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Platelet-derived microparticles augment the adhesion and neovascularization capacities of circulating angiogenic cells obtained from atherosclerotic patients

Masanori Ohtsuka [a], Ken-ichiro Sasaki [a,*], Takafumi Ueno [a], Ritsuko Seki [b], Takaharu Nakayoshi [a], Hiroshi Koiwaya [a], Yasuyuki Toyama [a], Shinji Yokoyama [a], Yoshiaki Mitsutake [a], Hidetoshi Chibana [a], Naoki Itaya [a], Takashi Okamura [b], Tsutomu Imaizumi [a]

[a] Division of Cardio-Vascular Medicine, Department of Internal Medicine, Kurume University School of Medicine, Japan
[b] Division of Hematology and Oncology, Department of Medicine, Kurume University School of Medicine, Japan

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ABSTRACT

Objective: The neovascularization-related capacities of circulating angiogenic cells (CACs) are impaired in atherosclerotic patients, which may explain the unsatisfactory effects of therapeutic angiogenesis with atherosclerotic patient-derived CACs. Platelet-derived microparticles (PMPs) were reported to augment the re-endothelialization capacity of CACs. Accordingly, we investigated whether PMPs could augment the neovascularization-related capacities of atherosclerotic patient-derived CACs *in vitro* and *in vivo* and if so, the associated mechanisms.

Methods and results: We isolated mononuclear cells and PMPs from atherosclerotic patient-derived peripheral blood and generated PMP-pretreated CACs (PMP-CACs) by co-culture of the mononuclear cells and PMPs. Although the migration capacity of PMP-CACs was similar to that of CACs, the adhesion capacity of PMP-CACs was greater. PMPs released RANTES into the culture medium, and the receptors were similarly expressed on the surfaces of CACs and PMP-CACs. Intravenous injection of PMP-CACs to rats with hindlimb ischemia augmented neovascularization of the ischemic limbs more than the injection of CACs. The number of PMP-CACs incorporated into the capillaries of the ischemic limbs was greater than that of incorporated CACs. The augmented adhesion and neovascularization capacities by PMP-CACs were canceled out by a RANTES neutralizing antibody.

Conclusions: PMP-secreted RANTES may play a role in the augmenting adhesion and neovascularization capacities of CACs. Injection of PMP-CACs may be a new strategy to augment the effects of therapeutic angiogenesis for limb ischemia in atherosclerotic patients.

1. Introduction

Cell therapy for augmenting neovascularization in ischemic tissues is a promising therapeutic option to treat patients with ischemic cardiovascular disease [1]. Although various stem/progenitor cells were effectively used in experimental models, peripheral blood-derived mononuclear cells (MNCs), bone marrow-derived MNCs, and circulating angiogenic cells (CACs) [2] have been used in clinical studies [3–5]. MNCs and CACs have been reported to contribute to neovascularization through a multistep process composed of the following neovascularization-related capacities of the cells: chemotaxis and adhesion to mature endothelial cells, migration and invasion to the intracellular space in adjacent endothelial cells, and secretion of cytokines to stimulate sprouting new capillaries from pre-existing arteries [6]. Thus, the effects of therapeutic angiogenesis with MNCs or CACs may depend on the neovascularization-related capacities of the cells. We and others have previously reported the effects and safeties of therapeutic angiogenesis with MNCs or CACs in patients with myocardial ischemia or critical limb ischemia in large-scale clinical trials [3–5]; however, the effects have been unsatisfactory. This may be due to the injection of atherosclerotic patient-derived MNCs or CACs with impaired neovascularization-related capacities [7]. Indeed, Heeschen et al. reported that the impaired migration capacity of

* Corresponding author. Department of Internal Medicine, Division of Cardio-Vascular Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. Tel.: +81 942 31 7562; fax: +81 942 33 6509.
E-mail address: sasaken@med.kurume-u.ac.jp (K.-i. Sasaki).

