의학석사 학위논문

인간 단핵세포와 혈관내피세포에서 PCSK9이 직접적으로 유발시킨 NF-kB에 의해 조절되는 염증에 관한 연구

PCSK9 directly induces NF-kB mediated inflammation in human monocytes and endothelial cells

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이 논문을 조성윤 석사학위논문으로 제출함

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Abstract

PCSK9 directly induces NF-kB-mediated inflammation in human monocytes and endothelial cells

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Atherosclerotic cardiovascular diseases (ACVDs) are the leading cause of death world widely and excess low density lipoprotein cholesterol (LDL-C) is their most important causal risk factor. Proprotein convertase subtilisin/kesintype-9 (PCSK9) increases plasma levels of low-density lipoprotein cholesterol (LDL-C) through binding to the LDL receptors (LDLR) and mediating its lysosomal degradation

on liver cells. PCSK9 also promotes atherosclerosis by increasing LDL-C levels through degradation of hepatic LDLR. Even though PCSK9 has been known to involve in atherosclerosis development, it has not been elucidated whether PCSK9 mediates local inflammation in the vessel wall, thereby directly effects on the plaque. To study the direct inflammatory effect of human PCSK9 (hPCSK9) on atherosclerotic lesion composition, we treated PCSK9 in the monocyte and endothelial cells. Interestingly, PCSK9 activates NF-kB and turns on the pro-inflammatory cytokines in the monocytes. Furthermore, treatment of PCSK9 increases the adhesion molecules; Integrins in the monocytes, and VCAM-1 and ICAM-1 in the endothelial cells. In mice, administration of PCSK9 (5ug/mouse; 24 hour period) via the peritoneal route induced a significant accumulation of monocytes (26.6% increase) and macrophages (23.2% increase) into the peritoneal cavity.

Taken together, we suggested that PCSK9 can directly induce inflammation through NF-kB activation, implying that PCSK9 directly accelerate atherosclerotic plaque progression by aggravating inflammatory conditions in the vessel wall through stimulating

monocyte infiltration and activating endothelial cells.

Keywords : PCSK9 , Atherosclerosis , Inflammation , NF-kB , Endothelial cell , Monocyte

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Introduction

Proprotein convertase subtilisin/kexin 9 (PCSK9), a member of the protein-converting enzyme family, is highly expressed in adult hepatocytes. PCSK9 determines low density lipoprotein cholesterol (LDL-C) level by regulating internalization and lysosomal degradation of LDL receptor (LDLR) and has recently emerged as a major drug target in cholesterol metabolism and atherosclerosis development.

Biological inhibitors of PCSK9 have reduced LDL-C levels and improved cardiovascular outcomes. LDLR enters the cell when bound to LDL-C, dissociates from LDL-C at the endosomes and recycles to the cell surface, whereas the LDL-C is directed to lysosomes for degradation. In contrast, when bound to PCSK9, LDLR is internalized and escorted to lysosomes for degradation through unknown mechanism. PCSK9 is known to be involved in cholesterol metabolism and atherosclerosis development, and its inhibitors are now used as new and cholesterol-lowering drugs.

PCSK9 reduces hepatic uptake of LDL by increasing the lysosomal degradation of LDLRs. PCSK9 deficient mice have low plasma LDL

cholesterol levels and are protected against atherosclerosis development. In contrast, transgenic mice over-expressing gain-of function mutants of PCSK9 developed hypercholesterolemia and atherosclerosis.

ACVDs are the leading cause of death world widely and excess LDL-C is their most important causal risk factor. Atherosclerosis is a multifaceted, progressive, inflammatory disease that affects mainly large and medium-sized arteries. It is characterized by the formation and build-up of atherosclerotic plaques that consist of a well-defined structure of lipids, necrotic cores, calcified regions, inflamed smooth muscle cells, endothelial cells, immune cells and foam cells; consequently, atherosclerosis is associated with cardiovascular disease.

Vascular inflammation plays an important role in pathogenesis of atherosclerosis, ranging from the initiation through progression to the formation of thrombotic complications. Therefore, the molecular mechanisms underlying atherosclerotic inflammation should be elucidated for developing effective preventive and therapeutic approaches.

Atherosclerosis is a complex disease in which lipid accumulation and increased inflammation promote lesion growth and affect its composition. Mutations in PCSK9 are strongly associated with levels of low-density lipoprotein cholesterol in the blood plasma and related to occurrence or resistance to atherosclerosis and coronary heart disease. In this study, we hypothesized that PCSK9 itself has a primary role in atherosclerotic plaque development and is independent to lipid changes. Many investigators have proposed that PCSK9 is expressed in endothelial cells (EC), vascular smooth muscles cells (VSMC), and in other areas of the human atherosclerotic plaque. Local inflammation in the vessel wall is major cause to develop atherosclerotic plaque formation, however, there is no direct evidence whether PCSK9 might be triggering inflammation in the vessel as a signal modulator directly to the monocyte or endothelial cells.

Here we provide the first evidence that PCSK9 can induce inflammation through NF-kB activation directly to the monocytes and endothelial cells. Our study suggested that PCSK9 can accelerate atherosclerotic plaque progression by aggravating inflammatory

conditions in the vessel wall through stimulating monocyte infiltration and activating endothelial cells.

Results

Inflammation-mediated PCSK9 gene expression in human monocyte.

First, we investigated whether there is a correlation between the expression level of PCSK9 and immune response in a pathological condition in human monocyte cell-line. We found that gene expression level of PCSK9, was increased upon stimulation with pro-inflammatory cytokines including Lipopolysaccharides (LPS), Interleukin 1 beta (IL-1β), and Tumor necrosis factor alpha (TNF-α) post 6h and 24hr.

To determine whether PCSK9 , LPS , IL-1β, and TNF-α affect the synthesis and secretion of PCSK9, THP-1 cell were treated with PCKS9 , LPS , IL-1β, and TNF-α, for 6h and 24 h. THP-1 cell showed a rapid (within 6-24 hours) increase in PCSK9 expression in genetic level.

