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Localization and Characterization of a Novel Secreted Protein, SCUBE2, in the Development and Progression of Atherosclerosis

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Inhibition of atherosclerosis progression has long been the subject of intensive pathophysiologic investigations. The identification of a novel target molecule to redeem the cellular processes remains a major challenge in cardiology. Signal peptide CUB domain EGF-like repeat protein (SCUBE) family has been detected on human tissues and cultured cells. Two members of the SCUBE family, SCUBE1 and SCUBE3 are reported to play a role in cardiovascular diseases. The other member, SCUBE2 has been reported to mediate Hedgehog (Hh) protein signaling and is expressed in major blood vessels during mouse embryogenesis. However its involvement in cardiovascular diseases is not known yet. The aim of this study was to investigate SCUBE2 expression and localization in diffuse intimal thickening (DIT), an early event of atherosclerosis and in advanced lesion of atherosclerotic plaque.

Carotid artery ligation in C57BL/6J mice was performed to induce intimal thickening, mimicking DIT in human. After 2 weeks of ligation, mRNA level of SCUBE2 increased significantly, while in LDLr$^{-/-}$ mice fed with high fat diet, a human atherosclerosis model, mRNA level of SCUBE2 expression markedly increased 8 weeks after start of the high fat diet. Our findings were confirmed by the observation of SCUBE2 expression in human coronary artery with DIT and advanced lesion of atherosclerotic plaque. Previous investigations described that SCUBE2 mediates Hh signaling pathway. We have observed that SCUBE2 expression is associated with Sonic Hedgehog (Shh) and its receptor, Patched (Ptc), both in DIT and advanced plaque lesion. Our results suggested that SCUBE2 is a new target molecule in atherosclerosis and might play an important role in atherosclerotic plaque progression via Hh signal transduction.

INTRODUCTION

Atherosclerosis is known as the highest contributing factor of death caused by cardiovascular diseases. Atherosclerosis involves complex interactions of molecules. Many experimental and clinical approaches have attempted to identify several candidate molecules and pathways responsible for the progression of the atherosclerotic plaque. Over several years, the importance of inflammation during all stages of atherosclerosis, from its inception through its progression, has greatly increased. It is believed that the inflammatory molecules in early atherogenesis play a role for the fate of advanced lesion of atherosclerotic plaque formation (9, 16). Experimental studies have demonstrated that an inflammatory subset of monocytes/macrophages preferentially accumulate in inflammatory plaque and produce pro-inflammatory cytokines. Accumulating experimental evidence supports a key role for inflammation as a link between risk factors for atheroslcerosis and the biology that underlies the complications of this disease. But still the atherogenesis event remains challenging to be elucidated (10, 14).

The epidermal growth factor (EGF) superfamily is a group of growth factors, cytokine-like mediators, and extracellular matrix proteins. One gene family named signal peptide CUB-EGF-like domain containing protein (SCUBE) encodes secreted proteins harboring nine copies of EGF-like repeats, a spacer region, three cysteine-rich domains, and one CUB domain at the C terminus. The SCUBE family is composed of three independent genes, SCUBE1, 2, and 3. SCUBE1 to 3 are expressed in various types of tissues and cells of human, mouse and zebrafish. (4, 25, 27, 28, 29, 31). Little is known on how the SCUBE family is involved in

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cardiovascular diseases. The gene SCUBE1 was first identified in human vascular endothelial cells (31) and has been reported to be expressed in platelets and human atherosclerotic vascular lesion. SCUBE1 is released by activated platelets, and enhances platelet – platelet adhesion and agglutination (25). Recent investigation revealed that SCUBE1 expression is elevated in patients with acute coronary syndrome and acute large-vessel atherothrombotic stroke (4). However, the biological function of SCUBE1 in atherosclerosis or thrombus formation is still unclear. Like SCUBE1, SCUBE2, a protein closely related to SCUBE1, was described as cell surface protein on the vascular endothelium. The expression of both SCUBE1 and SCUBE2 are rapidly downregulated by interleukin-1β or TNF-α (31) which indicates a potential involvement of the SCUBE proteins in inflammation. In humans, SCUBE1 was only detected in endothelial cells and platelets while SCUBE2 is expressed in a broad spectrum of human tissues including cardiovascular tissues (28, 31). SCUBE2 might therefore have some functions in cardiovascular diseases. SCUBE2 was initially reported to play a role in embryogenic development. On the basis of genetic studies, it was suggested that *you* gene, the zebrafish orthologue of the mammalian SCUBE2 gene, acts as a permissive factor for Hedgehog (Hh) signaling upstream of Smoothened (SMO), the Hh receptor signal component (11, 13, 26). Tsai et al, reported that SCUBE2 forms a complex with Sonic hedgehog (Shh) and its receptor Patched-1 (Ptc1) in vitro to promote the Shh-induced signaling. SCUBE2 represents a novel positive component in the propagation of the Hh signal (3, 12, 24) and may have significant implications for Hh-mediated biological processes during development and Hh-related human pathologies.

