Effects of vimentin protein transduction on biological function of vascular endothelial cells

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Effects of vimentin protein transduction on biological function of vascular endothelial cells

A Master's Thesis Submitted to the Department of Graduate Program in Science For Aging and the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master in Science for Aging

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ABSTRACT

Effects of vimentin protein transduction on biological function of vascular endothelial cells

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(Directed by Professor Yangsoo Jang)

The cytoskeleton plays a central role for the integration of biochemical and biomechanical signals across the cell required for complex cellular function. Recent studies indicate that the intermediate filament vimentin is necessary for

endothelial function. Vimentin is one of the intermediate filaments that had been considered to be related with cellular integrity and endothelial functions such as cell-cell adhesion, migration and tube formation. It was studied the potential endothelial function of vimentin as a therapeutic protein in HUVECs and its pathways during pHis/TAT-vimentin transduction, using protein delivery system.

The results indicated that pHis/TAT-vimentin was effectively transduced in HUVECs and had strong angiogenesis effects though tube formation assay. And it was shown that pHis/TAT-vimentin induced endothelial function is tightly linked to phosphorylation of VASP and activation of protein kinase A (PKA) during tube formation. It acts as stability effects of the cytoskeleton and influences tube formation of endothelial cells.

Previous studies found that vasodilator-stimulated phophoprotein (VASP), actin binding protein, has multiple serind/theronine phosphorylation sites. VASP localizes to endothelial junction complexes and co-localizes with ZO-1. To address the role of phospho-VASP in endothelial function, A phospho-specific VASP antibodies targeting phosphorylation site (Ser 157) were used. The site was preferred by PKA. Transduction of pHisTAT-vimentin induced VASP phosphorylation was attenuated by PKA inhibitors in HUVECs. Overall, pHisTAT-vimentin had strong angiogenesis effects and these effects were inhibited by blocking PKA.

Key Words: Vimentin, angiogenesis, Protein transduction domain, vasodilator-stimulated phosphoprotein, protein kinase A.

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I. INTRODUCTION

Endothelial cells play a wide variety of critical roles related with vascular function. The functional properties of the endothelial cells shifted their role from a passive membrane or barrier to a complex tissue with complex functions. Hence, it participates to all aspects of the vascular homeostasis also to physiological or pathological processes like thrombosis, inflammation, or vascular wall remodeling. Endothelial cells participate in role in blood vessel

formation, in coagulation in the regulation of vascular tone and in inflammatory reactions and in tumor neoangiogenesis

Because of these characters, endothelial cells are suitable for a diverse range of physiological processes, such as angiogenesis and as the selective blood barrier; and pathophysiological processes, including arterial disease and cancer development. Among endothelial cells, Human Umbilical Vein Endothelial Cells (HUVEC) were used in this study. It was isolated from normal human umbilical vein. They are responsive to cytokine stimulation in the expression of cell adhesion molecules. These cell systems are commonly used for physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, and fibrinolysis.

Many studies make an effort to find angiogenesis pathway to related to regenerate endothelial cells and protects an inner blood vessels. The growth of new capillary blood vessels in the body is an important natural process in vascular healing. It helps regeneration of damaged or lost tissues.[4, 5] The somatic maintenance system administrate the balance of angiogenesis modulators. The body controls angiogenesis by producing proper growth and inhibitory factors.

In angiogenesis, new blood vessels are formed by vascular endothelial cells. These neo-vessels supply oxygen and nutrients to the ischemic wound region. Fibroblasts grow and form transiently a new extracellular matrix (ECM) during

tissue formation. Also, collagen and fibronectin, which are the most critical proteins for the restoration of injured tissue was secreted in angiogenesis.[6, 7] Because of these, Angiogenesis-dependent diseases occur when new blood vessels either grow excessively or insufficiently.

Recent studies demonstrate that intermediate filament (IF) proteins play a role in angiogenesis. In many cells and tissue, IF proteins are essential for the integrity of cellular architecture and are protective against mechanical and other kinds of stress. Until recently, intermediate filaments had been considered to be relatively stable. It was proposed solely to provide cellular integrity and resistance against mechanical stresses. The IF proteins consist of greater than 50 members, constituting one of the larger gene families in the human genome. They all share a common predicted domain structure consisting of a central rod domain flanked by head and tail domains

Vimentin is one of the most established actins among the large IF protein family. It is the major IF protein in various cells and frequently used as a developmental marker of cells and tissue. It is a type Ⅲ intermediate filament protein that is expressed frequently in various cells. Vimentin is an important structural feature of endothelial cells. It makes up the cytoskeleton and exists as a dynamic structure. It consists of head, tail and central domain. Central domain and N-terminal domain (Head) plays an important role in intermediate filament

formation and stabilization. The role of the C- terminal domain (Tail) of vimentin is not pronounced. However, the removal of the C-terminal domain alters control of the assembling filament thickness. A structure of the vimentin monomer is presented in Fig. 1A. The amino acid sequence at this site is presented. The position of each amino acid in the heptad repeat pattern is indicated by notations of “a-g". This heptad repeat is thought to impose a coiled-coil assembly of polypeptide chains. It is studied that spin labels along the exterior surface of a dimer, and established in intact filaments that two dimers overlap at residue 348 in rod domain 2 (Fig. 1B). The overlap of two dimers at residue 348 in rod 2 presages an interaction between the free rod 1 domains. It was shown for such an overlap by introducing spin labels along the exterior surface of the dimer in the rod 1 region the broadening centered near position 191 indicates an interaction between dimers or higher level oligomers.

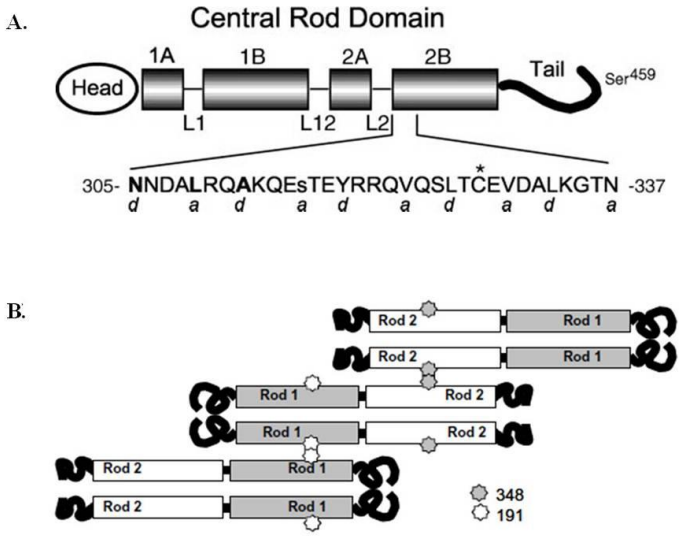

Fig. 1. **The structure of vimentin protein. (A)** The predicted vimentin molecule is shown diagrammatically, with the central rod domain emphasized. alpha-helical rod subdomains 1A, 1B, 2A, and 2B are shown. Hypothesized non-helical linker regions L1, L12, and L2 are drawn as thin lines. The region of rod subdomain 2B is expanded, and the sequence of this region is shown in single-letter amino acid abbreviations. Positions 305, 309, and 312 are bold; position 316 is lowercase. An asterisk marks the site of the single endogenous cysteine. Letters a and d below the amino acid sequence represent positions within individual heptad repeats in this area. The location of Ser459 is abstractly indicated near the end of the vimentin tail. (B) vimentin dimer interacts with rod domain 2 of one dimer (position 348), and rod domain 1 of another dimer (position 191). Both interactions are anti-parallel.