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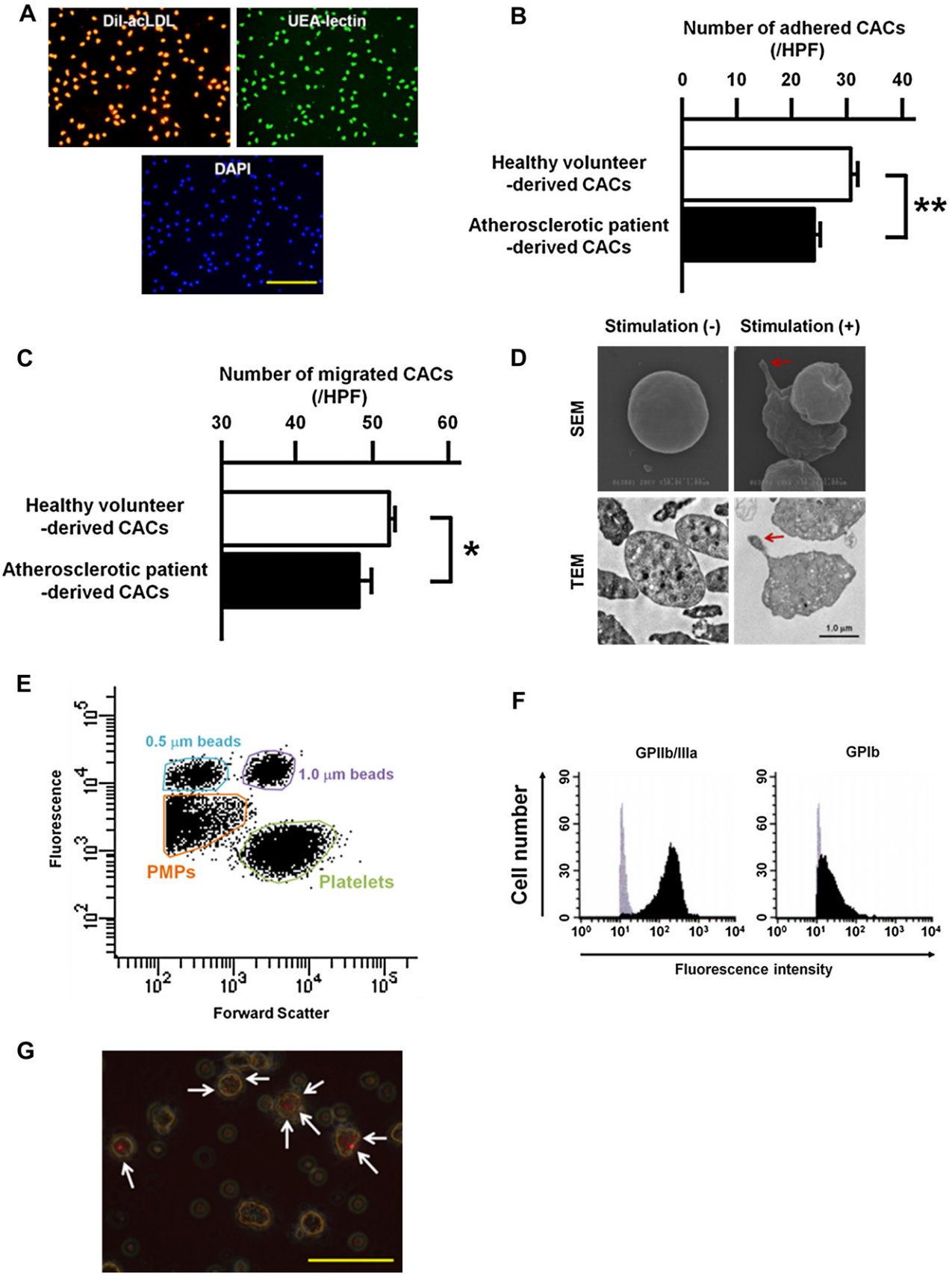

Fig. 1. (A) Representative microscopic images of CACs. CACs were stained by three fluorescent dyes Dil-acLDL (red), UEA-lectin (green), and DAPI (blue). The scale bar indicates 200 μm. (B) Bar graphs for pooled data of the number of CACs adhered to fibronectin. The number was significantly smaller for atherosclerotic patient-derived CACs than for healthy volunteer-derived CACs (**: $p < 0.005$, $n = 6$, each) (C) Bar graphs for pooled data of the number of migrated CACs for SDF-1α. The number was significantly smaller for

atherosclerotic patient-derived MNCs *in vitro* was closely correlated with impaired neovascularization capacity of the cells *in vivo* [8]. If we can augment the neovascularization-related capacities of the patient-derived MNCs or CACs before the injection, the effects of therapeutic angiogenesis with the cells may be more desirable.

Platelet-derived microparticles (PMPs) are small membrane vesicles released from activated platelets by a process of exocytic budding of the plasma membrane [9,10]. Janowska-Wieczorek et al. reported that PMPs increased the number of hematopoietic stem/progenitor cells adhered on human umbilical vein endothelial cells (HUVECs) [11]. Mause et al. reported that PMPs augmented the adhesion of CACs to endothelial cells and the migration of CACs to stromal cell-derived factor-1α (SDF-1α) and thereby accelerated re-endothelialization of denudated endothelial cells [12]. These studies reported the role of PMPs in re-endothelialization for vascular damage. However, there have been no reports about the effects of PMPs on the neovascularization-related capacities of CACs. Accordingly, we investigated whether atherosclerotic patient-derived PMPs could augment the neovascularization-related capacities of atherosclerotic patient-derived CACs *in vitro* and *in vivo* and if so, the associated mechanisms.

2. Materials and methods

Please see Supplementary Text) [9–24].

