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# Podoplanin expression in advanced atherosclerotic lesions of human aortas

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ABSTRACT

Thrombus formation on disrupted atherosclerotic lesion is a key mechanism of cardiovascular events. Podoplanin (Aggrus), expressed on the surface of several tumor cells, is an endogenous ligand for C-type lectin-like receptor 2 (CLEC-2), and is involved in tumor cell-induced platelet aggregation and its malignant potency. Podoplanin, which is also expressed in lymphatic endothelial cells, facilitates blood/lymphatic vessel separation. However, podoplanin expression in atherosclerotic lesion has not been investigated. To clarify podoplanin expression in atherosclerotic lesion and to assess its importance for the onset of cardiovascular events, we examined podoplanin expression in abdominal aortas obtained from 31 autopsy cases. Immunohistochemical analysis indicated that podoplanin was localized to smooth muscle cells and macrophages. Moreover, podoplanin immunoreactivity was increased in advanced atherosclerotic lesions containing necrotic core, many macrophages and smooth muscle cells, compared with early lesions composed of smooth muscle cells and small numbers of macrophages. Furthermore, Western-blot and real time-PCR analyses showed that podoplanin expression was significantly enhanced in advanced atherosclerotic lesions, compared with early lesions. These results suggest that podoplanin contributes to thrombotic property of advanced stages of atherosclerosis and that it might be a novel molecular target for an anti-thrombus drug.



## Introduction

Thrombus formation on disrupted atherosclerotic plaques leads to the onset of cardiovascular diseases such as acute myocardial infarction and arteriosclerosis obliterans [1]. It also contributes to atherosclerosis progression. The thrombogenicity of atherosclerotic lesions is mostly dependent on plaque components. Because the process of arterial thrombus formation is initiated by platelet adhesion and aggregation, the platelet activation in plaques is critical to the onset of clinical events [2].

Podoplanin (Aggrus), which is expressed on the surface of several tumor cells, is an endogenous ligand for C-type lectin-like receptor 2 (CLEC-2), and is involved in tumor cell-induced platelet aggregation [3,4]. Although podoplanin is reportedly expressed in normal tissues such as lymphatic endothelial cells, podocytes, and type I alveolar epithelia [5], podoplanin is also known to be overexpressed in various tumors such as squamous cell carcinomas, testicular seminomas, malignant brain tumors, osteosarcomas, fibrosarcomas, and malignant mesotheliomas [6–13]. Furthermore, previous reports show that podoplanin is associated with cell migration [14], epithelial–mesenchymal transition [15], and tumor metastasis [16,17]. Moreover, increased expression of podoplanin relates to tumor malignancy and poor clinical outcome [11,18–20]. To establish a therapy targeted to podoplanin, we generated a rat anti-human podoplanin monoclonal antibody (mAb), NZ-1 [8], which suppressed podoplanin-induced pulmonary metastasis through inhibition of tumor-induced platelet aggregation [17,21]. Furthermore, we showed that NZ-1 has not only high specificity and sensitivity but also high binding affinity against podoplanin, making it a candidate for radioimmunotherapy or immunotoxin therapy [22]. However, podoplanin expression in atherosclerotic lesions has not been investigated. CLEC-2 is expressed on platelets; therefore, expression of podoplanin in atherosclerotic lesions may be involved in the activation of platelets leading to thrombosis.

In this study, we investigated whether podoplanin is expressed in atherosclerotic lesions and whether it is critical to the onset of

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cardiovascular events. To this end, we examined podoplanin expression in abdominal aortas using immunohistochemistry, Western-blot, and real time-PCR analyses.

