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Recombinant Vascular Basement Membrane Derived Multifunctional Peptide Blocks Endothelial Cell Angiogenesis and Neovascularization

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ABSTRACT

Angiogenesis is an innovative target in the therapy of cancer and other diseases, but the effects of anti-angiogenic drugs have been rather modest in clinical trials. We have developed a small peptide, recombinant vascular basement membrane derived multifunctional peptide (rVBMDMP), which significantly inhibits endothelial cells in vitro. Here we test the mechanisms of rVBMDMP in angiogenesis balance in assays of tubule formation, colony formation, and apoptosis in HUVE-12 endothelial cells. We also analyzed the differential expression of phosphorylation proteins and related genes in a protein phosphorylation chip and extracellular matrix adhesion molecule cDNA microarray, and validated changes with Western blot or real-time quantitative PCR, respectively. rVBMDMP dose-dependently inhibited colony formation, induced apoptosis, and inhibited in vitro tubule formation. rVBMDMP increased the phosphorylation of 88 signal proteins, including caspase-3, death receptor 3, 4, and 5, and integrin αV, β1, and β3, and down-regulated 41 signal proteins, including EGFR, pEGFR, VEGFR-1, and survivin versus control. rVBMDMP upregulated 14 genes, including collagen 4, 7, and 27, and down-regulated 21 genes, including integrin αVβ3, MMP10, and MMP12. Our study suggests that rVBMDMP inhibits angiogenesis and may be a viable drug candidate in anti-angiogenesis and anticancer therapies. J. Cell. Biochem. 111: 453–460, 2010.

KEY WORDS: RECOMBINANT VASCULAR BASEMENT MEMBRANE DERIVED MULTIFUNCTIONAL PEPTIDE; ANGIOGENESIS INHIBITORS; ENDOTHELIAL CELL; NEOVASCULARIZATION

Angiogenesis, the formation of new capillaries from pre-existing capillaries, is a complex process that involves extracellular matrix remodeling, endothelia cell (EC) migration, proliferation, and the functional maturation of new ECs into mature blood vessels [Folkman, 1995; Senger et al., 1997]. Angiogenesis is important in embryonic development, wound healing, and rheumatoid arthritis, as well as in tumor growth and metastasis [Carmeliet and Jain, 2000]. Manipulating angiogenesis may therefore be a potential therapeutic target [Bisacchi et al., 2003; Pralhad et al., 2003]. Neovascularization depends on the balance between circulating endogenous pro-angiogenic factors (VEGF, FGF, PDGF, etc.) and endogenous angiogenesis inhibitors (anti-angiogenic peptides generated from the extracellular matrix (ECM) by proteases) [Auerbach and Auerbach, 1994]. Angiogenesis inhibitors include angiostatin [O'Reilly et al., 1994], endostatin [O'Reilly et al., 1997], canstatin [Kamphaus et al., 2000], arrestin [Colorado et al., 2000], and tumstatin [Hamano and Kalluri, 2005], with tumstatin being of particular interest [Maeshima et al., 2002].

Tumstatin, a 28 kDa NC1 domain fragment of type IV collagen α3 chain, possess two active regions. The region consisting of amino acids 74–98 has anti-angiogenic properties, and amino acids 197–215 has anti-tumor activity [Shahan et al., 1999; Maeshima et al., 2000a,b]. Tumstatin can inhibit the proliferation of endothelial cells and induce apoptosis in a αVβ3 integrin-dependent

Chengkun Wang and Jianguo Cao contributed equally to this work.

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manner [Maeshima et al., 2002]. However, tumstatin does not show a direct anti-tumor effect because its anti-tumor fragment (197–215 aa) is covered by another peptide [Maeshima et al., 2000a]. To target both proliferating tumor cells and endothelial cells, we have constructed a 55-aa fusion protein consisting of a human IgG3 upper hinge region and the above two tumstatin-derived specific sequences, termed recombined vascular basement membrane derived multifunctional peptide (rVBMDMP) [Peng et al., 2003]. rVBMDMP selectively inhibits human umbilical vein endothelial cell (HUVE-12) growth in vitro [Cao et al., 2006]. However, the mechanism of rVBMDMP in endothelial cells needs to be elucidated. Thus, in this study, we explored its roles in tubule formation, colony formation, and apoptosis, and investigated the angiogenesis balance changes using a protein phosphorylation chip and cDNA microarray in HUVE-12 cells. The present findings indicate that rVBMDMP inhibits angiogenesis by shifting endothelial cell neovascularization away from angiogenesis.

