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Effects of CXCL5 secreted from vascular endothelial cells by transcription factor Etv2 on smooth muscle cells

조선대학교 대학원

의 과 학 과

추 병 삼

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전사인자 Etv2 로 인해 혈관내피세포에서 분비된

CXCL5 의 혈관평활근세포에 대한 영향

2021년 2월 25일

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Abbreviations

CXCL5	C-X-C motif chemokine 5
Etv2	ETS variant transcription factor 2
HUVEC	Human umbilical vein endothelial cell
VSMS	Vascular smooth muscle cell
CVD	Cardiovascular disease
ERK	Extracellular-signal-regulated kinase
MMP2	Matrix metalloproteinase-2
MMP9	Matrix metalloproteinase-9
MMP13	Matrix metalloproteinase-13
GROa/b/g	Growth -regulated oncogene a/b/g
CXCL1	C-X-C motif chemokine 1
MCP1	Monocyte chemoattractant protein 1
MCP2	Monocyte chemoattractant protein 2
IL-6	Interleukin 6
IL-8	Interleukin 8
TNF-α	Tumor necrosis factor- alpha
KLF4	Kruppel-like factor 4
Cx43	Connexin 43
α -SMA	Alpha-smooth muscle actin

초 록

전사인자 Etv2로 인해 혈관내피세포에서 분비된 CXCL5의 혈관평활근세포에 대한 영향

추 병 삼

지도교수: 송 희 상

조선대학교 대학원 의과학과

심혈관질환(Cardiovascular disease,CVD)은 세계적으로 높은 유병률과 사망률을 차지하는 질병으로, 이 중 죽상동맥경화증은 대표적인 심혈관질환 발병의 주원인이다. 죽상동맥경화증은 저밀도지단백(low-density lipoprotein, LDL)이 혈관내피세포(endothelial cell)를 뚫고 들어가 산화되는 것을 시작으로 내피세포의 활성화와 함께 면역세포, 혈관평활근세포(vascular smooth muscle cell)들의 복잡한 염증반응으로 발생하는데, 특히 혈관내피세포 손상 후 혈관평활근세포의 과도한 이동과 증식은 죽상동맥경화의 진행뿐만 아니라 관상동맥 재협착의 주요 기전으로 알려져 있다.

Etv2(ETS Variant Transcription Factor 2)는 ETS family의 구성원으로 특이적으로 태아 발생과정 중에서 혈관형성을 위해 일시적으로 발현하고 성체에서는 발현되지 않는 것으로 알려졌다. 하지만, Etv2가 혈관내피세포에서

내인성 발현 및 손상 후에 혈관 재생의 목적을 위해 발현한다는 연구가 보고되면서, 병적인 상태에서 제한적으로 발현된 Etv2의 역할에 대한 연구가 다양한 질병에서 진행되고 있다. 혈관신생과 관련하여 혈관내피세포에서 Etv2에 관한 많은 연구가 이루어지고 있지만, 혈관 구성의 또 다른 중요한 세포인 혈관평활근세포에 관해서 Etv2의 역할은 거의 알려져 있지 않다. 또한 Etv2와 관련된 내피세포와 혈관평활근세포의 상호작용에 관한 연구는 보고된 바 없다. 따라서 본 연구에서는 Etv2에 의해 내피세포에서 분비된 cytokine이 혈관평활근세포에 이동과 증식에 미치는 영향을 확인하였다.

먼저, 내피세포(HUVECs)와 혈관평활근세포(VSMCs)에서 Etv2의 내인성 발현을 Western blot과 세포염색을 통해 확인한 결과, Etv2의 발현은 HUVECs의 핵에서 유의하게 발현하고 VSMCs에서는 전혀 발현하지 않는 것을 확인하였다. 이와 같은 결과를 바탕으로 HUVECs에서 Etv2에 의해 분비되는 cytokine을 확인하기 위해 먼저 Etv2를 과발현하는 아데노바이러스를 제작하였고, 72 시간 동안 Etv2를 과발현시킨 HUVECs-conditioned media(Etv2-CM)을 이용하여 cytokine array를 진행하였다. 그 결과 lacZ-CM 대조군과 비교하여 Etv2-CM에서 CXCL1, CXCL5, MCP1, MCP2 및 IL8의 발현이 현저하게 증가하고 IL-6와 angiogenin의 발현은 감소되는 것을 확인하였고, 이를 qPCR를 통해 교차 검증하여 Etv2에 의해 변화된 cytokine의 통계적 유의한 변화를 확인하였다. 또한, Etv2 과발현에 의해 HUVECs의 형태학적 변화를 확인할 수 있었는데, 현미경과 phalloidin 염색에서 Etv2가 기존 세포형태에서 날카로운 스핀들 모양으로 세포의 형태학적 변화를 유도하는 것을 관찰하였다. 이와 함께, Etv2의 과발현이 HUVECs의 증식을 유도하는 것을 MTT assay 결과를 통해서 확인할 수 있었다.

HUVECs에서 Etv2에 의해 분비된 cytokine이 VSMCs의 이동과 증식에 미치는

영향을 확인하기 위해서 VSMC 배양 배지를 Etv2-CM로 교체 후 세포의 이동과 증식능력을 조사하였다. 그 결과, Etv2에 의해 분비된 cytokine에 의해서 VSMCs의 이동이 증가되는 것을 확인하였고, 반면 증식에서는 통계적인 유의성이 없었다. 2D migration assay를 통해 Etv2에 의해 증가된 5개의 cytokine (CXCL1, CXCL5, MCP1, MCP2 및 IL8) 중 CXCL5가 VSMCs의 이동에 직접적으로 영향을 미치는 cytokine임을 확인하였고, 3D migration assay를 통해 CXCL5 농도 의존적으로 VSMCs의 이동이 촉진되는 것을 다시 한번 확인하였다. VSMCs의 이동과 증식에 관여하는 MMPs 계열의 발현과 활성에 CXCL5가 미치는 영향을 western blot과 zymography를 통해 확인한 결과, CXCL5 농도 의존적으로 MMP9과 MMP13의 발현 및 활성이 증가되었고, MMP2의 발현은 영향이 없는 것을 확인하였다. 기존 논문에서 CXCL5가 종양세포에서 Akt, ERK 및 p38의 활성화를 통해서 암의 증식 및 전이를 촉진시킨다는 연구가 보고됨에 따라, CXCL5의 MMP9/-13 증가에 의한 VSMCs 이동에 있어서 Akt, ERK, p38 그리고 c-jun 인산화 발현 변화를 조사하였다. 그 결과, VSMC에서 CXCL5는 Akt, p38, c-Jun 인산화 수준을 시간 의존적으로 증가 시키는 것으로 나타났으며 ERK의 인산화 변화는 확인 할 수 없었다. 또한, Akt, p38 억제제(A6730, SB203580)는 VSMC에서 CXCL5로 의해 증가된 이동을 감소 시킬 수 있음을 확인하였으며, CXCL5로 의해 증가된 MMP9, MMP13의 발현과 활성을 억제시켰다.

본 연구는 HUVEC에서 Etv2 과발현으로 인해 분비되는 cytokine을 연구했고 분비된 CXCL5는 VSMC에서 MAPK(Akt, p38, c-jun) 신호전달 경로를 통해 MMP9, MMP13의 발현 및 활성을 조절하여 세포에 이동을 유도 할 수 있음을 확인했다.

1. Introduction

Cardiovascular diseases (CVD) is a disease that occurs in the heart and arteries and includes high blood pressure, ischemic heart disease, coronary artery disease, angina, myocardial infarction, atherosclerosis , and stroke [1]. In particular, atherosclerosis is one of the diseases with high mortality and prevalence and it is so fatal and dangerous.

Atherosclerosis is a disease in which blood vessels are blocked due to the occurrence of plaque [2], and is caused by various factors such as lipid accumulation, miRNA expression, DNA damage, proliferation and migration of vascular smooth muscle cells(VSMCs) [3-5].

VSMCs promotes plaque formation through abnormal migration and proliferation, however it also helps the formation of fibrous caps to prevent rupture of the plaques in atherosclerosis [6]. It is suggested that in-stent restenosis of atherosclerosis may be curable by inhibiting migration and proliferation of VSMCs [7]. Therefore, migration and proliferation of VSMCs is crucial regulator in atherosclerosis. VSMCs migration and proliferation are related to MMPs [8]. In the arteriosclerosis model, it was found that MMP2 and MMP9 are activated, which is involved in VSMCs migration and proliferation [9, 10]. VSMCs exhibits motility when phenotypic changes are induced, and this phenotypic regulation occurs in a very complex process such as chemokinesis, chemotaxis, and interaction with ECM [8, 11].

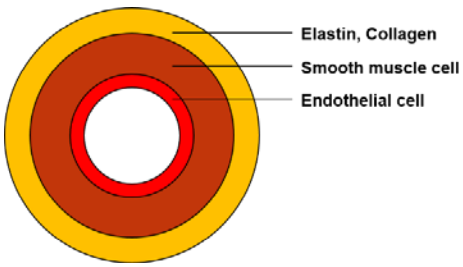

Figure 1. Vascular structure

As shown in Figure 1, The vascular wall is mainly composed of VSMCs and is

composed of one layer of endothelial cells (ECs) inside [12]. In addition, these two cells play an important role in vascular homeostasis, and they interact with each other to participate in the development and formation of the vascular system [12]. When blood vessels are formed, the initial interaction is the embryonic stage [12-14]. These primitive endothelial tubes recruit supporting cells, the pericytes or smooth muscle cells [6, 15]. When angiogenesis occurs, ECs damage occurs and VSMCs are recruited by inducing migration and proliferation [6]. Therefore, cytokines secreted from ECs by stimulation may affect VSMCs. However, the molecular mechanisms and ECs-VSMCs interactions that cause atherosclerosis are unknown. Therefore, in recent studies, many studies are being conducted on the interaction between endothelial cells and vascular smooth muscle cells as a key point of atherosclerosis.