Vimentin has key roles in adhesion by regulating integrin functions. Integrins are heterodimer transmembrane cell adhesion receptors that are expressed in all metazoan cell-types, except erythrocytes, and the number of integrins expressed increases with increased complexity of the organism. Its filaments are required for endolysosomal vesicle transport and positioning of endosomes and lysosomes. The vimentin is the major IF protein present in leukocytes. Regarding the role of the vimentin in the transmigration on the endothelial side, it has been suggested that an ICAM-1 and VCAM-1-based "transmigratory cup" would be involved in leukocyte diapedesis through individual vascular endothelial cells. Most likely vimentin-deficient mice and/or phosphomimetic vimentin mutant cells and mice will help dissect the roles of the still poorly characterized transmigratory extravasation route. The protein can also affect the operation of protein complexes on the cell membrane by regulating cytosolic components in a fashion resembling the keratin-mediated modulation of TNFreceptor signaling by sequestering TNF receptor-associated death domain protein (TRADD). The vimentin protein has been demonstrated to function as a potential regulator of transcription, as it is able to interact and sequester transcriptional determinants such as p53. The ability complex with p53 represents another paradigm on the participation of vimentin in regulation of cell death and survival through protein interactions.

Many kinases are recognized as substrate for vimentin, including protein kinase C, A, G (PKC, PKA, PKG), cGMP kinase, RhoA kinase and PAK and so on.[8] The cores of focal adhesions consist of integrin receptors, hetero-dimers of alpha- and beta-submits which are intracellular surrounded by many adapter proteins as VASP.[16, 17, 27] Vasodilator stimulating phosphoprotein (VASP) is focal adhesion proteins that transmit mechanically induced alterations in the intermediate filament network into biochemical information.

In the recent years, people found a kind of protein functional domain, they can guide the bio-macromolecule to permeate the plasma membrane and accumulate in the cells, so they were called protein transduction domain (PTD). A new powerful protein drug as PTD-fusion protein using the PTD system is used. Protein drugs are expected as effective and strong drug, because they can affect specific targets directly, reduce the period of drug development, and have high safety better then drugs using target gene or chemical substances. PTD-fusion proteins entering into cells occurred quickly and affected target protein properties. The molecules carried into the cells hold their native activity. They can do their native work. So PTD is very charming because the character. It is possible for the macromolecule protein or polypeptide to permeate cytoplasmic membrane because of the PTD. PTD accelerates the protein engineering and the exploitation of protein medicine. At present, many kinds of protein have been

carried into the different cells or the animals, they hold their native activity. PTD will be applicator to find new therapy method and new function of protein. This study was used to the PTD system for protein delivery. Protein transduction domain (PTD) in a novel therapeutic perspective has been introduced to deliver target protein into cells directly. One of PTDs is the human immunodeficiency virus (HIV-1) transactivated TAT protein. By using TAT protein, numerous target proteins can be delivered into cells. The interaction of TAT-PTD with cell surface causes the internalization of TAT-fusion proteins.

As described above, it was established that endogenous vimentin considerably influences migration and proliferation in cells. However, effects on tube formation by endogenous vimentin was unsatisfied. It seems that transduced external vimentin protein support form new blood vessel.

The studies of tube formation functions have not been adequate. Therefore the PTD-fusion vimentin protein is transduced as the possible regulator in tube formation in this study.

Ⅱ. Material and Method

1. Materials

Vimentin and ZO-1 antibodies were obtained from abcam (Cambridge, MA, USA). Phospho-VASP (Ser157) was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-VASP (Ser239) was obtained from Calbiochem (San diego, CA, USA). KT5823 (PKG inhibitor) was purchased from Tocris Bioscience (Cookson Inc., USA), and H-89 (PKA inhibitor) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitric oxide Detection Kit obtains from intronbio biotechnology. (Seoul, Korea). Anti-b-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). In Vitro Angiogenesis Assay Kit was purchased from CHEMICON. (Termecula, CA, USA). Horseradise peroxidase-conjugated secondary antibodies and enhanced cheminescense (ECL) Western blotting detection system were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Molecular cloning of human vimentin gene

To construct pHis/TAT-vimentin fusion protein, the sense primer was 5'- ATC TAC GGA TCC ACC AGG TCC GTG TCC TCG- 3' and the antisense primer was 5'- AGT GTG GTC GAC TTA TTC AAG GTC ATC GTG -3'. The PCR product was restricted with BamHI and SalI, and then subcloned into the BamHI and SalI sites of the pHis/TAT bacterial expression vector. The correct sequence of the pHis/TAT vector was confirmed by DNA sequencing using the universal T7 primer.

3. Expression and purification of pHis/TAT-vimentin fusion protein

Escherochia coli BL21 pLysS (Novagen, Madison, WI, USA) was transformed to the pHis/TAT-vimentin plasmid, and then grown for 24 h at 37 ℃ in LB broth supplied with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and while shaking at 200 rpm. Protein expression was induced by the addition of 1 mM β-D-1-thiogalactopyranoside (IPTG) for 4 h while shaking at 37 ℃. The pHis/TAT-vimentin fusion protein were then isolated using a urea-denaturing protein purification protocol. The bacterial pallet was isolated by centrifugation at 3000 rpm, resuspended in buffer Z (8 M urea, 100 nM NaCl and 20 mM

HEPES, pH 8.0) , and sonicated 6 times with 15 second pulse continually adding 1 mM phenylmethanesulphonylfluoride (PMSF). The sample was then clarified by centrifugation at 14,000 rpm 4 ℃ for 1 h. The clarified lysate was loaded to Ni-NTA, Ni-IDA and Ni-TED column at 1 ml/min and then the column was washed using buffer A (washing buffer) (50 mM NaH2PO4, 300 mM NaCl) for 1 h at 1 ml/min. The elute vimentin fusion protein, the column was loaded by using buffer B (elution buffer) contained increasing concentrations of imidazole (10-500 mM) at 1 ml/min. Concentration of the protein was quantified by the Bradford assay. (BioRad, Hercules, CA, USA), using bovine serum albumin (BSA) as the standard. The purity of the fusion protein was assessed by Coomassie Brilliant blue staining. The purified fusion proteins were dissolved in PBS 10 % glycerol were aliquot and stored at -80 ℃.