In our present study, we have found that SCUBE2 is expressed in the injured carotid artery and aortic sinus of two different animal models of DIT and atherosclerotic plaque (carotid artery ligation and $LDL^{-/-}$ mice fed with high fat diet) as well as in human atherosclerotic arteries. Expression of SCUBE2 was gradually elevated following plaque progression in aortic sinus in the atherosclerotic mouse model. Consistently we have also found SCUBE2 to be expressed in both human coronary artery with DIT and advanced lesion of atherosclerotic plaque. Macrophage presence was not detectable in DIT, as lesion progressed however, macrophage infiltration occurred. SCUBE2 was colocalized not only with macrophages, but also with ApoB, Shh, and Ptc1 in advanced plaque. SCUBE2 may act on macrophage through Hh signals and induce atherosclerosis progression. Our study implied that SCUBE2 might be a new target molecule that plays a role in atherosclerosis lesion.

MATERIAL AND METHODS

Carotid artery ligation

Nine male 10-week-old C57BL/6J mice weighing 20 – 25 grams were used in all experiments. The animals were anesthetized using isoflurane inhalation and the left carotid arteries were dissected and ligated near the bifurcation. One or two weeks after the injury, the ligated left carotid arteries and sham operated right carotid arteries were collected for subject of analysis.

Low-density lipoprotein receptor knockout ($LDLr^{-/-}$) mice fed with high fat diet

$LDLr^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The $LDLr^{-/-}$ mice were fed standard CRF-1 mouse chow (Charles River Laboratories International, Inc.) until 10 – 12 weeks of age. Subsequently, they were switched to western diet with 1.5% cholesterol to stimulate a high fat diet (D12079B, Research Diets, Inc., NJ, USA). Whole aortas were collected and used for analyzing mRNA expression. The heart containing aortic sinus was frozen in Tissue-Tek OCT (Sakura Finetek USA, Inc.) for cryosectioning.

This study was approved by the Kobe Pharmaceutical University Animal Care and Use Committee, and animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe Pharmaceutical University, Act on Welfare and Management of Animals (Law No. 105; 1973, revised 2006), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88, 2006), and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No. 71, 2006).

RT-PCR and Real-Time PCR

Total mRNAs were extracted from aorta of mice using RNAisol (Takara Bio.Inc., Tokyo, Japan). A ReverTra Ace RT PCR Kit (Toyobo Co. Ltd, Japan) was used for reverse transcription. Real-time PCR using a 7500 Real-Time PCR System (Applied Biosystems, Japan) and Rotor Gene Q Series (Qiagen, Japan) with a Two Step SYBR® RT-PCR Kit (Toyobo Co. Ltd, Japan). The following primers were used for amplification : mouse SCUBE2 : forward, AGA CTT ATG AAC GCC CCA TC and reverse, TAG AGC CTG CCA TCT CGA AC; mouse GAPDH: forward, TGT GTC CGT CGT GGA TCT GA and reverse, TTG CTG TTG AAG TCG CAG GAG.

Human autopsy material

Human coronary arteries were obtained from 20 autopsy cases (aged 15 – 84 years) after informed consent was provided by their families. The clinical investigation conformed to the principles outlined in the Declaration of Helsinki, and was approved by the Institutional Review Board of Miyazaki University Graduate School of Medicine. Formalin-fixed human coronary artery samples were sectioned and subjected to hematoxylin-eosin (H-E) staining, immunohistochemistry and immunofluorescence staining.

Immunostaining

Formalin-fixed human coronary artery sections were deparaffinized, hydrated and subjected to antigen retrieval by microwave heating with strong heat 5 minutes and weak heat 10 minutes. Serial sections of human coronary arteries were incubated overnight with goat polyclonal human anti-SCUBE2 antibody (Santa Cruz Biotechnology), α-actin smooth muscle (Invitrogen) and mouse monoclonal human anti-CD68 (Dako Corp., Japan). For immunofluorescence double staining, following the incubation of anti SCUBE2 overnight, rabbit polyclonal anti Shh (Santa Cruz Biotechnology Inc. USA) was assessed. Fluorescence signals were detected using anti goat Alexa 488 (Invitrogen) and anti rabbit Alexa 594 (Invitrogen). The nuclei were visualized using DAPI (Molecular Probes). Immunofluorescence microscopy was performed using Olympus IX 71.

