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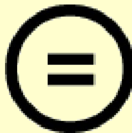

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The relationship between high-density lipoprotein associated proteins and its biological functions

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The relationship between high-density lipoprotein associated proteins and its biological functions

Directed by Professor Sang-Hak Lee

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degree of Master of Science for Aging

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ABSTRACT

The relationship between high-density lipoprotein associated proteins and its biological functions

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(Directed by Professor Sang-Hak Lee)

Low levels of high density lipoprotein (HDL) cholesterol are an important predictor for cardiovascular disease. Based on the anti-atherogenic property of HDL cholesterol, some scholars suggest that HDL particles may contribute to protective effects for cardiovascular disease. Recent proteomics studies showed that over 100 proteins are associated with HDL and these HDL proteins are related to several biological roles, including lipid transport, protease inhibitor activity, and complement pathways. However,

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specific HDL functions in relation to HDL-associated proteins remain unknown.

We examined the correlation between multiple parameters of HDL function and HDL-associated proteins. A total of 21 patients with coronary artery disease or risk factors participated in this study. The cholesterol efflux capacity and other anti-inflammatory functions were assessed, and preselected HDL proteins were measured based on HDLs from the participants. The results showed that the cholesterol efflux capacity positively correlated with apolipoprotein A1 (apoA1) and apoC3, while apoA1 and apoC1 had negative associations with VCAM-1 expression. In conclusion, we suggest that multiple HDL proteins show a relationship with HDL functions, and further study investigating the effect of more HDL-associated proteins on HDL function may be helpful for the development of novel therapeutics for cardiovascular disease.

Key Words: high-density lipoprotein, apolipoprotein, correlation, cholesterol efflux, VCAM-1 expression

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Ⅰ. INTRODUCTION

Epidemiological studies have shown an inverse association of high density lipoprotein (HDL) cholesterol levels with the risk of cardiovascular events [1]. Because of the protective effects of HDL cholesterol on cardiovascular disease (CVD) and its heterogeneous composition [2], some researchers have investigated whether HDL

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particles may contribute to the anti-atherogenic properties of HDL.

HDL has two significant roles involved in anti-atherogenic properties. First, it protects against atherosclerosis by regulating cholesterol efflux from peripheral tissues and by returning cholesterol to the liver [3,4]. HDL has also been shown to modulate inflammation. Rye et al. demonstrated that the expression of pro-inflammatory molecules related to leukocyte extravasation was inhibited by HDL in the endothelial cells of healthy individuals [5,6].

However, several clinical studies had shown that measuring HDL cholesterol levels to determine the risk for CVD is not the most-appropriate way [4] and does not indicate the properties of HDL. Therefore, recent studies have performed several cell culture experiments to quantitatively measure HDL functions. In one study, HDL from healthy or coronary artery disease (CAD) patients showed different endothelial anti-inflammatory effects from NO, superoxide, or VCAM-1 production [7].

Previous studies have suggested that the binding of HDL3 to its cell-surface receptor facilitates cholesterol efflux from cells [8]. Subsequent studies have shown that the interaction with the ATP-binding cassette transporter A1 (ABCA1), which is a cell-membrane protein, and HDL apolipoproteins removes cellular cholesterol [9]. These findings suggest that HDL particles perform an important role in diverse biological functions.

Recent proteomics studies have revealed that over 100 proteins are associated with

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HDL [10]. Interestingly, a gene ontology (GO) analysis demonstrated that HDL proteins are related to several categories, including lipid metabolism, protease inhibitor activity, complement regulation, and the acute-phase response [11]. However, specific HDL functions in relation to HDL-associated proteins remain unknown. The objective of this study is to identify the relationship between the multiple parameters of HDL function and HDL-associated proteins.

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Ⅱ. MATERIALS AND METHODS

1. Study participants

A total of 21 patients with a history of atherosclerotic cardiovascular disease, high cardiovascular risk or diabetes mellitus were included in this study. Participants who were pregnant or breast feeding were excluded. Other exclusion criteria were a history of acute cardiovascular or cerebrovascular disease within 3 months of enrollment, thyroid dysfunction, infection, serum transaminase level >2 times upper limit of normal, uncontrolled hypertension or diabetes mellitus, serum creatinine >1.5 mg/dL, or a history of cancer or adverse reaction to test drugs. All patients provided written informed consent.

