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T-CAM, a fastatin-FIII 9-10 fusion protein, potently enhances anti-angiogenic and anti-tumor activity via αvβ3 and α5β1 integrins

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Abbreviations: ECM, extracellular matrix; EPDIM, Glu-Pro-Asp-Ile-Met; FAS1, fasciclin I domain; FGF, fibroblast growth factor; FN, fibronectin; HIF-1, hypoxia inducing factor-1; PHSRN, Pro-His-Ser-Arg-Asn; RGD, Arg-Gly-Asp; VN, vitronectin; YH, Try-His

Abstract

We made fusion protein of fastatin and FIII 9-10, termed tetra-cell adhesion molecule (T-CAM) that can interact simultaneously with αvβ3 and α5β1 integrins, both playing important roles in tumor angiogenesis. T-CAM can serve as a cell adhesion substrate mediating adhesion and migration of endothelial cells in αvβ3 and α5β1 integrin-dependent manner. T-CAM showed pronounced anti-angiogenic activities such as inhibition of endothelial cell tube formation, endothelial cell proliferation, and induction of endothelial cell apoptosis. T-CAM also inhibited angiogenesis and tumor growth in mouse xenograft model. The anti-angiogenic and anti-tumoral activity of molecule like fastatin could be improved by fusing it with integrin-recognizing cell adhesion domain from other distinct proteins. The strategy of combining two distinct anti-angiogenic molecules or cell adhesion domains could facilitate designing improved anticancer agent of therapeutic value.

Keywords: angiogenesis inhibitors; angiostatic proteins; antineoplastic agents; cell adhesion molecules; integrin αvβ3; integrin α5β1

Introduction

Angiogenesis, the process by which small new blood vessels are derived from pre-existing blood vessels, is required for tumor growth and metastasis (Folkman, 2002). Given the crucial role of angiogenesis, various therapeutic approaches taken for tumors are targeted to tumor angiogenesis (Alessi *et al.,* 2004; Alghisi and Ruegg, 2006). It is believed that tumor angiogenesis is the result of disturbed balance between angiogenic factors (such as VEGF, FGF, PDGF and HIF-1) and endogenous inhibitors of angiogenesis (Nyberg *et al.,* 2005). A large number of known endogenous inhibitors of angiogenesis are derivatived from extracellualr matrix (ECM) proteins e.g. arresten, canstatin, endostatin and tumstatin (Nyberg *et al.,* 2005). Fastatin, which is the 4th FAS1 domain of βig-h3 protein represents one of the new members of ECM protein-derived endogenous inhibitor of angiogenesis (Nam *et al.,* 2005). The FAS1 domains have been identified in the secretory and membrane proteins of many organisms including mammals, insect, sea urchins, plants, yeast and bacteria (Thapa *et al.,* 2007). The FAS1 domain mediates cell adhesion and migration via interactions with different integrins (Kim *et al.,* 2000a; Park *et al.,* 2004; Lee *et al.,* 2005; Thapa *et al.,* 2007). Currently, only four FAS1 domain-containing human proteins have been identified (βig-h3, periostin, stabilin-1 and stabilin-2) and investigation of physiological and/or pathological source of fastatin and its regulatory mechanism has been the topic of intense investigation (Thapa *et al.,* 2007).

Various cell surface and intracellular molecules regulating tumor angiogenesis have been identified (Bicknell and Harris, 2004). The vascular integrins represent one of cell surface adhesion molecules that play a crucial role in mediating adhesion and migration, and intracellular signaling for angiogenic endothelial cells (Paulhe *et al.,* 2005). Among integrins highly expressed in angiogenic endothelial cells, α1β1, α5β1, αvβ3 and αvβ5 integrins have gained considerable attentions as their interventions by pharmacological means have already been proven promising approach in tumor therapy (Alghisi and Ruegg, 2006). Moreover, many endogenous inhibitors of angiogenesis are known to function via their ability to interact with distinct

integrins expressed on cell surface of angiogenic endothelial cells, e.g. arresten via $\alpha1\beta1$ (Sudhakar *et al.,* 2005), endostatin via $\alpha5\beta1$ (Sudhakar *et al.,* 2003) and tumstatin via $\alpha v\beta3$ (Sudhakar *et al.,* 2003) integrins. Previously, we demonstrated that anti-angiogenic and anti-tumoral activity of fastatin is mediated via its ability to interact with $\alpha v\beta3$ integrin in endothelial cells (Nam *et al.,* 2003).

Given the fact that multiple integrins play important roles in tumor angiogenesis (Mizejewski, 1999), targeting the multiple integrins and/or their interventions by therapeutic means may provide an elegant approach in tumor therapy (Mizejewski, 1999; Jin and Varner, 2004). With this rationale, herein, we pursued the study on anti-angiogenic and anti-tumoral activities of the fusion protein tetra-cell adhesion molecule (T-CAM) synthesized by fusing the 9th and the 10th type III domains of FN (FIII 9-10) to fastatin. Thus, T-CAM represents the fusion protein of cell adhesion domains from two prominent and distinct ECM proteins, βig-h3 and FN. The known-cell adhesion motifs present in fastatin are Glu-Pro-Asp-Ile-Met (EPDIM) and Try-His (YH), and are recognized by $\alpha3\beta1$ (Kim *et al.,* 2000a) and $\alpha v\beta3/\alpha v\beta5$ integrins (Kim *et al.,* 2002a; Park *et al.,* 2004; Lee *et al.,* 2005; Thapa *et al.,* 2005), respectively. The known-cell adhesion motifs present in the 9th and the 10th type III fibronectin domains are Pro-His-Ser-Arg-Asn (PHSRN) and Arg-Gly-Asp (RGD), respectively, and are known to interact with various integrins including $\alpha5\beta1$ and $\alpha v\beta3$ (Grant *et al.,* 1997). The combination of FAS1 and FIII 9-10 domains in T-CAM is expected to increase the repertoire of recognizing integrins, particularly, $\alpha v\beta3$ and $\alpha5\beta1$ which are highly expressed in angiogenic tumor vasculature (Kim *et al.,* 2000b). We assessed the ability of T-CAM to support adhesion, migration and proliferation of endothelial cells. The specific integrins mediating adhesion and migration of endothelial cells to T-CAM were identified. The anti-angiogenic and anti-tumor activity of T-CAM and its efficacy were examined and compared with that of both fastatin and FIII 9-10. With these results, we provide the model of fusion protein system containing multiple integrin-binding motifs that could have more effective anti-angiogenic and anti-tumoral activity than single integrin-targeting molecule. This strategy could facilitate designing the vascular integrin-targeting anti-cancer agents of therapeutic value.

