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Original Article

Circulating microparticles enhanced rat vascular wall remodeling following endothelial denudation

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Abstract: This study tested the hypothesis that circulating microparticles (MPs) exacerbated vascular wall (VW) remodeling after endothelial denudation by 0.014 wire in a rat model. Adult male Sprague Dawley rats (n = 40) were equally categorized into group 1 [sham-control (SC); 3.0 mL saline intravenous injection], group 2 [SC + intravenous MPs (1.0×10^7) derived from patients with carotid artery stenosis (CAS)], group 3 [femoral arterial endothelial denudation (FAED)], group 4 (FAED + MPs derived from healthy subjects), and group 5 (FAED + CAS-derived MPs). Animals were euthanized by day 28 after FAED procedure. The results demonstrated that neointimal area (NIA) (mm^2), medial area, and number of infiltrated cells in medial layer were highest in group 5 and lowest in groups 1 and 2, and significantly higher in group 4 than those in group 3 (all P<0.0001), but no differences were noted between groups 1 and 2. However, the ratio of luminal area to VW area showed an opposite pattern compared to that of NIA among five groups (P<0.0001). Immunofluorescent study showed an identical pattern of changes in the numbers of inflammatory (F4/80, CD14, CD40, IL-β) and proliferative (Ki-67, Cx43) cells in VW compared to that of NIA among the five groups (all P<0.00). The mNRA expressions of inflammatory (MMP-9, NF-κB, TNF-α, IL-1β, iNOS, PDGF) and cell activation (c-Fos, c-Myc, osteopontin, PCNA) biomarkers showed an identical pattern compared to that of NIA among all groups (all P<0.001). Take altogether, CAS-derived MPs further aggravated MP-mediated VW remodeling after endothelial damage compared to that observed after administration of MPS derived from healthy subjects.

Keywords: Endothelial denudation, vascular wall remodeling, inflammation, neointimal proliferation, circulating microparticles

Introduction

Not only is ischemic heart disease (IHD) one of the top-ranking causes of mortality worldwide [1-4], but it is also a leading contributor to heart failure (HF) that often develops as a complication of acute myocardial infarction (AMI) [1, 4]. Globally, AMI, the major consequence of IHD, is an important cause of premature death [2, 5]. As is well recognized, the majority of cardiovascular diseases start with an inflammatory process [6]. Indeed, abundant data have further indicated the participation of numerous inflammatory mediators in vascular wall at all stages of atherosclerosis, from initiation, progression, to the evolvement of plaque rupture and acute coronary syndrome [7-10]. However, the exact mechanisms of inflammation underlying the initiation and propagation of atherosclerosis in vessel wall remain unclear.

Intercellular communication is not only achieved through direct contacts, but it has also been found to be attained via the production of signaling molecules such as microparticles (MPs) [11, 12]. It is well recognized that MPs are "small plasma membrane fragments" that are shed from virtually all kinds of cells especially in active or apoptotic phase as well as in response to physical, toxic/endotoxin or pathological stimulation [12-19]. A substantial body of evidence indicates that MPs are involved in the pathophysiology of different cardiovascular disorders, including atherogenesis and thrombosis [16, 17, 19, 20]. Our recent experimental studies have further demonstrated that not only do MPs directly participate in the enhancement of angiogenesis [21], but they are also involved in cancer cell metastasis [22]. In addition, other studies have shown that MPs are involved in regulating inflammatory response and promoting the recruitment of inflammatory cells in the vascular wall [17, 23]. These studies, therefore, raise the hypothesis that circulating MPs may directly participate in neoitimal and medial layer proliferation as well as vessel wall remodeling after endothelial damage, thereby contributing to arterial obstruction. Accordingly, this study tested the hypothesis that human circulatory MPs may directly participate in arterial neoitimal and medial proliferation and obstruction by utilizing a rodent model of femoral arterial endothelial denudation of (FAED).

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. 2014062503) 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 (2°C and 12/12 light cycle).

Model of femoral artery injury

Pathogen-free, adult male Sprague-Dawley (SD) rats (n = 40) weighing 320-350 g (Charles River Technology, BioLASCO Taiwan Co. Ltd., Taiwan) were utilized in the present study. All animals were anesthetized by inhalational 2.0% isoflurane, placed in a supine position on a warming pad at 37°C for isolation of the right and left femoral arteries in the inguinal area.

Under flow control with vascular clamps, a small opening was created over proximal right and left femoral arteries (FAs) with a scalpel after adequate exposure in sterile condition. A coronary angioplasty wire with a diameter of 0.014 inches was used to pass through the small orifice and advanced into the common iliac artery and distal portion of abdominal aorta. The wire was then gently pushed and pulled forward and backward several times within the arteries. This method reliably produced FAED. Additionally, this method ensured the damage of only endothelial cells rather than smooth muscle during the procedure. Each FA was then ligated at the level of the orifice, followed by closure of the muscle and skin layers.

Blood samples for plasma microparticles

The procedure and protocol for collection of MPs have been described in details in our recent report [21]. For MPs derived from patients (n = 20) with symptomatic severe carotid artery stenosis (CAS) undergoing carotid stenting, the blood samples (20.0 mL for each patient) were obtained at catheterization room after radial arterial puncture. Additionally, age-matched healthy subjects (HS) (n = 6) who had no CAD risk factor were enrolled and served as control subjects. Peripheral blood samples (20.0 mL from each subject) were collected and prepared for MPs. Blood samples were collected in acid citrate dextrose (ACD) vacutainer tubes. To prepare platelet-rich plasma, the blood sample was centrifuged at 2500 × g at 4°C for 15 min without acceleration or brake. The plasma samples were thawed and centrifuged for 10 min at 19,800 × g at 4°C, and then collected for investigation of MPs smaller than 1.0 μm. Size calibration was conducted with 1.0 μm beads (Invitrogen, Carlsbad, CA). All buffers were sterile-filtered with a 0.2 μm filter. Pellets were used in the current study without

purification of the isolated MPs. Moreover, since a large number of MPs was needed for one animal, the MPs were obtained from a pool of patients with CAS.