2. Blood sampling and isolation of HDL

Blood samples were collected from each patient. Samples were analyzed within 4 h by a local laboratory, certified by the Korean Society for Laboratory Medicine. The lipid levels were measured by an auto-analyzer. Ultracentrifugation was used to isolate HDL as described below. Briefly, 0.12 g potassium bromide (KBr) and 0.045 g sucrose were added to 2 mL of serum sample and this mixture was transferred to a 12 mL-ultracentrifuge tube (Polyallomer, Beckman Coulter Korea Ltd, Seoul, Korea). Then, 2 mL of solution B (1 mL distilled water, sodium chloride [NaCl] 0.012 g, and KBr 0.135 g), 4 mL of solution A (1 mL distilled water, NaCl 0.012 g, and KBr 0.318 g), and 4 mL of distilled water were sequentially added. Next, a Beckman Coulter XL-100K Table Top

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Ultracentrifuge with a Beckman fixed-angle rotor (SW41Ti) conducted ultracentrifugation for 18 h at 35,000 rpm at 4 ℃. Then, lipoprotein with very low density and LDL, which contained supernatant, were removed, and HDL was isolated. Finally, the isolated HDL was desalted and concentrated with an Amicon 30k ultra-centrifugal filter device (Merck Millipore Korea, Korea) at 3000 rpm at 4 ℃.

3. Measurement of selected HDL proteins

Based on the previously reported preliminary proteomic analysis of HDL samples [12], we chose five HDL-associated proteins that were richly and reproducibly detected: apoA1, apoA2, apoC1, apoC2, and apoC3. The same amounts of HDL samples (0.5 – 10 μg) were separated on 15 % SDS-polyacrylamide gel and transferred to a PVDF membrane. After blocking the membranes, and incubated with primary antibodies against apoA1, apoA2 (Santa Cruz Biotechnology, Dallas, TX, USA), apoC1, apoC2 (Abcam, Cambridge, MA, USA) and apoC3 (Academy Bio-Medical Company Inc., Houston, TX, USA). Membranes were then incubated with appropriate HRP-conjugated bovine anti-mouse or goat anti-rabbit secondary antibodies (Santa Cruz). Chemiluminescence with ECL reagent (GE Healthcare, Piscataway, NJ, USA) was used to detect the signal and the band intensities were quantified by the ImageJ software.

4. Cell culture

Murine J774 macrophages were cultured in Dulbecco’s modified Eagle media (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1 % antibiotic-

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antimycotic in humidified 5 % CO2 at 37 ℃. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and grown in endothelial basal medium (EBM)-2, which contains EGM-2 growth factors, cytokines, and supplements with 2 % FBS. Cells were maintained in humidified 5 % CO2 at 37 ℃ and used for experiments between passage 5 and 7.

5. Cholesterol efflux assay

The J774 cells were grown in 24-well plates and radiolabeled with 2 μCi of ^{3}H-cholesterol/mL for 24 h. To up-regulate ATP-binding cassette transporter A1 (ABCA1) expression, the cells were equilibrated in medium containing 0.2 % bovine serum albumin (BSA) and 0.3 mM cyclic adenosine monophosphate (cAMP) for 2 h. Subsequently, for efflux induction, cells were incubated with 0.2 % BSA and HDL for 4 h. The steps were performed by treatment the cells with 2 μg/mL acyl-coenzyme A:cholesterol acyltransferases (ACAT) inhibitor. ^{3}H-cholesterol in cell lysates and medium was detected using a Scintillation counter (β-counter), respectively. Cholesterol efflux values were the percent efflux which was calculated % of ^{3}H-cholesterol (μCi) in medium relative to total ^{3}H-cholesterol (μCi) in medium and cells. The values were normalized to the efflux capacity of the pooled serum included on each plate.

6. Nitric oxide (NO) assay

HUVECs were plated in 12-well plates and grown until confluency. After overnight incubation in serum-free medium, cells were treated with 50 μg/mL HDL in the fresh

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serum-free medium for 4 h. The treated cells were washed with DPBS and lysed in a buffer containing 5 mM Tris-HCl (pH 6.8) and 0.1 % SDS. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 ℃ and then the supernatants were transferred to Amicon 10 kDa-cut off filter tubes (Merck Millipore Korea). After centrifuged at 40,000 g for 30 min at 4 ℃, the flow-through was collected. The nitrite level was assayed using a kit according to the manufacturer's instruction (Cayman Chemical, Ann Arbor, MI, USA).