For 6h PCSK9 treated group, there was a 9-fold increase compared to vehicle in the level of PCSK9 mRNA. The expression of PCSK9 mRNA increased by 6-fold when LPS and TNF-α was treated for

6h. Lastly, IL-1β led to up to a 2-fold increase of PCSK9 mRNA with 6h treatment. Similarly, the expression of PCSK9 mRNA level in those cytokine treated groups were further up-regulated in 24h than 6h of stimulation. Our data (Fig 1) indicate that rhPCSK9 induced autocrine production of PCSK9 expression in THP-1 cell.

To investigate the effects of PCSK9 on NK-kB activation, we evaluated the actvation of NF-kB and processing of PCSK9 in monocytes. THP-1 cells were treated rhPCSK9 (2ug/ml) for 24hr. The western data show that endogenous PCSK9 are highly expressed and induced PCSK9 processing, changing PCSK9 into the matured form. (Fig 2) As shown rhPCSK9 induced activating NF-kB in THP-1. These data suggest that PCSK9 promotes NF-kB activation.

PCSK9 directly activates NF-kB, and promotes the pro-inflammatory genes and adhesion molecule in monocytes and EC

We observed that PCSK9 of expression is induced by inflammation. To investigate further the role of PCKS9 in inflammation and potenti

al mechanisms, we speculated that expression of PCSK9 in monocytes and EC have a critical role in atherosclerosis.

We investigated that at the site of atherosclerotic lesion, adhesion molecules such as pro-inflammatory genes and integrin are upregulated.

Using realtime qPCR , Pro-inflammtory related genes were quantified and mRNA levels of pro-inflammatory genes increased along the concentration of PCSK9 treatment in THP-1 and HUVEC.

The expression of mRNA and protein were upregulated in a concentration-dependent manner with the highest expression resulting from treatments of 2000ng/ml PCSK9. (Fig 3)

Because PCSK9 is known to induce activation of NF-kB , our goal was to determine if the PCSK9-mediated increase adhesion molecule protein level in monocytes and EC.

We perform that adhesion molecules such as integrin and its counter part molecule VCAM, ICAM are upregulated in monocytes and EC.

The protein level of adhesion molecule were significantly increased with rhPCSK9 treatment in THP-1 and HUVEC. (Fig 4,5)

These data demonstrate that PCSK9 promotes pro-inflammatory gene and secretion of adhesion molecule response involving activation of

the NF-kB pathway in monocytes and EC.

PCSK9 induced activation of NF-kB and mediated of translocation NF-kB

To elucidate potential mechanisms implicated in NF-kB regulation of PCSK9 , we performed NF-kB gene promoter reporter assays. The NF-kB gene promoter region is inducible by PCSK9 and TNF-α. Results from co-transfection experiments of the luciferase NF-kB promoter-reporter construct in THP-1 cells are shown.

Cells were harvested 2 days post transfection and luciferase activity was measured. To find out whether PCSK9 activates NF-kB promotor, PCSK9 and TNF-α were treated in THP-1 cells and there were a large increase of NF-kB in THP-1 cells.

These data support that PCSK9 is a key protein for the activation of NF-kB pathway. (Fig 6)

It is well known that NF-kB translocation occurs when it is activated. We validate the effects of PCSK9 on key regulatory proteins associated with in nuclear- and cytosolic-enriched fractions of HEK 293T

cell. p65(NF-kB) was found to be mainly associated with cytosol in HEK 293T cell. We proved that the exogenously applied PCSK9 elicited a change in p65 localization from cytosol to nucleus surface.

Our data indicates that PCSK9 induces translocation of NF-kB from cytosol to nucleus. (Fig 7)

PCSK9 induced inflammation in a murine model

Consistent with our previous in-vitro study, we observed PCSK9 is induced by inflammation in mouse model. To examine whether PCSK9 modulates leukocytes extravasation, PCSK9 was injected to the peritoneal cavity.

Injection was performed with BL6 mice. 24 hours after PCSK9 injection, cells were harvested from the femur and tibia by flushing the BM cavity and were seperated in to mononuclear cells.

The extravasation of CD3 positive lymphocyte was 5.9% increased after PCSK9 injected. While control group was increased 1.1% after saline injected. CD11B positive monocyte was increased 22.6% after PCSK9 injected. Otherwise control group was increased 2% after sali

ne injected. F4/80, CD11B positive macrophage was increased 23.2% after PCSK9 injected while control group was increased 1.1% increase d after saline injected. (Lymphocyte:CD3 , monocyte: CD11B , macrophage : F4/80)

As a result, there were small difference in lymphocytes and the extr avasation of mono/macrophages was increased after PCSK9 injection. These data suggest that PCSK9 may be a key protein involved in inflammatory progression and in the distribution of cells in lesion area.

Discussion

Beyond the obvious association of PCSK9-induced LDLR degradation and hypercholesterolemia, emerging studies have uncovered other mechanisms by which PCSK9 may facilitate atherosclerosis development independent of its impact on lipids. PCSK9 induced lipid metabolism and ultimately plasma lipid levels are the most obvious mechanism that links PCSK9 to atherosclerosis. PCSK9 is highly expressed in the liver, followed by the intestine, kidney, pancreas, and the brain. Increasing evidence suggests that PCSK9 is expressed in human atherosclerotic plaques.

A recent line of investigation has sought, however, to determine if PCSK9 itself has a primary role in atherosclerotic plaque development, independent of lipid changes. Chronic inflammatory disease in the vascular wall, and its pathogenesis is associated with various inflammatory cytokines. The major pro-inflammatory cytokines that contribute to vascular inflammation and plaque destabilization, including IL-1β, TNF-α, are abundantly expressed in atherosclerotic lesions. (1) We found that human PCSK9 treatment of

human moncytes and EC directly causes dose-response increase in NF-kB expression in mRNA and protein level.