Animal grouping

After the FAED procedure, the animals were categorized into: (1) sham control (SC) (only incision on skin and muscle layer in the right inguinal area, followed by closure of the layers and intravenous injection of 3.0 mL normal saline from tail vein) (group 1), (2) SC + CAS-derived MPs (1.0×10^7) (by tail intravenous administration) (group 2), (3) FAED only (group 3), (4) FAED + healthy subject (HS)-derived MPs (group 4), and (5) (FAED + CAS-derived MPs) (group 5). The dosage and route of administration of MPs were based on our recent report with some modifications [21]. In details, the MPs (1.0×10^6 each time) were administered at three time points at (1) day 7, (2) day 14, and (3) day 21 after the FAED procedure. The animals in each group were euthanized by day 28 after FAED induction. The FA was collected from each animal for individual study.

Specimen collection

The detailed procedure and protocol have been described in our previous report [24]. Briefly, the femoral arteries in each rat were rapidly removed and immersed in cold saline. For immunohistofluorescent (IHF) study, 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.

Immunofluorescent (IF) analysis

The procedure and protocol of IF staining have been described in details in our previous reports [25, 26]. For IF staining, rehydrated paraffin sections were first treated with 3% H_2O_2 for 30 minutes and incubated with Immuno-Block reagent (BioSB, Santa Barbara, CA, USA) for 30 minutes at room temperature. Sections were then incubated with primary antibodies specifically against F4/80 (1:100, Abcam), CD14 (1:50, Santa Cruz), CD40L (1:100, Abcam), interleukin (IL)-1β (1:100, Aviva Systems Biology), connexin43 [(Cx43), 1:100, Abcam], Ki67 (1:100, Abcam), von Willebrand factor (vWF) (1:200, Millipore), 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.

Morphometric analysis

Sections of the injured femoral arteries 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 DP70 microscope digital camera attached to a light microscope (Olympus BX-51) and analyzed with Image Tool 3 (IT3) image analysis software (The 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) were calculated. The intimal area was defined as the luminal surface and IEL.

Real-time quantitative PCR analysis

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.

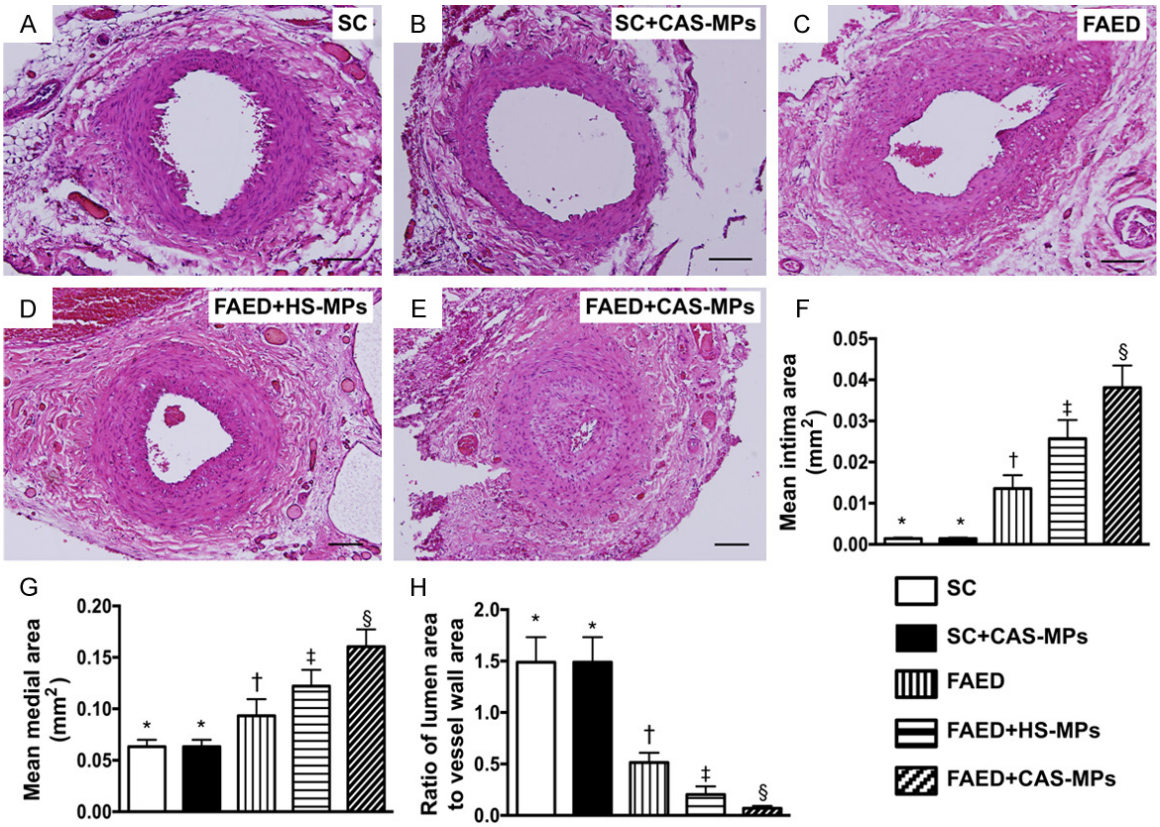

Figure 1. Vessel wall remodeling by day 28 after FAED procedure. A-E. Illustrating microscopic finding (100 ×) of H&E staining for identification of the proliferations of intimal and medial layer of femoral artery (FA). F. Analytic result of intimal area (i.e., area of neointimal proliferation), * vs. other groups with different symbols (†, ‡, §), P<0.0001. G. Analytical result of medial area, * vs. other groups with different symbols (†, ‡, §), P<0.0001. H. Analytical result of ratio of lumen area to vessel wall area (i.e., areas of intima + medium). * vs. other groups with different symbols (†, ‡, §), P<0.0001. Scale bars in right lower corner represent 100 μm. SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