4. Cell culture

Primary HUVEC cells (Cambrex Walkersville, MD, USA) were cultured in endothelial cell growth medium (EGM-2; Lonza, MD, USA) containing 2 % fetal bovine serum (FBS), 0. 4% hydrocortisone, 4 % hFGF-B, 0.1 & VEGF, 0.1 % R3-IGF, 0.1 % ascorbic acid, 0.1 % hEGF, 0.1 % GA-1000 and 0.1 % heparin, according to the manufacturer's instruction. Cells were grown in

atmosphere of 95 % air, 5 % CO_2 at 37 ℃. Sub-cultured HUVECs from passage 2 to 10 were used in these experiments.

5. Transduction of pHis/TAT-vimentin fusion protein into cells.

To transduce pHis/TAT-vimentin fusion protein, cells were grown to confluence in 60 mm dish or rotated in 1.5 ml tube. The culture was replaced with fresh containing 0.04 % FBS and supplement, then treated with various concentrations of pHis/TAT-vimentin fusion protein. The cells were washed by using PBS for 2 times while centrifuged. Cells were prepared for analysis by immunoblot.

6. Immunoblot analysis

At the termination of culture, the lysate were scraped into microcentrifuge tubes and centrifuged at 14,000 rpm for 15 min at 4 ℃. The supernatant were washing 2 times with PBS. And then, harvested cells were solubilized in cell lysis buffer containing 1 M HEPES (pH 7.5), 5 M NaCl, 0.5 M EDTA, Triton-X 100, protease inhibitor cocktail 1ea (Roche). Protein concentration was measured using a BSA. The same amounts of proteins from whole cell lysates

were loaded to 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-t 0.1 % tween 20) containing 10 % non-fat skim milk for 1 h at room temperature, washed with TBS-t at 4 times for each 7 min and incubated with primary antibodies for overnight at 4 ℃. The membranes were washed 4 times with TBS-T for each 7 min and HRP-conjugated secondary antibodies for 1 h at room temperature. After washing 4 times for each 7 min, the object size of proteins was detected using ECL Kits. (Millipore Co, Bedford, MA, USA and Santa Cruz Biotechnology).

7. Nitric oxide detection assay

NO activity was determined by the NO assay kit. This kit was composed of N1, N2 buffer, Nitric standard and plate. Cells were transduced with pHis/TAT-vimentin. And then, supernatant was collected. When supernatant was collected, Should be careful not to include cell debris. About 100 μl of supernatant was dispensed into wells in lines. Continually, dispense 100 μl of N1 buffer and incubate for 5-10 min at room temperature. After 10 min, final reaction was carried out by adding 50 μl of N2 buffer for 10min in room temperature. Finally,

measure the absorbance value between 520-560nm using a plate reader.

8**. Tube formation**

HUVEC tube formation assay was performed with an *In Vitro* Angiogenesis Assay Kit. ECMatrix gel solution was thawed at 4 ℃ overnight, mixed with ECMatrix diluent buffer and then placed in a 96-well plate at 37℃ for 1 h to allow the matrix solution to solidify. HUVECs were harvested with trypsin/ EDTA and 1×10^4 HUVECs were placed on a matrix solution with EGM-2 medium. It was incubated at 37 ℃ for 16 h. Tubule formation was inspected under an inverted light microscope (×100).

Ⅲ. RESULTS

1. Construction and purification of pHis/TAT-vimentin fusion protein.

The plasmid which produce artificial vimentin was designed as a cell permeable protein fused with histaq and TAT protein transduction domain. Constructed plasmid was transformed to Competent *Escherichia coli* BL21 plysS. The bacteria were cultured in LB medium with shaking at 37 ℃. 1 mM IPTG was added (O.D =) and incubated for additional 4 h in order to induce the expression of the recombinant proteins. Recombinant pHis/TAT-vimentin protein was purified by using Ni-NTA, Ni-IDA and Ni-TED affinity column chromatography. Or because pHis/TAT-vimentin was aggregated easily by elution buffer containing imidazole, it was not went through elution step and directly purified. The protein was analyzed by SDS-PAGE gel and stained with Coomassie brilliant blue. The purified protein was confirmed by immunoblot analysis with anti-vimentin antibody.

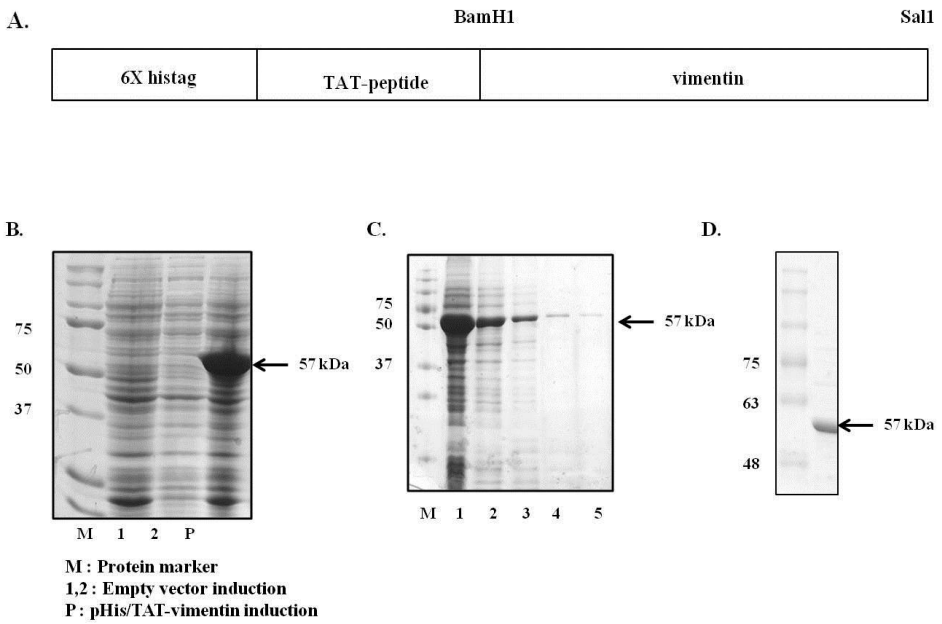

Fig. 2. Expression and purification of pHis/TAT-vimentin protein.

(A) TAT domain and vimentin-coding sequence were fused. (B) Recombinant pHis/TAT-vimentin transformed in BL21 pLysS was induced by 1 mM IPTG for 4 h. (C) pHis/TAT-vimentin was purified using NI-NTA affinity chromatography. Lane from 3 to 5 show continuous elution fraction. However,because pHis/TAT-vimentin was aggregated easily in elution buffer with imidazole, (D) pHisTAT-vimentin which went through elution step was purified directly using Ni-NTA, Ni-IDA and Ni-TED affinity chromatography.