Therefore, we conclude that PCSK9 may be an inflammatory mediator that promote atherosclerosis through a mechanism beyond LDL cholesterol regulation. The major novel finding of this study is that PCSK9 directly induces NF-kB mediated inflammation in monocytes and EC. These results demonstrate that PCSK9 increases the secretion of inflammatory cytokines at least partially by promoting the activation of the NF-kB pathway. We speculated that monocytes and EC have a critical role in atherosclerosis that is induced by PCSK9. The NF-kB signaling pathway is critical for atherogenesis because it regulates vascular inflammatory responses. PCSK9 can induce NF-kB activation that is linked to the transcription of many proinflammatory genes including TNFα, IL-1β and IL-6. PCSK9 and activated NF-kB have been observed in the atherosclerotic lesions. Our results provide that PCSK9 may play a proinflammatory role in development of atherosclerotic plaques. Additionally, protein level of adhesion molecules such as integrin, VCAM, and ICAM was up-regulated.

In conclusion, our study has provided the evidence that proves the increase of PCKS9 expression in atherosclerotic lesions. We have also shown that PCSK9 gene could directly increase vascular inflammation through NF-kB signaling pathway. These findings provide further insight into the roles of PCSK9 in atherosclerotic inflammation and suggest that therapeutic PCSK9 inhibition may offer vascular benefits in addition to plasma LDL reduction.

Figure 1. In monocytes, Inflammation induced by PCSK9

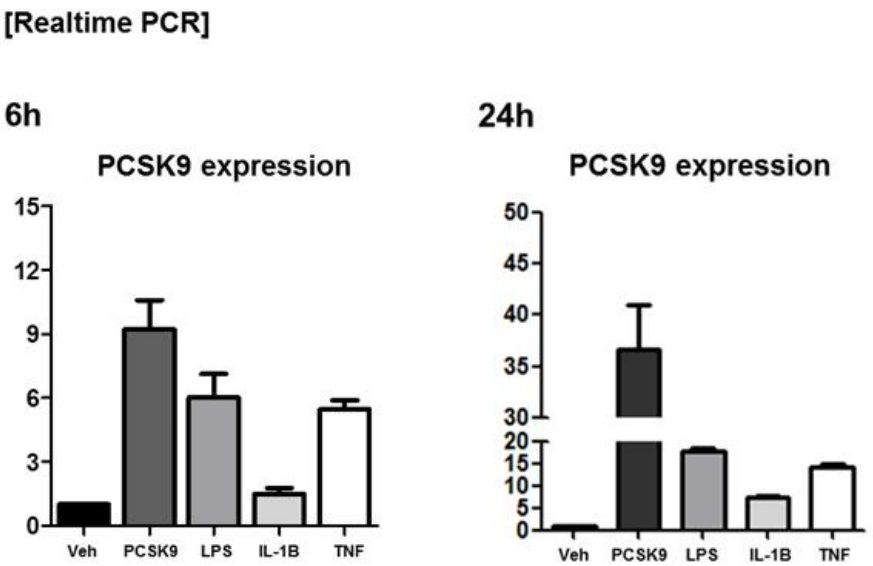


Fig1. Notably, rhPCSK9 induced autocrine production of PCSK9 expression in THP-1 cell.

Figure 2. In monocytes, PCSK9 is expressed and turn on NF-kB

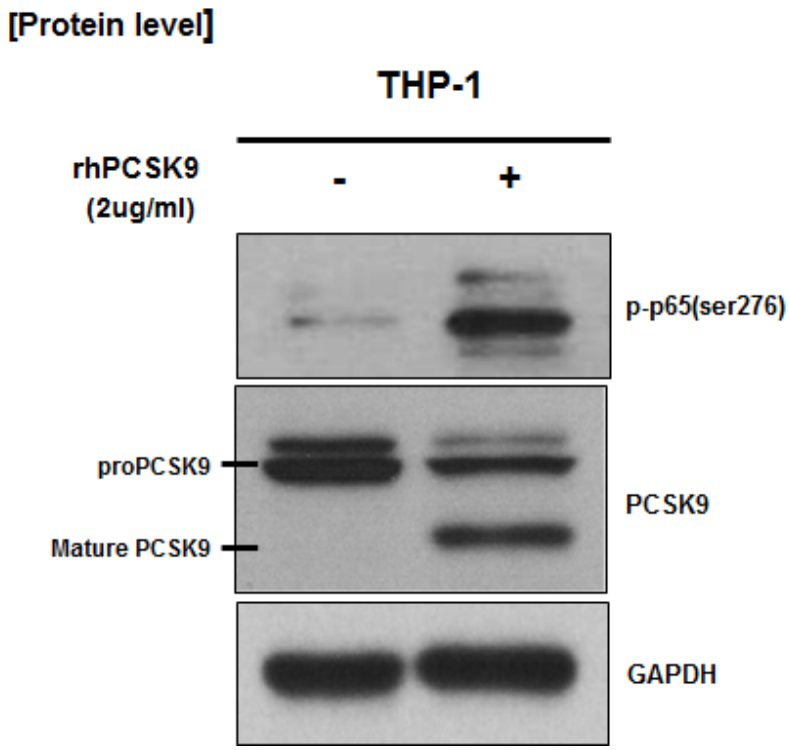


Fig 2. THP1 cells were treated with recombinant PCSK9 (2ug/ml) for 24h.

Figure 3. PCSK9 directly activates NF-kB, and turns on the pro-inflammatory genes.