3. Results

3.1. Generation of CACs

Most cells that adhered on the culture plate incorporated DiI-acLDL and bound UEA-lectin (Fig. 1A), indicating successful generation of CACs as reported in previous studies [2,15–17].

3.2. Adhesion and migration capacities of CACs

The number of CACs adhered to fibronectin was smaller for atherosclerotic patient-derived CACs than for healthy volunteer-derived CACs (Fig. 1B). Likewise, the number of migrated CACs for SDF-1α was smaller for atherosclerotic patient-derived CACs than for healthy volunteer-derived CACs (Fig. 1C). The baseline clinical characteristics of the patients and healthy volunteers are shown in Table 1. Given the clinical use of PMP-CACs for therapeutic angiogenesis, we generated PMP-CACs with atherosclerotic patient- but not healthy volunteer-derived MNCs and PMPs. Accordingly, we depict the effects of PMPs pretreatment on atherosclerotic patient-derived CACs hereafter.

3.3. Generation of PMPs and PMP-CACs

In the analyses with transmission- and scanning electron microscopy, membrane-/micro-vesicles were observed near the terminal end of the pseudopodium of U46619-activated platelets (Fig. 1D). The collected vesicles were smaller than platelets and 1.0 μm beads (Fig. 1E) and expressed GPIIb/IIIa and GPIb antigens on the surfaces in the flow cytometric analysis (Fig. 1F) as previously reported [8–11,13,17–19]. The percentage of GPIIb/IIIa expression on isolated PMPs was consistently more than 98%, suggesting that the purity of isolated PMPs was more than 98%. Co-cultured PMPs for generating PMP-CACs were incorporated into PMP-CACs (Fig. 1G).

Table 1
Baseline clinical characteristics of healthy volunteer- and atherosclerotic patient-derived CACs in adhesion and migration assays.

	Healthy volunteers ($n = 6$)	Atherosclerotic patients ($n = 6$)	
Age (yrs)	34.7 ± 8.0	56.8 ± 5.3	$P < 0.05$
Gender			
Male	6	4	N.S
Female	0	2	N.S
Hypertension	0	5	$P < 0.01$
Dyslipidemia	0	3	N.S
Diabetes mellitus	0	2	N.S
Smoking	0	3	N.S
Medication			
RASi	0	5	$P < 0.01$
Statin	0	3	N.S

RASi = renin-angiotensin system inhibitor. Data are presented as the mean ± SEM.

3.4. Adhesion and migration capacities of PMP-CACs

We generated PMP-CACs by the culture of MNCs with 10×10^2, 10×10^3, or 10×10^4 PMPs. The number of CACs adhered to HUVECs was greater for PMP-CACs than for CACs (Fig. 2A); the adhesion capacity of PMP-CACs was augmented dose-dependently by the co-culture of PMPs (Fig. 2B and C). However, the number of CACs migrated for SDF-1α was not different between CACs and PMP-CACs generated by the co-culture of MNCs and 10×10^4 PMPs (Fig. 2D).

3.5. Phenotype of PMP-CACs

In the flow cytometric analysis, the expressions of PMP markers GPIIb/IIIa and GPIb, hematopoietic stem cell markers CD133 and CD34, monocyte marker CD14, endothelial cell markers CD31, VE-cadherin and KDR, and SDF-1 receptor CXCR-4 were similar on the surfaces of CACs and PMP-CACs (Fig. 2E). These results indicated that: (1) PMPs did not attach on CACs, and (2) PMPs did not alter the phenotype of CACs.

3.6. Concentrations of PMPs-secreted cytokines

After 24 h incubation of 10×10^4 PMPs per culture well, the incubated-PMPs released 13.6 ± 5.8 pg/ml RANTES (mean ± SEM, $n = 4$). Other cytokines such as IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, b-FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, TNF-α, and VEGF were not measured in this study.

3.7. Adhesion capacity of PMP-CACs pretreated with RANTES neutralizing antibody and with RANTES receptor antagonists

Although CACs expressed RANTES receptors, CCR1/3 and CCR5 (Fig. 3A), the receptor expressions were not different between CACs and PMP-CACs (Fig. 3A). Interestingly, the adhesion capacity of PMP-CACs was dose-dependently attenuated by the application of

atherosclerotic patient-derived CACs than for healthy volunteer-derived CACs (*: $p < 0.05$, $n = 6$, each). (D) Representative electron microscopic images of platelets and platelet-derived microparticles. SEM and TEM indicate scanning electron microscope and transmission electron microscope, respectively. It appeared that membrane-/micro-particles (red arrows) were released from the terminal ends of pseudopodia of U46619-activated platelets. The scale bar indicates 1.0 μm. (E) Characterization of isolated PMPs by flow cytometric analysis. The population of PMPs (orange region) showed smaller cell size than that of platelets (green region) and that of 1.0 μm beads (purple region). The blue region indicates the population of 0.5 μm beads. (F) Characterization of isolated PMPs by flow cytometric analysis. PMPs expressed GPIIb/IIIa and GPIb on the surfaces (black histograms). The gray histograms indicate isotype-matched IgG. (G) Representative microscopic images of PMP-CACs with or without PMPs incorporation. Red fluorescent dye DiI-labeled PMPs were incorporated into the cytoplasm of PMP-CACs. The scale bar indicates 50 μm.