7. Immunoblot analysis

HUVECs were seeded on 12-well culture plates and incubated with 5 ng/mL of tumor necrosis factor-α in serum-free media for 19 h to induce VCAM-1 expression. Then, the cells were treated with 50 μg/mL of HDL for 4 h. Treated cells were washed twice in PBS and harvested in lysis buffer containing radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail tablet (Roche Applied Science, Penzberg, Germany). Protein concentrations were determined using a Pierce™ BCA protein assay kit (ThermoFisher Scientific, Rockford, USA). Proteins (7 μg/lane) were separated on 10 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were blocked with Tris-buffered saline-Tween20 (TBS-T, 0.5 % Tween-20) containing 5 % skim milk for 1 h at room temperature and incubated with anti-VCAM-1 (Abcam, Cambridge, MA, USA) and mouse anti-β-actin antibodies (Santa Cruz Biotechnology, CA, USA) for overnight at 4 ℃. The membranes were washed three times with TBS-T for 5 min, and incubated for 1 h at room temperature with HRP-conjugated secondary

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antibodies. After washing, the protein bands were detected using the SuperSignal West Pico Chemiluminescent substrate (ThermoFisher Scientific). The band intensities were quantified by the ImageJ software (National Institute of Health, Bethesda, MD, USA).

8. Measurement of reactive oxygen species (ROS) production

J774 were treated with 100 μg/mL of HDL for 24 h and ROS production was assayed by using dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, ThermoFisher Scientific). Briefly, cells were stained with 5 μM CM-H_2DCFDA in PBS for 24 min at 37 ℃ and incubated in the presence or absence of 100 M hydrogen peroxide for 20 min. Flow cytometer was used to detect the ROS production.

9. Statistical analysis

The correlations between the HDL functional parameters versus HDL-associated protein levels were assessed with the Spearman correlation analysis. All the analyses used two-tailed tests and P values lower than 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA).

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Ⅲ. RESULTS

1. Patient's characteristics and laboratory values

The clinical characteristics and laboratory values of the study participants were listed in Table 1. The median age of the patients was 58 years and 86 % were males. Among the 21 participants, 14 (67 %) and 3 (14 %) had hypertension and diabetes, respectively. In addition, a total of 62 % of the participants were Coronary artery disease. The median level of HDL-C was estimated to be 42 mg/dL while the LDL-C level was 128 mg/dL.

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Table 1. Clinical characteristics of the study subjects

	All Participants (N=21)
Age, years	58 (49, 63)
Male	18 (86)
Medical history	
Hypertension	14 (67)
Diabetes mellitus	3 (14)
LDL-C ≥160mg/dL	4 (19)
Smoking	13 (62)
Coronary artery disease	13 (62)
Body mass index, kg/m^2	24.6 (23.4, 26.2)
Laboratory values, mg/dL	
Total cholesterol	201 (186, 223)
Triglyceride	154 (105, 198)
HDL-C	42 (38, 50)
LDL-C	128 (99, 142)
Medications	
Aspirin	14 (67)
β-blockers	9 (43)
Calcium channel blockers	6 (29)
RAS inhibitors	11 (52)

Variables are expressed as median (25th percentile, 75th percentile) or number (%); HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; RAS: renin-angiotensin system

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2. The four parameters of HDL function

The HDL function of 21 patients was measured using four cell culture experiments: cholesterol efflux capacity, endothelial nitric oxide (NO) production, vascular cell adhesion molecule-1 (VCAM-1) expression, and production of reactive oxygen species (Figure 1). The median value of the cholesterol efflux capacity was 13.7 when the patient's HDL sample was treated with macrophage (Figure 1A). The values of NO production and VCAM-1 expression on endothelial cells were 96 and 109, respectively (Figure 1B and Figure 1C). ROS production's median value from the macrophage was 23 (Figure 1D).