MATERIALS AND METHODS

REAGENTS

rVBMDMP was produced in *Escherichia coli* JM109 using pGEX-4T-1-VBMDMP, and purified as previously described [Peng et al., 2003], with a molecular weight of 6.4 kDa. All images were taken with a Canon digital camera and developed with Kodak 400 DK-coated TMAM.

CELL CULTURE AND LABORATORY ANIMALS

HUVE-12 cells (No. CRL-2480) were obtained from the Center of China Typical Culture Collection (Wuhan, China). The cells were cultured with DMEM supplemented with 10% fetal bovine serum, 100 IU ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin in a 37°C and 5% CO_2 incubator. The same batch of HUVE-12 cells was used for parallel experiments. Female C57BL/6 mice, weighing 19–21 g with an age range of 6–8 weeks, were purchased from Beijing Vital River Laboratory Animal Technology Company. All the mice were maintained under a barrier environment with light for 12 h (7 a.m. to 7 p.m.), temperature of 25–28°C, and 45% relative humidity. Mice were fed a standard pellet laboratory diet and were provided with water ad libitum. Animal protocols were approved by the BIDMC Institutional Animal Care and Use Committee.

COLONY FORMATION ANALYSIS

About 400 cells were plated in each well containing 0.5 mL of culture medium of a 12-well culture plate and incubated with rVBMDMP. Each group contained 4 wells of dispersed cells. After 12 days of incubation, colonies were fixed with a 3:1 mixture of methanol/acetic acid and stained with crystal violet. Only colonies with more than 50 cells were counted. Two separate experiments were performed.

FLOW CYTOMETRIC DNA ANALYSIS WITH ANNEXIN V-FITC/PI STAINING

Cells were centrifuged to remove the medium, washed once with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ in *aqua dest.*), and stained with 5 μl Annexin V-FITC at room temperature for 15 min. Propidium iodide (PI, 10 μl at 20 μg ml^{-1}) was added and incubated at 4°C for 10 min and cells were analyzed by flow cytometry. Viable cells were negative for both PI and Annexin-V; apoptotic cells were positive for Annexin-V and negative for PI, whereas late apoptotic dead cells displayed both high Annexin-V and PI labeling. Necrotic cells were positive for PI and negative for Annexin-V.

DETECTION OF DNA LADDER

Cells were seeded at 5×10^2 cells mm^{-2} in a 25 cm^2 flask and cultured for 24 h. The medium was changed to DMEM containing 0.1% bovine serum albumin and 1.0 μmol L^{-1} rVBMDMP and continually cultured for 48 h. DNA was extracted with the DNA Ladder Extraction Kit (Beyotime, Haimen, China) according to the manufacturer's instructions. DNA was dissolved in TE buffer and electrophoresed in a 1.0% agarose gel containing ethidium bromide and photographed under UV light [Rosl, 1992].

TUBULE FORMATION ASSAYS IN VITRO

Matrigel (BD Bioscience, Bedford, MA) was added to a 48-well plate (150 μl) and allowed to solidify at 37°C for 30 min, followed by 2.5×10^4 HUVE-12 cells in DMEM (Invitrogen, Carlsbad, CA) without antibiotic. Cells were treated with rVBMDMP, synthetic peptides, and BSA, and all assays were performed in triplicate. The cells were incubated for 24 h and viewed using a CK 2 Olympus microscope (4× ocular, 10× objectives). The cells were then photographed using 400 DK-coated TMAM (Eastman Kodak Co.). The images were analyzed using Image-pro Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD). Quantitation was done by counting the number of branch points at both ends in ten fields, an available method that has been shown to measure endothelial cell reorganization into a capillary-like network [Guidolin et al., 2004]. The control tube numbers were defined as 100% tube formation, and the percent increase in tube formation as compared with control was calculated for each sample.

