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Smad-induced alterations of matrix metabolism by a myristoyl tetra peptide

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Regulation of extracellular matrix (ECM) components is essential for tissue homeostasis and function. We screened a small peptide that induces ECM protein synthesis for its usefulness in protecting keratinocytes. In this report, we demonstrate that myristoyl tetrapeptide Ala-Ala-Pro-Val (mAAPV) stimulates the expression of ECM proteins and inhibits the expression of metalloproteinases (MMPs) that degrade ECM proteins in Hs68 human fibroblast cells. In order to elucidate the underlying molecular mechanisms for the effects of mAAVP, we investigated the changes in gene expression in the presence of mAAPV using a cDNA microarray. Treatment with mAAPV resulted in decreased expression of MMP-related genes such as *MMP1*, *MMP3*, *TIMP1* and *TIMP3* and increased expression of collagen genes, including *COL1A1*, *COL1A2*, *COL3A1*, *COL5A1* and *COL6A3*. The pattern of gene expression regulated by mAAPV was very similar to that of gene expression induced by transforming growth factor (TGF)-β, indicating that the TGF-β signaling pathway is crucial for simultaneous activation of several ECM-related genes by mAAPV. We examined whether the activation of SMAD, a downstream protein of TGF-β receptor, is involved in the signal transduction pathway induced by mAAPV. The results demonstrate that mAAVP directly activates SMAD2 and induces SMAD3 to bind to DNA. In conclusion, our results demonstrate that mAAPV both enhances the expression of collagen and inhibits its degradation via production of protease inhibitors that prevent enzymatic breakdown of the ECM. The results suggest that mAAPV would be a useful ECM-protecting agent. Copyright © 2014 John Wiley & Sons, Ltd.

KEY WORDS—myristoyl tetrapeptide; transforming growth factor-β signaling; SMAD; procollagen; microarray

INTRODUCTION

Exposure of skin to stress leads to alterations in the skin through stimulation of multiple signal transduction pathways, leading to activation of various transcription factors or target genes.[1] Specifically, ultraviolet (UV) light induces the expression of metalloproteinase (MMP)-1, -3 and -9 in normal human epidermis *in vivo*.[2] Among these proteins, MMP-1, which degrades collagen, is thought to be the major contributor to aging of the skin.[3] Studies have shown that the down-regulation of transforming growth factor (TGF)-β receptor (TbR) II mRNA and protein expression leads to down-regulation of type I procollagen gene expression in human skin *in vivo*.[4,5] Oxidative stress caused by exposure to UV irradiation, ozone, hydrogen peroxide and free radicals may lead to activation of AP-1, thereby increasing expression of MMPs and resulting in collagen degradation.[6] The published data suggest that human skin aging is related to down-regulation of TGF-β-induced procollagen gene expression.

TGF-β is a multifunctional cytokine that regulates cell proliferation and differentiation, tissue remodelling and repair.[7] In the dermis, TGF-β acts as a positive growth factor, inducing the synthesis of mRNAs encoding extracellular matrix (ECM) proteins, including procollagen.[7–9] Over-expression of TbR I or II increases collagen promoter activity in fibroblasts.[10] Prolonged TGF-β1 stimulation induces mesenchymal cells to secrete collagens, decrease protease production and increase the secretion of protease inhibitors such as TIMPs and SERPINE1, also known as plasminogen activator inhibitor 1 (PAI-1).[11–15] TGF-β1 is also a key pro-fibrotic cytokine that is involved in cell signaling and other processes, including cell proliferation, differentiation, adhesion and migration, ECM deposition, apoptosis, embryonic development and immune responses.[12,13,16–20] TGF-β1 exerts its effects through the TGF-β1/SMAD3 signal transduction pathway operating between cell surface receptors for TGF-β1 and the gene regulatory machinery in the nucleus.[21,22] In humans, there are eight members of the SMAD family of transcription factors.

Upon activation, TGF-β binds to serine/threonine kinase receptors. Following ligand activation, signaling from TbRI to the nucleus occurs predominantly as a result of

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phosphorylation of cytoplasmic mediators belonging to the SMAD family.[8,9,23] Type I receptors specifically recognize and phosphorylate ligand-specific receptor-activated SMADs, particularly SMAD2 and SMAD3 downstream of TGF-β. Upon phosphorylation by TbRI, SMAD3 forms a heteromeric complex with co-SMADs, such as SMAD4. SMAD3/SMAD4 complexes are then translocated into the nucleus, where they may function as transcription factors, binding DNA either directly or in association with other DNA-binding proteins.[8,9,23–25]

In this study, the myristoyl tetrapeptide AAPV (mAAPV) promoted the expression of ECM proteins such as procollagen I and fibronectin while suppressing the expression of MMP-1 and MMP-3. We investigated changes in gene expression in cells cultured in the presence of mAAPV using a microarray analysis in order to identify targets and molecular pathways associated with mAAPV. The pattern of gene expression regulated by mAAPV was very similar to the pattern of gene expression induced by transforming growth factor (TGF)-β, indicating that the TGF-β signaling pathway is important for simultaneous activation of several ECM-related genes by mAAPV. The foreskin fibroblast cell line, Hs68, is a derivative of primary cells that respond to TGF-β. Therefore, using the Hs68 cell line, we investigated the early events involved in peptide- and TGF-β-induced cellular signaling. Genome-wide identification of targets and molecular pathways associated with mAAPV enhanced our understanding of its functional roles in multiple cellular processes correlated with the TGF-β1/SMAD3 pathway.