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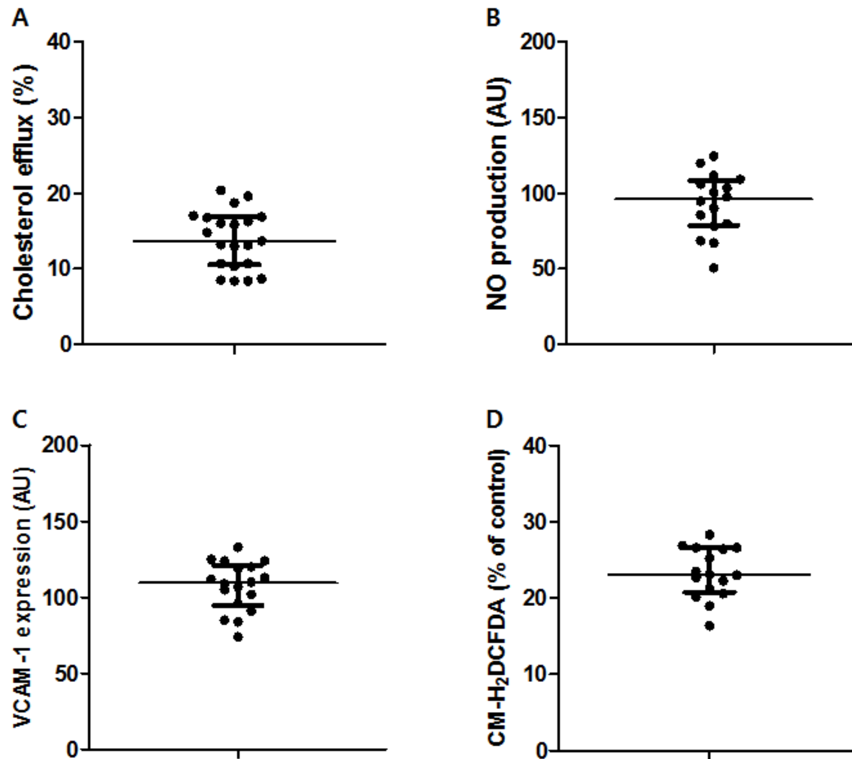

Figure 1. The parameters of high-density lipoprotein (HDL) function. The HDL function of 21 patients was measured using four experiments: (A) cholesterol efflux capacity, (B) nitric oxide (NO) production, (C) vascular cell adhesion molecule-1 (VCAM-1) expression, and (D) reactive oxygen species (ROS) production. Data were expressed as the median and interquartile range; n = 16 to 21.

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3. The expression of selected HDL-associated proteins

Figure 2 shows the results of Western blot of HDL samples from 21 patients. The differences in expression levels of five apolipoproteins that had been selected previously are shown for each patient. The last line in the figure represents a control group.

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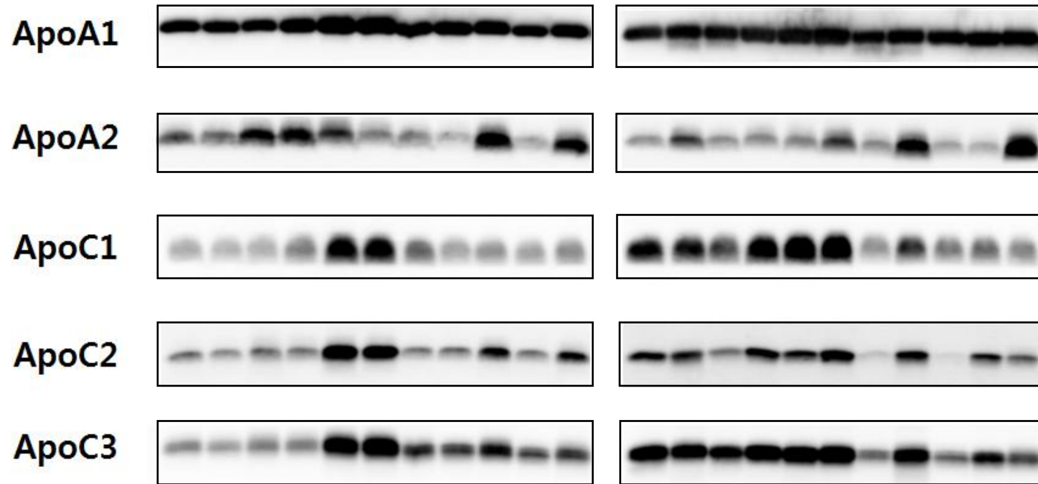

Figure 2. The expression of selected high-density lipoprotein (HDL)-associated proteins. The HDL samples from 21 patients were assayed for proteins by Western blot analysis. The same amounts of HDL samples (0.5 – 10 μg) were loaded. The bands are shown for five HDL-associated proteins. The last line represents a control group.