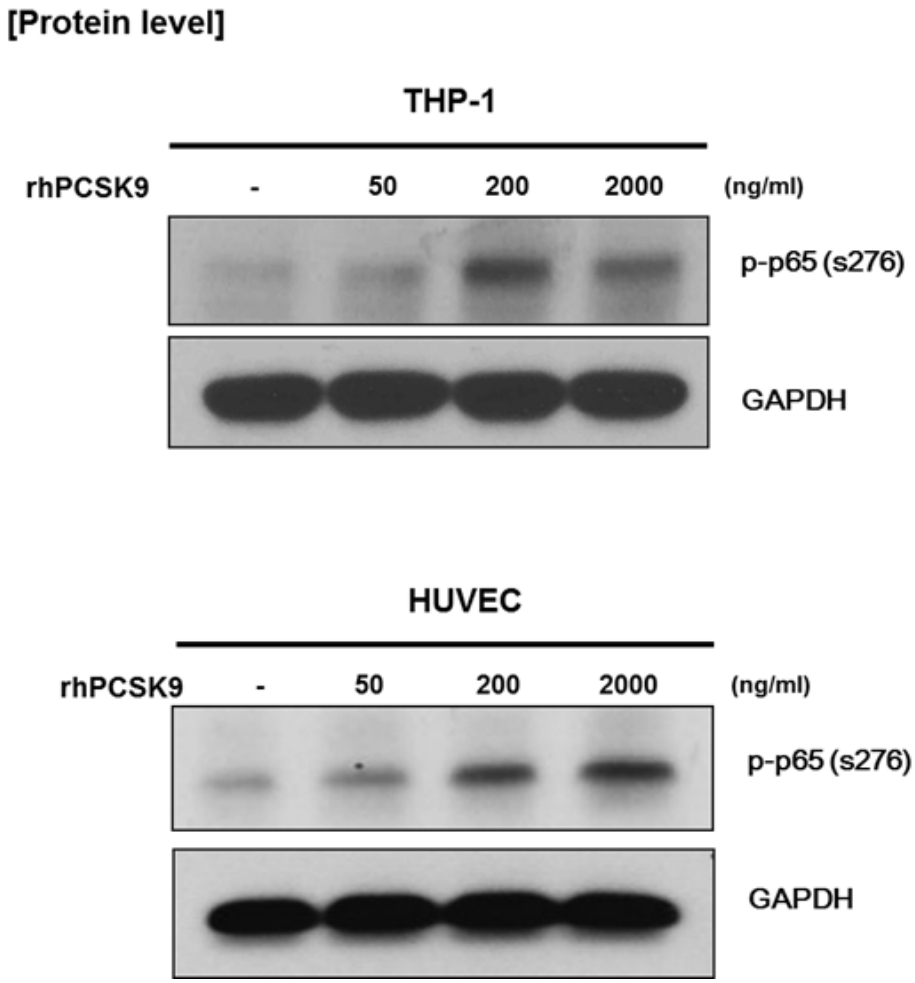


Fig3. Dose dependent change in NF-kB protein expression of monocyte and EC treatment with PCSK9

Figure 4. Inflammatory cytokines in monocytes were induced by PCSK9

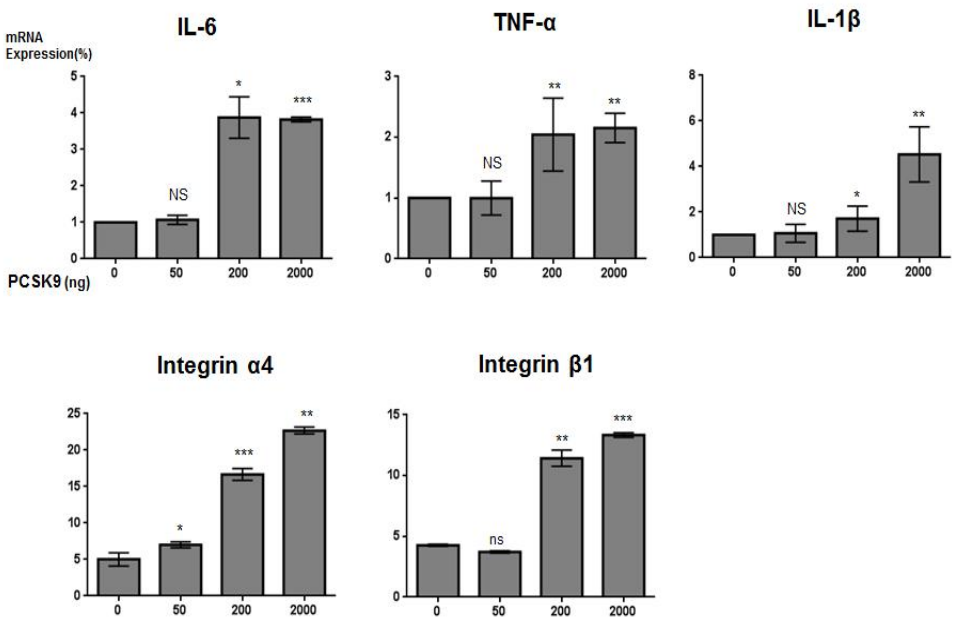


Fig4. Dose-dependant changes in inflammtory mRNA level of monocyte and EC after treatment with PCSK9 and EC treatment with PCSK9