## Materials and methods

### *Specimens*

We examined the abdominal aortas of 31 patients (24 male, seven female; 18–83 years of age, mean 66 years) autopsied at University of Miyazaki Hospital and Miyazaki Medical Association Hospital (Table 1). The respective Institutional Ethics Committees approved the study protocol. Postmortem abdominal aortas were removed as described [23]. Several fresh aortic tissues (2×2 cm) were taken from various degrees of atherosclerotic lesions. Each tissue was cut into two specimens. In one specimen of each tissue, the intima of the aortas were separated mechanically from the media, and were stored at −80°C until just before using for Western-blot and real time-PCR analyses [23,24]. Another specimen was frozen in OCT compound. Then the tissue sections were stained with hematoxylin and eosin (HE). Atherosclerotic lesions were categorized histologically in three lesions as two early lesions (diffuse intimal thickening (DIT)/initial lesion, fatty streak) and advanced lesions according to AHA classification [25]. The DIT, that is considered almost as normal intima of artery, is composed exclusively of proliferated smooth muscle cells and extracellular matrix. Fatty streak lesion is DIT with an accumulation of lipid-laden macrophages. The advanced lesion is atheromatous plaque, which contains proliferated smooth muscle cells, lipid-laden macrophages, a large amount of extracellular matrix, and the central necrotic core.

### *Immunohistochemistry*

With the primary antibodies, 4-μm thick serial sections of 31 aortic intima were stained immunohistochemically [24]. Briefly, after blocking endogenous peroxidase activity in 3% hydrogen peroxide in methanol for 20 min, the sections were incubated with NZ-1, anti-α-smooth muscle actin (SMA, clone 1A4; Dako, Glostrup, Denmark), and anti-CD68 (clone PGM-1; Dako). Then, the sections were immunostained with the EnVision + (Dako) or LSAB kit (Nichirei Corp., Tokyo, Japan). Horseradish peroxidase activity was visualized with 3, 3′- diaminobenzidine containing hydrogen peroxide. The negative control contained normal mouse IgG or rat serum instead of the primary antibody. Podoplanin-positive cells were identified with immunohistochemical or immunofluorescent double stain as reported previously [23,26]. To perform semiquantitative analysis of podoplanin immunoreactivity in various stages of atherosclerosis, the slides stained with NZ-1 were evaluated by an experienced, board-certified pathologist on a four-tiered scale (0–3) for percentage of intimal cells that were positive for podoplanin (staining in more than 30% of intimal cells was scored as 3+; staining in more than 5% but fewer than 30% of intimal cells was scored as 2+; staining in fewer than 5% of intimal cells was scored as 1+; lack of staining was scored as 0).

**Table 1**
Profiles of autopsy cases.

| | |
|---|---|
| Number | 31 |
| Age (y; mean ± SD) | 66.4 ± 15.2 |
| Male, n (%) | 24 (77) |
| Cardiovascular death, n (%) | 7 (23) |
| Acute myocardial infarction, n | 2 |
| Rupture of aortic aneurysm, n | 1 |
| Cerebral infarction, n | 1 |
| Cerebral hemorrhage, n | 1 |
| Subarachnoid hemorrhage, n | 1 |
| Cardiomyopathy, n | 1 |
| Non-cardiovascular death, n (%) | 24 (77) |
| Malignancy, n | 14 |
| Idiopathic pulmonary fibrosis, n | 4 |
| Gastrointestinal bleeding, n | 2 |
| Renal failure, n | 1 |
| Pulmonary hemorrhage, n | 1 |
| Others, n | 2 |

### *Western-blot analysis*

The frozen tissues of 10 aortic intima (early lesions including three DIT lesions and four fatty streak lesions, and three advanced lesions) were solubilized with lysis buffer (1% Triton in phosphate-buffered saline (PBS) and 50 mg/ml aprotinin). They were then electrophoresed under reducing conditions on 10–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd., Osaka, Japan). The separated proteins were transferred to a nitrocellulose membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with a rat monoclonal antibody to podoplanin (1 μg/ml; clone NZ-1) [8] or mouse anti-β-actin antibody (1/5,000 dilution; Sigma-Aldrich Corp., St. Louis, MO); then it was incubated with peroxidase-conjugated secondary antibodies (1/1,000 diluted; GE Healthcare, Buckinghamshire, UK). The proteins were subsequently developed for 3 min using ECL reagents (GE Healthcare) with X-Omat AR film (Eastman Kodak Co., New York, NY).