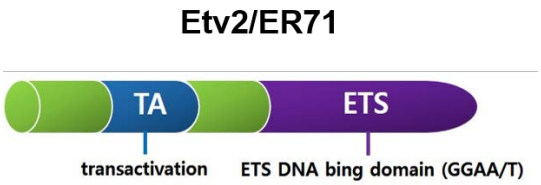

Figure 2. Domain structure of Etv2

Etv2 (Ets variant 2), also known as ER71 (ETS related 71), belongs to the ETS family of transcription factors. Etv2 has a consensus sequence (5'-GGAA/T-3') in the region of the enhancer or promoter that can be the target of ETS factor (Figure 2) [16]. Etv2 is transiently expressed up to 9 days during embryogenesis, but is turned off in mature embryo and adult tissues [17-19]. During embryonic development, Etv2 functions as key regulator of haematopoietic and vascular development by activating blood and endothelial cell lineage specifying genes, such as FLK1 (VEGF-R2), and enhancing VEGF signaling [20, 21]. Etv2 deficiency leads to a complete block in haematopoietic and vascular formation and embryonic lethality [17-19]. However, recent studies have demonstrated that although Etv2 is not known

to be expressed in adult, it reactivated following tissue injury or pathological conditions [17-24]. For example, Etv2 expression is increased in vascular endothelial cells of the hind limb ischemia model, and is essential for improving and remodeling blood vessel regeneration through FLK1, the transcriptional target of Etv2 [22]. Etv2 expression was remarkably increased in vascular endothelial cells of tumor angiogenesis, and tumor formation and angiogenesis were remarkably suppressed by treatment with Etv2 inhibitor nanoparticles [23]. Injection of Etv2 adenovirus into the heart of an animal model in which myocardial infarction is induced improves cardiac function through regeneration of vascular endothelial cells [24]. Etv2, JMJD2A and JMJD2D may jointly promote neuroendocrine prostate tumors [25]. ETS factors may inhibit tumor angiogenesis and reduce tumor growth [26]. Etv2 mediates endothelial transdifferentiation of glioblastoma [27]. Etv2 directly converts human fibroblasts into functional endothelial cells [28, 29]. Etv2 is expressed endogenously in endothelial cells [21]. However, there is no obvious role of VSMCs.

Cytokines are a family of signaling proteins that are secreted by cells and are small, 8-12 kDa. It is largely classified into four types (C, CXC, CC, CX3C chemokine) and interacts with several transmembrane proteins. Also, cell proliferation, migration and invasion are essential [30-32]. CXCL5 (C-X-C motif chemokine ligand 5) is a chemokine also called ENA-78 (Epithelial Neutrophil-Activating Peptide-78). It is a structure in which one other amino acid is inserted into the cysteine and cysteine signs [33, 34]. Overexpression of CXCL5 is found in a variety of cancers and promotes tumor growth and metastasis [35, 36]. CXCL5 promote metastasis of breast cancer cells via upregulation of Snail [37]. CXCL5 induces EMT and promotes cell migration, invasion, and lung metastasis, in nasopharyngeal carcinoma [38]. CXCL5 promotes neutrophil activity and EMT-induced gastric cancer metastasis [39]. In addition, CXCL5 reduces symptoms by inhibiting the formation of foam cells in atherosclerosis [40]. CXCL5 plays a protective role in coronary artery disease [41]. CXCL5 is being studied a lot in cancer. However, the mechanism of CXCL5 in cardiovascular diseases

(CVD) is unknown.

Accordingly, the objective of the present study was to examine the role of CXCL5 secreted by Etv2 in VSMCs. This study found that CXCL5 secreted by Etv2 in HUVECs induces migration to VSMCs. In addition, it was identified that the related mechanism signals by regulating MAPK (Akt and p38), and the relationship with MMP9 and MMP13 was also identified. These results suggest that CXCL5 secreted by Etv2 in HUVECs will play a positive role in cardiovascular diseases by interacting with ECs-VSMCs.

2. Materials and Methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVECs) obtained from Promo cell and were cultured in endothelial cell growth medium (Promo Cell) supplemented with growth medium supplement mix (Promo Cell). Vascular smooth muscle cells (VSMCs) were extracted from 6 weeks old male Sprague-Dawley rat thoracic aorta and were cultured in DMEM (Capricorn) supplemented with 10% FBS (Capricorn), 5% SMGS (Gibco). All the cells were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2 incubator.

2.2. Construction of adenoviral vector

Recombinant adenoviruses that expressing full-length human Etv2 with a C- terminal Flag tag (named as Ad-Etv2) were constructed using a ViraPower™ Adenovirus Expression System (Invitrogen). An adenoviral vector expressing lacZ-b-galactosidase (named as Ad-lacZ) was used as an infection control. The constructs were then used in a LR recombination reaction with the pAd/CMV/V5/DEST gateway vector to generate an adenoviral expression clone. Then, the constructed adenoviral vector was transfected into 293A cells using lipofectamine 2000 reagent (Invitrogen life Technologies) after digesting with the Pac I restriction enzyme. Adenoviral particles were tittered using the Adeno-X™ qPCR titration kit (Takara, Clontech).

2.3. Chemical reagent, inhibitor and antibodies

Recombinant human CXCL5 (Prospecbio) was used to simulate VSMCs(30,100ng/ml, 24h). Other cytokines were also purchased from Prospecbio. Specific Akt, ERK and P38 pathway inhibitors A6730 , U0126 and SB203580 were used at a concentration of 10μM, 10μM and 20μM. Antibody used in the experiment was Etv2 (ab181847), MMP2 (ab37150), MMP9

(ab76003) MMP13 (ab39012), p-Akt (CST #9271) Akt (CST #9101), p-ERK (CST #9272), ERK (Santacruz-135900), p-p38 (CST #9211), p38 (CST #9102), p-c-Jun (CST #9164), c-Jun (CST #9102), c-Jun (CST #9165), p38 (CST #9212).

2.4. Cytokine array

HUVECs were seeded in a 6-well cell culture plate at 3.3×10^5 cells/well for 12h with serum-starved (EC serum free media : EC media = 8:2), then treatment Etv2 virus for 72 hours. The cell culture supernatants were collected. cytokine in the supernatant were then analyzed using a RayBio® C-Series Human Cytokine Antibody Array C3 (Cat: AAH-CYT-3-4, RayBio), according to the manufacturer's instructions.

2.5. Western blot analysis

Vascular smooth muscle cells (VSMCs) were lysed in lysis buffer (phosphate-buffered saline containing 1% Triton X-100, protease inhibitor cocktail, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). Protein concentrations were determined using a Bradford protein assay kit. Equal quantities of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel via electrophoresis and transferred to a polyvinylidene difluoride (PVDF, Bio-Rad) membrane. The membranes were incubated with primary antibodies then, the membranes were incubated with a HRP-conjugated secondary antibody for 1h at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) system (Intron). The Image J software was used for quantification.

2.6. RNA extraction and Real-time quantitative PCR(qPCR)

RNA was isolated using TRIzol reagent (Invitrogen, USA), and 1 μg of RNA was subjected to reverse transcription using PrimerScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's protocol. Quantitative-PCR was conducted using a Bioneer program with Power SYBR™ Green PCR Master Mix (Thermo Fisher, USA) to determine the mRNA expression level of genes of interest. PCR primers used in this study are listed in Table 1.

2.7. Cell viability

For cell viability assay, experimental cells were evaluated by both the MTT and trypan blue assay. MTT assay was measured by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The absorbance was measured at 490 nm using an ELISA reader (TECAN, infinite M200 PRO). For Trypan blue assay, cells were detached by trypsinization and stained with 0.4% Trypan blue dye. The number of viable cells was counted under microscope using a hemocytometer counter. The viability of the control was regarded as 100%.

2.8. BrdU assay

Cell proliferation was measured using BrdU Cell Proliferation Assay kit (Cell Signaling, 6813) and performed following the manufacturer's instructions. Absorbance was read at 450nm using an ELISA reader (TECAN, infinite M200 PRO).

2.9. Immunofluorescence staining

HUVECs and VSMCs were plated in 24-well cell culture slides (BD Biosciences) with cover glass and cultured at 37ºC for 24h. After 24 hours, cells were infected with Ad-lacZ, Ad-ETV2

for 48h.Cells were immobilized with 2% paraformaldehyde for 15min followed by incubation with 0.5% Triton X-100 for 15min at room temperature. After permeabilization, cells were washed 3times with PBS then blocked with 2% bovine serum albumin (BSA) in PBS 1 hours. Cells were incubated with primary antibody overnight at 4ºC, and then stained with secondary at RT for 1h in dark. The cell's nucleus was stained with 0.2μg/ml DAPI (Sigma-Aldrich) for 1min. Finally, the cells were visualized by laser scanning confocal microscope (Fluoview FV1000, Olympus). For Phalloidin staining, cells were stained with phalloidin-conjugate working solution 30min at room temperature in dark. The cell's nucleus was stained with 0.2μg/ml DAPI (Sigma-Aldrich) for 1min. Finally, the cells were visualized by laser scanning confocal microscope (Fluoview FV1000, Olympus).

2.10. Cell migration assay

VSMCs migration assay was measured using Wound healing assay and Boyden chamber assay. For Wound healing assay, the cells ($6x10^5$/2ml) were collected and seeded into 6 wells. After 12 hs, scrape the center with a scraper. After washing with PBS, it was changed to DMEM (1% FBS and 5% SMGS), and treated with CXCL5. Boyden chamber (Costar®) is an 8.0 μm pore, 6.5 mm diameter transwell chamber used for 24 well plates. For Boyden chamber assay, gelatin coating is performed on the polycarbonate filter of the upper chamber. After coating 1% gelatin for 1 hour, the number of cells in DMEM is measured, and $3x10^4$ cells/400 μl inside the insert of the upper chamber, and 600ul of DMEM containing 1% FBS was added to each well in a low chamber, followed by incubation for 6hours and 12hours in a 37°C, 5% CO_2 incubator. After that, the lower part of the filter was fixed, stained with hematoxylin and eosin stain, and then carefully wiped with a cotton swab on the insert part to remove unmoved cells, and observed with an optical microscope at 200x magnification.

