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Letter to the Editors-in-Chief

Podoplanin expression on endothelial cells promotes superficial erosive injury and thrombus formation in rat carotid artery: Implications for plaque erosion

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Atherosclerotic plaque erosion, a morphological pattern of plaque disruption, is characterized by a denuded plaque surface and thrombus formation, and defined by the lack of disruption of the fibrous cap and exposure of necrotic core. The morphologic characteristics include an abundance of smooth muscle cells (SMCs) and proteoglycan matrix and disruption of surface endothelium. Plaque erosion contains relatively few inflammatory cells compared with plaque rupture [1]. Although many mechanisms including disturbed blood flow, toll-like receptor signaling, leukocyte activation and modification of sub-endothelial matrix have been proposed, the precise mechanisms of plaque erosion and subsequent thrombus growth still remain unclear [2,3].

Podoplanin is an endogenous ligand for C-type lectin-like receptor (CLEC)-2, which is novel platelet activation receptor [4]. In the physiological condition, podoplanin is expressed on lymphatic but not on arterial and venous endothelial cells in the vascular system. In the past report, we presented that podoplanin was expressed significantly in atherosclerotic plaques suggesting that this protein contributes to the thrombotic property of atherosclerotic lesions [5]. However, the mechanisms and rules of podoplanin expression in atherosclerotic lesions remain unclear. In this study, we examined whether overexpression of podoplanin in arterial wall influences superficial arterial injury and thrombus formation in vivo and investigated factors which influence podoplanin expression in cultured arterial endothelial cells in vitro.

We evaluated thrombosis formation using a rat model of vascular occlusion induced superficial arterial injury in carotid artery [6]. Recombinant adenoviruses expressing human podoplanin (Ad-Podoplanin) or β-galactosidase (Ad-LacZ) were constructed according to previously described and infected to rat carotid arteries after distal ligation [7]. Immunofluorescent micrographs of the vascular wall one day after gene transfer of human podoplanin showed that arterial endothelial cells were positive for human podoplanin (Fig. 1A). Four days after the gene transfer, small mural thrombi developed in all arteries infected with Ad-LacZ (Fig. 1B) ($n = 6$), whereas occlusive thrombus formed in those infected with Ad-Podoplanin (Fig. 1C) ($n = 6$). Ratios of thrombus area to luminal area in the rat carotid arteries infected with Ad-Podoplanin were larger than in those infected with Ad-LacZ (86.0 ± 12.9% and 14.4 ± 3.8%, respectively, n = 6 each, $p < 0.05$). Immunohistochemical staining revealed detachment and involvement of the human podoplanin expressing cells in the occlusive thrombus (Fig. 1D). The podoplanin expression colocalized with CD34, a marker for vascular endothelial cells (Fig. 1E), but not with smooth muscle actin, a SMC marker (data not shown). The findings suggest that overexpression of podoplanin in endothelial cells induces endothelial detachment and further thrombus growth. The histological features of endothelial detachment and occlusive thrombus formation are similar to the plaque erosion in human [1]. Although the mechanisms of endothelial detachment are unclear, endothelial binding of flowing platelets could physically denudate the endothelial cells. We previously reported that podoplanin was localized to smooth muscle cells and macrophages in atherosclerotic lesions and podoplanin protein was increased as arteriosclerosis progresses [5]. In addition, vascular SMCs in advanced atherosclerotic lesions expressed S100A13, which activated platelets through platelet CLEC-2 and S100A13 interaction [8]. The evidences suggest that subsequent erosive injury activate platelet CLEC-2 receptor via podoplanin-dependent and independent manners.