### *Quantitative real-time PCR analysis*

Total RNAs were prepared from frozen tissues of 29 aortic intima (16 DIT lesions, three fatty streak lesions, and 10 advanced lesions) with TRIzol (Life Technologies Corp., Grand Island, NY). The initial cDNA strand was synthesized using SuperScript III transcriptase (Life Technologies Corp.) by priming an oligo-dT primer according to the manufacturer's instructions. We performed PCR using oligonucleotides: human podoplanin sense (5'-GGAAGGTGTCAGCTCTGCTC-3') and human podoplanin antisense (5'-CGCCTTCCAAACCTGTAGTC-3'); human β-actin sence (5'-GGCATCCTCACCCTGAAGTA-3') and human β-actin antisense (5'-AGGTGTGGTGCCAGATTTTC-3'). Real-time PCR was conducted using the QuantiTect SYBR Green PCR (Qiagen Inc., Hilden, Germany). The PCR conditions were 95 °C for 15 min (1 cycle) followed by 40 cycles of 94 °C for 15 s, 53 °C for 20 s, and 72 °C for 10 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. A standard curve for podoplanin templates was generated through serial dilution of PCR products ($1\times10^8$ copies/μl to $1\times10^2$ copies/μl). The expression level of podoplanin was normalized by β-actin. The statistical significance of podoplanin mRNA expression in tissues was determined using single-tailed, Student's *t*-tests.

## Results

### *Immunohistochemical analysis against human atherosclerotic lesion using an anti-podoplanin antibody*

Podoplanin possesses platelet-aggregating activities, which play crucial roles in thrombosis/hemostasis, tumor metastasis, and

**Table 2**
Results of podoplanin staining by NZ-1 (31 cases).

| Stage | No. of cases | Podoplanin immunostaining | | | |
|---|---|---|---|---|---|
| | | 0 | 1+ | 2+ | 3+ |
| DIT | 10 | 5 | 5 | 0 | 0 |
| Fatty streak | 6 | 2 | 3 | 1 | 0 |
| advanced | 15 | 0 | 4 | 5 | 6 |

lymphangiogenesis [3,4]. To date, no report in the literature has described a study of podoplanin expression in human atherosclerotic lesions. We previously produced NZ-1, a rat anti-human podoplanin monoclonal antibody (mAb), to investigate the relation between podoplanin expression in human cancer and tumor-induced platelet aggregation [8]. Although other anti-podoplanin antibodies (D2-40, 18H5, AB3) do not possess neutralizing activities, NZ-1 suppressed podoplanin-induced pulmonary metastasis through inhibition of the tumor-induced platelet aggregation [17,21]. Furthermore, we showed that NZ-1 has not only high specificity and sensitivity but also high binding affinity against podoplanin; $K_D$ of NZ-1 is $\sim 1 \times 10^{-10}$ M, whereas $K_D$ of clone P2-0 is $\sim 1 \times 10^{-8}$, indicating that the binding affinity of NZ-1 is about 100 times higher than that of P2-0, although P2-0 also possesses neutralizing activity [22,27]. Therefore, we selected NZ-1 among several anti-podoplanin mAbs (D2-40, 18H5, AB3, P2-0, NZ-1) to analyze podoplanin expression in human atherosclerotic lesions.

We first performed immunohistochemical analysis using NZ-1 antibody against the abdominal aortas of 31 patients (Table 2). In our