MATERIALS AND METHODS

Synthesis and myristoylation of tetrapeptide AAPV

The AAPV tetrapeptide was synthesized according to Merrifield's solid phase synthesis method.[26] Briefly, the method involves attaching the first amino acid of the chain to a solid polymer by a covalent bond, followed by the addition of succeeding amino acids one at a time in a stepwise manner until the desired sequence is assembled. Finally, the peptide is removed from the solid support. 2-Chlorotrityl chloride resin was used as the attachment polymer. The N-termini of the three amino acids composing the tetrapeptide (Ala, Pro, Val) were protected with a 9-fluorenylmethyoxy carbonyl group. After synthesis of the myristoyl tetrapeptide, the derivatives were liberated from the resin using trifluoroacetic acid.

Cell culture and drug treatments

Hs68 human skin fibroblasts were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% antibiotic mixture under standard cell culture conditions (37 °C, 5% CO_2 in a humidified incubator). Peptides were dissolved in dimethyl sulfoxide (DMSO). The non-treated control was actually the vehicle control. All data from cells treated with mAAPV tetrapeptide was compared to DMSO treated control.

Total cell lysate extraction and Western blot analysis

Cells (1×10^6) were harvested and suspended in 1 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 ng/ml of phenylmethanesulfonyl fluoride, 0.03% aprotinin and 1-μM sodium orthovanadate) and incubated on ice for 30 min. The protein content of the final extracts was estimated using a BCA protein assay kit according to the manufacturer's protocol (Bio-Rad, Richmond, CA). Total cell lysates were used for measuring the expression of procollagen I and phosphorylation of SMAD. Anti-phospho-SMAD (Cell Signaling Technology, Beverly, MA), anti-SMAD (Cell Signaling Technology) and anti-procollagen I (Cell Signaling Technology) antibodies were used. Cell extract proteins were separated using 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After transfer, the membrane was blocked by incubation with 5% (*w/v*) non-fat dry milk in Tris-buffered saline (TBS)–0.1% Tween-20 (TBST) at 4 °C for 1 h and then incubated with the appropriate antibody overnight at 4 °C. After washing with TBST, the membrane was incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). After 60 min of incubation at room temperature, the membrane was washed three times with TBST, and the blot was developed using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Anti-tubulin (Santa Cruz Biotechnology) or polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) antibodies were used for normalization. Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA) for detection of secreted MMP-1

Cells (1×10^6) were cultured in a 6-well plate for 24 h, after which the medium was replaced with serum-free medium containing peptide or vehicle. After 48 h of incubation, the medium was removed and transferred into wells coated with anti-MMP1 or anti-fibronectin antibody (Merck, Whitehouse Station, NJ) in a 96-well plate. After 2 h of incubation at 37 °C in the dark, the plate was washed with phosphate-buffered saline (PBS)-0.05% Tween-20 and treated with horseradish peroxidase (HRP)-conjugated secondary antibody. After 1 h of incubation, the plate was washed with PBS-0.05% Tween-20, and the reaction was quenched with 3,3′,5,5′-tetramethylbenzidine and 1 M sulfuric acid. Absorbance of the wells was measured at 450/595 nm using a microplate reader (Tescan, St. Petersburg, Russia). ELISA data were normalized to cell numbers.

RNA isolation and polymerase chain reaction (PCR)

Hs68 cells (1×10^6) were cultured in a 6-well plate for 24 h, after which the medium was replaced with serum-free

medium containing peptide or vehicle. After 6 h (TbRI), 24 h (procollagen I) or 48 h (MMP-1 and MMP-3) of incubation, total RNA was extracted by homogenization of cells in Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (50 μg) was reverse-transcribed into double-stranded cDNA using oligo (dT) primers (iNtRON, Gyeonggi-Do, Republic of Korea) and reverse transcriptase (iNtRON). For PCR, 40 ng of cDNA was mixed with 0.5 μg of each primer in PCR premix (Bioneer, Daejeon, Republic of Korea). PCR was performed using a CFX 96 system (Bio-Rad) using the following thermal profile: (95 °C, 30 s; 60 °C, 30 s; 72 °C, 120 s) × 30 cycles. All amplifications were run in triplicate. Real-time quantitative PCR (qPCR) assay was performed on a real-time PCR system (Mx3000P, Stratagene, Tokyo, Japan) using SYBR PreMix ExTaq (Takara Bio, Shiga, Japan) or Premix ExTaq (for the use of TaqMan probes; Takara Bio) in accordance with the manufacturers' protocols. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen) was carried out with DNase-treated total RNA using oligo 2 primers. GAPDH was selected as the endogenous control. Primer pairs were designed for procollagen I, MMP-1, MMP-3, TbR1 and GAPDH. The information for each probe was obtained from the Stanford Online Universal Resource for Clones and expressed sequence tags (ESTs) (SOURCE; http://www.source.stanford.edu), which compiles information from several publicly accessible databases, including UniGene, dbEST, Swiss-Prot, GeneMap99, RHdb, GeneCards and LocusLink. The sequences of primers (Bionics, Republic of Korea) used in the PCR analyses were as follows: GAPDH: 5′-CAA AAG GGT CAT CAT CTC TG-3′ and 5′-CCT GCT TCA CCA CCT TCT TG-3′; Procollagen I: 5′-CTC GAG GTG GAC ACC ACC CT-3′ and 5′-CAG CTG GAT GGC CAC ATC GG-3′; MMP-1: 5′-ATT CTA CTG ATA TCG GGG CTT TGA-3′ and 5′-ATG TCC TTG GGG TAT CCG TGT-3′; MMP-3: 5′-CTT TCC TGG CAT CCC GAA GTG-3′ and 5′-GGA GGT CCA TAG AGG GAC TG-3′; TGF-βR1: 5′-TTA AAA GGC GCA ACC AAG AAC-3′ and 5′-GTG GTG ATG AGC CCT TCG AT-3′.