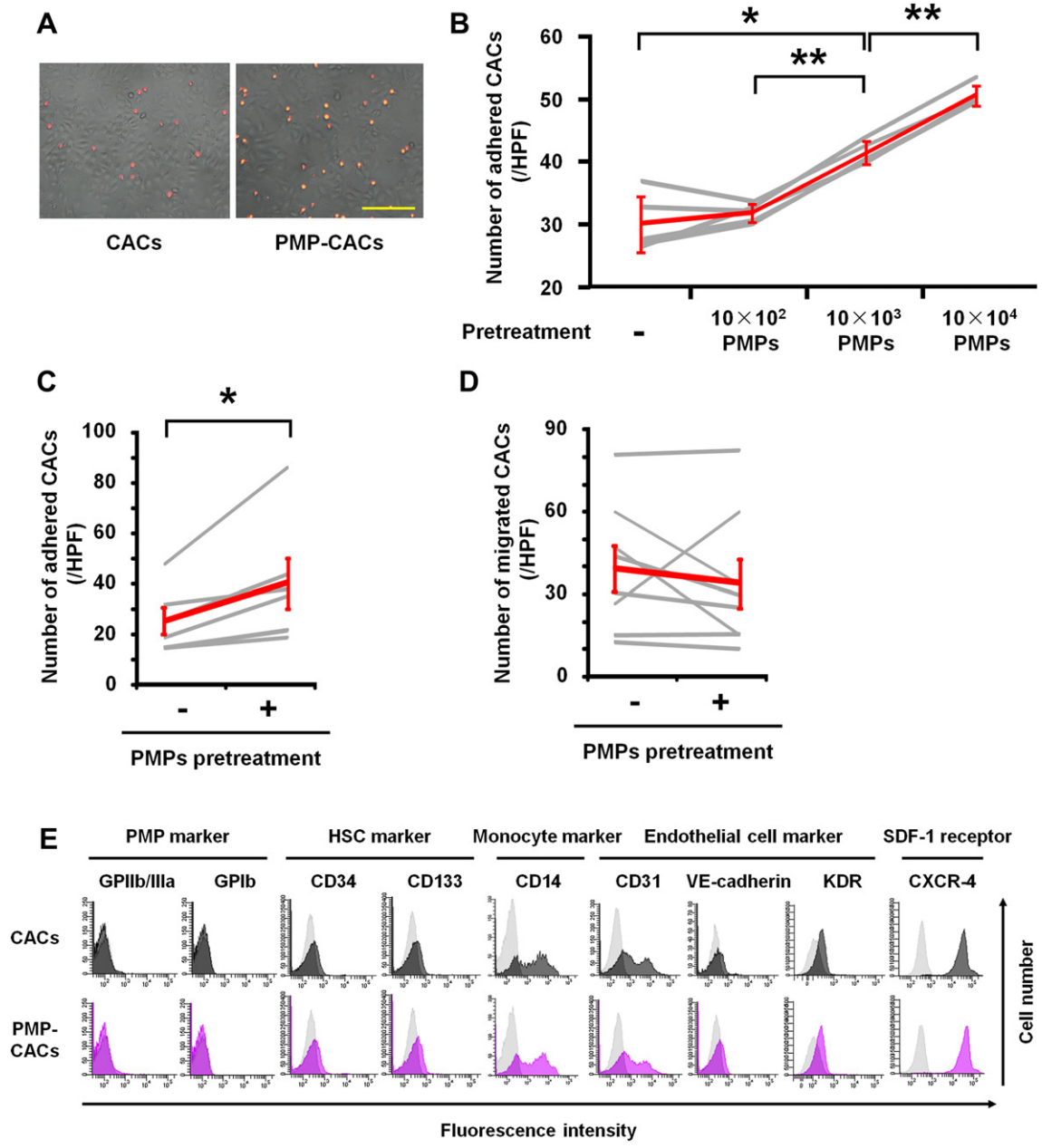

Fig. 2. (A) Representative fluorescence microscope photos of Dil-labeled CACs adhered to HUVEC-coated culture plates. The number of Dil-labeled PMP-CACs was greater than that of Dil-labeled CACs. The scale bar indicates 100 μm. (B) Adhesion capacity of PMP-CACs generated by the co-culture of MNCs and PMPs in several mixture ratios. The number of PMP-CACs adhered to HUVEC increased proportionally with the number of co-cultured PMPs (*: $p < 0.01$, **: $p < 0.001$, $n = 5$, each). Bold red line indicates the average. (C) The number of PMP-CACs adhered to HUVECs was significantly greater than that for CACs (*: $p < 0.05$, $n = 6$, each). Bold red line indicates the average. (D) The number of migrated CACs for SDF-1α was similar to that for PMP-CACs ($n = 8$, each). Bold red lines indicate the average. (E) In the flow cytometric analysis, there were no differences in the surface antigens shown between CACs (black histograms) and PMP-CACs (pink histograms). The gray histograms indicate isotype-matched IgG. HSC indicates hematopoietic stem cell. Similar results were shown in five experiments.