4. Relationship between the parameters of HDL function and HDL-associated proteins

ApoA1 levels (r_s = 0.52, p = 0.02) and apoC3 (r_s = 0.49, p = 0.03) revealed positive correlations with cholesterol efflux capacities, but not with the apoA2, apoC1, and apoC2 proteins (Figure 3). VCAM-1 expression was negatively associated with the apoA1 (r_s = -0.73, p = 0.001) and apoC1 (r_s = -0.53, p = 0.02) (Figure 5). However, none of the five HDL-associated proteins had a correlation with NO or ROS production (Figure 4 and Figure 6). The correlations between the parameters of HDL function and selected HDL-associated proteins are summarized in Table 2.

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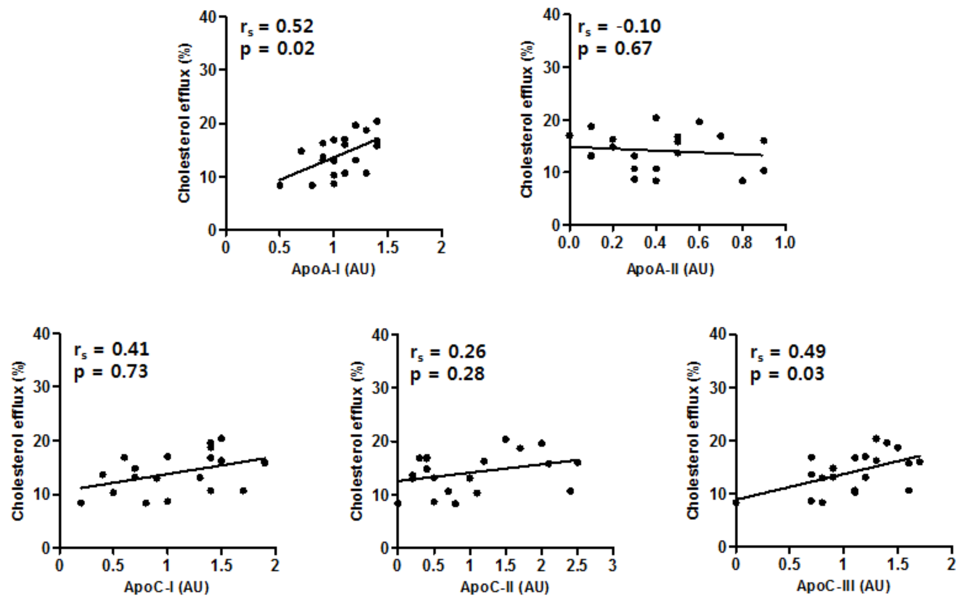

Figure 3. Correlation between cholesterol efflux and selected HDL-associated proteins. A Spearman correlation analysis was performed to analyze the association of cholesterol efflux and apoA1, apoA2, apoC1, apoC2, and apoC3. Cholesterol efflux was significantly associated with apoA1 (Spearman correlation coefficient = 0.52, $P < 0.05$) and apoC3 (Spearman correlation coefficient = 0.49, $P < 0.05$). The lines indicate linear regression analysis results; n = 19 to 20

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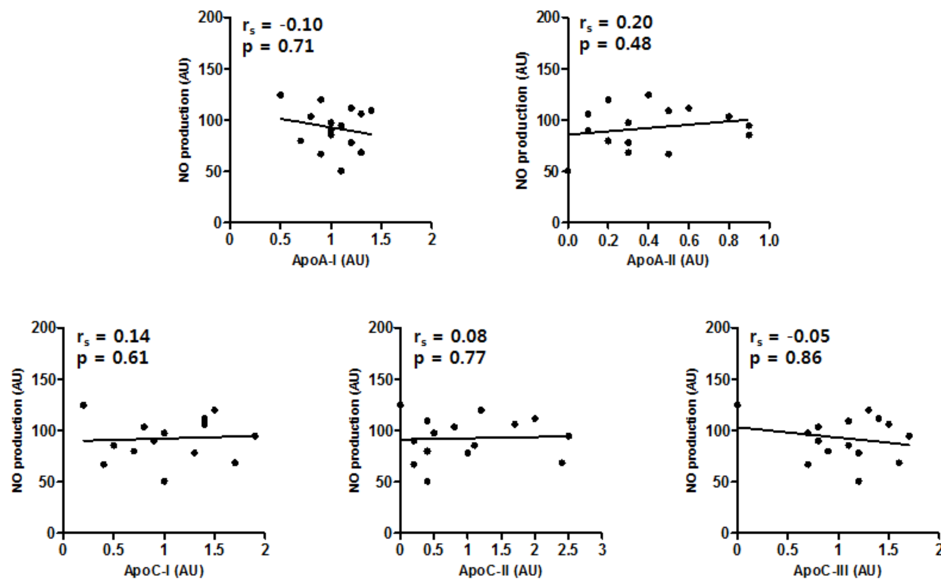