Statistical analyses

Quantitative data are expressed as mean ± SD. Statistical analysis was performed by ANOVA followed by Bonferroni multiple-comparison *post hoc* test. All analyses were conducted using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

Microscopic identification of neointimal and medial layer proliferations and cell infiltration in vessel wall on day 28 after FAED procedure

The intimal and medial areas were largest in FAED + CAS-derived MPs treatment group (group 5) and smallest in SC (group 1) and the SC + CAS-derived MPs treatment group (group 2), and significantly larger in FAED + HS-derived MPs treatment group (group 4) than those in the FAED only group (group 3) (**Figure 1**), but there was no difference between groups 1 and 2. On the other hand, the ratio of lumen area to the vessel wall area (i.e., intima + medium) showed an opposite pattern compared to that of intimal area among the five groups (**Figure 1**). These findings suggest the MPs were involved in arterial proliferation and obstruction only after endothelial damage. Besides, CAS-derived MPs had a stronger influence as compared with that of HS-derived MPs on the induction of proliferations in neoitimal and medial layers. Furthermore, the number of infiltrated cells in the vessel wall, an indicator of the severity of inflammation/proliferation, exhibited a pattern identical to that of changes in intimal area among the five groups (**Figure 2**).

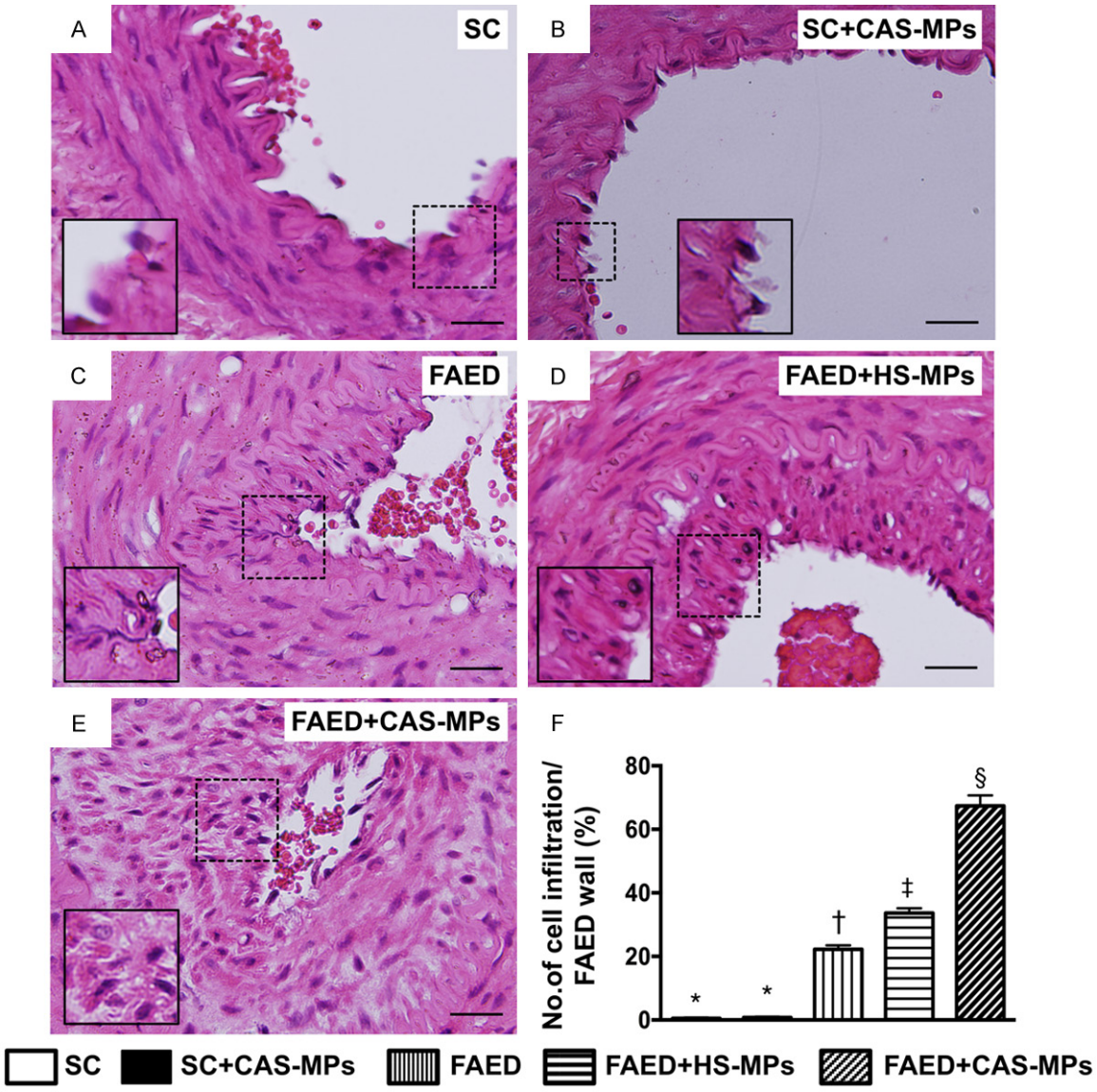

Figure 2. Cellular infiltration in vessel wall on day 28 after FAED procedure. A-E. Showing microscopic finding (400 ×) of cellular infiltration in FAED wall (black color of nuclei). The small dotted-line square box was magnified into large solid-line square box for the purpose of more easily to identify the distribution of number of cell nuclei. F. Statistical analysis of number of cell distribution in FAED wall. * vs. other groups with different symbols (†, ‡, §), P<0.0001. Scale bars in right lower corner represent 100 μm. SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