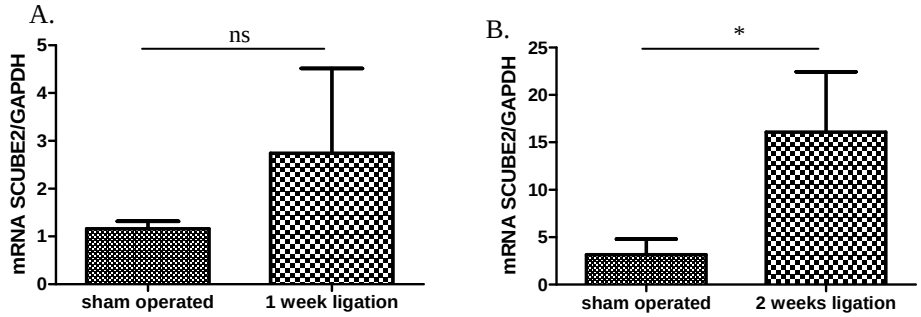
Murine carotid arteries were embedded in OCT compound immediately after euthanasia, section and fixed in cold acetone for 20 minutes. Carotid arteries and serial sections of sinus aortic were incubated overnight with rabbit polyclonal anti SCUBE2 antibody (Abcam Plc.), α-actin smooth muscle (invitrogen) and Mac3 (BD Pharmingen).

Statistical analysis

All data are presented in mean and standard error of mean. Statistically significant differences of SCUBE2 expression between mice with carotid ligation and sham operated mice was analyzed using the Mann-Whitney *U* test. Differences of SCUBE2 expression in normal diet, 4 weeks, and 8 weeks after beginning of high fat diet in $LDLr^{-/-}$ were compared using one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) test with equal or unequal variances. Values of $P < 0.05$ were considered significant.

RESULTS

Diffuse intimal thickening (DIT) is an early stage of atherosclerosis in humans. In order to know whether SCUBE2 is related to DIT, we performed left carotid artery ligation in mice as an animal model of human DIT. Ligated carotid arteries and sham operated control arteries were collected to study gene expression of SCUBE2 from 1 week and 2 weeks after injury. After 1 week ligation, SCUBE2 expression level started to increase (Figure 1A). Extended to 2 weeks after ligation, mRNA level of SCUBE2 was noticeably up-regulated compared to sham operated control (Figure 1B).



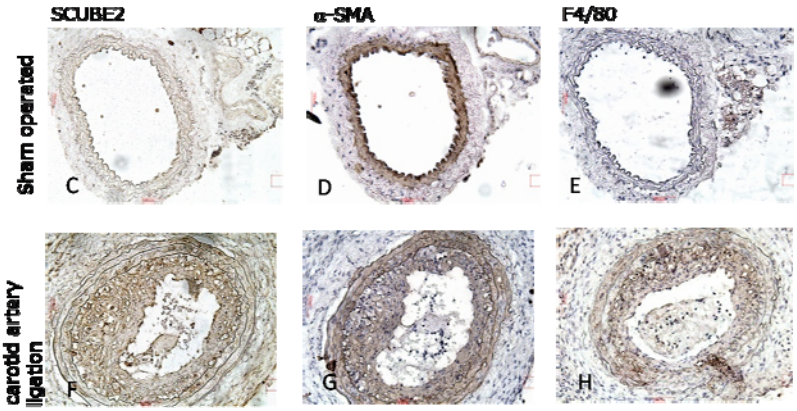


Figure 1. mRNA expression level of SCUBE2. One week after left carotid artery ligation, mRNA expression level of SCUBE2 started to increase (A). Extended to two weeks after ligation, mRNA level of SCUBE2 was significantly up-regulated (B). Immunohistochemistry serial section of SCUBE2, α-SMA, and F4/80 in sham operated carotid artery (C, D, E). SCUBE2, α-SMA, and F4/80 serial section two weeks after carotid artery ligation in C57BL/6J mice. At this stage, SCUBE2 co-localized dominantly with α-actin SMA, and to a lesser extent with F4/80 (F, G, H).

Further, we investigated SCUBE2 expression in DIT using serial section of immunohistochemistry in the carotid artery (Figure 1C-H). Two weeks after carotid artery ligation, thickened intima expressed SCUBE2 abundantly and a lesser amount was found in the medial layer (Figure 1F). Thickened intima layer which predominantly consisted of an α-actin positive cells, exhibited a co-localization with SCUBE2. F4/80 positive cells were in small amount and some of them were incorporated with SCUBE2 (Figure 1H). We identified the SCUBE2 expression in smooth muscle cells (SMCs) abundantly in the mice which underwent the model of DIT.