SIGNAL TRANSDUCTION ANTIBODY ARRAY ANALYSIS

Serum-starved HUVE-12 cells treated with 1 μmol L^{-1} rVBMDMP, an optimal level for inhibiting endothelial cell proliferation, for 30 min and lysed in 0.5% Triton X-100 buffer. This concentration of rVBMDMP was determined as optimal in inhibition of endothelial cell proliferation [Cao et al., 2006]. The antibody array membrane (HM3000 Signal Transduction Antibody Array; Hypromatrix, Worcester, MA) was treated in blocking buffer containing 0.01% Tween-20 followed by incubation with sample diluted in 1% dry milk/phosphate-buffered saline for 2 h at room temperature with slow shaking. After antibody filters were incubated with supernatant protein solution at room temperature for 2 h, the antibody array filter was washed with TBST and blotted with HRP-conjugated anti-phosphotyrosine monoclonal antibody for 2 h. Anti-phosphotyrosine reactivity was visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to X-ray film. The gray-scale chip scanogram was analyzed with chip image analysis software (QuantArray, Packard Biochip Technologies, Inc., USA) to correct protein signals. Immunoreactivity on the chip incubated with lysate from control-treated cells was set to 1 for each spotted

antibody. Ratios of phosphorylation >2 or <0.5 were considered significant.

EXTRACELLULAR MATRIX AND ADHESIVE MOLECULE cDNA MICROARRAY ANALYSIS

A human extracellular matrix and adhesive molecule cDNA microarray (SuperArray, MA) was used to screen gene expression (n = 128). Chip hybridization and chemoluminescence detection were performed as recommended. The exposure image of X-ray film was obtained with a desktop scanner. Integral chip data and the gray-scale scanogram were analyzed with integrated GEArray Expression Analysis Suite.

WESTERN BLOTTING AND REAL-TIME-PCR

Western blot analysis [Zhang et al., 2008], RNA extraction and RT-PCR [Lv et al., 2007; Zhang et al., 2008] were performed as previously described. PCR primer sequences are presented as Supplementary Material 1.

IMMUNOPRECIPITATION

Whole-cell lysates were prepared in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture [Roche Diagnostics, Mannheim, Germany], pH 7.5). After centrifugation (12,000*g* for 30 min at 4°C), supernatants were transferred to fresh tubes. Cell lysates (1 mg of protein each) were incubated with 1 μg rVBMDMP at 4°C for 6 h. The mixtures were precipitated using anti-rVBMDMP rabbit antiserum (HuaCheng Biotechnology, Xi'an) at 4°C overnight. Thereafter, 25 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was added, and the mixture was incubated at 4°C for 4 h. Beads were washed three times with the lysis buffer and two times with 1× phosphate-buffered