Materials and Methods

DNA constructions and protein purifications

The DNA sequence encoding fastatin (amino acids 368 to 506) were amplified by PCR using βigh3 cDNA as template and specific primers (5'-ATGGAGATATCGCTGACCCCCCCA-3' and 5'-TC-CTGCTCGAGGTTGGCTGGAGGC-3'). The PCR products were cloned into the *Eco*RV and *Xho*I sites of pET29b vector (Novagen, Madison, WI). Similarly, cDNA fragment for the type III 9th-10th domain of fibronectin (FIII 9-10) (amino acids 1130 to 1513) was generated by PCR using specific primers (FIII 9-10 primer 5'-ATTCGATATCGGT-GTTCGGTAATT-3' and 5'-AGACAGATATCCGG-TCTTGATTCC-3'). The PCR product, after blunting, inserted into *Eco*RV site of pET29b vector. The fastatin-FIII 9-10 fusion (T-CAM) gene was created by inserting the *Nde*I and *Nsi*I fragment of FIII 9-10 at the *Eco*RV site of Fastatin. All the constructs were verified by DNA sequencing. His-tagged recombinant proteins were expressed in BL21 (DE3) cells, harvested, and purified using nickel/ nitrilotriacetic acid/agarose column (Qiagen, Inc., Valencia, CA) as described previously (Nam *et al.,* 2005). Endotoxin was removed by using plymixin B agarose (Pierce, Rockford, IL) and was not detected by the Limulus Amebocyte Lysate (Sigma Chemical Co., Louis, MO) test.

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) and murine melanoma cells (B16F10) were cultured as described previously (Nam *et al.,* 2005). Briefly, HUVECs were cultured in M199 (Sigma Chemical Co., Louis, MO) supplemented with 20% FBS. B16F10 cells were cultured in RPMI 1640 (Gibco BRL., Gaitherburg, MD) containing 25 mM HEPES with 10% FBS. HEK293 (Human embryonic kidney) cells stably transfected with an empty vector (pcDNA3) or a human integrin $\beta3$ expressing vector were kindly provided by Dr. Jeffrey Smith (Burnham Institute, San Diego). $\beta3$/HEK293 and $\beta5$/ HEK293 were cultured in DMEM (Gibco BRL., Gaithersburg, MD) containing high glucose with 10% FBS and 100 U/ml of penicillin- streptomycin.

Cell adhesion and inhibition assay

Cell adhesion assay was performed as described previously (Kim *et al.,* 2000a). Briefly, flat-bottomed 96-well ELISA plates (Costar, Corning, Inc., NY) were incubated overnight at 4^{o}C with 10 μg/ml of indicated protein and blocked with 2% BSA in PBS for 1 h at room temperature. Cells were suspended in medium at a density of 3×10^5 cells/ml, and 0.1 ml of the cell suspension was added to each wells of the coated plates. After incubation for 20 min at

37°C, unattached cells were removed by rinsing twice with PBS. Attached cells were then incubated for 1 h at 37°C in 50 mM citrated buffer, pH 5.0, containing 3.75 mM *p*-nitrophenyl-*N*-acetyl-D-glycosaminide and 0.25% Triton X-100. Enzyme activity was blocked by adding 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA, and the absorbance was measured at 405 nm in a Bio-Rad model 550 microplate reader. For inhibition assay, cells were preincubated with indicated concentration of protein before adding to plate-coated with FN or VN Purchased from Promega (Madison, WI). To identify the receptor for the indicated proteins, HUVEC cells in 0.1 ml of the cell suspension (3×10^5 cells/ml) were preincubated at 37°C with monoclonal antibodies (5 μg/ml) specific to different integrins (Chemicon, Temecula, CA) for 30 min. The cells were then transferred onto plates precoated with indicated recombinant proteins and incubated for additional 30 min at 37°C. The attached cells were then quantified as described above. Function-blocking monoclonal antibodies to the following integrin subunits were used: α5 (P1D6), αv (P3G8), β1 (6S6), α5β1 (JBS5), αvβ3 (LM609), αvβ5 (P1F6).

Migration assay

Cell migration assays were performed in transwell plates (8 μm pore size, Costar, Cambridge, MA). The undersurface of the membrane was coated with 10 μg/ml of indicated proteins at 4°C then, blocked with 2% BSA in PBS for 1 h at room temperature. Cells were suspended in medium at a density of 3×10^5 cells/ml, and 0.1 ml of the cell suspension was added to the upper compartment of the filter with or without the indicated concentrations of each protein. In some experiments, cells were preincubated at 37°C for 30 min with function-blocking monoclonal antibodies. Cells were allowed to migrate for 6-8 h at 37°C. Migration was terminated by removing the cells from the upper compartment of the filter with a cotton swab, and the filters were fixed with 8% glutaraldehyde and stained with crystal violet. The extent of cell migration was determined by light microscopy. Cell counting were performed in five randomly selected microscopic high power fields.

Proliferation assay

The measurement of cell viability was performed using the mitochondrial reduction assay (Nam *et al*., 2005). A suspension of cells (3,000 cells per well) were serum starved for 24 h. The next day, cells were incubated for 48 h with or without the indicated concentrations of each protein and then MTT (Sigma Chemical) was added to each well. Cells were lysed with DMSO and quantified by the measurement of A_{570} nm using an ELISA reader.

Apoptosis assay

Cell apoptosis was assessed by FITC-Annexin V staining. Cells were serum starved for 24 h and incubated with or without the indicated proteins for 48 h, followed by incubation with FITC-Annexin V (Santa Cruze Biotechnology) according to manufacturer's instruction. Cells were immediately analyzed at 488 nm on the flow cytometer FACScalibur system (BD Biosciences) equipped with a 5-W laser.

In vitro and *in vivo* angiogenesis assays

An *in vitro* endothelial tube formation assay was performed as described previously (Nam *et al*., 2005). Matrigel (BD Bioscience, San Jose, CA) was added (100 μl) to each well of a 96-well plate and allowed to polymerize. Cells were suspended in medium at a density of 3×10^5 cells/ml, and 0.1 ml of the cell suspension was added to each well coated with matrigel with or without the indicated proteins. Cells were incubated for 8 to 10 h at 37°C. The cells were then photographed, and branch points from 4 to 6 high-power fields (× 200) were counted and averaged. Each group consisted of three or four matrigels. An *in vivo* matrigel plug assays were performed as described previously (Nam *et al.,* 2005). Briefly, Matrigel was mixed with 20 U/ml heparin, 0.15 μg/ml basic fibroblast growth (bFGF) factor (R&D Systems, Inc., McKinley, NE), and indicated poteins. The Matrigel mixture (500 μl) was injected subcutaneously into 5- to 6- week-old male C57BL/6 mice. After 7 days, mice were sacrificed, and the Matrigel plugs were removed and fixed in 4% paraformaldehyde. Paraffin sections were prepared and stained with H&E. Sections were examined by light microscopy, and the number of erythrocyte-filled blood vessels from 4 to 6 high-power fields (× 200) were counted and averaged. Each group consisted of five or six Matrigel plugs.

