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Original Article

Endothelial progenitor cells, rosuvastatin and valsartan have a comparable effect on repair of balloon-denudated carotid artery injury

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Abstract: Endothelial cell (EC) dysfunction plays a crucial role for arterial obstructive disease. This study tested the therapeutic role of autologous endothelial progenitor cells (EPCs)/rosuvastatin-(Rosu)/valsartan-(Val) on repair of injured carotid ECs. Male Sprague-Dawley rats (n = 60) were categorized into five groups [sham-control (SC), left common carotid artery injury induced by balloon denudation (LCA^{BD}), LCA^{BD} + Rosu (10 mg/kg/day), LCA^{BD} + Val (20 mg/kg/day), and LCA^{BD} + EPC (1.2×10^6)]. By day 5, the LCA was harvested from each rat (n = 6/each time interval in group) after the procedure. Carotid-ring angiogenesis was significantly lower in LCA^{BD} than the other groups (all $P < 0.001$). Compared with LCA^{BD}, the number of EC was significantly higher in LCA^{BD} treated with adipose-derived mesenchymal stem cells (ADMSCs) and more significantly higher in LCA^{BD} treated with EPCs (all $P < 0.001$). Gene expression of EC (CD31/vWF), EPC (SDF-1α/CXCR4) and angiogenesis (VEGF/VEGF-receptor/angiopoietin/eNOS) and EC intercellular junction (VE-cadherin) biomarkers were significantly lower in LCA^{BD} than in groups LCA^{BD} + Rosu to LCA^{BD} + EPC (all $P < 0.001$). Conversely, the gene expression of inflammatory (VCAM-1/MMP-9/TNF-α), oxidative-stress (NOX-1/NOX-2), apoptosis (cleaved caspase-3/PARP) and thrombin cofactor (thrombomodulin) biomarkers were significantly higher in LCA^{BD} than in other groups (all $P < 0.001$). By day 14, the neointimal-layer area and cellular expressions of (CD40+/CD68+) were highest in LCA^{BD}, lowest in SC, significantly higher in LCA^{BD} + Val than in LCA^{BD} + Rosu and LCA^{BD} + EPC (all $P < 0.001$). In conclusion, EPCs were comparable to rosuvastatin and valsartan in upregulation of angiogenesis and repair of injured carotid ECs.

Keywords: Balloon denudation, carotid artery, endothelial cell damage, angiogenesis, inflammation

Introduction

Cardiovascular disease (CVD) remains the leading cause of death worldwide [1-7]. It is well recognized that the causal etiology of CVD is multifactorial. Coronary artery disease (CAD), one of causal etiologies of CVD, is an extremely important contributor of CVD [1-7]. Despite numerous advances in treatment of CAD, including the mature surgical technique of coronary artery bypass grafting (CABG) [5], refinement of instruments and techniques for catheter-based per-

cutaneous coronary intervention (PCI) [6, 8, 9], renewal of education [10, 11] and guidelines for the strategic treatment of CAD [12, 13], CVD has remained the second most common cause of death in the world without change over the past two decades.

Many investigations have shown that the initiation and propagation of endothelial cell dysfunction play pivotal roles in the development of CAD and peripheral arterial obstructive disease [14-16]. Plaque formation and rupture that ultimately causes acute arterial occlusion syndrome is a major contributor to unacceptably high morbidity and mortality in CVD patients [14-19]. Accordingly, to develop a more complete revolution of CAD treatment, the understanding of the intrinsic vessel wall response to endothelial damage and the time course of repair capacity of exogenous treatment to the vascular wall damage are believed to be of paramount importance for scientists and physicians.

It is well known that endothelial progenitor cell (EPC) plays an essential role in the cardiovascular repair, capillary growth, formation of collaterals and vasculogenesis [20, 21]. Abundant data have shown that compared with healthy subjects, CAD patients have significantly lower numbers of circulating EPCs [22]. Interestingly, many studies have revealed that EPC therapy improved ischemia-related organ dysfunction [23-26] mainly via angiogenesis/repair of senile/damaged ECs in vessels [21-26]. Additionally, statin therapy not only upregulates the circulating number of EPCs [27, 28] but also suppresses atherosclerotic obstructive arterial disease through lowering the low-density lipoprotein (LDL) and inflammation as well as oxidative stress [29]. Furthermore, increasing amounts of data also show that angiotensin II type I receptor (ARB) blockade therapy offers benefit to patients with arteriosclerotic obstructive diseases through the underlying mechanisms of upregulation of EPC numbers and stabilization of the plaque lesion by means of inhibiting the oxidative stress and renin angiotensin aldosterone system (RAAS) [30-34]. Based on the aforementioned issues [20-35], this study tested the hypothesis that EPC, rosuvastatin and valsartan might have comparable effect on upregulation of angiogenesis and repair of injured carotid ECs in rats after balloon denudation (BD) injury. This study further detected the time points of reendothelization in the damaged endothelial layer and assessed the EC repaired capacity between EPCs and mesenchymal stem cells.

Materials and methods

Ethics

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2015092902) and performed in accordance with the Guide for the Care and Use of Laboratory Animals [The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011)].

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility in our hospital with controlled temperature and light cycle (24°C and 12/12 light cycle).

Model of left common carotid artery injury

The procedure and protocol were as described in our previous report [36] with minimal modification. Pathogen-free, adult male Sprague-Dawley (SD) rats (n = 60) weighing around 350 g (Charles River Technology, BioLASCO, Taiwan) were utilized in the present study. All animals were anesthetized by inhalational of 2.0% isoflurane, placed in a supine position on a warming pad at 37°C for isolation of the left external and common carotid arteries in the left neck area.

Under flow control with vascular clamps, a small opening was created over the proximal left external carotid artery with a scalpel after adequate exposure in sterile conditions. A coronary angioplasty wire with a diameter of 0.014 inches was used to pass through the small orifice and advance into the left common carotid artery (LCA) and distal to the aorta. A 1.5 × 15 mm angioplasty balloon along the wire was then gently pushed forward and inflated, followed by dilatation within the arteries. This method reliably produced carotid artery endothelial denudation. Each external carotid artery was then ligated at the level of the orifice, fol-

lowed by closure of the muscle and skin layers. In sham control animals, only skin and muscle layers were opened followed by closing these two layers.

Animal grouping

The animals (n = 60) were categorized into sham controls (SC), left common carotid artery injury induced by balloon denudation (LCA^{BD}), LCA^{BD} + Rosu (orally 10 mg/kg/day for 5 days), LCA^{BD} + Val (orally 10 mg/kg/day for 5 days), and LCA^{BD} + autologous EPCs (1.2×10^6 by intravenous administration) once at 24 h after the balloon denudated procedure. An equal number of animals was euthanized in each group (i.e., n = 6 in each group) at two-time points (i.e., at days 5 and 14 after LCA^{BD} procedure). The dosage of EPCs [26], rosuvastatin [37] and valsartan [38] to be utilized in the present study were based on our previous reports [26, 37, 38] with minimal modification.

Collected peripheral blood for EPC culture and labeling

The procedure and protocol have been described in our previous report [39]. In brief, except for the SC group, rats in other groups were anesthetized by inhalational of 2.0% isoflurane at day 21 prior to LCA^{BD} procedure for the collection of 3 mL of peripheral blood from the tail vein. The isolated mononuclear cells from peripheral blood were cultured in a 100-mm diameter dish with 10 mL EGM2 culture medium containing 10% FBS. At culture day 21, 1.2×10^6 autologous EPCs were obtained from each animal in group 3. Rats in the SC group were anesthetized by inhalational of 2.0% isoflurane and only received an identical procedure of peripheral blood sampling without further culture.