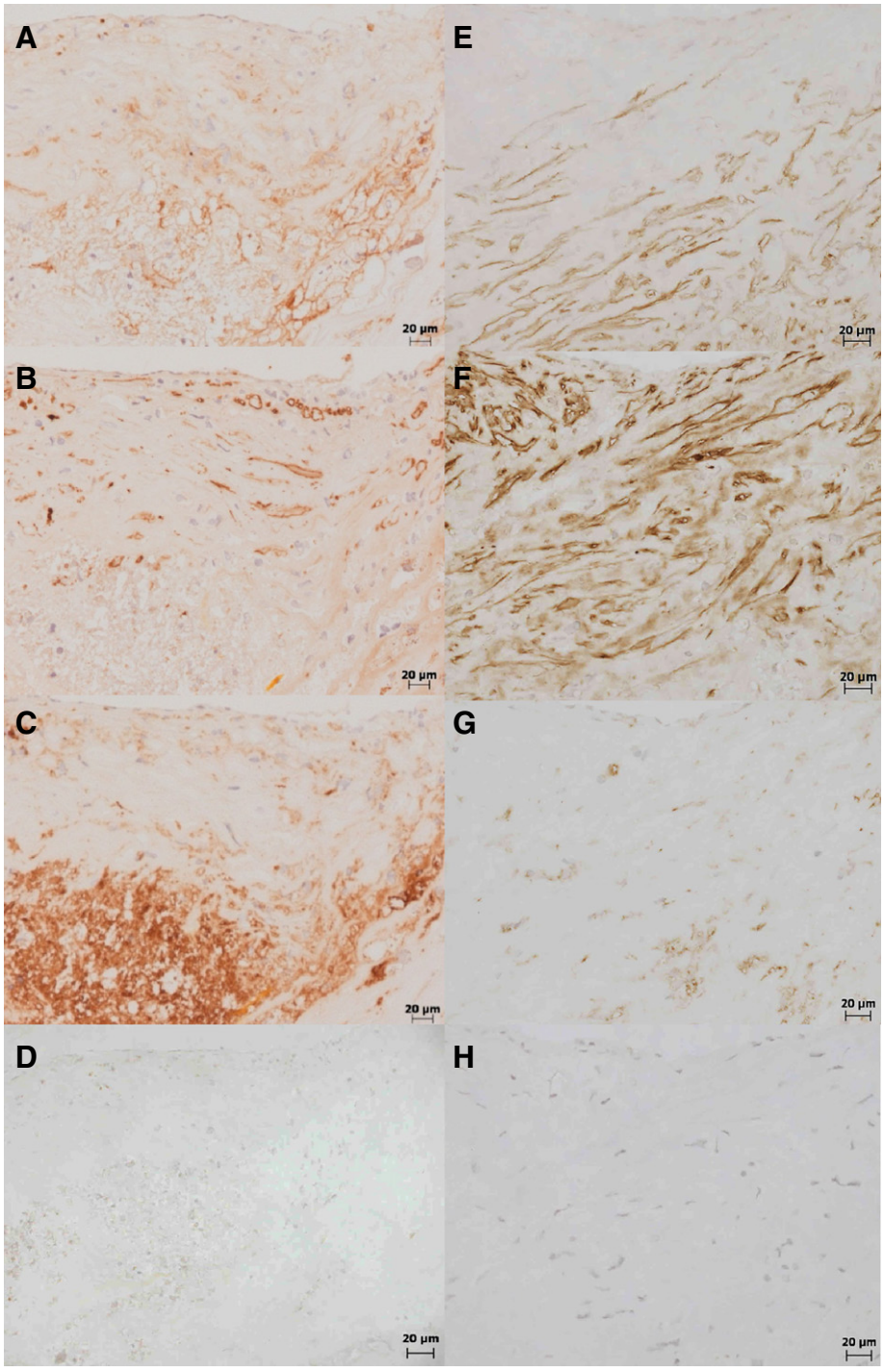


**Fig. 1.** Representative immunohistochemical results of advanced atherosclerotic lesions (A-D or E-H are serial sections, respectively) in abdominal aortas. Specimens were stained by NZ-1 (A, E), anti-SMA (B, F), anti-CD68 (C, G), or rat serum (D, H). Macrophages and smooth muscle cells are positive for podoplanin in advanced lesions (atheromatous plaques). Original magnification × 400.

previous study, NZ-1 detected the podoplanin protein of tumor cells clearly in a membranous staining pattern [8]. As depicted in Fig. 1, podoplanin protein was highly detected in advanced lesions in a membranous or cytoplasmic staining pattern (Fig. 1A and E), whereas none or less than 1% of the intimal cells was immunopositive for podoplanin in early lesions (Fig. 2A and E). In fatty streak, less than 1% of the intimal cells were immunopositive for podoplanin (Fig. 2E). In advanced lesions, many macrophages (Fig. 1C: serial section of Fig. 1A) and smooth muscle cells (Fig. 1F: serial section of Fig. 1E) in the plaques were stained by an anti-CD68 mAb or an anti-SMA mAb, respectively. These data demonstrated that podoplanin is expressed in advanced atherosclerotic lesions and that it is localized in macrophages or smooth muscle cells. In serial sections of Fig. 1A, a few smooth muscle cells were found (Fig. 1B), whereas few macrophages were found in serial sections of Fig. 1E (Fig. 1G). Immunohistochemical and immunofluorescent double staining indicated that podoplanin-positive cells are macrophages or smooth