*p < 0.05 **p < 0.01 ***p < 0.01

Gene level Normalized to GAPDH

Figure 5. Adhesion molecules were induced by PCSK9

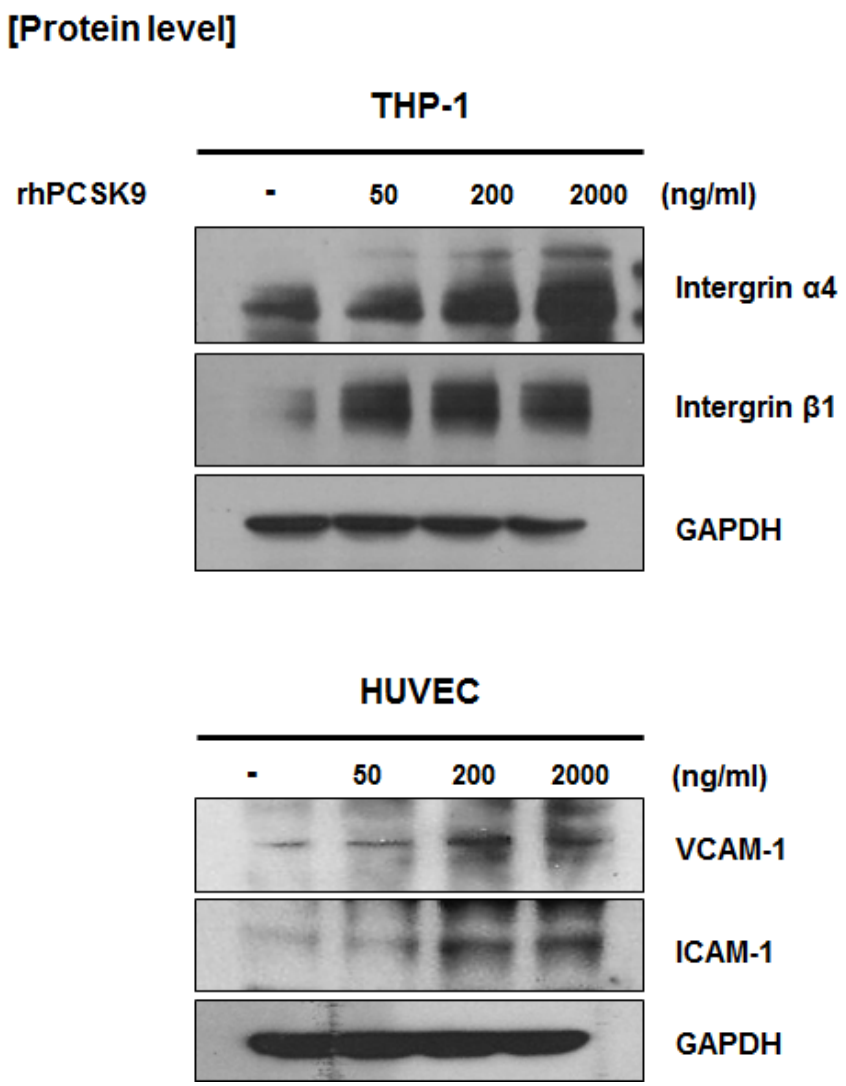


Fig5. Dose-dependant changes in adhesion molecule protein level of monocyte and EC after treatment with PCSK9

Figure 6. PCSK9 mediated NF-kB activation

[Luciferase assay]

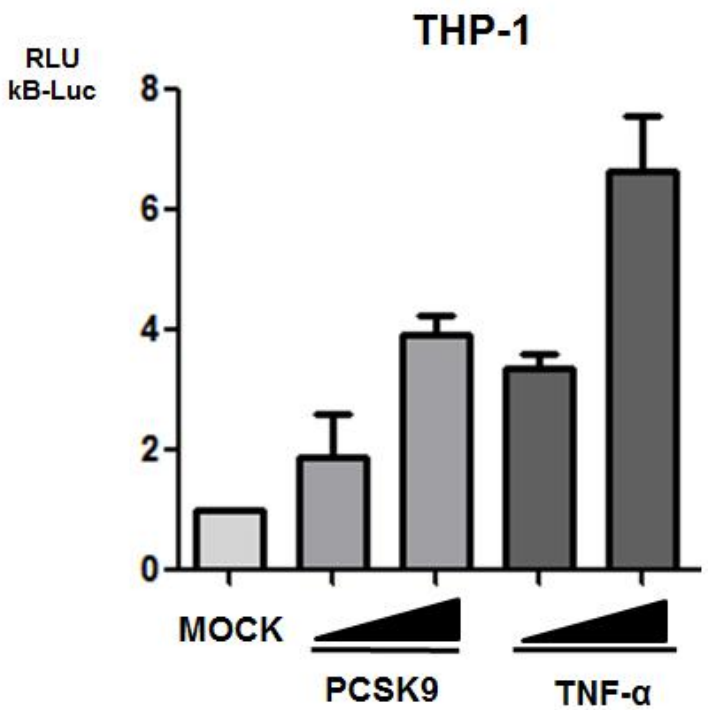


Fig6. The NF-kB gene promoter region is inducible by PCSK9 and TNF-α. Result from transfection experiments of the luciferase NF-kB promoter-reporter consturct in THP-1 cells are shown.THP-1 cells were transfected with the luciferase NF-kB promoter-reporter constructfor 48h. PCSK9 : 20 , 2000ng/ml TNFα : 2, 10ng/ml