Anti-tumor assay

Male BALB/c nude mice (4-5 weeks old) were implanted with 1×10^6 B16F10 cells into the flank subcuits. Experimental groups were i.p. injected daily with indicated proteins (1 μM) in a total volume of 0.1 ml PBS. The control group was given an equal volume of PBS each day. Each

experimental group consisted of six to eight mice. Indicated proteins for injection was mixed with polymixin B-agarose (Sigma Chemical) for 2 h at 4°C to remove endotoxin. Tumor sizes were measured using Vernier calipers every 2 to 3 days, and the volumes were calculated using the standard formulation: width^2 × length × 0.52.

CD31 immunostaining

Intratumoral microvessel density (MVD) was analyzed on frozen sections of B16F10 tumor using a rat anti-mouse CD31 monoclonal antibody (PharMingen, San Diego, CA). Immunoperoxidase staining was done using the Vectastain avidin-biotin complex Elite reagent kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with methyl green. MVD was assessed initially by scanning the tumor at low power, followed by identification of three areas at the tumor periphery containing the maximum number of discrete microvessels, and counting individual microvessels at a low magnification field (× 40).

Statistical analysis

All values are expressed as mean ± SE. The statistical significance of differential finding between experimental and control groups was determined by Student's *t* test. $P < 0.05$ was considered statistically significant and is indicated with an asterisk over the value.

Results

Expression and purification of recombinant proteins

Schematic diagrams of fastatin, FIII 9-10 and T-CAM are illustrated in Figure 1A. The position of known cell adhesion motifs present in fastatin (EPDIM and YH) and FIII 9-10 (PHSRN and RGD motif) are indicated in the diagrams. The T-CAM, in total, has four cell adhesion motifs. All of these recombinant proteins were produced in *Escherichia coli* using a pET29b vector expression system and purified using Ni-NTA resin. The integrity and purity of proteins were assessed by SDS-PAGE and coomassie staining (Figure 1B).

T-CAM supports adhesion and migration of endothelial cells through αvβ3 and α5β1 integrins

The ability of T-CAM to serve as an adhesion substrate for endothelial cells was tested and compared with that of fastatin and FIII 9-10. All of these proteins exhibited comparable cell adhesion activity to HUVEC cells in a dose-dependent manner (Figure 2). However, no additive activity of FAS1 domain and FIII 9-10 was observed in T-CAM for HUVEC cell adhesion. The cells were well spread with a very few cells remaining rounded and were morphologically similar when plated onto any of these proteins (data not shown). Endothelial migration is an essential feature of angiogenesis. We examined the migration of HUVEC cells to fastatin, FIII 9-10 and T-CAM in a dose-dependent manner using a transwell system. Unlike cell adhesion,

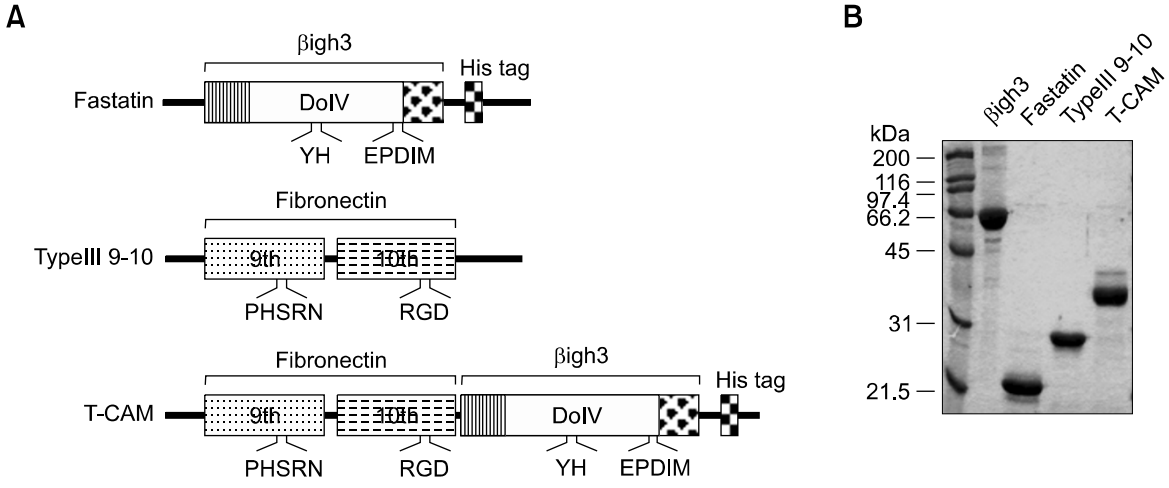


Figure 1. Generation of T-CAM. (A) Schematic diagrams of fastatin, FIII 9-10 and T-CAM. The position of YH and EPDIM motifs in fastatin, and PHSRH and RGD motifs in 9th and 10th FIII 9-10 are shown. The T-CAM consists N-terminus FIII 9-10 fused to C-terminus FAS1 domain. (B) The purity and integrity of protein used are shown by SDS-PAGE and coomassie staining.

