Suppressive Mechanism of a Novel Disintegrin (Saxatilin) in Vascular Smooth Muscle Cell Proliferation

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Suppressive Mechanism of a Novel Disintegrin (Saxatilin) in Vascular Smooth Muscle Cell Proliferation

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Abbreviations

bFGF: basic fibroblast growth factor

CAS : Crk-associated substrate

ECM : extra cellular matrix

EGF: epidermal growth factor

ERK : extracellular signal regulated kinase

DTT : dithiothreitol

FAK : focal adhesion kinase

FBS : fetal bovine serum

GAPDH : glyceraldehydes-3-phosphate dehydrogenase

HCASMC: human coronary artery smooth muscle cell

HPLC : high performance liquid chromatography

PDGF : platelet derived growth factor

PKB : protein kinase B

PMSF : phenylmethyl sulfonyl fluoride

PTCA : percutaneous transluminal coronary angioplasty

RT-PCR: reverse transcription polymerase chain reaction

SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrohoresis

ABSTRACT

Suppressive Mechanism of a Novel Disintegrin (Saxatilin) in Vascular Smooth Muscle Cell Proliferation

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Restenosis occurs in approximately 30-40% of patients following percutaneous transluminal coronary angioplasty (PTCA) for treatment of atherosclerosis. The stimulation of vascular smooth muscle cell is critical steps in the development of the neointimal tissues that contribute to restenosis of arterial wall. RGD-containing peptides are able to inhibit the binding of ligands to certain β3 integrins, αIIbβ3, and αvβ3, both of which are involved in the neointimal hyperplasia. Most of the disintegrins contain Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequence, which is a structural motif

recognized by the platelet fibrinogen receptor GP IIb-IIIa, αvβ3, and α5β1. The purpose of this study was to examine the biological effects of saxatilin, a novel disintegrin from the venom of a Korean snake (*Gloydius saxatilis*) on vascular smooth muscle cells. To perform this work we first needed to express and produce large amounts of the active form of recombinant saxatilin in yeast *Pichia pastoris*. The recombinant saxatilin was highly expressed as a biologically active form in *Pichia pastoris*, and it was successfully purified from the culture media to be homogeneity by the combination of a phenyl-Sepharose chromatography and a reverse-phase HPLC. The overall yield was approximately 150 mg/L. The molecular and biological properties of the purified recombinant protein were almost same with its natural from. It interacted with integrin αvβ3, and significantly suppressed the adhesion of human coronary artery smooth muscle cells (HCASMC) to vitronectin with an IC_{50} of 2.5uM. Half maximal-inhibited concentration of saxatilin for growth factors (PDGF-BB or b-FGF) induced-proliferation was an approximately 25uM. Saxatilin disassembled actin cytoskeleton of focal adhesion and induced cells to being rounded and detached, but did not alter microtubule structure in the early stage of cells to being shrunk. This disassembly of focal adhesion in saxatilin-treated HCASMCs involved caspase-induced paxillin

degradation. Focal adhesion kinase (FAK) and ERKs of saxatilin-treated SMCs were temporally phosphorylated. Saxatilin affected cell cycle progression of HCASMCs by increasing CDK inhibitors (p21 & p27) and reducing cyclins (D1/2 and E). While SMC was proliferated normally on plates without saxatilin, cells treated with saxatilin eventually underwent anoikis. These results may provide new insights into role of integrin in SMC pathophysiology as well as role of SMCs in the process of neointimal hyperplasia and also suggest significant implications for integrin antagonistic therapy of the treatment for arteriosclerosis and restenosis.

Key words: integrin, disintegrin, recombinant, saxatilin, platelet aggregation, adhesion, proliferation, smooth muscle cells, restenosis

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I. INTRODUCTION

Vascular smooth muscle cell proliferation is an important process during normal vascular development, but the abnormal activation of SMC migration and proliferation are also major contributors to the pathogenesis of several important cardiovascular disease states including atherosclerosis,

hypertension, and restenosis [1, 2, 3, 4]. A large number of extrinsic stimulations (growth factors, extracellular matrix, cell-cell interactions, etc.) have been identified that regulate SMC growth and migration[5, 6]. However, the precise cellular signaling mechanisms involved are not completely understood, and very little is known about how these signaling pathways are integrated. Adhesion of cells to the extracellular matrix (ECM) is mediated by binding of cell surface integrins to the ECM, which activate intracellular signaling cascades and tension-dependent changes in cell morphology and cytoskeletal structures[6, 7, 8]. The primary ECM binding site of integrins was defined as arginine-glycine-aspartate (RGD) tri-peptide motif. RGD-containing peptides inhibit the binding of tumor cells to the ECM by competing with ECM proteins. Ligation and activation of integrin lead to the activation and autophosphorylation of focal adhesion kinase (FAK), a tyrosine kinase prominently localized at focal adhesions. FAK is tightly coupled to the

assembly of focal adhesions by recruiting talin, vinculin, and paxillin and by binding to integrin cytoplasmic domains[9, 10]. Activated FAK is autophosphorylated at tyrosine residue 397 and then binds to the SH2 domain of Src or Fyn. The Src family kinase then phosphorylates a number of FAK-associated proteins including paxillin, tensin, and p130CAS. Phosphorylation of these focal adhesion proteins may be involved in the regulation of focal adhesions and potentially regulates cell proliferation by signaling to the mitogen-activated protein kinases (MAPKs), extracellular signaling regulated kinases (ERKs), and the Jun amino-terminal kinase (JNK). In addition to FAK, a recently identified serine/threonine kinase, integrin-linked kinase (ILK), binds to the cytoplasmic domains of both β1 and β3 integrins and phosphorylates PKB/Akt. ILK also phosphorylates and inhibits glycogen synthase kinase 3 (GSK-3), which, in turn, activates β-catenin and AP-1. The activation of PKB/Akt and the inhibition of GSK-3

by ILK lead to the suppression of apoptosis and the promotion of cell survival.

Vascular Integrins

Of the approximately 24 known integrins, 16 have been reported to have involvement in some aspect of vascular biology. Of these 16, α1β1, α2β1, α3β1, α5β1, α6β1, α6β4, αvβ3 and αvβ5 are known to be present in endothelial cells, while VSMCs have been reported to have α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, αvβ1, αvβ3, αvβ5, and α6β4 [5, 6, 10]. At present, the role for all of these integrins in the regulation of vascular tone has not been investigated. Furthermore, the extent to which different ECM proteins are involved is incompletely defined. Considering that both endothelial and vascular smooth muscles cells, *in situ*, they are embedded in a three-dimensional network of interacting ECM proteins, such

as type I, III, IV, V and VI collagens, fibronectin, vitronectin, thrombospondin, elastin, tenascin, osteopontin and several types of laminin, it is conceivable that there may be broad interactions taking place that directly or indirectly influence vascular control. At present a unified theory of integrin involvement in vascular control is absent.

Disintegrins

Platelet aggregation plays a key role in hemostasis and occurs by binding of fibrinogen to glycoprotein (GP) IIb-IIIa receptor complex located on the platelet surface membrane, where the Arg-Gly-Asp (RGD) sequence of fibrinogen is essentially engaged[11, 12]. The RGD sequence was identified as the cell recognition site on fibronectin and is present in a variety of proteins including fibrinogen, von Willebrand factor, thrombospondin, collagen, vitronectin, and osteopontin. The particular tripeptide sequence is thought to

mediate adhesive function of those proteins. Disintegrins isolated from snake venoms and leeches are cysteine-rich, low molecular weight proteins. Most of the disintegrins contain Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequence, which is a structural motif, recognized by the platelet fibrinogen receptor GP IIb-IIIa, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ [13, 14, 15]. These proteins bind to the fibrinogen receptor GP IIb-IIIa on the surface of platelets, resulting in the inhibition of fibrinogen-dependent platelet aggregation[16, 17, 18, 19, 20, 21]. Disintegrins also inhibit the adhesion of human umbilical vein endothelial cell (HUVEC) to fibrin or to immobilized extracellular matrix (ECM) through the blockade of $\alpha_v\beta_3$ integrin. Because the endothelial $\alpha_v\beta_3$ integrin plays a major role in angiogenesis as described above, the anti-adhesive function of disintegrins between endothelial cells and ECM may be a key factor to inhibit angiogenesis. In our previous studies, we have found and characterized the novel disintegrin, saxatilin, derived from Korean snake (*Gloydius saxatilis*)

venom. Saxatilin inhibits fibrinogen-dependent platelet aggregation, experimental tumor metastasis, tumor growth and bFGF-induced angiogenesis without affecting the proliferation of normal endothelial cells. Until recently, we were limited to produce an active form of recombinant disintegrin in *E.coli* expression system [27, 28, 29]. The bacterial expression system expressed the target proteins as an inactive and insoluble fusion protein, and thus required protein refolding and cleavage reaction for the purification process. Therefore, we tried to express and produce large amounts of the active form of recombinant disintegrin in yeast *Pichia pastoris* system.

In this thesis, the aims of the study were to demonstrate mass production of saxatilin and their biological effects on vascular smooth muscle cells.

Ⅱ. MATERIALS AND METHODS

1. Mass production of recombinant saxatilin and its biological activity

1.1. Construction of *Pichia pastoris* expression systems for recombinant saxatilin

The cDNA cloning of saxatilin has been described[31, 32]. Saxatilin was cloned from the cDNA library of the Korean snake *Gloydius saxatilis* venom gland using the sense primer 5'-gaggccggagaagaatgtgactgtggc-3' and the antisense primer 5'-ggcatggaagggatttctgggaca-3' in polymerase chain reaction. The amplified PCR fragment was cloned into pBluescriptKS (+) and its nucleotide sequence was analyzed using the ALF automatic sequencing system (Amersham Bioscience, Uppsala, Sweden). This saxatilin cDNA was modified by PCR for the *Pichia pastoris* expression vector pPIC9 (Invitrogen, San Diego, CA, USA). The N-terminal primer for saxatilin (5'-cc gctcgagaaaagagaggccggagaagaatgt-3') contains a *Xho*I recognition site and a

KEX2 endo-peptidase cleavage site. The C-terminal primer (5'-cggaattctcattaggcatggaaggga-3') adds two stop codons and an *Eco*RI restriction site. The thermal cycling reaction was performed with 30 cycles of steps [denaturation for 30 seconds at 94℃, annealing for 30 seconds at 55℃, and extension for 30 seconds at 72℃] and 5 minutes incubation at 72℃. After the PCR reaction, the amplified fragment was subcloned into the pBluescript KS (+) *Eco*RV site and analyzed for nucleotide sequence. From this modified cDNA of saxatilin was inserted into an *Xho*I and an *Eco*RI site of pPIC9, which forms a fusion protein of the α–mating factor's signal peptide and saxatilin. The pPIC-saxatilin vector (*pPSAX*) was digested with a *Sal*I restriction enzyme. This linearized plasmid was transformed into electro-competent GS115 cells (Invitrogen, San Diego, CA, USA) using GenePulser (Bio-Rad Laboratories, Hercules, CA, USA). The His^+ *pPSAX* -transformed colonies were analyzed for their ability of methanol utilization

by replica selection on minimal dextrose and minimal methanol plate. Finally, His^+ and Mut^+ cells were collected for saxatilin expression.

1.2. Expression of recombinant saxatilin

A single colony of transformant was inoculated to 30 ㎖ of phosphate-buffered (pH 6.0) minimal glycerol medium, and grown overnight at 30℃. The fully-grown culture was determined using the cell density measured at an absorbance of 600 nm. These cultures were harvested and re-suspended at an A_{600} 1.0 in phosphate-buffered (pH 6.0) minimal methanol (0.5%) medium, and induction of saxatilin was performed at 30℃ for up to 72 hours. The supernatants of yeast culture were analyzed using SDS-polyacrylamide gel electrophoresis and coomassie blue staining.

