1996). Pathophysiological evidence has established that PAH, which is caused by various diseases (Abenhaim et al., 1996; Pousset et al., 1997; Gaine and Rubin, 1998; Archer and Rich, 2000; Galiè et al., 2002; Runo and Loyd, 2003), is characterized by a progressive increase in pulmonary vascular resistance resulting from vascular cell proliferation and obliteration of pulmonary microvasculature, leading to right side heart failure if the condition is severe (Pietra et al., 1989).

The treatment of PAH is a formidable challenge to clini-
ciations. Although several management strategies have been proposed, including long-term therapy with oxygen or inhaled nitric oxide (Anand et al., 1998), diuretic therapy, anticoagulants, vasodilators (Barst et al., 1999), calcium channel blockers (Kalra and Bone, 1993), intravenous prostacyclin (Barst et al., 1996; Badesch et al., 2000), phosphodiesterase inhibitors, and endothelin antagonists (Badesch et al., 2000; Channick et al., 2001; Rubin et al., 2002), these regimens remain unsatisfactory due to either limited efficacy or serious side effects. In view of the mechanistic basis of vascular cell proliferation and vascular occlusion, inhibition of vascular smooth muscle proliferation, together with restoration of endothelial function and integrity of pulmonary microvasculature, may be a novel and effective therapeutic strategy for the re-establishment of normal pulmonary hemodynamics in patients with PAH.

Cilostazol, a phosphodiesterase III inhibitor approved by the United States Food and Drug Administration for treatment of intermittent claudication (Thompson et al., 2002), has been shown to reduce smooth muscle proliferation (Pan et al., 1994), limit intimal hyperplasia after endothelial injury (Kubota et al., 1995), and lower restenotic rate of endovascular intervention (Douglas et al., 2005). Recently, our team found that cilostazol therapy can partially reverse monocrotaline (MCT)-induced PAH in a rat model (Chang et al., 2008). In addition, mounting data indicate that bone marrow contains multipotent progenitor cells that can differentiate into various cell types, including endothelial progenitor cells and myogenic cells (Davani et al., 2003; Reffelmann and Kloner, 2003; Yoon et al., 2005). Moreover, recent studies have shown that circulating bone marrow-derived endothelial progenitor cells (BMDEPCs) participate directly in postnatal vasculogenesis and angiogenesis in systemic and pulmonary vascular beds and play an essential role in the repair of endothelial damage (Luttun et al., 2002; Asahara and Kawamoto, 2004; Yoon et al., 2005; Rocher et al., 2006; Schächinger et al., 2006). Therefore, bone marrow-derived progenitor cells seem to be highly advantageous for cell therapy (Davani et al., 2003; Reffelmann and Kloner, 2003; Yoon et al., 2005; Rocher et al., 2006; Schächinger et al., 2006). It is interesting that a recent study has also shown that implantation of BMDEPCs can partially rescue MCT-induced PAH in rats (Zhao et al., 2005). Accordingly, we tested the hypothesis that early combined treatment with cilostazol and autologous BMDEPC implantation exerts therapeutic synergism compared with either cilostazol or BMDEPC transplantation alone in attenuating MCT-induced PAH in a rat model.

Materials and Methods

Ethics. All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, Republic of China and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, National Academies Press, Washington, DC, revised 1996).

Bone Marrow Cell Isolation and Cultures. On day 18 prior to MCT-induced PAH, pathogen-free male adult Sprague-Dawley (SD) rats (n = 24), weighing 275 to 300 g (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taipei, Taiwan), were anesthetized by intraperitoneal injections of chloral hydrate (35 mg/kg). After careful separation of the ligament from the patella, an electric rotator with a diameter of 0.2 mm was used to screw straight into the femoral bone from the distal end. A sterile 22-gauge needle syringe was then used to aspirate the bone marrow through the orifice. The bone marrow cells were buffered in 10 ml of culture medium (Iscove’s modified Dulbecco’s medium and 10% fetal bovine serum, centrifuged at 400 g for 5 min, and then digested for 40 min with 0.01% collagenase B and DNase 1. The cells were then filtered through a 30-μm nylon mesh and centrifuged at 400 g for 5 min in Ficollicolution. The bone marrow-derived mononuclear cells (BMDMNCs) were then isolated by Ficollic-Paque (GE Healthcare, Chalfont St. Giles, UK) density-gradient centrifugation. Finally, BMDMNCs in interphase were collected, and PBS was used to wash these cells twice. Finally, cells were centrifuged at 400 g for 5 min. Approximately 2.0 × 10⁶ BMDMNCs were obtained from each animal (both femoral bones) via this method. These cells were plated on gelatin-coated tissue culture flasks; cultured in differential endothelial cell culture medium (endothelial cell basal medium-2; Cambrex, East Rutherford, NJ) with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM l-glutamine (Invitrogen, Burlington, ON, Canada) with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (10 ng/ml); and incubated at 37°C with 5% CO₂ for 21 days. Culture medium was changed every 48 h. By day 21, ~2.0 × 10⁶ spindle-shaped cells with cobblestone-like morphology typical of endothelial cells were found attached on the plate (Fig. 1A).