RANTES neutralizing antibody (RANTES NA) to the co-culture medium (Fig. 3B). The adhesion capacity of PMP-CACs did not change in the application of the negative control iso-IgG antibody (Fig. 3B). In addition, the antagonist of CCR5 but not CCR1/CCR3 suppressed the RANTES-mediated effect for augmenting the adhesion capacity of PMP-CACs (Fig. 3C).

3.8. Neovascularization capacity of PMP-CACs in vivo

At 14 days after intravenous injection of CACs to the rats with hindlimb ischemia, the blood flow and capillary density of the ischemic limbs were greater than in those receiving injection of PBS (Fig. 4A–D). The injection of PMP-CACs further increased the blood flow and capillary density (Fig. 4A–D). Dil-positive cells corresponded to CD31-positive capillaries of the ischemic limbs (Fig. 4B), indicating the incorporation of Dil-labeled CACs into the capillaries. The number of Dil-positive capillaries of the ischemic limb was greater for the injection of PMP-CACs than for the injection of CACs (Fig. 4B, E). The augmented *in vivo* neovascularization after the injection of PMP-CACs was reversed to the level after the injection of CACs pretreated by the application of RANTES NA to the co-culture medium (Fig. 4A–E).

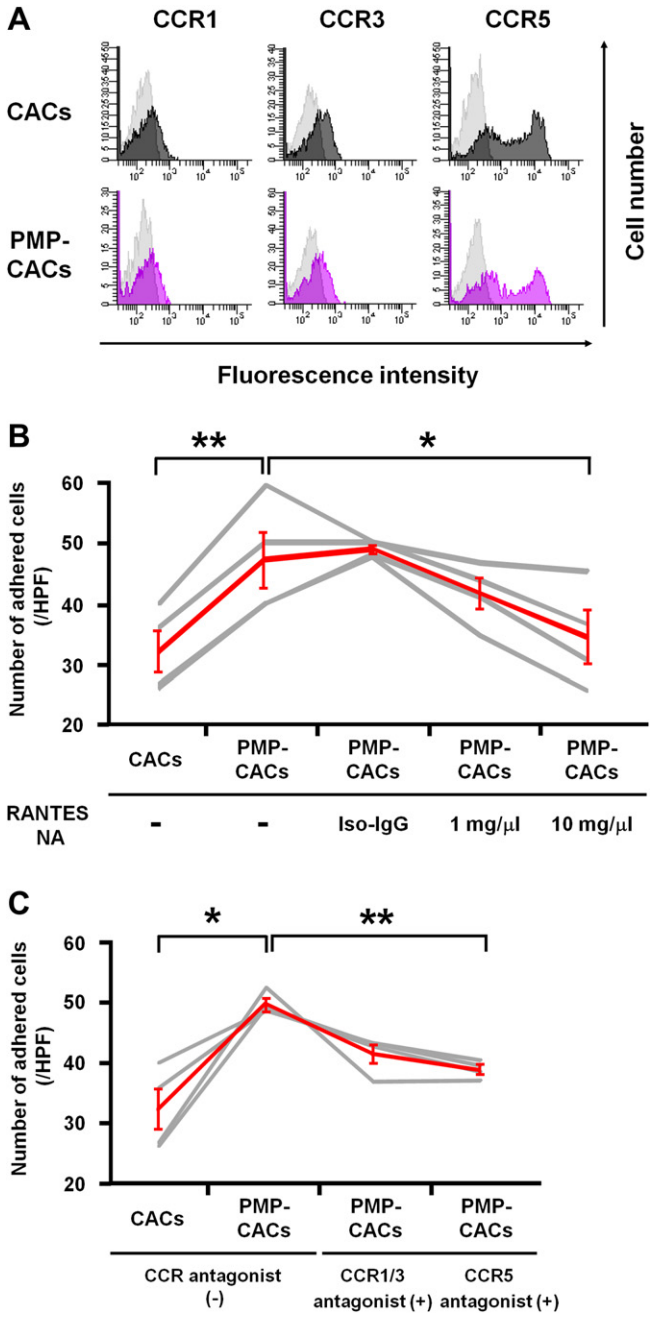

Fig. 3. (A) In the flow cytometric analysis, RANTES receptors CCR1, CCR3, and CCR5 were expressed on the surfaces of CACs (black histograms). The gray histograms indicate isotype-matched IgG. The receptor expressions were similar between CACs and PMP-CACs (pink histograms). Similar results were seen in four experiments. (B) Adhesion capacity of CACs and PMP-CACs with or without RANTES neutralizing antibody (RANTES NA) pretreatment. Iso-IgG indicates isotype-matched IgG for negative control. There were no differences in the adhesion capacities shown between PMP-CACs and PMP-CACs with Iso-IgG pretreatment ($n = 4$). The number of PMP-CACs adhered to HUVECs was greater than that of adhered CACs to HUVECs (**: $p < 0.005$, $n = 5$). The pretreatment with RANTES NA dose-dependently decreased the numbers of PMP-CACs adhered to HUVECs (*: $p < 0.05$; no pretreatment vs. 10 mg/μl pretreatment, $n = 5$). There were no differences in the adhesion capacities shown between CACs and PMP-CACs with 10 mg/μL RANTES-NA pretreatment ($n = 5$), suggesting that the augmented adhesion capacity of PMP-CACs was canceled out by the RANTES-NA pretreatment. (C) Adhesion capacity of CACs and PMP-CACs with or without the antagonist of CCR1/3 and CCR5. The number of PMP-CACs adhered to HUVECs was greater than that of adhered CACs to HUVECs (*: $p < 0.05$, $n = 4$, each). The CCR5 but not CCR1/3 antagonist pretreatment for PMP-CACs decreased the number of PMP-CACs adhered to HUVECs (**: $p < 0.01$, $n = 4$, each).

4. Discussion