Microarray analysis

Hs68 cells (1×10^6) were cultured in a 6-well plate for 24 h, after which the medium was replaced with serum-free medium containing peptide or vehicle. After 24 h of incubation, total RNA was extracted from the cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and the extracted RNA was treated with DNase I (RNase-free DNase kit, Qiagen, Hilden, Germany) for 15 min at room temperature to remove genomic DNA. Each 200 ng of RNA was labeled with Cy3 and Cy5 using a Low Input Quick Amp labeling kit (Agilent Technologies, Palo Alto, CA). Gene expression was analysed using an Agilent SurePrint G3 Human GE 8 × 60 K array (Agilent Technologies). Hybridization was performed under a Gasket 8-plex slide (Agilent Technologies) for 17 h at 65 °C. The resulting hybridization intensity data were converted into presence/absence calls for each gene, and changes in the level of gene expression between experiments were assessed by comparison analysis. The data were further analysed using Gene Spring (Silicon Genetics, Redwood City, CA, USA) to extract the significant genes.

Isolation of nuclear extracts and electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Fremont, CA) according to the manufacturer's instructions. Hs68 cells were treated with lysis buffer (Panomics) for 10 min at room temperature with gentle mixing and then removed from the culture dish by scraping. After centrifugation, the pellet was resuspended in extraction buffer (Panomics) and incubated on ice for 1 h. Nuclear extracts were used in the EMSA. EMSA was performed using a gel-shift kit according to the manufacturer's protocol (Panomics). Nuclear extract (4 μg) was incubated for 5 min at room temperature with poly dI-dC (1 μg), 5× binding buffer and protease inhibitors. Next, biotin-labeled SMAD binding probe (5′-AGTATGTCTAGACTGA-3′) was added and the mixture was incubated for 30 min at 15 °C in a thermal cycler. The samples were analysed by non-denaturing 6% PAGE in 0.5× Tris/borate/ethylenediamine tetraacetic acid buffer at 120 V for 55 min. Separated proteins were transferred onto a Biodyne B nylon membrane (Pall Life Science, Port Washington, NY) for 1 h at 300 mA. Target proteins were visualized by autoradiography using streptavidin-HRP and substrate solution. Western analysis was performed for the presence of histone H2A, a nuclear marker and the absence of cytoplasmic β actin to confirm the purity of the nuclear extract and validate gel shift results.

Quantitative analysis

Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad). In all experiments, statistical significance was calculated for the data from three or four independent experiments using one-way analysis of variance or the Student *t*-test (Sigma Plot, LaJolla, CA). Error bars in figures represent the standard deviations of the mean.

RESULTS

mAAPV induces procollagen synthesis in cultured human fibroblast Hs68 cells

In this study, the Hs68 cells were incubated with mAAPV for 48 h and the cell lysates were collected and analysed using Western blot. The results show that collagen I expression was induced by mAAPV in Hs68 cells. Incubation of cells with 0.01, 0.04 and 0.2-μM mAAPV for 24 h induced 1.4, 1.9 and 2.1-fold increase in collagen I expression compared with the non-treated control in a concentration-dependent manner (Figure 1A). Next, to determine whether peptide-induced collagen I expression occurs through *de novo* synthesis, a transcriptional inhibitor (actinomycin D) and a translational inhibitor (cyclohexamide) were used. We found that pretreatment of Hs68 fibroblasts with either

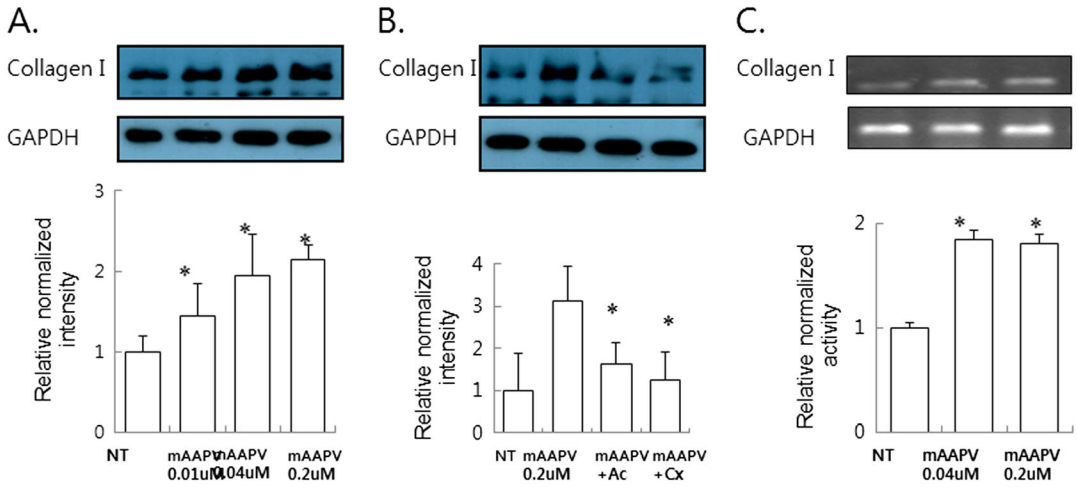

Figure 1. Collagen I protein expression induced by mAAPV in Hs68 fibroblasts. (A) Cells were incubated with vehicle, 0.01-μM, 0.04-μM and 0.2-μM mAAPV for 24 h. Cells were lysed and then immunoblotted with antibodies specific for collagen I or GAPDH. Equal loading in each lane is shown by similar intensities of GAPDH. Quantitative analysis was performed using densitometry, and results are expressed as activity relative to that of the untreated control (NT). Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant differences between treatment and untreated control. * $p < 0.05$, Student's *t*-test compared with the mAAPV-treated group. (B) Cells were pretreated for 30 min with an equivalent vehicle control (DMSO), 1-μM actinomycin D (ActD), or 1-μM cycloheximide (CHX) and then stimulated with mAAPV (0.2 μM) for another 24 h. Cells were lysed and then immunoblotted with antibodies specific for collagen I or GAPDH. The values represent results from three independent experiments, which are presented as the mean ± SD. * $p < 0.05$, compared with the mAAPV-treated group. (C) Cells were treated with 0.04 and 0.2-μM mAAPV for another 24 h. Total RNA was extracted and PCR was carried out as described in the Materials and Methods section. The values represent results from three independent experiments