Identification of Endothelial-Like Progenitor Cell Pheno-
type. The EPCs were characterized as adherent cells positive for acetylated low-density lipoprotein (acLDL) uptake by direct fluorescent staining. In brief, adherent EPCs (5 × 10⁶/ml) on glass slides were first incubated with 10 μg/ml 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled acLDL (Invitrogen) for 4 h at 37°C, followed by washing with PBS three times before being fixed in absolute methanol at −20°C for 10 min. Nuclei were counterstained with 200 μl of 0.02% 4'-6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO) for 30 min. The mounted slides were then observed under fluorescent microscopy for positively stained cells (Fig. 1B).

Flow Cytometric Quantification of Endothelial Progenitor Cells Based on Surface Markers. To identify the population of EPCs after 21-day cell culture, the cells were trypsinized, washed twice with PBS, and immunostained for 30 min on ice with monoclonal antibodies against primary antibodies CD31 (Serotec, Oxford, UK), CD34 (Serotec), kinase insert domain-containing receptor (Neomarkers, Fremont, CA), VEGF, and von Willebrand factor (vWF) (Abcam plc, Cambridge, UK). Secondary detection was done using appropriate Alexa Fluor 488 (Molecular Probes). Isotype-identical antibodies (IgG) served as controls. After staining, the cells were fixed with 1% paraformaldehyde (PFA). Flow cytometric analyses were performed by using a fluorescence-activated cell sorter (FC500 flow cytometer; Beckman Coulter, Fullerton, CA) (Fig. 1, C–G). Each analysis included 100,000 cells per sample. Assessment in each sample was performed in duplicate, with the mean level reported. List Mode Data files were exported and analyzed using the accompanying software.

Animal Models of PAH. On day 0 of PAH induction, 44 adult male SD rats, including the 24 SD rats having previously undergone bone marrow aspiration, were given a subcutaneous injection of MCT (70 mg/kg; Sigma-Aldrich) (Chang et al., 2008). On day 3, MCT-treated animals were assigned to one of four experimental groups: group 2 (MCT alone; n = 10); group 3 [MCT plus daily oral cilostazol (20 mg/kg/day); n = 10] (Chang et al., 2008); group 4 [MCT plus a single intravenous bolus of BMDEPCs (2.0 × 10³ cells) transplantation; n = 14]; and group 5 (MCT plus a combination of cilostazol and BMDEPCs; n = 10). Another group of 10 SD rats (group 1) receiving only subcutaneous injection of 3 ml of physiological saline served as normal controls. Individual treatment was performed immediately after the assignment until sacrifice on day 42 after MCT administration. To evaluate whether BMDEPCs were trapped and engrained...
endothelial lineage after 21-day cell culture (n = 3). Four SD rats in group 4 were sacrificed on day 5 after MCT treatment.

Identification of Fluorescence-Labeled BMDEPCs in Pulmonary Arteries. To investigate whether the infused BMDEPCs were localized in pulmonary arteries, CM-Dil (Vybrant Dil cell-labeling solution; Invitrogen)-labeled BMDEPCs (1.2 × 10⁶) were intravenously injected from tail vein into pulmonary circulation of another four rats in group 4 on day 3 after MCT-induced pulmonary artery injuries. Five days after injection, the rats were sacrificed, and the lung tissue was collected for examination.

Hemodynamic Studies. On day 42 after MCT treatment, the rats were anesthetized by intraperitoneal injections of chloral hydrate (35 mg/kg). After being shaved on the chest, each animal was endotracheally intubated with positive-pressure ventilation (180 ml/min) with room air by using a small animal ventilator (SAR-830/A; CWE Inc.). The heart was exposed by left thoracotomy. A sterile 20-gauge, soft-plastic-coated needle was inserted into the right ventricle and femoral artery of each rat to measure the right ventricular systolic pressure (RVSP) and arterial pressure, respectively. The pressure signals were first transmitted to pressure transducers (model 1050; UFI, Morro Bay, CA) and were then exported to a bridge amplifier (ML866 PowerLab 4/30 data acquisition systems; ADInstruments Pty Ltd., Castle Hill, NSW, Australia) where the signals were amplified and digitized. The data were recorded and later analyzed with the LabChart software (ADInstruments Pty Ltd.).