Many previous reports of animal and human studies have suggested that atherosclerotic risk factors impair the migration and neovascularization capacities of CACs/MNCs and decrease the effects of therapeutic angiogenesis by the injection of atherosclerotic patient-derived CACs/MNCs [7,8,17,25]. In the present study, the *in vitro* adhesion and migration capacities of atherosclerotic patient-derived CACs were inferior to those of healthy volunteer derived-CACs. We recently demonstrated that mechanical [17] and pharmacological [25] pretreatments of atherosclerotic patient-derived CACs/MNCs augmented the migration and neovascularization capacities of CACs/MNCs *in vitro* and *in vivo*, respectively. This may suggest that pretreatment of atherosclerotic patient-derived CACs/MNCs can offer a new strategy to augment the effects of therapeutic angiogenesis by the injection of atherosclerotic patient-derived CACs/MNCs. In the present study, we generated PMP-CACs by the co-culture of patient-derived MNCs and autologous PMPs and investigated whether the pretreatment of atherosclerotic patient-derived CACs with PMPs could augment the *in vitro* adhesion, migration capacities, and the *in vivo* neovascularization capacities in rats with hind-limb ischemia. As shown in Fig. 1D–F, the size and phenotype of our PMPs were similar to those of PMPs shown in previous reports [9–12,14,18–21], indicating that we obtained appropriate PMPs for the co-culture. We isolated MNCs and PMPs from 50 ml peripheral blood; the maximum number of stably-provided PMPs was 10×10^4 per co-culture. Consequently, several mixture ratios such as 10×10^6 MNCs with 10×10^2, 10×10^3, or 10×10^4 PMPs per culture were actually tested for the co-culture; the co-culture of 10×10^6 MNCs with 10×10^4 PMPs per culture yielded the highest adhesion capacity of CACs. Although no mixture ratio altered the migration capacity of CACs, a smaller number of PMPs than MNCs for the co-culture might result in a lack of PMP-mediated augmentation of the migration capacity of CACs. Accordingly, we adopted this ratio of MNCs to PMPs for the subsequent experiments.

In order to examine the mechanisms by which PMP augmented the adhesion but not migration capacity of CACs, we examined the surface antigens of PMP-CACs and measured the cytokines released from PMPs. Baj-Krzyworzeka et al. reported that PMPs transferred the surface antigen GPIIb/IIIa onto hematopoietic cells and thereby augmented the adhesion of hematopoietic cells to fibrinogen [14]. PMP-CACs did not express PMPs surface antigens GPIIb/IIIa and GPIb, indicating that PMPs did not attach on CACs or transfer GPIIb/IIIa and GPIb antigens onto CACs. Barry et al. reported that PMPs increased the expressions of CD11a and CD11b on monocytes and thereby modulated the adhesion of monocytes to HUVECs [18]. Although we examined the changes in expressions of integrins such as CD11a, CD11b, CD18, and CD49d/CD29, which are receptors to mediate cell–cell and cell–matrix interaction, on the surfaces of CACs and PMP-CACs, the expressions did not change between CACs and PMP-CACs (data not shown). Thus, the augmented adhesion capacity of PMP-CACs was not brought about by these mechanisms. CXCR-4, which is the chemokine receptor of SDF-1, is expressed on CACs and involved in migration of CACs [26]. PMP-CACs had the same expression of CXCR-4 as CACs, which may explain the unchanged migration capacity of CACs by PMPs.