2. Transduction of pHis/TAT-vimentin fusion protein into HUVECs

HUVECs were treated with pHisTAT-vimentin for 15 min or 2 h to determine efficient time of transduction. Efficiency of transduction was measured by immnoblot analysis. The immnoblot images presented that pHis/TAT-vimentin was effectively transduced into HUVECs either in short or long expose. However, transduction tendency was fluctuant in short expose. Additionally, vimentin was unstabilized in high concentration which led to apoptosis or hypertrophy of HUVECs. Taken together, the vimentin dosage was appointed at 5 μM to satisfy subjected conditions. Fig. 3C. was shown that pHis/TAT-vimentin fusion protein was greater transduced at 5 μM for 2 h.

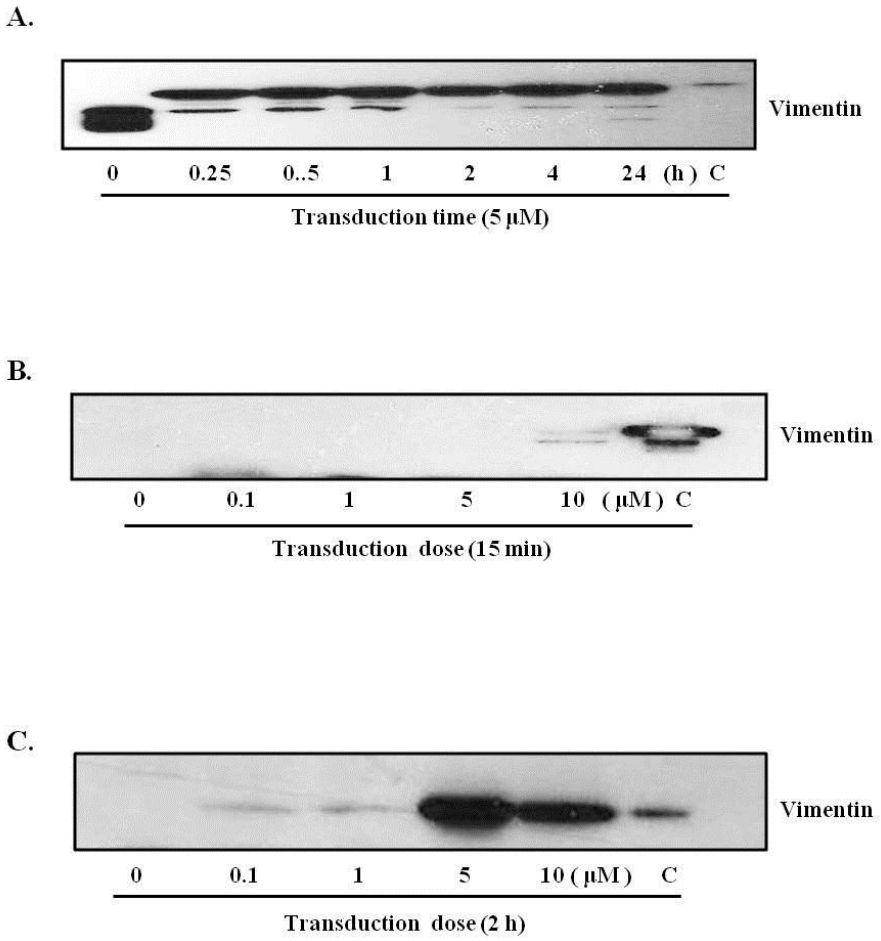

Fig. 3A. Vimentin protein transduction into endothelial cells.

HUVECs were incubated in EGM-2 with pHis/TAT-vimentin. Immunoblot analysis was performed using anti-vimentin antibody. (A) pHis/TAT-vimentin (5 μM) was continuously transduced in HUVECs as time-denepent manner. (B) The cells were treated with pHis/TAT-vimentin (0.1, 1, 5, 10 μM) for 15min or 2 h.

3. Effects of vimentin protein transduction on the tube formation.

Vimentin has been reported to be effective in endothelial functions such as adhesion, migration and proliferation. [9, 10, 11] However effect of tube formation was unsatisfied and there was a lack of research on tube formation effects of the vimentin. HUVECs were incubated with pHis/TAT-vimentin to study further tube formation function of the constructed protein. The vimentin-transduced cells was seeding on ECMatrix gel condition. After 22 h, tube formation patterns by vimentin were measured by optical microscopy (CKX41, OLYMPUS, Tokyo, Japan). It was shown that the pHis/TAT-vimentin increased tube formation as dose-dependent manner in the cells

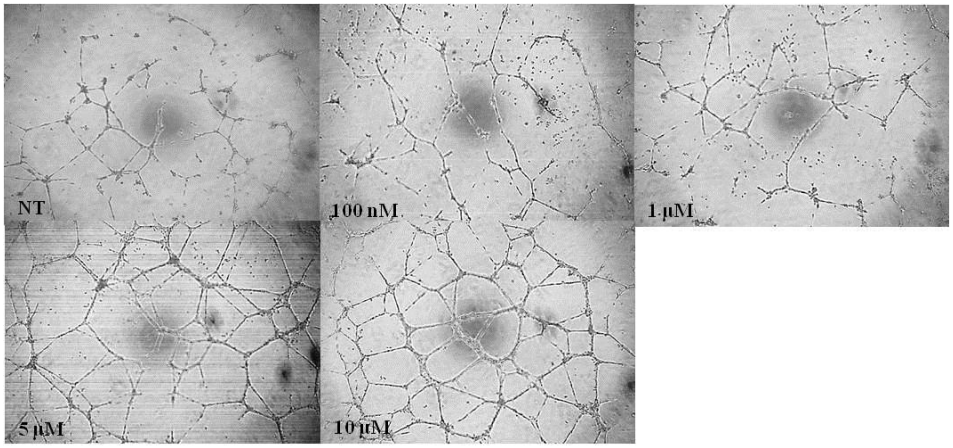

Fig. 4. Vimentin protein transduction promotes tube formation of endothelial cells.

HUVECs were treated with pHis/TAT-vimentin (0.1, 1, 5 and 10 μM) for 2 h After 20 h, each well was measured with microscope. pHis/TAT-vimentin increased tube formation on the cells as dose-dependent manner.

4. Vimentin protein transduction increases VASP phosphorylation.

Previous data have showed that the transduced pHis/TAT-vimentin induced tube formation. Many studies indicate that Vasodilator-stimulated phosphoprotein (VASP) plays a central role in endothelial barrier function. [27, 28,] The focal adhesion protein induced alterations in the intermediate filament network into biochemical information [30, 37, 40] The VASP was regulated through Nitric oxide or many pathways in endothelia function. [41] Hence, it was further more studied the relation between transduced pHis/TAT-vimentin and phosphorylation of VASP. An immnoblot analysis was examined to determine whether phosphorylation of VASP was regulated by the pHis/TAT-vimentin.