Semiquantitative Analysis of Lung Parenchymal Structure by Microsphere Particle Injection into Trachea. The protocol for fluorescent microsphere preparation was modified from that reported previously (Dutly et al., 2006). In brief, low melting point agarose (100 mg) (Sigma-Aldrich) was first dissolved in 9 ml of distilled water at 80°C, followed by the addition of 1 ml of greenish yellow fluorescence polystyrene 0.2-µm microspheres (505-nm excitation, 515-nm emission) (FluoSpheres; Invitrogen) into the agarose solution. Three rats in each group were anesthetized by intraperitoneal chloral hydrate (35 mg/kg). After being shaved on the chest and abdomen, each animal was endotracheally intubated with positive-pressure ventilation (180 ml/min) with room air using a small animal ventilator (SAR-830/A; CWE Inc.). A median incision was made on the abdomen and extended through the sternum. The right ventricle was injected with 20 ml of prewarmed normal saline, accompanied by the severance of the abdominal aorta and inferior vena cava. The right ventricle was then injected with 3 ml of prewarmed agarose-head mixture with 0.2-mm-diameter yellow-green polystyrene microspheres. Filled syringes containing normal saline and agarose-head mixture were maintained in a 45°C water bath until use. After complete injection, ventilation was stopped, and the lungs were inflated at a pressure of 20 to 30 cm H2O. The rat's chest was covered with crushed ice to enhance gelling of the perfusate, followed by intratracheal injection of 6 ml of ice-cold paraformaldehyde. The heart-lung block was then removed. After 24 h of fixation in 4% buffered PFA, the specimen was rinsed and stored in 70% ethanol. Samples were then sent to The Animal Disease Diagnosis Center, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, where cross-sections (80 µm in thickness) of the left lobe of all lungs were cut using a vibrating-blade microtome (VT1000SR; Leica, Nussloch, Germany) (Fig. 2, C–G).

Real-Time Quantitative PCR Analysis of Lung Tissue and RV Myocardium. Real-time polymerase chain reaction (PCR) was conducted using LightCycler TaqMan Master apparatus (Roche Diagnostics, Mannheim, Germany) in a single capillary tube according to the manufacturer’s guidelines for individual component concentrations. Forward and reverse primers were each designed in a different exon of the target gene sequence, eliminating the possibility of amplifying genomic DNA. The sequences of the primers used in this study are listed in Table 1. A positive result was determined by identifying the threshold cycle value at which reporter dye emission
occurred above background. If fluorescence signal was not detected within 55 cycles, the sample was considered negative.

Distribution of Alveolar Sacs and Vessels in Lung Parenchyma. Immunohistochemical staining of H9251-smooth muscle actin (Sigma-Aldrich) was performed to determine the number of alveolar sacs and arterioles according to manufacturer’s instructions. Three lung sections from each rat were analyzed and three randomly selected high-power fields (HPFs) (100× objective) were examined in each section. The mean number per HPFs for each animal was then determined by summation of all numbers divided by 9.

Statistical Analysis. Data are expressed as mean values ± S.D. The significance in differences in the data between two groups was determined by t test. The means among groups were compared by one-way analysis of variance followed by Duncan’s multiple comparison procedure. Statistical analysis was performed using SAS statistical software for Windows, version 8.2 (SAS Institute, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

In Vitro Characteristics of BMDEPCs after 21-Day Culture. After 21 days of culture in endothelial growth medium, BMDMNCs exhibited spindle-shaped or cobblestone-like morphology typical of endothelial cells (Fig. 1A). In addition, endothelial cell phenotype was characterized by assessing Dil-acetylated LDL uptake (Fig. 1B, yellow arrows). Furthermore, the cells were also positive for a panel of endothelial cell markers, including kinase insert domain-containing receptor, VEGF,
vWF, CD31, and CD34 by flow cytometry (Fig. 1, C–G). The cells were therefore confirmed as BMDEPCs.