PMPs released RANTES. In addition, CACs expressed RANTES receptors CCR1, CCR3, and CCR5 on the surface. RANTES is a CC-chemokine contributing to the recruitment of leukocytes to endothelial cells [27]. von Hundelshausen et al. reported that RANTES promoted monocytes arrest on endothelial cells [28]. Mause et al. reported that PMP-released RANTES recruited monocytes to endothelial cells [29]. Although several reports described the presence of RANTES receptor on various cells, this is the first report describing the presence of RANTES receptors on CACs. Interestingly,

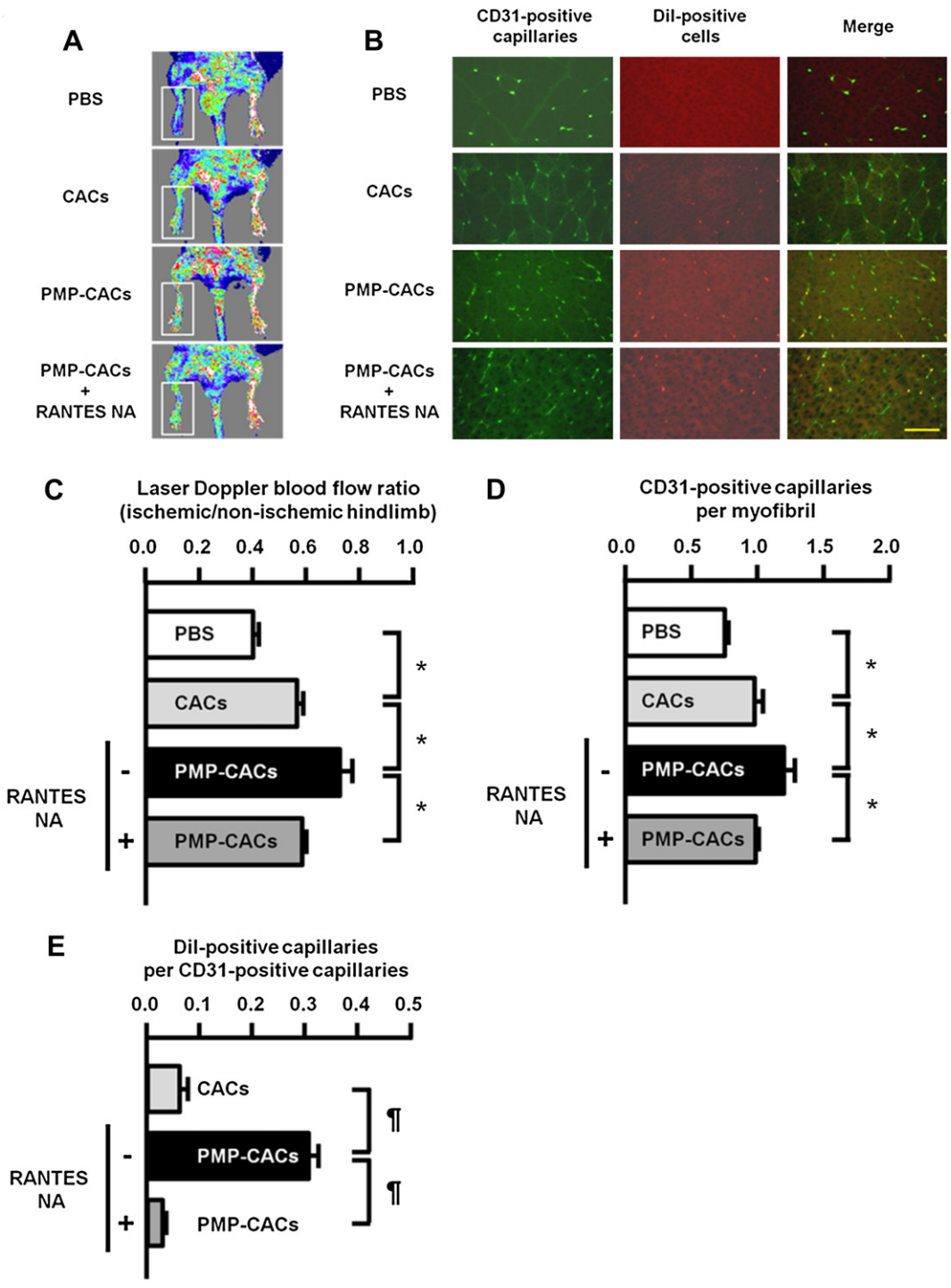

Fig. 4. (A) Representative laser Doppler blood flow images of athymic nude rats with ischemic hindlimb at 14 days after intravenous injection of PBS, CACs, PMP-CACs, or RANTES NA-pretreated PMP-CACs. CACs were labeled with a fluorescent red reagent, Dil. The hindlimbs enclosed by white squares indicate right ischemic limbs. Red to white color and dark blue on the images indicate high and low perfusion signals, respectively. (B) Representative fluorescence microscope photos of the ischemic limbs. Green and red cells indicate CD31-positive capillaries and Dil-positive cells, respectively. It was considered that Dil-labeled CACs were incorporated into capillaries. The scale bar indicates 100 μm. (C) Bar graphs for pooled data of the ischemic/non-ischemic hindlimb blood flow ratio. (D) Bar graphs for pooled data of CD31-positive capillary density of the ischemic limbs. (E) Bar graphs for pooled data of Dil-positive capillary density (i.e. eEPC-incorporated capillary density) of the ischemic limbs. The injection of CACs significantly increased the perfusion signal, the blood flow ratio, and the CD31-positive capillary density compared with the injection of PBS (*: $p < 0.05$; $n = 9$ and $n = 12$, respectively). The injection of PMP-CACs significantly augmented the neovascularization effects of the injection of CACs (*: $p < 0.05$; $n = 9$, each) and significantly increased the eEPC-incorporated capillary density compared with the injection of CACs (¶: $p < 0.0001$; $n = 9$, each). However, RANTES NA pretreatment for PMP-CACs reversed the neovascularization effects of the injection of PMP-CACs (*: $p < 0.05$, ¶: $p < 0.0001$; $n = 9$, each).

the augmented adhesion capacity of PMP-CACs was dose-dependently inhibited by the application of RANTES NA to the co-culture medium. This suggested that PMP-released RANTES played an important role in augmenting the adhesion capacity of CACs *in vitro*. However, the augmented adhesion capacity of PMP-CACs was not brought about by upregulation of the RANTES receptors on CACs because expressions of the receptor were similar between CACs and PMP-CACs. The CCR5 antagonist pretreatment for PMP-CACs diminished the augmented adhesion capacity of PMP-CACs (Fig. 3C), suggesting that RANTES-CCR5 signaling from outside of CACs plays a role in augmenting the adhesion capacity of CACs. On the other hand, co-cultured PMPs were incorporated into PMP-CACs (Fig. 1G), suggesting that PMP-released RANTES stimulation from inside of CACs plays a role in augmenting the adhesion capacity of CACs. However, we were not able to clarify which mechanism was essential for the augmentation.