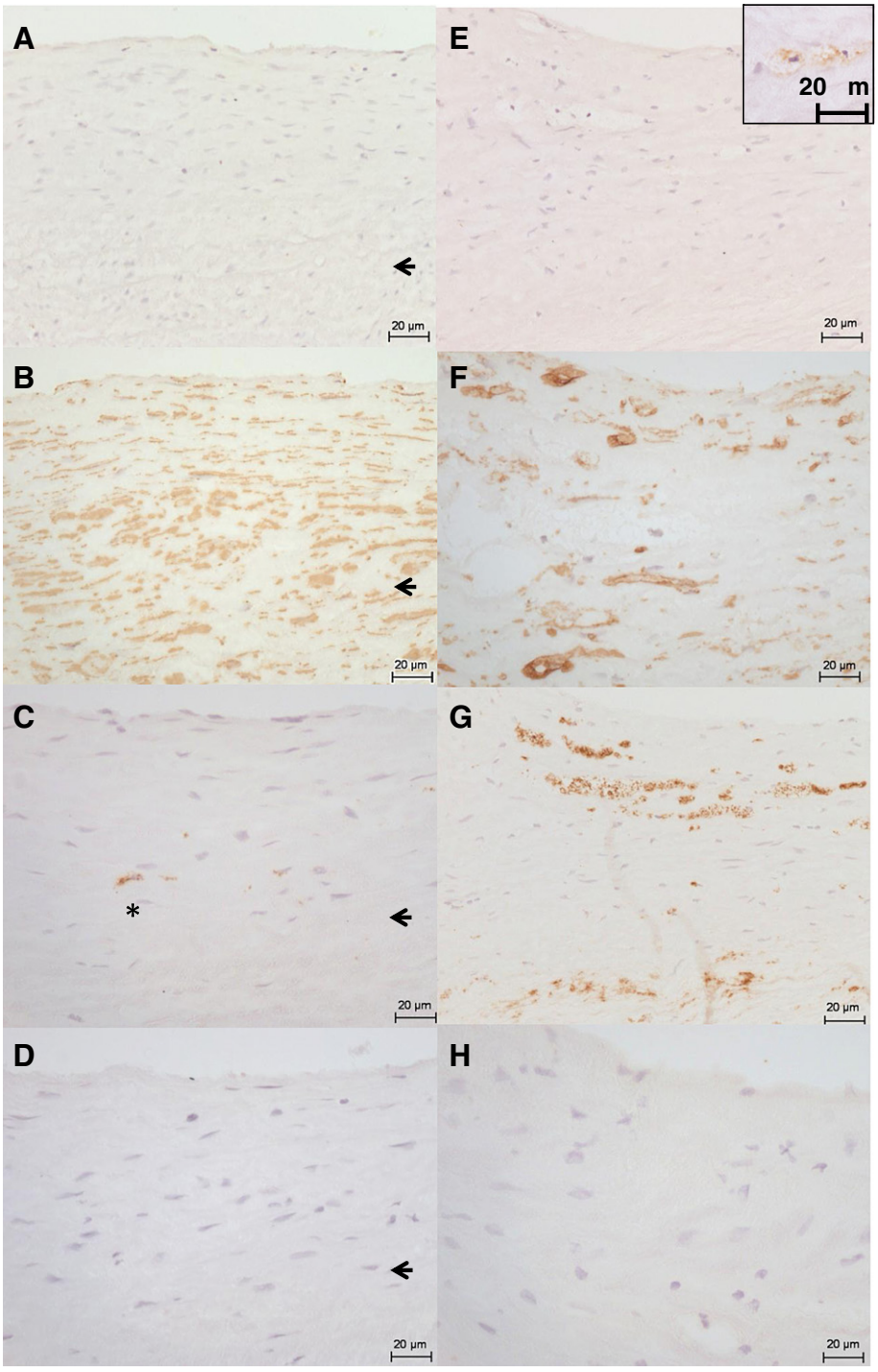


**Fig. 2.** Representative immunohistochemical results of early atherosclerotic lesions (A-D, diffuse intimal thickening; E-H, fatty streak) in abdominal aortas (A-D or E-H are serial sections, respectively). Specimens were stained by NZ-1 (A, E), anti-SMA (B, F), anti-CD68 (C, G), or rat serum (D, H). Almost all of the intimal cells are negative for podoplanin (A, E) in early lesions, although less than 1% of cells show positive reaction for podoplanin (inset in E). The intima of early atherosclerotic lesions is mainly composed of smooth muscle cells (B, F), focally with small numbers of macrophages (C, asterisk indicates macrophage) in diffuse intimal thickening/initial lesion or accumulation of macrophages (G) in fatty streak. (Arrows indicate the internal elastic laminae, Original magnification × 400).

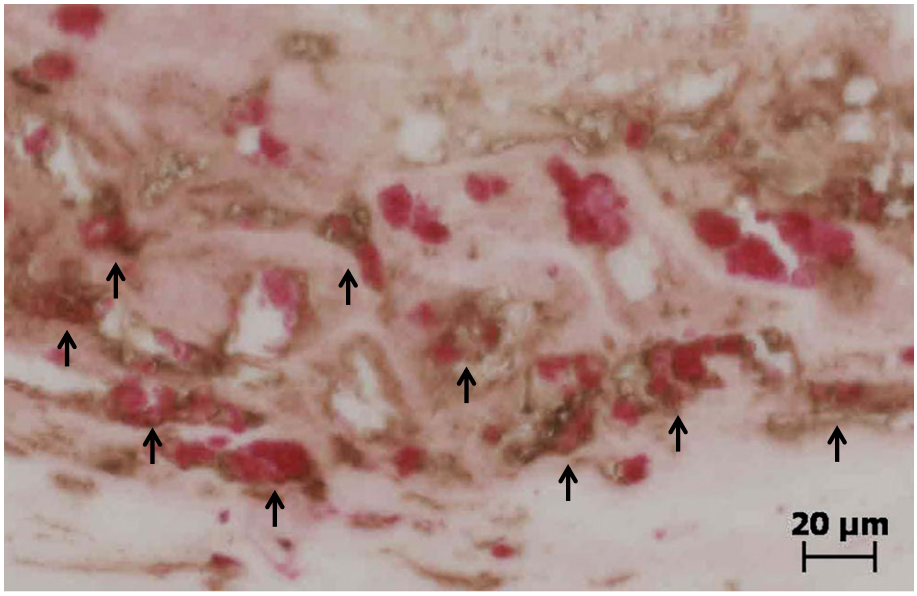


**Fig. 3.** Double immunohistochemical staining of podoplanin and macrophages in an atheromatous plaque. Macrophages (brown) are positive for podoplanin (red). Arrows show double positive cells for anti-CD68/PGM-1 (macrophages) and NZ-1 (podoplanin). Original magnification×400.

muscle cells (Figs. 3 and 4). Furthermore, macrophages and smooth muscle cells do not always express podoplanin in these specimens (Fig. 1). Therefore, small populations of macrophages or smooth muscle cells might be activated and express podoplanin.

*Western-blot and quantitative real-time PCR analyses against human atherosclerotic lesions*

