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In Vitro Analysis of SCUBE1 Expression in Aortic Vascular Smooth Muscle Cells on Atherosclerosis Progression

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ABSTRACT

Background: *Signal peptide-CUB-EGF domain-containing protein-1 (SCUBE1) is a platelet–endothelial glycoprotein implicated in thrombosis and vascular inflammation, and has been detected within atherosclerotic lesions. Whether vascular smooth muscle cells (VSMCs) upregulate SCUBE1 under inflammatory stimuli remains unclear.* ***Objective:*** *To quantify SCUBE1 mRNA expression in rat aortic VSMCs following lipopolysaccharide (LPS) exposure in an organ-culture model of atherogenesis.* ***Methods:*** *This study aimed to analyze SCUBE1 expression in aortic VSMCs in atherosclerosis using real-time PCR. This study was a true experimental-post test control group design using organ culture. The aorta from six wistar rats were divided into two groups, consisting of five samples that were not induced with LPS and five samples exposed to LPS at a concentration of 10* μg/mL for 48 hours. *Expression of the SCUBE1 gene was then analyzed using real-time PCR. The data was then processed using the dependent T-Test.* ***Results:*** *The result showed LPS increased SCUBE1 transcript levels compared with control (mean ± SD: 0.78 ± 0.22 vs 0.44 ± 0.35), representing a non-significant trend toward induction (p=0.07). These preliminary data are consistent with SCUBE1's inducibility in inflammatory states and its presence in atherosclerotic tissue.* ***Conclusion:*** *This study revealed increased SCUBE1 gene expression in aortic VSMCs in the LPS-treated group. These data suggest that SCUBE1 has the potential to be a marker of endothelial dysfunction in atherosclerosis.*

1. INTRODUCTION

Cardiovascular diseases are the most common cause of death worldwide and the most significant contributor to the global burden of disease among non-communicable diseases. In 2019, there were approximately 56.5 million deaths worldwide, of which cardiovascular disease accounted for 32.9% (18.6 million deaths).[1] Based on Indonesia's 2018 national report, Riset Kesehatan Dasar (Riskesdas), 1.5%, or around 1,017,290 people, were diagnosed with heart disease.[2]

The pathophysiology of atherosclerosis is complex and does not only involve lipid infiltration into the intima.[3] This pathological state is characterized by the accumulation of lipids in the blood vessels accompanied by the proliferation and migration of vascular smooth muscle cells (VSMCs) as well as extracellular matrix (ECM) synthesis, which ultimately contributes to plaque

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progression. The inflammatory process plays an essential role in the progression of atherosclerotic lesions and is thus a chronic inflammatory disease.[4] The theory that atherosclerosis is an inflammatory disease was first proposed by Russell Ross in 1999, based on the discovery of circulating monocytes in fatty streaks.[5] Atherosclerosis begins with dysfunctional changes in the endothelium, which leads to endothelial and platelet activation, monocyte adhesion to the activated endothelium, and differentiation of these cells into proinflammatory macrophages. These cells absorb oxidized LDL (oxLDL) and transform into foam cells, enhancing the inflammatory response.[6] Inflammation in the intima also results in the migration and proliferation of VSMCs.[7]

Based on their contractile ability, VSMCs play a crucial role in maintaining vascular tone and regulating blood pressure.[8] In the development of atherosclerosis, VSMCs are significantly involved in plaque and ECM formation. Research has shown that the stability of plaques depends on the strength of the fibrous cap, which is synthesized by VSMCs from the media layer. Furthermore, studies have demonstrated that VSMCs exhibit plasticity in changing their phenotype, which can positively and negatively impact disease progression. The complex nature of VSMC plasticity and its implications in atherosclerosis are still the subject of ongoing research. Understanding VSMC plasticity and its role in vascular diseases, including atherosclerosis, is an evolving study area with potential implications for developing targeted therapies.[9]

Given the central role of VSMCs in plaque development and vascular remodeling, profiling *SCUBE1* expression in VSMCs was considered mechanistically relevant in this study. VSMCs are not merely passive structural cells, but active participants, in atherogenesis through migration, proliferation, ECM remodeling, and fibrous cap formation.[9] This makes them a biologically important cell population for evaluating molecules that may contribute to inflammatory vascular remodeling. In addition, *SCUBE1* is functionally linked to pathways relevant to VSMC biology, particularly BMP-related signaling, which has been implicated in vascular inflammation, remodeling and lesion progression.[10,11] Although *SCUBE1* has more commonly been associated with platelets, endothelial cells, and the subendothelial matrix, its expression in aortic VSMCs remain insufficiently characterized. Therefore, evaluating *SCUBE1* in VSMCs may help clarify whether this molecule participates more directly in vascular wall inflammation and remodeling during atherogenesis.

