치의학석사 학위논문

The effects of recombinant vitronectin fragments on the human osteosarcoma cell line behavior

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2012년 6월

서울대학교 대학원

치의과학과 종양및발달생물학 전공

김 오 복

김오복의 석사학위논문을 인준함

2012년 7월

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논문제목 : The effects of recombinant vitronectin fragments on the osteosarcoma cell line behavior

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-Abstract-

The effects of recombinant vitronectin fragments on the human osteosarcoma cell line behavior

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Vitronectin (VN) regulates various cellular responses, such as cell adhesion, spreading, and migration through ligand-cell surface receptor in vascular epithelial cells. In this study, three recombinant vitronectin fragments were cloned and expressed as soluble fusion individual proteins, and their biological function was analyzed. Among these, recombinant vitronectin fragment I (rVN-FI; amino acids 1-130) protein promoted HOS cell adhesion, spreading, and migration compared to recombinant vitronectin fragment II (rVN-FII; amino acids 131-303) protein and recombinant vitronectin fragment III (rVN-FIII; amino acids 304-459) protein. Moreover, function-blocking inhibition assay using monoclonal antibody against integrin subunits revealed that αV integrin subunit played key a role in HOS cell adhesion, and αVβ3 and αVβ5 integrins acted as receptor of rVN-FI protein. rVN-FI-derived synthetic peptides containing Arg-Gly-Asp (RGD) motif, P6 (AECKPQVTRGDV) and P7 (PQVTRGDVFTMP) completely blocked the adhesion of HOS cell to VN. These results

indicate that rVN-FI protein showed biological functions on osteoblast-like HOS cells. Thus, rVN-FI protein of human vitronectin may have potential application on bone tissue regeneration-related tissue engineering.

Keywords : vitronectin, HOS cells, cell behavior, integrin, bone regeneration

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Introduction

Vitronectin (VN) is a Arg-Gly-Asp (RGD) motif-containing adhesive glycoprotein present in blood plasma and in other body fluids as well as in the extracellular matrix (ECM) of many tissues including mineralized bone matrix (1-3). It regulates cellular important functions, such as cell adhesion, spreading, and migration (4-6). Through its various domains, VN interacts with multiple binding partners. A well-characterized domain in VN is the somatomedin B domain (SMB), a compact 4 disulfide bond knot at the N-terminus (7). That binds to the plasminogen activator inhibitor 1 (PAI-1) and urokinase receptor (uPAR) and plays a key role in wound healing (8,9). Immediately adjacent to the SMB domain is the RGD motif which is an integrin binding site regulating cell adhesion and migration (10). Integrin partners that recognize this RGD motif in VN are αVβ1, αVβ3, and αVβ5 integrins (11). Connecting region of VN containing RGD motif also contains a putative collagen-binding site (12). C-terminal domain of VN has four tandem repeat sequences with some homology to sequences in hemopexin (1). In the second hemopexin-like domain, positively charged amino acids (348-370) responsible for the glycosaminoglycan binding capacity of vitronectin were located (13). Interaction of ligands, such as plasminogen activator inhibitor-1 (PAI-1), may triggers conformational changes in VN that expose cryptic binding sites for integrins or heparins (12).

Bone ECM regulates bone cells by providing ECM - integrin interaction that enables the formation of adhesive structures and activate signaling pathways, which regulate cell spreading, survival,

and differentiation (14). In bone osteoblasts express a number of integrins, including αV, α1, α2, α3, α5, α6, α8 and β1, β3, β5 (15-19). Among these, αVβ1, αVβ3, and αVβ5 integrins are mainly expressed on endothelial and smooth muscle cells, on platelets, and leukocytes including osteoblast-like cell types (20).

Difficulties in obtaining a stable attachment to the material, immunogenicity, relatively higher cost, large size, instability, and sterilization problem, limited the application of ECM proteins in bone tissue engineering (21). Another approach for tissue engineering to overcome these disadvantages is the use of small peptides or proteins containing core binding sequence of the ECM proteins. N-terminal fragment of VN (amino acids 20-143) was less effective than fibronectin fragment containing type III modules coated on titanium of newborn mouse calvaria-derived MC3T3-E1 immature osteoblast-like cells behavior such as cell adhesion, differentiation, and proliferation (22). Furthermore, recombinant rVN143 (amino acids 20-143) peptide enhances human gingival fibroblasts adhesion and proliferation comparable to native VN by 70% (23). Full-length VN purified from *E. coli*, bound to heparin, and promoted cell adhesion, spreading, and growth of fibronectin-null mouse embryonic myofibroblasts similar contents to plasma VN (24). Thus, recombinant VN fragment or itself, especially N-terminal VN, can improve biological function of osteoblast-like cells or fibroblasts.

In this study, three recombinant fragments of human VN were expressed as soluble fusion proteins in *E. coli* to clarify the molecular determinants of the multiple biological functions of human VN. Functional studies revealed that recombinant VN fragment I (rVN-FI) promoted HOS cell adhesion, spreading and migration activities

relative to recombinant VN fragment II (rVN-FII) and fragment III (rVN-FIII). Furthermore, function blocking inhibition assay using monoclonal antibody against integrin subunits showed that αVβ3 and αVβ5 integrins act as receptor for rVN-FI. Peptide inhibition analysis determined that rVN-FI-derived P6 (AECKPQVTRGDV) and P7 (PQVTRGDVFTMP) peptides as important binding site for HOS cell adhesion onto VN.