At day 21 after the LCA^{BD} procedure, Cell tracker (Molecular probes) (1 μM) was added to the culture medium [i.e., cells (10^6-10^7 cells) in 200 μl non-phenol red DMEM medium + cell tracker 5 μl] and incubated at 37°C for 30 minutes before cell transfusion for EPC labeling.

Isolation and culture of autologous adipose-derived mesenchymal stem cells

The procedure and protocol were as described in our previous report [40]. In brief, 200-300 μL of sterile saline was added to every 0.5 g of adipose tissue to prevent dehydration. The tissue was cut into < 1 mm^3 size pieces using a pair of sharp, sterile surgical scissors. Sterile saline (37°C) was added to the homogenized adipose tissue in a ratio of 3:1 (saline: adipose tissue), followed by the addition of stock collagenase solution to a final concentration of 0.5 units/mL. The centrifuge tubes with the contents were placed and secured on a Thermaline shaker and incubated with constant agitation for 60 ± 15 minutes at 37°C. After incubation for 40 minutes, the content was triturated with a 25-mL pipette for 2-3 minutes. The cells obtained were placed back on a rocker for incubation. The contents of the flask were transferred to 50 mL tubes after digestion, followed by centrifugation at 600 g for 5 minutes at room temperature. The fatty layer and saline supernatant from the tube were poured out gently in one smooth motion or removed using vacuum suction. The thus obtained cell pellet was resuspended in 40 mL saline and then centrifuged again at 600 g for 5 minutes at room temperature. After being resuspended again in 5 mL saline, the cell suspension was filtered through a 100 μm filter into a 50-mL conical tube to which 2 mL of saline was added to rinse the remaining cells through the filter. The flow-through was pipetted into a new 50 mL conical tube through a 40 μm filter. The tubes were centrifuged for a third time at 600 g for 5 minutes at room temperature. The cells were resuspended in saline. An aliquot of cell suspension was then removed for cell culture in Dulbecco's modified Eagle's medium (DMEM)-low glucose medium containing 10% FBS for 14 days. Approximately 2.0-3.0 × 10^6 ADMSCs were obtained from each rat. The dosage of ADMSCs used in the present study was based on our previous report [40].

Specimen collection

By day 5 after the LCA^{BD} procedure, the LCA in each group was harvested for individual study. The detailed procedure and protocol have been described in our previous report [36]. Briefly, the LCA in each rat was rapidly removed and immersed in cold saline. The vessels were rinsed with PBS, embedded in OCT compound (Tissue-Tek, Sakura, Netherlands) and snap-frozen in liquid nitrogen before being stored at

-80°C. For hematoxylin-eosin staining, vessels were fixed in 4% formaldehyde and embedded in paraffin.

Carotid-ring culture and method for determining rat carotid-ring angiogenesis

The procedure and protocol have been described in our previous report [41]. In detail, by day 5 after the LCA^{BD}, some of the harvested carotid ring in each group of animals was utilized for culture for individual study. No additional treatment with EPC, rosuvastatin or valsartan was performed during carotid-ring culturing.

Carotid-ring angiogenesis assay was conducted in twenty-four-well tissue culture plates embedded with 150 μl of 1 mg/ml type I collagen (BD Biosciences, NJ) and allowed to gel for 60 minutes at 37°C and 5% CO_2. The carotid was excised from 6-rats/each group then all extraneous tissue and branching vessels were removed with forceps and a scalpel. The aorta was cut into 1 mm cross-sections, placed on collagen-coated wells then filled with 500 μl of serum free MCDB131 medium. These rings were incubated at 37°C and 5% CO_2 for 5 days and photographed with 12.5 × magnification. The number and length of sprouting vessels were quantified by ULYMPUS DP72 software. Experiments were repeated twice.

Morphometric analysis

The procedure and protocol have been described in our previous reports [36, 42]. In brief, sections of the injured LCA stained with hematoxylin-eosin were morphometrically measured using a computer-assisted image analysis system. For each animal, the segment of the injured artery was analyzed. Digital images were acquired with a DP70 microscope digital camera attached to a light microscope (Olympus BX-51) and analyzed with Image Tool 3 (IT3) image analysis software (University of Texas Health Science Center in San Antonio, UTHSCSA, Image Tool for Windows, Version 3.0, USA) (100 ×, scale bar; 1 μm = 4.64 pixels). The areas of lumen, intimal layer (i.e., internal elastic lamina, IEL), neointima of IEL, and medial layer (i.e., external elastic lamina, EEL) were calculated. The intimal area was defined as the luminal surface and IEL.

Immunofluorescent (IF) analysis

The procedure and protocol of these staining have been described in details in our previous reports [41, 43, 44]. In brief, sections were then incubated with primary antibodies specifically against CD68 (1:100, Abcam), CD40L (1:100, Abcam), von Willebrand factor (vWF) (1:200, Millipore), CD31 (1:100, Bio-Rad), CD34 (1:125, Abcam) while sections incubated with the use of irrelevant antibodies served as controls. Three sections of femoral artery specimen from each rat were analyzed.

For quantification, three randomly selected HPFs (200 × or 400 × for IHC and IF studies) were analyzed in each section. The mean number of positively-stained cells per HPF for each animal was then determined by summation of all numbers divided by 9.

Real-time quantitative PCR analysis

The procedure and protocol has been described in our previous reports [36]. Briefly, real-time polymerase chain reaction (RT-PCR) was conducted using LighCycler TaqMan Master (Roche, Germany) in a single capillary tube according to manufacturer guidelines for individual component concentrations. Forward and reverse primers were designed from sequences of different exons of the target gene to avoid amplifying genomic DNA.

During PCR, the probe was hybridized to its complementary single-strand DNA sequence within the PCR target. As amplification occurred, the probe was degraded due to exonuclease activity of Taq DNA polymerase, thereby separating the quencher from reporter dye during extension. During the entire amplification, cycle light emission increased exponentially. Positive result was identified by the threshold cycle value at which reporter dye emission appeared above background.

Statistical analysis

Quantitative data are expressed as means ± SD. Statistical analysis was adequately performed by ANOVA followed by Bonferroni multiple-comparison post hoc test. Statistical analysis was performed using SPSS statistical software for Windows version 22 (SPSS for Windows, version 22; SPSS, IL, U.S.A.). A value