In order to mimic human atherosclerosis process, we fed LDLr$^{-/-}$ mice with high fat diet for 4 weeks as an early stage of atherosclerosis and for 8 weeks as a progressive stage of atherosclerosis. SCUBE2 mRNA level of whole aorta was increased significantly in LDLr$^{-/-}$ mice after eight weeks feeding with high fat diet (Figure 2A). To investigate which cells expressed SCUBE2, we performed immunohistochemistry staining with serial section of aortic sinus of LDLr$^{-/-}$ mice fed with high fat diet after 4 weeks (Figure 2E, F, G) and 8 weeks (Figure 2H, I, J). Plaque formation in aortic sinus was abundantly found in LDLr$^{-/-}$ after 8 weeks fed with high fat diet (Figure 2H, I, J). Both α-actin positive cells (Figure 2F) and Mac3, macrophage marker positive cells (Figure 2G) showed to express SCUBE2 (Figure 2E) 4 weeks after beginning of high fat diet. After 8 weeks with high fat diet, plaque lesion progressed and SCUBE2 was strongly expressed in the plaque lesion (Figure 2F, I, J).

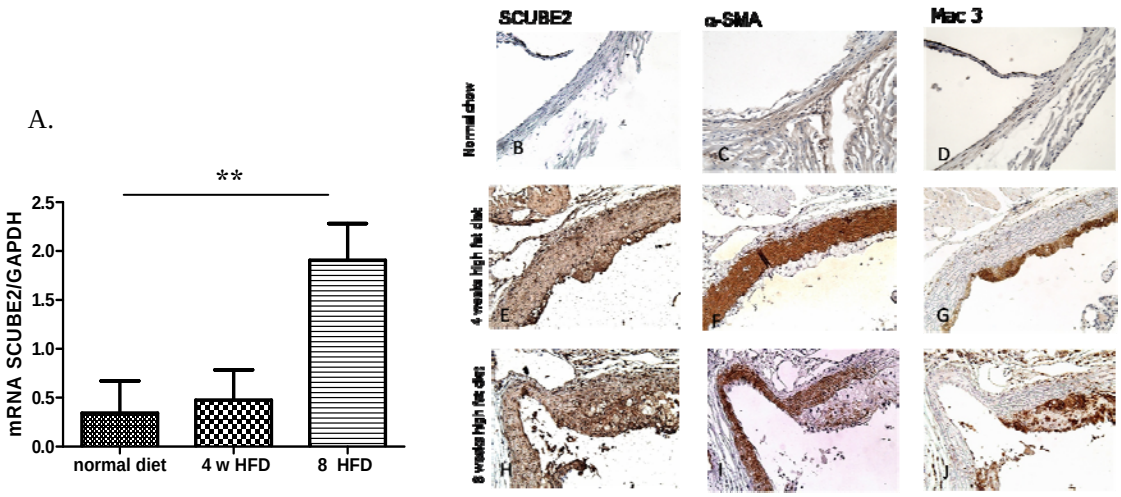
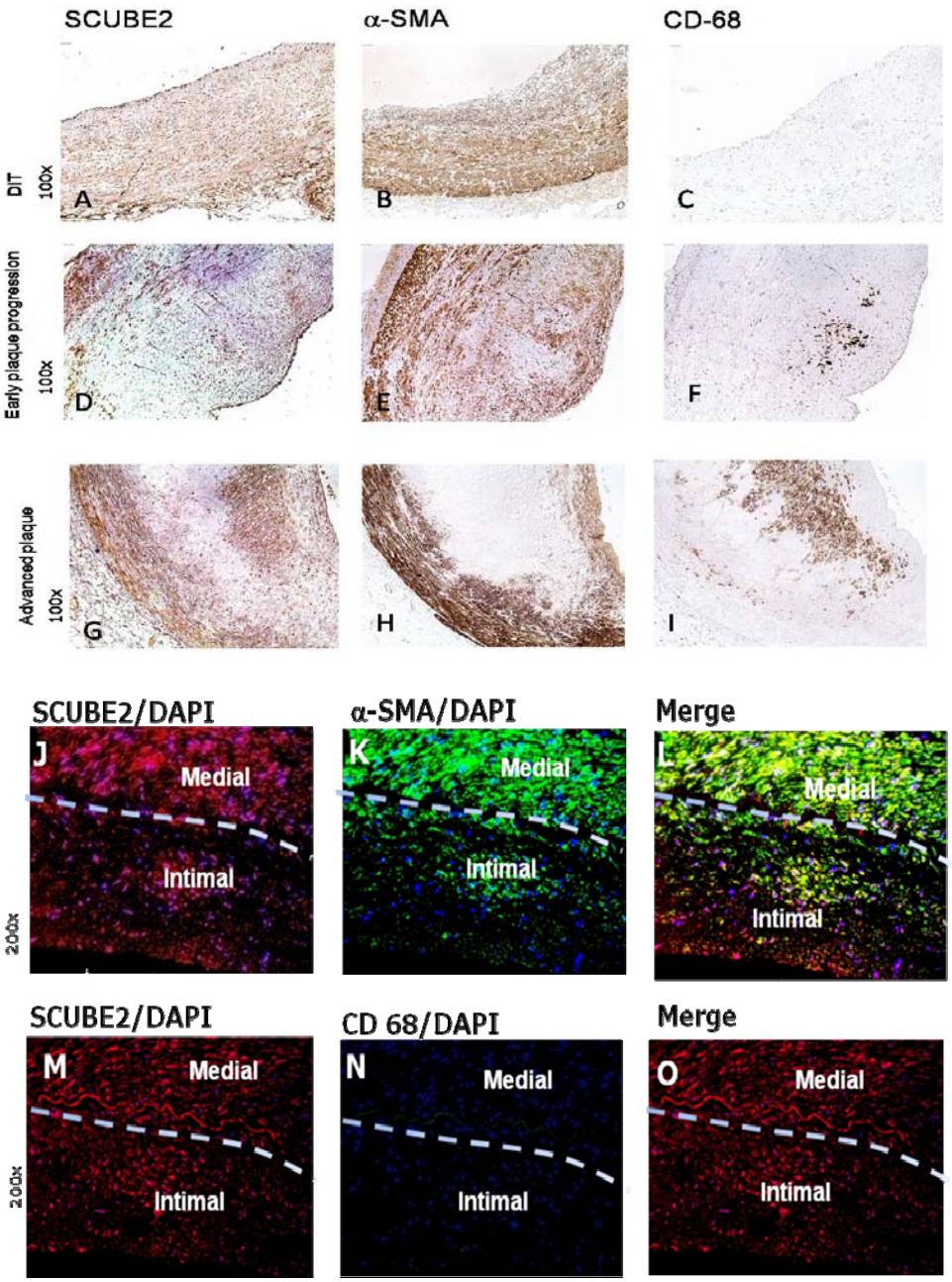