2.11. Zymography

Gelatin zymography can confirm the activity of MMP2 and MMP9 in conditioned cultured medium, and collagen zymography proceeds to confirm the activity of MMP13.Proteolytic enzyme MMP decomposes gelatin and collagen in the gel to quantify the degree of activity of the enzyme in the band that appears when gelatin is stained. Each experimental group was treated in VSMCs and cultured for 24h was collected, and the supernatant was obtained, loaded on 10% SDS-polyacrylamide gel with 0.8% gelatin and 0.3mg/ml collagen, and electrophoresed at 4℃ for 5h. After washing the gel after electrophoresis with 2.5% Triton X-100 twice at room temperature for 30minutes, incubation buffer for activating MMP2and MMP9 and incubation buffer for activating MMP13, reacted in an incubator at 37°C. Coomassie blue After staining with, the gel was checked after washing until the gelatin band decomposed with the destaining buffer was visible. The Image J software was used for quantification.

2.12. Statistical analysis

All quantified data from at least triplicate samples were analyzed with SPSS 13.0 software. Data are expressed as mean ± SD. Statistical comparisons between two groups were performed using Student's t-test. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA). A two tailed $P < 0.05$ was considered statistically significant.

Table 1. The sequences of primers used for qPCR analysis.

Gene	Forward(5'-3')	Reverse(5'-3')	Size(bp)
Etv2	ATTCAGCTGTGGCAGTTCCT	CCGAAGCGGTACGTGTACTT	242
CXCL1	GCGCCCAAACCGAAGTCATA	ATGGGGGATGCAGGATTGAG	70
CXCL5	CGGGAAGGAAATTTGTCTTGA	AGCTTAAGCGGCAAACATAGG	283
IL-6	GCACTGGCAGAAAACAACCT	TCAAACTCCAAAAGACCAGTGA	119
IL-8	CTCTTGGCAGCCTTCCTGATT	ACTCTCAATCACTCTCAGTTCT	171
MCP-1	TTCCCCTAGCTTTCCCCAGA	TCCCAGGGGTAGAACTGTGG	204
Angiogenin	TTCCTGACCCAGCACTATGATG	CGTCTCCTCATGATGCTTTCAC	74
KLF4	TCAAGAGCTCATGCCACCGG	CTCGCCTGTGTGAGTTCGCA	175
CX43	GGCCTTCCTGCTCATCCA	GGGATCTCTCTTGCAGGTGTAGA	74
Myocardin	CAGAAAGTGACAAGAACGATACAG	TGAAGCAGCCGAGCATAGG	265
α-SMA	AACTGGTATTGTGCTGGACTCTGG	CACGGACGATCTCACGCTCAG	188

3. Results

3.1. Construction of Etv2 adenoviral vector

This study constructed adeno virus vector that overexpresses Flag-Etv2 in order to identify the cytokine secreted from HUVECs by Etv2 overexpression. In order to insert Etv2 into a vector that overexpresses Etv2, pcDNA 6.1Flag-Etv2 was subjected to enzyme cutting and ligation with pENTR vector. The insertion site of the pENTR vector (Flag-Etv2) is inserted into the pAd/CMV/V5-DEST vector through LR recombination with the pAd/CMV/V5-DEST vector. The insertion site of the pENTR vector (Flag-Etv2) was inserted into the DEST vector by LR recombination with the DEST vector, and then cut with pac1 and transfected into 239A to construct adenovirus (Figure 3A). The expression level of the constructed Etv2 adenovirus was confirmed (Figure 3B-C). Etv2 adenovirus treatment in HUVECs increased the Etv2 expression level by about 4 times compared to lacZ in qPCR results (Figure 3B). Etv2 dose dependent adenovirus treatment in VSMCs confirmed the dose dependent Etv2 expression level (Figure 3C). As shown in the results, we could check the endogenous level in HUVECs, but the endogenous level could not be confirmed in VSMCs.

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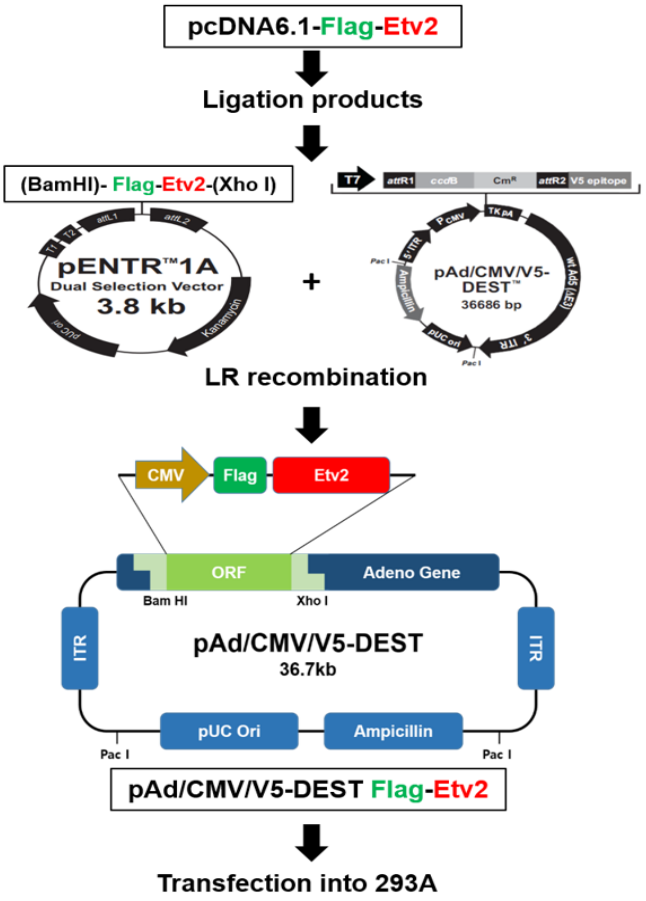

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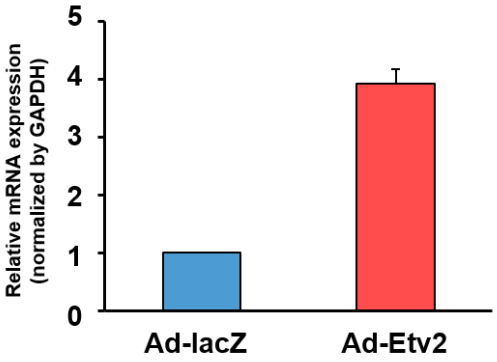

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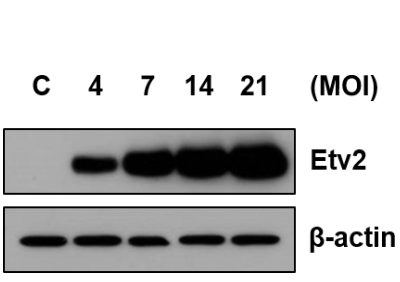


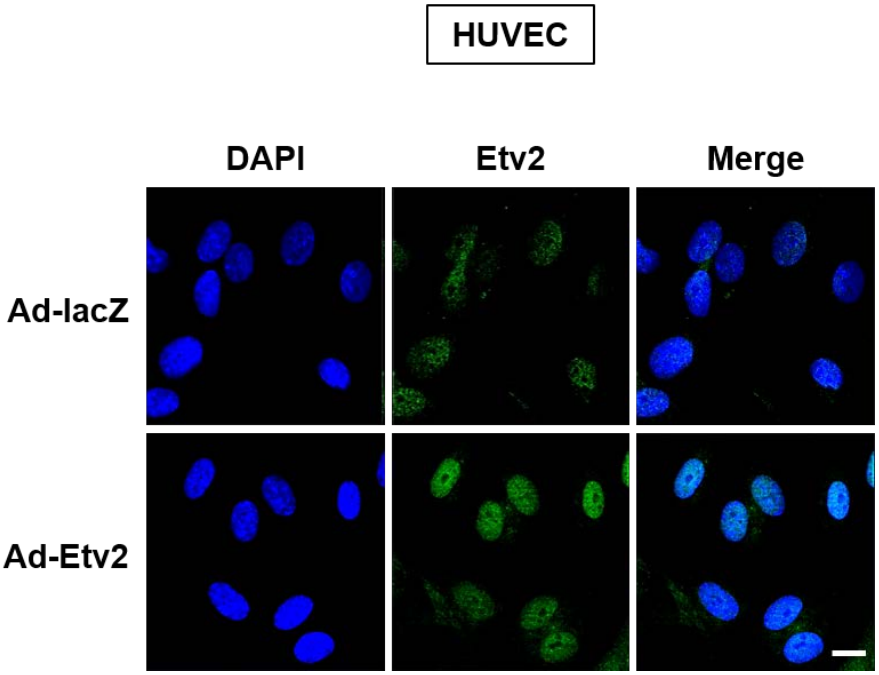
Figure 3. Construction of Etv2 adenoviral vector

(A) Development of adenoviral-vectored through a direct-cloning strategy. (B) Effect of Etv2 overexpression in HUVECs. qPCR confirmation of mRNA expression. Gene expression was normalized to GAPDH. Fold change was calculated relative to Ad-lacZ control. (C) Effect of dose dependent Etv2 overexpression in VSMCs. Representative western blots showing Etv2 expression in VSMCs. Protein levels were normalized to β-actin levels.

3.2. Expression of transcription factor Etv2

Etv2 is a transcription factor expressed in nucleus in ECs. It is expressed only up to 9 days during embryo development and is not expressed at all in mature embryo. In this experiment, immunofluorescence staining was performed by overexpressing Etv2 in HUVECs and VSMCs (Figure 4A-B). As shown in Figure 4, Etv2 expression in HUVECs was also expressed in endogenous, but VSMCs was not expressed in endogenous. And when Ad-Etv2 was processed, increased Etv2 expression could be confirmed.