Identification of Trapping and Engraftment of Fluorescence-Labeled BMDEPCs. The immunofluorescence imaging study revealed trapping and engraftment of the fluorescence-labeled BMDEPCs in the pulmonary arterioles (Fig. 2, A and B) on day 5 after implantation. In contrast, no fluorescence-positive cells were identified in RV or inferior vena cava (data not shown).

Semiquantitative Analysis of Lung Parenchymal Structure. Confocal imaging study (Fig. 2, C–G) showed distorted microscopic architecture of the lung in group 2 (i.e., MCT only without treatment) (Fig. 2D) compared with other groups, including compact lung parenchyma with thickened alveolar septa and reduced number of alveoli.

Hemodynamic Parameters, Body Weight, Ratio of RV to Whole Heart Weight and to Left Ventricle plus Septum Weight. The initial and final body weight did not differ among the five groups (Fig. 3). There was also no significant difference in whole heart weight among group 1 (i.e., normal controls), group 3 (i.e., MCT plus cilostazol), group 4 (i.e., MCT plus BMDEPCs), and group 5 (i.e., combined treatment), whereas whole heart weight was significantly higher in group 2 than in groups 1 and 5 (Fig. 4A). In contrast, although RV weight did not differ between groups 1 and 5 and between groups 3 and 4, it was notably higher in group 2 than in groups 1 and 3 to 5 and significantly higher in groups 3 and 4 than in groups 1 and 5 (Fig. 4B). There was no remarkable difference in RV-to-whole heart weight ratio between groups 1 and 5, among groups 3 to 5, and among groups 2 to 4, whereas the ratio was notably higher in group 2 (i.e., MCT only without treatment) than in groups 1 and 5 and significantly higher in groups 3 and 4 than in group 1 (Fig. 4C). The RV-to-left ventricle plus septum weight ratio was similar between groups 1 and 5 and between groups 3 and 4. However, this ratio was significantly higher in group 2 than in other groups and notably higher in groups 3 (i.e., MCT plus cilostazol) and 4 (i.e., MCT plus BMDEPCs) than in groups 1 and 5 (i.e., combined treatment) (Fig. 4D).

Although the femoral arterial systolic blood pressure did not differ among the five groups (Fig. 4F), the RVSP was substantially higher in groups 2 to 4 than in groups 1 and 5 on day 42 and notably higher in group 2 than in groups 3 and 4 (Fig. 4E). These findings suggest that early treatment of MCT-induced pulmonary artery injury by combined autologous BMDEPC transplantation and cilostazol treatment is superior to BMDEPC or cilostazol only for preventing MCT-induced PAH.

mRNA Expressions of Bcl-2 and Caspase-3 in RV and Lung. On day 42 after MCT treatment, Bcl-2 mRNA expression in RV (Fig. 5A) was significantly higher in groups 1 and 3 to 5 than in group 2. In addition, Bcl-2 expression was significantly higher in group 5 than in group 4, whereas it was similar among groups 1, 3, and 5 and between groups 3 and 4. The Bcl-2 expression in lung (Fig. 5B) did not differ between groups 1 and 5 and between groups 3 and 4. However, this mRNA expression was notably higher in groups 1 and 5 than in groups 2 to 4 and notably higher in groups 3 and 4 than in group 2.

On day 42 after MCT treatment, caspase 3 mRNA expression in RV (Fig. 5C) did not differ among groups 1, 3, 4, and 5. However, this mRNA expression was significantly higher in group 2 than in other groups. In addition, the expression in lung (Fig. 5D) was significantly higher in groups 2 to 4 than in groups 1 and 5 and notably higher in group 2 than in groups 3 and 4. No significant difference was noted between groups 1 and 5 and between groups 3 and 4. These findings indicate that combined treatment with BMDEPCs and cilostazol provide further protection against cellular apoptosis in both RV and lung.

mRNA Expressions of eNOS, MMP-9, and TNF-α in RV and Lung. On day 42 after MCT treatment, the endothelial nitric-oxide synthase (eNOS) mRNA expression (Fig. 6A) did not differ between groups 1 and 5, between groups 3 and 4, and between groups 4 and 5. However, this mRNA expression were markedly decreased in groups 2 to 4 com-
pared with group 1, significantly lower in group 2 than in groups 3 to 5, and also notably lower in group 3 than in group 5. Moreover, eNOS expression in lung tissue (Fig. 6B) was significantly lower in group 2 than in other groups and significantly lower in group 3 than in groups 1 and 5, but no remarkable difference was noted among groups 1, 4, and 5 and between groups 3 and 4.