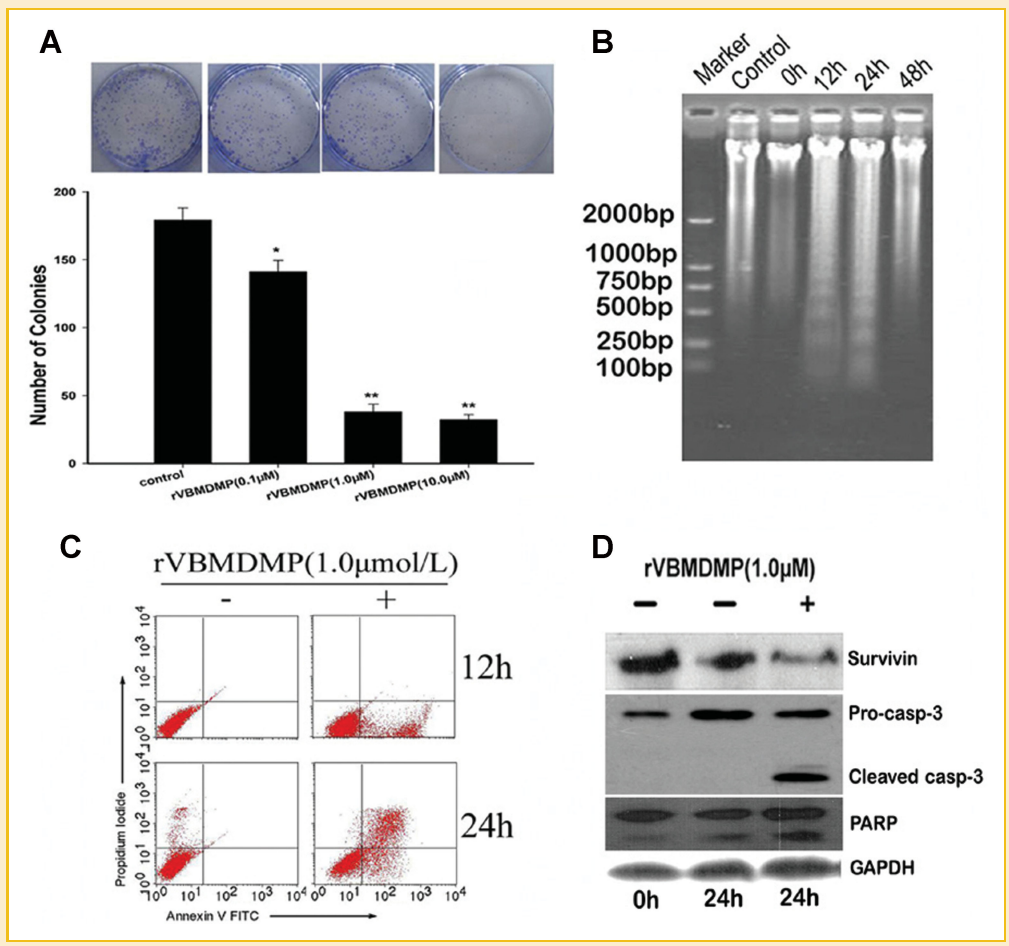

Fig. 1. rVBMDMP inhibits proliferation and induces apoptosis of HUVE-12 cell. A: Plating efficiency assay reveals changes in colony formation. Cells (4×10^2) from each of the cell pools were plated in 12-well plates. After 10 days, the colonies were stained with crystal violet, counted, and photographed. All experiments were performed at least three times and data are shown as means ± SD. The number of colonies in untreated groups served as the 100% control value. ANOVA was used to evaluate the significance (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. B: Agarose gel electrophoresis of DNA extracted from HUVE-12 cells treated with 1.0 μmol L^{-1} rVBMDMP for 0, 12, 24, 48 h. C: Effects of rVBMDMP on HUVE-12 cells in an Annexin-V-FITC/PI staining assay. Representative dot plots of Annexin-V-FITC/PI staining are shown. The lower left quadrant contains the viable (double negative) population. The lower right quadrant contains the apoptotic (Annexin V^+/PI) population. The upper left quadrant contains the necrosis (Annexin V^-/PI^+) population. The upper right quadrant contains the late apoptotic dead cells (Annexin V^+/PI^+). D: HUVE-12 cells were treated with rVBMDMP (1.0 μmol L^{-1}) and incubated 24 h. Survivin, Caspase-3, and PARP were detected by Western blot analysis. GAPDH was used as an equal loading control. rVBMDMP decreases survivin expression but increases cleaved caspase-3 (17 kDa) and PARP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

saline, boiled in Laemmli loading buffer, separated by SDS–polyacrylamide electrophoresis in 10% gels, and blotted with anti-αV and anti-β3 antibodies.

STATISTICAL ANALYSIS

Continuous data were expressed as mean ± SD. Comparisons between groups were performed by a Student's *t*-test. Analysis of variance was used to examine differences in response to treatments and between groups. *P* values of <0.05 were considered statistically significant.

RESULTS

rVBMDMP INHIBITS COLONY FORMATION AND INDUCES APOPTOSIS IN HUVE-12 CELLS

We performed a colony formation assay using the HUVE-12 cell line. After 12 days of culture, the percentages of colonies formed in control and rVBMDMP-incubated groups were compared. rVBMDMP dose-dependently inhibited colony formation, with 1.0 μmol L^{-1} rVBMDMP inhibiting colony formation by 60% (Fig. 1A). rVBMDMP (1.0 μmol L^{-1}) treatment of HUVE-12 cells for 24 or 48 h produced a typical DNA "ladder" indicative of apoptosis (Fig. 1B). Apoptosis was also measured at 0, 12, and 24 h by Annexin-V-FITC-propidium iodide staining and flow cytometry. rVBMDMP (1.0 μmol L^{-1}) increased the total apoptotic rate (both positive for Annexin-V-FITC and PI), early-stage apoptotic rate (positive for Annexin-V-FITC and negative for PI), and late-stage apoptotic rate (positive for PI and negative for Annexin-V-FITC) compared to controls (Fig. 1C). Total apoptosis and early-stage apoptosis peaked at 24 h, in contrast to late-stage apoptosis.