1-μM actinomycin D or 1-μM cyclohexamide almost completely inhibited peptide-induced collagen I protein expression (Figure 1B). This result was supported by the finding that mAAPV induced collagen I mRNA expression, as determined by PCR (Figure 1C). Altogether, these results suggest that increased collagen I expression in Hs68 cells in response to mAAPV occurs through *de novo* synthesis.

mAAPV inhibits MMP-1 expression in cultured human fibroblast Hs68 cells

To investigate the expression of MMP protein, Hs68 cells were incubated with mAAPV (0.04 μM and 0.2 μM) for 48 h. The secreted proteins were analysed using ELISA. With respect to MMP-1 expression, we found that mAAPV inhibits the expression of MMP-1 in human fibroblast Hs68 cells. Treatment with mAAPV significantly inhibited the expression of MMP-1, reducing the level to 78 ± 11.2 and 70 ± 7.7% of the control at 0.04 and 0.2-μM mAAPV, respectively (Figure 2A). The results of real time PCR analyses demonstrated that mAAPV has an inhibitory effect on MMP-1 gene expression (Figure 2B).

The TGF-β signaling pathway is involved in mAAPV-induced collagen I expression

The results of cDNA microarray analysis indicated that the expression of 716 genes was significantly up-regulated (>2-fold increase in expression relative to the control) in Hs68 cells treated for 24 h with mAAPV. The expression

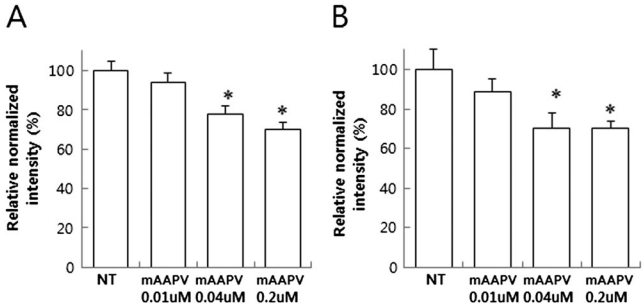

Figure 2. Effect of mAAPV on expression of MMP-1 in Hs68 fibroblasts. (A) After cells were treated with vehicle, 0.01, 0.04 and 0.2-μM mAAPV for 48 h, the medium was transferred to wells of plates coated with anti-MMP-1 antibody. After treatment with HRP-conjugated secondary antibody, absorbance was measured using a microplate reader. (B) Cells were treated with vehicle, 0.01, 0.04 and 0.2-μM mAAPV for 24 h. Total RNA was extracted and real time PCR was carried out as described in the Materials and methods section. In all cases, quantitative analysis was performed using densitometry, and results are expressed as activity relative to that of the untreated control (NT). Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant differences between treatment and untreated control. * $p < 0.05$, Student's *t*-test compared to the control without mAAPV

of 1042 genes was significantly down-regulated (>0.5-fold decrease in expression relative to the control) following 24-h treatment with mAAPV.

A number of secreted matrix protein and cytoskeletal protein genes were highly up-regulated, including cartilage oligomeric matrix protein, annexin, matrix Gla protein, elastin, asporin, ectodermal-neural cortex 1, fibronectin 1 and filamin C. As shown in Table 1, treatment with mAAPV for 24 h resulted in decreased expression of MMP-related genes, such as *MMP1*, *MMP3*, *TIMP1* and *TIMP3* and increased expression of collagen genes, including *COL1A1*, *COL1A2*, *COL3A1*, *COL5A1* and *COL6A3*. It has been known that up-regulation of procollagen I, fibronectin and TbRI gene expression is related with TGF-β activation. Isoforms of TGF-β induce the expression of ECM proteins in mesenchymal cells and stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM.[15] To examine whether TGF-β activation is involved in the signal transduction pathway leading to collagen I expression induced by mAAPV, we performed cDNA microarray analysis and compared with the results. The pattern of gene regulation was comparable to the change induced by TGF-β in Hs68 cell line. Most notably, these results indicate that TGF-β signaling plays a crucial role in the simultaneous effect mAAPV exerts on the expression of several fibrosis-related genes, such as those encoding collagen and MMPs. As shown in Figure 3 and Table 1, the gene expression microarray results for TGF-β and mAAPV were consistent with each other.

A total of 853 of the 938 genes that were significantly up-regulated by TGF-β were also up-regulated by mAAPV.