Figure 7. PCSK9 mediated NF-kB translocation

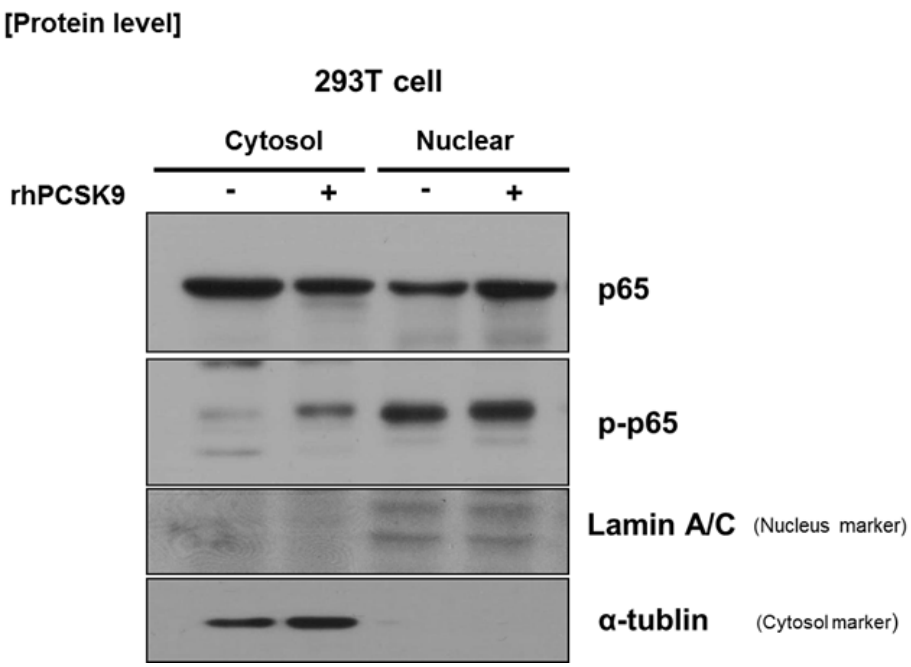
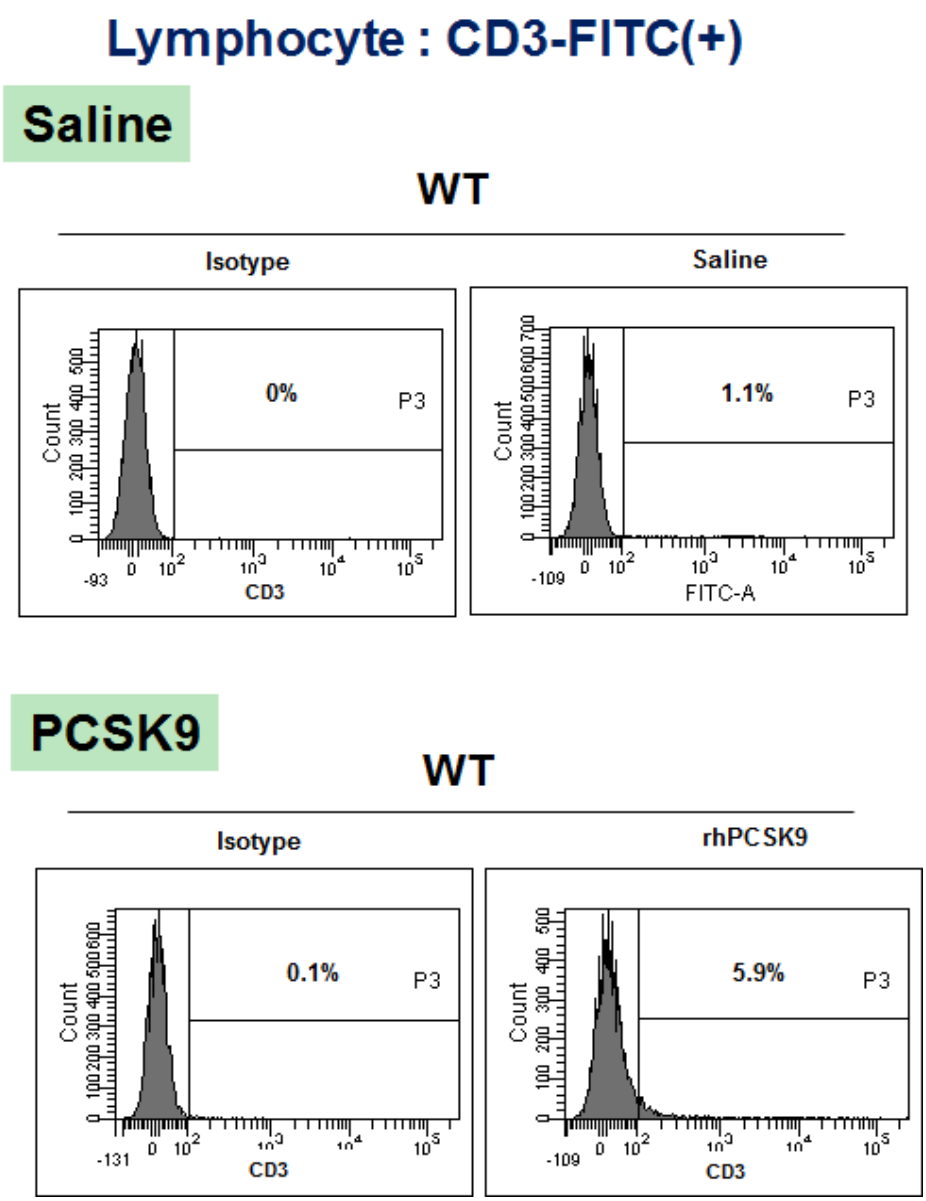


Fig7. Reprsentative western blotting detecting p65 in the nuclear fr action of HEK 293T cells as well as cytosol. Importantly, the exog enously applied PCSK9 elicited a change p65 localization to nuclear to cytosol. PCSK9 : 500ng/ml

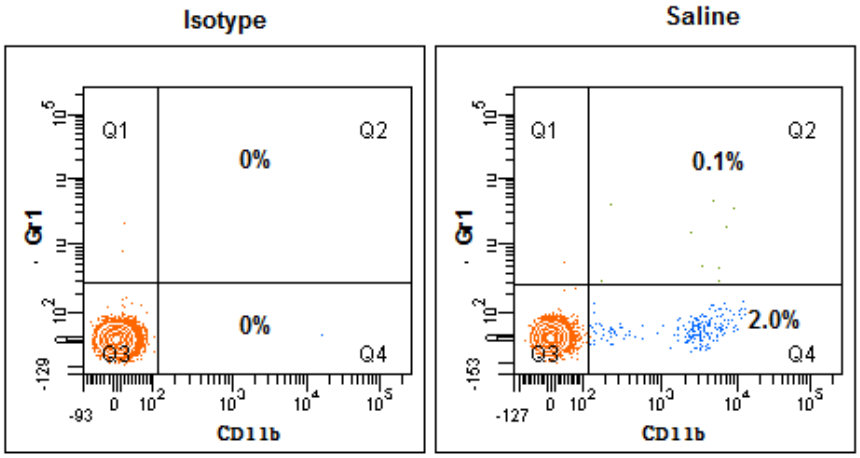
Figure 8. PCSK9 induced inflammation in a murine model



Monocyte : CD11b-FITC (+) / Gr1-APC (-)
Neutrophil : CD11b-FITC (+) / Gr1-APC (+)