A



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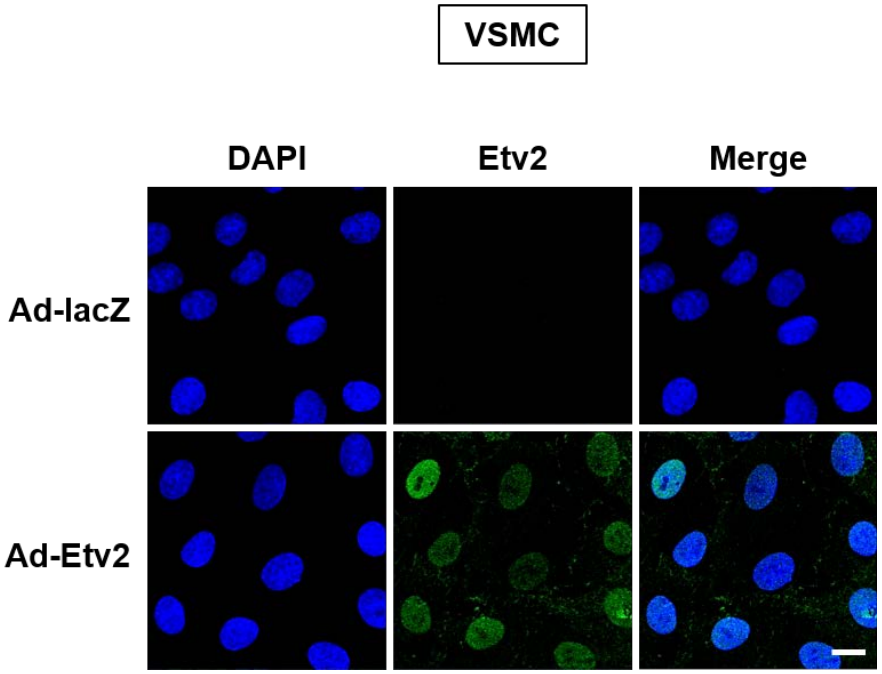


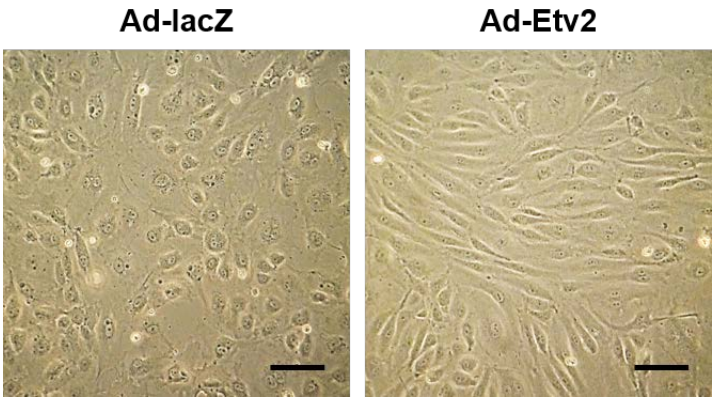
Figure 4. Etv2 expression and subcellular distribution in HUVECs and VSMCs

(A), (B) Representative fluorescence image of Etv2 staining (green) for Ad-lacZ- and Ad-Etv2-infected-HUVECs and VSMCs. Nuclei were stained with DAPI (blue). Scale bar, 10 μm.

3.3. Morphological change by Etv2 in HUVECs

To investigate the effect of Etv2 overexpression in HUVECs, cells were infected with Ad-Etv2 for 72h. As shown in Figure 5, the morphology of Etv2-infected cells was changed. Etv2-infected cells began to change morphology sharply after 48h and exhibited a sharp morphology at 72h. Moreover, it was confirmed that the cell morphology was clearly changed by the overexpression of Etv2 in the phalloidin staining to visualize the change in cellular actin filaments. MTT assay was performed to confirm the proliferation of Etv2 overexpression in HUVECs. Etv2 overexpression increased viability compared to lacZ (Figure 6). Therefore, the effect on Etv2 overexpression in HUVECs will increase proliferation while inducing phenotype change.

A



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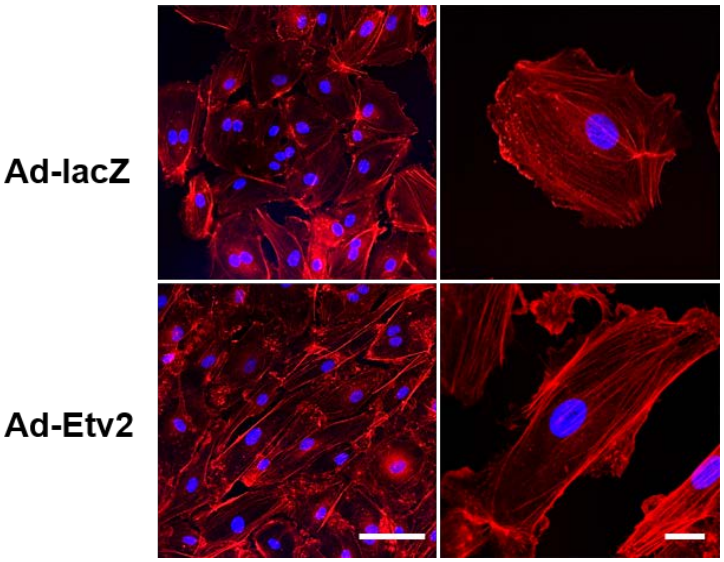


Figure 5. Effects of morphological change by Etv2 overexpression in HUVECs

(A), (B) Cell morphology pictures taken before and after Ad-Etv2 infection. (A) Phase-contrast microscopy of the changes in HUVECs morphology induced by Ad-Etv2. Scale bar (100 μm). (B) Confocal microscopy of phalloidin staining for changes in HUVECs morphology induced by Ad-Etv2. Scale bar (left 50 μm. right 100 μm). The image shown are representative of 3 independent experiments.

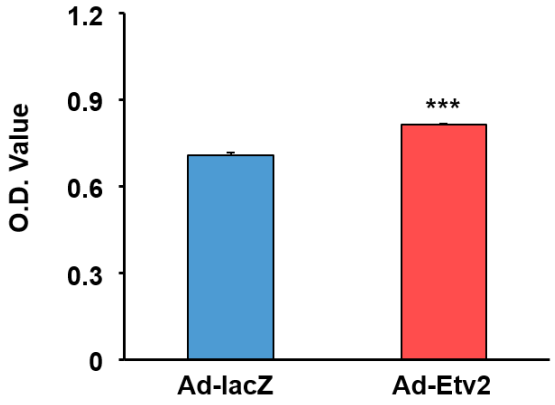
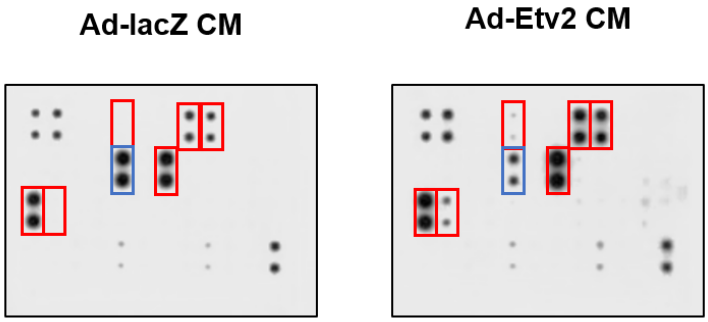


Figure 6. Effect of cell proliferation by Etv2 overexpression in HUVECs

MTT assay in Ad-lacZ and Ad-Etv2-infected HUVECs. Cell proliferation was normalized to Ad-lacZ control.

3.4. Identification of cytokines induced by Etv2 in HUVECs

Etv2 overexpression in HUVECs confirmed the effects of Etv2 overexpression: morphology changes and increased proliferation. Therefore, when Etv2 is overexpressed, a cytokine array is performed to determine which cytokine is secreted and decreased on HUVECs. The medium overexpressed by Etv2 for 72h in HUVECs was harvested, It was analyzed using RayBio® C-series human cytokine antibody array C3 (Cat: AAH-CYT-3-4, RayBio) according to the manufacturer's instructions. As shown in the figure, compared with lacZ CM, Etv2 CM increased GROa/b/g, CXCL1, CXCL5, MCP1, MCP2, and IL-8, and decreased IL-6, TNF-a, and Angiogenin (Figure 7). As a result, Etv2 CM altered several cytokines. Etv2 overexpression in HUVECs secreted several cytokines, and we examined whether it is the same as the change in mRNA expression level. Cells were harvested 24h after Etv2 overexpression in HUVECs. It was subjected to RNA extraction and qPCR. As with the previous results, compared with lacZ, Etv2 increased CXCL1, CXCL5, and IL8, and angiogenin decreased. Therefore, it was confirmed that the cytokines secreted by HUVEC and the mRNA expressed by cells were similar.