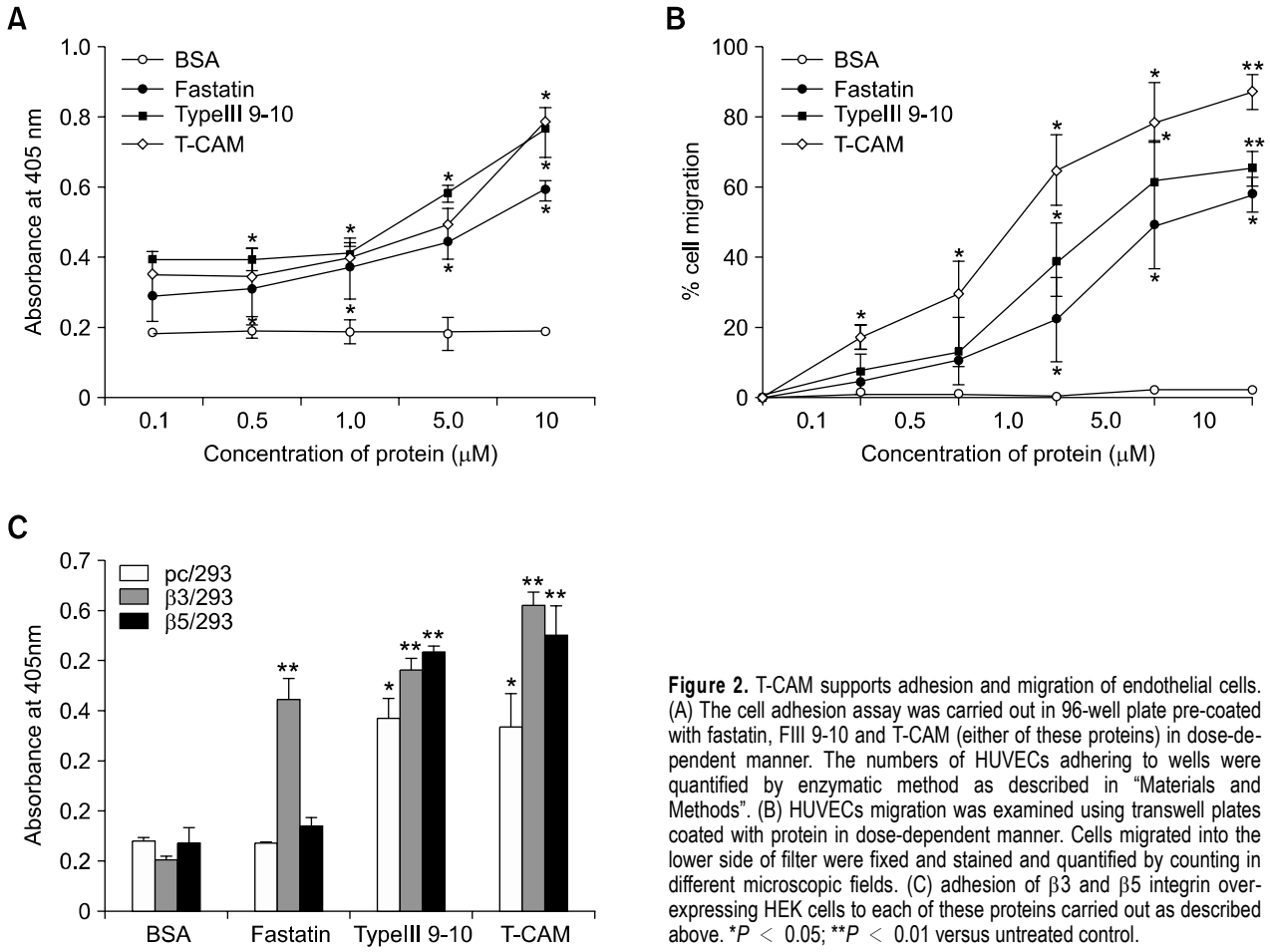


Figure 2. T-CAM supports adhesion and migration of endothelial cells. (A) The cell adhesion assay was carried out in 96-well plate pre-coated with fastatin, FIII 9-10 and T-CAM (either of these proteins) in dose-dependent manner. The numbers of HUVECs adhering to wells were quantified by enzymatic method as described in "Materials and Methods". (B) HUVECs migration was examined using transwell plates coated with protein in dose-dependent manner. Cells migrated into the lower side of filter were fixed and stained and quantified by counting in different microscopic fields. (C) adhesion of β3 and β5 integrin over-expressing HEK cells to each of these proteins carried out as described above. *$P < 0.05$; **$P < 0.01$ versus untreated control.

T-CAM potently induced migration of HUVEC cells, and its migration-promoting activity was superior to that of both fastatin and FIII 9-10 (Figure 2B). In addition, each of these proteins was tested for adhesion of β3 and β5 integrin overexpressing HEK cells described previously (Nam *et al.,* 2005). The adhesion of HEK cells irrespective of β3 or β5 integrin overexpression was higher to FIII 9-10 and T-CAM whereas only β3/HEK cells showed higher adhesion to fastatin (Figure 2C).

To identify the integrin responsible for endothelial cell adhesion and migration to fastatin, FIII 9-10 and T-CAM, we used several integrin function blocking antibodies. The adhesion of HUVEC cells to fastatin (Figure 3A, a) and to FIII 9-10 (Figure 3B, b) were inhibited by antibody against αvβ3 and α5β1 integrins, respectively. These results were in concurrence with that of our previous observation (Nam *et al.,* 2005) and with that of Kim *et al.* (2000b) who showed adhesion and migration of HUVEC cells to fibronectin were dependent on α5β1 integrin. However, antibodies against both αvβ3 and α5β1 integrins were required for effective inhibition of HUVEC cells adhesion to T-CAM, and treatment of single antibody against αvβ3 or α5β1 integrin alone was not effective (Figure 3A, c).

In addition to adhesion, integrin responsible for endothelial cell migration to these proteins were examined. Like cell adhesion, migration of HUVEC cells to fastatin and FIII9-10 were significantly inhibited by antibodies against αvβ3 and α5β1 integrins, respectively (Figure 3B, a and b). However, antibodies against both αvβ3 and α5β1 integrin were required to observe significant inhibition of HUVEC cell migration to T-CAM; partially inhibited by antibody against αvβ3 or α5β1 integrin individually (Figure 3B, c). These results suggest T-CAM enhances endothelial cell adhesion and migration through αvβ3 and α5β1 integrins and indicate that integrin-binding specificity and integrity of both fastatin and FIII 9-10 are intact in T-CAM.