Figure 4. Correlation between nitric oxide (NO) production and selected HDL-associated proteins. A Spearman correlation analysis was performed to analyze the association of nitric oxide (NO) production and apoA1, apoA2, apoC1, apoC2, and apoC3. No proteins revealed any correlation with NO production. The lines indicate linear regression analysis results; n = 19 to 20

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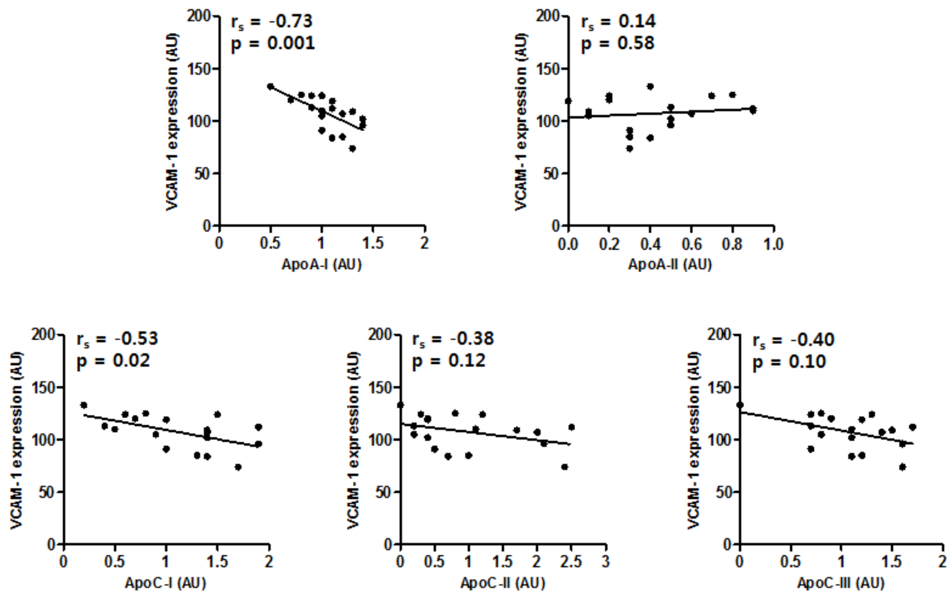

Figure 5. Correlation between VCAM-1 expression and selected HDL-associated proteins. A Spearman correlation analysis was performed to analyze the association between VCAM-1 expression and apoA1, apoA2, apoC1, apoC2, and apoC3. VCAM-1 expression was significantly associated with apoA1 (Spearman correlation coefficient = -0.73, $P < 0.05$) and apoC1 (Spearman correlation coefficient = -0.53, $P < 0.05$). The lines indicate linear regression analysis results; n = 19 to 20