POS	POS	NEG	NEG	CXCL5	GCSF	GM-CSF	GROa/b/g	CXCL1	I-309	IL-1 alpha	IL-1 beta
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-gamma
MCP-1	MCP-2	MCP-3	M-CSF	MDC	MIG	MIP-1 delta	CCL5	SCF	SDF-1	CCL17	TGF beta 1
TNF alpha	TNF beta	EGF	IGF-1	Angiogenin	OSM	TPO	VEGF-A	PDGF-BB	Leptin	NEG	POS

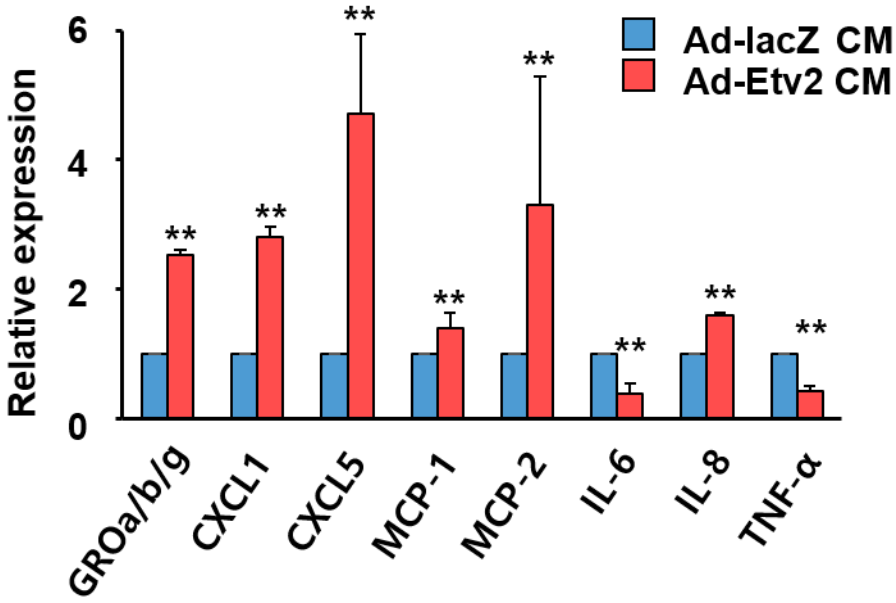


Figure 7. The level of cytokines secreted by Etv2 in HUVECs

(A) Cytokine array for HUVECs-CM from Ad-lacZ and Ad-Etv2. Each cytokine was detected in duplicate. Target cytokines are indicated using a square frame. Increased cytokines are red and decreased cytokines are blue; their names are listed below. (B) Grey density analyses indicating the levels of cytokines in HUVECs-CM using Image J software. The mean value (M) of the grey density of positive controls was calculated, and the grey density for each point of a target cytokine was divided by Etv2(M)/lacZ(M), and this ratio was normalized.

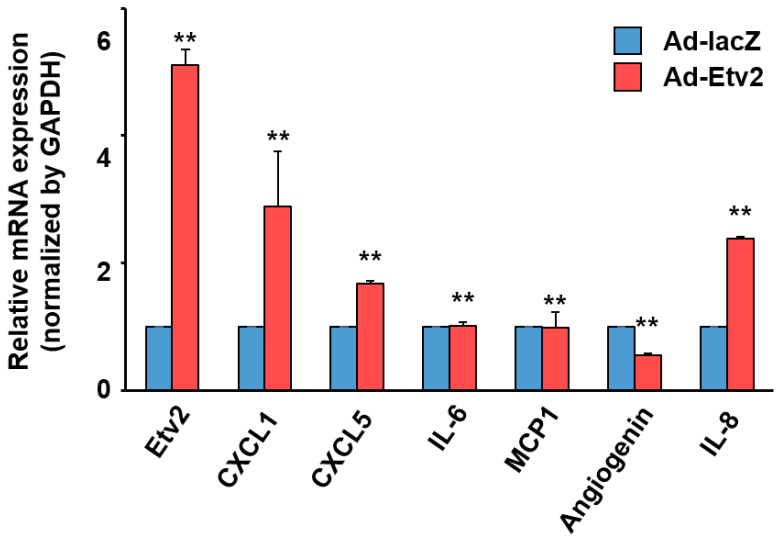


Figure 8. The mRNA expression levels of cytokines secreted by Etv2 in HUVECs

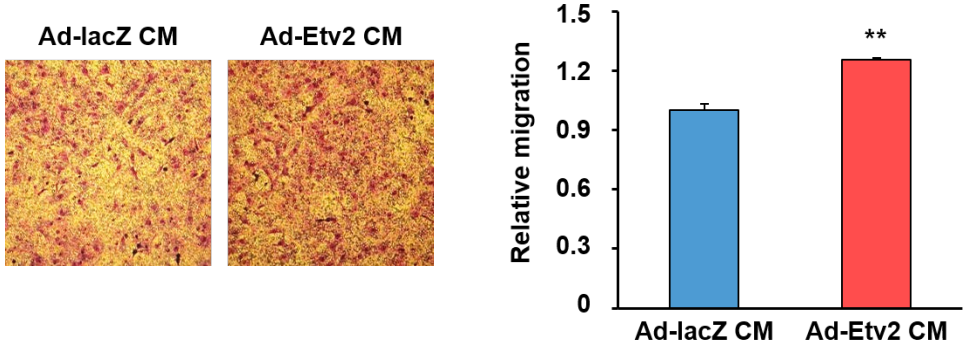
Cytokine expression by Etv2 overexpression in HUVECs. qPCR confirmation of cytokine mRNA expression. Gene expression was normalized to GAPDH. Fold change was calculated relative to Ad-lacZ control.

3.5. Effect of conditioned medium from Etv2-induced HUVECs on VSMCs migration

Migration of VSMCs from the media to intima is an essential part for pathogenesis of atherosclerosis. The effect of Etv2 Conditioned Media (CM) on VSMCs migration was investigated. CM from HUVECs infected with Ad-lacZ or Ad-Etv2 was prepared, and its role in VSMCs migration and the underlying molecular mechanism were investigated.

Ad-Etv2 CM was harvested from HUVECs. Boyden chamber assay was performed in VSMCs using the harvested medium. As a result, the migration was increased in Ad-Etv2 CM than in Ad-lacZ CM. In Figure 7B, the migration was confirmed by Wound healing assay, and the same as the Boyden chamber assay result, the increased migration was confirmed. Therefore, it is expected that cytokines secreted by Etv2 overexpression in HUVECs could induce migration to VSMCs.

A



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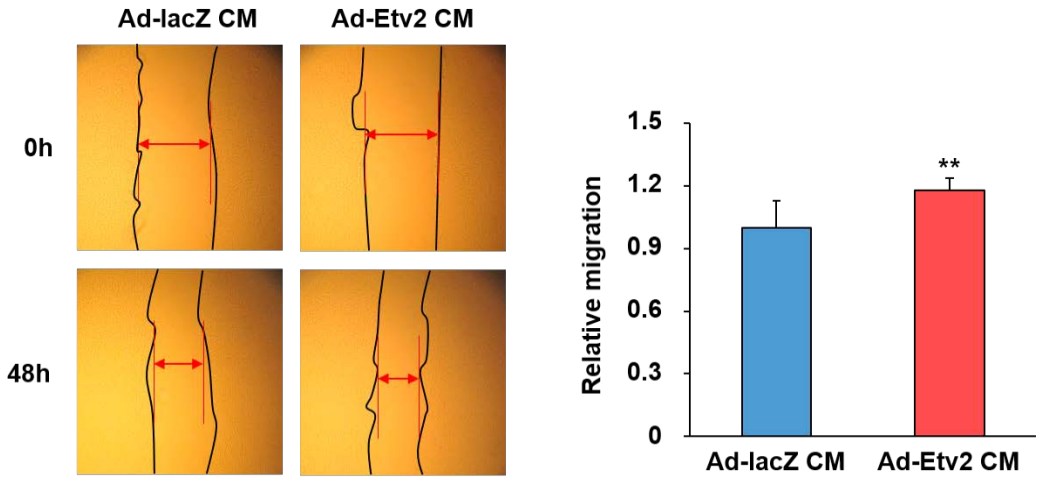


Figure 9. Effect of conditioned media from Etv2-induced HUVECs on VSMCs migration

(A) Cell migration by HUVECs-CM as a Boyden chamber assay. (B) Cell migration by HUVECs-CM as a wound healing assay. Each half of HUVECs-CM and serum free media were mixed to analyze the migration effect. The images were analyzed using the Image J software to evaluate the scratch by quantification of the areas occupied by the lesion.

3.6. Effect of CXCL5 on VSMCs migration and proliferation

The effect of CXCL5 secreted by HUVEC by Etv2 was investigated. To reduce the target further, each cytokine was treated to study 2D migration CXCL5 and MCP1 showed a significant increase in migration of VSMCs (Figure 10A). However, in the case of MCP1, there are already known papers, so we chose CXCL5 for novelty. VSMCs confirmed migration and proliferation by CXCL5 (Figure 10). With the Wound Healing Assay, CXCL5 treatment in VSMCs was able to confirm increased migration than control (Figure 10A). Similarly, with Boyden chamber assay, CXCL5 treatment in VSMCs was able to confirm increased migration (Figure 10B). However, CXCL5 did not significantly change cell viability and proliferation (Figure 10C). These results show that CXCL5 was not involved in VSMC proliferation, but may be involved in migration.