We then performed Western-blot and real-time PCR analyses to investigate the podoplanin expression in atherosclerotic lesions. As presented in Fig. 5, podoplanin was detected in seven of 10 atherosclerotic lesions, which include four fatty streak lesions and three advanced lesions, although no podoplanin expression was observed in DIT. Furthermore, the expression level of podoplanin in three advanced lesions is much higher than that of four fatty streak lesions, suggesting that podoplanin expression is associated with the progression stage of atherosclerosis. In human cancers, podoplanin expression is also associated with malignant progression and poor clinical outcome [11,18–20]. It is particularly interesting that the molecular weight of podoplanin in advanced lesions is apparently larger than that in streak lesions, indicating that podoplanin in advanced lesions is highly glycosylated. Our previous studies showed that glycosylation of podoplanin, especially the disialyl-core 1 structure at Thr52, is critical for its platelet-aggregating activity [28,29]. Therefore, not only high expression levels of podoplanin, but also a high glycosylation level of podoplanin in advanced lesions might be involved in the increased thrombogenicity of atherosclerotic plaques and progression of atherosclerosis. Next, we confirmed the expression level of podoplanin using quantitative real-time PCR analysis. As presented in Fig. 6, the podoplanin mRNA level in advanced lesions ($n=10$) was significantly higher ($p<0.05$) than that of DIT ($n=16$).