IF staining for identification of inflammatory cell infiltration in vessel wall on day 28 after FAED procedure

IF microscopic analysis demonstrated that the numbers of cells with expressions of F4/80 and CD14 (**Figure 3**) as well as CD40 and IL-β (**Figure 4**) in the vessel wall, four indices of inflammation, were significantly higher in group 5 than those in other groups, significantly higher in group 4 than those in groups 1 to 3, and significantly higher in group 3 than those in groups 1 and 2, but no difference was noted between groups 1 and 2. These findings imply that the inflammation was elicited after endothelial cell damage and further enhanced after treatment with MPs.

IF staining for identification of proliferative cells in vessel wall on day 28 after FAED procedure

IF staining demonstrated that cellular expressions of Ki-67 and Cx43 in the vessel wall, two

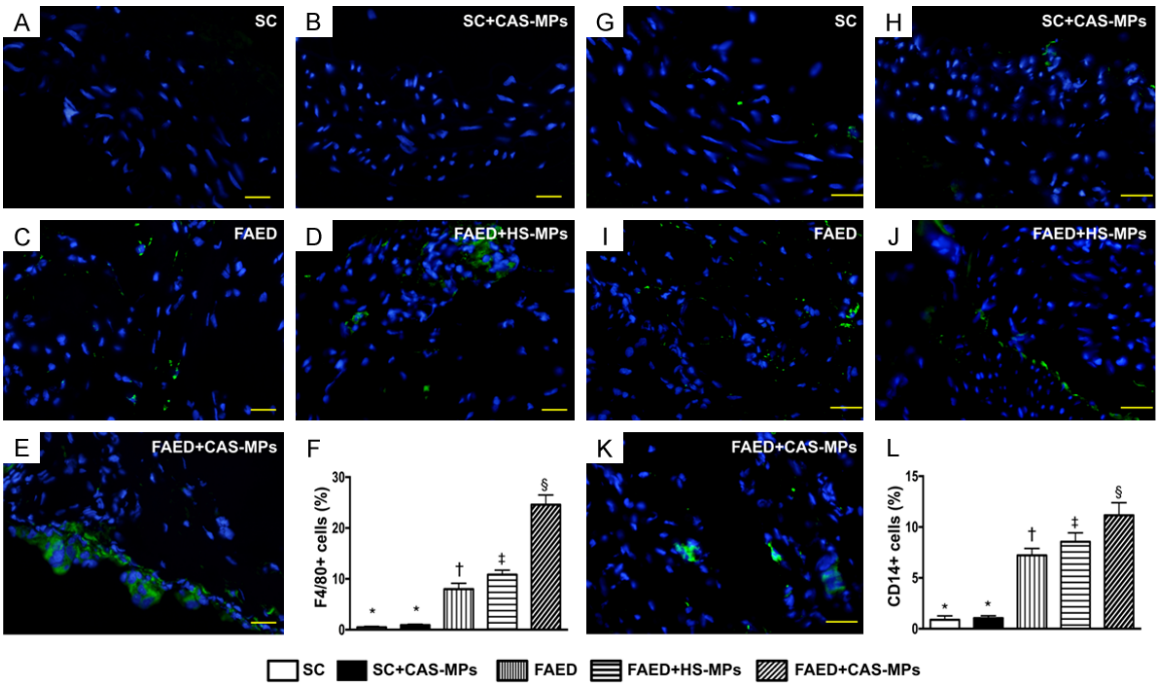

Figure 3. F4/80+ and CD14+ cell infiltration in vessel wall on day 28 after FAED procedure. A-E. Exhibiting the immunofluorescent (IF) microscopic finding (400 ×) for identification of F4/80+ cells localized in FAED wall (green color). F. Statistical analysis of number of F4/80+ cells in FAED wall, * vs. other groups with different symbols (†, ‡, §), P<0.0001. G-K. Exhibiting the IF microscopic finding (400 ×) for identification of CD14+ cells localized in FAED wall (green color). L. Statistical analysis of number of CD14+ cells in FAED wall. * vs. other groups with different symbols (†, ‡, §), P<0.0001. Scale bars in right lower corner represent 20 μm. SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