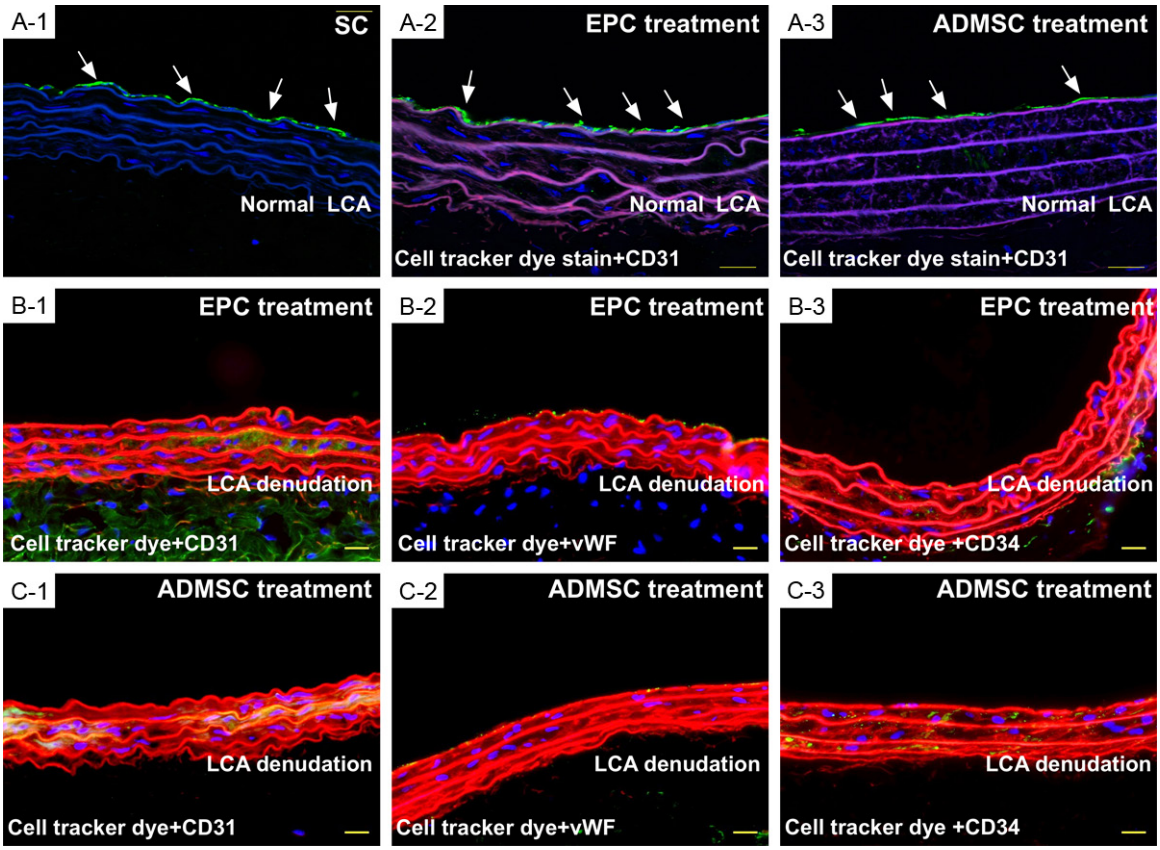

Figure 1. No stem cell adhered into the normal epithelial layer in intact vessel and in early phase of cell therapy. (A-1 to A-3) Immunofluorescent (IF) microscopic finding (400 ×) of CD31 satin for identification of exogenous administration of EPC (A-2) and ADMSC (A-3) in epithelial layer of normal LCA. No any cell tracker positively-stained cell was identified in epithelial layer of LCA. White arrows indicate the intact of intrinsic epithelial cells. These findings in (A-1 to A-3) suggest that, EPC/ADMSC did not deposit in the epithelial layer of normal LCA. (B-1 to B-3) IF microscopic findings (400 ×) (n = 3) of double stains of cell tracker dye + CD31 (B-1), cell tracker dye + vWF (B-2) and cell tracker dye + CD34 (B-3) for identifying whether the EPC-derived EC (i.e., CD31+ and vWF+ cells) or EPC (i.e., CD34+ cells) was present at BD-injured epithelial layer of LCA at early treatment phase (i.e., at day 3 after stem cell therapy). (C-1 to C-3) IF microscopic findings (400 ×) (n = 3) of double stains of cell tracker dye + CD31 (C-1), cell tracker dye + vWF (C-2) and cell tracker dye + CD34 (C-3) for identifying whether the ADMSC-derived EC (i.e., CD31+ and vWF+ cells) or EPC (i.e., CD34+ cells) was present at BD-injured epithelial layer of LCA at early treatment phase (i.e., at day 3 after stem cell therapy). The results (B & C) showed that no any of these biomarkers was identified in the injured epithelial layer of LCA. Scale bars in right lower corner represent 20 μm. EC = endothelial cell; EPC = endothelial progenitor cell; ADMSC = adipose derived mesenchymal stem cell; vWF = von Willebrand factor; LCA = left common carotid artery; BD = balloon denudation.

of $P < 0.05$ was considered as statistically significant.

Results

*No exogenous stem cell adhered into the epithelial layer of intact left common carotid artery (LCA) or at early treatment phase of injured LCA (**Figure 1**)*

To elucidate whether the stem cells (i.e., adipose-derived mesenchymal stem cells (ADMSCs) and EPCs) were found in the endothelial layer of normal LCA (i.e., without balloon denudation), the cells were labelled with Cell tracker dye and were intravenously administered into the animals (n = 3 for each group). The results showed no exogenic therapeutic cells (i.e., AMDSCs and EPCs) in the epithelial layer of normal LCA, suggesting that cell-based therapy did not offer benefit to the normal epithelial layer of vessels.

To clarify whether AMDSC- or EPC-derived endothelial cells (EC) [i.e., CD31+ and von Willebrand factor (vWF)+ cells] or EPC (i.e., CD34+

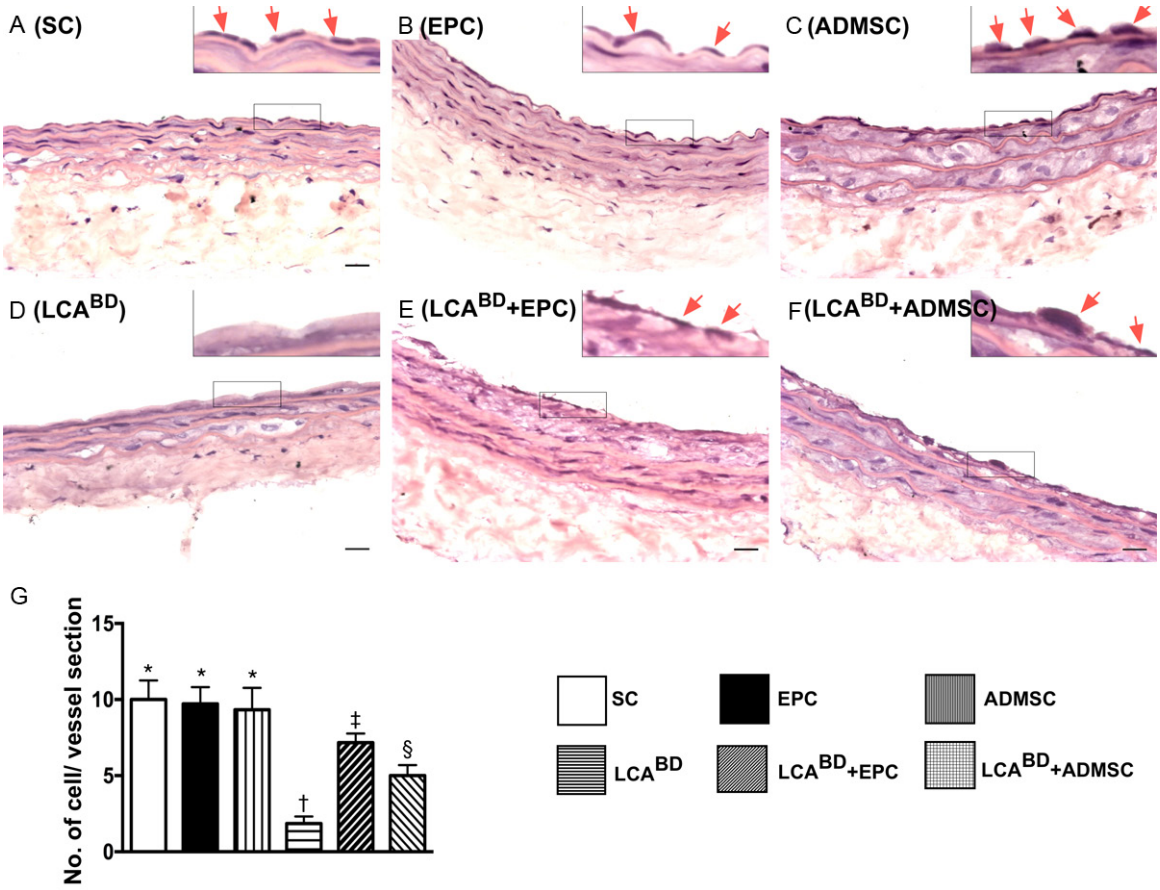

Figure 2. To compare the ability of EPCs and ADMSC on repair of EC layer in balloon denudated (BD) LCA at day 5 after BD procedure. A-F. Illustrating the pathological finding of H.E., stain (400 ×) for identification of the number of ECs in the epithelial layer of LCA (refer to small and magnified squares) among the six groups. Red arrows indicate EC. G. Analytical result of number of EC in the epithelial layer of LCA, *vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. Scale bars in right lower corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §) indicate significance at the 0.05 level. SC = sham control; EC = endothelial cells; EPC = endothelial progenitor cell; ADMSC = adipose-derived mesenchymal stem cell; LCA = left common carotid artery; BD = balloon denudation.

cells) was present at BD-injured epithelial layer of LCA at early treatment phase (i.e., at day 3 after stem cell therapy), immunofluorescence (IF) stain of the harvested LCA was performed for identification of these biomarkers. The results showed that no any of these biomarkers was identified in the injured epithelial layer of LCA.