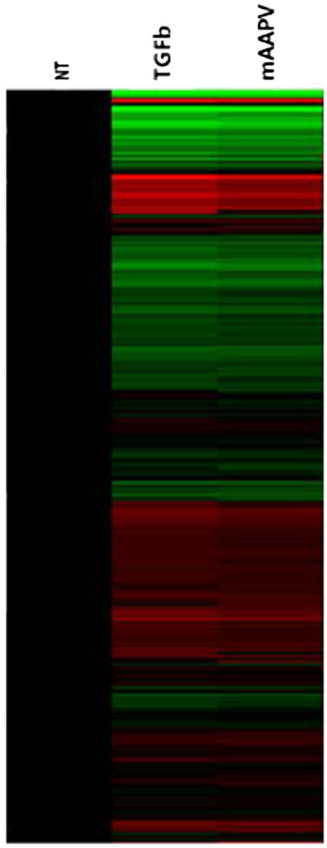

Figure 3. Expression profiles for gene clusters induced by TGF-β and mAAPV

Table 1. List of genes classified as ECM-related protein gene clusters

	Description	Gene symbol	TGFβ/NT intensity ratio	mAAPV/NT intensity ratio
NM_000088	Collagen, type I, alpha 1 (COL1A1), mRNA	COL1A1	2.6	2.5
NM_000089	Collagen, type I, alpha 2 (COL1A2), mRNA	COL1A2	1.6	1.7
NM_000090	Collagen, type III, alpha 1 (COL3A1), mRNA	COL3A1	1.8	1.8
NM_000093	Collagen, type V, alpha 1 (COL5A1), mRNA	COL5A1	6.5	6.2
NM_004369	Collagen, type VI, alpha 3 (COL6A3), transcript variant 1	COL6A3	3.2	2.7
NM_182943	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2)	PLOD2	3.3	3.1
NM_000501	Elastin (ELN), transcript variant 1, mRNA	ELN	3.3	2.5
NM_054034	Fibronectin 1 (FN1), transcript variant 7, mRNA	FN1	11.4	10.7
NM_178232	Hyaluronan and proteoglycan link protein 3 (HAPLN3), mRNA	HAPLN3	3.1	2.8
NM_005328	Hyaluronan synthase 2 (HAS2)	HAS2	2.5	1.8
NM_002210	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	ITGAV	1.9	1.8
NM_133376	Integrin, beta 1 (fibronectin receptor, beta polypeptide)	ITGB1	1.8	1.8
NM_003254	TIMP metallopeptidase inhibitor 1 (TIMP1)	TIMP1	1.4	1.7
NM_000362	TIMP metallopeptidase inhibitor 3 (TIMP3)	TIMP3	2.7	2.1
NM_000660	Transforming growth factor, beta 1 (TGFB1)	TGFB1	3.2	2.7
NM_003238	Transforming growth factor, beta 2 (TGFB2), transcript variant 2	TGFB2	5.2	4.7
NM_002421	Matrix metallopeptidase 1 (interstitial collagenase) (MMP1)	MMP1	0.0	0.1
NM_002422	Matrix metallopeptidase 3 (stromelysin 1, progelatinase) (MMP3)	MMP3	0.2	0.2
NM_001032280	Transcription factor AP-2 alpha	TFAP2A	0.5	0.6
NM_003222	Transcription factor AP-2 gamma	TFAP2C	0.4	0.5

Treatment of Hs68 cells with TGF-β or mAAPV for 24 h resulted in significant up-regulation (>2-fold increase relative to the control) of the expression of 938 and 716 genes, respectively, as determined by cDNA microarray analysis.

And, 669 of the 716 genes that were significantly up-regulated by mAAPV were also up-regulated by TGF-β. A total of 1127 of the 1203 genes that were significantly down-regulated by TGF-β were also down-regulated by mAAPV. Over 93% of the genes examined were commonly regulated by TGF-β and mAAPV. The examined genes were also classified according to function. Significantly up-regulated genes involved in mediating cell adhesion are listed in Table 2. Genes that were highly up-regulated (i.e. >tenfold increase in expression) by either TGF-β or mAAPV are listed in Table 3.

mAAPV activates the SMAD signaling pathway

Following ligand activation, signaling between TGF-β receptor1 and the nucleus occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the SMAD family.[8,9,23] Downstream of the TGF-β receptor1, SMAD2 and SMAD3 are specifically phosphorylated. To examine whether SMAD activation is involved in the signal transduction pathway leading to collagen I expression induced by mAAPV, phosphorylation of SMAD was investigated using Western blot analysis. Figure 4 shows the phosphorylation of SMAD2 in Hs68 cells treated with mAAPV for 10 min, 30 min and 1 h. Phosphorylation of SMAD2 increased beginning at 30 min after treatment with either 0.04 or 0.2-μM mAAPV and increased further until 1 h.

Binding of SMAD transcription factor to DNA is involved in mAAPV-induced signaling

Upon phosphorylation by TbRI, SMAD2 or SMAD3 form heteromeric complexes with various co-SMADs, such as SMAD4. SMAD3/SMAD4 complexes are then translocated into the nucleus via a mechanism involving the cytoplasmic protein importin.[24,25] The SMAD complexes may then function as transcription factors, binding to DNA either directly or in association with other DNA-binding proteins.[8,9,23]

The direct binding of a transcription factor to DNA is an important step with respect to the factor's activity. Therefore, we performed EMSAs to investigate whether peptide treatment affects the binding of SMAD complexes to DNA. Hs68 cells were treated with mAAPV for 1 h, and nuclear extracts were prepared and incubated with SMAD binding element (SBE) consensus DNA *in vitro*. When excess unlabeled SBE was competed with labeled SBE, the bands for which mobility was retarded disappeared (Figure 5, lane 4). This result suggests that the shifted DNA/protein complex is specific for SMAD complexes binding to the consensus DNA. As shown in Figure 6, treatment with 0.04-μM and 0.2-μM mAAPV enhanced the formation of SMAD/SBE complexes *in vitro* by approximately 1.2- and 1.9-fold, respectively. This result was consistent with the notion that TGF-β signaling through the SMAD pathway plays a role in ECM gene expression and regulation, leading to ECM remodelling and wound healing.