We also analyzed the expression of caspase-3, PARP, and survivin proteins in Western blots. rVBMDMP decreased survivin expression, but up-regulated the expression of the apoptotic markers, cleaved caspase-3 and PARP (Fig. 1D). These results suggested that rVBMDMP induces apoptosis in HUVE-12 cells.

rVBMDMP INHIBITS HUVE-12 CELL TUBE FORMATION IN VITRO

We used an endothelial tube formation assay to measure anti-angiogenic activity. Wells were treated with BSA (control) or rVBMDMP, for 24 h and tube formation was measured. rVBMDMP dose-dependently inhibited tube formation, with almost complete inhibition at 10.0 μmol L^{-1} (Fig. 2).

rVBMDMP CHANGES SIGNALING PROTEIN PHOSPHORYLATION

We next examined the effects of rVBMDMP on the phosphorylation of 400 signaling proteins using a protein phosphorylation chip (Fig. 3A,B). rVBMDMP increased phosphorylation (defined as a twofold or higher increase compared to control) of integrin subunits (integrin αV, integrin β1, integrin β3), Grb 2, the executive molecule of apoptosis (caspase-3), and death receptors (DR3, DR4, DR5). rVBMDMP decreased phosphorylation (defined as a 0.5-fold or more decrease compared to control) of EGFR, p-EGFR, VEGFR-1, and survivin (Table I). Western blot analysis confirmed that 30 min rVBMDMP treatment was sufficient to inhibit FAK and EGFR phosphorylation (Fig. 3C,D) and increase the phosphorylation of DR5 and integrin αV (Fig. 3E,F). These results were concordant with antibody microarray data (Supplementary Material 2).

rVBMDMP CHANGES GENE EXPRESSION OF THE EXTRACELLULAR MATRIX AND ADHESIVE MOLECULES IN HUVE-12 CELLS

We next measured mRNA expression changes for 128 extracellular matrix and adhesion molecules using a cDNA microarray (Fig. 4). rVBMDMP (1.0 μmol L^{-1}) treatment for 4 h upregulated (defined as a twofold or higher increase compared to control) Collagen 4α2, Collagen 6α2, Collagen 7α1, Collagen 15α1, and Collagen 27α1 (Table II). rVBMDMP decreased (defined as a 0.5-fold or more

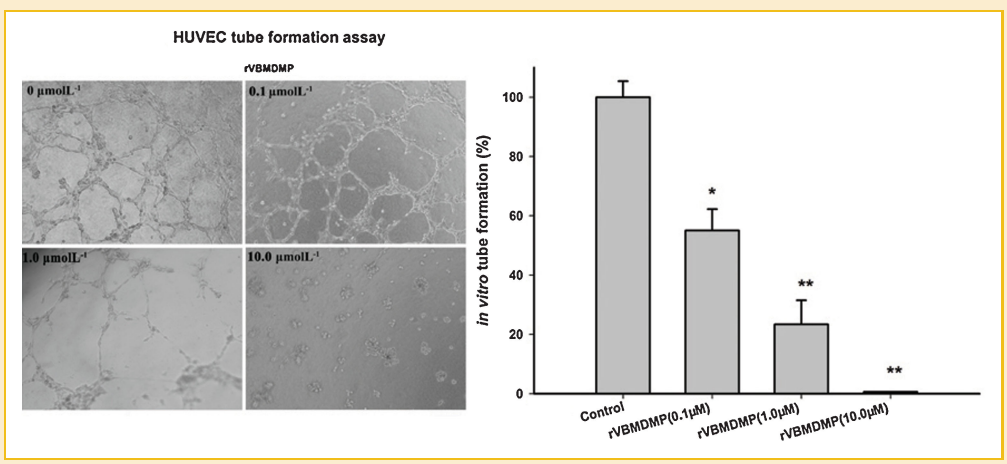

Fig. 2. rVBMDMP inhibits HUVE-12 tube formation in vitro. HUVEC-12 cells in the index growth phase were seeded onto 96-well plates coated with growth factor-reduced Matrigel. Cells were then treated with FBS or rVBMDMP for 24 h, and tube formation was observed under an inverted microscope. Images were captured with a Canon camera. Only intact tubules with branch points at both ends were counted as positive. The data in control group was set as 1. Bars represent mean ± SD (n = 3) normalized to control (FBS). $^{*}P<0.05$, $^{**}P<0.01$ versus control.