Figure 2. SCUBE2 expression in LDLr-/- mice fed with high fat diet (HFD). mRNA expression level of SCUBE2 increased markedly in LDLr$^{-/-}$ mice after 8 weeks fed with HFD (n = 3 – 6) (A). Serial section of SCUBE2, α-SMA, and Mac 3 in LDLr-/- mice fed with and without high fat diet. SCUBE2 expression seemed to increase after 4 weeks HFD and progressed after 8 weeks HFD compared to control (B, E, H). Co-localization of SCUBE2 with α-actin SMA and macrophage marker, Mac 3, showed strongly in 4 weeks and 8 weeks after HFD (C, F, I, D, G, J)

We extended our study on the role of SCUBE2 in human atherosclerosis by using human coronary artery with several stages of atherosclerosis. DIT is dominantly composed of SMCs both in intimal layer and medial layer. Macrophage infiltration has not been detected in this stage. SCUBE2 was detected in both intimal layer and medial layer of DIT in human coronary artery (Figure 3A, B, C). In an early plaque progressive stage of human coronary artery, macrophage infiltration increased and plaque formation was detected (Figure 3D, E, F). SCUBE2 was expressed scanty in the plaque area (Figure 3D). α-SMA positive cells were also expressed scanty and co-localized with SCUBE2 (Figure 3E). CD68 positive cells were apparent, infiltrated in the plaque area and co-localized with SCUBE2 strongly (Figure 3F).

As atherosclerosis progressed, plaque area was expanded. SCUBE2 was observed strongly in near-surface parts of the intimal layer. In other parts of the plaque, SCUBE2 was expressed scanty. α-SMA positive cells were detected in the area where SCUBE2 was expressed scanty (Figure 3G, H). In contrast, CD68 positive cells were strongly expressed with SCUBE2 in near-surface parts of intimal layer (Figure 3G, I). We confirmed using immunofluorescence staining that cells expressing SCUBE2 were co-localized with α-actin positive SMCs in DIT (Figure 3J-O). As lesion progressed, SCUBE2 stained cells were co-localized with α-actin positive SMCs and CD68 in some parts (Figure 3P-U).