Materials and Methods

Cell Culture

The human osteosarcoma (HOS) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained under a humidified atmosphere of 5% CO_2 at 37℃in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Antibodies and reagents

Function-blocking monoclonal antibodies (mAbs) against the human integrin α1, α2, α3, α5, αV, β1, β3, β4, β5 subunits and αVβ3, αVβ5 integrins were obtained from Chemicon (CA, USA). Human plasma VN was obtained from Millipore (Billerica, MA, USA).

Construction, expression, and purification of human vitronectin fragments

The human VN cDNA was cloned using a reverse transcriptase-polymerase chain reaction with reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions by using mRNA isolated from HepG2 cell lines. Three VN fragments (rVN-FI, rVN-FII, and rVN-FIII) were amplified by polymerase chain reaction using the VN cDNA as a template and ligated into either the

pGEM-T Easy vector. The polymerase chain reaction primers used were as follows: rVN-FI, 5'-GGATCCGACCAAGAGTCATGCAAG-3' (sense) and 5'-GAATTCTCAGGGCTGAGGTCTCC-3' (antisense); rVN-FII, 5'-GGATCCCCAGCAGAGGAGGAGC-3' (sense) and 5'-GAATTCTCACCAGAAGAGAAGCTCGAAT-3' (antisense); rVN-FIII, 5'-GGATCCGGCAGAACCTCTG-3' (sense) and 5'-GAATTCTCACAGATGGCCAGGAGCTG-3' (antisense). Nucleotide sequences of all of the plasmid constructs were confirmed by sequence analysis. The pGEM-T Easy vector containing VN cDNA fragments were digested with appropriate restriction enzymes. These cDNA fragments were subsequently cloned into either the *Bam*HI-*Eco*RI site of the mammalian expression plasmid vector pET-32a(+) (Novagen, Darmstadt, Germany), or into the *Bam*HI-*Eco*RI site of the mammalian expression plasmid vector pRSET (Invitrogen, Carlsbad, CA, USA). Correct orientation of the inserts was verified by sequence analysis. The molecular weights of the expected rVN-FI, rVN-FII and rVN-FIII proteins were 32, 23, and 35 kDa, respectively.

Induction of protein expression in *Escherichia coli* strain BL21 grown to the midlog phase in Luria - Bertani medium was carried out using 1 mM isopropyl-β-D-thiogalactopyranoside (RV Haarlem, Netherland). After protein induction for 5 h at 30℃, the cells were harvested by centrifugation at 6000 x g for 10 min. Cell pellets were stored at -80℃ until use. For rVN-FI protein purification, pellets were thawed and resuspended in ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). For rVN-FII and rVN-FIII protein purification, pellets were thawed and resuspended in ice-cold lysis buffer (100 mM NaH_2PO_4, 10 mM Tris - HCl, 8 M urea, pH 8.0). Cell

lysis was carried out by passing the cell suspension through a 10 ml pipette, using caution to avoid foaming. The clarified cell lysates were applied to a Ni^{2+}-nitrilotriaceticacid agarose column (QIAGEN, Valencia, CA, USA). The column was washed with 10 mM imidazole in lysis buffer and eluted with 250 mM imidazole in lysis buffer. Then purified recombinant histidine (His_6)-tagged rVN-FI protein was stored at -80℃until use. Purified recombinant histidine (His_6)-tagged rVN-FII and rVN-FIII proteins were dialyzed sequentially against a solution containing 10 mM Tris-HCl, 100 mM NaH_2PO_4, 1 mM phenylmethylsulfonylfluoride, and 3, 2, 1, or 0.5 M urea, pH 3.0. Finally, the proteins were dialyzed with 1 mM phenylmethylsulfonylfluoride in phosphate-buffered saline (PBS; pH 3.0). The dialyzed recombinant rVN-FII and rVN-FIII proteins were stored at -80℃until use. The protein concentration was determined using a BioRad protein assay kit (BioRad, Hercules, CA, USA).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed as described by Laemmli (25). The purified proteins were prepared in non-reduced or reduced forms. For the preparation of non-reduced protein samples, sample buffer was added to the protein sample. For the preparation of reduced protein samples, reduced sample buffer (100 mM DTT in sample buffer) was added to the protein samples. The mixture was boiled at 100℃ for 10 minutes. Electrophoresis was performed using minigel system apparatus (Biorad) at a constant voltage of 80 volts when samples were in the stacking gel. When the dye front reached the resolving

gel, voltage was increased to 120 volts. The gel was then visualized by Coomassie staining.

Synthetic peptides

All peptides were synthesized by the Fmoc (9-fluorenylmethoxycarbonyl) -based solid-phase method with a C-terminal amide by using a Pioneer peptide synthesizer (Applied Biosystems, Foster City, CA, USA), purified, and characterized at Peptron (Daejeon, Korea). The purity of all peptides used in this study was more than 90%, as determined by high-performance liquid chromatography.