Vimentin (5 μM) was transduced for 15min because phosphorylation of VASP peaks before 1 h and lasted during 24 h. [30] Compared to control, the protein-transduced cells shown increased phosphorylation of VASP as dose-dependent manner. It was presented that vimentin transduction induces elevation of phosphorylation of VASP.

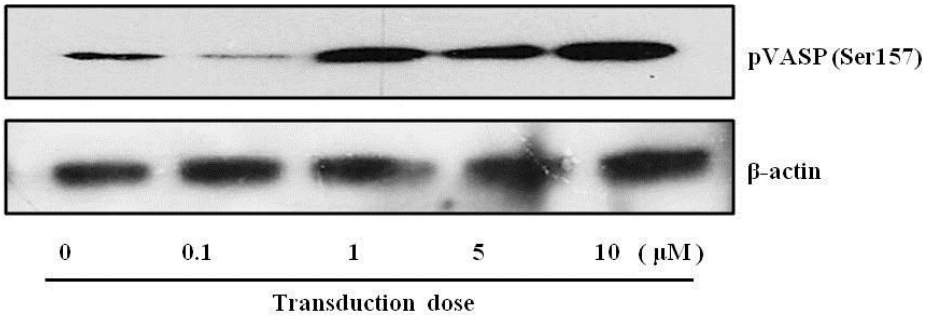

Fig. 5. VASP (Ser157) is phosphorylated by vimentin protein transduction.

The cells were transduced with pHis/TAT-vimentin (5μM) for 15min. Cell lysates were analyzed with anti-phospho VASP (Ser157). And it was quantified by anti- β -actin antibody. Phosphorylation of VASP was increased as dose-dependence compared to control.

5. Effects of PKA inhibitor on vimentin-induced tube formation.

Many studies indicated that phosphorylation of VASP is related to protein kinase A (PKA) [29, 31, 50] VASP is originally identified as a substrate of PKA which recognize Ser157 site of VASP. The PKA has been reported to involve in endothelial function. [40, 48] H-89 was used as PKA inhibitor to study whether the vimentin affect PKA activity on angiogenesis. Although previous studies used H-89 for 30 min or 1 h, [35] it didn't block PKA activity in this study. Therefore H- 89 was treated for 2 h before treatment of vimentin (5 μM)

Tube formation was reduced as concentration-dependent manner of H-89. (1, 10 and 20 μM). Results were presented that vimentin-induced tube formation was related to PKA activity.

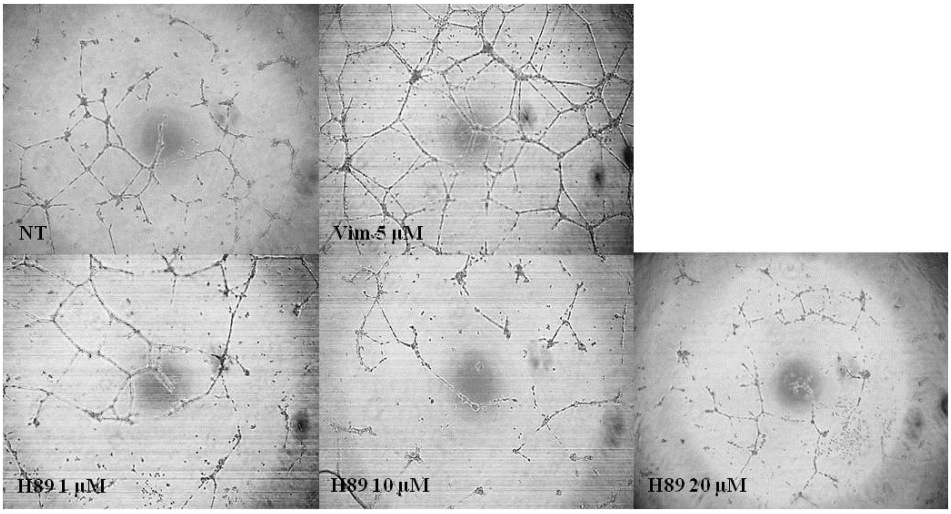

Fig. 6. PKA inhibitor suppresses vimentin-induced endothelial cell tube formation.

Cells were treated with PKA inhibitor (1, 10 and 20 μM) for 2h before the cells were incubated with pHis/TAT-vimentin (5 μM) and PBS (control) for 2 h

Tube formation assay was performed using a ECMatrix gel kit. Each well was photographed under microscope at X100 magnification. pHis/TAT-vimentin-mediated increased tube formation was significantly diminished by PKA inhibitor treatment.

6. Effects of PKA inhibitor on vimentin-induced VASP phosphorylation.

Recent studies reported that VASP was structurally localized at endothelial junction and rapidly phosphorylated (Ser157) by agent which activates PKA. [29, 35, 40, 48] Immunoblot assay was used to elucidate whether the vimentin induced-PKA has an effect on phosphorylation of VASP. Cells were pretreated with PKA inhibitor for 2 h and then of pHis/TAT-vimentin (5 μM) was added for 2 h As tube formation assay, VASP phosphorylation (Ser157) was increased by pHis/TAT-vimentin. The phosphorylation of VASP (Ser157) was decreased after H-89 exposure as dose-dependent manner.

The result shown that pVASP (Ser157) was associated with PKA activity in pHis/TAT-vimentin transduced HUVECs.

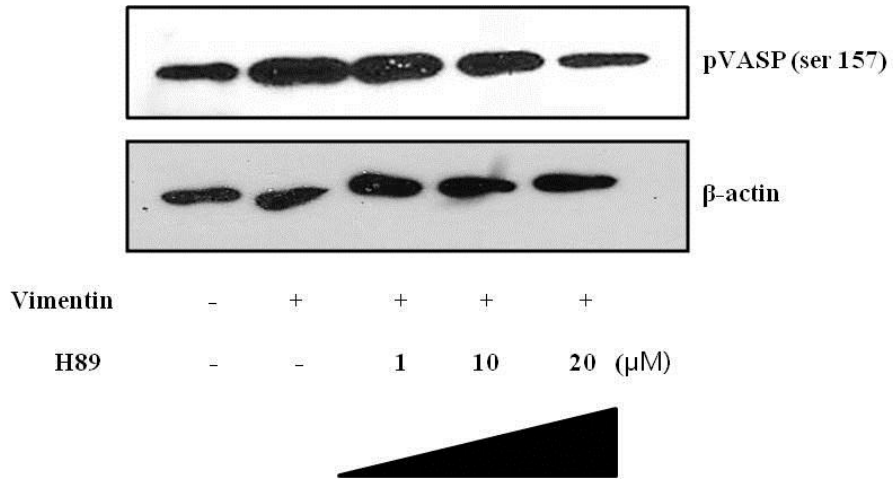

Fig. 7. Effects of PKA inhibitor on vimentin-induced VASP phosphorylation.