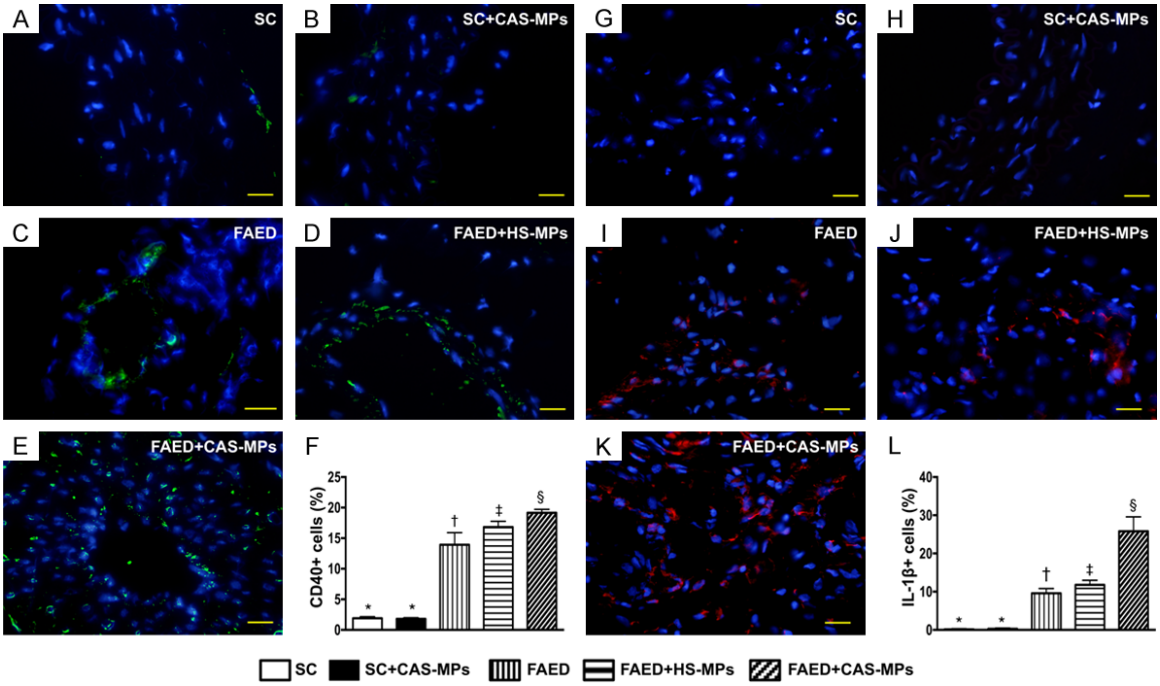

Figure 4. CD40+ and IL-1β+ cell infiltration in vessel wall on day 28 after FAED procedure. A-E. Exhibiting the immunofluorescent (IF) microscopic finding (400 ×) for identification of CD40+ cells localized in FAED wall (green color). F. Statistical analysis of number of CD40+ cells in FAED wall, * vs. other groups with different symbols (†, ‡, §), P<0.0001. G-K. Exhibiting the IF microscopic finding (400 ×) for identification of interleukin (IL)-1β+ cells localized

in FAED wall (red color). L. Statistical analysis of number of IL-1β+ cells in FAED wall. * vs. other groups with different symbols (†, ‡, §), P<0.0001. Scale bars in right lower corner represent 20 μm. SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

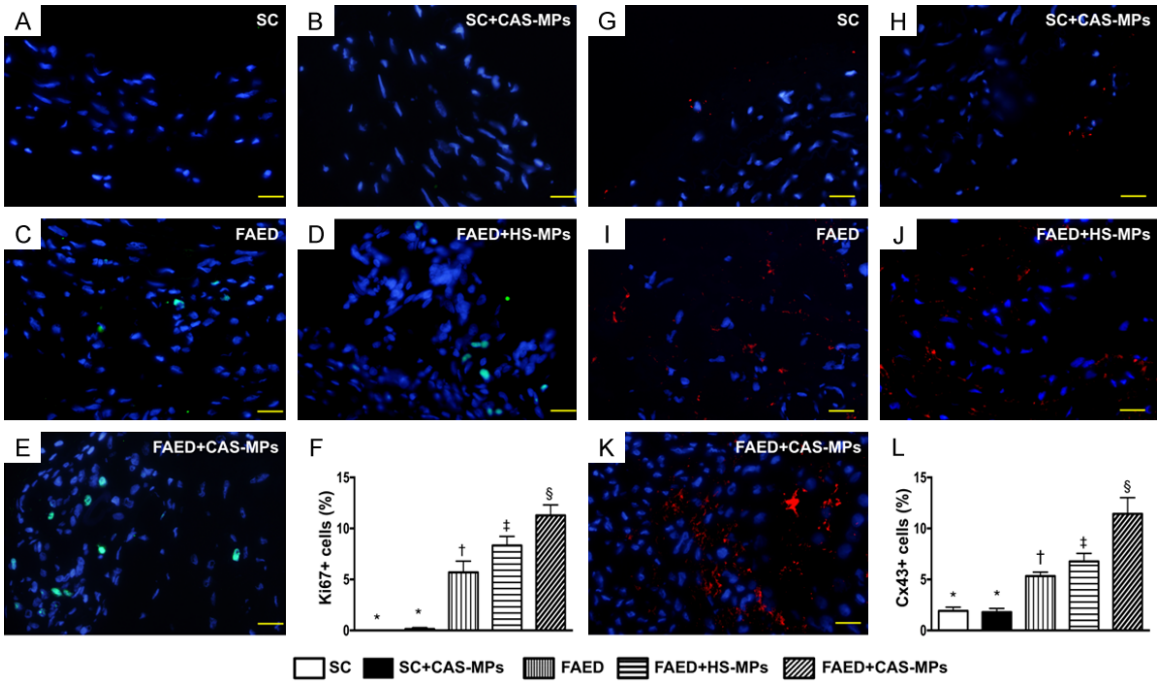

Figure 5. Expressions of proliferative cells in vessel wall on day 28 after FAED procedure. A-E. Showing the immunofluorescent (IF) microscopic finding (400 ×) to identify Ki67+ cells localized in FAED wall (green color). F. Statistical analysis of number of Ki67+ cells in FAED wall, * vs. other groups with different symbols (†, ‡, §), P<0.0001. G-K. Illustrating the IF microscopic finding (400 ×) for identification of connexin (Cx)43+ cells localized in FAED wall (red color). L. Statistical analysis of number of Cx43+ cells in FAED wall. * vs. other groups with different symbols (†, ‡, §), P<0.0001. Scale bars in right lower corner represent 20 μm. SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

indices of smooth muscle proliferation, were significantly higher in group 5 than those in other groups, significantly higher in group 4 than those in groups 1 to 3, and significantly higher in group 3 than those in groups 1 and 2, but no difference was noted between groups 1 and 2 (**Figure 5**). These findings suggest that treatment with MPs, especially those derived from patients with CAS, enhanced medial layer proliferation and promoted arterial obstruction.