Cell adhesion, spreading and migration assays

The cell adhesion assay was performed as described previously (26). Briefly, 48-well culture plates (Nunc, Roskilde, Denmark) were coated with 1 ㎍/㎖ human plasma VN (Millipore) or 5 ㎍/㎖ rVN-FI, rVN-FII, and rVN-FIII proteins for 12 h at 4℃. The substrate-coated plates were blocked with 1% heat-inactivated bovine serum albumin (BSA) in PBS for 1 h at 37℃ and then washed with PBS. HOS cells were detached by 0.05% trypsin and 0.53 mM EDTA in PBS and resuspended in serum-free culture media. HOS cells (5 x 10^4cells/250 ㎕) were added to each plate and incubated for 1 h at 37℃. After incubation, unattached cells were removed by rinsing with PBS. The attached cells were fixed with 10% formalin for 15 min and then stained with 0.5% crystal violet for 1 h. Plates were gently washed

with distilled water three times and dissolved with 2% SDS for 5 min. Absorbance was measured at 570 nm with a microplate reader (BioRad). To determine cell spreading assays, the attached cells were fixed with 10% formalin and then stained with 0.5% crystal violet for 1 h. Plates were gently washed with distilled water three times. The surface area of cells was measured with Image-Pro plus software (Version 4.5; Media Cybernetics, Silver Spring, MD, USA). Cell migration assays were performed by using transwell migration chambers (pore size, 8 mm; Corning, Pittston, PA, USA) as described previously (27). The lower side of the transwell filters was coated with VN (1 or 5 ㎍/㎖) or rVN fragments (5 ㎍/㎖) for 12 h at 4℃. The lower side of the transwell filters was blocked with 1% BSA in PBS for 1 h at 37℃. HOS cells (2 x 10^5cells/㎖) were suspended in DMEM containing 0.5% FBS and 0.1% BSA. This suspension (100 ㎕) was seeded in the upper chamber of a transwell filter. HOS cells were allowed to migrate for 24 h at 37℃. Cells were then fixed with 10% formalin for 15 min and stained with 0.5% crystal violet. Unmigrated cells in the upper side of transwell filters were removed with a cotton swab, viewed under a light microscope, and counted.

Adhesion inhibition assay

To identify the receptor of the HOS cells for rVN-FI, 5 ㎍/㎖ monoclonal antibodies (mAbs) to different types of integrins or 5 mM EDTA and to identify major binding site for HOS cells for VN, various concentration of synthetic peptides were preincubated individually with HOS cells in 250 ㎕ incubation solution (2 x 10^5cells/㎖) for 10 min at 37℃. The preincubated cells were then

transferred onto plates precoated with either 5 ㎍/㎖ of rVN-FI protein or 1 ㎍/㎖ of plasma VN and incubated for 1 h at 37℃. Attached cells were then quantified by the cell adhesion assay described above.

Statistical analysis

Statistical analysis of data was performed using the STATISTICA 6.0 software package. The results were compared by an analysis of variance (ANOVA). When significant differences were found, pairwise comparisons were performed using a Scheffe's adjustment. p-values less than 0.01 were considered significant.

Results

In vitro expression of three recombinant fragments of human vitronectin

For identification of binding motif, human vitronectin was expressed as three different fragments in *E. coli*, without overlapping amino acid residues. The corresponding amino acid positions of the VN recombinant proteins are shown in Fig. 1A. The molecular weights of the expected rVN-FI, rVN-FII, and rVN-FIII proteins were 32, 23, and 35 kDa, respectively (Fig. 1B). To determine solubility, the soluble fraction and insoluble fraction of *E. coli* expressed rVN proteins were prepared under reducing conditions and then subjected to SDS-PAGE analysis. As a result, rVN-FI protein was mainly expressed as soluble protein and rVN-FII and rVN-FIII proteins as insoluble protein (data not shown). Because rVN-FII and rVN-FIII proteins were expressed as insoluble protein, induced *E. coli* were lysed in 8 M urea-containing lysis buffer and then applied to sequential dialysis for refolding against solutions containing 10 mM Tris - HCl, 100 mM NaH_2PO_4, 1 mM phenylmethylsulfonylfluoride, and 3, 2, 1, or 0.5 M urea, pH 3.0, followed by a final dialysis against PBS containing 1 mM phenylmethylsulfonylfluoride, pH 3.0. The recombinant VN fragments were expressed as N-terminal His_6-tagged fusion proteins to provide easy to control for the protein purification and identification assays (Fig. 1B). The recombinant VN fragment proteins were purified with Ni^{2+}-nitrilotriaceticacid-agarose under denaturing conditions or non-denaturing conditions. All three rVN fragment proteins were obtained almost equally in same culture volume.