## Discussion

Acute cardiovascular event usually involves thrombus formation at the site of a disrupted atherosclerotic plaque. Therefore, thrombogenicity of exposed plaque constituents is critical to the onset of clinical events. Atherosclerotic lesions show predominant expression of type I and III collagens, which are potent platelet activators [30], and significant decrease of CD39, a major metabolic enzyme of extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) [31]. Those data indicate that advanced atherosclerotic lesions have high platelet-aggregating activity. In addition, tissue factor, a trigger of extrinsic coagulation pathway, is also abundantly expressed in advanced atherosclerotic lesions [23].

In this study, we showed that the podoplanin expression might be related to thrombogenicity of advanced atherosclerotic lesions. Because podoplanin is a platelet aggregation factor, the evidence suggests that disruption of advanced atherosclerotic lesions promotes thrombus formation, which leads to the onset of cardiovascular events. Recent reports of some studies have described that podoplanin is also expressed in stromal myofibroblasts and that it might promote cell migration and invasion [20,32], suggesting that podoplanin expression in atherosclerotic plaques is associated with vascular remodeling and the progression of atherosclerosis. However, the podoplanin expression mechanisms in advanced atherosclerotic lesions remain unclear in this study. Results of recent studies showed that inflammatory cytokines such as transforming growth factor-β (TGF-β) and interleukin-3 (IL-3), both of which are known to participate in plaque progression, up-regulate podoplanin expression in stromal cells and endothelial cells [12,33]. Therefore, inflammatory responses might play a critical role in podoplanin expression in atherosclerotic plaques, although further studies are necessary to confirm this speculation.

Although many anti-platelet drugs have been developed, direct anti-platelet drugs might leads to bleeding side effects. Identification of the function and the mechanism of podoplanin expression might support the use of podoplanin as a molecular target to prevent atherothrombosis.

## Conflict of interest statement

We have no conflict of interest to declare.

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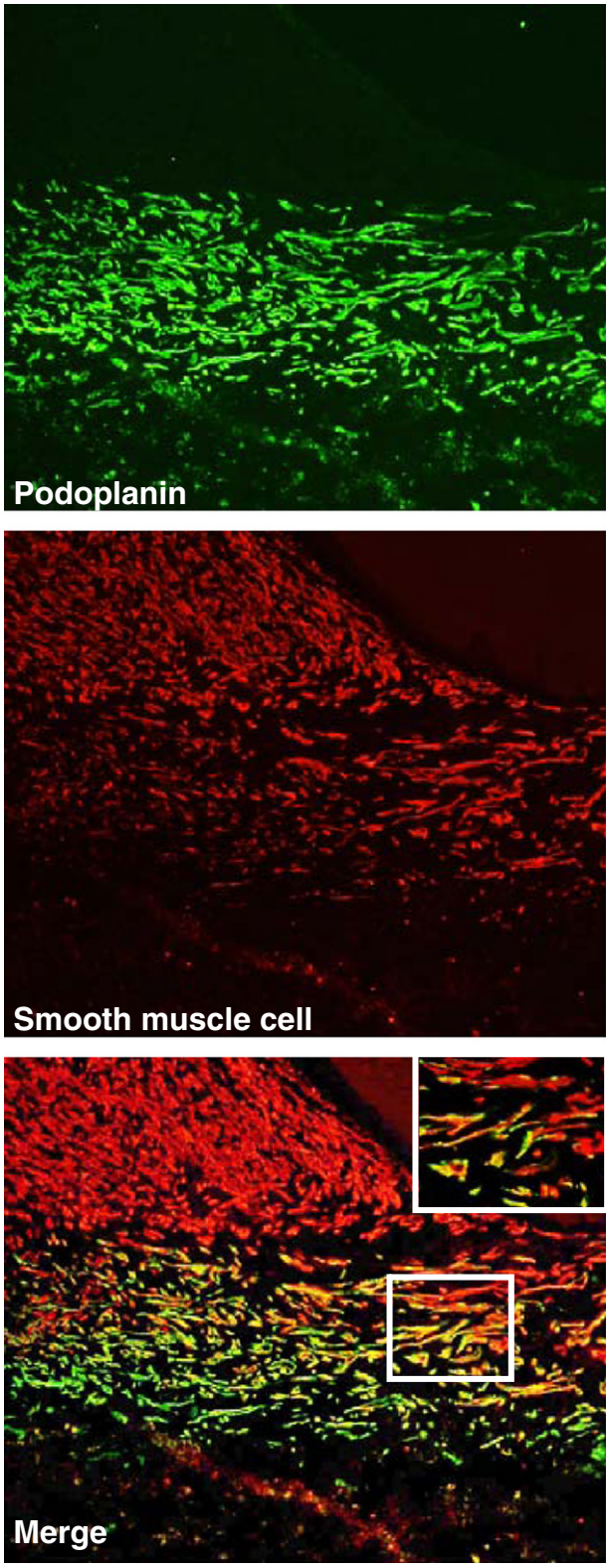


