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CD9 expression in vascular aging and atherosclerosis

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Summary. CD9 is a transmembrane glycoprotein belonging to the tetraspanin family. CD9 expression has been reported to be associated with cellular signaling, cell adhesion, cell migration, and tumor related processes. The aim of this study was to examine the immunohistochemical expression of CD9 in vascular senescence and atherosclerosis. One hundred and twenty samples of normal young arteries (obtained from individuals aged 0-60 years), 40 samples of normal old arteries (obtained from individuals aged 61-80 years), and 67 samples of atherosclerotic arteries were obtained from surgically resected specimens. Tissue microarray blocks were prepared for immunohistochemical staining. Immunohistochemical staining detected CD9 expression in 10.8% (13 of 120 samples) of normal young arteries and 30.0% (12 of 40 samples) of normal old arteries. CD9 expression was absent or mildly present in the smooth muscle cells and endothelial cells of normal arteries. Normal old arteries showed significantly higher expression of CD9 than normal young arteries ($P<0.01$). Atherosclerotic arteries showed moderate or strong CD9 expression (65 of 67 samples, 97.0%), which was observed in the smooth muscle cells, endothelial cells, macrophages, and atheromatous plaques. CD9 was significantly expressed in the atherosclerotic arteries compared to normal young and old arteries ($P<0.01$). The results suggest that CD9 expression may play an important role in the vascular senescence and pathogenesis of atherosclerosis.

Key words: CD9, Immunohistochemistry, Artery, Senescence, Atherosclerosis

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Introduction

CD9 is a cell-surface glycoprotein comprising two extracellular domains and four hydrophobic transmembrane domains (Maecker et al., 1997). It is a member of the transmembrane 4 superfamily, also known as the tetraspanin family (Scherberich et al., 1998). CD9 is commonly expressed in neural tissues, hematopoietic cells, and various tumor cells (Romanska and Berditchevski, 2011). It is involved in biologically important cellular processes, such as platelet activation and aggregation, cell adhesion, migration, differentiation, and proliferation (Brosseau et al., 2018).

Atherosclerosis is the major cause of cardiovascular diseases. The pathological process leading to atherosclerosis is complex. Atherosclerosis is known as an inflammatory disease of the vascular wall that is associated with lipid and other metabolic alterations (Hansson, 2005; Barquera et al., 2015). Aging is an independent risk factor for the development of atherosclerosis (Klein-Soyer et al., 2000; Wang and Bennett, 2012).

Few published studies have addressed CD9 expression in blood vessels. Klein-Soyer et al. (2000) demonstrated that CD9 participated in endothelial cell migration during wound repair *in vitro*. CD9 was found to be expressed in the smooth muscle cells of the atherosclerotic coronary arteries and aorta, as assessed by immunohistochemical analysis (Nishida et al., 2000). Kim et al. (2008) demonstrated that CD9 mRNA and protein expression levels were increased in the endothelial cells of old human umbilical veins *in vitro* and revealed that CD9 expression may be involved in cellular senescence of the endothelial cells. Kotha et al. (2009) demonstrated that CD9 expression was uniformly present in the vascular smooth muscle cells of the neointima of injured carotid arteries. Although few studies have suggested that CD9 may be involved in

atherogenesis, the function of CD9 in vascular senescence and atherosclerosis is still unknown.

This study aims to investigate the role of CD9 in vascular senescence and atherosclerosis. The expression of CD9 in normal young, normal old, and atherosclerotic arteries was analyzed by immunohistochemistry.