Table 2. Classification of genes according to function

Genbank accession	Description	Gene symbol	TGFβ/NT intensity ratio	mAAPV/NT intensity ratio
NM_000095	Cartilage oligomeric matrix protein (COMP), mRNA	COMP	590.1	585.7
NM_004750	Cytokine receptor-like factor 1 (CRLF1), mRNA	CRLF1	90.0	76.5
NM_016931	NADPH oxidase 4 (NOX4), transcript variant 1	NOX4	33.0	18.5
NM_001945	Heparin-binding EGF-like growth factor (HBEGF)	HBEGF	30.4	16.8
NM_020182	Prostate transmembrane protein, androgen induced 1	PMEPA1	29.6	13.2
NM_004455	Exostoses (multiple)-like 1	EXTL1	27.5	17.8
NM_031866	Frizzled family receptor 8 (FZD8)	FZD8	23.9	16.8
NM_000399	Early growth response 2 (EGR2)	EGR2	22.7	20.7
NR_024607	Hypothetical protein MGC16121 (MGC16121), non-coding RNA	MGC16121	18.6	10.6
NM_001630	Annexin A8-like 2 (ANXA8L2)	ANXA8L2	17.7	10.3
NM_001190839	Matrix Gla protein (MGP), transcript variant 1	MGP	17.7	18.1
NM_000501	Elastin (ELN), transcript variant 1, mRNA	ELN	16.5	10.0
NM_000428	Latent transforming growth factor beta binding protein 2	LTBP2	16.5	12.7
NM_002899	Retinol binding protein 1, cellular (RBP1)	RBP1	16.4	14.4
NM_020182	Prostate transmembrane protein, androgen induced 1	PMEPA1	15.7	7.9
NM_000900	Matrix Gla protein (MGP), transcript variant 2	MGP	15.0	16.4
NM_000029	Angiotensinogen (serpin peptidase inhibitor, clade A)	AGT	14.9	13.1
NM_017680	Asporin (ASPN), transcript variant 1, mRNA [NM_017680]	ASPN	14.6	10.6
NM_003612	Semaphorin 7A, GPI membrane anchor	SEMA7A	13.3	11.5
NM_003633	Ectodermal-neural cortex 1	ENC1	12.9	7.4
NM_001905	CTP synthase (CTPS)	CTPS	12.5	7.1
NM_000641	Interleukin 11 (IL11)	IL11	11.6	6.0
NM_054034	Fibronectin 1 (FN1), transcript variant 7	FN1	11.4	10.7
NM_002517	Neuronal PAS domain protein 1	NPAS1	11.4	7.5
NM_001030059	Phosphatidic acid phosphatase type 2 domain containing 1A	PPAPDC1A	11.4	8.5
NM_020692	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like, transcript variant 2,	GALNTL1	11.2	7.2
NM_001458	Filamin C, gamma (FLNC), transcript variant 1, mRNA	FLNC	10.7	8.1

Table 3. Genes highly up-regulated (>10-fold increase in expression relative to the control) by either TGF-β or mAAPV

Accession no.	Definition	Accession no.	Definition	Accession no.	Definition
NM_000095	Cartilage oligomeric matrix protein	NM_001004439	Integrin, alpha 11	NM_152890	Collagen, type XXIV, alpha 1
NM_006475	Periostin, osteoblast specific factor	NM_001144058	Neurotrimin	NM_003247	Thrombospondin 2
NM_001190839	Matrix Gla protein	NM_002587	Protocadherin 1	NM_001393	Extracellular matrix protein 2, female organ and adipocyte specific
NM_000900	Matrix Gla protein	NM_005202	Collagen, type VIII, alpha 2	NM_003247	Thrombospondin 2
NM_000029	Angiotensinogen	NM_001855	Collagen, type XV, alpha 1	NM_002473	Myosin, heavy chain 9, non-muscle
NM_054034	Fibronectin 1	NM_000094	Collagen, type VII, alpha 1	NM_001937	Dermatopontin
NM_022131	Calsyntenin 2	NM_021641	ADAM metallopeptidase domain 12	NM_181501	Integrin, alpha 1
NM_001901	Connective tissue growth factor	NM_001005388	Neurofascin	NM_005086	Sarcospan
NM_001040058	Secreted phosphoprotein 1	NM_182966	Neural precursor cell expressed, developmentally down-regulated 9	NM_003259	Intercellular adhesion molecule 5, telencephalin
NM_000093	Collagen, type V, alpha 1	NM_002821	PTK7 protein tyrosine kinase 7	NM_004040	Ras homolog gene family, member B
NM_004385	Versican	NM_016533	Ninjurin 2	NM_024767	Deleted in liver cancer 1
NM_181847	Adhesion molecule with Ig-likedomain 2	NM_021641	ADAM metallopeptidase domain 12	NM_001102	Actinin, alpha 1
NM_003474	ADAM metallopeptidase domain 12	NM_004369	Collagen, type VI, alpha 3	NM_002318	Lysyl oxidase-like 2
NM_002404	Microfibrillar-associated protein 4	NM_201266	Neuropilin 2	NM_002473	Myosin, heavy chain 9, non-muscle
NM_004791	Integrin, beta-like 1 (with EGF-like repeat domains)	NM_002206	Integrin, alpha 7		
NM_004791	Integrin, beta-like 1 (with EGF-like repeat domains)	NM_005202	Collagen, type VIII, alpha 2		
NM_001792	Cadherin 2, type 1, N-cadherin	NM_178232	Hyaluronan and proteoglycan link protein 3		
NM_016269	Lymphoid enhancer-binding factor 1	NM_006500	Melanoma cell adhesion molecule		
NM_001792	Cadherin 2, type 1, N-cadherin	NM_004796	Neurexin 3		
NM_004598	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1	NM_182643	Deleted in liver cancer 1		