The search for genetic determinants of atherosclerosis has been a longstanding endeavor. Current research in this field is focused on identifying potential genes involved in the atherogenic pathway and conducting experimental studies to evaluate their role in the pathogenesis of atherosclerosis.[12] The *SCUBE1* gene have been increasingly recognized for their potential involvement in various biological processes and development of diseases characterized by inflammation. It is located on the 22q1312 chromosome and is the first isoform of the SCUBE protein. This molecule comprises an N-terminal signal peptide sequence, nine EGF-like sequential repeats, a spacer region, three cysteine-rich repeat motifs, and a CUB domain.[13–17] The EGF-like sequences and cysteines in *SCUBE1* are also found in various growth factors, transmembrane receptors, adhesion molecules, signaling proteins, and ECM components. The CUB domain, which consists of three proteins, has a role in interactions between proteins and has been found in a set of proteins involved in developmental processes such as embryogenesis or organogenesis.[18]

The bone morphogenetic protein (BMP) is a member of the transforming growth factor-β (TGF-β) superfamily, which plays a vital role in the regulation of inflammation in various types of cells, including endothelial cells, macrophages, T-cells, fibroblasts, and VSMCs. It is well-established that BMPs carry out vital functions during embryogenesis, bone morphogenesis, vascular remodeling, and the development of various organs. With the advancement of research, BMPs have been found to have a close relationship with cardiovascular diseases, particularly atherosclerosis.[10] *SCUBE1* protein can form a complex with BMP2, one of TGF-β ligands, serving as a BMP co-receptor to enhance BMP signaling activity.[11] In a mouse animal model of early atherosclerosis, BMP2 synthesized by VSMCs from atherosclerosis lesions can increase monocyte migration and accelerate the inflammatory process in the disease via BMP2 receptor activation.[10]

Furthermore, an in vivo study by Yang RB et al. has shown that *SCUBE1* gene expression increases 48 hours after LPS induction, suggesting the potential role of *SCUBE1* in the inflammatory process.[17,18] With this, the authors aim to investigate the possibility of an increase in *SCUBE1* gene expression in aortic VSMCs to determine whether *SCUBE1* plays a role in the inflammatory process of atherosclerosis.

2. METHODS

This research was conducted at the Biomedical Laboratory, Faculty of Medicine, Universitas Andalas, with the approval from the Ethics Committee of the Faculty of Medicine through the Certificate of Passing the Ethics Review No. 579/UN.16.2/KEP-FK/2023.

Under sterile conditions, anesthetized wistar rats (*Rattus norvegicus*) were placed supine, and the chest was opened. The descending aorta was removed and transferred to a 15ml falcon tube containing cold DMEM (4°C). After removal of fatty tissue around the aorta, the artery was cut longitudinally into segments of approximately 1 mm and placed in a 24-well plate containing 500 µl DMEM. The aortic segments were then allocated into two groups consisting of five samples each: a control group without LPS and a treatment group exposed to LPS at a concentration of 10 µg/mL. All samples were incubated in a CO_2 incubator at 37°C for 48 hours.[19,20]

Total RNA from tissues of all experimental groups was isolated using TRIzol reagent (Thermo Fisher Scientific, CA, USA). Tissues were homogenized using a homogenizer with 1 mL of TRIzol reagent per 10 mg of tissue in the sample. Then, 200 µl chloroform were added, the tube was inverted and incubated for 5 minutes at room temperature. Next, centrifuge at 12,000×g at 4°C for 15 minutes. The aqueous layer was transferred to a new sterile microtube. 2x isopropanol were added and incubated again for 10 minutes at room temperature. Centrifuge again at 12,000×g at 4°C for 10 minutes. Then, the supernatant was discarded, and the pellet was washed with 70% ethanol for 350 µl. Next, the tube was inverted and vortexed gently. Centrifuge again at 7,500×g at 4°C for 5 minutes. Then, discard the supernatant and vacuum it for 10 minutes. After the vacuum is complete, resuspend the pellet in RNAse Free Water 25-40 µl (depending on the number of pellets). Then, the RNA was quantified and equalized at a concentration of 1000 ng.

The cDNA synthesis was performed using a synthesis kit (Sensifast Bioline). The composition of total cDNA synthesis was 10 µl total RNA, 4 µl reverse transcriptase, 1 µl iScript, and 5 µl RNAse Free W ater with a reaction volume of 20 µl. Total cDNA synthesis was performed at ice temperature according to the working protocol following the kit manual.