Gene expressions of inflammatory biomarkers in vessel wall by day 28 after FAED procedure

The mRNA expressions of matrix metalloproteinase (MMP)-9, nuclear factor (NF)-κB, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), platelet-derived growth factor (PDGF), six indicators of inflammation, were significantly higher in group 5 than those in other groups, significantly higher in group 4 than those in groups 1 to 3, and significantly higher in group 3 than those in groups 1 and 2, but there were no differences between groups 1 and 2 (**Figure 6**). These findings once again indicate that inflammation was initiated after endothelial cell damage and was further augmented in vessel wall after receiving MP treatment.

Gene expressions of cell proliferation biomarkers in vessel wall by day 28 after FAED procedure

The mRNA expressions of c-Fos, c-Myc, osteopontin, proliferating cell nuclear antigen (PCNA), four indicators of cell activation/proliferation, were significantly higher in group 5 than those in other groups, significantly higher in group 4 than those in groups 1 to 3, and significantly higher in group 3 than those in groups 1 and 2,

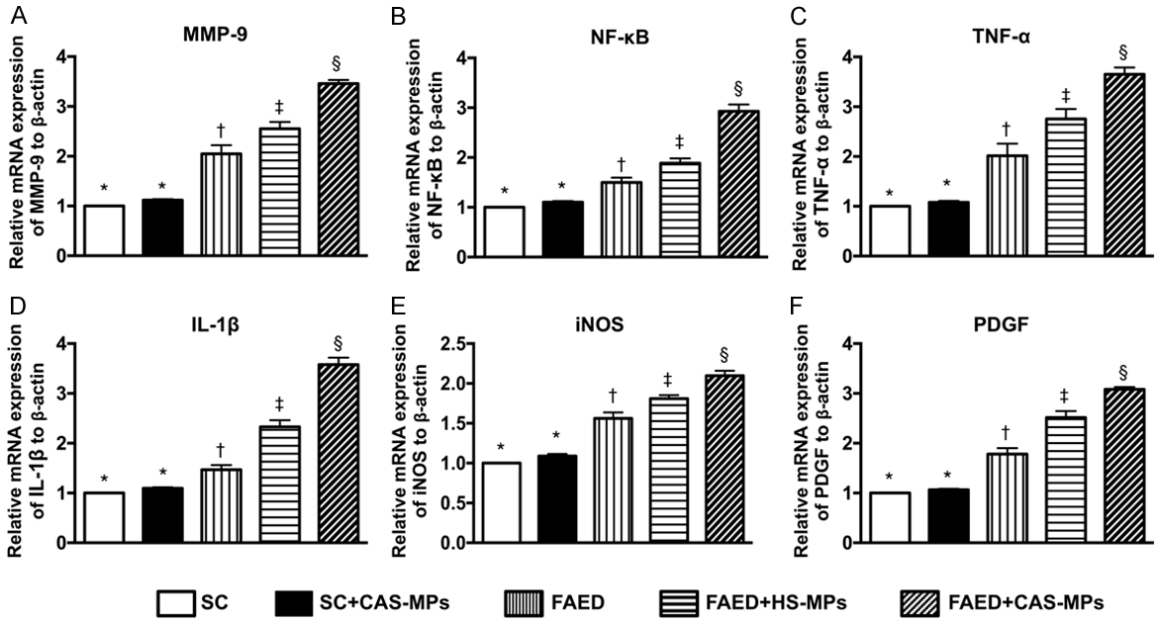

Figure 6. The gene expressions of inflammation of FA by day 28 after FAED procedure. (A) The mRNA expression of matrix metalloproteinase (MMP)-9. (B) The mRNA expression of nuclear factor (NF)-κB. (C) The mRNA expression of tumor necrosis factor (TNF)-α. (D) The mRNA expression of interleukin (IL)-1β. (E) The mRNA expression of inducible nitric oxide synthase (iNOS). (F) The mRNA expression of platelet-derived growth factor (PDGF). * vs. other groups with different symbols (†, ‡, §), P<0.001 for (A-F). SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

but no significant differences were noted between groups 1 and 2 (**Figure 7**). Additionally, the mRNA expressions of tropoelastin, gamma-smooth muscle actin (γ-SM actin), calponin, phospholamban, and matrix Gla protein (MGP), five biomarkers of calcium-binding protein regulation, showed an identical pattern compared to that of cell proliferation markers among the five groups (**Figure 8**).

Discussion

This study, which investigated the impact of human circulating MPs (i.e., both HS- and CAS-derived) on femoral arterial wall remodeling after endothelial denudation, yielded several striking implications. First, as compared with the control group, the degree of vessel wall remodeling (i.e., proliferations of neoitimal and medial layers) was remarkably higher in the FAED group. Second, as compared with animals receiving only FAED, the severity of vessel wall remodeling was markedly increased in the animals with FAED treated by HS-derived MPs and further aggravated in those receiving CAS-derived MPs. Third, compared with the control group, the expressions of inflammatory biomarkers in vessel wall were significantly higher in the FAED only group, further increased in the FAED + HS-derived MPs group, and further enhanced in the FAED + CAS-derived MPs group.