In order to further investigate whether PMP-CACs had greater neovascularization capacity than CACs *in vivo* and to investigate the contribution of RANTES, we conducted experiments in rats with hindlimb ischemia. As we reported previously [16], intravenous injection of CACs increased the blood flow and capillary density of rat ischemic limbs compared with the injection of PBS. The neovascularization by the injection of CACs was further augmented by the injection of PMP-CACs. In addition, the number of CACs incorporated into capillaries of the ischemic limbs was greater for the injection of PMP-CACs than for the injection of CACs. The increased incorporation of PMP-CACs into capillaries might be due to the augmented adhesion capacity of PMP-CACs to endothelial cells, because the increased incorporation of PMP-CACs and the augmented adhesion capacity of PMP-CACs were canceled out by the addition of RANTES NA to the co-culture medium. Thus, it is suggested that PMP-released RANTES may have played an essential role in the greater neovascularization capacity of PMP-CACs in the ischemic limbs by the augmented adhesion capacity of PMP-CACs to endothelial cells. Nevertheless, we must consider mechanisms other than RANTES for the augmented adhesion and neovascularization capacities brought about by PMP-CACs because it was reported that intra-PMP angiogenic cytokines such as VEGF, b-FGF, and PDGF augmented angiogenesis *in vivo* [30]. The greater neovascularization capacity by PMP-CACs was not likely to be brought about by PMPs themselves because PMPs did not attach on the surface of PMP-CACs *in vitro* and because the *in vivo* injected PMP-CACs were not contaminated with PMPs. Moreover, VEGF, b-FGF, PDGF, and other cytokines were not released from 10×10^4 PMPs.

This study had some limitations. First, CACs were generated from peripheral blood-derived MNCs but not bone marrow-derived MNCs. If PMP-CACs were generated from bone marrow-derived MNCs, different results might have been achieved. Second, the precise mechanisms by which PMP-released RANTES augmented the adhesion capacity of CACs have remained unclear.

In conclusion, therapeutic angiogenesis by the injection of PMP-CACs potentially offers a new strategy for treatment of patients with critical limb ischemia. PMP-CACs are generated by the co-culture of autologous MNCs, PMPs, and serum, suggesting no possibility of graft versus host disease after the injection.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.01.040.

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