Characterization of rVN fragments

Because cloned and expressed mammalian proteins in prokaryotes were not properly modified after translation, we previously purified and eluted rVN-FII and rVN-FIII proteins under denaturing condition and applied these proteins to sequential dialysis to refold denatured rVN-FII and rVN-FIII proteins. Further we determined whether an intramolecular disulfide bonds were formed in the rVN fragments. We subjected the purified rVN proteins to SDS-PAGE under reducing or nonreducing conditions and observed mobility differences. Treatment of rVN proteins with 100 mM dithiothreitol prior to SDS-PAGE caused a small but reproducible reduction in gel mobility, suggesting that intramolecular disulfide bonds are present in all three recombinant proteins (Fig. 2). However, rVN-FII showed different pattern of band intensity. This may be due to significant portion of purified rVN-FII forming a three-dimensionally compact structure by intramolecular hydrophobic interactions that migrate at lower molecular weight under non-reducing condition. This result suggests that the bacterially expressed rVN proteins from vitronectin may be form structured conformation to support potential functional activity.

Determination of cell adhesion, spreading, and migration activities of rVN proteins

Vitronectin is known to mediate cell adhesion to various types osteoblast-like cell (20). To determine whether rVN proteins affect cellular responses, such as cell adhesion, spreading, and migration of HOS cells, rVN proteins were assessed by functional analysis. In a

preliminary experiments, adhesion effect of 0.1 - 5 ㎍/㎖ VN on HOS cell adhesion was tested on the culture plate surface. We found that 1 ㎍/㎖ of VN had the maximum effect (data not shown). rVN-FI and rVN-FII showed the adhesion activity of the HOS cells (Figs. 3A and 3B). HOS adhesion on rVN-FI and rVN-FII proteins was dose-dependent, with the maximum adhesion occurring at a 1 ㎍/㎖ and 15 ㎍/㎖, respectively (Figs. 3A and 3B). At equal or greather coating concentrations, the adhesion activity of rVN-FIII protein showed slight adhesion activity compared to BSA control (Figs. 3A and 3C). The level of HOS cell adhesion using 5 ㎍/㎖ rVN-FI protein was reduced approximately 24% and that of rVN-FII protein (5 ㎍/㎖) was reduced 50% compared to the 1 ㎍/㎖ VN-coated control (Fig. 3C). Further studies were carried out to determine the spreading and migration activities of rVN proteins. As expected, VN highly promoted cell spreading (Fig. 3D) and migration (Fig. 3E) of HOS cells. Although the levels of cell adhesion to rVN proteins were lower than to VN, HOS cells were adhered to rVN-FI and rVN-FII proteins, but not to rVN-FIII protein. However, rVN-FII did not promote cell spreading and migration, and rVN-FIII produced marginal effects on cell adhesion, spreading, and migration of HOS cells. Taken together, rVN-FI protein harbored VN's biological activities, such as adhesion, spreading, and migration, except adhesion to rVN-FII protein of HOS cells.

Cell adhesion to rVN-FI protein through αVβ3 and αVβ5 integrins

It is well-known that ligand-integrin interaction requires a divalent cation (28). Firstly the role of cation was determined by using metal-chelating reagent EDTA on HOS cell adhesion to rVN-FI protein-coated plates. Cell adhesion to rVN-FI protein was almost completely inhibited by 5 mM EDTA (Fig. 4A). This result indicates that the cell surface receptor for rVN-FI protein might be one of the integrin that require divalent cations for their interaction with the ligands. Next, inhibition assay using mAbs to integrin subunits on the adhesion of HOS cells to a surface coated with rVN-FI was tested. Adhesion to the rVN-FI protein-coated surface was specifically inhibited by antibodies to the αV and β3 subunits, but not by the antibodies to the α1, α2, α3, α5, β1, and β4 subunit. Treatment of HOS cells with the anti-integrin αV, β3 antibodies inhibited the cell adhesion activities of rVN-FI by 90% and 36%, respectively. Furthermore, HOS cell adhesion to the rVN-FI protein-coated surface was also inhibited by antibodies against the α Vβ3, αVβ5, and both integrins by 55%, 35% and 80%, respectively. These results suggest that the inhibitory effects of the antibodies to the rVN-FI-coated surface were mediated by interaction with αVβ3 and αVβ5 integrins of HOS cells.

Identification of human vitronectin sequence active for cell adhesion

Above results indicate that rVN-FI protein promotes cell adhesion, spreading, and migration of HOS cells. The RGD motif (amino acids 46-48) of VN N-terminus is also commonly found in the other ECM

proteins and also mediates cell adhesion of various cell types. Furthermore, RGD motif in VN was well conserved in mouse, rat, bovine, donkey, pig, and chicken. Eight overlapping 12-mer peptides derived from rVN-FI were synthesized (Fig. 5A). To determine which concentration of peptide inhibits HOS cell adhesion onto VN, various concentrations of P6 and P7 peptides which covering RGD motif were tested preferentially. As a result, P6 peptide almost completely inhibited HOS cell adhesion to VN at 250 ㎍/㎖, whereas P7 inhibited HOS cell adhesion at 25 ㎍/㎖ (Fig. 5B). Because of differences in peptide solubility, the result of peptide inhibition assay was compared separately. Moreover, among these 8 peptides, P6 (AECKPQVTRGDV) and P7 (PQVTRGDVFTMP) inhibited VN-mediated HOS cell adhesion compared to other rVN-FI-derived peptide (Fig. 5C).