We investigated physiological stimuli on the podoplanin expression in endothelial cells according to the previous report [9]. We examined the effects of interleukin (IL)-3, -6, transforming growth factor (TGF)-β, and vascular endothelial growth factor (VEGF)-A on podoplanin expression in human aortic endothelial cells (HAOECs, Cell Application Inc., San Diego, CA, USA). After changing to the serum-free medium overnight, HAOECs were incubated with these cytokines up to 24 h. The administration of VEGF-A (VEGF165, 500 ng/mL) but not IL-3, -6, or TGF-β, induced podoplanin mRNA and protein expression (Fig. 1F). Podoplanin expression was detected by immunofluorescent staining 24 h after VEGF stimulation. (Fig. 1G). VEGF-A is a potent growth factor/vascular permeability factor that induces neovascularization in neoplastic lesions, ischemia, and hypoxia. VEGF-A is expressed in SMCs, macrophages and endothelial cells in the advanced atherosclerotic lesions of human coronary artery and also in superficial SMCs beneath occlusive thrombus [10]. Therefore, it is possible that VEGF-A derive from superficial SMC affects endothelial podoplanin expression and thrombus formation in advanced atherosclerotic lesions.

To investigate the contribution of VEGF-A stimulated endothelial cells to platelet aggregation, we assessed cell-mediated platelet aggregation with an aggregometer (PA-20, Cowa, Nagoya, Japan). Rat

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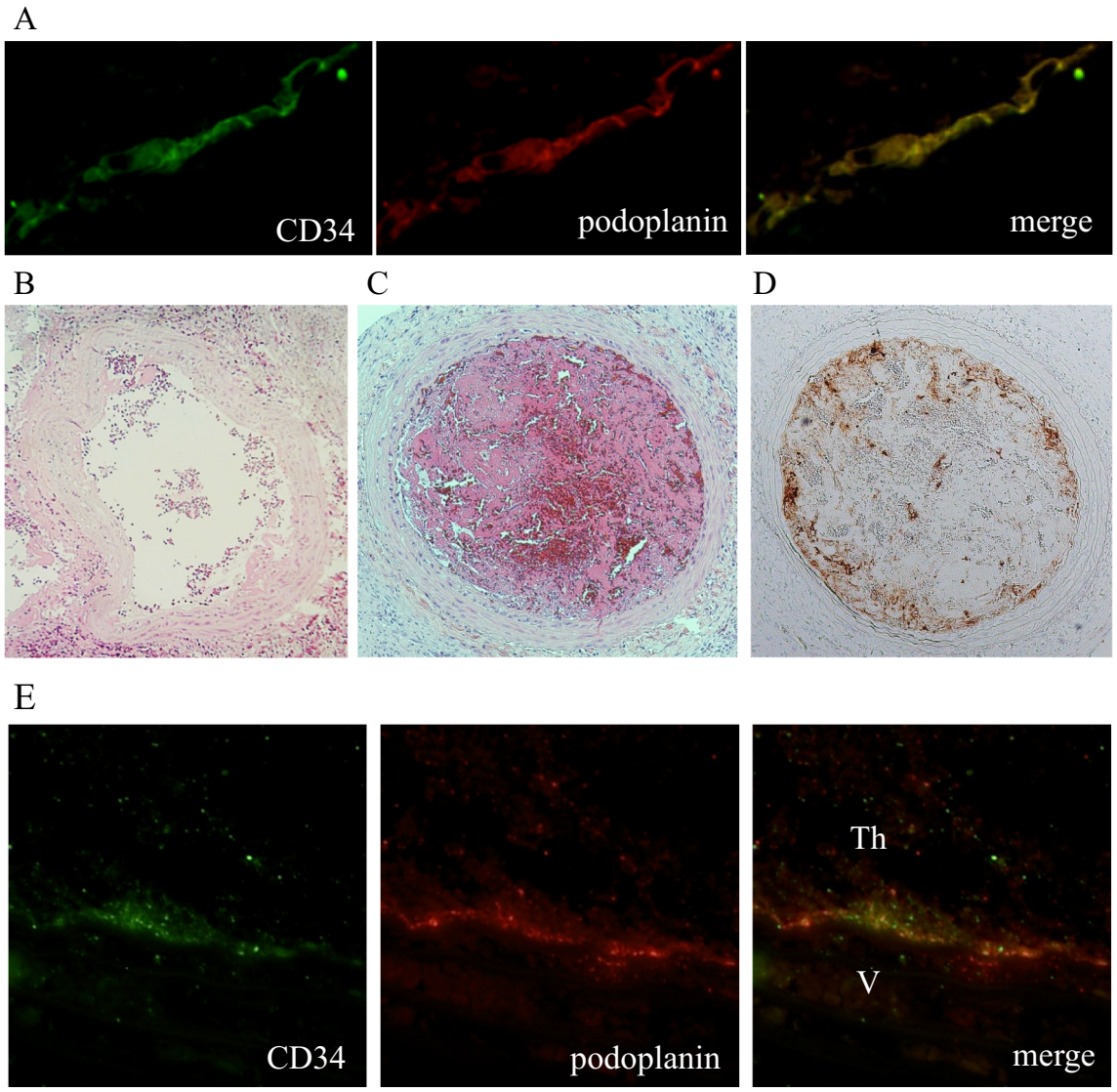