Materials and methods

Case selection

Twenty specimens of normal arteries were collected from individuals of each of the following age groups: 0-10 years old, 11-20 years old, 21-30 years old, 31-40 years old, 41-50 years old, 51-60 years old, 61-70 years old, and 71-80 years old. Sixty-seven samples of atherosclerotic arteries were selected. A total of 160 normal muscular arteries were collected from the specimens resected by neoplasms and benign lesions of the spleen, stomach, rectosigmoid, pancreas, kidney, and testis from 2000 to 2010 at the Yeungnam University Hospital. All patients had no history of diseases such as atherosclerosis, hypertension, diabetes, and other specific vascular diseases. The arteries collected from individuals less than 60 years old were classified as normal young arteries, while those collected from individuals more than 60 years old were classified as normal old arteries. The 67 atherosclerotic arteries included carotid arteries (31 specimens), abdominal aorta (30 specimens), and femoral arteries (6 specimens). All the specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Hematoxylin and eosin staining was performed for histological examination. Tissue microarrays were prepared made from representative tissue blocks for each specimen. One core with 5 mm diameter was extracted and trans¬ferred to the recipient tissue microarray blocks. This study was approved by the Institutional Review Board of the Yeungnam University Hospital (YUH 2019-10-034).

Immunohistochemistry of CD9

Tissue sections of 4-μm thickness were deparaffinized in xylene and rehydrated with serial ethanol solutions. Heat-induced antigen retrieval was performed using EDTA buffer (pH 8.0). CD9 monoclonal antibody (EPR2949, Abcam, Cambridge, UK) was used at 1:100 dilution. Immunohistochemical staining was performed on the automated immunostaining system using an I-View detection kit (Benchmark XT, Ventana Medical Systems, Tucson, AZ, USA). Diaminobenzidine was used as a chromogen. The normal kidney tissues were used as positive controls.

Evaluation of CD9 expression

CD9 expression was scored semi-quantitatively by evaluating the proportion of stained cells and their staining intensity. The proportion of stained cells was rated on a scale from 0 to 3 as follows: 0 (no positively stained cells), 1 (<10% positively stained cells), 2 (10-50% positively stained cells), or 3 (>50% positively stained cells). The staining intensity was rated on a scale from 0 to 3 as follows: 0 (no stain), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The scores were then added to give a total score between 0 and 6. A total score of 3 or higher was counted as positive staining for CD9.

Statistical analysis

The correlation of CD9 expression in normal young arteries, normal old arteries, and atherosclerotic arteries

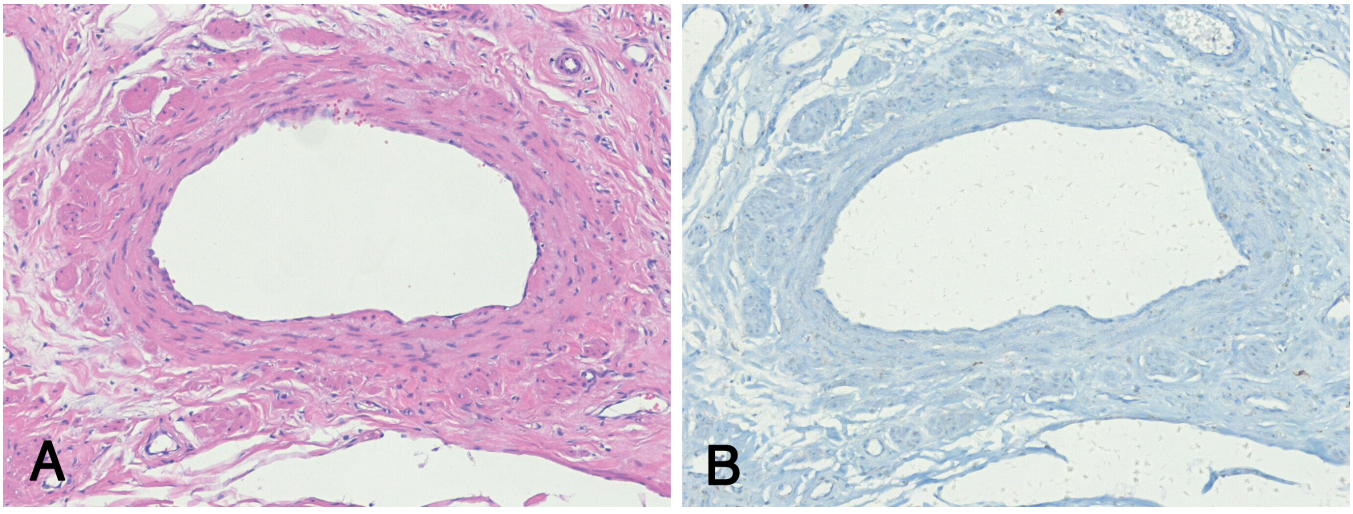

Fig. 1. A. The image shows a normal young artery (hematoxylin-eosin stain). **B.** CD9 expression is not present (immunohistochemical stain). x 100.