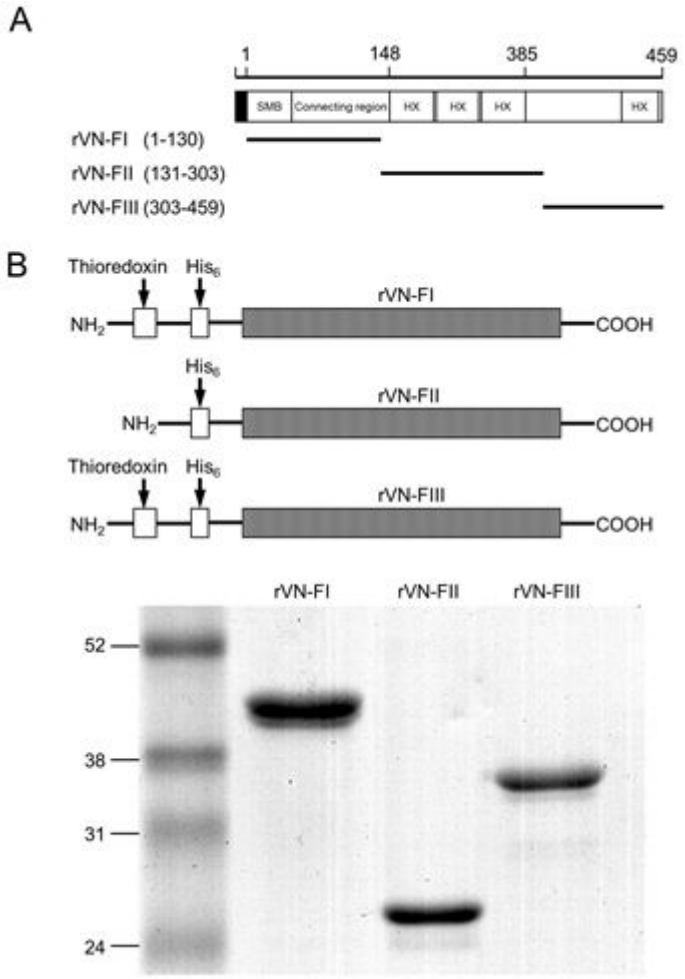

Figure 1. Analysis of purified rVN fragments of the human vitronectin by SDS-PAGE. (A) Schematic diagram of recombinant VN fragments used in this study. The amino acid scale is shown on the top. The domain structures of the mature VN (open column) are indicated by SMB, Connecting region, and HX. The shaded portion indicates the signal peptide. The closed bars represent the positions of the recombinant proteins. Numbers in parentheses indicate the corresponding amino acid positions of the recombinant proteins in the whole VN protein. (B) Schematic diagram and SDS-PAGE analysis of rVN proteins in the VN. The three rVN proteins were expressed

as His_6-tagged fusion proteins. In the rVN-FI and rVN-FIII proteins, expressed with thioredoxin-His_6-tagged fusion proteins in the pET-32a(+) expression. In the rVN-FII protein, expressed only with His_6-tagged fusion proteins. The rVN proteins were subjected to SDS-PAGE analysis (10% acrylamide, reducing condition) and visualized by Coomassie staining. SMB, Somatomedin B domain; HX, Hemopexin-like repeat.

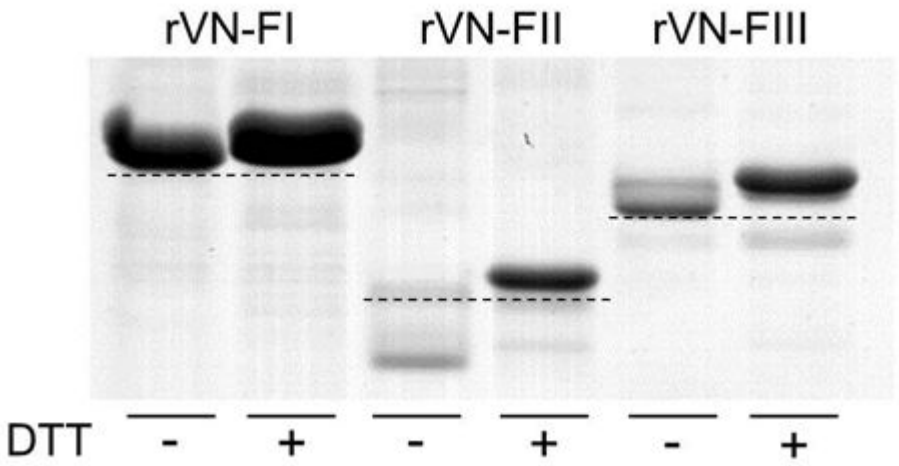

Figure 2. Analysis of rVN proteins of human vitronectin by SDS-PAGE. Gel mobilities of purified rVN proteins treated with dithiothreitol (DTT) were compared with nontreated rVN proteins under 10% SDS-PAGE conditions and visualized by Coomassie staining. Reduction of rVN proteins prior to electrophoresis resulted in reproducible decrease in gel mobility.