*Comparison of EPCs and ADMSCs for repair of endothelial cells (EC) in balloon denudated (BD) carotid artery at day 5 after BD procedure (**Figures 2** and **3**)*

To compare the ability of EPCs (i.e., 1.2×10^6 cells) and ADMSCs (i.e., 1.2×10^6 cells) (i.e., EPC vs. MSC) to repair ECs, BD left common carotid arteries were treated with one of these two types of the cells (once at 24 h after BD procedure by intravenous administration) and were harvested by day five after BD procedure. The microscopic findings of hematoxylin and eosin (H&E) staining showed that the number of ECs in the epithelial layer of the LCA was highest in the sham control (SC), followed by SC + EPC and SC + ADMSC, lowest in LCA^{BD}, and significantly higher in LCA^{BD} + EPC than in LCA^{BD} + ADMSC, but showed no difference among the former three groups (**Figure 2**). Additionally, the confocal microscopic findings showed that the number of CD31+ cells, an indicator of integrity of ECs, exhibited identical findings of H&E stain in the epithelial layer (**Figure 3**). These findings suggest EPCs are superior to ADMSCs for endo-

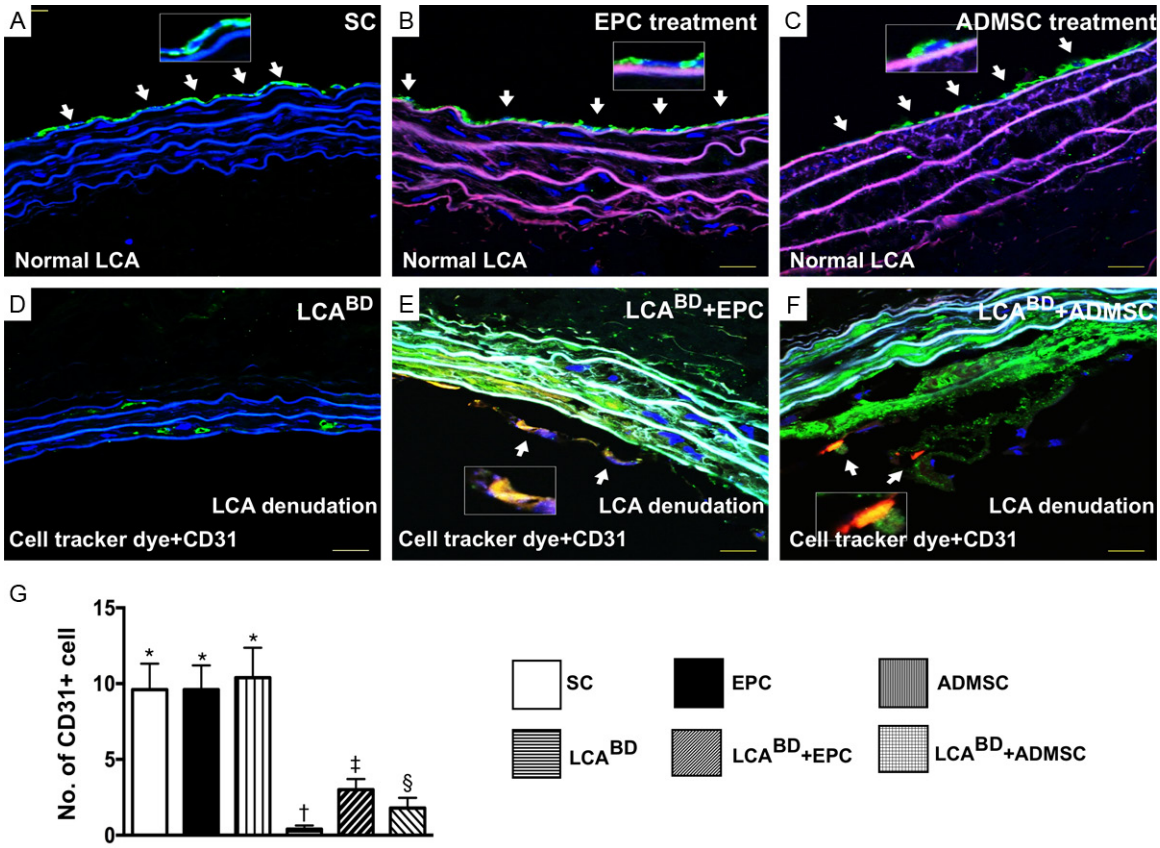

Figure 3. Confocal study for expression of CD31+ cells in intimal layer of left common carotid artery by day 5 after balloon denudation procedure. A-F. Illustrating the confocal finding (600 ×) for identification of the expression of CD31+ cells (white arrows) (green color in SC, SC-EPC and SC-ADMSC; red-yellow color in BD-EPC and BD-ADMSC) in intimal layer of LCA. Red-yellow color indicated a doubled staining (i.e., cell tracker dye + CD31 stain) in BD-EPC and BD-ADMSC groups, suggesting that only those of extrinsic stem cells (i.e., intravenous administration of cell tracker dye stained EPCs and ADMSCs). G. Analytical result of number of CD31+ cells in the epithelial layer of LCA, *vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. Scale bars in right lower corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §) indicate significance at the 0.05 level. SC = sham control; EPC = endothelial progenitor cell; ADMSC = adipose-derived mesenchymal stem cell; LCA = left common carotid artery; BD = balloon denudation.

thelial cell repair after epithelial layer damage in the vascular wall.

*Comparison of the therapeutic potential of EPCs, rosuvastatin and valsartan treatment for carotid-ring angiogenesis at day 5 after BD procedure (**Figure 4**)*

The sprout area, an indicator of angiogenesis ability, was highest in SC, lowest in LCA^{BD}, significantly higher in LCA^{BD}-Rosu than in LCA^{BD}-EPC and LCA^{BD}-Val and significantly higher in LCA^{BD}-EPC than in LCA^{BD}-Val. Additionally, mean sprout front distance, another parameter for measuring the angiogenesis capacity, was highest in SC, lowest in LCA^{BD}, significantly higher in LCA^{BD}-EPC than in LCA^{BD}-Rosu and LCA^{BD}-Val, and significantly higher in LCA^{BD}-Rosu than LCA^{BD}-Val.

*Gene expression of angiogenesis biomarkers at day 5 after BD procedure (**Figure 5**)*

The mRNA expression of CD31 and von Willebrand factor (vWF), two indicators of EC surface markers, were highest in the SC, lowest in the LCA^{BD}, and significantly higher in the LCA^{BD}-EPC than in the LCA^{BD}-Rosu and LCA^{BD}-Val and significantly higher in the LCA^{BD}-Rosu than in the LCA^{BD}-Val group.