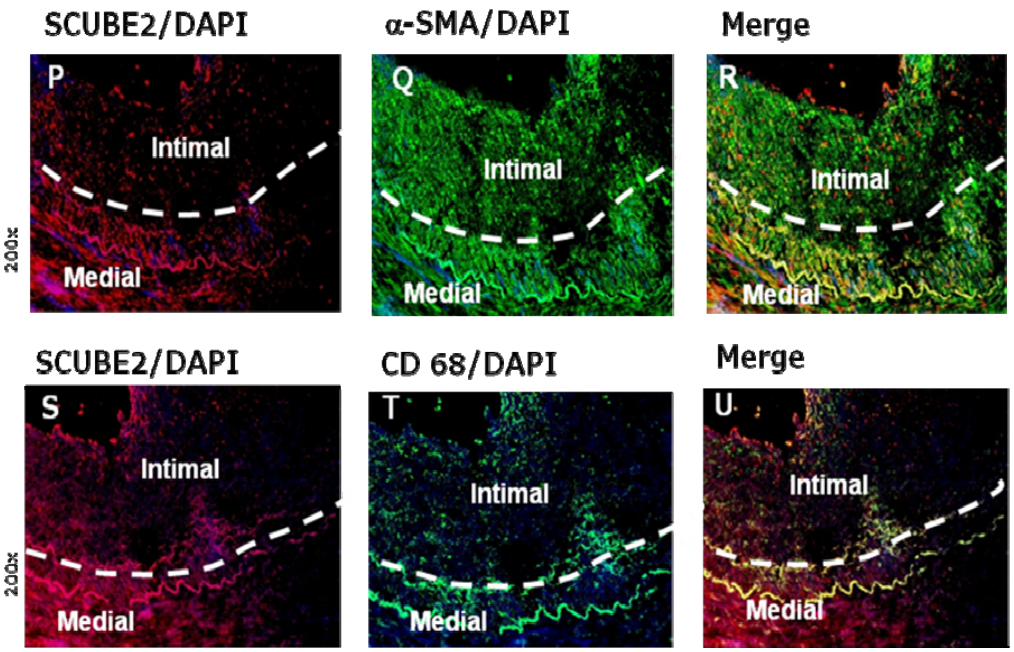


Figure 3. SCUBE2 expression in human coronary artery. Serial section of SCUBE2, α-SMA, and CD68 in human coronary artery consisting DIT, progressed lesion and advanced plaque, 100x magnification (A-I). 200x magnification of diffuse intimal thickening in human coronary artery (J-O). SCUBE2 co-localized with α-actin positive SMCs (J-L). Macrophage was not detected at this stage (M-O). 200x magnification of advanced lesion of atherosclerotic plaque (P-U). Macrophage infiltration was detected (S-U). Dotted lines indicate the intimal : medial boundary.

We have found that SCUBE2 was co-localized with CD68 positive cells in the advanced lesion of atherosclerotic plaque. Next, we confirmed whether SCUBE2 was co-localized with ApoB. We have detected that SCUBE2 expressing cells also showed an ApoB positive staining in human coronary artery (Figure 4).

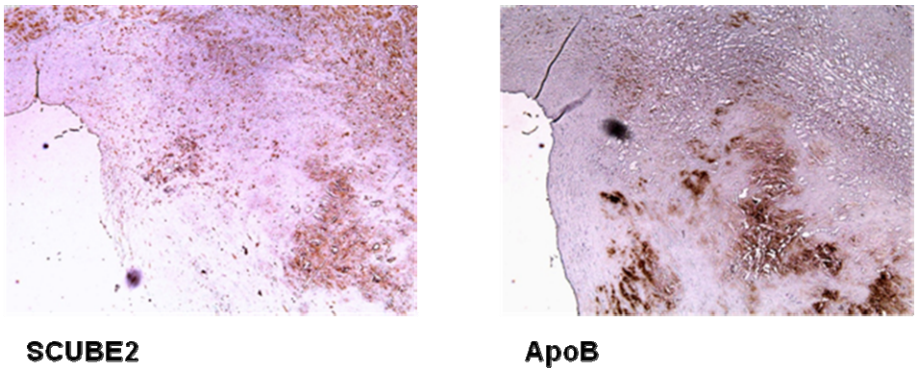


Figure 4. SCUBE2 co-localization with ApoB positive cells in human coronary artery. SCUBE2 was co-localized with apoB accumulation in advanced atherosclerosis plaque of human coronary artery.

As previously reported, SCUBE2 is regarded to mediate Shh signal transduction. We therefore investigated whether SCUBE2 was incorporated with Shh and its receptor, Ptc. Using serial section of immunohistochemistry staining on human coronary artery, we have found that SCUBE2 was co-localized with Shh in DIT (Figure 5A, B) and also in advanced plaque lesion (Figure 5C, D). Further, we confirmed our findings using immunofluorescence double staining of SCUBE2 and Shh. We have found that SCUBE2 was incorporated with Shh as well (data not shown).