Fig. 1. Podoplanin gene-transfer and thrombus formation in rat carotid arteries (A–E), podoplanin expression on cultured endothelial cells (F, G), and platelet aggregation in presence of VEGF-A gene-transfected endothelial cells (H).

A. Immunofluorescent images for CD34 (a endothelial marker, green), podoplanin (red), and merged image (yellow) of the rat carotid artery one day after gene transfer of human podoplanin.

B, C. Microphotographs four days after vascular ligation and gene transfer, the carotid artery infected with control Ad-LacZ (B) or Ad-podoplanin (C) shows mural thrombus formation (B) or occlusive thrombus formation (C).

D. The human podoplanin expressing cells are involved in the occlusive thrombus.

E. Immunofluorescent images for CD34 (green), podoplanin (red), and merged image (yellow) in rat carotid artery thrombus 4 days after gene transfer of human podoplanin. Th: thrombus, V: vascular wall.

F. The effect of VEGF-A on podoplanin expression in cultured HAOECs. HAOECs were stimulated with VEGF-165 at indicated times and the podoplanin mRNA and protein were measured by real-time PCR and ELISA. $*p < 0.01$ vs. control (n-4, in each, One-way ANOVA).

G. Podoplanin expression detected by fluorescent staining 24 h after VEGF stimulation.

H. ADP induced rat platelet aggregation in presence of VEGF-A or LacZ gene-transferred HAOECs. Rat platelet-rich plasma and HAOECs were co-incubated for 2 min and were stimulated with ADP. VEGF-A but not LacZ overexpression on HAOECs enhances ADP-induced platelet aggregation. Anti-human podoplanin antibody (LpMab12) inhibits the enhanced platelet aggregation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

platelet-rich plasma was co-incubated with VEGF165 gene transfected HAOECs for 2 min, then was stimulated with ADP. ADP-induced platelet aggregation was enhanced in the presence of VEGF165 gene transfected HAOECs, and the aggregation was inhibited with anti-human podoplanin antibody (LpMab12) (Fig. 1H). While, LacZ transfected HAOECs did not affect the platelet aggregation. The results support the notion that VEGF-A stimulated endothelial cells can induce platelet aggregation via podoplanin-CLEL-2 interaction.

It is reported that VEGF-A is induced by hypoxia or inflammation [11]. In atherosclerotic lesions, smooth muscle cells and macrophages can express VEGF-A. The advanced atherosclerotic lesions, especially large plaques, are hypoxic, because oxygen supply from the blood vessel lumen and vasa vasorum is insufficient. The hypoxic cells are thought to express VEGF-A and other various factors within the atheroma. In addition to hypoxia, inflammatory cytokines can enhance the expression of VEGF-A. Because eroded plaque is characterized by rich in SMCs with or without inflammation but not large lipid rich plaques, inflammation rather than hypoxia could contribute to VEGF-A