The mRNA expressions of stromal cell-derived factor (SDF)-1α and CXCR4, two indices of EPC surface markers, were highest in the LCA^{BD}-

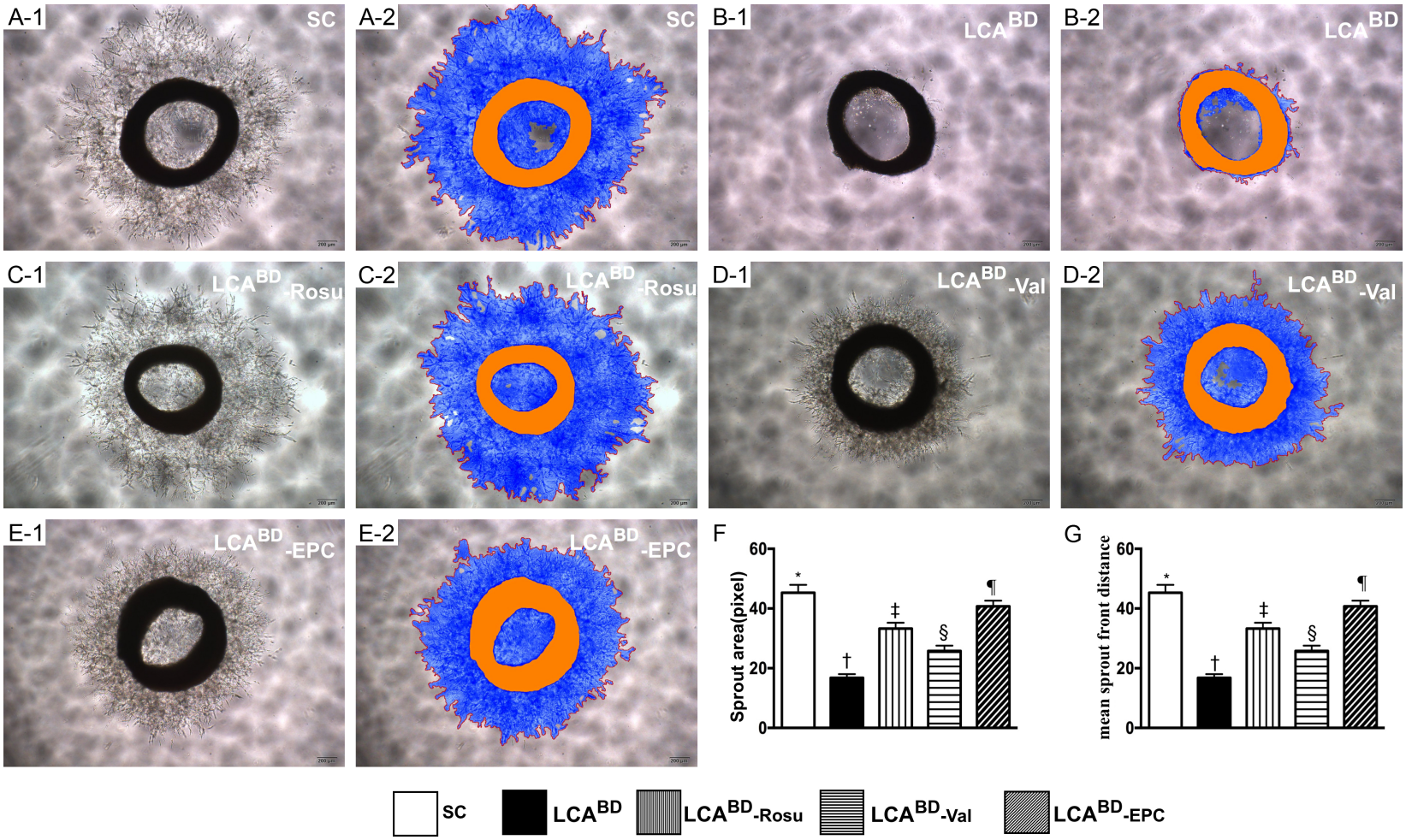

Figure 4. Therapeutic effects of EPC, rosuvastatin and valsartan on angiogenesis of culture carotid ring by day 5 after BD procedure. A-E. Illustrating microscopic findings of aortic-ring culturing in SC (A-1, A-2), LCA^{BD} (B-1, B-2), LCA^{BD}-Rosu (C-1, C-2), LCA^{BD}-Val (D-1, D-2) and LCA^{BD}-EPC (E-1, E-2), respectively. F. Analytical results of sprout area, * vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. G. Analytic result of mean sprout front distance, *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance at the 0.05 level. SC = sham control; EPC = endothelial progenitor cell; ADMSC = adipose-derived mesenchymal stem cell; LCA = left common carotid artery injury; BD = balloon denudation.

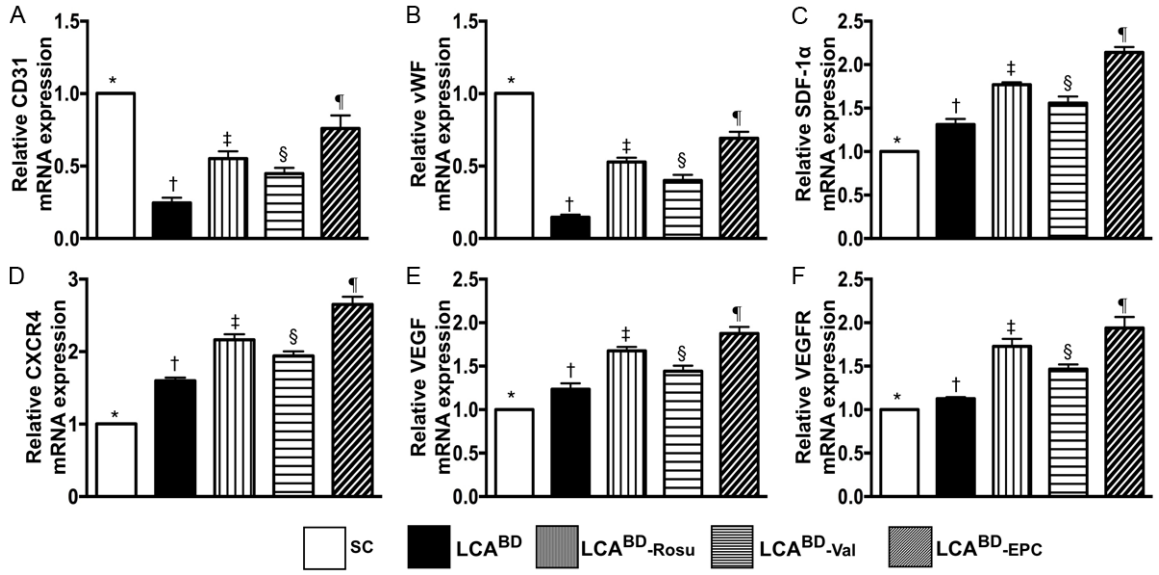

Figure 5. Gene expression of angiogenesis biomarkers at day 5 after BD procedure. A. mRNA expression of CD31, *vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. B. mRNA expression of von Willebrand factor (vWF), *vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. C. mRNA expression of stromal cell-derived factor (SDF)-1α, *vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. D. mRNA expression of stromal cell-derived factor CXCR4, *vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. E. mRNA expression of vascular endothelial cell growth factor (VEGF), *vs. other groups with different symbols (†, ‡, §, ¶), P<0.0001. F. mRNA expression of VEGF-receptor (R), *vs. other groups with different symbols (†, ‡, §, ¶), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; EPC = endothelial progenitor cell; LCA^{BD} = left common carotid artery injury by balloon-denudation; Val = valsartan; Rosu = rosuvastatin.