HUVECs were pre-incubated with H-89 (1, 10, 20 μM) for 2h before it was transduced with pHis/TAT-vimentin 5 μM for 2 h. Lane 2 indicated vimentin-induced pVASP (Ser157) and Lane 3-5 treated with H-89 showed decrease as concentration-dependent manner.

7. Effects Adenylate cyclase inhibitor on vimentin-induced tube formation

It is known that adenylate cyclase alter ATP to cAMP that activates PKA. The cells were pretreated with adenylate cyclase inhibitor (AC inhibitor) for 2 h before pHis/TAT-vimentin was transduced for 2 h to determine the protein induced-adenylate cyclase. After 24 h, tube formation was not reduced regardless of concentration of AC inhibitor. It was shown that PKA induced-tube formation was not affected by adenylate cyclase. Namely pHis/TAT-vimentin directly affects PKA activation.

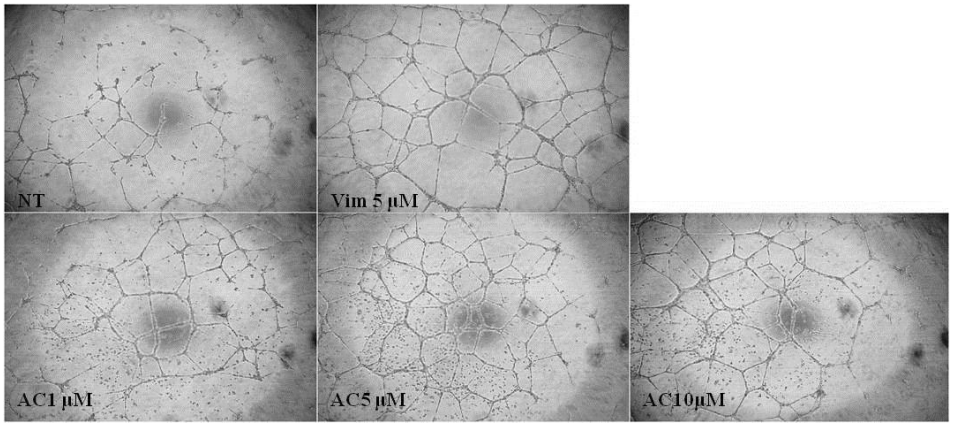

Fig. 8. Adenylate cyclase inhibitor did not affect vimentin-induced tube formation.

HUVECs were incubated with adenylate cyclase (AC) inhibitor (1, 5 and 10 μM) for 2 h. And then pHis/TAT-vimentin (5 μM) was treated on the cells for 2 h. pHis/TAT-vimentin induced tube formation lasted though AC inhibitor. It indicated that pHis/TAT-vimentin directly influenced PKA activation.

8. Effects of vimentin protein transduction on nitric oxide production.

It is famous that Nitric oxide plays crucial roles in the endothelial function and influence VASP phosphorylation [41, 42] NO production assay was performed using NO production kit to show the effect of pHis/TAT-vimentin on the production. Cells were treated with the protein for 15 min or 2 h as dose-dependent manner. Nitric oxide level in the supermant was measured

Results were shown that the fusion protein didn't influence Nitric oxide production. The phosphorylation of VASP by PKA was regardless of nitric oxide.

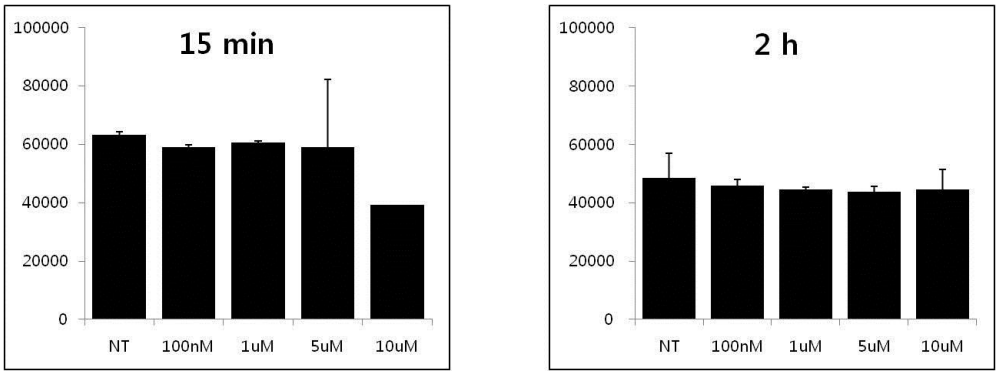

Fig. 9. The effect of transduced pHis/TAT-vimentin fusion on NO production.

HUVECs were treated with pHisTAT-vimentin (5μM) for 15 min or 2 h. Nitric level in the culture media with vimentin was measured using the NO production Kit. The statistical analysis was performed using the synergy H4.

9. Expression of junctional protein by vimentin transduction.

ZO-1 is known as a dominant tight junction-associated phosphoprotein and associate VASP. [40] ZO-1 is associated with actin microfilaments and was thought to influence alterations in actin distribution within endothelial cells as junction markers. PKA inhibitor was pretreated on HUVECs and pHis/TAT-vimentin was added to elucidate expression of ZO-1 by vimentin-induced PKA. ZO-1 was expressed in the fusion protein-transduced cells as Fig, 9 shown. The expression by vimentin was decreased by PKA inhibitor. This result indicated that vimentin-induced PKA expressed ZO-1 protein.

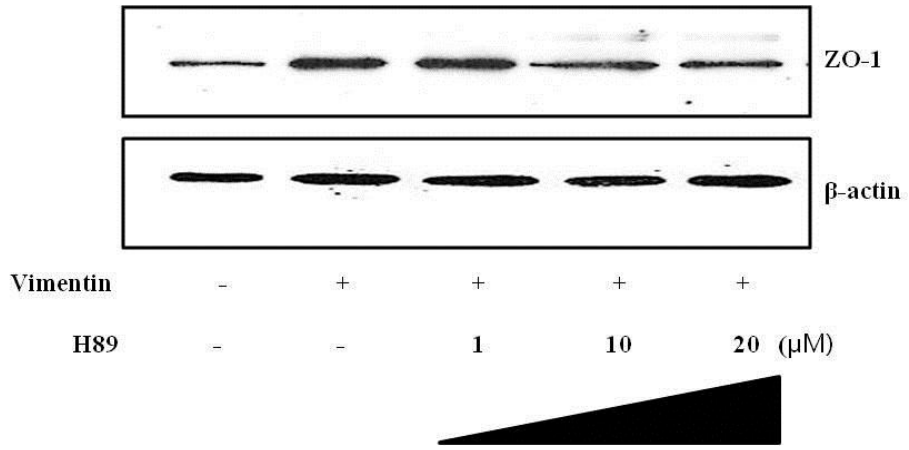

Fig. 10. pHis/TAT-vimentin induced ZO-1 expression was decreased by H-89. HUVECs were pre-incubated with H-89 (1, 10, and 20 μM) for 2h before pHisTAT-vimentin (5 μM) was added for 2 h to determine whether PKA expressed ZO-1 in the cells. Results showed that vimentin-induced ZO- expression was decreased by H-89.