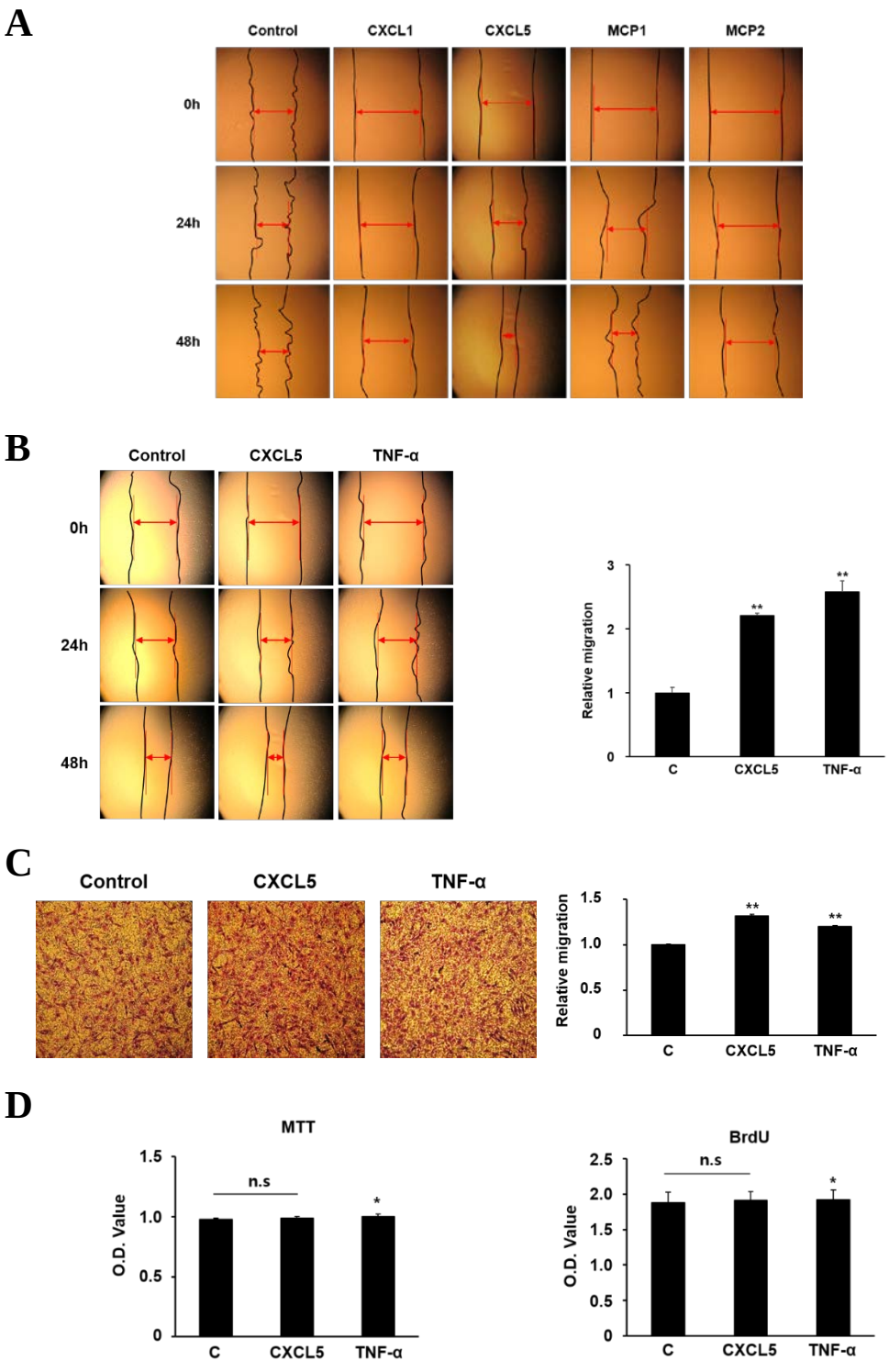


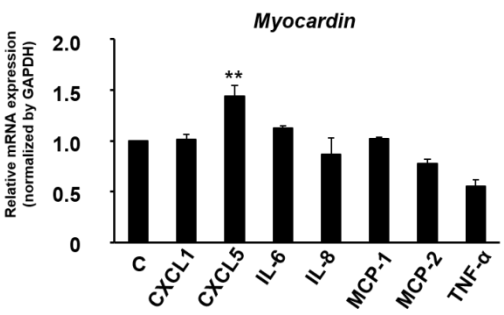
Figure 10. Effect of CXCL5 on VSMCs migration and proliferation

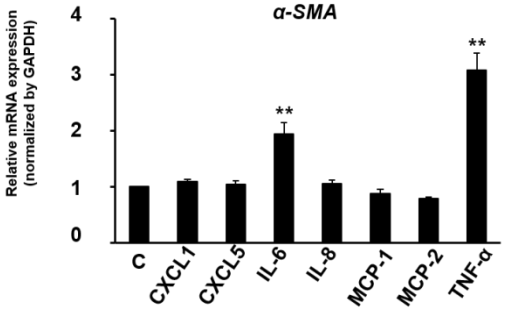
(A, B) Cell migration by CXCL5 as a wound healing assay. (C) Cell migration by CXCL5 as a Boyden chamber assay. The images were analyzed using the Image J software to evaluate by quantification of the areas occupied by the migration. (D) MTT assay and BrdU assay in CXCL5 treatment VSMCs. Absorbance was read at 450nm using an ELISA reader (TECAN, infinite M200 PRO). Cell proliferation was normalized to control.

3.7. Effect of cytokines on VSMCs phenotypic change

There is a way to check the migration and proliferation of VSMCs by checking the expression of phenotypic markers. The expression level of phenotypic markers related to proliferation was investigated. After treatment with recombinant protein in VSMCs, the levels of contractile marker Myocardin and α-SMA were investigated (Figure 11A). As a result, the levels of Myocardin and α-SMA showed significant changes in CXCL5, IL6, and TNF-α, but there was no significant difference. And the level of the synthetic markers KLF4 and CX43 were investigated (Figure 11B). As a result, there was no significant change in the levels of KLF4 and CX43, but there was a significant change in TNF-α. Therefore, it supports that there is no change in the proliferation effect by CXCL5 as in the previous results.

A





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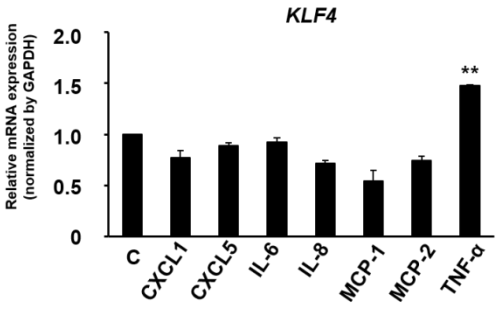


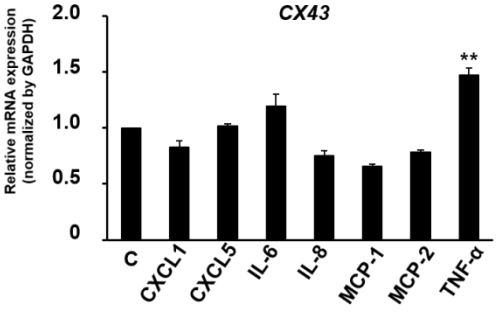


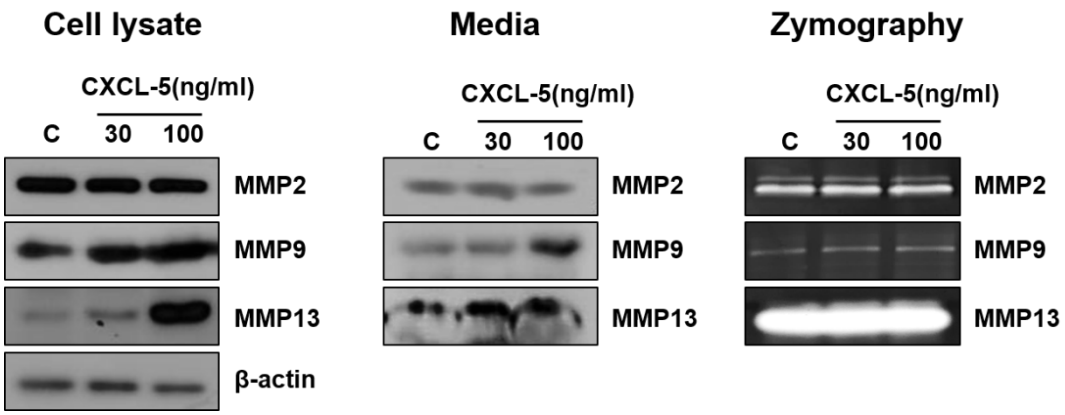
Figure 11. mRNA expression of phenotypic genes in cytokines-induced VSMCs

phenotypic genes expression by cytokine treatment in VSMCs. (A) Synthetic type marker mRNA expression. (B) Contractile marker mRNA expression. Gene expression was normalized to GAPDH. Fold change was calculated relative to control.

3.8. Effects of CXCL5 on expression of MMP2/9/13 in VSMCs

The activation of MMPs affects cell migration and proliferation in VSMCs. Also, as mentioned earlier, there were cases where MMP2 and MMP9 were increased in the atherosclerosis model. Treatment with CXCL5 confirmed the change in the expression level of the MMPs family. CXCL5 treatment in the cell lysate showed no change in MMP2 compared to the control, but it was confirmed that MMP9 and MMP13 increased (Figure 12A). The change in MMP level was the same in media and zymography (Figure 12B-C). These results show that CXCL5 increases the expression and activation of MMP9/13, which may be involved in the migration of VSMCs by CXCL5.