EPC, lowest in SC, significantly lower in the LCA^{BD} than in LCA^{BD}-Rosu and the LCA^{BD}-Val and significantly lower in the LCA^{BD}-Val group than in LCA^{BD}-Rosu group, suggesting an intrinsic response to EC damage in the epithelial layer that was further enhanced after the therapy. Additionally, the mRNA expressions of vascular endothelial growth factor (VEGF) and VEGF-receptor (R), two indicators of angiogenesis, displayed an identical pattern of EPC surface markers among the five groups.

*Gene expressions of inflammatory and oxidative stress biomarkers at day 5 after BD procedure (**Figure 6**)*

The mRNA expressions of vascular cell adhesion molecule (VCAM)-1, matrix metalloproteinase (MMP)-9 and tumor necrosis factor (TNF)-α, three indicators of inflammation, were highest in LCA^{BD}, lowest in SC, significantly higher in LCA^{BD}-Val than LCA^{BD}-Rosu and LCA^{BD}-EPC and significantly higher in LCA^{BD}-Rosu than in LCA^{BD}-EPC. Additionally, the mRNA expressions of NOX-1 and NOX-2, two indices of oxidative stress, showed an identical pattern of inflammation.

*Gene expressions of EC integrity/intercellular junction, apoptosis and anticoagulant biomarkers at day 5 after BD procedure (**Figure 7**)*

The mRNA expression of endothelial nitric oxide synthase (eNOS), an indicator of EC integrity and vascular endothelial (VE)-cadherin, an indicator of EC intercellular junctions, were highest in SC, lowest in LCA^{BD}, significantly higher in LCA^{BD}-EPC than in LCA^{BD}-Rosu and LCA^{BD}-Val and significantly higher in LCA^{BD}-Rosu than in LCA^{BD}-Val.

The mRNA expressions of caspase-3 and poly (ADP-ribose) polymerase (PARP), two indicators of apoptosis, demonstrated an opposite pattern of eNOS among the five groups. Additionally, mRNA expression of thrombomodulin, serves as a cofactor for thrombin, displayed an identical pattern of apoptosis among the five groups, suggesting in intrinsic response to EC damage that was suppressed by the treatment.

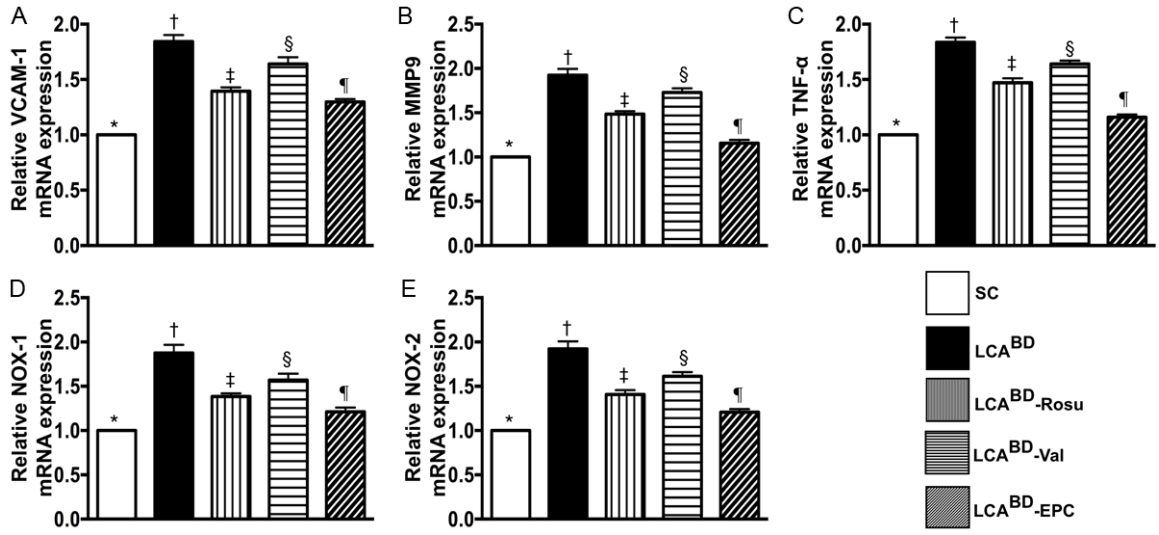

Figure 6. Gene expressions of inflammatory and oxidative stress biomarkers at day 5 after BD procedure. A. mRNA expression of vascular cell adhesion molecule (VCAM)-1, *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. B. mRNA expression of matrix metalloproteinase (MMP)-9, *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. C. mRNA expression of tumor necrosis factor (TNF)-α, *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. D. mRNA expression of NOX-1, *vs. other groups with different symbols (†, ‡, §, ¶), $p<0.0001$. E. mRNA expression of NOX-2, *vs. other groups with different symbols (†, ‡, §, ¶), $P<0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; EPC = endothelial progenitor cell; LCA^{BD} = left common carotid artery injury by balloon-denudation; Val = valsartan; Rosu = rosuvastatin.

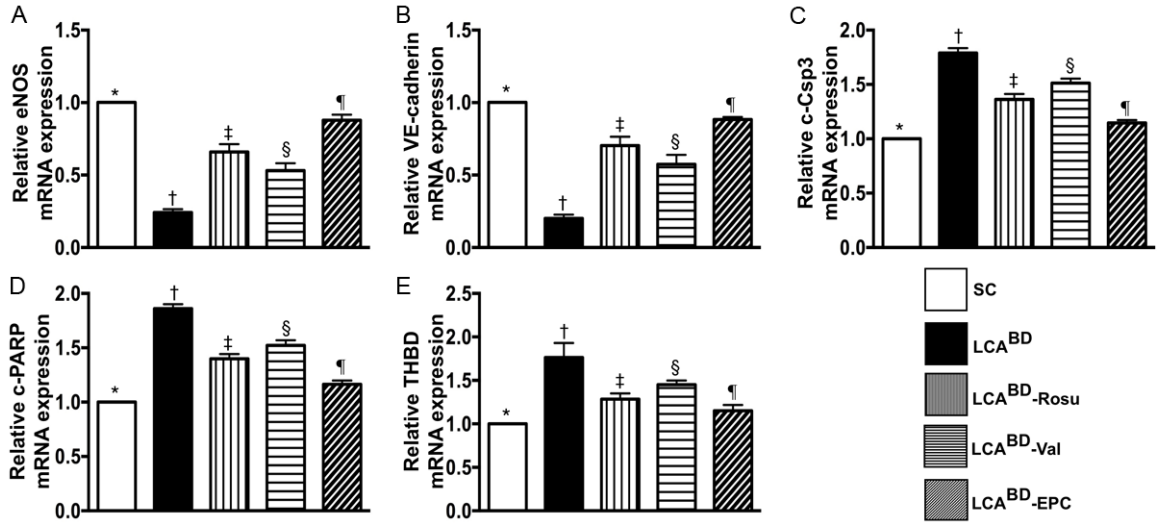

Figure 7. Gene expressions of EC integrity/intercellular junction, apoptosis and anticoagulant biomarkers at day 5 after BD procedure. A. mRNA expression of endothelial nitric oxide synthase (eNOS), *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. B. mRNA expression of vascular endothelial-cadherin ((VE-cad), *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. C. mRNA expressions of caspase-3 (Casp 3), *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. D. mRNA expression of poly (ADP-ribose) polymerase (c-PARP), *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. E. mRNA expression of THBD (thrombomodulin), *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; EPC = endothelial progenitor cell; LCA^{BD} = left common carotid artery injury by balloon-denudation; Val = valsartan; Rosu = rosuvastatin.