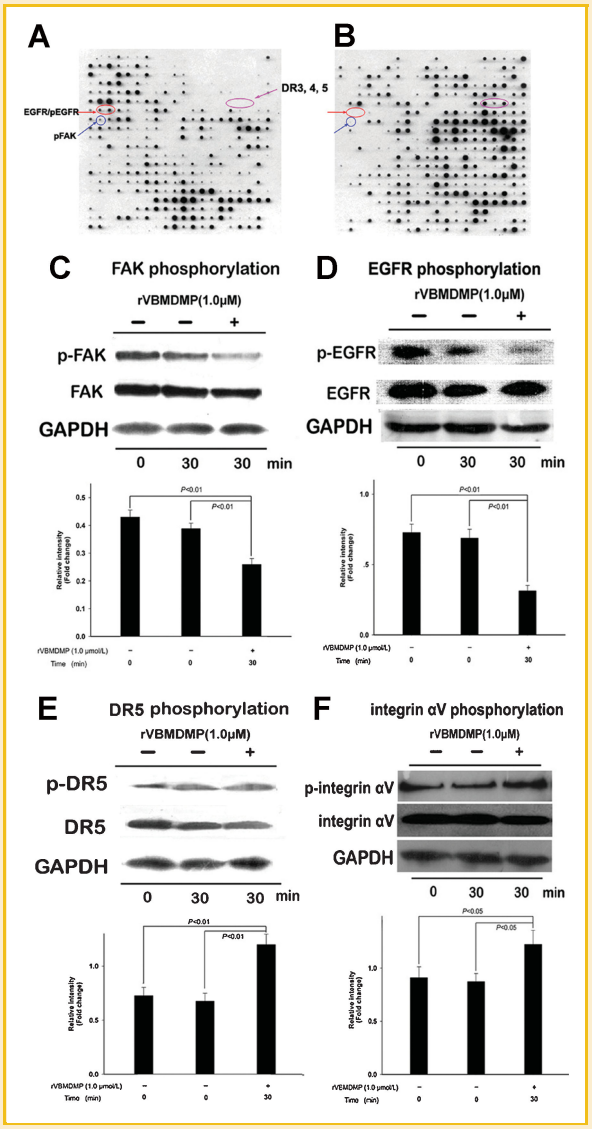

Fig. 3. Signal Transduction Antibody Array and Immunoblots. A,B: X-ray film of protein phosphorylation antibody chips (A: 0 μmol L^{-1} rVBMDMP as the control, B: 1.0 μmol L^{-1} rVBMDMP). C,D: Immunoblot for phospho-FAK and phospho-EGFR indicate that rVBMDMP inhibits phosphorylation of FAK and EGFR (p-FAK and p-EGFR). E,F: Immunoblot for phospho-DR5 and phospho-integrin αV demonstrate that rVBMDMP significantly increased the phosphorylation of DR5 and integrin αV. Expression of each protein was normalized to GAPDH level, and control = 1. The means of three separate experiments are shown. Student's *t* test was used to evaluate the significance (n = 3).

decrease compared to control) MMP12, MMP7, MMP11, and MMP1 (Table II, Supplementary Material 3).

rVBMDMP CHANGES mRNA EXPRESSION OF INTEGRIN αV, INTEGRIN *B*3, MMP-1, MMP-12, AND COLLAGEN 7α1

To confirm these changes in mRNA expression, we used quantitative RT-PCR to calculate Ct values for Integrin αV, Integrin β3, MMP-1, MMP-12, COL7A1, and GAPDH, and then calculated fold change according to the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001]. rVBMDMP dose-dependently decreased Integrin αV, Integrin β3, MMP-1, and MMP-12 expression, but increased collagen 7α1 expression compared with controls (Fig. 4 B), consistent with the gene chip data.

rVBMDMP INTERACTS WITH INTEGRINS ON THE ENDOTHELIAL CELL SURFACE

Tumstatin directly interacts with members of the integrin family, including αV and β3 [Sudhakar et al., 2003]. Therefore, we tested the physical interaction between rVBMDMP and αVβ3 integrin using immunoprecipitation and immunoblotting (Fig. 5) using an anti-rVBMDMP antibody for immunoprecipitation and anti-αV or anti-β3 antibodies for immunoblotting. rVBMDMP interacted with both the human αV and β3 integrin, but the negative group did not (Fig. 5).

DISCUSSION

rVBMDMP is a novel, 55-amino acid bioactive peptide obtained by connecting two fragments of the non-collagenous domain of the α3 chain and type IV collagen from the vascular basement membrane with a human IgG3 upper hinge sequence [Peng et al., 2003; Cao et al., 2006]. The two rVBMDMP peptides can fully extend and selectively inhibit HUVE-12 cell growth and human pulmonary adenocarcinoma (A549) cell proliferation [Cao et al., 2006; Wang et al., 2010].