On day 42 after MCT administration, matrix metalloproteinase (MMP)-9 in RV (Fig. 6C) and lung (Fig. 6D) did not differ among groups 1, 3, and 5 and between groups 3 and 4. However, this mRNA expression in both RV and lung was notably higher in group 2 than in other groups and significantly higher in group 4 compared with groups 1 and 5.

On day 42 after MCT administration, the mRNA expression of tumor necrosis factor (TNF-α) in RV (Fig. 6E) showed no significant difference among groups 1, 3 and 5 or between groups 3 and 4. In contrast, it was significantly higher in group 2 than in other groups and notably higher in group 4 than in groups 1 and 5. Moreover, this mRNA expression in lung tissue (Fig. 6F) was significantly higher in group 2 than group 5.
in groups 1, 3, and 5. However, this mRNA expression in lung tissue did not differ between groups 1 and 3 to 5 and between groups 2 and 4.

**Connexin43 Expression.** Cx43 protein expression in RV (Fig. 7) did not differ between groups 1 and 5 and between groups 3 and 4, whereas it was significantly higher in groups 1 and 5 compared with groups 2 to 4 and notably higher in groups 3 and 4 than in group 2. These findings imply that pressure overload in RV, an indicator of MCT-induced PAH, markedly suppressed Cx43 expression in RV. Furthermore, the results indicate that BMDEPC plus cilostazol treatment was more effective than either BMDEPC transplantation or cilostazol alone in preserving Cx43 protein expression in RV on day 42 after MCT treatment.

**Quantitative Analysis of Small Arteriolar Density and Numbers of Alveolar Sac in Lung Parenchyma.** The number of small vessels (<100 μm) in lung parenchyma did not differ among groups 1, 3, and 4 (Fig. 8). However, the number of small vessel was substantially decreased in group 2 compared with other groups on day 42 after MCT administration. In addition, the number of small vessels was significantly lower in groups 1, 3, and 4 than in group 5. Furthermore, histopathological findings revealed that arteriolar wall thickness was notably increased in group 2 compared with the other groups and increased in groups 3 to 5 compared with group 1. Moreover, microscopically, the lung parenchyma was more dense, and the septum was more thickened in group 2 than in groups 1 and 3 to 5 on day 42 after MCT treatment (Fig. 9). Furthermore, the number of alveolar sacs was substantially lower in group 2 than in groups 1 and 3 to 5, significantly lower in groups 3 and 4 than in group 5, and notably lower in group 5 than in group 1 (Fig. 9).

**Discussion**

The in vitro parts of the present study, including flow cytometric examination and immunofluorescence imaging study, demonstrated a high proportion of BMDEPCs after 21-day cell culturing. In addition, the implanted BMDEPCs were clearly identified in the pulmonary arteries, and some of them were found to have engrafted into the pulmonary arterioles by the 5th day after implantation. These findings imply that the BMDEPCs in pulmonary vascular beds may play an essential role in the reparation of endothelial damage. Our suggestion was supported by the in vivo parts of this study that demonstrated elevations in eNOS mRNA expression and the number of small vessels in the lung in group 4 (MCT treated by BMDEPCs) compared with those in group 2 (MCT only). Our recent study (Yip et al., 2008) also showed that BMDEPCs identified in the pulmonary vascular beds 5 days after implantation from rat tail vein effectively attenuated MCT-induced PAH. Therefore, our present findings reinforce those of Yip et al. (2008).