1.3. Fermentation of recombinant saxatilin

The 2.5㎖-frozen stock of transformant was inoculated into a 250㎖ of yeast nitrogen base (YNB)-glycerol media, and it was incubated in shaker with 240 rpm for 24h at 30℃. The entire 250㎖ volume of culture was transferred into a 5 L fermenter vessel containing 2.5L of basal salt medium (H_3PO_4 27㎖/L, $CaSO_4$ $2H_2O$ 0.9g/L, K_2SO_4 18g/L, $MgSO_4 7H_2O$ 15g/L, KOH 4.13g/L) plus a PTMI trace metal solution and glycerol 40 g/L. Temperature and D.O. setting-point were controlled at 30℃ and 30%, respectively. The set point of pH (5.0) was automatically adjusted by the addition of ammonium hydroxide (30%) solution. After 18 hours of batch culture, the optical density (at 600nm) reached to 40, following, which the fed-batch processing of glycerol was initiated. The feeding medium consisted of 50% glycerol and 12㎖/L of the trace elements mixture. The feeding rate of this medium was 24㎖/L/hour. Pure oxygen was automatically supplied to the culture to

maintain the D.O. level at the setting point after the agitation speed reached to maximum (1000 rpm). Thereafter, the growth phase, a half-hour of carbon source starvation period, was established before the culture was switched to the production phase. The production phase started after 48h of growth. The feed medium consisted of 100% methanol and 12㎖/litre trace metal solution.

1.4. Purification of recombinant saxatilin

The recombinant saxatilin was purified from the culture supernatant of *Pichia pastoris*, which was cloned to produce saxatilin. The culture supernatant was collected by centrifugation and treated with an ammonium sulfate to a final concentration of 2.0M. The ammonium sulfate mixture was loaded to a phenyl-Sepharose column (Amersham Pharmacia Biotech, Pitscataway, NJ, U.S.A.). The protein fraction was eluted with a 1M of ammonium sulfate solution. From this fraction, we further purified

recombinant saxatilin with an acetonitrile gradient of Source 30 reverse phase column. (Amersham Pharmacia Biotech, Pitscataway, NJ, USA). Fractions that showed platelet aggregation inhibition activity were collected and lyophilized for further experiments.

1.5. Mass spectrometry and N-termianl amino acid sequence analysis

MALDI-MS analysis for the purified recombinant saxatilin was obtained by a Kratos Kompact model II mass spectrometer. N-terminal amino acid sequence of the recombinant protein was carried out in the Applied Biosystem Precise Protein Sequencing system (Perkin-Elmer, Boston, MA, USA).

1.6. Platelet aggregation assay

Platelet aggregation assay was performed in human platelet-rich plasma (PRP). The platelet concentrate was diluted to 300,000 platelets per microliter

of platelet-poor plasma. Ten ㎕ of sample added to 450㎕ of plasma and then incubated for 3 minutes in a incubation tube of Chrono-Log Aggregometer (Chrono-Log Co., Havertown, PA) at 37℃. The impedance was recorded, and ADP (20μM) was added to initiate platelet aggregation. The inhibition of platelet aggregation was measured at the maximum aggregation response.

1.7. Inhibition assay of fibrinogen-GPIIb/IIIa complex formation

Fibrinogen-GPIIb/IIIa ELISA was performed by a modified method of Nachman and Leung[27, 28, 29]. The 96-well plates were coated with purified human fibrinogen (10μg/㎖). After blocking with 1% bovine serum albumin in TACTS for 1 hour, the plate was washed, and each protein sample to be tested was added immediately by the addition of purified GPIIb/IIIa (40 μg/㎖) (Calbiochem, La Jolla, CA, USA) in TACTS containing 0.5% bovine

serum albumin. After 2 hours of incubation, the plate was washed and mouse anti-human GP IIIa antibody (CHEMICON, Temecula, CA, USA) was added. Following an additional 1 hr incubation and washing steps, goat anti-mouse IgG-conjugated to horseradish peroxidase (BIO-RAD, Hercules, CA, USA) was added. A final wash was performed and a developing substrate (1-Step ABTS, PIERCE, Rockford, IL, USA) solution was added. Then, the plate was incubated for about 10 minutes until color developed. The reaction was stopped with 3M HCl followed by absorbance measurement at 492 nm.

1.8. Test drug

The purity of saxatilin was over than 99%, which was confirmed by HPLC analysis. For the *in vitro* and *in vivo* test grade, saxatilin was resolved in pyrogen-free sterile saline and filtered using a 0.2㎛-pore syringe membrane (Adventec MFS, Inc. CA, USA).

2. The effects of saxatilin on vascular smooth muscle cells

2.1. Vascular smooth muscle cells

Human coronary artery (CC-2583), aortic smooth muscle cells (CC-2571) and Clonetics SmGM-2 Bullet kit (CC-3182) medium were purchase from Biowhittaker Inc. (Walkersville, MD, USA). Clonetics SmGM-2 bullet kit contains smooth muscle cell basal medium (modified MCDB 121) and growth supplements include insulin, bFGF, EGF, and final 5% fetal bovine serum (FBS). Cultures were maintained in a humified atmosphere of 5% CO_2 at 37'C. All kinds of smooth muscle cell used in these studies were from passages 3 to 7 and their identity was proved by immunofluorescence microscopy result of α-SM actin-positive and von Willenbrand factor (vWF)-negative characteristics.

Rat aortic smooth muscle cells (RAoSMCs) were isolated by a modification of the method of Chaley-Campbell *et al* [34]. The thoracic aortas

from 6- to 8-week-old Sprague-Dawley rats were removed and transferred on ice in serum-free Dulbecco's modified Eagle' medium (DMEM; Invitrogen Co, Carlsbad, CA, USA) containing 100 units/㎖ of penicillin and 100 ㎍/㎖ of streptomycin. The aorta was freed from connective tissue, transferred into Petri dish containing 5 ㎖ of an enzyme dissociation mixture containing DMEM with 1 ㎎/㎖ of collagenase type I (Sigma, St. Louis, MO, USA) and 0.5㎍/㎖ elastase (USB Bioscience, Cleveland, OH, USA), and incubated for 30 min and at 37°C. The aorta was the transferred into DMEM and the adventitia was stripped off with forceps under a binocular microscope. The aorta was transferred into a plastic tube containing 5 ㎖ of the enzyme dissociation mixture and incubated for 2h at 37°C. The suspension was centrifuged (1,500 rpm for 10 min) and the pellet was resuspended in DMEM with 10% fetal bovine serum (FBS). Cells were cultured over several passages (up to 10). RAoSMCs were cultured in DMEM supplement with 10% FBS,

100 IU/㎖ penicillin, 100 ㎍/㎖ streptomycin in 75-㎠ flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (Forma Scientific, Inc., Marjetta, OH, USA.)

2.2. Cell proliferation assay

Cells were seeded in triplicate at a concentration of $1x10^3$ cells/well in 100 ul of DMEM containing 10% (v/v) fetal bovine serum in 96-well flat-bottom plates (Costar, Corning, NY, U.S.A.). After 24 h incubation, the complete medium was replaced with DMEM containing 0.2% FBS, and incubated for an additional 72h. And then, the cells were treated with or without 20uM saxatilin in 100㎕of DMEM containing PDGF (5ng/㎖) or 5% (v/v) fetal bovine serum for 24h. SMC proliferation was determined using a colorimetric assay kit based on the uptake of WST by viable cells (Premix WST-1 cell proliferation assay system, Takara Bio Inc, Otsu, Japan). The assay kit is

dependent on the reduction of tetrazolium salt WST-1, which results in formation of a dark red formazan product, by various mitochondrial dehydrogenases of viable cells. The absorbance of a dark red formazan product was read at 450 and 540 nm using a microplate reader (Model 550, BioRad Laboratories, Hercules, CA, USA).

2.3. Cell adhesion assay

The vascular smooth muscle cells were maintained in Dulbecco's modified eagle medium (DMEM, GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 units/㎖), and streptomycin (100 μg/㎖). 96-Well plates were pre-coated with vitronectin (1 μg/well) at 4°C overnight. The plates were washed with PBS and incubated for 1 hr with 1 % of bovine serum albumin to block remaining protein binding sites. The plates were washed with PBS before use. SMCs were dissociated by

treatment with trypsin-EDTA, washed three times in PBS, and resuspended were suspended in serum-free DMEM with 0.25% BSA. Prior to addition of the cells to each well, the cells (5×10^4) were pre-incubated with saxatilin or anti-$\alpha_v\beta_3$ monoclonal antibody for 30 min at 37°C. After the incubation, the cells were transferred to each well and incubated for 1 hr at 37℃ in 5 % CO_2 and 95% air. Unattached cells were removed by washing with PBS. Attached cells were fixed and stained with crystal violet. Absorbance at 570 nm of the individual well was measured to determine the relative cell number.

2.4. RT-PCR for integrin expression on SMCs

The expression levels of integrin transcripts were determined using RT-PCR protocol. For total RNA isolation from vascular smooth muscle cells were performed with Ultraspec II RNA isolation kit (Biotecx, Houston, Texas, USA). The cDNA was synthesized from total RNA using AMV reverse

transcriptase of Reverse transcription system (Promega Co, Madison, WI, USA). A 5 ㎕ of RT-reaction end-product mixture used as a template of polymerase chain reaction for integrins. PCR primers for integrins and control gene are; sense of αv; 5'-agatctggaccaggatggtt-3', antisense of αv; 5'-atctgtggctcctttcattg-3', sense of β3; 5'-accactgatgccaagactca-3', antisense of β3; 5'-gcatcaacaatgagctggag-3', sense of α5;5'-ccaggatggctacaatgatg-3', antisense of α5; 5'-cccacaatcagatcaggata-3', sense of β1; 5'-gttacacggc tgctggtgtt-3', antisense of β1;5'-ctactgctgacttagggatc-3', sense of GAPDH (control); 5'-accacagtccatgccatcac-3', and antisense of GAPDH; 5'-tcc accaccctgttgctgta-3'. The thermal cycling reaction was performed with 30 cycles of steps in Robocycler™ 96 (Staratagene Inc. La Jolla, CA USA).

2.5. Flow cytometric analysis of DNA content

For DNA content analysis, SMCs in suspension were collected and cells attached to the dishes were removed with trypsin (0.01%), and pooled with

the detached cells. The cells were harvested by centrifugation (160g) for 5 min at 4℃. The pellet of cells was washed with PBS and then cells were adjusted to $5x10^6$ cells/㎖ of 70% of ethanol in PBS at 4℃, overnight. Ethanol was then removed by centrifugation and DNA of the cells was stained with PI staining solution (100 ㎍/㎖ propidium iodide, 0.1% Triton-X, 1 mM EDTA in PBS) in the presence of an equal volume of DNase-free RNase (200 ㎍/㎖) and analyzed immediately by FACS Calibur™ (Becton Dickinson, Franklin Lakes, NJ USA). For cell cycle analysis, PBS control, or saxatilin-treated cells were maintained in the presence of 10% FBS DMEM for the observation of cell cycle progression. At the indicated time, treated cells were fixed and stained as described above. Debris and clumps were excluded by monitoring DNA area (FL2-A) and peak (i.e., signal height and FL2-W). Fluorescence signals from 20,000-gated cells were collected. Data were analyzed by using the ModFit program (Version 2.0, Verity Software

House, Inc., Topsham, ME, USA) to estimate the percentage of G2-M phase.

2.6. DNA fragmentation assay

Briefly saxatilin- or PBS-treated VSMCs ($5x10^7$ cells) were collected by centrifugation, and the genomic DNA was isolated using Accuprep® Genomioc DNA Extraction Kit (Bioneer Co. Daejeon, South Korea). The concentration of the genomic DNA was determined using Biophotometer (Eppendorf Inc. Hamburg, Germany). After treatment of loading dye, the reaction mixture was subjected to 1% agarose gel electrophoresis. DNA ladders stained by etidium bromide were visualized under ultraviolet light.

2.7. Visualization of actin cytoskeleton

Vascular smooth muscle cells (5×10^3 cells/well) plated in 4 well chamber slides (Nalgen Nunc International, Rochester, NY, USA) were

treated with the respective experimental conditions. Cells were fixed with 3.7% paraformaldehyde in PBS for 5min and permeabilized in PBS containing 0.1% Triton X-100, then blocked with 1% bovine serum albumin (BSA) in PBS for 60 min at room temperature. For F-actin staining, cells were stained with 1 μg/㎖ tetramethylrhodamine B isothiocyanate conjugated-phalloidin (Sigma-Aldrich Co, St. Louis, MO, USA) and 100 ng/㎖ DAPI solutions for 30min in the dark. After extensive washing, cells on the cover-slides were mounted with Gelmount™ solution (Sigma-Aldrich Co, St. Louis, MO, USA). Microscope observation was performed using the Metamorph ™ Image analysis software provided with the Zeiss LSM-510 series microscope. All illustrations were assembled and processed digitally using Adobe Photoshop 7 (Adobe Inc., San Jose, CA, USA).