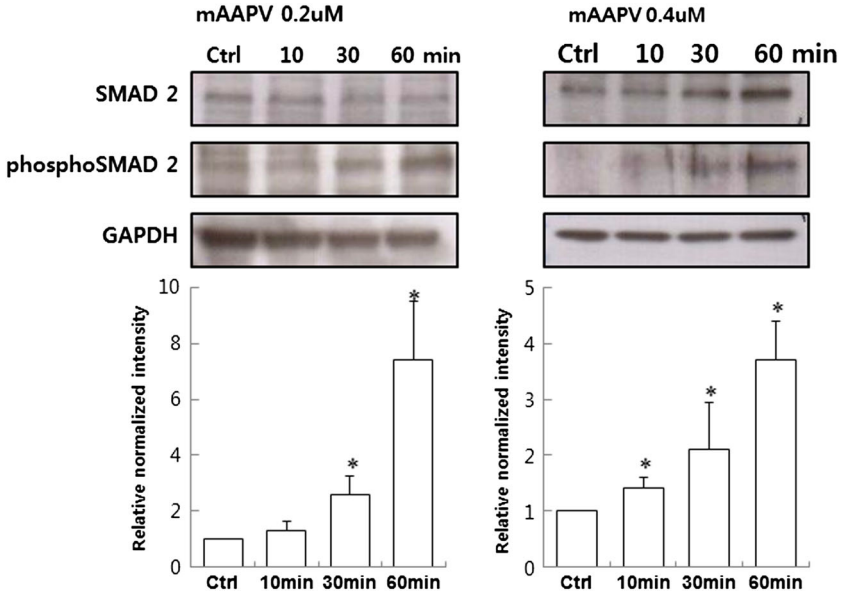

Figure 4. Effect of mAAPV on phosphorylation of SMAD2 in Hs68 cells. Cells were incubated with 0.04 and 0.2-μM mAAPV for 10, 30 or 60 min. Levels of total and phosphorylated SMAD were measured by Western blot analysis. Cells were lysed and then immunoblotted with antibodies specific for SMAD2, phosphoSMAD2 and GAPDH. Equal loading in each lane is shown by similar intensities of GAPDH. Quantitative analysis was performed using densitometry, and results are expressed as activity relative to that of the untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant differences between treatment and untreated control. *$p < 0.05$, Student's *t*-test compared with the mAAPV-treated group

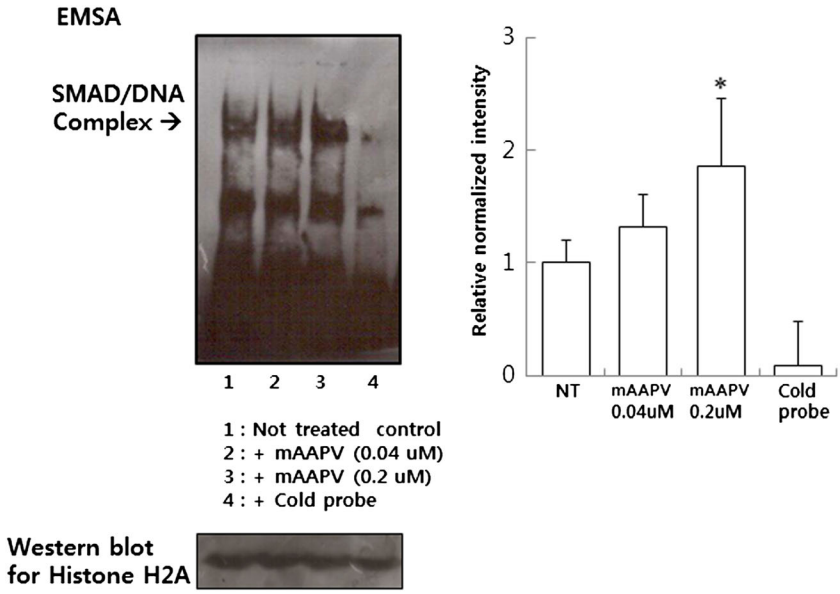

Figure 5. Effect of mAAPV on the binding of SMAD complexes to DNA. Hs68 cells were treated with 0.04 or 0.2-μM mAAPV for 3 h, after which nuclear extracts were prepared. EMSA was performed with 4 mg of nuclear extract with or without mAAPV. Lane 4 shows that a tenfold excess of unlabeled Tcf-binding oligonucleotide used as a competitor inhibits the binding of SMAD to oligonucleotide containing the SMAD-binding region. Shifted bands indicate binding to a labeled SMAD-binding region. Quantitative analysis was performed using densitometry, and results are expressed as activity relative to that of the untreated control (NT). Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. *$p < 0.05$, Student's *t*-test compared with the mAAPV-treated group

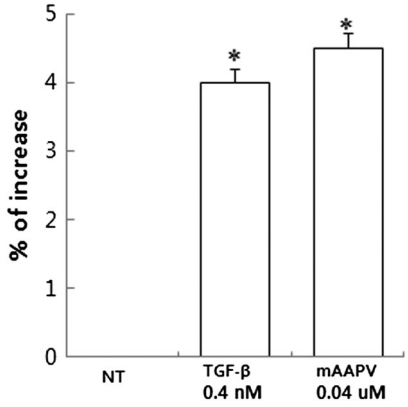

Figure 6. Effect of mAAPV on expression of fibronectin in Hs68 fibroblasts. After cells were treated with vehicle, 0.4 nM TGF-β, or 0.04-μM mAAPV for 48 h, the medium was transferred to wells of plates coated with anti-fibronectin antibody. After treatment with HRP-conjugated secondary antibody, absorbance was measured using a microplate reader. Quantitative analysis was performed using densitometry, and results are expressed as activity relative to that of the untreated control (NT). Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. * $p < 0.05$, Student's *t*-test compared with the control without mAAPV or TGF-β stimulation

mAAPV induces fibronectin and TbRI synthesis in cultured human fibroblast cells

To further elucidate the effect of mAAPV related with TGF-β pathway, the induction of fibronectin and TbRI that are known by TGF-β was investigated. mAAPV induced expression of fibronectin and TbRI in Hs68 cells. Incubating cells with mAAPV (0.04 μM) for 48 h resulted in a 4.5% increase in the expression of fibronectin, as determined by ELISA (Figure 6). With respect to TbRI gene expression, incubating cells with mAAPV (0.04 μM) for 48 h resulted in a 2.2 fold increase compared to the control, as determined by PCR (Figure 7).