Ⅳ. DISCUSSION

Intracellular delivery of vimentin fusion protein-fused to a protein transduction domain TAT was performed to induced angiogenesis. The fusion protein was produced as TAT-fusion vimentin protein containing the 11-amino acid transduction domain of TAT. It was constructed by recombinant technology. (Fig.2) Recent studies demonstrate that vimentin has been shown to participate in a number of critical functions and is related to organization of proteins that are involved in adhesion, migration and cell signal. The protein has key roles in angiogenesis by regulating integrin functions. It has also been shown to play a functionally important role in the regulation of cell-cell contacts in endothelial cells.[9, 10, 11] . Endogenous vimentin obviously affects migration and proliferation on HUVECs. Presence and absence of vimentin IFs markedly affects the organization and expression of surface molecules critical for adhesion or angiogenesis.[17, 20, 21, 22] However tube formation by the vimentin was comparative unsatisfied. Because the ability of endothelial cells to form capillary-like tubule structure is a necessary precursor for angiogenesis, it was studied that whether inserted vimentin increased tube formation in HUVECs.

Fig. 3 showed pHisTAT-vimentin fusion protein transduces into HUVECs for 15 min or 2 h continually. The vimentin protein was unstabilized in high concentration which led to hypertrophy and produce debris of the protein on the

cells.

Recent studies found that vimentin increases PKA levels in HUVECs and leads to phosphorylation of VASP protein on serine 157 (Fig. 4) Vasodilator stimulating phosphoprotein (VASP) is a focal adhesion protein that induces alteration in the intermediate filament network into biochemical information. The phosphoprotein associates with focal adhesion and areas of dynamic membrane activity and regulates actin polymerization. Furthermore, it is known to be a regulator of fibroblast migration, protrusion formation and adhesion. In endothelial cells, VASP is involved in tube formation and phosphorylated by vimentin.[27, 28, 30, 37] Previous studies show that vimentin is essential for VASP localization to the cell membrane. The VASP is phosphorylated by cAMP dependent kinase (PKA) and cGMP dependent kinase (PKG) in endothelial cells.[29, 31,32] VASP contains three phosphorylation sites: Ser239, Ser157 and Thr278. The equivalents of Ser157 and Ser239 but not Thr278 are found in separated by a divergent central proline-rich region, whereas Ser157 is the only site found in EVL(Ena/VASP homology). (Fig.11) Phosphorylation of VASP proteins at the conserved N-terminal (EVH1) serine (Ser157) leads to a major conformational change, which results in an angiogenesis and alterations in protein-protein interactions. PKA regulates filopodia formation in endothelial cells and increases VASP phosphorylation at Ser157.[31, 32, 33, 38, 40] In vitro, VASP

phosphorylation at Ser239 and Thr278 decreases its anti-capping and filament-bundling activity, and its ability to bind F- and G-actin. Phosphorylation at Thr278 is associated with decreased F-actin content, suggesting that it has a negative role in F-actin assembly, whereas phosphorylation at Ser239 in response to NO treatment which disrupts VASP localization in lamellipodia that leads to loss of lamellipodial protrusions.[41, 42, 43, 44, 45, 46] Phosphorylation at Ser157 is correlated with activities such as filopodia formation and regulation of protein-protein interactions. Namely, vimentin scaffold is essential for VASP localization to the cell membrane and VASP phosphorylation by PKG and PKA in vessels and endothelial cells. VASP was originally indicated as substrate of both cAMP and cGMP-dependent kinase and localized at actin-rich sites which regulate actin cytoskeleton dynamics. [27, 29, 31, 32, 40, 48, 50, 51]

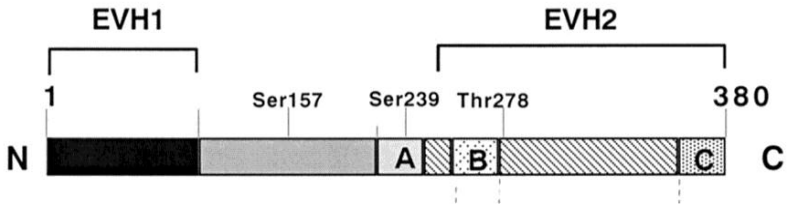

Fig. 11. The structure of vasodilator-stimulated phosphoprotein. VASP is a member of a family of proline-rich proteins. VASP family members are organized into three distinct domains. The homologous N-terminal (Ena/VASP

homology domains-EVH1) and COOH-terminal domains (EVH2) are separated by a divergent central proline-rich region . 1) The EVH1 region is responsible for VASP binding to proline-rich motif proteins, such as the adhesion proteins. 2) The central VASP domain binds the G-actin regulatory protein profilin that can promote the polymerization of F-actin. 3) The EVH2 region binds and bundles F-actin and localizes to stress fibers. VASP has three phosphorylation sites (Ser157, Ser239, and Thr278). Both PKA and PKG recognize and phosphorylate all three sites, but with different specificities and kinetics. Ser157 is the site preferred by PKA, whereas Ser239 is phosphorylated first by PKG. In this study, VASP is a key regulator of endothelial permeability elicited by PKA activation.[40]

Vimentin-induced phosphorylation of VASP was inhibited by the PKA-specific inhibitor H-89. (Fig. 6) . It is known that VASP is involved with PKA and cAMP-mediated PKA activation is an important signaling pathway for endothelial barrier stabilization. [50] PKA inhibitor completely blocked vimentin-induced increased pVASP. Inhibition of PKA by H-89 blunted vimentin-mediated tube formation of endothelial cells, providing evidence that this signaling pathway was PKA dependent under this experimental condition.

These data underline the significant role of PKA activation for endothelial tube formation and demonstrate that recruitment of VASP and PKA are required for this process. Elevated PKA activation promotes cell-cell, cell-matrix association, intracellular gap gomation, and permeability that are regulated by phosphorylation of complex-associated proteins. Activity of PKA plays a key role for endothelial tube formation. Fig. 5 showed that PKA inhibitor before treatment of vimentin prevented tube formation of HUVECs. Continually, Fig. 6 shown that pHisTAT-vimentin induced phosphorylation of VASP (Ser157) was decreased by H-89. It was demonstrated that increased PKA by vimentin led to strong phosphorylation of VASP (Ser157) in endothelial cells. It has been proposed that VASP phosphorylation at Ser157 by PKA induce tube formation and relaxation of cytoskeletal tension. This phosphorylation of VASP stabilizes endothelial function. [27, 28, 30, 37] It is known that cAMP activates the PKA that is essential to actin-based responses. The cAMP is mediated by adenylase cyclase. The adenylyl cyclase is an enzyme and integral membrane proteins that consist of two bundles of six transmembrane segments. Two catalytic domains extend as loops into the cytoplasm, as depicted in the figure to the right. A soluble (non-membrane bound) form of adenylyl cyclase has recently been characterized in mammalian sperm. This form of the enzyme appears to be activated by bicarbonate ion. When adenylyl cyclase is activated, it catalyses

the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which leads to an increase in intracellular levels of cyclic AMP. However, the fusion vimentin-induced tube formation was not affected by adenylase cyclase inhibitor. It was shown that pHis/TAT-vimentin directly affects PKA activity regardless of adenylase cyclase.(Fig. 7)