2.8. Flow cytometric analysis

VSMCs in monolayer culture treated with or without saxatilin were washed with PBS, trypsinized, and collected by centrifugation. Then, 1x 10^6 cells/㎖ were re-suspended in PGB buffer (1% bovine serum albumin and 20mM glucose-containing PBS, pH7.4). After a wash, cells were labeled with primary anti-αvβ3 (LM609) or anti-α5β1 (JBS5) integrin monoclonal antibodies (Chemicon, Temecula, CA., USA) (1:100, final 10㎍/㎖) or control non-immunized IgG (as a negative control) for 30min at room temperature. Labeled cells were washed with 1% bovine serum albumin containing PBS and then incubated with secondary FITC-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, USA) at room temperature for 30 min with a continuous shaking. After incubation, cells were washed, re-suspended in PBS, and analyzed immediately by FACS Calibur (Becton Dickinson, USA) using excitation and emission wavelengths at 488 and 525 nm, respectively.

Fluorescence signals from 10,000 cells were collected to calculate mean fluorescence intensity of a single cell.

2.9. Determination of apoptosis by annexin V- FITC staining

Cells were stained with annexinV-fluorescein isothiocyanate (FITC) (Oncogene Research Products, Cambridge, MA, USA), which measures the externalization of phosphatidyl serine. Briefly, cells were treated with drug and at the end of 8 h treatment; both floating and adherent cells were collected, washed, and resuspended in media. Cell suspensions were adjusted to 1×10^6 cells/㎖. Annexin V-FITC was added to cell suspension with 0.5 ㎖ cold 1X binding buffer. After the reaction was incubated in the dark for 15min at room temperature, cells were harvested and re-suspended in cold 1 X binding buffer and then added propidium iodide (PI). After sample tubes place on ice and away from light, immediately tubes were analyzed by flow cytometry. Data

acquisition and analysis were performed with FACS Calibur™ (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer.

2.10. Immunoblots

Vascular smooth muscle cells were cultured for various experimental conditions with saxatilin. At the end of the various treatments, cells were suspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_2EDTA, 1mM EGTA, 1% Triton, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 ㎍/㎖ leupeptin) for 15 min on ice. Soluble extracts were prepared by centrifugation at 14,000 rpm for 15min at 4°C and the protein lysate concentrations were measured by BCA protein assay kit (Pierce Biotechnology Inc, Rockford, IL, USA). The same amounts and proportions of proteins from whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) and transferred onto methanol treated PVDF membranes. After blocking the Immobilon-P transfer membrane (Millipore Co, Bedford, MA, USA) with PBS containing 10% (w/v) skim milk (Difco, Spark, MD, USA) for 60 min at room temperature, they were washed twice with PBS and incubated with primary antibodies for 2h to overnight at room temperature or at 4℃. The membranes were washed three times with 0.2% (v/v) Tween-20 in PBS for 10min, and then incubated for 45min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the membranes were detected by enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). The primary antibodies for Paxillin ERK, p-ERK pCAS130, p21, Bax, p53, Bcl 2, and p-Tyr (PY99) were purchased from Santa Cruz (Santa Cruz Biotech Inc., CA., USA). The primary antibodies for p-FAK Tyr397, ILK, β-catenin cyclin D1/2, cyclin E, and p27 were purchased

from Upstate Biotech (Upstate Biotech Inc., NY, USA). And the rest of antibodies using this studies are β-actin, α-tubulin (Sigma, Saint Louis MI, USA), p-p38 (Biosource International Inc. Camarillo, CA, USA), and capase-3 (Chemicon, Temecula, CA, USA).

2.11. Statistical analysis

All values expressed as group means ± SD. Significance of difference was determined by student's t-test or analysis of variance (ANOVA) with Duncan's multiple range test using Win SPSS ver 11.0 (Statistical Package for the Social Science, SPSS Ins., Chicago, IL, USA). $P < 0.05$ and $p < 0.01$ was considered significant.

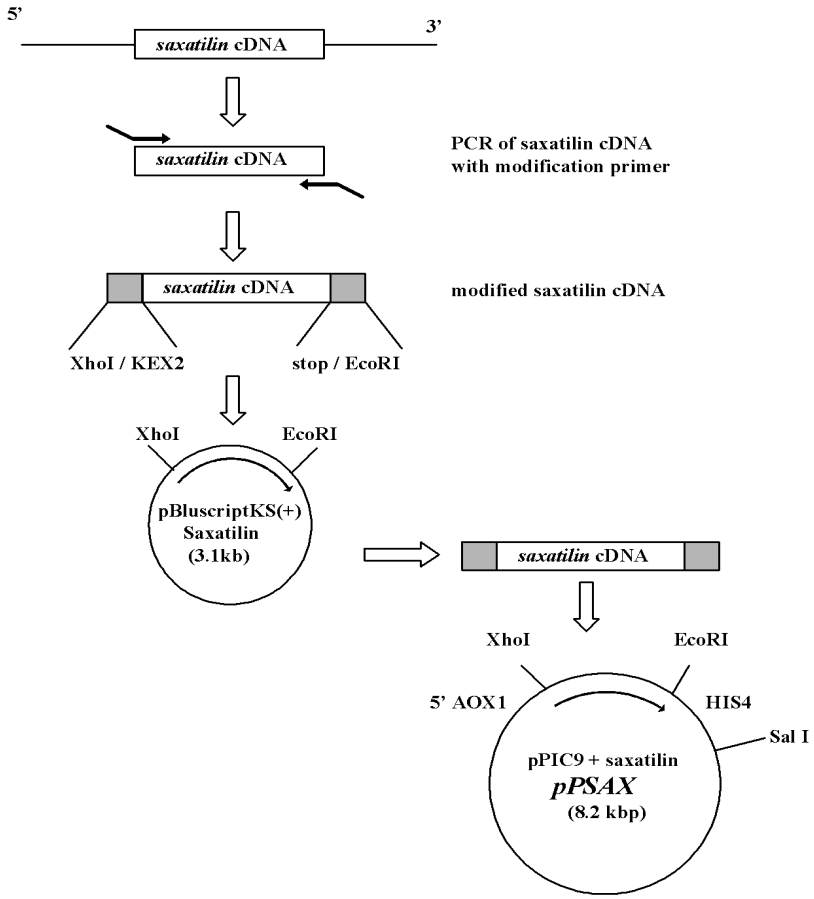
Ⅲ. RESULTS

1. Mass production of recombinant saxatilin and its biological activity

1.1. Production of recombinant saxatilin in *Pichia pastoris*

Designated as *pPSAX,* an expression vector for *saxatilin* was constructed (Fig 1). Yeast colonies transformed with *pPSAX* were screened on a yeast nitrogen base-dextrose minimal plate without histdine. Integration of the expression cassette into *his4* locus on *P. pastoris* genome was determined by PCR using the primers set for saxatilin (data not shown). The His+, Mut+ clones were cultured in minimal glycerol medium and induced in a 0.5% methanol medium for 96 hours (Fig.2). The SDS-PAGE analysis of supernatant revealed that transformed *P. pastoris* had produced a detectable level of a secreted 7.7 kDa protein (Fig.3). We presumed that methanol-induced 7.7kDa protein was recombinant saxatilin. To prove this

suggestion, the supernatants of methanol induced and non-induced culture were collected, and platelet aggregation assay was performed. The supernatants of yeast culture were compared with a purified native saxatilin and we confirmed that the methanol- induced culture of *pPSAX* had only platelet aggregation inhibition activity like a native saxatilin.

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1   GAG GCC GGA GAA GAA TGT GAC TGT GGC GCT CCT GCA AAT CCG TGC TGC GAT GCT GCA ACC
1   E   A   G   E   E   C   D   C   G   A   P   A   N   P   C   C   D   A   A   T
61  TGT AAA CTG AGA CCA GGG GCG CAG TGT GCA GAA GGA CTG TGT TGT GAC CAG TGC AGA TTT
21  C   K   L   R   P   G   A   Q   C   A   E   G   L   C   C   D   Q   C   R   F
121 ATG AAA GAA GGA ACA ATA TGC CGG ATG GCA AGG GGT GAT GAC ATG GAT GAT TAC TGC AAT
41  M   K   E   G   T   I   C   R   M   A   R   G   D   D   M   D   D   Y   C   N
181 GGC ATA TCT GCT GGC TGT CCC AGA AAT CCC TTC CAT GCC TAA
61  G   I   S   A   G   C   P   R   N   P   F   H   A
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Fig. 1. Construction of recombinant saxatilin expression vector and its cDNA sequence.

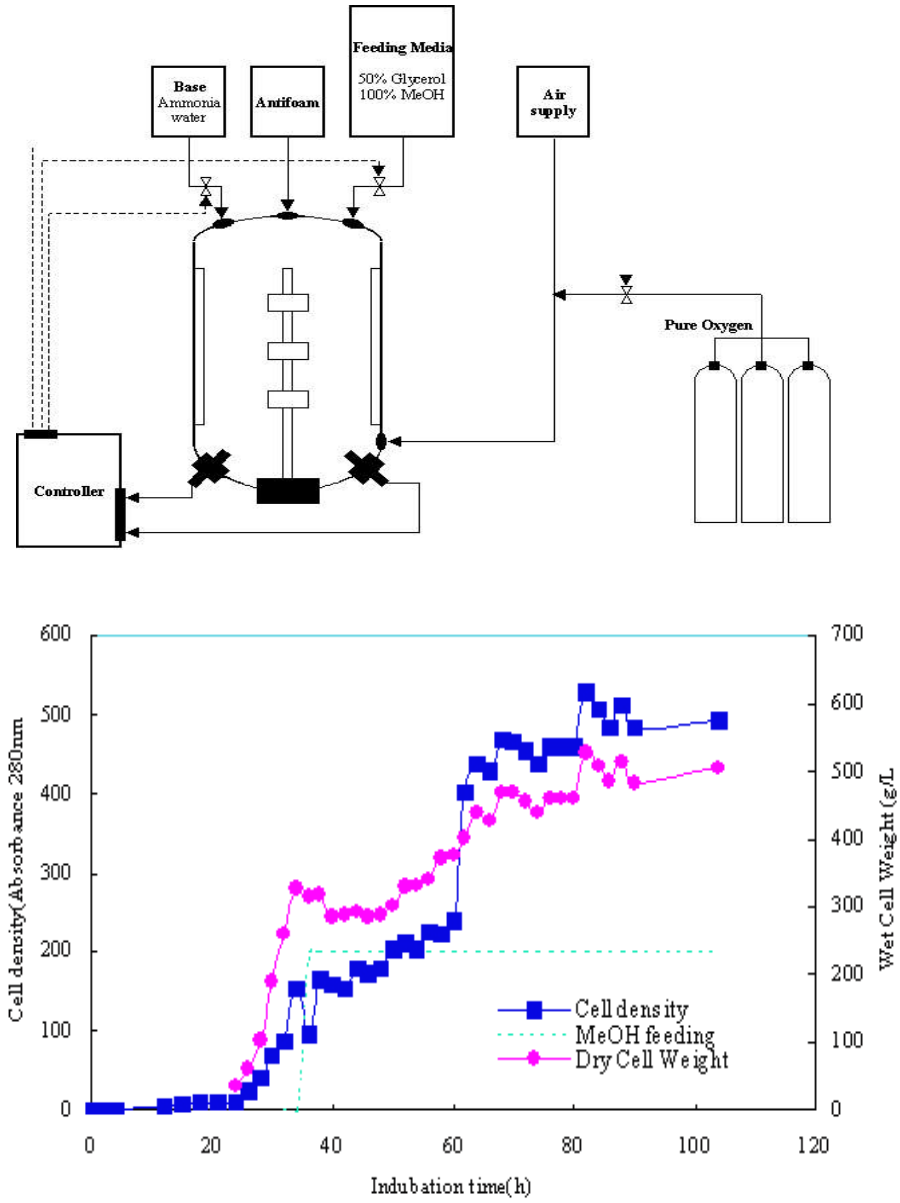

Fig. 2. Schematic outline of the fermentation process and the high-density fermentation profile of recombinant saxatilin.