In contrast, the current study showed that caspase-3 mRNA expression, an index of apoptosis in both RV and lung, was notably higher in group 2 than in group 1 (control group),
whereas Bcl-2 mRNA expression, an index of antiapoptosis in RV and lung, was remarkably lower in group 2 than in group 1. Interestingly, compared with group 2, these changes in mRNA expressions were found to be reversed in groups 3 (MCT treated by cilostazol) and 4 (MCT treated by BMDEPCs). Moreover, eNOS mRNA expression, an index of NO production, was markedly decreased in group 2 compared with group 1, whereas the mRNA expression of TNF-α and MMP-9, two indexes of inflammation, were substantially increased in group 2 compared with group 1. These changes in mRNA expressions, however, showed a notably reversed change in groups 3 and 4 compared with group 2. Recently, Thum et al. (2005) proposed that stem cell therapy modulates immune reactivity by down-regulating both innate and adaptive immunity. Accordingly, we recently demonstrated that stem cell therapy exerted both antiapoptotic and anti-inflammatory actions (Sun et al., 2009). The findings of the present study, therefore, not only reinforce the results of these studies (Thum et al., 2005; Sun et al., 2009) but also provide insight into the mechanisms underlying the BMDEPC-induced improvement in PAH, at least in part through the inhibition of inflammatory reaction and cellular apoptosis as demonstrated in this study. In accordance with our recent finding that cilostazol can effectively attenuate MCT-induced PAH (Chang et al., 2008), other studies have demonstrated that pentoxifylline, a phosphodiesterase-3 and -4 inhibitor, significantly alleviates TNF-α-mediated inflammatory responses in vascular smooth muscle cells (Chen et al., 2003, 2004). Therefore, the findings of those previous studies (Chen et al., 2003, 2004) also support the results of this study. Of importance is that, compared with groups 3 and 4, further anti-inflammatory and antiapoptotic activities were noted in group 5 (MCT plus BMDEPCs and cilostazol) as reflected in the mRNA expressions of Bcl-2, caspase-3, eNOS, MMP-9, and TNF-α. These findings support that combined BMDEPC-cilostazol treatment exerted synergistic therapeutic actions in the amelioration of inflammatory response and apoptosis in a rat model of MCT-induced PAH.

Gap junctions, which consist of Cx subunits, provide pathways of minimal resistance for electrical coupling between cardiomyocytes (Yeh et al., 2001). Changes in Cx expression patterns have been identified in a variety of cardiac pathologies and contribute to the development of cardiac arrhythmia (Kirchhoff et al., 1998; Lerner et al., 2000; Yeh et al., 2001). The current study demonstrated that compared with the normal controls, Cx43 protein expression in RV was significantly decreased in rats after PAH induction without treatment. However, Cx43 protein expression was significantly increased after either cilostazol or BMDEPC treatment. It is important that further increase in Cx43 protein expression was noted after BMDEPC-cilostazol combined treatment. It is interesting that the association between RV hypertrophy and down-regulation of Cx43 expression has been identified (Uzzaman et al., 2000; Chang et al., 2008; Yip et al., 2008). Our findings, in addition to supporting the findings of those studies (Uzzaman et al., 2000; Chang et al., 2008; Yip et al., 2008), also suggest that combined BMDEPC-cilostazol therapy offer a synergic effect on the preservation of the integrity of Cx43 expression in RV.

Our recent studies (Chang et al., 2008; Yip et al., 2008) have identified a distorted pulmonary architecture, including a marked reduction in both the numbers of small vessels and alveolar sacs in lung parenchyma, as a key histological feature after MCT treatment. As expected, in this study, these pathological features were notably improved after either cilostazol or BMDEPC treatment. It is noteworthy that compared with either cilostazol or BMDEPC alone, combined treatment using BMDEPC-cilostazol further prevented the increase in alveolar septal thickness and distortion in lung parenchymal architecture as well as effectively preserved the number and integrity of alveolar sacs and the pulmonary arteriolar-capillary density. It is important that combined treatment using BMDEPC-cilostazol successfully normalized RV blood pressure, an index of pulmonary arterial blood pressure, and final RV mass, an index of RV hypertrophy due to pressure overload, to a level comparable with that of the normal control group. These findings, together with the lack of significant fluctuation in body weight and systolic blood pressure of the animals during the course of the experiment, imply that the changes in RV mass and hemodynamics were not secondary to primary systemic impacts caused by either MCT or the treatment regimens. Accordingly, the findings of the present study, in addition to strengthening those of our recent studies (Chang et al., 2008; Yip et al., 2008), further establish the therapeutic efficacy of the combined BMDEPC-cilostazol regimen against PAH.

In conclusion, early combined treatment with BMDEPCs and cilostazol after MCT-induced pulmonary arterial injury is not only superior to either BMDEPC or cilostazol alone in the prevention of MCT-induced PAH but also can successfully abolish PAH-induced hemodynamic impacts on RV in rats. These findings may raise the need for further prospective studies on assessing the therapeutic potential of combined BMDEPC-cilostazol regimen in human subjects with
PAH. The proposed mechanisms underlying the potential impacts of combined BMDEPC-cilostazol therapy against MCT-induced PAH in rats are summarized in Fig. 10.

References

Address correspondence to: Dr. Hon-Kan Yip, Division of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital, 123 Ta Pei Rd., Niao Sung Hsiang, Kaohsiung Hsien 83301, Taiwan. E-mail: hann.gung@msa.hinet.net.