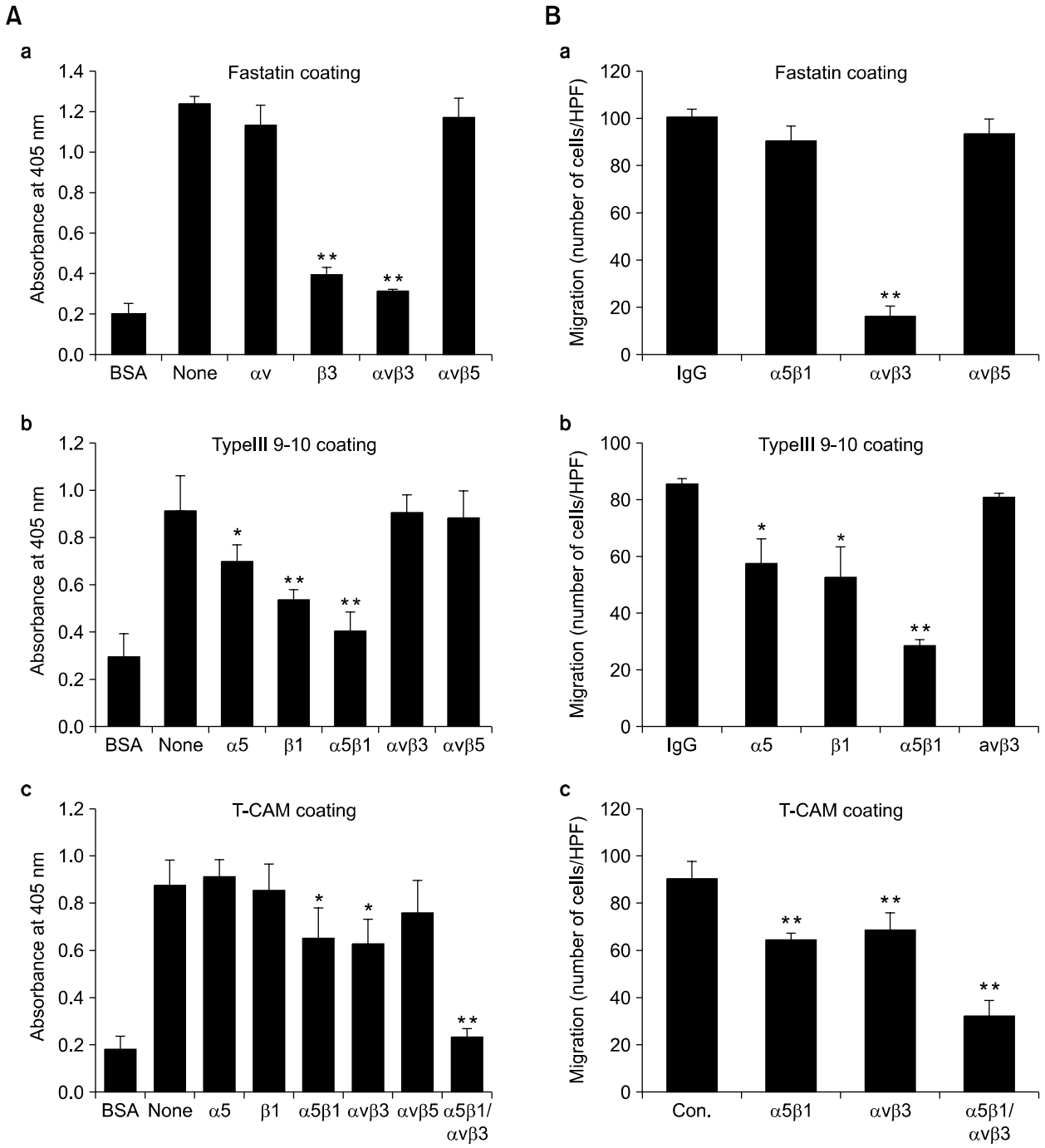


Figure 3. Identification of integrins mediating adhesion and migration of HUVECs to T-CAM. (A) HUVECs were preincubated with the function-blocking monoclonal antibodies to integrins and then added to 96-well plates precoated with fastatin (a), FIII 9-10 (b) and T-CAM (c). The numbers of attached cells were quantified by enzymatic methods as described above. (B) HUVECs migration were assayed using transwell plates coated with either of these proteins (a, fastatin; b, FIII 9-10; c, T-CAM). Cells were preincubated with function-blocking monoclonal antibody before adding cells into the upper wells of the transwell plates. The number of cells migrated into the lower chamber of filter were quantified after fixing and staining the cells. *$P < 0.05$; **$P < 0.01$ versus untreated control.

T-CAM potently inhibits angiogenesis, both *in vitro* and *in vivo*

We examined the effect of fastatin, FIII 9-10 and T-CAM in cell adhesion of HUVECs to FN and VN. The inhibitory activity of fastatin to HUVEC cell adhesion to both FN and VN is consistent with that