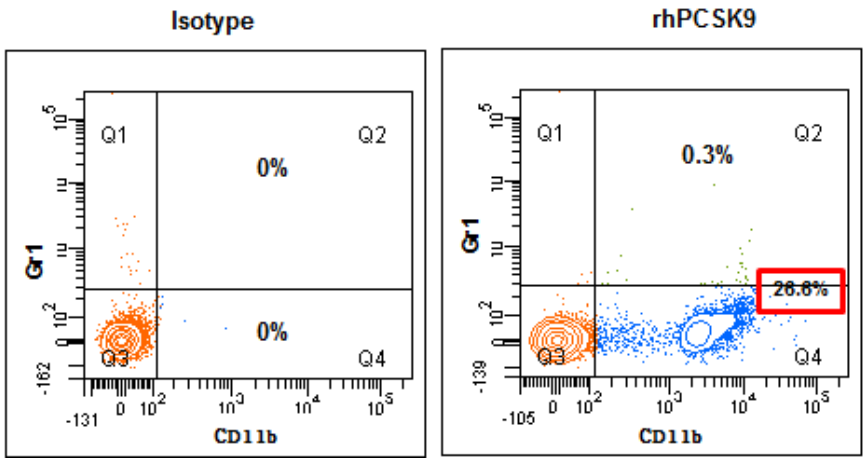
Saline

WT



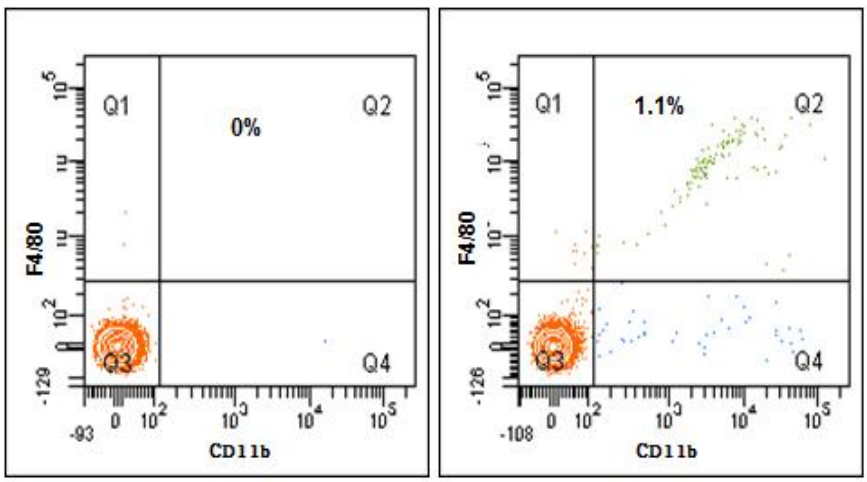
PCSK9

WT



Macrophage : CD11b-FITC (+) / F4/80-APC (+)

Saline **WT**



PCSK9 **WT**

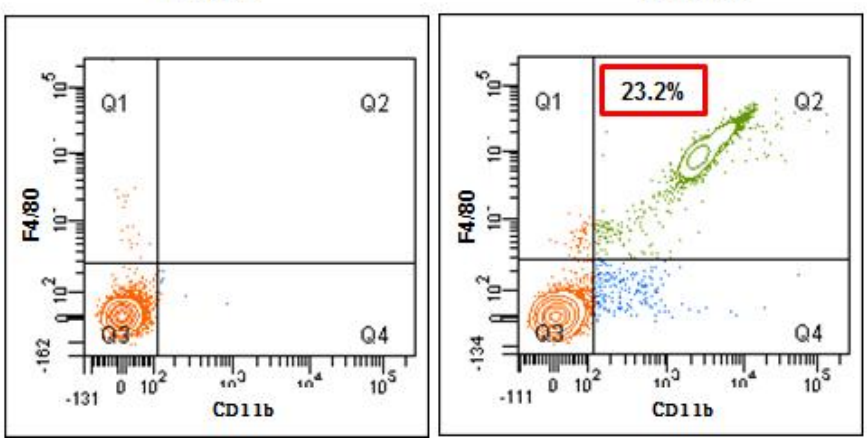


Fig8. FACS analysis of CD3,F4/80 positive popuation in PCSK9 grou p. the mono/macro population was assessed by anti-F4/80-APC, CD1 1b-FITC. After treat, F4/80, CD11B positive cell dramatically increase d extravasation compared with saline group.

The lymphocyte population was assessed by anti- CD3. PCSK9 treate d CD3 positive cells had no significant difference.

Materials and Methods

Antibodies and Reagents

Primary antibodies used in this research were as follows: anti NF-κB p65 (SantaCruz, sc-109), anti-His tag (Cell Signaling, cat#2365), anti-LaminA/C (SantaCruz, sc-6215), anti Integrin (SantaCruz, sc-100917) (SantaCruz, sc-134637), anti ICAM-1 (SantaCruz,) (SantaCruz, sc-5310), anti ICAM-1 (SantaCruz, sc-7891), anti-GAPDH (Sigma Aldrich, G9545), anti-VCAM-1 antibody (SantaCruz, sc-5310). Anti-NF-kB (Cell Signaling, cat#85813)

Cell culture and transfection

Pooled human umbilical vein endothelial cells (HUVECs) were purchased at Lonza and cultured in EGM-PLUS SingleQuots Kit (CC-4542) that formulates 500 ml of EBM- PLUS Basal Medium to EGM- PLUS Growth Medium; contains BBE, 1.0 ml; L-Glutamine, 25.0 ml; Ascorbic Acid, 0.5 ml; Hydrocortisone, 0.5 ml; rhEGF, 0.5 ml;

Heparin 0.5 ml; GA, 0.5 ml with 10% Fetal bovine serum (GIBCO, #16000). All HUVECs used in this research were passage 6 to 7.

Cell culture and treatments. THP-1 cells C grown in a monolayer were maintained in RPMI-1640 medium containing 10% fetal bovine serum at 37°C in 5% CO2 S1 was cultured in RPMI high glucose(wellgene)supplemented with 10% Fetal bovine serum (Gibco) and 1X Antibiotics - antimycotics (Gibco).