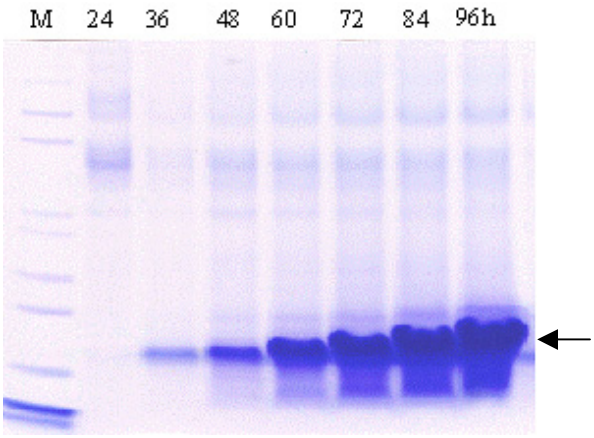

Fig. 3. Time course of the production of saxatilin induced by methanol feed. All lanes are 1㎖ of concentrated culture media. The arrow is indicating the 7.7kD band of recombinant saxatilin.

1.2. Purification and characterization of recombinant saxatilin

Two steps of purification were performed for recombinant saxatilin from a *Pichia pastoris* culture supernatant. Consequently, recombinant saxatilin was purified to be homogeneity using phenyl-Sepharose and Source-30^{TM} reverse phase chromatography (Fig.4) as described in Materials and Methods. MALDI-MS analysis of purified recombinant protein was determined that the molecular mass of saxatilin is 7,712 (Fig.5). To confirm the purified protein, it was determined by amino terminal sequence analysis. Fifteen residues of the N-terminal sequence were detected to be EAGEECDCGGAPANPC of saxatilin. From this data implicated that recombinant saxatilin was correctly processed by yeast KEX2 endo-peptidase during the secretion event.

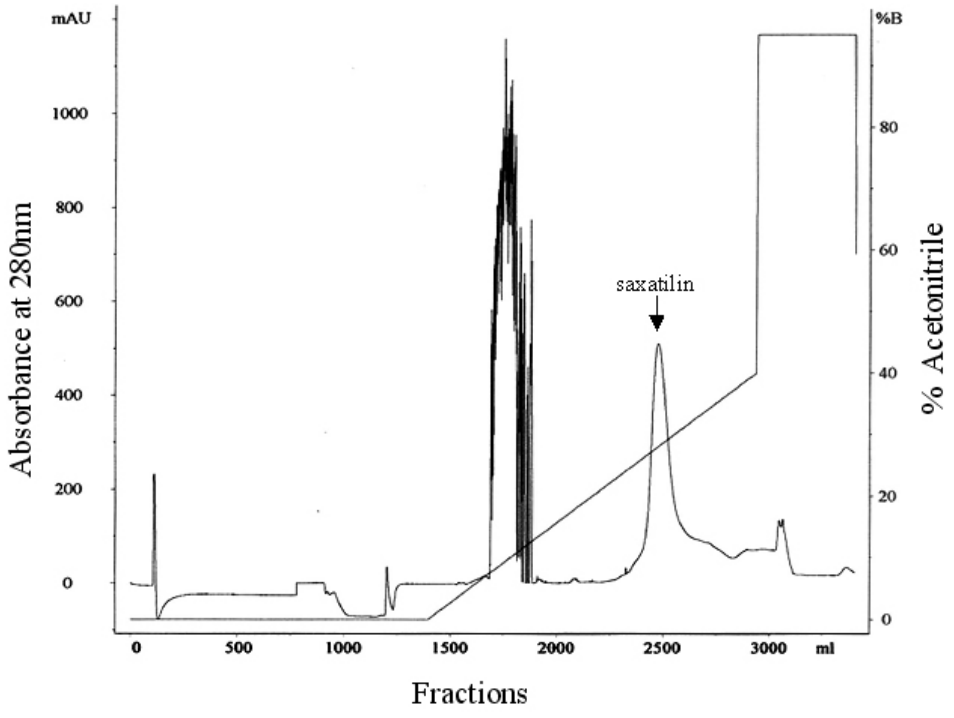

Fig. 4. Purification of recombinant saxatilin; by reverse-phase HPLC column chromatography.

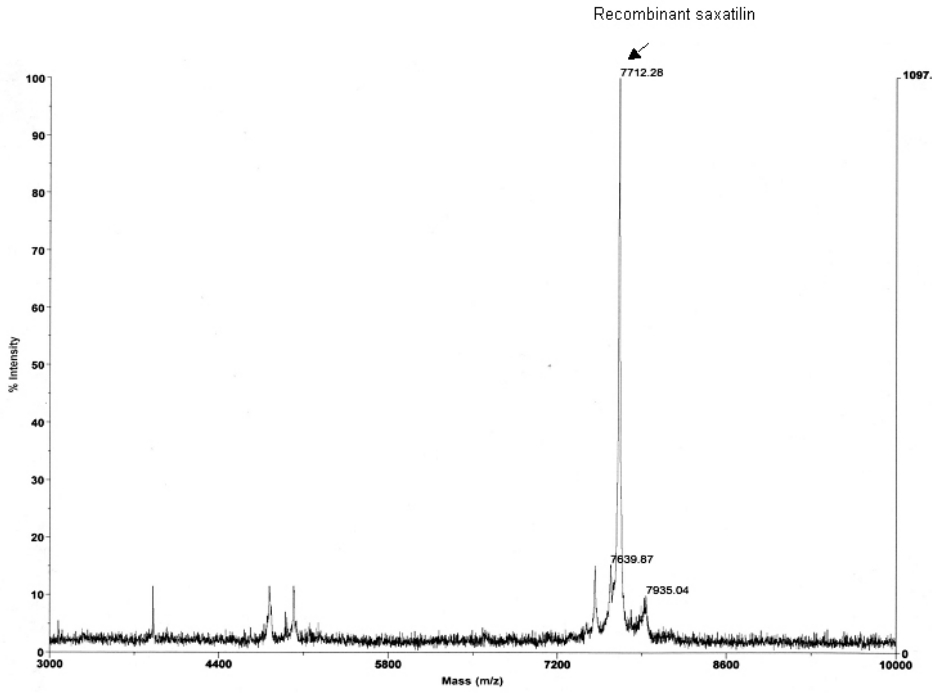

Fig. 5. Mass spectrometry analysis of purified recombinant saxatilin.

1.3. Inhibition of platelet aggregation

Dose-dependent inhibitions of human platelet aggregation by purified recombinant and native saxatilin were also measured in an ADP–induced platelet aggregation assay. Previously, platelet aggregation inhibition of saxatilin was compared with other disintegrins from a snake venom origin such as kistrin and flavoridin[28, 29, 30, 31]. The IC_{50} value of recombinant saxatilin is the same as the native form, which was approximately 139 nM, and complete inhibition concentration was observed at 500 nM (Fig.6).

1.4. Inhibition of fibrinogen-GPIIb/IIIa complex formation

The complex formation of GPIIb/IIIa with immobilized fibrinogen as measured by the solid-phase fibrinogen-GPIIb/IIIa binding assay was inhibited directly by recombinant saxatilin and its native form. The specific activities of recombinant and native saxatilin were determined in the binding

inhibition assay. The IC_{50} values of native and recombinant saxatilin for the inhibition of fibrinogen–GPIIb/IIIa binding were in the range of 2.2 to 2.7nM (Fig. 7). Previously, we confirmed that saxatilin is a much more effective inhibitor of fibrinogen–GPIIb/IIIa binding than the synthetic peptide GRGDS.

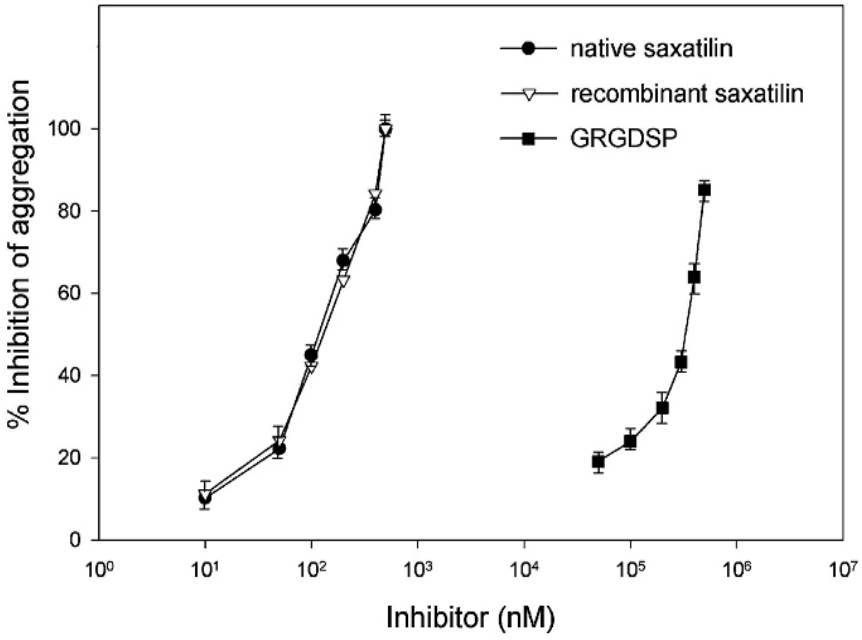

Fig. 6. Inhibition of ADP-induced platelet aggregation. The IC_{50} values were 131 M (native saxatilin), 139 nM (recombinant saxatilin), and 270 uM (GRGDSP), respectively

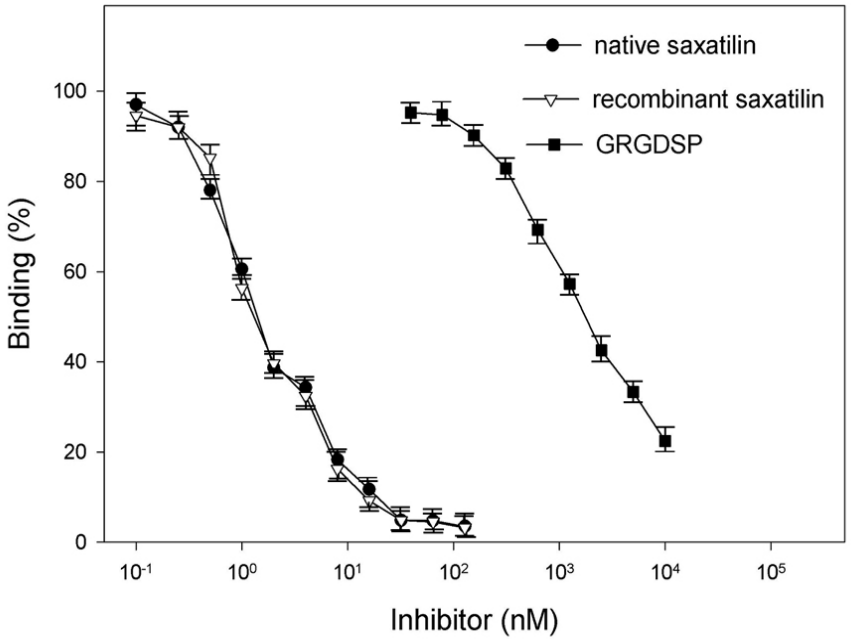

Fig. 7. Inhibition of fibrinogen-GPIIb/IIIa complex formation. The IC_{50} values were 2.2nM (native saxatilin), and 2.7nM (recombinant saxatilin) respectively.

2. The effects of saxatilin on vascular smooth muscle cells

2.1. Integrin expression profile of vascular smooth muscle cell

To characterize the expression profile of integrin on human vascular smooth muscle cells, we compared that of human coronary and aortic SMCs. As shown in Fig.8, the surface expression of the essential integrins αvβ3 and α5β1 on smooth muscle cells were indicated to be about 1:2 ratio at growing condition. To investigate the change of expression level of known integrins of HCASMCs, we tested it using reverse-transcription polymerase chain reaction for integrin's mRNA of serum-starved and growth factor-stimulated cells. As shown in Fig.9, the quiescent (serum-starved) SMCs majorly expressed α5 and β1 subunit. However, PDGF-stimulated synthetic SMCs highly expressed αv subunit for αvβ3 (Fig.9.). These results showed that α5β1 plays important role in cell survival, and αvβ3 is target of proliferation and migration stimulation on SMCs.