All PCR processes were performed in an amplification range of 40 amplification cycles consisting of a pre-denaturation step of 95°C for 3 minutes, initial denaturation of 94°C for 5 minutes, annealing of 50-60°C for 30 seconds, then extension of 72°C for 45 seconds.

After cDNA synthesis is complete, RT-PCR using gene primers will be used according to the design and temperature optimization that has been done. The gene primer sequence and temperature used for gene expression analysis can be seen in Table 1.

Table 1.
SCUBE1 and GAPDH primers

Gene	Forward primer	Reverse primer	Temperature
SCUBE1	5'- CGCAAAC TCTGGAT CCAGTTC- 3'	5'- GAACATC TCCTTGGA CTCCTGG- 3'	58°C
GAPDH	5'- ACACATT GGGGGTA GGAACA-3'	5'- AAGGGCT CATGACC ACAGTC-3'	58,3°C

Table 2.
Real time PCR components

Components	Reaction
Sybr	5
Nucleus-free water (NFW)	3,2
Forward	0,4
Reverse	0,4
cDNA	1
Total	**10**

The Livak equation or delta-delta Ct method (2-ΔΔCt) was used to analyze the gene expression. The 2-ΔΔCt method involves calculating the difference between the Ct values of the target gene (*SCUBE1*) and the reference gene (GAPDH) for LPS-induced and non-LPS-induced samples.

The results of this study were processed by conducting a normality test using SPSS. The Shapiro-Wilk test was used to determine whether the data were normally distributed. Normally distributed data was then analyzed with parametric statistical tests using the dependent T-Test test to determine the difference between the control and treated groups.

3. RESULTS

SCUBE1 expression in aortic VSMCs of non-LPS- induced control group

The control group (K1, K2, K3, K4, and K5) were aortic tissue samples not given LPS. Based on real-time PCR results (Table 3), *SCUBE1* expression in the non-LPS-induced control group showed a mean value of 0,44 ± 0,35.

Table 3.
SCUBE1 gene expression levels in control group

Group	*SCUBE1* gene expression	Mean ± SD
K1	1	
K2	0,13	
K3	0,41	0,44 ± 0,35
K4	0,16	
K5	0,50	

SCUBE1 gene expression in aortic VSMCs of LPS- induced group

The treated group (P1, P2, P3, P4, and P5) were aortic tissue samples given LPS. The mean *SCUBE1* gene expression in the treatment groups can be seen in Table 4.

Table 4.
SCUBE1 gene expression levels in treated group

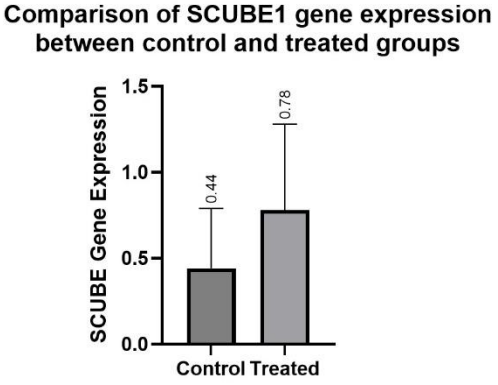
Group	*SCUBE1* gene expression	Mean ± SD
P1	1,33	
P2	0,56	
P3	0,38	0,78 ± 0,22
P4	0,32	
P5	1,31	

The LPS-induced treatment group in this study showed higher *SCUBE1* gene expression than the control group. This study's results align with an in vivo study by Yang RB et al., which showed that *SCUBE1* gene expression in mice increased 48 hours after systemic LPS injection.[18] These data suggest that *SCUBE1* is involved in the inflammatory response with LPS stimulus.

Comparison of SCUBE1 gene expression between control and treated groups

This study showed increased *SCUBE1* gene expression in aortic VSMCs before and after LPS induction, as shown in Figure 1.

Figure 1.
Comparison of SCUBE1 gene expression between control and treated groups



This study is the first to analyze *SCUBE1* gene expression by real-time PCR in aortic VSMCs. The data obtained proved to be insignificant after statistical analysis. The aortic organ culture method employed in this study entails intracellular interactions, and the dosage of LPS is suboptimal for stimulating *SCUBE1* gene expression.