**Fig. 4.** Representative immunofluorescent micrographs of an atheromatous plaque. Upper: staining with fluorescein isothiocyanate-labeled podoplanin (green). Middle: staining with Cy3-labeled smooth muscle actin (red). Lower: merged immunofluorescent image. Co-localized area of podoplanin and smooth muscle actin is stained yellow.

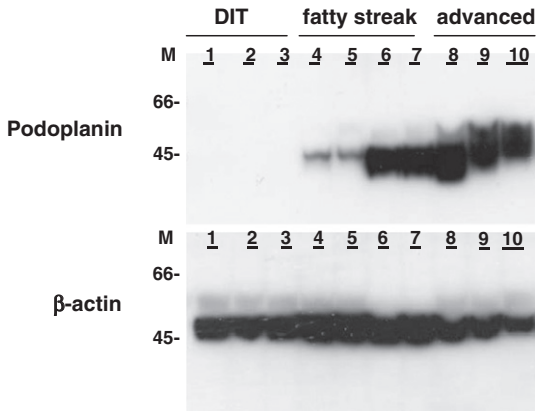


**Fig. 5.** Western-blot analysis of podoplanin in atherosclerotic lesions of abdominal aortas. Tissue homogenates from DIT lesions (lanes 1–3), fatty streak lesions (lanes 4–7), and advanced lesions (lanes 8–10) were electrophoresed under reducing conditions using 10–20% gels, and were Western-blotted with NZ-1 (upper; about 36 kDa) and anti-β-actin (lower).

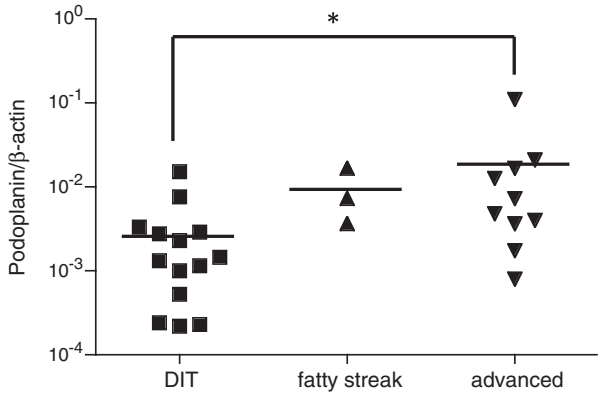


**Fig. 6.** Quantitative real-time PCR analysis of podoplanin transcripts in atherosclerotic lesions of abdominal aortas. First-strand cDNA samples derived from 29 aortic intima (16 DIT lesions, three fatty streak lesions, and 10 advanced lesions) were used as real-time PCR templates. The respective expression levels of podoplanin were normalized to β-actin, as described in Materials and Methods. The statistical significance of podoplanin mRNA expression in tissues was determined using single-tailed, Student's *t*-tests. $^{*}p<0.05$.

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