A



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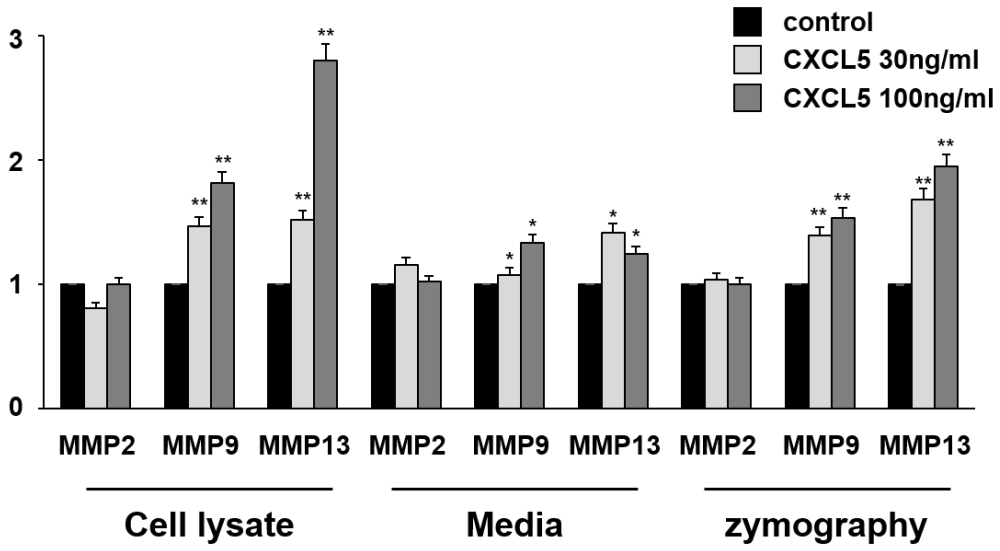


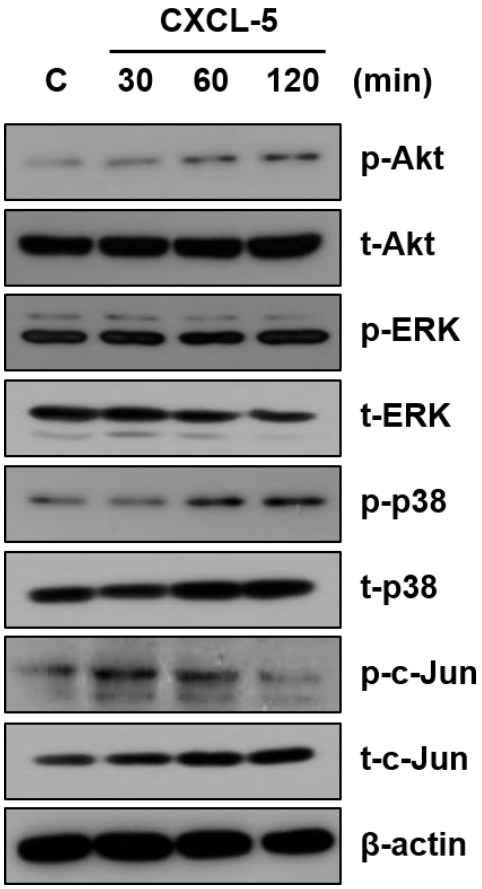
Figure 12. Altered MMPs expression by CXCL5 in VSMCs

(A) MMPs expression and activity by dose dependent CXCL5 in VSMCs. Western blots showing MMPs expression and activity in VSMCs. Protein levels were normalized to β-actin levels. Media. is concentrated acetone and performed by SDS page, and zymography is performed by using gelatin for MMP2 and MMP9 and collagen for MMP13. (B) The (A) were analyzed using the Image J software.

3.9. Effect of CXCL5 on Akt, ERK, p38 and c-Jun signaling pathway

CXCL5 is known to induce migration, proliferation and invasion in cancer through the MAPK pathway of Akt, ERK and p38. So, we investigated the signaling pathway of MAPK caused by CXCL5 (Figure 13). As a result, CXCL5 increased Akt and p38 phosphorylation levels, but there was no change in ERK phosphorylation levels. It also increased the level of phosphorylation in c-Jun. These results show that CXCL5 activates the phosphorylation of Akt, p38 and c-Jun, which shows that Akt and p38 pathways may be involved in the migration of VSMCs by CXCL5.

A



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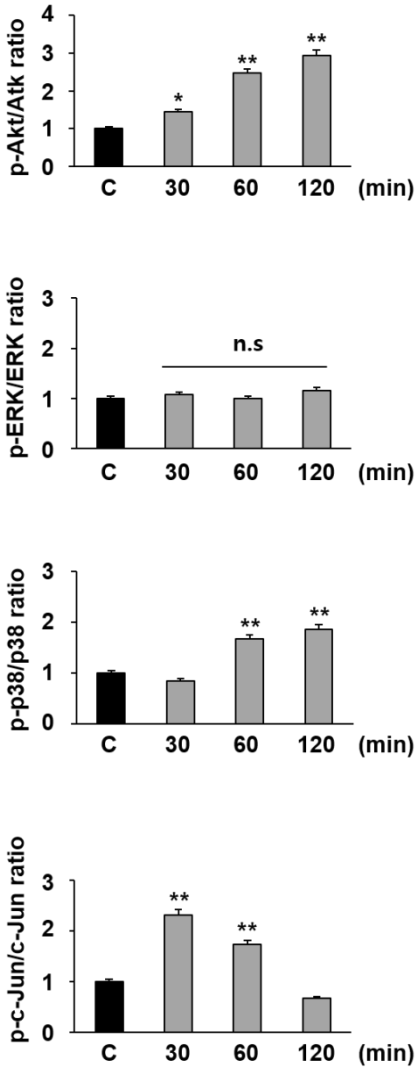


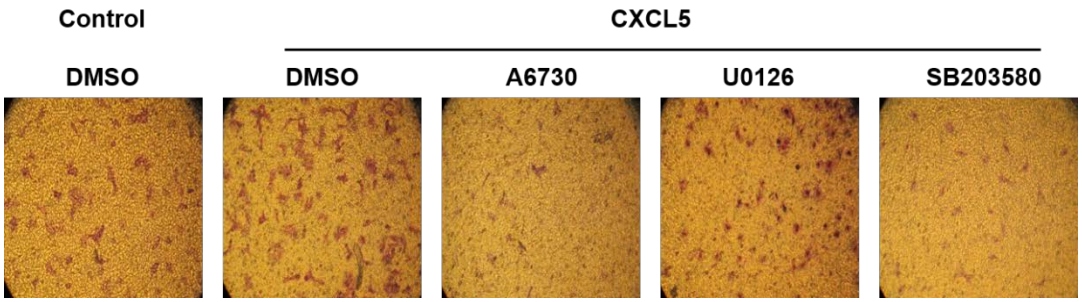
Figure 13. The phosphorylation levels of MAPKs in CXCL5 treated VSMCs

(A) Time-dependent analysis of Akt, ERK, p38 and c-Jun phosphorylation levels by CXCL5 in VSMCs. Western blots showing Akt, ERK, p38 and c-Jun activity in VSMCs. (B) Total protein normalization for western blot analysis.

3.10. Effects of Akt, ERK and p38 activity inhibition on VSMCs migration

Inhibitor experiments were conducted to confirm whether the migration of VSMCs proceeds through the MAPK signaling pathway. Boyden chamber analysis confirmed that VSMCs CXCL5 treatment increased migration, and when Akt inhibitor (A6730) treatment, increased migration due to CXCL5 decreased. In addition, the increased migration due to CXCL5 was reduced when p38 inhibitor (SB203580) was treated. However, ERK inhibitor (U0126) did not reduce the increased migration to CXCL5. These results show that CXCL5 increases migration, which shows that Akt and p38 pathways may be involved in the migration of VSMCs by CXCL5.

A



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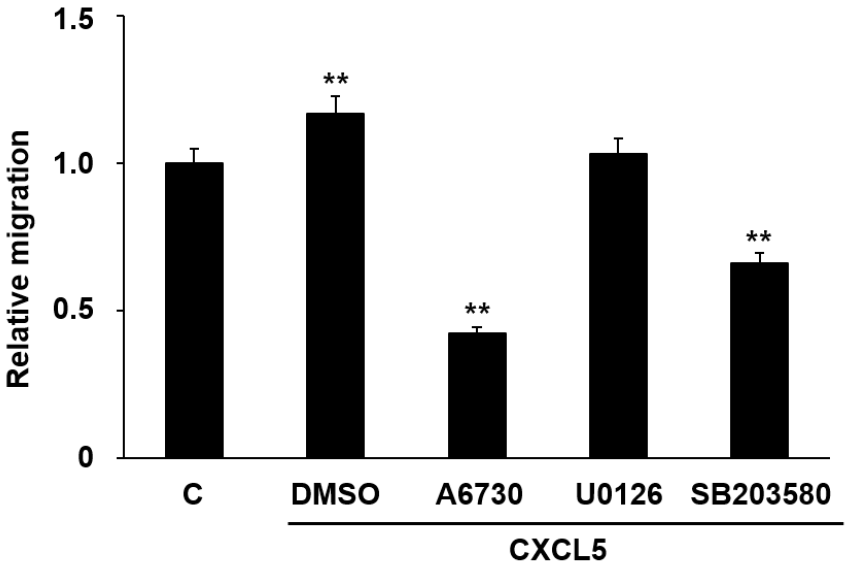


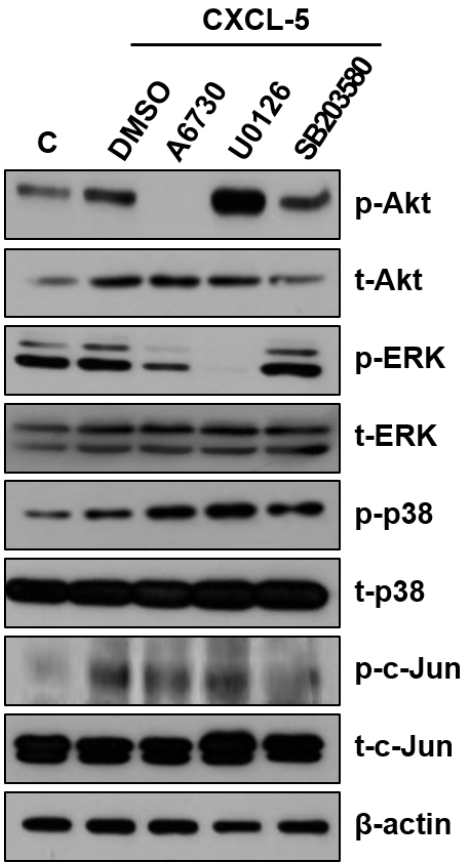
Figure 14. Effect of MAPKs inhibitors on VSMCs migration

Cell migration by CXCL5 as Boyden chamber assay. Akt (A6730), ERK (U0126) and p38 (SB239063) activity inhibitors were treated with CXCL5 after 30 min pretreatment to confirm VSMCs migration. The images were analyzed using the Image J software to evaluate by quantification of the areas occupied by the migration.