4. DISCUSSION

To this day, no studies have been conducted on the expression of *SCUBE1* in aortic VSMCs; therefore, the data we obtained are preliminary. Smooth muscle cells account for an average of 90-95% of the cellular component in early-stage atherosclerotic lesions and nearly 50% in advanced stages. In response to various stimuli, such as inflammation, VSMCs from the tunica media migrate to the intima layer and proliferate. This process is the initial step of atherosclerotic lesion formation.[21] Under normal conditions, smooth muscle cells in the aorta are maintained in a secretory state, indicating that these cells not only have contractile properties but are also capable of producing ECM, which is essential for maintaining the typical structure of the aorta. LPS induction was shown to have various effects on VSMCs. Research by Jiang et al. showed that LPS promotes the proliferation of rat aortic VSMCs, which may contribute to the development of cardiovascular disorders.[22] Lower *SCUBE1* gene expression in the non-LPS-induced group suggests that LPS regulates *SCUBE1* gene expression in aortic VSMCs.

The present findings should be interpreted with caution because several factors may attenuate or obscure measurable SCUBE1 expression in VSMCs. This study used an organ culture method, which involves complex interactions among multiple vascular wall cell types; therefore, the measured transcript may not fully represent a purely VSMC-specific response. In addition, although all aortic segments were processed uniformly, SCUBE1 expression may still be influenced by differences in vascular wall composition, regional heterogeneity of the aorta, and phenotypic switching of VSMCs under inflammatory and ex vivo culture conditions. Another important consideration is that SCUBE1 has been more consistently associated with endothelial cells, platelets, and the subendothelial matrix than with constitutive smooth muscle expression, which may partly explain a relatively low basal signal in VSMCs. Therefore, while the observed

increase after LPS induction supports a possible role of SCUBE1 in vascular inflammation, the possibility of confounding factors affecting the final expression profile should be acknowledged.

Atherosclerosis is a pathological condition characterized by chronic inflammation of the arterial wall. Characteristic of chronic inflammation involves the accumulation of lipids and increased inflammatory cells within atherosclerotic lesions.[23] Apart from activating inflammatory pathways, abnormal proliferation of VSMCs also plays a role in vascular diseases such as atherosclerosis.[24] Recent studies have revealed that exposure to LPS can increase the expression of inflammation-related genes. For example, a study conducted in mice by Palladino et al. found that LPS-induced inflammation can increase the expression of hypoxia-related genes, such as ANGPTL4, EGR1, IER3, PAI1, and GLUT1, as well as increase genes that contribute to inflammation, such as CCL12, CC13, CD14, CXCL10, ICAM1, IL-10, IL-1β, IL-6, NFKBIA, TLR2, AND TNF.[25] The study by Dervishi et al. supports this theory with their research, which concluded that exposure to LPS in mice impacts the expression pattern of inflammation-related genes.[26] The increased expression of *SCUBE1* after LPS induction suggests that *SCUBE1* may be involved in the inflammatory process in response to LPS and illustrates the potential of this gene in its regulation.

The dose of LPS used in this study is based on in vitro studies that have proven that LPS at a dose of 10 μg/ml can trigger the proliferation of VSMCs, which is one of the significant stages in the progression of atherosclerosis.[24] To date, no literature has yet discussed the dose of LPS needed to stimulate *SCUBE1* gene expression optimally.

Localization of the *SCUBE1* protein in atherosclerotic lesions was first performed by Tu CF et al. using immunohistochemical analysis. The study, however, did not detect any *SCUBE1* protein localized in SMCs.[27] Using real-time PCR, this research found increased *SCUBE1* gene expression in aortic VSMCs induced with LPS in vitro. This discrepancy between protein and gene detection highlights the importance of using multiple methods to validate findings and the need to consider the limitations of each technique when interpreting results.

Experimental studies often use time-dependent and dose-dependent designs to better characterize biological responses to a stimulus. Time-dependent analysis evaluates response kinetics at different time points, while dose-dependent analysis determines the concentration required to induce a biological effect. Future studies should therefore investigate varying concentrations of LPS and incubation periods to better characterize *SCUBE1* gene expression.

Variability in real-time PCR measurements may arise from experimental factors such as contamination, pipetting errors, or unstable reference genes. In this study, the variability in *SCUBE1* expression may be related to the use of a single reference gene that may not be stable across different experimental conditions. Future studies are therefore recommended to use multiple reference genes to improve normalization accuracy.[28] Another limitation of this study is the use of an organ culture model involving complex interactions among multiple cell types. Future studies using VSMC cultures may enable a more specific evaluation of *SCUBE1* gene expression at the cellular level.

5. CONCLUSION

This study revealed increased *SCUBE1* gene expression in aortic VSMCs in the LPS-treated group, however the difference was not statistically significant. These data suggest that *SCUBE1* has the potential to be a marker of endothelial dysfunction in atherosclerosis.

6. ACKNOWLEDGMENTS

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