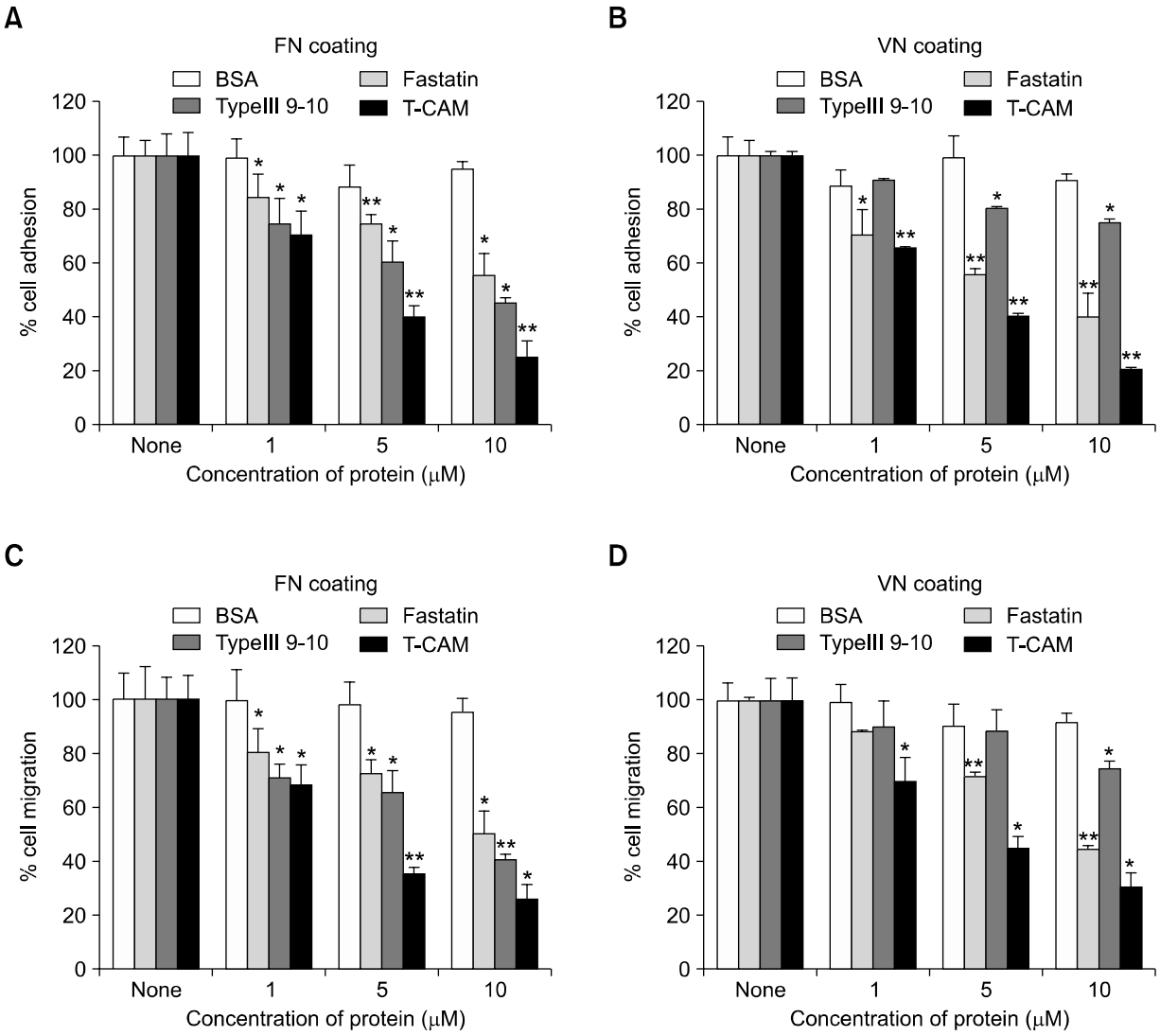


Figure 4. Inhibition of adhesion and migration of HUVECs to FN and VN by T-CAM. Inhibition of HUVECs adhesion and migration by fastatin. FIII 9-10 or T-CAM were tested in dose-dependent manner. Cells were preincubated with either of these proteins before adding to 96-well plate coated with FN (A) or VN (B) for cell adhesion and quantified as above. For migration, cells were preincubated with either of these proteins before to transwell plate coated with FN (C) or VN (D). The numbers of migrating cells were quantified as described above. *$P < 0.05$; **$P < 0.01$ versus untreated control.

of our previous results (Nam *et al.,* 2005). Similarly, FIII 9-10 significantly inhibited adhesion of HUVEC cells to FN but marginally to VN. These results were expected because HUVEC cell adhesion to FIII 9-10 is dependent on $\alpha5\beta1$ integrin and to VN is more dependent on $\alpha v\beta3$ integrin (Kim *et al*., 2000b). However, T-CAM significantly inhibited the adhesion of HUVEC cells to both FN and VN (Figure 4A and B). Similar results were obtained when migration assays were performed (Figure 4C and D).

Next, we examined and compared the ability of fastatin, FIII 9-10 and T-CAM to disrupt endothelial cell tube formation *in vitro* and blood vessel formation *in vivo* using matrigel plug assay. Fastatin and FIII 9-10 partially inhibited endothelial cell tube formation whereas T-CAM completely disrupted endothelial cell tube formation. The microscopic picture and quantitation of numbers of tube branches formed per high power field are shown in Figure 5A and B. To confirm the anti-angiogenic activity of T-CAM *in vivo*, we measured the extent of blood vessel invasion into matrigel plugs in presence of these proteins. Similar to the data obtained from *in vitro* tube formation assay, the extent of blood vessel invasion in matrigel plug assay was almost completely inhibited by T-CAM (Figure 5C and D) and was more effective than

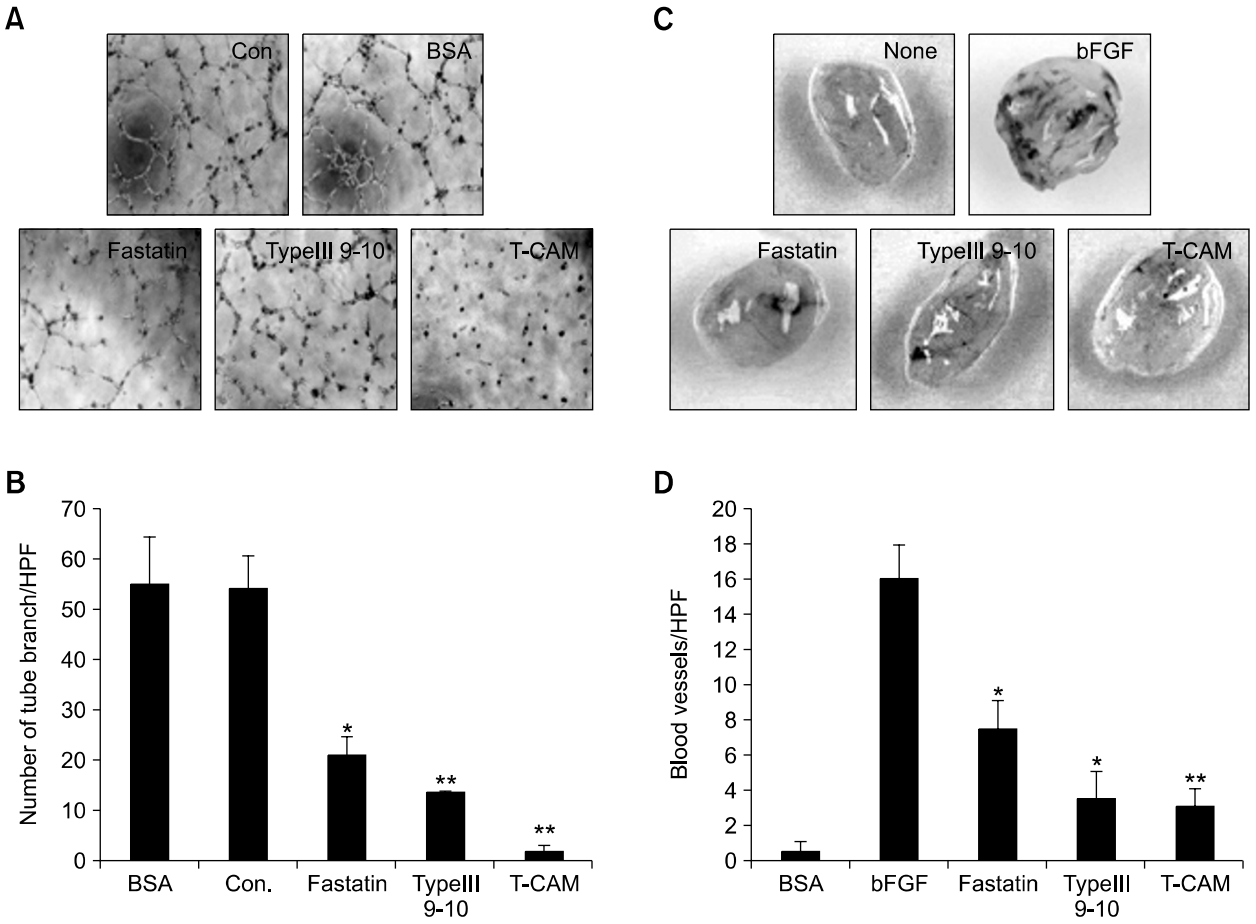


Figure 5. *In vitro* and *in vivo* angiogenic activities of T-CAM. For *in vitro* angiogenesis, HUVECs were seeded on matrigel in the absence or presence of either of these proteins (fastatin, FIII 9-10 and T-CAM). Cells were photographed (A) and quantified by observing in microscope ($\times$ 200) (B). For *in vivo* angiogenesis, bFGF containing matrigel plug were mixed with either of these proteins (fastatin, FIII 9-10 or T-CAM) and injected into the flank region of mouse. After removing, section of each matrigel plugs were stained with H&E (C) and quantified by examining the number of blood vessels formed (D). $^{*}P < 0.05$; $^{**}P < 0.01$ versus untreated control.

that of fastatin or FIII 9-10 alone.