Despite the anti-angiogenesis activity of rVBMDMP, the molecular mechanism of anti-tumor growth is still not clear [Cao et al., 2006]. The peptide from the NC-1 domain of the α3 chain (185–203 aa) of type IV collagen (197–215 aa of tumstatin) binds specifically to the αVβ3 integrin receptors [Shahan et al., 1999] and αVβ3 integrin may be the putative receptor for tumstatin [Maeshima et al., 2000a,b, 2001a,b]. In the present study, we showed that rVBMDMP interacts with integrin αVβ3 and maybe mediates rVBMDMP-induced inhibition of proliferation. The NC1 domain of α3 (collagen 4) may exert anti-angiogenic activity via binding to αVβ3 integrins [Petitclerc et al., 2000]. There is a tumstatin (54–132 aa) peptide binding site on αVβ3 integrin, and tumstatin specifically interacts with the β subunit [Maeshima et al., 2000a, 2001a]. rVBMDMP contains this same tumstatin domain, which could then interact with αVβ3 integrins.

Stimulation of death receptors, including DR3, DR4, and DR5, with death receptor ligands initiates apoptotic signaling via receptor oligomerization as well as recruitment and phosphorylation of Fas-associated death domain-containing protein [Matsuyoshi et al., 2006] and caspase activation [Shimada et al., 2004]. Caspase-3, the active form of procaspase-3, stimulates apoptosis when it is phosphorylated and active [Voss et al., 2005]. The downstream substrates of caspase-3 include caspase-activated DNase (DFF45, 40). rVBMDMP upregulates the phosphorylation of DR3, 4, and 5 and their downstream signaling molecules, including caspase-3 and DFF45, and reduces survivin, thereby inducing apoptosis. We also observed a dramatic upregulation of TRAF4 phosphorylation (ratio: 11.6) in HUVE-12 cells. TRAF4 is a member of the TRAF family of adaptor proteins that bind to members of the tumor necrosis family receptor superfamily and IL-1/toll-like receptor super family

TABLE I. Ratios of Phosphorylation Levels of Cell Signaling Proteins

Spot	Symbol	Fold change	Description and function
NP_004392	DFF-45	2.03	DNA fragmentation factor subunit alpha
NP_683871	DR3	5.017	Death receptor 3
NP_003835	DR4	3.084	Death receptor 4
NP_671716	DR5	3.718	Death receptor 5
NP_002077	GRB2	3.024	Growth factor receptor-bound protein 2
NP_002201	Integrin αV	2.356	Integrin alpha subunit
NP_000203	Integrin β3	3.709	Integrin beta subunit
NP_004286	TRAF 4	11.592	TNF receptor-associated factor 4
NP_005219	*EGFR*	0.278	Epidermal growth factor receptor
NP_001159	*Survivin*	0.344	Baculoviral inhibitor of apoptosis repeat-containing 5
NP_002010	*VEGFR-1*	0.353	Vascular endothelial growth factor receptor 1
NP_722560	*FAK*	0.493	Focal adhesion associated protein-tyrosine kinase

Cells were treated with rVBMDMP (1.0 μmol L^{-1}) for 30 min. Ratios were included when >2 or <0.5.

[Bradley and Pober, 2001; Chung et al., 2002]. Overexpression of TRAF4 induces apoptosis and suppresses colony formation in multiple tumor cell lines, suggesting a role for TRAF4 in p53-mediated apoptosis [Sax and El-Deiry, 2003]. rVBMDMP also blocked phosphorylation of angiogenesis factors, including EGFR, pEGFR, and VEGFR-1, indicating changes in growth factor signaling. These changes in growth factor and death receptor signaling may inhibit growth or induce apoptosis, leading to inhibition of neovascularization in early angiogenesis.

rVBMDMP increased expression of extracellular matrix molecules, including collagen7 α1, collagen4 α2 and collagen6 α2, but decreased levels of collagen degrading proteins, including MMP-7,