*Proliferations of neointimal layer in the left common carotid artery by day 14 after BD procedure (**Figure 8**)*

The microscopic findings of H&E stain revealed that the neointimal-layer area, an indicator of intimal proliferation in response to EC damage, was highest in the LCA^{BD}, lowest in the SC, significantly higher in LCA^{BD}-Val than in LCA^{BD}-EPC and LCA^{BD}-Rosu, and significantly higher in LCA^{BD}-Rosu than in LCA^{BD}-EPC. Additionally, the medial-layer area was significantly higher in LCA^{BD} and LCA^{BD}-Val than in SC, LCA^{BD}-Rosu and LCA^{BD}-EPC but it showed no difference between the former two groups or among the later three groups.

*Expressions of inflammatory cell infiltrations in left common carotid artery by day 14 after BD procedure (**Figure 9**)*

The expression of CD68+ cells, an indicator of inflammation, was highest in the SC group, lowest in LCA^{BD} group, significantly higher in the LCA^{BD}-Val group than in the LCA^{BD}-EPC and LCA^{BD}-Rosu group, and significantly higher in the LCA^{BD}-Rosu group than in LCA^{BD}-EPC group. Additionally, the expression of CD40L+ cells, another indicator of inflammation, demonstrated a similar pattern of CD68+ cells among the five groups.

Discussion

Previous studies [20, 21, 45] have shown that angiogenesis/neovascularization is a connate response for repair of senile/damaged ECs and vascular damage. However, whether exogenous EPC administration to the vessels with intact ECs in healthy subjects would offer additional benefit is currently unclear. One important finding in the present study was that no EPCs or ADMS were found to be present in the intimal layer of intact left common carotid artery, suggesting cell therapy does not offer additional benefit for normal vessels. Our finding, therefore, extends the findings of the previous studies [20, 21, 45].

Our previous study has shown that CD34+ cell therapy improved heart function for patients with severe diffuse coronary artery disease unsuitable for coronary intervention and with poor response to pharmacotherapy [24]. Surprisingly, when we look at the literatures, we don't find any report in term of directly proving intravenous administration of EPCs can repair damaged endothelial layer of artery. The novel finding in the present study was that the endothelial layer of LCA was well repaired by day 5 after intravenous administration of EPCs in animals with setting of LCA damage by balloon dilatation. In this way, our experimental finding may support the findings of our previous clinical study [24]. Of distinctively important finding in the present study was that the neointimal-layer and medial-layer formation/proliferation were markedly suppressed in LCA^{BD} animals after receiving EPCs therapy, suggesting that this resulted from the successful repairmen of endothelial layer.

Although abundant data have demonstrated that EPC or ADMSC therapy improved ischemia-related organ dysfunction via the effects of angiogenesis/neovascularization [24, 26, 35, 40], anti-inflammation [40], reduction of oxidative stress and immunomodulation [40], surprisingly, to the best of our knowledge, no specific study has addressed the therapeutic ability of EPCs vs. ADMSCs on repair of intimal layer after EC damage. An essential finding in the present study was that when we looked at this issue, we found that EPC therapy was superior to ADMSCs to adhere to damage to the intimal layer for re-endothelialization. Our finding, in addition reinforcing the results of previous reports [24, 26, 35, 40] suggests that the endothelial linage may be better than the MSC lineage for repair of damaged/senile ECs in vessels.

While the therapeutic role of EPCs on repair of damaged ECs in the intimal layer and angiogenesis has been fully investigated [20, 21, 24, 26, 35], the time courses and the exact time interval for the relatively completely healing process of endothelial layer (i.e., re-endothelization) after EPC treatment has not yet been reported. A principal finding in the present study was that the optimal time point, i.e., treatment day 5, was found not only in EPCs but in rosuvastatin and valsartan. Additionally, the better carotid-ring angiogenesis was found at day 5 of EPC-Rosu-Val treatment.

Accordingly, as compared with previous studies [20, 21, 24, 26, 35], our findings present some new information to better understand the endothelial healing process after receiving cell,

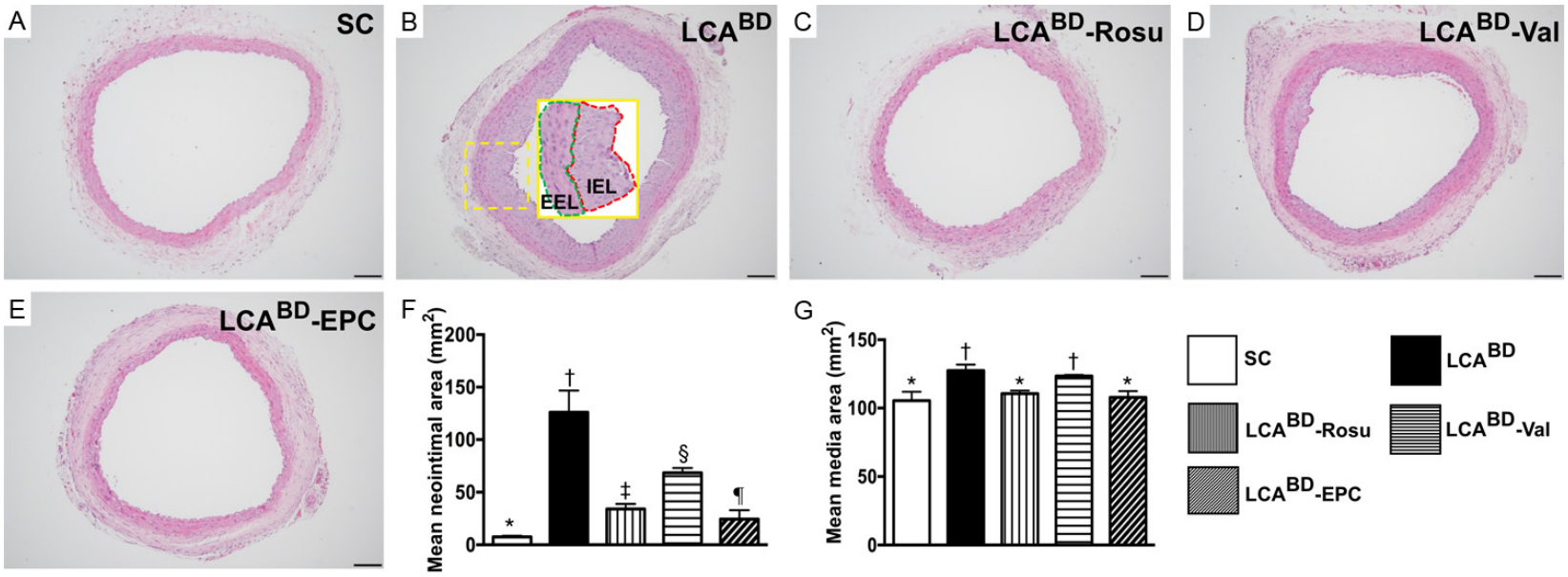

Figure 8. Proliferations of neointimal layer in the left common carotid artery by day 14 after BD procedure. A-E. Pathological finding of H.E. stain (100 ×) for identifications of intimal and medial layers of LCA. The large square (yellow solid line) (400 ×) is magnified from small square (yellow dot-line). Neointimal hyperplasia in internal elastic lamina (IEL) (i.e., intimal layer) (red dot-line) was found to be more severe in LCA^{BD} than in other groups. Additionally, the medial layer (green dot-line) (i.e., external elastic lamina, EEL) hyperplasia was also found in LCA^{BD} and LCA^{BD}-Val than other groups. F. Neointimal layer area, *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. G. Medial layer area, *vs. †, $p<0.01$. Scale bars in right lower corner represent 100 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; EPC = endothelial progenitor cell; LCA^{BD} = left common carotid artery injury by balloon-denudation; Val = valsartan; Rosu = rosuvastatin.