T-CAM potently inhibits tumor growth

To analyze whether the anti-angiogenic effects of fastatin, FIII 9-10 and T-CAM are associated with inhibition of tumor growth *in vivo,* we examined inhibitory effect of these proteins in tumor growth. We implanted B16F10 melanoma cells into the flanks of BALB/c nude mice and monitored tumor growth and neovascularization after systemic treatment with fastatin, FIII 9-10 and T-CAM. These proteins (1 μM) were i.p. injected everyday from 6 days after tumor cell implantation. As shown in Figure 6A, T-CAM significantly inhibited tumor growth, compared with that of fastatin and FIII 9-10. The density of microvessels in control (PBS-treated) and exogenous protein-treated tumors were quantified after immunostaining with CD31 antibody. The reduced sizes of tumors were consistent with decrease in CD31-positive microvessels in treated groups (Figure 6B and C). No significant differences in body weight were observed between the groups (data not shown).

T-CAM potently induced endothelial cell apoptosis

Recently, we reported that fastatin inhibited endothelial cell proliferation and induced apoptosis (Nam *et al.,* 2005). We examined whether FIII9-10 and T-CAM also affect endothelial cell growth and survival. FIII9-10 inhibited proliferation of endothelial cell more slightly than the fastatin whereas T-CAM potently inhibited proliferation in a dose-dependent manner (Figure 7A). Next, we examined the apoptosis of endothelial cells when in-

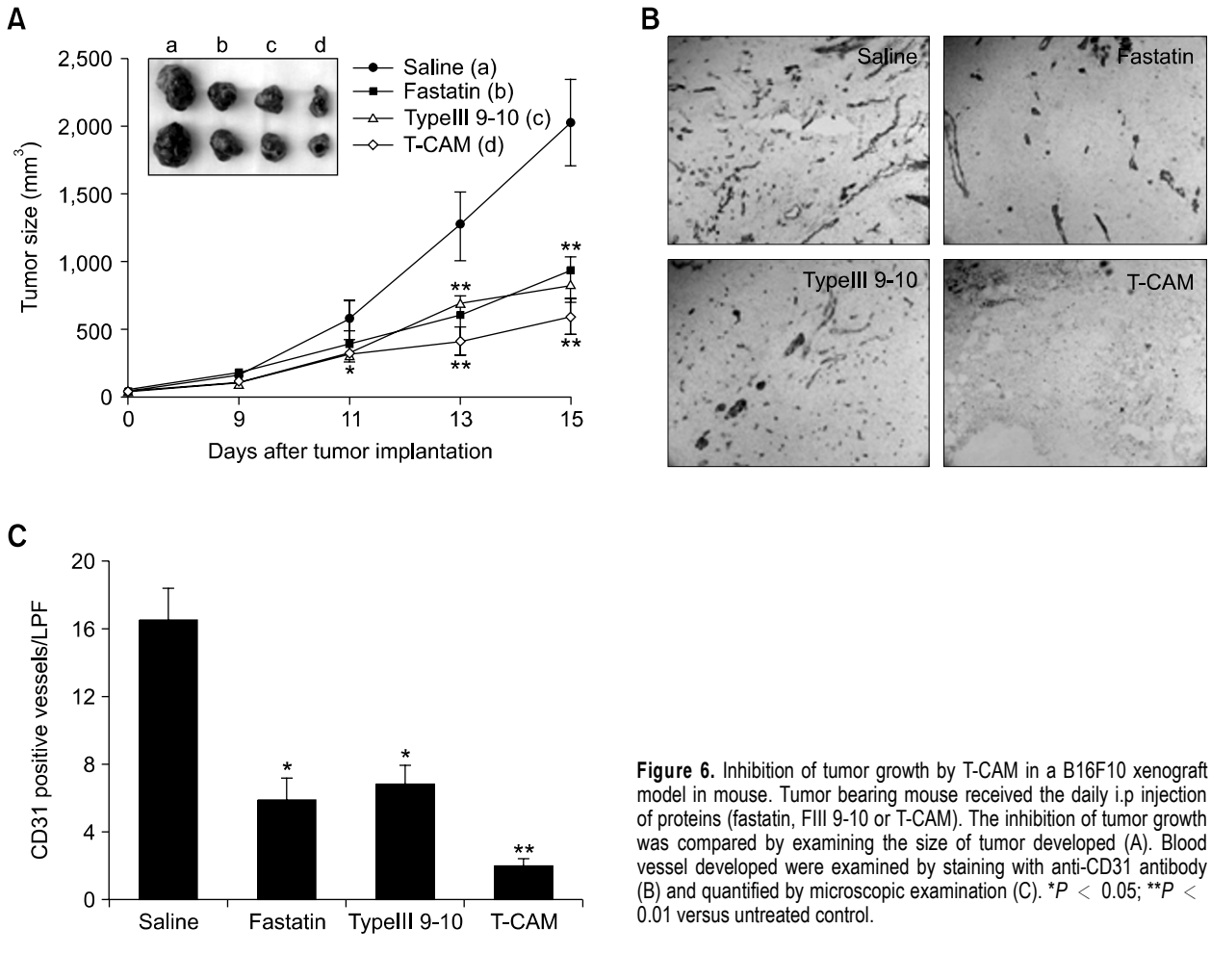
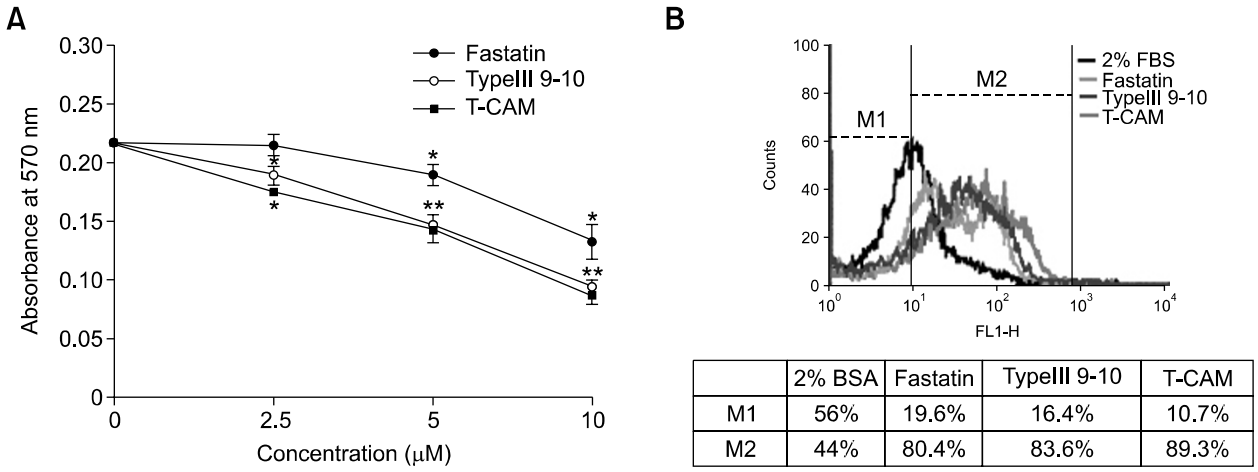