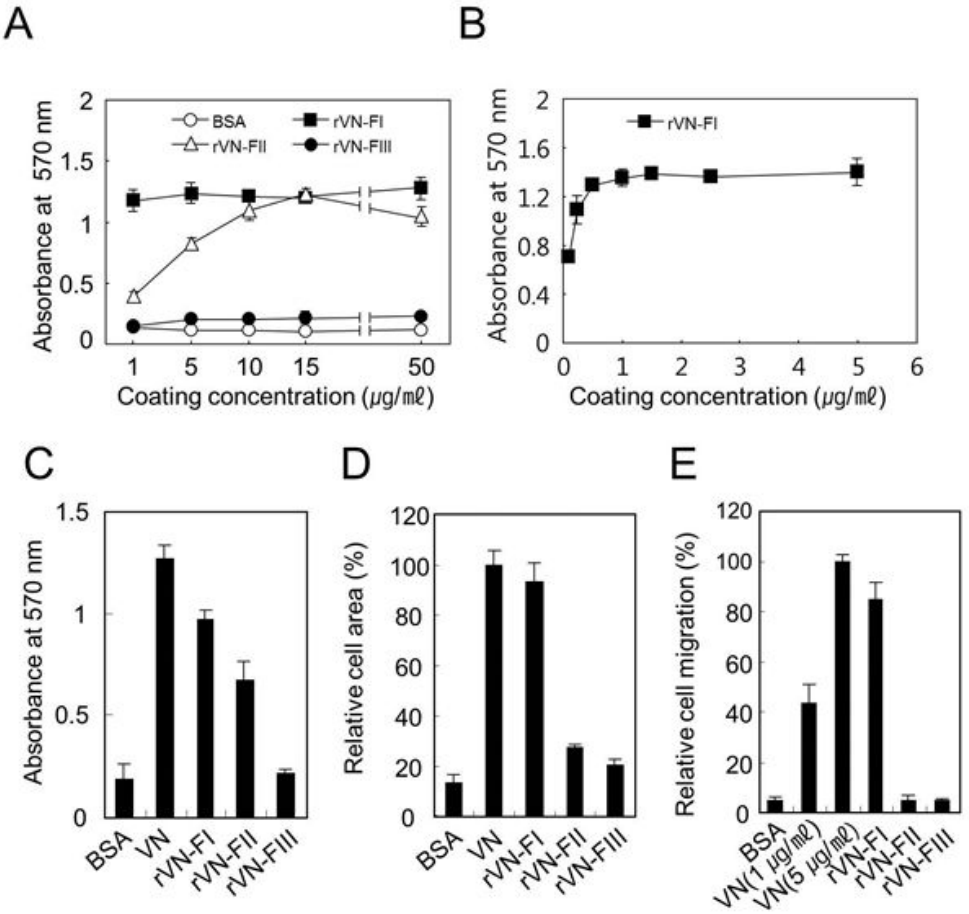

Figure 3. rVN proteins in human vitronectin support cell adhesion, spreading, and migration. Purified His_6-tagged rVN proteins were coated onto 48-well plates and HOS cells were allowed to adhere for 1h in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained with crystal violet, and solubilized. The absorbance was read at 570 nm. (A) Dose-dependent cell adhesion to the immobilized rVN proteins. The recombinant proteins were coated onto 48-well plates at the indicated concentrations. (B) Cell adhesion to the immobilized rVN-FI protein at low concentrations. (C) Cell adhesion to the immobilized rVN proteins that were coated onto 48-well plates at 5 μg/mℓ and human plasma VN (VN) at 1 μg/mℓ.

(D) Spreading of HOS cells seeded on plates coated with VN, rVN proteins for 3 h in serum-free medium. (E) Migration of HOS cells induced by VN and rVN proteins.