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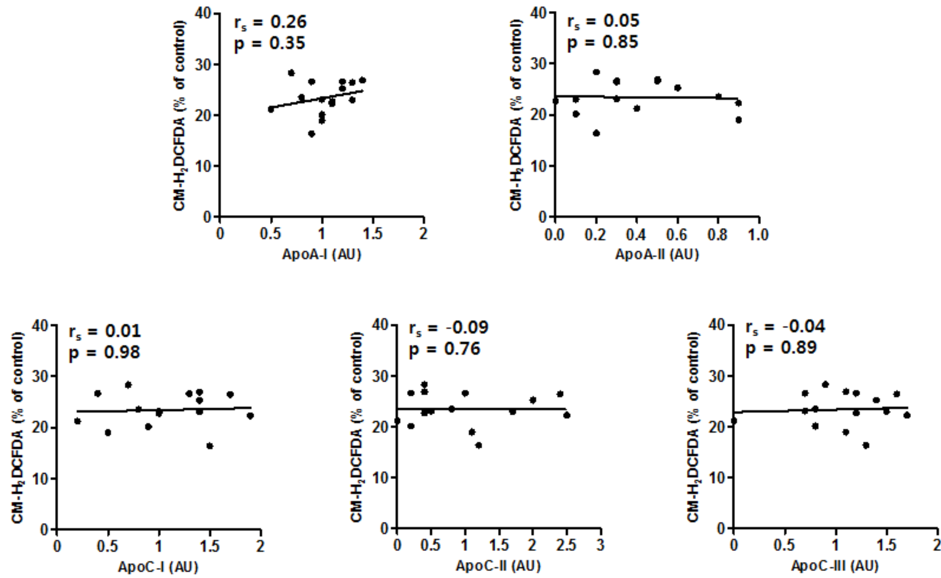

Figure 6. Correlation between reactive oxygen species (ROS) production and selected HDL-associated proteins. A Spearman correlation analysis was performed to analyze the association of reactive oxygen species (ROS) production and apoA1, apoA2, apoC1, apoC2, and apoC3. No proteins revealed any correlation with ROS production. The lines indicate linear regression analysis results; n = 19 to 20

Table 2. Correlation between the parameters of HDL function and selected HDL-associated proteins

	Cholesterol efflux		NO production		VCAM-1 expression		ROS production	
	r_s	p	r_s	p	r_s	p	r_s	p
ApoA1	0.52	0.02	-0.10	0.71	-0.73	0.001	0.26	0.35
ApoA2	-0.10	0.67	0.20	0.48	0.14	0.58	0.05	0.85
ApoC1	0.41	0.73	0.14	0.61	-0.53	0.02	0.01	0.98
ApoC2	0.26	0.28	0.08	0.77	-0.38	0.12	-0.09	0.76
ApoC3	0.49	0.03	-0.05	0.86	-0.40	0.10	-0.04	0.89

NO: nitric oxide; VCAM: vascular cell adhesion molecule; ROS: reactive oxygen species; r_s: Spearman rho coefficient

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Ⅳ. DISCUSSION

The present study revealed that an assessment of parameters of the HDL function, including cholesterol efflux and modulation of inflammation, was feasible. The cholesterol efflux capacity had a positive correlation with apoA1 and apoC3, while apoA1 and apoC1 were negatively associated with VCAM-1 expression.

In this study, we performed four experiments to test HDL functions: 1) cholesterol efflux capacity, 2) endothelial nitric oxide (NO) production, 3) vascular cell adhesion molecule-1 (VCAM-1) expression, and 4) the production of reactive oxygen species (ROS). Previous studies had shown that cholesterol efflux capacity [13] and the anti-inflammatory properties [7] of HDL could be evaluated using in vitro experiments. Based on these studies, we applied the similar methods in our study and assessed HDL function through cell experiments. In addition, we examined the expression of five HDL-associated proteins from 21 patients. The quantities of each apolipoprotein varied among individuals. Our data suggested that the variations in expression of proteins were associated with the functional heterogeneity of HDL particles.

Although the number of study participants was small, we found that clinical factors regarding sex, age, and with or without disease were not associated with five HDL-associated proteins in the study population. In this study, apolipoproteins seemed to have no clinical significance. We also discerned the relationships between the biological functions of HDL and proteins. First of all, our study confirmed that apoA1 had similar

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effects in relation to HDL functions as previously reported data. ApoA1 is the predominant HDL apolipoprotein, and represents about 70% of total HDL proteins [2,14]; it plays a key role in cholesterol efflux [15]. Several studies have suggested that apoA1 can directly interact with ABCA1 in mediating lipid efflux [9,16]. Furthermore, in vivo experiments have shown that the overexpression of apoA1 can accelerate reverse cholesterol transport [17]. In addition, previous studies have reported that reconstituted HDLs containing apoA1 can inhibit the expression of various cell adhesion molecules [18,19]. Undurti et al. showed that the oxidation of HDL by myeloperoxidase (MPO) gained a pro-inflammatory function regarding the upregulation in VCAM-1 expression, and they demonstrated that the pro-inflammatory activity of oxidized HDL is mediated by apoA1 [20].