was analyzed by either Chi-square test or Fisher's exact test. Statistical analysis was performed using IBM SPSS 25.0 for Windows (SPSS Inc., Chicago, IL, USA). A P value of less than 0.05 was considered statistically significant.

Results

CD9 expression was observed in 5.0% (1 of 20 samples) of normal 1-10 years of age, 10.0% (2 of 20 samples) of normal 21-30 years of age, and 35% (7 of 20 samples) of normal 71-80 years of age (Table 1). CD9 expression was absent or mildly present in the cytoplasm of the smooth muscle cells and endothelial cells, and vascular walls of normal arteries (Figs. 1, 2). CD9 expression tended to increase with age (Table 1), but no significant correlation was found (P=0.251). However, when compared with CD9 expression in normal young arteries (less than 60 years of age) and normal old arteries (more than 60 years of age), CD9 expression was observed in 10.8% (9 of 120 samples) of normal young arteries and 30.0% (12 of 40 samples) of normal old arteries. Normal old arteries showed significantly higher expression of CD9 than in normal young arteries (P<0.01). Atherosclerotic arteries showed moderate or strong CD9 expression in 97.0% of the samples tested

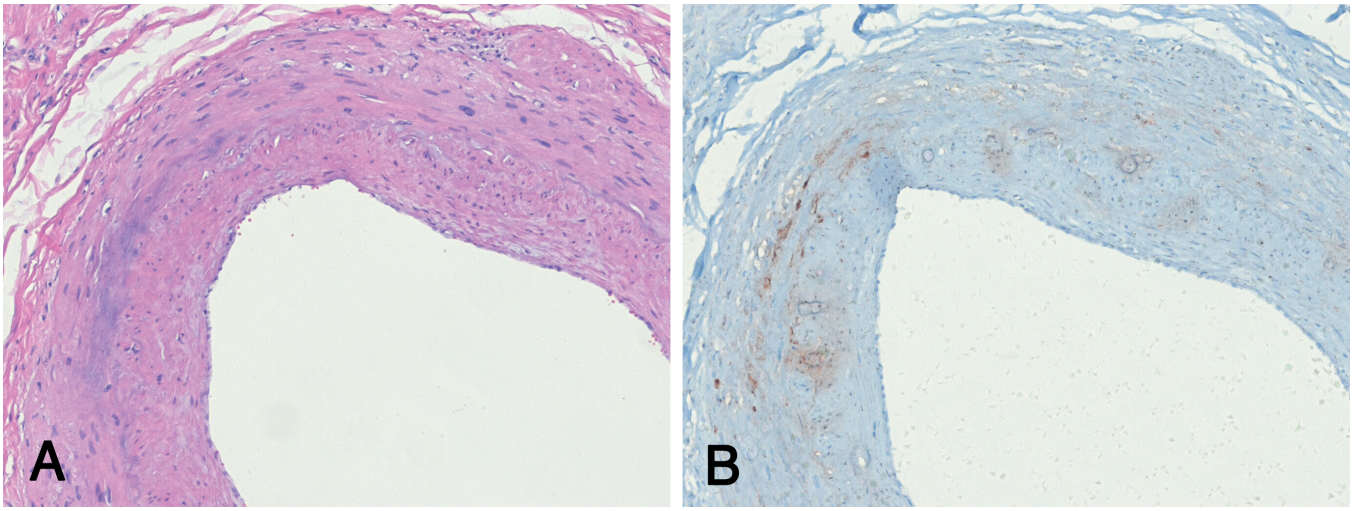

Fig. 2. A. The image shows a normal old artery (hematoxylin-eosin stain, stain). **B.** CD9 is mildly expressed in the endothelial cells, smooth muscle cells, and artery walls (immunohistochemical stain). x 100.