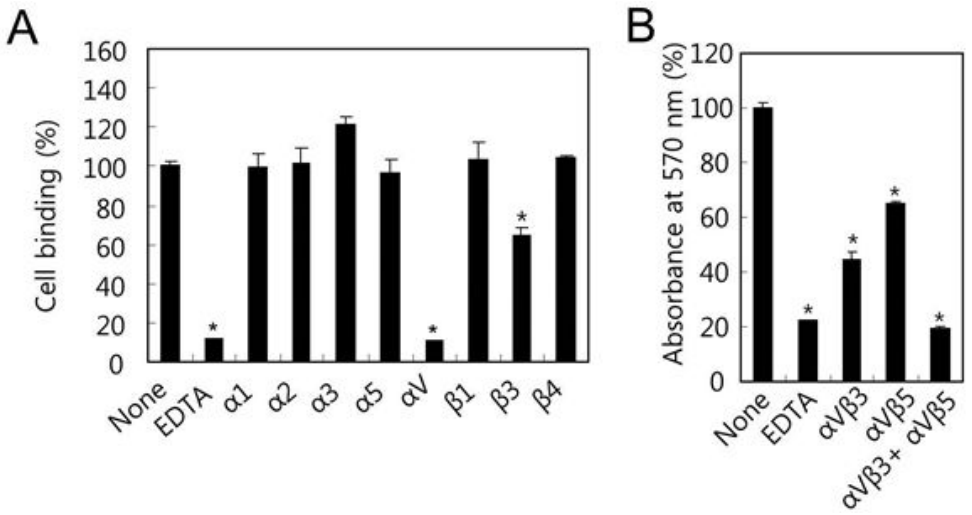

Figure 4. HOS cell adhesion to rVN-FI protein is blocked by antibodies to αVβ3 and αVβ5 integrins. (A) HOS cells were preincubated with either 5 mM EDTA or 5 ㎍/㎖ of function-blocking mAbs to integrin α1, α2, α3, α5, αV, β1, β3, β4 subunits and (B) αVβ3 and αVβ5 for 10 min at 37°C. They were then seeded on plates precoated with 5 ㎍/㎖ of rVN-FI protein and incubated for 1 h. Unbound cells were washed off; adherent cells were fixed, stained with crystal violet, and counted. The average adhesion activity of the control cultures (None; HOS cells preincubated without EDTA or integrin antibodies) was considered 100%. Data are expressed as mean ± SD (n = 3). *P < 0.01 for EDTA- or integrin antibody-untreated cells.

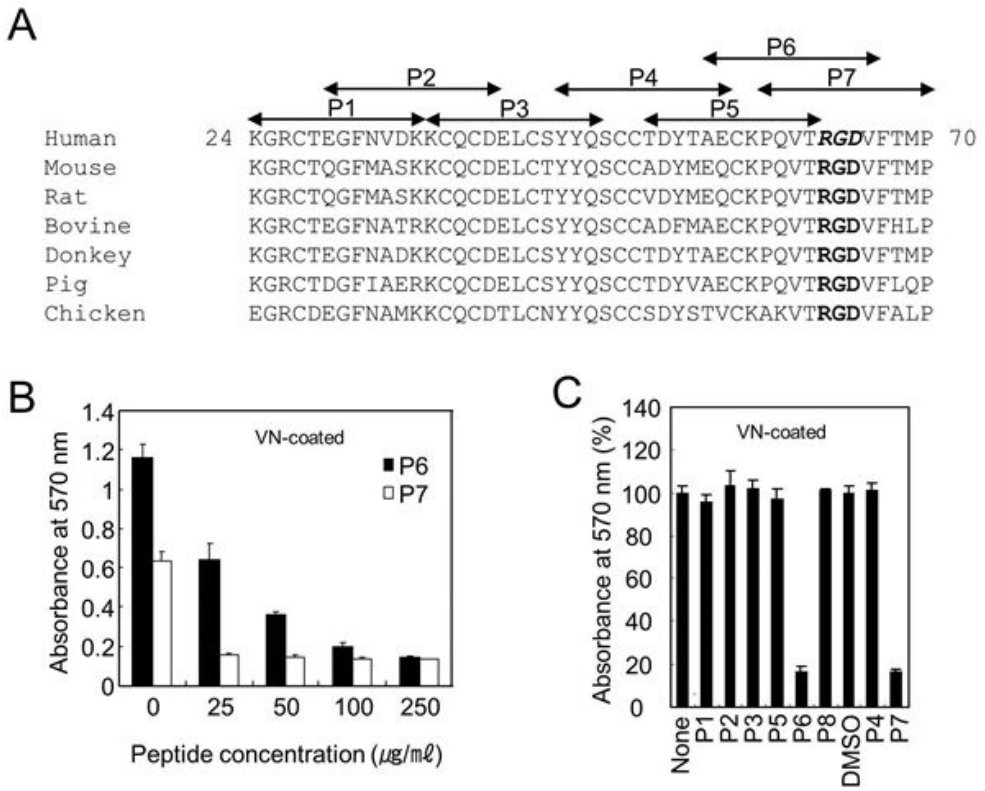

Figure 5. Inhibition of HOS cell adhesion by rVN-FI-derived peptides. (A) Amino acid sequence alignment of part of the vitronectin of humans, mice, rats, bovines, donkeys, pigs, and chickens. The arrows indicate the locations of the synthetic peptides. (B) Inhibition of HOS cell adhesion onto the VN by peptide P6 and P7. VN (1 μg/mℓ) were coated onto 48-well plates. HOS cells were preincubated with the various peptide concentrations (0, 25, 50, 100, 250 μg/mℓ) for 10 min at 37℃. The preincubated cells were then added to VN-coated plates for 1 h in serum-free medium. The unbound cells were washed off and the adherent cells were fixed and stained with crystal violet. The average adhesion activity of the control cultures (None; HOS cells preincubated without peptide) was considered 100%. (C) Cell adhesion to vitronectin was inhibited by peptide P6 and P7. Data are expressed as mean ± SD (n = 3).