Previous studies have shown that circulating MPs play multiple pathophysiological roles, including angiogenesis [21], tumor growth and metastasis [22], thrombosis and atherogenesis [16, 17, 19, 20], promotion of inflammation [17, 23], and serving as a useful predictor of the prognostic outcome of lung cancer patients [27, 28]. However, whether circulating MPs were a direct contributor to or simply a biomarker of arterial occlusion remains uncertain. The most important finding is that the degree of femoral arterial remodeling (i.e., the proliferation of neoitimal and medial layers resulting in luminal obstruction) was notably increased in animals with FAED than that in sham-operated controls. This finding reinforces the concept that endothelial dysfunction contributes to arterial atherosclerotic obstruction that ultimately leads to the development of arterial occlusive syndrome. Another important finding in the present study is that arterial remodeling in animals with FAED was substantially enhanced after receiving of CAS-derived MPs.

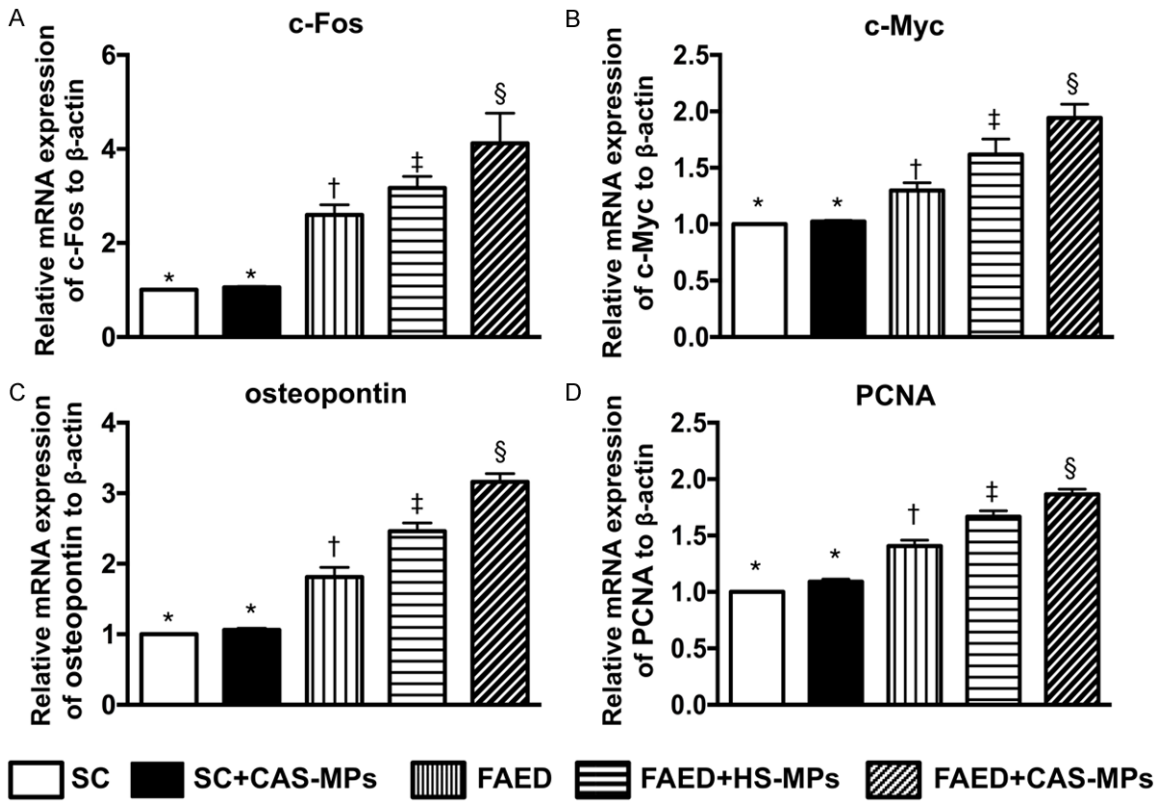

Figure 7. The gene expressions of cell activation/proliferation biomarkers at vessel wall by day 28 after FAED procedure. (A) mRNA expression of c-Fos. (B) mRNA expression of c-Myc. (C) mRNA expression of osteopontin. (D) mRNA expression of proliferating cell nuclear antigen (PCNA). * vs. other groups with different symbols (†, ‡, §), P<0.001 for (A-D). SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

Interestingly, arterial remodeling was also notably aggravated in FAED animals treated with HS-derived MPs, although the degree of enhancement was much less severe compared with that in FAED animals receiving CAS-derived MPs treatment. These findings highlight two important novel issues: (1) MPs shed from endothelial cells, platelets or monocytes/lymphocytes [15, 18, 29-31] into circulation can directly participate in arterial obstruction/vessel remodeling only on condition of endothelial damage; (2) MPs derived from patients with arterial atherosclerotic obstruction are more powerful biological mediators for enhancing arterial obstruction/vessel remodeling compared to those derived from healthy subjects.

The links among inflammation, endothelial dysfunction, and atherosclerosis have been well established in previous studies [7-10]. One important finding in the present study is that the expressions of inflammatory markers at gene and protein levels were consistently higher in normal controls than those in animals with FAED. These findings corroborated those of previous studies [7-10]. Of particular importance is that the expressions of these biomarkers in arterial wall were remarkably higher in animals with FAED treated by HS-derived MPs and further significantly increase after CAS-derived MP treatment. These findings could, at least in part, explain the highest degree of obstruction in animals with FAED receiving CAS-derived MPs compared with that in other groups.