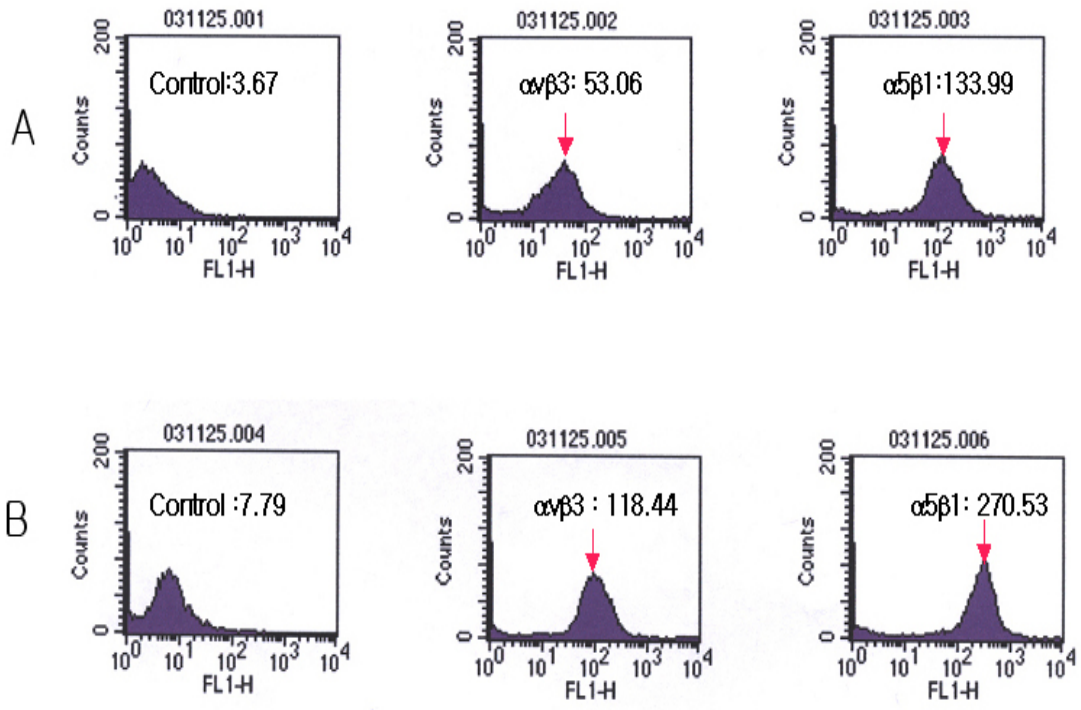

Fig. 8. Comparison of integrin expression on human coronary and aortic SMCs. A; human coronary SMC, B; human aortic SMC. The growth condition of smooth muscle cells was 5% FBS, SmGM2 (described in materials and methods). Indirect flow cytometry performed with mouse IgG as a control, LM609 as an anti-αvβ3, and JBS5 as an anti-α5β1 respectively.

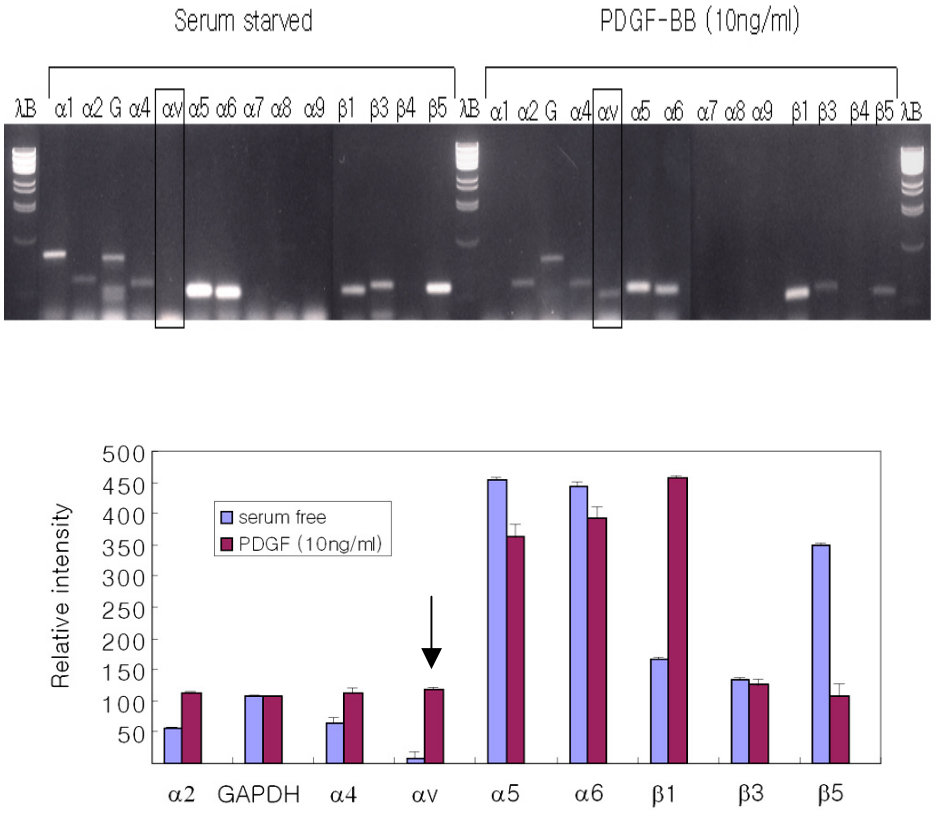

Fig. 9. Expression profile of integrins on HCASMCs by RT-PCR.

Relative intensities were normalized using by GAPDH. The serum-starved (for 48 h) HCASMC was treated with PDGF-BB (10ng/ml) for 1 h. Sizes of PCR product were indicated; internal control GAPDH (422bp), αv (197bp), α5 (222bp), β3 (296bp), and β1 (264bp).

2.2. Adhesion inhibition of HCASMC by recombinant saxatilin

SMC adhesion to immobilized vitronectin was examined *in vitro* in the presence of saxatilin. As shown in Fig.9, saxatilin inhibited human coronary artery SMC adhesion to vitronectin in a dose-dependent manner. SMC adhesion was inhibited to 50% in the presence of 2.5uM of saxatilin in the assay system. Monoclonal antibody known to inhibit cell adhesion to vitronectin by blocking $\alpha_v\beta_3$ integrin was used as a positive control. The results showed that the adhesion of VSMCs to vitronectin is highly suppressed by shortly pre-incubation the cells with saxatilin. Much higher concentration was needed to prevent the VSMC adhesion to fibronectin or type I collagen (more than 100uM) (Fig.10). These results suggested that the major binding target of saxatilin is $\alpha_v\beta_3$ integrin on VSMCs and thereby blocks the integrin-mediated cell adhesion.

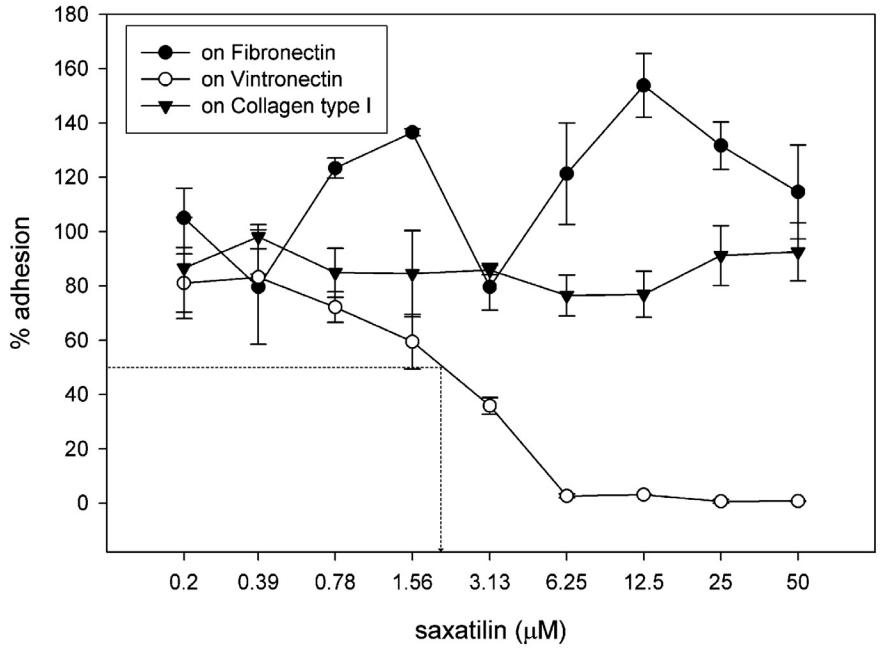

Fig. 10. Adhesion inhibition of HCASMCs on ECM proteins by saxatilin.

Recombinant saxatilin inhibited human coronary artery SMC adhesion to vitronectin in a dose-dependent manner (IC_{50} to vitronectin was 2.5uM). However, the HCASMCs adhesion to fibronectin or type I collagen was not affected by saxatilin in this range of concentration.

2.3. Proliferation inhibition of HCASMCs by recombinant saxatilin

To examine whether the biological function of saxatilin is correlated with restenosis, we employed proliferation assay of HCASMCs. Saxatilin was able to inhibit the growth factors-induced proliferation of HCASMCs in a dose-dependent manner. The half-maximal inhibition concentrations of saxatilin to growth factors (bFGF, or PDGF-BB)-induced HCASMCs proliferation were determined to be approximately 25 uM (Fig.11). Interestingly, further experimental investigation revealed that low concentration (1nM to 500nM) of saxatilin stimulated HCASMC proliferation rather than inhibited its proliferation (data not shown). Taken together these results, it is possible to postulate that saxatilin binds to $\alpha_v\beta_3$ integrin, which is closely associated with growth factor- integrin linked proliferation.

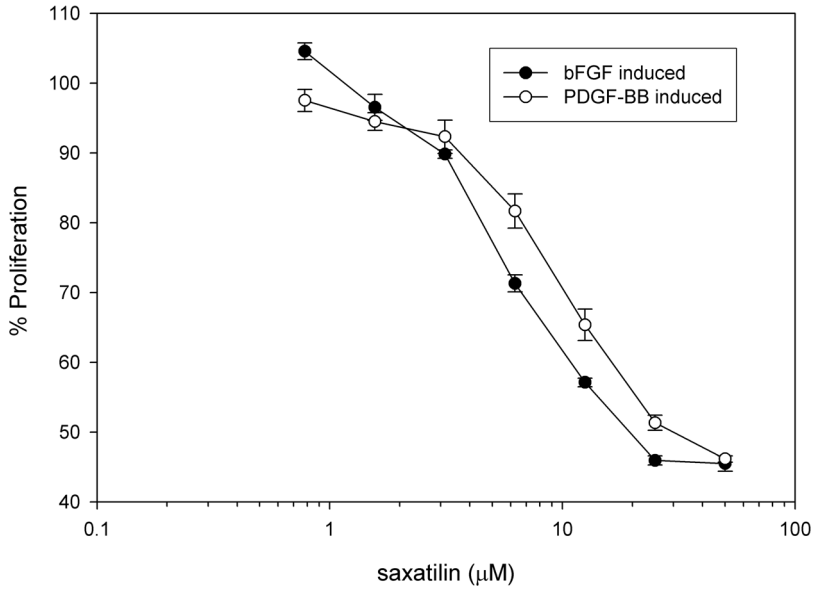

Fig. 11. Proliferation inhibition of HCASMCs by recombinant saxatilin.

Growth factors (20ng/㎖ of b-FGF and 10ng/㎖ of PDGF-BB) were added to HCASMCs pre-treated with various concentration of saxatilin. The control was not treated with saxatilin. (IC_{50} of saxatilin to growth factors was determined to be approximately 25uM.)

2.4. Disintegrin-induced morphological change and detachment of HCASMCs

Exposure of HCASMC cells to saxatilin in monolayer culture led cells to be retracted, rounded-up, and detached, forming gaps among cells and multicellular aggregates. Time-course microscopic analysis showed that lower density cells were retracted or rounded-up (Fig.12-A). However, highly confluent cells were pulled neighboring cells, forming muticellular aggregates (Fig.12-B). Saxatilin induces detachment of proliferating SMC cells, but the serum starved (-growth arrested) cells were insensitive to disintegrin-linked morphological changes.