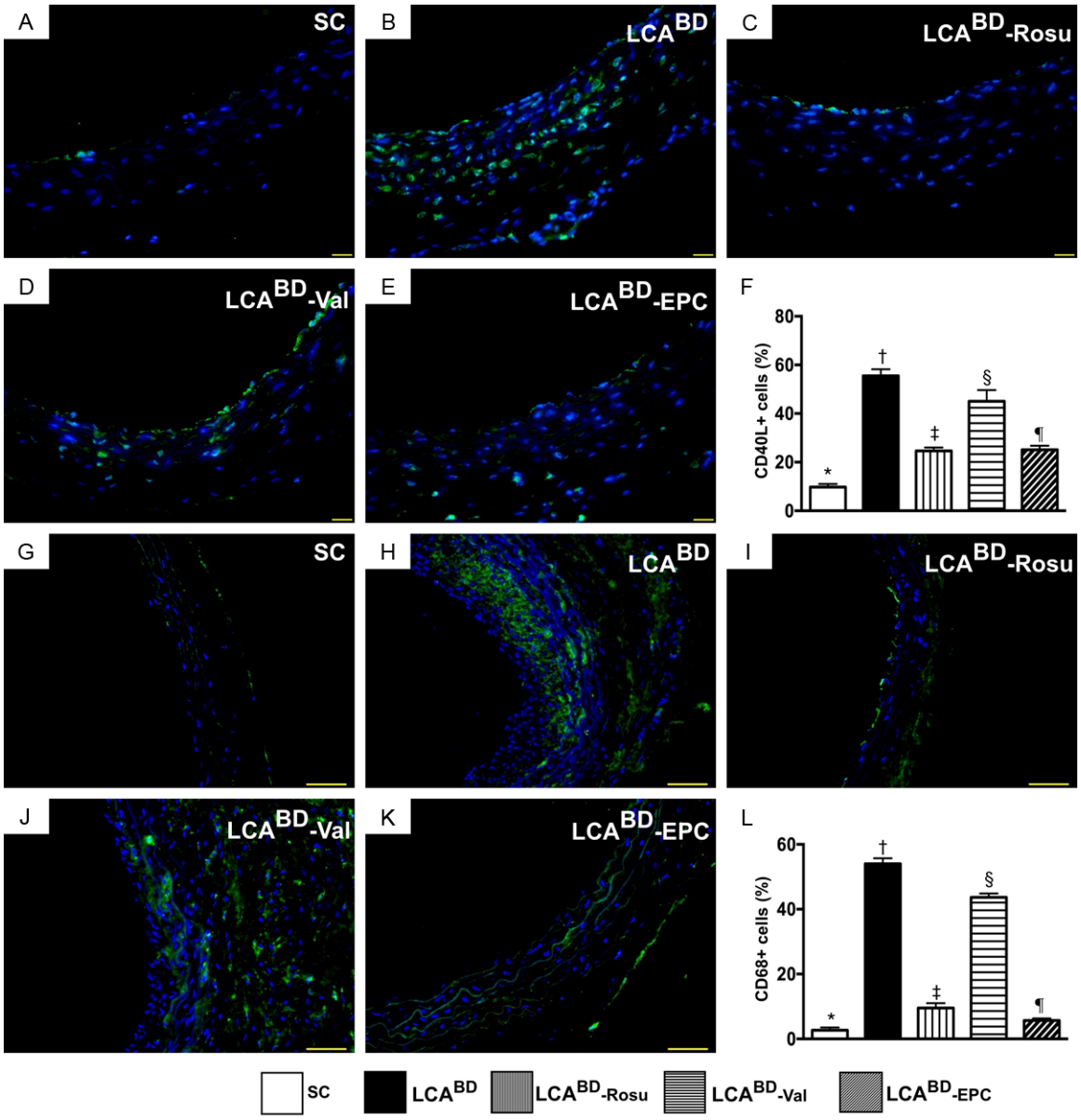

Figure 9. Expressions of inflammatory cell infiltrations in left common carotid artery by day 14 after BD procedure. A-E. Microscopic finding (400 ×) of immunofluorescent (IF) stain for identification of CD40L+ cells (green color) infiltration in vascular wall. F. Analytical result of number of CD40L+ cells, *vs. other groups with different symbols (†, ‡, §), $P < 0.0001$. G-K. IF microscopic finding (400 ×) for identification of CD68+ cells (green color) in vascular wall. L. Analytical result of number of CD68+ cells, *vs. other groups with different symbols (†, ‡, §, ¶), $P < 0.0001$. Scale bars in right lower corner represent 20 μm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §, ¶) indicate significance (at 0.05 level). SC = sham control; EPC = endothelial progenitor cell; LCA^{BD} = left common carotid artery injury by balloon-denudation; Val = valsartan; Rosu = rosuvastatin.

statin and ARB inhibitor treatment. A concern and debated issue in our clinical practice is how long a patient should take the double antiplatelet agents after receiving a drug eluting stent. This experimental study may provide some useful information for interventional cardiologists and physicians in their clinical practice.

Previous studies have shown that rosuvastatin and valsartan therapies upregulated the circulating level of EPCs [27, 28, 34]. Additionally, statin therapy has been established to augment angiogenesis [46]. The most important finding in the present study was that angiogenesis capacity and reendothelization in balloon damaged left common carotid artery were com-

parable in EPCs, rosuvastatin or valsartan therapy. With respect to rosuvastatin and valsartan, the repair (i.e., reendothelization) of the damaged intimal layer could be due to the enhancement of the mobilization of EPCs from bone marrow to circulation or ECs grew from relatively normal endothelial lineage to denudated intimal layer (i.e., endogenous growth).

Another important finding in the present study was that serious neointimal and medial-layer proliferations were identified in the LCA^{BD} animals. Endothelial dysfunction has been clearly recognized to play a key role on smooth muscle proliferation [36] and development of arterial atherosclerosis [36, 47]. Our finding is consistent with the findings of previous studies [36, 47]. Of importance was that rosuvastatin and valsartan therapy remarkably attenuated the proliferation of the intimal layer and upregulated the re-endothelialization in this layer. Additionally, our previous study has shown that rosuvastatin significantly inhibited the high-cholesterol diet induced rabbit aortic atherosclerosis and stiffness as well as smooth muscle growth [29]. Furthermore, other previous study has demonstrated that angiotensin II has been found to play a crucial role on vascular smooth muscle cell migration and proliferation [48]. In this way, these studies [29, 48] could reinforce the findings of our present study.

Study limitation

This study has limitations. First, as compared with autologous EPC therapy, rosuvastatin and valsartan therapy were also able to enhance repair of the intimal layer and stimulate carotid-ring angiogenesis in animals after receiving the LCA^{BD} procedure which could be an indirect effect. However, the underlying mechanism is still unclear. Additionally, despite the essential role of EPC on repairmen of vessel damage and replacement of senile endothelial cells have been identified by abundant reported data, whether EPC therapy on attenuating the balloon-denudated carotid artery injury was also through the same mechanistic basis was not delineated by the present study. Second, the optimal dosage of these three different kinds of therapy has not been tested; therefore, whether the EPC therapy is superior to rosuvastatin or valsartan or vice versa remains uncertain.

Conclusion

In conclusion, EPC-Rosu-Val therapy was found to be effective in repairing the intimal layer and stimulating carotid ring angiogenesis as well as inhibiting the neointimal formation in a setting of LCA^{BD} in rodents. These findings suggest that not only statins and ARB inhibitors but also EPCs may be considered as therapeutic options for patients who have endothelial dysfunction and arteriosclerotic obstructive syndrome.

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Disclosure of conflict of interest

None.

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