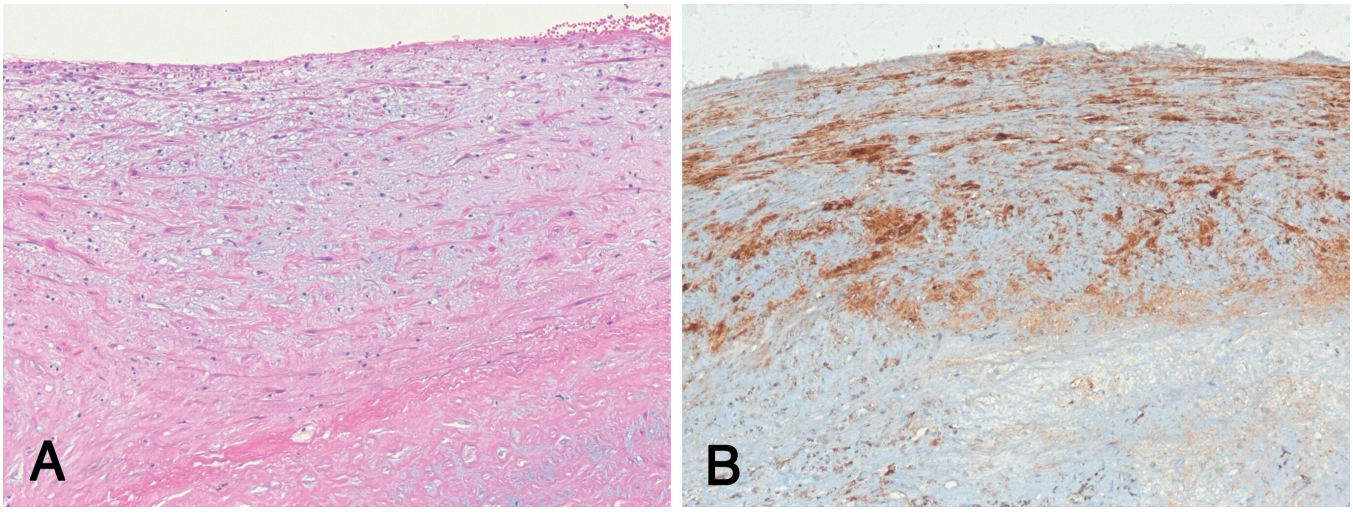

Fig. 3. A. The smooth muscle cells and extracellular matrix of an atherosclerotic artery (hematoxylin-eosin stain). **B.** CD9 is strongly expressed in the cytoplasm of smooth muscle cells (immunohistochemical stain). x 200.

(65 of 67 samples). CD9 was expressed in the endothelial cells, smooth muscle cells, macrophages, and atheromatous plaques (Figs. 3, 4). CD9 was expressed predominantly in the cytoplasmic granules. CD9 expression was significantly higher in the atherosclerotic arteries compared to that in the normal young arteries ($P<0.01$) and normal old arteries ($P<0.01$).

Discussion

Atherosclerosis is a leading cause of mortality worldwide (Barquera et al., 2015). It is an age-related disease; its incidence and prevalence increase with age (Uryga and Bennett, 2016). It is important to understand the cellular and functional changes that occur in the arteries during aging and development of atherosclerosis (Hansson, 2005). We studied the immunohistochemical expression of CD9 in normal young, old arteries, and atherosclerotic arteries to elucidate the role of CD9 in vascular senescence and atherosclerosis.

In the present study, CD9 expression was observed in 10.8% of normal young arteries and 30.0% of normal

Table 1. CD9 expression in normal arteries.