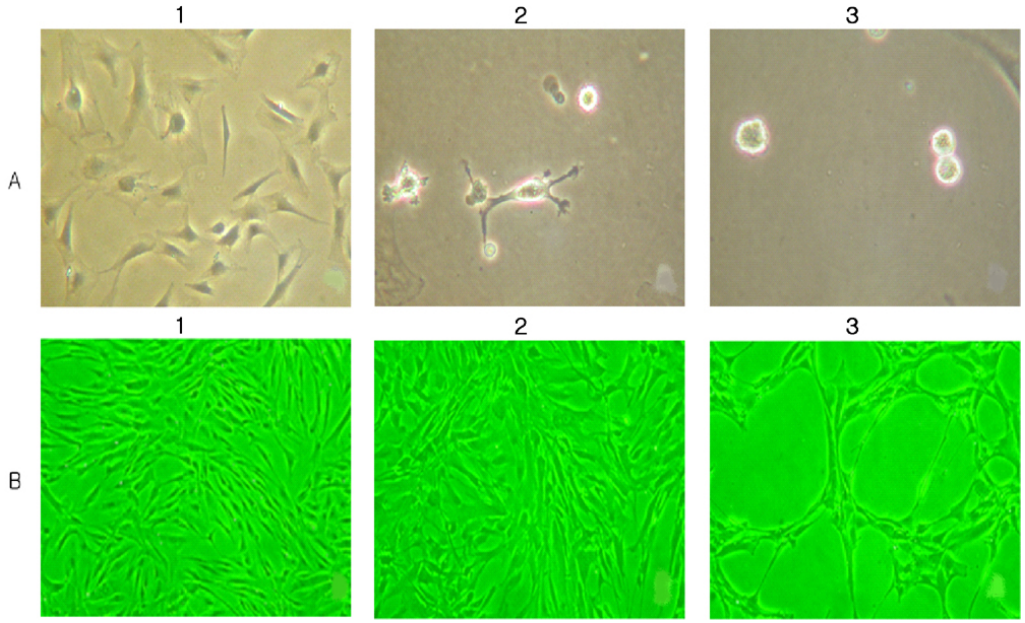

Fig. 12. Disintegrin-induced morphological change and detachment of HCASMCs. The panel A1 is a control of HCASMCs. The panel A2 and A3 are 20uM of saxatilin-treated cells (for 6 to 24hours). The panel A-3 was fully detached as a single cell and rounded up (100x). The panel B1 is a high-density control of HCASMCs (10% FBS supplied). The B2 and B3 are HCASMCs treated with 20uM of saxatilin after 6 to 24hours. The B2 pulled neighboring cells, the B3 were forming muticellular aggregates (40x).

2.5. Disassembly of focal adhesion of HCASMCs by disintegrin

Saxatilin disassembled the cytoskeleton of F-actin and focal adhesion complex on HCASMCs. HCASMC cells not treated with saxatilin were fully spread and had well-organized F-actin fibers, as monitored by TRITC-phalloidin staining. In the presence of saxatilin, cortical actins of the HCASMC cells began to shrink into the center at cellular focal contacts and were completely disassembled at 120min, resulting in an almost rounded morphology (Fig. 13 A). However, not much change in microtubule structures was observed in the early stage of cells being rounded by saxatilin treatment, suggesting that saxatilin would not directly affect microtubule structures (Fig. 13 B). Paxillin is one of the proteins recruited to focal adhesions when cells are attached to the matrix through integrins[33]. The change of paxillin was analyzed using immunoblot of the saxatilin-treated HCASMC cells. Paxillin was time dependently down regulated by saxatilin treatment (Fig.14)

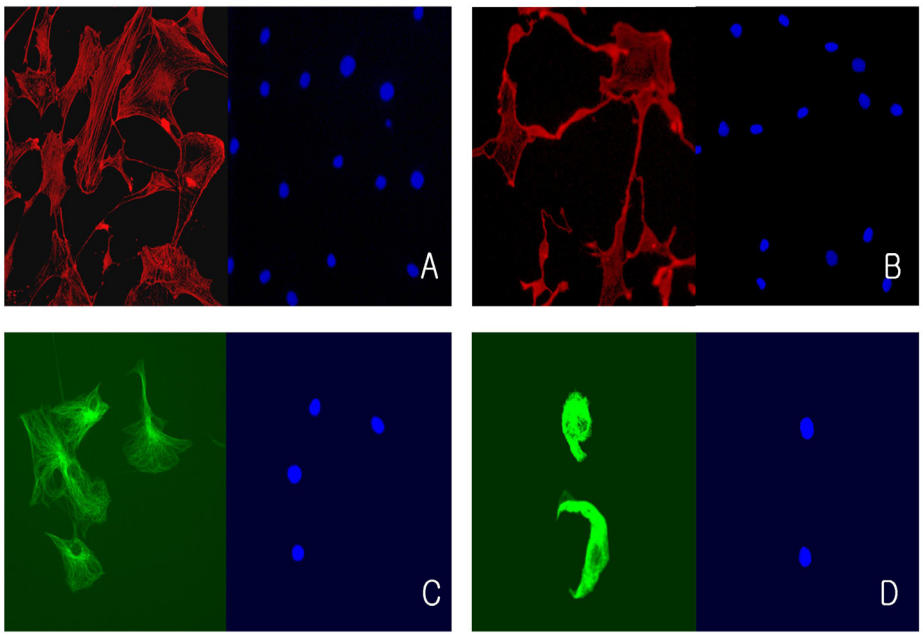

Fig. 13. Disassembly of the F-actin cytoskeleton and focal adhesions of HCASMCs by saxatilin. HCASMC cells grown on a slide-chamber were incubated with (B, D) or without (A, C) 40uM of saxatilin for 90min. Cells were fixed and stained with DAPI and either TRITC conjugated rhodamine phalloidin or a-tubulin polyclonal antiserum to visualize the nucleus (blue), F-actin (red: A, B) and tubulin (green: C, D).

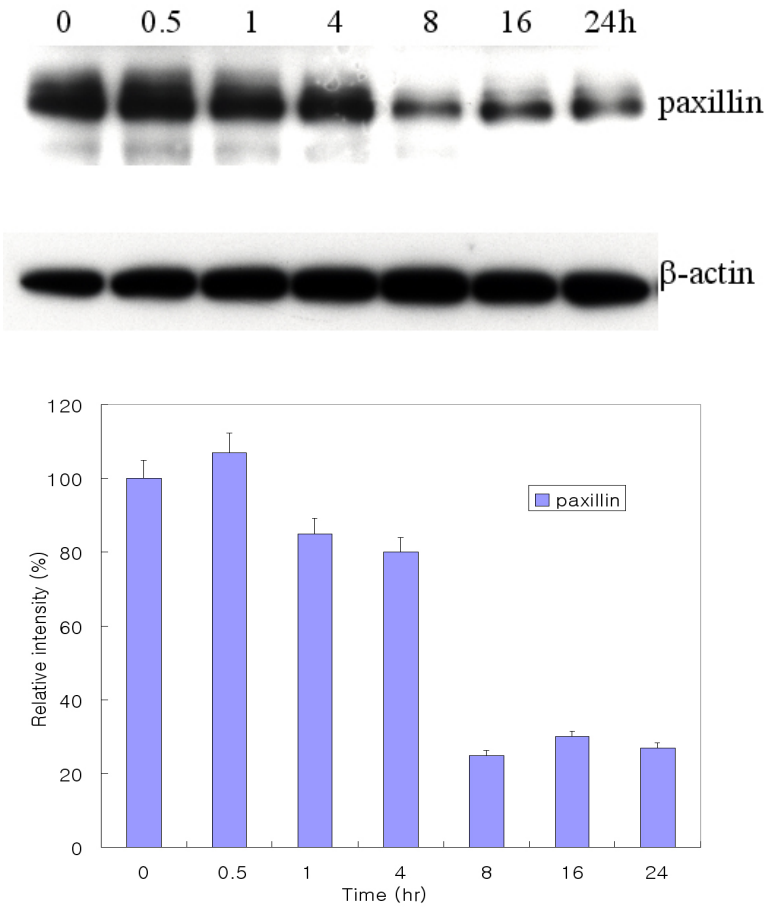

Fig. 14. Changes of paxillin in HCASMCs by saxatillin treatment. HCASMCs were treated with 20uM of saxatilin. The degradation profile of paxillin was analyzed by immunoblot. The intensities of bands were normalized using signals of β-actin.

2.6. Caspase-mediated degradation of paxillin

Recently, there was a report that caspase degraded paxillin during apoptosis process[33]. Paxillin plays a critical role in cell survival signaling and its degradation by caspases might be crucial event for focal adhesion disassembly during the anoikis of adherent cells. To investigate the linkage of caspases and paxillin degradation in the saxatilin treated HCASMCs, pan-caspase inhibitor; Z-VAD-FMK (50uM) was pre-treated with SMC culture. From the result of immunoblot analysis, saxatilin-treated HCASMC showed completely degraded paxillin, while inhibitor pre-treated and control cells showed an intact 68 kDa of paxillin (Fig.15).

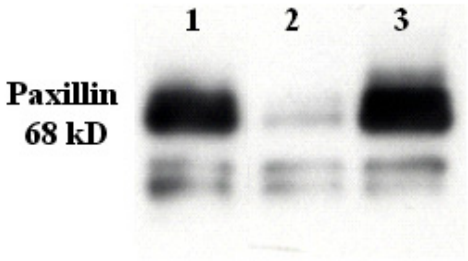

Fig. 15. Caspase induced paxillin degradation by saxatilin treatment on HCASMCs. Lane1; HCASMC control, lane2; HCASMC treated with 25uM of saxatilin, lane3; HCASMC pre-treated Z-VAD-FMK plus 25uM of saxatilin. HCASMCs were incubated with or without Z-VAD-FMK in the presence of saxatilin. Control was not treated with saxatilin. After 24 hours, samples were performed with immunoblot analysis for paxillin.

2.7. Effect of saxatilin on focal adhesion kinase (FAK) stimulation

Saxatilin inactivated focal adhesions through the FAK-dependent signaling pathway. The observation that saxatilin binds to integrin αvβ3 [31, 32] and induces the disassembly of focal adhesions led us to investigate the integrin-mediated signaling pathways. Since activation and phosphorylation of FAK (pp125) are tightly coupled to the assembly of focal adhesions by the recruitment of other proteins including paxillin, p130CAS, talin, and vinculin [5, 6, 7]. To elucidate the expression and phosphorylation of FAK, immunoblot analysis of HCASMC cells were performed after saxatilin treatment. Serum-starved quiescent SMCs were pre-treated with saxatilin for 10 min before serum stimulation. Detachment of HCASMC cells was started at 3 h of incubation and reached to around 60% at 12 h of incubation. As shown in Fig. 16, saxatilin had little effect on the expression of FAK but decreased the level of phosphorylated FAK. Serum starved-quiescent SMCs were temporally

phosphorylated Tyr397 of FAK (Ras-ERK pathway linked residue). Activated FAK (Tyr397) started to decrease at 3 h and was dramatically decreased until 24 h when most cells were detached. It is possible to be suggested that reduction of phosphorylation of FAK by disintgrin treatment due to disassembly of focal adhesion via paxillin degradation.

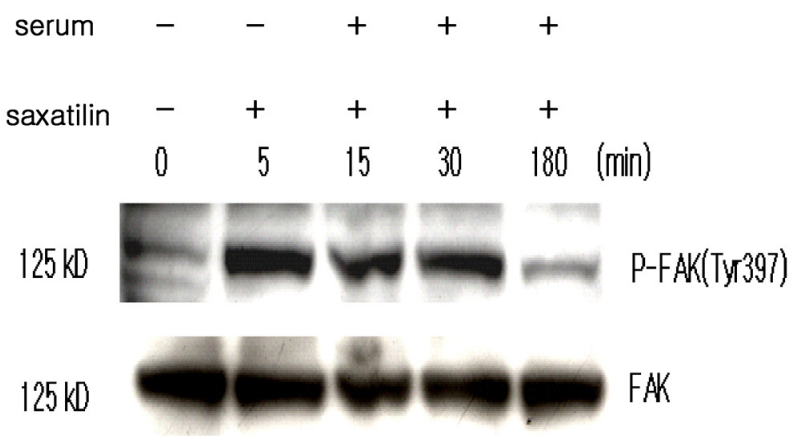

Fig. 16. Effect of saxatilin on focal adhesion kinase (FAK) expression and activation. Serum-starved HCASMCs were treated with saxatilin as indicated periods and stimulated with serum at 10 min. The cells were analyzed with immunoblot against phosphor-FAK (pp125) and total FAK.