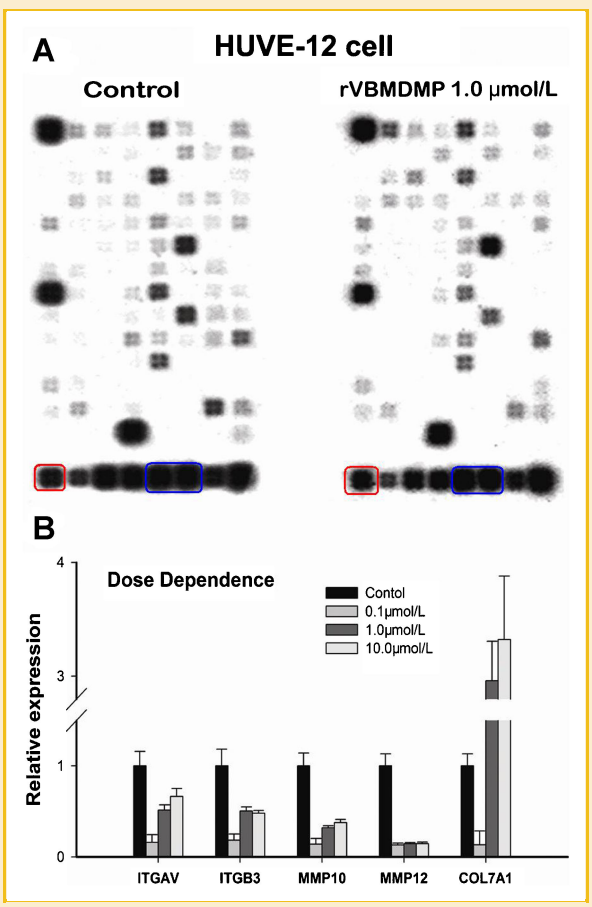

Fig. 4. Extracellular matrix and adhesion molecules cDNA microarray and real-time PCR. A: X-ray film of cellular matrix and adhesion molecules in cDNA microarrays of HUVE-12 cells treated with 1.0 μmol L^{-1} rVBMDMP for 4 h (0 μmol L^{-1} rVBMDMP as control). The red frames represent GAPDH and blue frames represent β-actin. B: Dose response of mRNA expression of selected genes in HUVE-12 cells following 4 h treatment with rVBMDMP. Cells were treated with 0 (control), 0.1, 1.0, and 10.0 μmol L^{-1} rVBMDMP. Bars represent mean ± SD (n = 4) normalized to control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II. Ratios of mRNA Expression After rVBMDMP Treatment for 4 h

GeneBank ID	Symbol	Description	Fold change
NM_000094	COL7A1	Collagen 7α1	3.15
NM_001846	COL4A2	Collagen 4α2	3.23
NM_032888	COL27A1	Collagen 27α1	3.93
NM_001855	COL15A1	Collagen 15α1	5.86
NM_001849	COL6A2	Collagen 6α2	19.24
NM_002426	*MMP12*	Matrix metalloproteinase 12	0.03
NM_002210	*ITGAV*	Integrin, alpha V	0.05
NM_000889	*ITGB7*	Integrin, beta 7	0.06
NM_005940	*MMP11*	RAS p21 protein activator 1	0.12
NM_002423	*MMP7*	Matrix metalloproteinase 7	0.13
NM_002214	*ITGB8*	Integrin, beta 8	0.15
NM_002206	*ITGA7*	Integrin, alpha 7	0.20
NM_000211	*ITGB2*	Integrin, beta 2	0.37
NM_002421	*MMP1*	Matrix metalloproteinase 1	0.49

Cells were treated with rVBMDMP (1.0 μmol L^{-1}) for 4 h. Ratios were included when >2 or <0.5.

Fig. 5. Co-immunoprecipitation of rVBMDMP with human integrins. Co-immunoprecipitation and immunoblot. HUVE-12 cells treated with rVBMDMP (1.0 μmol L^{-1}) for 30 min were lysed and immunoprecipitated with anti-rVBMDMP polyclonal antibody. Western analyses of the precipitate for bound rVBMDMP show two specific bands at 115 kDa (integrin αV) and 110 kDa (integrin β3) that are absent in lysates of untreated cells.

MMP-11, and particularly MMP-12. These changes are generally conducive to angiogenesis [John and Tuszynski, 2001; Nyberg et al., 2005] and play a key role in neovascularization. Therefore, rVBMDMP may block angiogenesis by reducing processes that generate it.

In summary, angiogenesis factors activate endothelial cells, and rVBMDMP effectively inhibits endothelial cells to form capillaries through inhibiting endothelial cell proliferation and inducing apoptosis to inhibit angiogenesis. Changes in phosphorylation levels of signal proteins and transcription levels of extracellular matrix and matrix metalloproteinase shift the balance away from angiogenesis. Combined with an anti-tumor effect, rVBMDMP may be suitable for application in clinical treatment.

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