Normal age groups	Cases	CD9 (-) (%)	CD9 (+) (%)	Positive scores for CD9				P value
				3	4	5	6	
1-10 years old	20	19 (95.0)	1 (5.0)	1	0	0	0	0.251
11-20 years old	20	18 (90.0)	2 (10.0)	2	0	0	0	
21-30 years old	20	18 (90.0)	2 (10.0)	2	0	0	0	
31-40 years old	20	17 (85.0)	3 (15.0)	2	1	0	0	
41-50 years old	20	18 (90.0)	2 (10.0)	1	1	0	0	
51-60 years old	20	17 (85.0)	3 (15.0)	1	2	0	0	
61-70 years old	20	15 (75.0)	5 (25.0)	2	3	0	0	
71-80 years old	20	13 (65.0)	7 (35.0)	2	4	1	0	

Table 2. Comparison of CD9 expression in normal young, normal old, and atherosclerotic arteries.

	Cases	CD9 (-) (%)	CD9 (+) (%)	Positive scores for CD9				P value
				3	4	5	6	
Normal young arteries	120	71 (89.2)	13 (10.8)	9	4	0	0	
Normal old arteries	40	28 (70.0)	12 (30.0)	4	7	1	0	*
Atherosclerotic arteries	67	2 (3.0)	65 (97.0)	3	7	26	29	**

* $P<0.01$ versus normal young arteries, ** $P<0.01$ versus normal young arteries and normal old arteries.

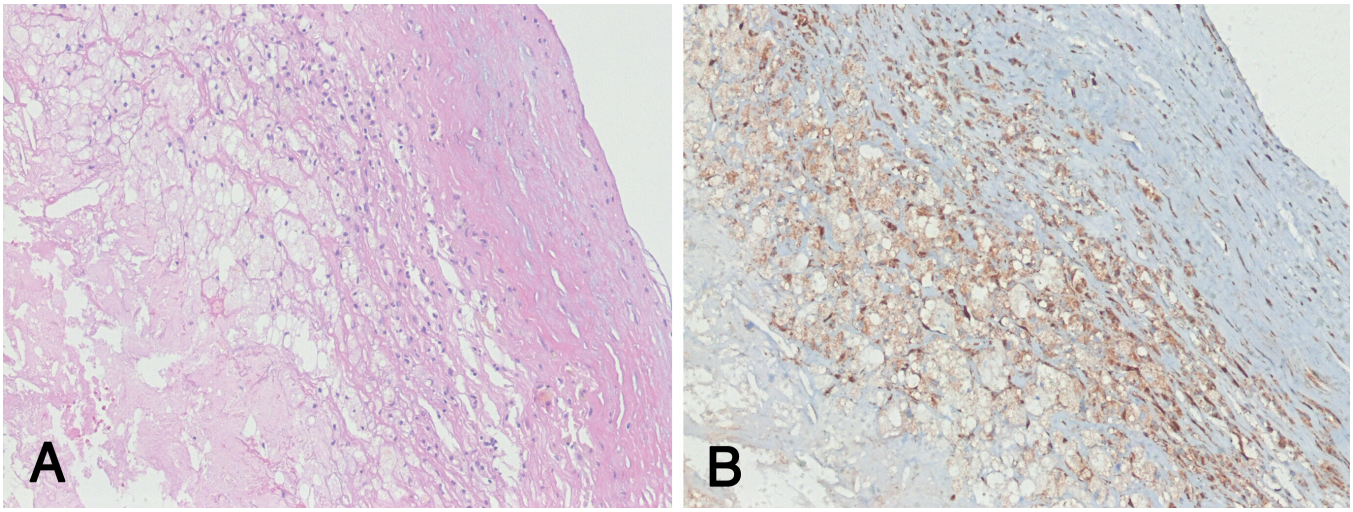

Fig. 4. A. Cholesterols and foamy macrophages observed in an atheromatous plaque (hematoxylin-eosin stain). **B.** CD9 is expressed present in the atheromatous plaque and foamy macrophages (immunohistochemical stain). x 200.

old arteries. Normal old arteries showed higher CD9 expression than normal young arteries ($P<0.01$). CD9 was mildly expressed in the cytoplasm of smooth muscle cells and endothelial cells of normal old arteries. Kim et al. (2008) reported increased expression levels of CD9 mRNA and protein levels in old endothelial cells and suggested that CD9 may play a role in cellular senescence. Our results show that CD9 expression in normal old arteries support a potential role of CD9 in vascular senescence. There is emerging evidence that cellular senescence may contribute to the pathogenesis of atherosclerosis (Minamino and Komuro, 2007). Senescent vascular cells accumulate in atheroma tissues and exhibit various features (Minamino and Komuro, 2007).