Discussion

The extracellular matrix (ECM) consists of a complex mixture of structural and functional macromolecules and serves an important role in tissue and organ morphogenesis and in the maintenance of cell and tissue structure and function. The ECM is important during growth, development, and especially, wound repair. ECM also mediates signals from extracellular part to inside of the cell to adhere, spread, proliferate, differentiate, and migrate (29). Numerous studies revealed that most of ECM proteins have various binding sites for cells for their biological function (29). VN also presents in ECM and provides cells various binding sites for cells, such as SMB domain, RGD motif, heparin binding domain that promotes biological functions of cell interacting with these partners (30). Thus, VN functions as multitalented adhesive glycoprotein in ECM or blood plasma. In these regards, author hypothesized that VN must have several core sequences that promote cell adhesion, spreading, and migration. As a result, rVN-FI (a.a. 1-130) that covers N-terminal fragment of VN promoted HOS cell adhesion and rVN-FII (a.a. 131-303) also promoted HOS cell adhesion but to a lesser degree than rVN-FI. In contrast, rVN-FIII did not promote HOS cells behavior in this study even though rVN-FIII (a.a. 304-459) covers heparin binding domain of VN. This is because heparin binding domain of VN covered by rVN-FIII was cryptic (5).

VN promotes cell adhesion, spreading, and migration through the interaction of VN's binding sites with cellular integrins including αVβ3, αVβ5, αVβ1, and αIIbβ3 (30). Function-blocking inhibition assay

using mAbs against integrin subunits and integrins revealed that αVβ3 and αVβ5 integrins act as receptor for rVN-FI of HOS cell adhesion. On the other hand, mAbs against the integrin β3 and β5 subunits blocked HOS cell adhesion onto rVN-FI to a lesser degree than mAb against integrin αV. These results indicate that αV integrin is crucial for rVN-FI-mediated HOS cell adhesion though αVβ3 and αVβ5 integrins.

Recent studies have investigated the biological activities of several peptides in tissue regeneration (31,32), suggesting that peptides may have potential applications. Because short peptides can promote various cellular responses without inducing host-immune responses (21), applying these peptides to bone regeneration-related tissue regeneration is highly promising. Bioactive peptides derived from various ECM proteins including VN were isolated and studied for bone regeneration (33). For examples, KRSR (B-B-X-B motif) peptide within VN enhances rat calvarial osteoblast cell adhesion (34), and RGD containing peptide, well-known as cell adhesion sequence via cell-membrane integrin receptors, also binds to osteoblasts (35). Recently, a nonapeptide (351-359)HVP, patterned on VN and presenting a X-B-B-B-X-B-B-X motif, also represents a rat femoral osteoblast-specific adhesive peptide able to improve proteoglycan-mediated mechanism (21). In this study, synthesized peptides derived from rVN-FI were screened and analyzed by inhibition assay for HOS cell adhesion onto VN. P6 and P7 containing RGD motif within rVN-FI inhibited VN-mediated HOS cell adhesion. In this result, P6 and P7 peptides can occupy VN binding site of cell surface and inhibited HOS cell adhesion onto VN. Although P6 and P7 have RGD sequence, different inhibition efficiencies were appeared

when various peptide concentrations were pretreated. This may because of the diversity of peptide function derived from its structural or functional nature that caused by their amino acid arrangements.

In summary, rVN-FI of human VN had a key role in HOS cell adhesion relative to rVN-FII and rVN-FIII through its receptor αVβ3 and αVβ5 integrins, and also showed cell spreading and migration activities. Further studies are required to isolate the core sequence of adhesion activity within rVN-FII and the signal transduction pathways induced by rVN-FI protein.

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-국문초록-

비트로넥틴 단편 재조합단백질이 사람골모세포주의 세포거동에 미치는 효과

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세포외기질단백질인 비트로넥틴은 리간드-세포 표면 수용체 결합을 통하여 혈관내피세포의 부착, 퍼짐, 이동 등 다양한 세포반응을 조절한다. 본 연구에서는 사람 비트로넥틴을 세 개의 단편으로 클로닝하여 발현시키고, 각각의 단편이 가지는 생물학적 기능을 사람 골모세포주인 HOS 세포를 사용하여 분석하였다. HOS 세포주의 세포 부착, 퍼짐 및 이동을 분석한 결과, 비트로넥틴 단편 I 재조합단백질 (rVN-FI; 아미노산 1-130)이 단편 II 재조합단백질 (rVN-FII; 아미노산 131-303)과 단편 III 재조합단백질 (rVN-FIII; 아미노산 304-459) 보다 HOS 세포주의 세포 부착, 퍼짐 및 이동을 촉진시킨다는 사실을 규명하였다. 또한, 인테그린 단일클론 항체들을 이용한 세포부착 억제실험 결과 HOS 세포주에서 αVβ3 및 αVβ5 인테그린이 수용체로 작용함을 규명하였다. rVN-FI에서 유래된 펩타이드 중 Arg-Gly-Asp (RGD) 모티프를 포함하는 P6

(AECKPQVTRGDV)와 P7 (PQVTRGDVFTMP) 펩타이드가 HOS 세포 부착에 핵심적인 역할을 하였다. 이상의 결과에서, *E. coli*에서 발현시킨 세 개의 재조합단백질 중 rVN-FI이 골모세포의 부착, 퍼짐 및 이동 등 다양한 생물학적 기능을 나타냄을 알 수 있었다. 이는 비트로넥틴의 rVN-FI 도메인이 골조직 재생관련 조직공학에 응용 가능하다는 것을 시사한다.

주요어 : 비트로넥틴, 골모세포주, 세포거동, 인테그린, 골재생

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