In our study, ApoC1 had a negative correlation with endothelial VCAM-1 expression. Studies on the role of apoC1 in HDL function have been very limited, and the results have not been coherent. A previous study demonstrated that apoC1 is involved in lipopolysaccharide (LPS)-induced atherosclerosis, which relates to increased inflammation [21]. This showed that there is a relation between apoC1 and chronic inflammation. Conversely, apoC1 has been reported to have an important function in promoting an early inflammatory reaction to LPS [22] and in suppressing pro-inflammatory cytokine production from murine immune cells [23]. Moreover, the inhibitory effect of HDL on inflammation by LPS decreases in lecithin-cholesterol acyltransferase (LCAT)-deficient mice [24]. It is also reported that apoC1 activates the

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LCAT required for HDL maturation [25]. Based on these studies, apoC1 may have an effect on vascular inflammation through LCAT activation and HDL maturation.

ApoC3 is reportedly the most plentiful C apolipoprotein in human plasma [25], and it may contribute to the development of hypertriglyceridemia by elevating plasma triglycerides levels [26]. However, the HDL function regarding the role of apoC3 remains unknown. Recently, proteomic analysis of murine HDL showed a correlation between apoC3 and cholesterol efflux capacity [27]. Our study also found this relationship in human HDLs. This clinical relevance is currently unclear. However, this association was also shown by the relationship between apoC3 and cholesterol efflux in our study.

This study was limited in that we selected candidate proteins that were abundantly and reproducibly detected. We chose five HDL-associated proteins, since small quantities of other proteins may not have performed well in the experiments. Thus, the quantities of other proteins would not have been large enough to elucidate a relationship with the biological function of HDL.

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Ⅴ. CONCLUSION

In conclusion, the results suggest that multiple HDL-associated proteins, not only apoA1, presented correlations with HDL functions. Further study investigating the effects of more HDL-associated proteins on HDL function may be helpful in the development of refined biomarkers or novel therapeutics for cardiovascular disease.

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ABSTRACT (IN KOREAN)

고밀도 지단백 관련 단백질과 생물학적 기능의 관계

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노혜민

고밀도 지단백 (HDL) 콜레스테롤의 낮은 수치는 심혈관 질환의 중요한 예측 인자이다. 고밀도 지단백 콜레스테롤의 항 죽상경화 특성에 기초하여, 일부 연구에 따르면 고밀도 지단백의 입자가 심혈관 질환의 보호 효과와 관련되어 있음이 제시되었다. 최근의 프로테옴 분석 연구에 의하면 100 개가 넘는 단백질들이 고밀도 지단백과 관련되어 있으며, 이러한 고밀도 지단백 관련 단백질은 지질 수송, 프로테아제 억제 활동, 보체 경로 등 여러 가지 생물학적

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기능들과 관련되어 있었다. 그러나 고밀도 지단백 관련 단백질에 대한 특정 기능은 아직까지 잘 알려져 있지 않다.

우리는 고밀도 지단백 관련 단백질들과 생물학적 기능의 여러 매개 변수 사이의 상관관계를 조사하였다. 이 연구에는 관상 동맥 질환이나 그 위험 인자를 가진 총 21 명의 환자가 참여하였다. 실험 참가자들의 고밀도 지단백을 이용하여 콜레스테롤 유출 용량 및 기타 항염증 기능을 평가하였고, 고밀도 지단백 관련 단백질들 중에서 사전에 선택된 아포지질단백질을 측정하였다. 결과는 콜레스테롤 유출 용량이 아포지질단백질 A1 (apoA1), apoC3과 양의 상관관계가 있는 반면, apoA1과 apoC1은 VCAM-1 발현과 음의 상관관계가 있음을 보여 주었다. 결론적으로 우리는 여러 가지 고밀도 지단백 관련 단백질이 고밀도 지단백의 기능과 관계가 있음을 보여주며 더 많은 고밀도 지단백 관련 단백질이 그 기능에 미치는 영향을 연구하는 것은 심혈관 질환에 대한 새로운 치료제 개발에 도움이 될 수 있음을 제시한다.

핵심되는 말: 고밀도 지단백, 아포지질단백질, 상관관계, 콜레스테롤 유출, VCAM-1 발현