An essential finding in the present study is that the expressions of biomarkers of cell activation and proliferation and the expression of calcium binding protein were substantially increased in the FAED only group, further substantially increased in animals with FAED treated with HS-derived MPs, and further notably elevated in FAED animals treated with CAS-derived MPs. These findings once again support that proliferation of smooth muscle cells and fibroblasts contributed to arterial obstruction after endo-

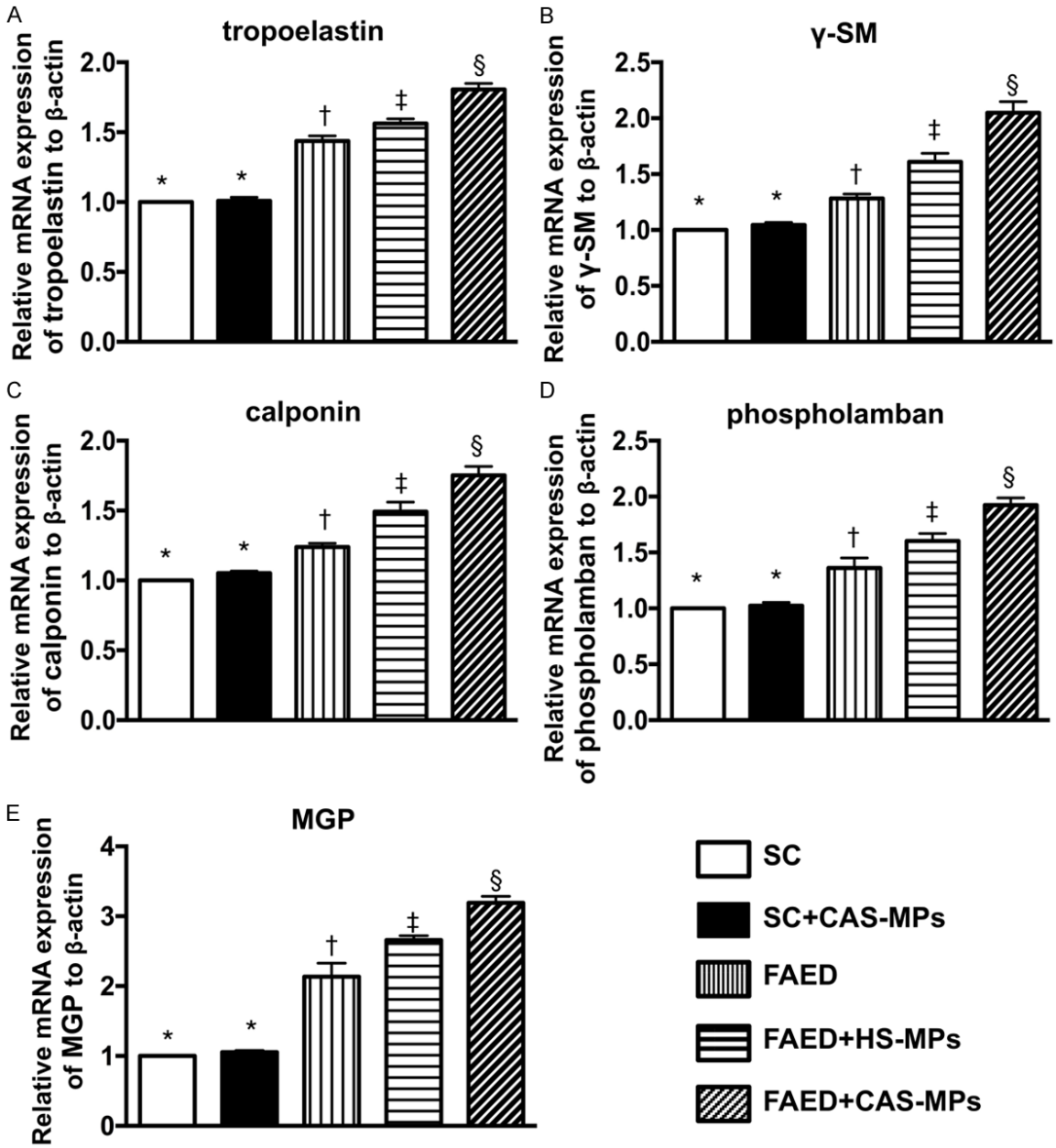

Figure 8. The gene expressions of calcium-binding protein regulators at vessel wall by day 28 after FAED procedure. (A) mRNA expressions of tropoelastin. (B) mRNA expression of gamma-smooth muscle actin (γ-SM actin). (C) mRNA expression of calponin. (D) mRNA expression of phospholamban. (E) mRNA expression of matrix Gla protein (MGP). * vs. other groups with different symbols (†, ‡, §), P<0.001 for (A-E). SC = sham control; HS = heath subject; FAED = endothelial denudation of femoral artery; CSA = carotid artery stenosis; MPs = microparticles.

thelial damage that was aggravated after HS-derived MP administration and further exacerbated after CAS-derived MP stimulation.

Study limitation

This study has limitations. First, although this experimental study was designed to mimic the clinical scenario of arterial atherosclerotic obstruction, the mechanisms of endothelial damage of the two settings are different. Second, although it is well recognized that statin possesses anti-inflammatory properties that can inhibit the propagation of arterial atherosclerotic obstruction, this study did not test whether statin therapy would offer an additional benefit in inhibiting MP-induced vessel wall remodeling.

In conclusion, the results of the present study demonstrated that circulating MPs, regardless of their sources, would participate in vessel remodeling following endothelial damage in an experimental setting.

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Disclosure of conflict of interest

None.

Authors' contribution

FY Lee and HK Yip participated in the design of the study, data acquisition and analysis as well as drafting the manuscript. HI Lu, HT Chai, JJ Sheu, SY Chung, PH Sung were responsible for the laboratory assay and troubleshooting. YL Chen, TH Huang, GS Kao, SY Chen, HW Chang participated in data acquisition, analysis, and interpretation. FY Lee, MS Lee and HK Yip conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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