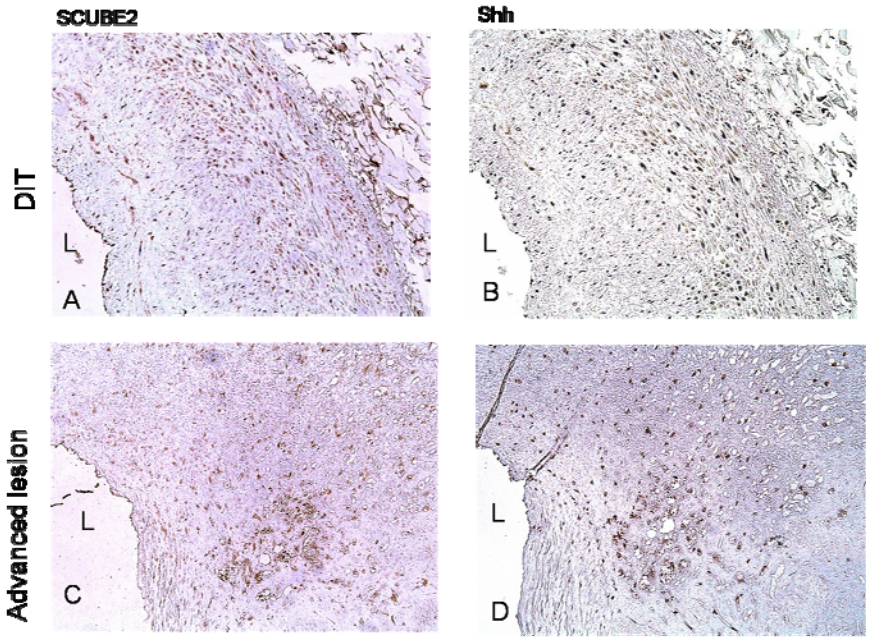


Figure 5. SCUBE2 co-localization with Shh. Immunohistochemistry staining of SCUBE2 and Sonic Hedgehog (Shh) co-localization in human coronary artery consisting DIT and advanced plaque

Ptc is known as hedgehog protein receptor. To confirm whether SCUBE2 was also incorporated with Ptc, we performed serial section of immunohistochemistry staining on human coronary artery. Consistent to hedgehog signaling, we have observed that SCUBE2 was also co-localized with Ptc both in DIT (Figure 6A, B) and in advanced plaque lesion (Figure 6C, D).

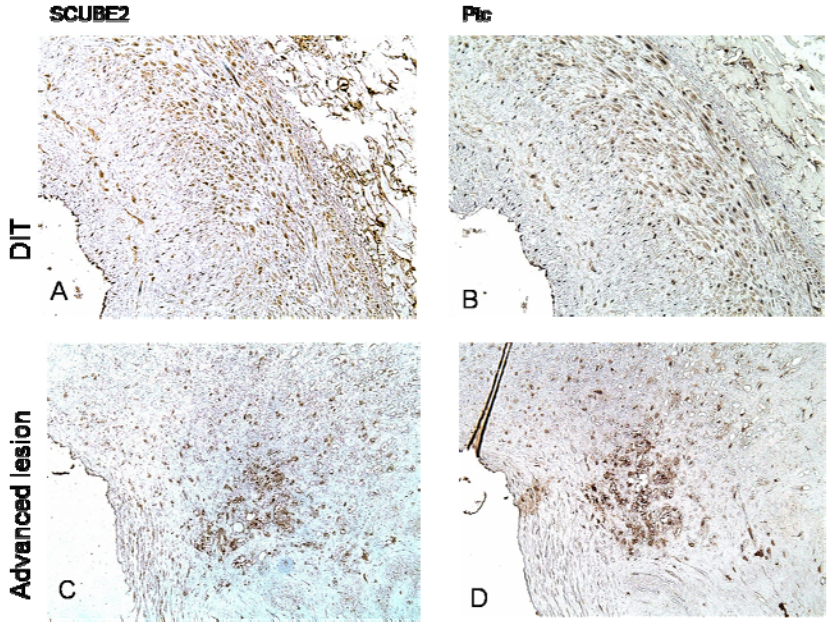


Figure 6. SCUBE2 incorporated with Patched (Ptc) receptor. Immunohistochemistry staining of SCUBE2 and patched receptor co-localization in human coronary artery with DIT and advanced plaque.

DISCUSSION

In this study we demonstrated that SCUBE2 was related to the development and progression of atherosclerosis in human coronary artery. Moreover, we have observed that SCUBE2 was co-localized with Shh and also with its receptor Ptc especially in the advanced plaque where macrophage infiltration and foam cell formation were found abundantly. This is the first evidence of the involvement of SCUBE2 in atherosclerosis.

At first, we used a mouse model of DIT or neointima formation, which is a model for an early stage of atherosclerosis in human. Unlike in humans, intimal layer in mice aorta is thin. Carotid artery was ligated to mimic DIT or neointima formation. After ligation of carotid artery, it is believed that in response to vascular injury, SMCs migrated into the intima layer and proliferate there like they do in the medial layer (18, 19, 20, 22). SCUBE2 co-localization with α-actin positive smooth muscle cells indicated that up-regulation of its expression was related to SMCs migration and proliferation in neointima formation.