3.11. Effect of inhibitors on phosphorylation levels of MAPKs in CXCL5-treated VSMCs

Inhibitor experiments were conducted to determine whether the migration of VSMCs proceeds through the MAPK signaling pathway. In the experiment, the prepared cells were treated with CXCL5 after 30 min of pretreatment with inhibitor and harvested after 2h. Inhibition of p-Akt and p-ERK was well performed, but p-p38 was not. p38 inhibitor (SB203580) is a chemical that prevents autophosphorylation of p38, and further studies on p38 are required. And p-c-Jun level was confirmed that the phosphorylation level increased by CXCL5 decreased when treated with Akt and p38 inhibitors.

A



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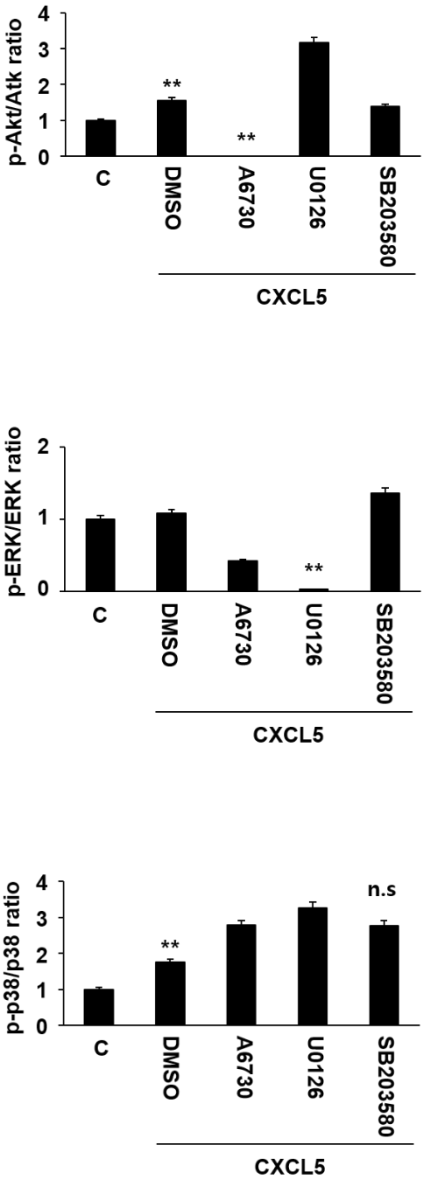
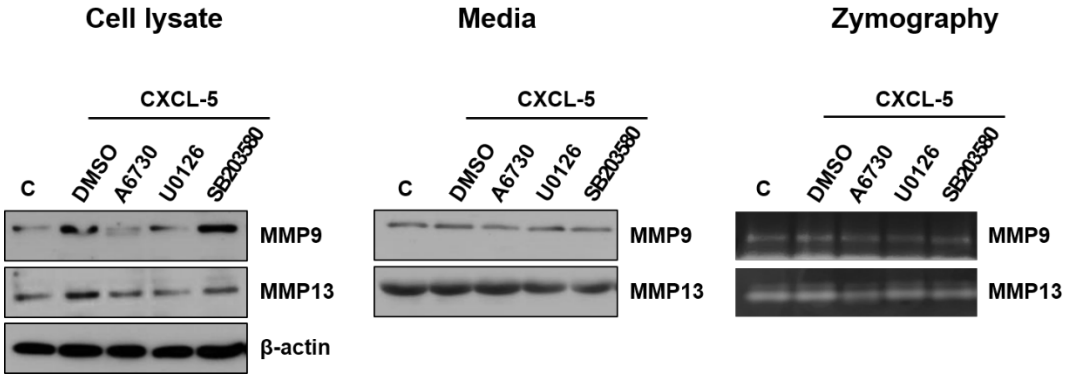


Figure 15. Effect of MAPKs inhibitors on phosphorylation levels of in CXCL5-treated VSMCs (A) Akt, ERK and p38 phosphorylation levels treated with CXCL5 after inhibitors of Akt, ERK and p38 activity. (B) Total protein normalization for western blot analysis.

3.12. Effect of MAPKs inhibitors on expression of MMP9/13 in CXCL5-treated VSMCs

It was confirmed that the levels of MMP9/13, which were increased by CXCL5, can be reduced by inhibitor treatment. Cell lysate confirmed that increased MMP9 due to CXCL5 was reduced by Akt inhibitor, but p38 inhibitor had no effect. In addition, the expression of MMP9 in media showed significant results, but the expression of MMP13 was not. zymography result confirmed that the activity of MMP9/13, which was increased by CXCL5, was reduced by Akt and p38 inhibitors. Therefore, CXCL5 is expected to be able to regulate the expression and activation of MMP9/13 through Akt and p38 signaling.

A



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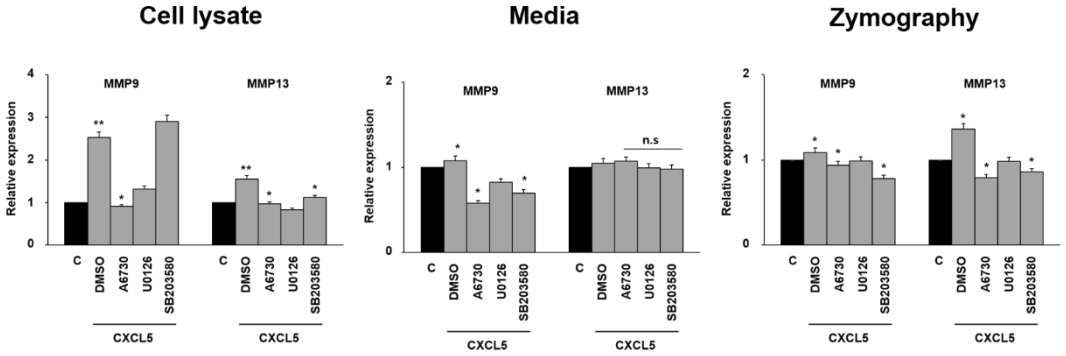

Figure 16. Altered expression of MMPs by CXCL5 after inhibition of Akt, ERK and p38 activity in VSMCs

(A) MMPs expression and activity by CXCL5 in VSMCs. Western blots showing MMPs expression and activity in VSMCs. Protein levels were normalized to β-actin levels. Media is concentrated acetone and performed by SDS page, and zymography is performed by using gelatin for MMP2 and MMP9 and collagen for MMP13. (B) The (A) were analyzed using the Image J software.

4. Discussion

The roles of Etv2 known so far are as follows. Etv2 is a member of the ETS family and is known as a transcription factor [16]. Etv2 is expressed until the 9th day of embryogenesis and is not expressed in mature embryos [17, 18]. It is known to play a key role in regulating angiogenesis and differentiation of blood cells during embryonic development[17-21]. In pathological conditions, Etv2 is expressed and, in particular, when blood vessels are damaged, the expression increases in ECs and is known to induce angiogenesis [22, 23]. In addition, it was found that treatment with Etv2 inhibitor in the same pathological condition inhibited angiogenesis [23]. Etv2, expressed in the ECs, plays an important role in angiogenesis [21]. However, no studies have been conducted on the ECs-VSMCs interaction by Etv2. The interaction of ECs-VSMCs plays an important role in vascular homeostasis and is involved in the development and formation of the vascular system [12]. For example, in the early stages of angiogenesis, the primitive endothelial tube recruits VSMCs, and when angiogenesis occurs, it causes VSMCs migration and proliferation [6, 15]. However, when pathological conditions come, the balance of ECs-VSMCs interactions is disrupted, causing abnormal VSMCs migration and proliferation [6]. Therefore, we evaluated which mechanisms of Etv2 can affect VSMCs. As a result of conducting a cytokine array with a medium overexpressing Etv2 in ECs, increased expression of CXCL5 was confirmed. In addition, as a result of treatment of Etv2 overexpressing HUVECs medium on VSMCs, the increased migration could be confirmed by Wound healing assay and Boyden chamber assay.

CXCL5 (C-X-C motif chemokine ligand 5) is a chemokine also called ENA-78 (Epithelial Neutrophil-Activating Peptide-78) [33, 34]. Overexpression of CXCL5 is found in a variety of cancers and promotes tumor growth and metastasis [34, 35]. For example, CXCL5 induces EMT and promotes cell migration, invasion, and lung metastasis in nasopharyngeal carcinoma [38]. CXCL5 promotes neutrophil activity and EMT-induced gastric cancer metastasis [39]. In addition, CXCL5 alleviates atherosclerosis by inhibiting the formation of

foam cells [40]. CXCL5 plays a protective role in coronary artery disease [41]. As such, CXCL5 is being studied in cancer, but the detailed mechanism of CXCL5 in cardiovascular disease (CVD) is unknown. Therefore, the experiment was performed by targeting CXCL5. The cause of atherosclerosis is known to be the primary cause of the migration of VSMCs. We evaluated CXCL5 ability to induce migration and proliferation in VSMCs. The migration of VSMCs was increased by CXCL5, but there was no significant change in the evaluation of the proliferative effect by MTT and BrdU. In addition, there was an increase only in the contractile marker myocardin, and there was no change in the synthetic markers CX43 and KLF4. Therefore, it supports that there is no change in the proliferation effect by CXCL5 as in the previous results. In an atherosclerosis model, MMP2 and MMP9 were found to be activated and involved in VSMCs migration. Therefore, the expression and activity of MMP2, MMP9 and MMP13 were confirmed by Western blotting and zymography by CXCL5 treatment. As a result, the expression and activity of MMP9 and MMP13 were increased. Therefore, in order to determine which signaling pathway is transmitted, the increased phosphorylation level was confirmed as a result of the activity of MAPK (Akt, p38), and the increased levels of MMP9 and MMP13 due to CXCL5 were reduced in inhibitor experiments.

This study confirmed that CXCL5, secreted by Etv2 in HUVECs, plays a role in the migration of VSMCs. Future studies need to further clarify the relationship with ECs-VSMCs by confirming the expression of Etv2 and CXCL5 in patient samples. We will also investigate the morphological changes in the underlying mechanism of HUVEC by Etv2 in the previous experiment.

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