Among recent studies, Redistribution of VASP to filopodia was response to a Nitric Oxide donor. NO is recognized as a mediator of angiogenesis, emerged as an important cellular regulator, and plays important roles in diverse physiologic and pathologic processes. [41] Even though nitric oxide (NO) signaling has anti-inflammatory effects in the vasculature, NO production were not increased by vimentin transduction in this study. (Fig. 8). Also, it was known that VASP was localized to endothelial junctional complexes and co-localized with ZO-1, occlidin, and junctional adhesion molecule-1 (JAM-1). [40] Studies whether ZO-1 was expressed by vimentin were performed because PKA-induced phosphorylation of VASP appeared at cell-cell junction where ZO-1 was expressed. [40]. PKA inhibitor was pretreated as concentration-dependent manner before vimentin was added. Fig. 9 showed vimentin-induced ZO-1 expression level was decreased by PKA inhibitor. These evidences show how vimentin affects the angiogenesis. [10, 11, 14]

To summarize these results, angiogenesis is induced via phosphorylation of

VASP (ser157) occurred from PKA activation. The fusion vimentin protein directly activates PKA which phosphorylate VASP (Ser157) and continuously expressed ZO-1 at cell-cell junction. This signal led to tube formation on HUVECs. Therefore, discovering a variety role of vimentin and its expressing mechanism or studying agents that express vimentin will be a solution to improve angiogenesis-dependent diseases

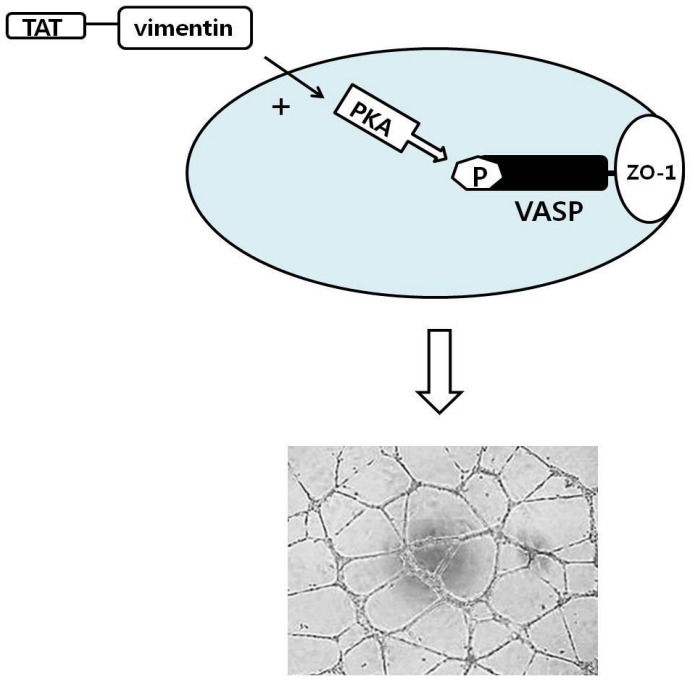

Fig. 12. The signal of vimentin-induced tube formation. The pHis/TAT-vimentin fusion protein was constructed using PTD-system. The protein was

transduced into HUVECs and it directly activated PKA without other stimulation. The activated PKA phosphorylated VASP and expressed ZO-1. It induced tube formation on HUVECs.

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ABSTRACT (KOREAN)

비멘틴 단백질 전달이 혈관내피세포 기능에 미치는 영향

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김은숙

세포골격은 복합적인 세포의 기능을 위하여 생화학적이고 생체역학적인 신호전달의 중요한 역할을 하고 있다.

최근 연구들은 중간미세섬유인 비멘틴 단백질은 혈관내피에 중요한 역할을 한다고 말한다. 세포에 내재되어 있는 비멘틴은 대표적인 중간미세섬유로 세포의 상태와 세포-세포 접합, 이동 그리고 증식과

같은 내피 기능과 관련이 있다고 알려져 있다. 그러나 혈관형성의 가장 기본적인 특성인 신생혈관형성에 관한 연구는 활발하게 이루어지지 않고 있다.

본 연구에서는 생체 물질 전달 시스템 중의 하나인 단백질 전달 도메인 (protein transduction domain, PTD) 을 이용하여 비멘틴 단백질을 인간 제대 정맥 내피세포로 전달하여 생물학적 효능을 관찰하였다. 인간의 비멘틴 DNA는 단백질 전달 도메인 TAT-PTD에 결합되어 세포 내로 침투가 가능한 재조합 단백질인 TAT-비멘틴으로 제조된다.

본 연구를 통해서 비멘틴 단백질이 인간 제대 정맥 내피세포에 전달 될수록 내재된 비멘틴과 시너지 효과를 이루어 신생혈관이 효과적이게 형성되는 것을 확인하였다. 그리고 신생혈관이 형성되는 동안 프로테인 카이네이즈 A과 프로테인 카이네이즈 G와 같은 프로테인을 활성화 시키는 인자들이 관련되어 있다는 것을 확인하였다. 최근 연구들을 통하면, 액틴과 결합하는 혈관 확장 조절 인단백질은 액틴의 구조과 세포막의 활성을 조절한다고 알려져 있다. 이 것은 세린 157과 세린 239와 같은 다양한 활성화 부위로 이루어져 있는데, 이 부위에 각각 프로테인 카이네이즈 A와 프로테인

카이네이즈 G가 결합하여 혈관 확장 조절 인단백질을 활성화 시킨다. 본 실험에서, 인간 제대 정맥 내피세포에 프로테인 카이네이즈 A와 프로테인 카이네이즈 G를 억제하고 비멘틴 단백질을 전달시켜보니, 혈관 확장 조절 인단백질의 활성이 감소하는 것을 관찰하였다.

결론적으로, 이 연구를 통해 단백질 전달 기술을 통한 비멘틴 단백질의 신생혈관형성 효과를 확인함으로써 필요한 단백질을 부가적으로 충족시켜주어 상처치유 가능성을 제시해 주었으며, 이것이 프로테인 카이네이즈 A, 프로테인 카이네이즈 G에 의해 억제되는 것을 확인함으로써, 세포 내로 전달된 단백질에 의해 형성되는 신생혈관형성을 조절할 수 있는 가능성을 제시 하였다.

핵심 되는 말: 비멘틴 프로테인, 신생혈관형성, 단백질 전달 도메인, 혈관 확장 조절 인단백질, 단백질 효소 A, 단백질 효소 G