DISCUSSION

In the present study, mAAPV plays roles in inducing the production of ECM proteins and in protecting ECM components from degradation. The function of the peptide is associated with the TGF-β/SMAD signaling pathway.

The TGF-β family of growth factors controls an immense number of cellular responses in development and homeostasis in most human tissues.[27] TGF-β exerts its effects on cell proliferation, differentiation and migration in part through its capacity to modulate the deposition of ECM components. Some growth factors, such as epidermal growth factor, have been used clinically to enhance wound healing in a variety of tissues.[28] A small 11-residue peptide (WCKPKPKPRCH) was suggested as a potential biomaterial for the development of a novel wound-healing agent by Tang *et al.*[28] This peptide appears to promote the release of TGF-β and IL-6. However, our myristoyl tetrapeptide increased the downstream signaling of TbR.

Because SMAD activation and nuclear translocation occur within minutes, SMAD/DNA complexes can be observed as soon as 10 min after the addition of TGF-β to

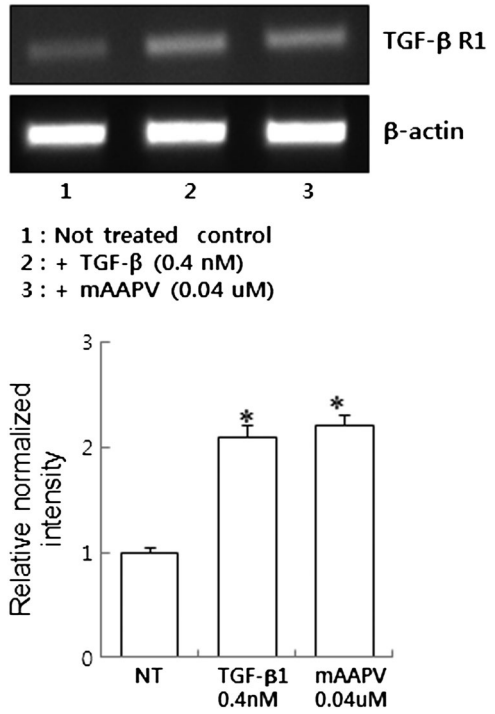

Figure 7. Effect of mAAPV on expression of TGF-β receptor I in Hs68 fibroblasts. Cells were treated with TGF-β or 0.04-μM mAAPV for another 24 h. Total RNA was extracted and RT-PCR was carried out as described in the Materials and Methods section. Quantitative analysis was performed using densitometry, and results are expressed as activity relative to that of the untreated control (NT). Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. * $p < 0.05$, Student's *t*-test compared with the control without mAAPV or TGF-β stimulation

fibroblast culture medium.[29] In our experiment, mAAPV induced phosphorylation of SMAD2 within 10 min as well. Moreover, treatment with mAAPV resulted in the formation of SMAD/DNA complexes. TGF-β and mAAPV regulated many of the same genes in Hs68 cells, indicating that the observed activation of several fibrosis-related genes (e.g. collagen) by mAAPV can be ascribed to activation of the TGF-β signaling pathway.

cDNA microarray analysis studies by Verricchia[30] suggested that TGF-β has an effect on fibroblast ECM-related gene expression based. However, they focused only on early time points in order to determine which genes are activated rapidly by TGF-β SMAD. The gene targets identified to date that are downstream of TGF-β include type VII collagen (*COL7A1*), plasminogen activator inhibitor-1 (*PAI1*), cyclin-dependent kinase inhibitor p21, JunB, *COL1A2*, c-Jun, immunoglobulin germline C, *SMAD7*, human germline IgA, *PDGF*, integrin, apoCIII, cyclin-dependent kinase inhibitor p15 and *COL1A1*, *COL3A1*, *COL5A2*, *COL6A1*, *COL6A3* and *TIMP1*. All the above genes were suggested to be responsive at each of either time points tested (30, 60, 120 and 240 min).[29–42]

The genes modulated by TGF-β have been classified into clusters based upon temporal profiles of activation.[28] The results of our experiments suggest that some of the previously reported immediate-early TGF-β target genes remain activated in Hs68 cells for as long as 24 h after treatment with TGF-β or our peptide. These genes could be classified into the cell matrix interaction or cell-cell interaction categories, and included genes such as integrin alpha-3, -4, -5, integrin β-5, plasminogen activator inhibitor-1, *IGFBP3*, versican (isoforms V1, V2, V3), *TIMP1*, *TIMP3*, *MMP2*, *MMP11*, *COL1A2*, *COL3A1*, *COL6A1*, *COL6A3*, *COL16A1*, *COL8A1*, fibronectin, notch group protein and desmoplakin 1.

In addition to the previously reported immediate-early TGF-β target genes, here we identified and suggested secondary activated genes that may be involved in protein/transcription factor neosynthesis. Our results were obtained from chip analyses performed at 24 h. The putative secondary activated ECM genes included *COL15A1*, *COL13A1*, *COL7A1*, *COL11A2*, *COL24A1*, *COL8A2*, *COL6A3*, actinin (*ACTN1*) and heparan sulfate proteoglycan (*HSPG2*).

In conclusion, mAAPV represents a possible candidate for ECM protecting agent by playing roles in inducing the production of ECM proteins and in protecting ECM components from degradation. The function of the peptide is associated with the TGF-β/SMAD signaling pathway.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGEMENTS

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