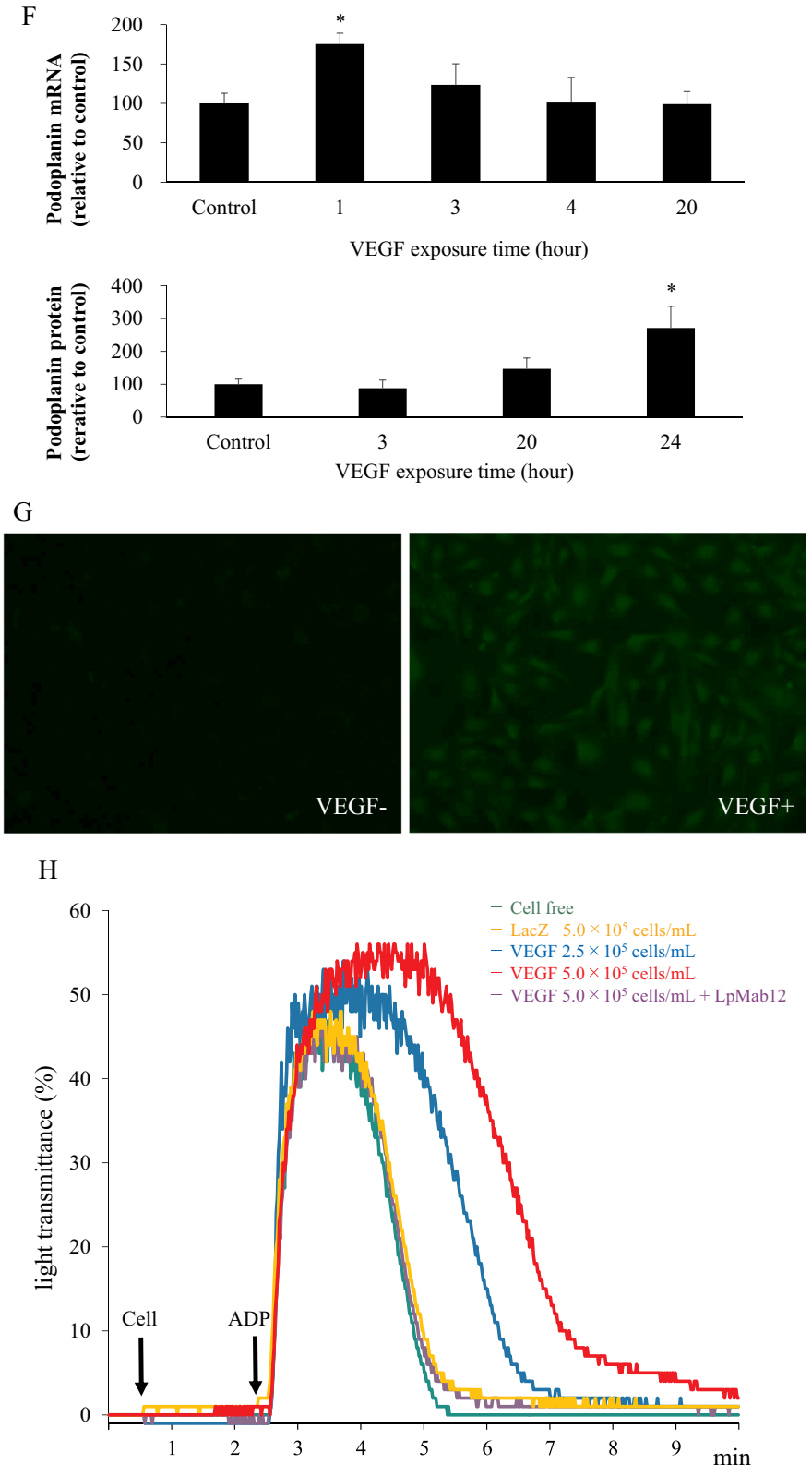

Fig. 1. (*continued*)

expression in plaque erosion.

A limitation of the study was the concentration of VEGF used in podoplanin is high (500 ng/mL) relative to the physiological conditions. Because the podoplanin expression varied at the doses of 100 and 200 ng/mL, we used 500 ng/mL of VEGF in this study.

In conclusion, this study demonstrated that overexpression of podoplanin in endothelial cells enhanced erosive injury and thrombus formation in rat carotid arteries and that VEGF-A mediated podoplanin expression in HAOECs enhance platelet aggregation via podoplanin-CLEL-2 interaction. Our results indicate that podoplanin and VEGF-A play a key role to thrombotic property of erosive arterial lesion. Although the further recognition of the mechanism of these proteins in

atherothrombosis is needed, it may provide novel insight into the mechanisms of plaque erosion.

Declaration of competing interest

The authors declare no conflict of interest.

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