In our study, moderate or strong expression of CD9 was observed in 97.7% of atherosclerotic arteries. CD9 expression was found in the cytoplasm of smooth muscle cells and endothelial cells, macrophages, and atheromatous plaques. CD9 was significantly expressed in atherosclerotic arteries compared to normal young and old arteries. These results suggest a significant role of CD9 in the development of atherosclerosis. In addition, comparison of CD9 expression in normal and atherosclerotic arteries indicates that CD9 is a potential marker of the atherosclerotic state of vessels (Scherberich et al., 1998). In recent experimental model study, CD9 expression increased in arterial tissues from humans and rats with age and in atherosclerotic plaques in humans and mice, and anti-mouse CD9 antibody prevented the formation of atherosclerosis in *ApoE*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice (Cho et al., 2020). These results suggest that CD9 may play critical roles in the control of endothelial cell senescence and the formation of atherosclerosis."

Nishida et al. (2000) demonstrated that in the aortas of atherosclerotic individuals, CD9-positive cells were mainly localized in the plaque shoulder, fibrous cap, and around the internal elastic lamina. In the present study, CD9 immunostaining was observed in a cytoplasmic granular pattern in the endothelial cells, macrophages, smooth muscle cells, and atherosclerotic plaques. In the process of atherogenesis, CD9 may be involved in the proliferation of smooth muscle cells (Scherberich et al., 1998; Romanska and Berditchevski, 2011). Vascular smooth muscle senescence can promote both atherosclerosis and features of plaque vulnerability (Wang et al., 2015; Grootaert et al., 2018). CD9 is also expressed by endothelial cells and different subtypes of leukocytes (Reyes et al., 2018). Although the exact mechanism by which CD9 takes part in atherosclerosis is not known, CD9 expression may contribute to oxidative stress, inflammation, foam cell formation, nitric oxide production, and autophagy; thereby, playing a role in the pathogenesisi of atherosclerosis (Kitada et al., 2016; Lee et al., 2017). Additionally, CD9 expression has been implicated in cell fusion, migration, cancer progression, and chemoresistance (Reyes et al., 2018; Ullah et al., 2019). A full understanding of vascular smooth muscle behavior in atherosclerosis is critical to identify therapeutic targets for both prevention and treatment of atherosclerosis (Bennett et al., 2016).

There are limitations in this study. Most cardiovascular drugs can modify vascular inflammation. The population of patients was not further specified particularly with respect to the therapies used. Another limitation of this study is that we did not examine the cellular and physiological functions of CD9 in normal young, normal old, and atherosclerotic arteries. Little is known regarding the functional significance of CD9 in vascular senescence and atherosclerosis. Further studies on the mechanism of cellular senescence may provide new insights into the pathogenesis of age-associated vascular disorders and clarify the potential of anti-senescence therapy for vascular senescence (Minamino and Komuro, 2007; Lee et al., 2017). Additional studies will be necessary to determine the functional implication of CD9 in the development of atherosclerosis. The use of state of the art techniques, such as next-generation sequencing, proteomics, and metabolomics can help advance our understanding of complex biologic processes involved in atherogenesis (Khan et al., 2017). In the future, new strategies to target CD9 will be developed and implemented for prevention and treatment of atherosclerosis.

Conclusion

The immunohistochemical expression of CD9 was investigated in normal young, normal old, and atherosclerotic arteries. We found that CD9 was strongly expressed in atherosclerotic arteries, which suggests that CD9 may play a role in the vascular senescence and pathogenesis of atherosclerosis.

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Conflicts of interest. The authors declare that they have no conflict of interest.

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