2.8. Effect of saxatilin on cell cycle of HCASMCs

To elucidate the mode of action of saxatilin to cause vascular smooth muscle cell anoikis, we analyzed cell cycle distribution (i.e., DNA content) at the treatment of disintegrin. As shown in Fig. 17, control cells and low concentration of saxatilin (5 to 20uM)-treated cells were relatively quiescent at 4 and 9h, but entered G2-M phase after 6 h in the treatment of 40 uM of saxatilin and 20 h in the treatment of 20 uM of saxatilin, respectively (summarized in Table1 and Table 2). Treatment of saxatilin promoted cells to progress to (or arrest at) G2-M phase (Fig. 17 A and B). The proportions in the G2-M phase cells were significantly increased by saxatilin treatment compared with control (Fig.17 C and D). However, G2-M and S-phase were decreased when cells underwent anoikis (Fig. 17 F).

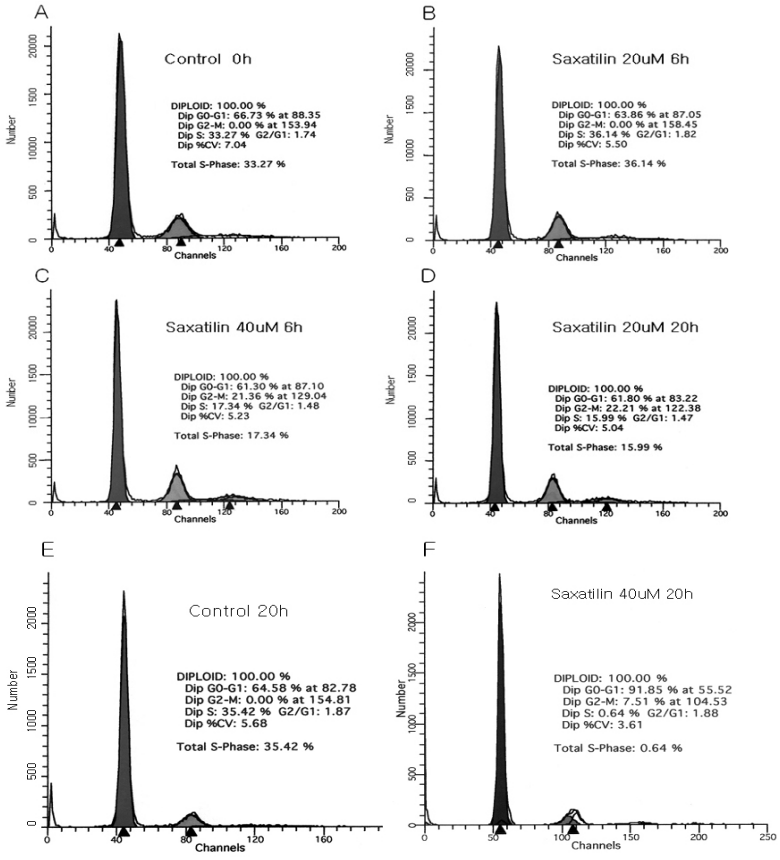

Fig. 17. Flow cytometry analysis for cell cycle distribution. (A) Untreated - normal HCASMC control, (B) saxatilin (20 uM for 6 h), (C) saxatilin (40 uM for 6 h), saxatilin (20 uM for 20 h), (E) untreated control 20 h, and (F) saxatilin (40 uM for 20 h)- treated cells. The samples were collected at the indicated time fixed, and stained with propidium iodide. The fluorescence of individual cell was measured by flow cytometry.

Table1. Cell cycle distribution of HCASMCs treated with various concentrations of saxatilin: (6 h treated)

Treatment	G0/G1 (%)	S (%)	G2/M (%)
Control	66.73	33.27	0
5uM	0.58	39.42	0
10uM	59.61	40.39	0
20uM	63.86	36.14	0
40uM	61.3	17.34	21.36

Table2. Cell cycle distribution of HCASMCs treated with various times of saxatilin

Treatment	G0/G1 (%)	S (%)	G2/M (%)
Control 20 h	64.58	35.42	0
20 uM 4 h	62.46	37.54	0
20 uM 6 h	63.86	36.14	0
20 uM 20 h	61.8	15.99	22.21
40 uM 20 h	91.85	0.64	7.51

2.9. Effect of saxatilin on the expression of cell cycle-regulating proteins in HCASMCs

To investigate the molecular events involved in the effect of disintegrin on cell cycle progression, the changes of cyclins and CDK inhibitors were determined by immunoblot analysis. As shown in Fig. 18, saxatilin treatment significantly down regulated the cell cycle stimulator cyclin D1/2 and cyclin E. And also, cell cycle inhibitor $p21^{WAF/Cip1}$ and $p27^{kip1}$ were increased (Fig. 19.). These results indicate that saxatilin-mediated inhibition of HCASMCs proliferation was controlled by expression of cell cycle regulators.

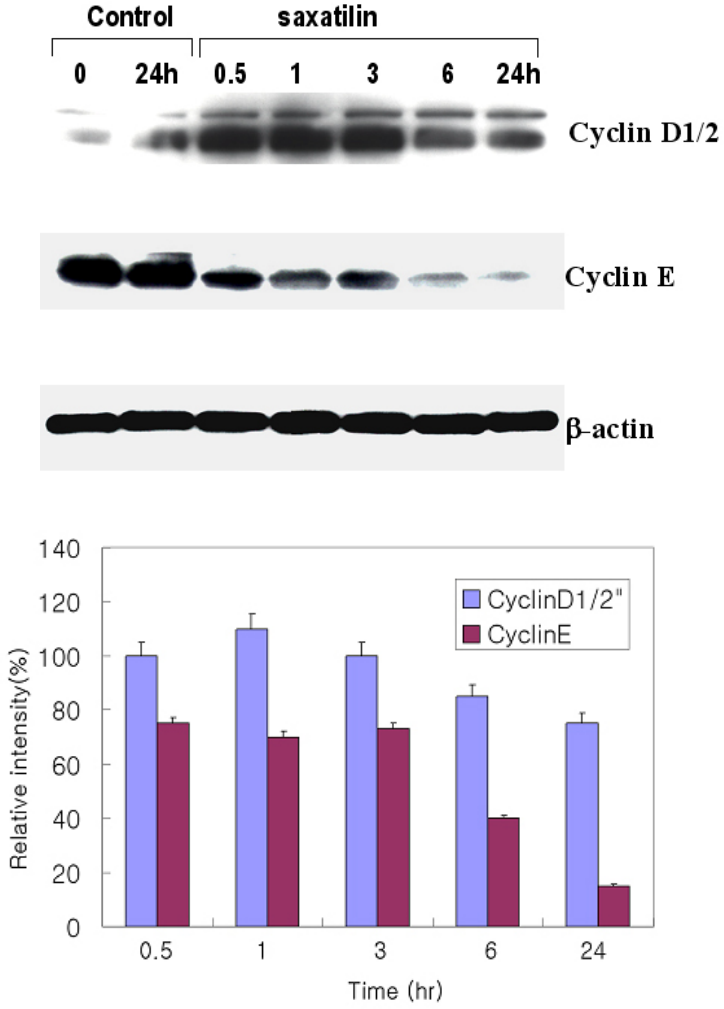

Fig. 18. Effect of saxatilin on the expression of cell cycle regulators in HCASMCs. Immunoblot analysis for cyclin was performed in the treated of HCASMC with 20uM of saxatilin. All bands were normalized with intensity of β-actin.

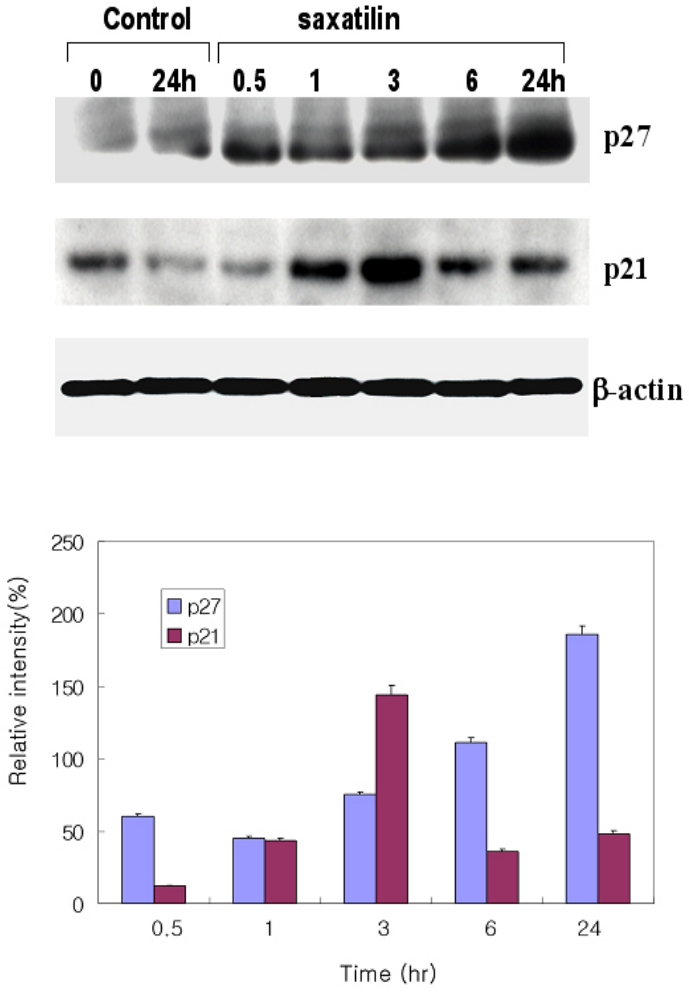

Fig. 19. Effect of saxatilin on the expression of cell cycle inhibitors in HCASMCs. Immunoblot analysis for cell cycle inhibitor was performed in the treated of HCASMC with 20uM of saxatilin. All bands were normalized with β-actin intensity.

2.10. Effect of saxatilin on activation of ERK

To investigate the effect of saxatilin on MAPK pathway of HCASMCs, the activation of ERK was detected by immunoblot against phospho-ERK1/2 (pp44/42). Serum-starved HCASMCs were treated with a various concentrations of disintegrin for 10 min. Saxatilin stimulated dose-dependently activation of ERK1/2 without growth factor (Fig.20). Effect of saxatilin on growth factor-induced ERK activation was confirmed with PDGF-BB-stimulated HCASMCs. ERK1/2 activation of HCASMCs stimulated by PDGF was typically short, but that of disintegrin pre-treated cells was prolonged until detachment. After the anoikis, ERK activation was rapidly reduced (Fig. 21)

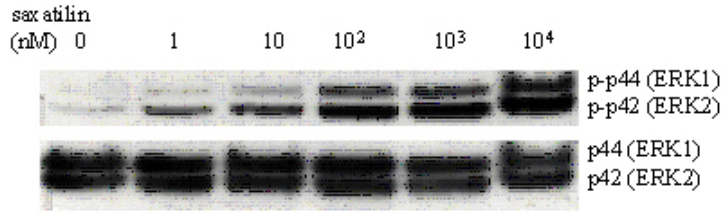

Fig. 20. Dose response of saxatilin on the phosphorylation of ERK 1 and 2 in HCASMCs. (10 min treated).

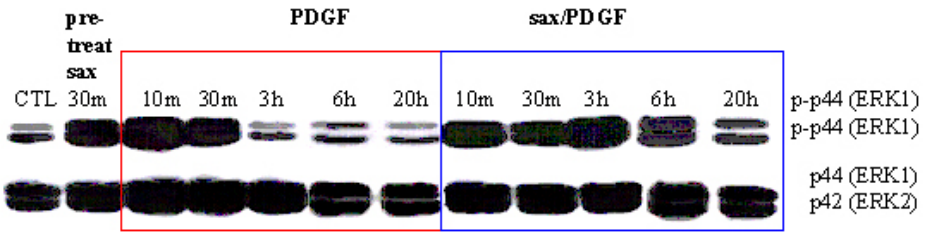

Fig. 21. Effects of saxatilin on PDGF-induced ERK activation

HCASMCs were treated with or without saxatilin (20uM), and then cells were stimulated by PDGF-BB (10ng/ml). Samples were harvested at indicated time and performed with immunoblot against p-ERK1/2 and total ERK1/2.