Further, we focused on SCUBE2 expression during atherosclerosis process. Mimicking human atherosclerosis process, $LDLr^{-/-}$ mice treated with high fat diet were found to progressively form a plaque in aortic sinus 8 weeks after beginning of the high fat diet. Expression of SCUBE2 was gradually elevated together with the plaque formation in aortic sinus. This significant difference in SCUBE2 mRNA expression between 4 weeks and 8 weeks after beginning of high fat diet might be correlated with plaque formation. SCUBE2 was strongly expressed in the plaque area and was co-localized with macrophage marker positive cells. These results indicated that SCUBE2 was strongly related to the accumulation of macrophages and the formation of plaque.

Using this atherosclerosis mouse model, we could provide evidences that SCUBE2 played a role in atherosclerosis. Furthermore, we investigated SCUBE2 in DIT as an early atherosclerotic event and extend our investigation to advanced lesion of atherosclerotic plaque. We detected SCUBE2 in DIT. At this early atherosclerotic stage, SCUBE2 was diffusely distributed in the thickened intima and showed a co-localization with α-actin positive SMCs. Following an early plaque progressive lesion, SCUBE2 was distributed in the vicinity of atherosclerotic plaque and showed co-localization with α-actin positive SMCs. SCUBE2 was also associated with macrophages. The co-localization of SCUBE2 and macrophage became prominent in advanced lesion of atherosclerotic plaque.

Previous studies reported that SCUBE2 was found to mediate Shh protein release and activated Hh signal transduction. SCUBE2 also enhanced Shh binding to its receptor, Ptc (3, 11, 12, 13, 24, 26). We could confirm these observations with our immunochemical study where detection of SCUBE2 was associated with Shh and its receptor Ptc both in DIT and advanced lesion. The relation of hedgehog signaling with cardiovascular diseases is still disputed (5). Kusano et al reported that Shh-induced vasculature in Shh-treated diabetic rats showed a significantly larger vessels and greater number of α-actin positive cells (15). This study was then supported by Morrow et al, who investigated the effect of Shh overexpression in both rat and human smooth muscle cells (SMCs). They have observed that hedgehog stimulation resulted in a significant elevation of SMCs proliferation. It is believed that Hedgehog stimulation on SMCs growth is induced through Notch signaling. Further, after vascular injury by ligation of carotid artery in mice, intima SMCs expressed abundantly Ptc1 receptor and Gli (an Hh target gene) implying Shh activation in these cells (17).

Moreover, a study by Dunaeva et al demonstrated that Shh induced human monocyte chemotaxis obtained from diabetes mellitus (DM) patients with and without coronary artery disease (CAD) as well as a higher expression of Ptc among them compared to control (6). Recent findings from Athanasiadis et al on genetic determinants of plasma β_2-glycoprotein I levels (β_2-GPI) phenotype revealed that SCUBE2 is strongly related with this gene (1). In atherosclerotic lesion, β_2-GPI has been reported to be expressed in endothelial cells and monocyte/macrophages. This molecule also has been identified in human carotid plaques after carotid endarterectomy and a higher level was found in plasma of patients with ischemic heart disease and unstable angina (7, 8). How SCUBE2 and β_2-GPI are connected in atherosclerosis is not described yet.

Based on previous evidences indicating that SCUBE2 is upstream the Shh signaling pathway, we can speculate that SCUBE2 upregulation in early stage of atherosclerosis may activate Shh signal transduction. Bijlsma et al hypothesized that Hh signaling is transferred from LDL particles located in thickened intima, induces SMCs proliferation and migration, and modulates T-lymphocyte cell progression, activation, and cytokine production (2). In addition, an observation in drosophila indicates that lipophorin are required for the transport of highly hydrophobic Hh molecules. In mammals, LDL might also be loaded with Hh and play a role as carrier protein (21). These facts might be put in perspective with our finding on SCUBE2. We may hypothesize that the abundant LDL particles present in thickened intima, might upregulate SCUBE2 expression in this stage. An increased release of Shh and its receptor Ptc might thus occur. The elevated Shh may induce SMCs proliferation, increase monocyte chemotaxis, and upregulate several growth factors as previously investigated.

In conclusion, we have shown that the secreted and membrane anchored SCUBE2 was present in diffuse intima thickening and advanced lesion of atherosclerotic plaque. Even though the atherosclerosis processes in humans and mice differ in term of vessel size, structure, hydrodynamics, embryonic origin, abundance of SMCs and elasticity (23), we could observed the same pattern of SCUBE2 expression during the process in both species. Taken together, SCUBE2 might represent a new target molecule for atherosclerosis. The expression of SCUBE2 in the SMCs and macrophages/foam cells of human coronary artery indicated a potential role in SMCs proliferation, migration or cell differentiation and inflammation reaction through hedgehog signaling pathway in

atherosclerotic plaque. Further investigation of the potential implications of SCUBE2 in atherosclerosis is needed.

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