Cytosol, nuclear and membrane fractionation

To prepare cytosolic extracts, cells were carefully collected by using cytosol lysis buffer (10mM HEPES pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.1mM DTT, 1mM Na3VO4). Samples have been lysed by adding 0.6% NP40 for a minute and quickly centrifuged at 13,000rpm. Supernatant was obtained as cytosolic extracts and remained pellets were lysed in nuclear lysis buffer(25mM HEPES pH7.9, 0.4M NaCl, 0.5mM EDTA, 0.5mM EGTA, 0.1mM DTT, 1mM Na_3VO_4)for30minute - incubation at 4°C.

Membrane extracts were conveniently obtained by using Qproteome Cell Compartment Kit (QIAGEN, #37502).

Immunoprecipitation and Western blot

Membrane extracts prepared by Qproteome Cell Compartment Kit (QIAGEN, #37502) were incubated with 1ug of each antibody for overnight. Normal IgGs were bought from SantaCruz (sc-2025 and sc-2027). After incubation, the lysates were incubated with protein A/G agarose beads (SanatCruz, sc-2003) for 3 hours at 4°C on an end-over-end rotator. After washing 4 times with cold PBS, proteins and beads were dissociated through boiling at 100°C with 2x SDS sample buffer (Intron, BS002). Beads were removed by centrifugation and samples were immunoblotted on SDS-PAGE gel and PVDF membrane.

Gene expression analysis

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription PCR was performed as previously described. Briefly, cDNA was synthesized using a Primescript 1st strand cDNA synthesis kit

(Takara) and oligo-dT primer.

Semi-quantitative PCR was performed with Maxime PCR Pre-Mix (Intron) according to manufacturer's instructions and real-time PCR was performed with Power SyBR Green I Mastermix (Applied Biosystems) using an ABI PRISM TM 7500 Sequence Detection System (Applied Biosystems).

Flow cytometry

Under sterile conditions, the femur and tibia were excised and all connective tissue was removed from bones. BM cells were harvested from the femur and tibia by flushing the BM cavity with DPBS. BM cells were dispersed by passing through a 40um strainer (FALCON). after suspended BM cells were obtained, cells were seperated in to mononuclear cells by Histopaque-1083(Sigma-aldrich). Collected mononuclear cells were washed once at 1800 rpm at 4℃ , and suspended in facs buffer.Flow cytometry was performed as previously described with slight modification. Flow cytometry analaysis and sorting(BD cantoⅡ, LSRⅡand FACS ariaⅢ) were performed using several antibodies specific for following CD11b (553310 BD) ,

Gr-1(17-9668-82E-BIOSCIENCE), F4/80(17-4801-82 E-BIOSCIENCE), CD3 (561798 BD).

In vivo Analysis of Extravastion Leukocytes

All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Clinical Research Institute in Seoul National University Hospital, Korea. Age-matched (9-to10-week-old) male BL6 mice in their monocyte/macrophage lineages were used in this study.

Peritonitis model was made by intra-peritoneal injection of PCSK9 and extravasated leukocytes were detected by its own marker.

We harvested bone marrow and spleen from BL6 mice and then isolated the mononuclear cells using panning procedures and cell sorting.

Statistical Analysis

All experiments were repeated at least three times, and all data were calculated as mean ± SEM. Group comparisons were performed by Student's t test, and $p < 0.05$ was considered statistically significant. The error bars represent SEM.

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요약 (국문초록)

인간 단핵세포와 혈관내피세포에서 PCSK9이 직접적으로 유발시킨 NF-kB에 의해 조절되는 염증에 관한 연구

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죽상경화성심혈관질환(ACVDs)은 전 세계적으로 사망의 주요 원인이며 초과된 저밀도 지단백 콜레스테롤 (LDL-C)은 가장 중요한 위험 요인이다. Proteinin 전환 효소 subtilisin / kesintype-9 (PCSK9)은 LDL 수용체(LDLR)에 결합하여 간세포에서의 라이소좀 분해를 매개하여 저밀도 지단백 콜레스테롤 (LDL-C)의 혈장 농도를 증가시킨다. PCSK9은 또한 간 LDLR의 분해를 통해 LDL-C 혈장농도를 증가시킴으로써 죽상 동맥경화증을 촉진한다. 또한 PCSK9이 죽상경화성심혈관질환 발달에 관여하는 것으로 알려져 있음에도 불구하고, PCSK9이 플라크에 직접 영향을 미치는지 , 혈관 벽의 국소 염증을 매개하는지에 대한 기작이 밝혀지지

않았다. 인간 PCSK9 (hPCSK9)의 죽상 경화 병변 구성에 대한 직접적인 염증 효과를 연구하기 위해서 우리는 단핵구와 내피 세포에서 PCSK9를 처리했다. 흥미롭게도, PCSK9은 NF-κB를 활성화시키고 단핵구에서 전구 염증성 사이토카인을 활성화시킨다. 또한, PCSK9을 처리했을 때 단핵구의 integrins, 내피 세포의 VCAM-1 및 ICAM-1 같은 접착성 분자들을 증가시킨다. 쥐에 PCSK9을 (5ug / mouse, 24 시간)를 복강 내로 투여하면 PCKS9에 의해 유도된 단핵구 (26.6 % 증가)와 대식세포 (23.2 % 증가)가 복강 내로 유의적으로 축적 되었다.

종합적으로 우리는 PCSK9이 NF-kB 활성화를 통해 직접 염증을 유도할 수 있다고 제안함으로써, PCSK9이 직접적으로 단핵구의 침윤을 자극하고 내피 세포를 활성화시킴으로써 혈관벽의 염증 상태를 악화시키고 죽상 경화 플라그의 진행을 직접적으로 촉진 시킬 것이다.

주요어 : PCSK9 , 죽상 동맥 경화증 , 염증 , NK-kB , 혈관 내피세포 , 단핵세포

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