IV. DISCUSSION

Restenosis occurs in approximately 30-40% of patients following percutaneous transluminal coronary balloon angioplasty (PTCA) for treatment of atherosclerosis[1, 2, 3, 4]. SMC proliferation, migration, and adhesion are critical initial steps in the development of the neointimal hyperplasia that contribute to the restenosis in the arterial wall. It has been also reported that interactions between vascular cell integrin and ECM protein play key role in angiogenesis. Integrins are heterodimeric transmembrane glycoproteins, composed of α and β subunits. These αβ heterodimers bind to vitronectin, fibronectin, laminin, collagen, and other matrix proteins and different isoforms of the α and β chains determine the ligand-binding specificity of various integrins. Integrin receptors primarily mediate cell-cell and cell-matrix interaction followed by activation of intracelllular signaling pathways through the cytoplasmic carboxyl terminus [5, 6].

Disintegrins are a family of cystein-rich, low-molecular-weight protein isolated from various snake venoms[16, 17, 18, 19, 20, 21]. These proteins contain the integrin-binding Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequence, which is the structural motif specifically recognized by the integrins αIIbβ3, α5β1, and αvβ3 expressed on platelets and other cells including vascular endothelial cells and some tumor cells. Cell adhesion to the ECM is, in part, mediated by binding of integrin to an integrin-recognition RGD motif found on some ECM components such as fibronectin, vitronectin and fibrinogen [23, 24, 25, 26].

In our previous report, it was demonstrated that the novel disintegrin, saxatilin, derived from Korean snake (*Gloydius saxatilis*) venom strongly inhibits human platelet aggregation, bFGF-induced proliferation of HUVEC and SMC migration. Saxatilin is a single chain polypeptide composed of 73 amino acids including 12 cysteines as well as the tripeptide sequence RGD [31,

[32].

In this study, the recombinant saxatilin was highly expressed as a biologically active form in *Pichia pastoris*, and it was successfully purified to be homogeneity from the culture media. The overall yield was approximately 150 mg/L. The molecular and biological properties of the purified recombinant protein were almost same with its natural from. It interacts with integrin αvβ3, and significantly suppresses the adhesion of human coronary artery smooth muscle cells to vitronectin with an IC_{50} of 2.5uM and inhibits the proliferation of HCASMC induced by growth factors (IC_{50} of saxatilin to growth factors was approximately 25uM for bFGF or PDGF). Saxatilin disassembled actin cytoskeleton of focal adhesion and induced cells to being rounded and detached, but did not alter microtubule structure in the early stage of cells being shrunk. Focal adhesion kinase (FAK) and ERKs of saxatilin treated SMCs were temporally phosphorylated. While SMC was

normally proliferated on plates without saxatilin, that HCASMC treated with saxatilin eventually underwent to anoikis. Anoïkis is defined as programmed cell death induced by the loss of cell/matrix interactions[36, 37].

Adhesion to structural glycoproteins of the extracellular matrix is necessary for survival of the differentiated adherent cells in the cardiovascular system, including endothelial cells, smooth muscle cells, fibroblasts, and cardiac myocytes. Adhesion is also a key factor for the differentiation of mesenchymal stem cells. In particular, fibronectin is considered a factor of survival and differentiation for many adherent cells. Adhesion generates cell tensional integrity (tensigrity) and repression of apoptotic signals, whereas detachment has the opposite effect. Anoïkis plays a physiological role by regulating cell homeostasis in tissues. However anoïkis can also be involved in pathological processes, as illustrated by the resistance to anoïkis in cancer and its enhancement in degenerative tissue remodeling. Extracellular

mediators of anoïkis include matrix retraction, leading to loss of tensegrity in fibroblasts, pharmacological disengagement of integrins by RGD-like peptides and fragments of fibronectin, and focal adhesion disassembly by fragments of thrombospondin, plasminogen activator-1, and high-molecular-weight kininogen. In addition to binding of the RGD peptide by integrins, the engagement of the heparin binding sites of adhesive glycoproteins with glycosaminoglycans on the cell surface is also involved in the prevention of cell detachment–induced apoptosis. Anoïkis is probably involved in pathological remodeling of cardiovascular tissues, including cardiac myocyte detachment in heart failure, de-endothelialization and plaque rupture in atherosclerosis, and smooth muscle cell disappearance in aneurysms and varicose veins.

Saxatilin disassembled actin cytoskeleton of focal adhesion involved caspase induced paxillin degradation. Paxillin is a 68kDa adaptor protein

discovered in focal adhesion as a substrate of Src kinase in Src-transformed cells. Recent researches have shown that focal adhesions are disassembled and some of their constituents, such as FAK and p130CAS, are cleaved by caspase during apoptosis [33]. However cleavage of these particular components does not appear to be critical for focal adhesion disassembly during apoptosis, because release of FAK from focal adhesion precedes cleavage of FAK by caspase[33]. Possible candidate for the caspase target that is crucial to focal adhesion disassembly is paxillin, because binding to LD motifs and tyrosine phosphorylation sites at the N-terminal half of paxillin is essential for recruiting other critical focal adhesion proteins, such as FAK, vinculin, Crk, p130 CAS, and actopaxin to focal adhesion complex. As shown in result, a pan-caspase inhibitor (Z-VAD-FMK) pre-treated HCASMCs had resistance to saxatilin induced degradation of paxillin.

Many cases were reported that disintegrin induced apoptosis involved

caspase3 activation on vascular endothelial cells, such as HUVECs [31, 35] and BCE cells[31]. Previous study has shown that echistatin induces apoptosis of epithelial cell line prior to cell detachment[40], which is different from our observation. One of the differences may result from the different cells they used, since different cells may exhibit different susceptibilities to drugs. As demonstrated by other research groups [35], poly- (HEME)-induced apoptosis of endothelial cells occurred within 4h, while in the epithelial cell line they used it occurred after 24-36hours of suspension. Furthermore, another report showed that echistatin detached melanoma cells grown on fibronectin, but they did not describe that echistatin-induced apoptosis within 6h of treatment [40]. Although different integrins are involved in these two experiments, it demonstrates that echistatin does not directly induce apoptosis. We tried to get the direct evidence for apoptosis in detached HCASMC cell by saxatilin. However, serials of data for apoptosis tests include the annexin -V staining,

PARP degradation, activity assay of caspase 3 and immunoblot were not significant to control apoptotic treatment (known SMC apoptosis induced drug such as curcuminoid; data not shown).

Recently reported that ERK1 associates with αvβ3 integrin and regulates cell spreading on vitronectin. On the other hand, numerous reports in the literature show that ERK activation plays a protective role in anoikis. We suggest the reason for unclear apoptotic evidence of detachment of disintegrin induced smooth muscle cell caused by prolonged activation of ERK activation. This growth factor independent- and disintegrin-stimulated ERK activation linked with focal adhesion kinase and Akt/ PKB pathway[5, 6, 41]. However, the integrin stimulated Akt/PKB pathway is known to positive controlled survival of adherent cells.

The engagement disintegrin to smooth muscle cell induces improper tensional integrity stress to cells. As shown in the result from RT-PCR of

integrin, the major integrin population of quiescent smooth muscle cell was $\alpha 5\beta 1$[39], but stimulations of growth factor lead high expression of integrin $\alpha v\beta 3$. This vitronectin receptor has key role in transitional function to the state of vascular smooth muscle cell. The perfect details of the integrin role in smooth muscle cells remain to be worked out. These studies may provide new insights into role of integrin in SMC pathophysiology as well as role of SMCs in the process of neointimal hyperplasia and have significant implications for integrin antagonistic therapy of the treatment for atherosclerosis and restenosis.

V. CONCLUSION

The recombinant saxatilin was expressed as a biologically active form in *Pichia pastoris*, and it was successfully purified to be homogeneity from the culture media. The overall yield was approximately 150mg/L. The molecular and biological properties of the purified recombinant protein were almost same with its natural from. It interacts with integrin $\alpha v\beta 3$, and significantly suppresses the adhesion of human coronary smooth muscle cells to vitronectin with an IC_{50} of 2.5uM. Half maximal inhibited concentration of saxatilin for growth factors (PDGF-BB or b-FGF) induced-proliferation was an approximately 25uM. Saxatilin disassembled actin cytoskeleton of focal adhesion and induced cells to being rounded and detached, but did not affect microtubule structure. This disassembly of focal adhesion in saxatilin treated HCASMs involved caspase induced paxillin degradation. Focal adhesion kinase (FAK) and ERKs of saxatilin treated SMCs were temporally

phosphorylated. Saxatilin affected cell cycle progression of HCASMCs by increasing CDK inhibitors (p21 & p27) and reducing cyclins (D1/2 and E). While SMC proliferated normally on plates without saxatilin, cells treated with saxatilin eventually underwent anoikis.

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Abstract (in Korean)

신규 디스인테그린(Saxatilin)의 평활근 세포증식 억제 기전

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동맥경화 치료를 위한 관상동맥 풍선도자성형 시술 환자의 30-40%에서 혈관의 재협착이 발생한다. 동맥혈관벽의 재협착에 있어서 신생조직의 발달에 혈관 평활근세포의 자극이 매우 중요한 과정으로 인식되고 있다. RGD 서열를 함유하고 있는 펩타이드들은 신생내막증식에 관여하는 β3-인테그린류인 αIIbβ3나 αvβ3등과 결합하여 목표하는 리간드와의 결합을 길항하는 것으로 보고되고 있다. 대부분의 디스인테그린은 혈소판 피브리노젠 수용체인 GPIIb-IIIa과 αvβ3등에 의해 인식되는 motif인 RGD 또는 KGD서열을 포함하고 있다. 본 연구의 목적은 한국산 칠점사의 독으로부터

분리된 새로운 디스인테그린인 saxatilin의 생물학적 활성을 규명하고자 효모로부터 대량생산을 시도하였다. *Pichia pastoris* 효모의 배양액에서 phenyl-Sepharose와 RPC-HPLC 크로마토그라피의 조합으로 생물학적으로 완전한 활성을 갖는 재조합 saxaxlin을 균일상태로 순수 정제하였다. 이때 수율은 리터당 약 150mg이 도달했으며 이 재조합 단백질의 생물활성과 분자적 특성이 천연물에서 얻은 것과 동일함을 확인하였다. 이 재조합 saxatilin은 인테그린 αvβ3에 반응하여 인간의 관상동맥유래 평활근세포와 vitronectin과의 결합을 강하게 저해했으며 IC_{50} 농도는 2.5uM이었다. 세포성장인자들에 의한 세포의 증식억제 IC_{50} 농도는 PDGF-BB 또는 bFGF에 대하여 약 25uM로 측정되었다. Saxatilin은 focal adhesion의 actin 세포골격망을 분해시키고 세포의 형태를 변형시켜 부착이 저해되도록 작용하지만, 이 같은 세포의 형태수축이 일어나는 동안 미세 tubulin 구조의 변화는 일으키지 않았다. 이러한 focal adhesion의 해체과정에서 caspase효소에 기인된 paxillin의 분해가 관여하는 것으로 확인되었다.

Saxatilin을 처리한 세포에 일시적인 FAK와 ERK 인산화가 일어났으며, 세포주기조절에 관여하는 CDK저해단백질 (p21, p27)은 증가하지만 cyclin들 (D1/2, E)은 오히려 감소하는 경향을 보였다.

또한, saxatilin을 처리하지 않은 평활근세포가 정상적으로 성장하는 동안 saxatilin처리 세포는 시간경과에 따라 세포탈착(anoikis)에 이르게 하였다.

이상의 결과들은 동맥경화와 혈관재협착 과정에 중요한 평활근세포의 신생내막증식 과정에서의 인테그린의 병리생리학적 역할에 대한 새로운 여러가지 정보를 제공하고 있다. 또한, 특정 인테그린에 대한 길항제를 이용한 동맥경화의 예방과 혈관재협착의 치료에 이와 같은 정보가 유용하게 활용될 수 있으며 동물시험을 통한 생체 내 활성이 입증된다면 saxatilin이 신약개발의 좋은 모델물질로 인식될 것으로 기대된다.

핵심되는 말: integrin, disintegrin, recombinant, saxatilin, platelet aggregation, adhesion, proliferation, smooth muscle cells, restenosis