Figure 6. Inhibition of tumor growth by T-CAM in a B16F10 xenograft model in mouse. Tumor bearing mouse received the daily i.p injection of proteins (fastatin, FIII 9-10 or T-CAM). The inhibition of tumor growth was compared by examining the size of tumor developed (A). Blood vessel developed were examined by staining with anti-CD31 antibody (B) and quantified by microscopic examination (C). *$P < 0.05$; **$P < 0.01$ versus untreated control.



	2% BSA	Fastatin	TypeIII 9-10	T-CAM
M1	56%	19.6%	16.4%	10.7%
M2	44%	80.4%	83.6%	89.3%

Figure 7. T-CAM inhibits endothelial cell proliferation and induces apoptosis. HUVECs after serum-starvation were incubated in the presence of protein (fastatin, FIII 9-10 or T-CAM) in dose-dependent manner. Cell proliferation was quantified by the MTT assay (A). The induction of apoptosis by either of these proteins were examined by FITC-annexinV staining and FACS analysis (B). *$P < 0.05$; **$P < 0.01$ versus untreated control.

cubated with equal molar concentration of either of these proteins by FACS analysis after staining with annexin V. FIII9-10 has slightly more apoptosis-inducing activity than that of the fastatin. T-CAM treatment potently induced apoptosis as observed by distinct shift in FACS analysis (Figure 7B).

Discussion

Integrins have gained considerable attention as a target molecule for tumor therapy. Several integrins play a role in tumor angiogenesis and tumor metastasis, and targeting these integrins by various means (e.g. antibodies, peptides, small molecules and integrin silencing by siRNA) represent effective therapeutic approaches to the cancer treatment (Alghisi and Ruegg, 2006). Nevertheless, many endogenous inhibitors of angiogenesis including fastatin are known to show anti-angiogenic and anti-tumor activity via their ability to interact with distinct integrins expressed in angiogenic endothelial cells of tumor vasculature (Alessi *et al.,* 2004). In the present study, we made a recombinant fusion protein of fastatin and FIII 9-10, two cell adhesion domains from βig-h3 and FN. The fastatin is the 4th FAS1 domain of βig-h3 and contains two known-cell adhesion motifs (YH and NKDIL). FIII 9-10 represents the central cell-binding domain of FN and contain PHSRN and RGD motifs which are known to act in synergistic manner to interact with α5β1 integrin (Redick *et al.,* 2000). However, unlike anastellin which is 10-kDa first type III repeat of FN, anti-angiogenic and anti-tumoral activity of FIII 9-10 is not known except its role in integrin recognition (Yi and Ruoslahti, 2000). We assumed that combination of fastatin and FIII 9-10 in T-CAM could target, simultaneously, both α5β1 and αvβ3 integrins that could have more potent anti-angiogenic and anti-tumoral activity. These integrins are highly expressed in angiogenic tumor vasculature (Kim *et al.,* 2000b).

T-CAM, as a cell adhesion substrate supported adhesion and migration of endothelial cells. However, additive cell adhesion activity of FAS1 domain and FIII 9-10 was not observed in T-CAM. Unlike cell adhesion, T-CAM promoted the enhanced migration of endothelial cells and was superior to that of fastatin or FIII 9-10 alone. The adhesion and migration of endothelial cells to FIII 9-10 was inhibited by α5β1 integrin. Moreover, integrin-binding specificity of both fastatin and FIII 9-10 were preserved in T-CAM because function blocking of both α5β1 and αvβ3 integrins were required to observe the significant inhibition of HUVEC cell adhesion/migration to T-CAM; the inhibition of either integrin alone was not sufficient. In addition, we assessed the ability of each of these proteins in soluble forms to inhibit the adhesion and migration of HUVEC cells to FN and VN. The inhibitory activity of fastatin to adhesion and migration of HUVEC cells to FN and VN is consistent with our previous observation (Nam *et al.,* 2005). The inhibitory effect of FIII 9-10 to FN was more noticeable than to VN, whereas T-CAM effectively inhibited the adhesion and migration of HUVEC cells to both FN and VN. These results are consistent with the facts that FIII 9-10 harbors the α5β1 integrin binding sites that mediate adhesion and migration of endothelial cells to FN. However, adhesion and migration of endothelial cells to VN is mediated by αvβ3 integrin. Presence of FAS1 and FIII 9-10 domains contributes T-CAM to exhibit the inhibitory effects on adhesion and migration of HUVEC cells to FN and VN.

The different mechanisms exist for inhibition of angiogenesis by angiogenesis inhibitors (Nyberg *et al.,* 2005) but, most of them are associated with inhibition of endothelial cells proliferation and migration, and induction of apoptosis. For example, canstatin inhibits endothelial cell proliferation and migration (Kamphaus *et al.,* 2000); tumstatin inhibits endothelial cell proliferation and promotes apoptosis, process mediated by αvβ3 integrin (Sudhakar *et al.,* 2003); endostatin inhibits endothelial migration and its activity mediated by α5β1 integrin (Sudhakar *et al.,* 2003). All of these processes contribute to inhibition of tumor angiogenesis and, then to tumor growth. T-CAM bears all the anti-angiogenic properties of fastatin such as induction of apoptosis, inhibition of endothelial proliferation, tube formation and *in vivo* angiogenesis. It is interesting to note that recombinant protein FIII 9-10 not only serve as an independent cell adhesion substrate but can display an anti-angiogenic and anti-tumoral property comparable with that of fastatin. All the activities of T-CAM were noticeably increased which were as reflected by more potent anti-angiogenic and anti-tumoral activity of T-CAM than that of fastatin or FIII 9-10. Since, fastatin and FIII 9-10 are two distinct domains, more potent anti-angiogenic and anti-tumoral activity of T-CAM could be because of their additive activities or or due to their independent activities converging to affect angiogenesis and its related processes. In addition, intracellular signaling cascades that underlie the anti-angiogenic activities of T-CAM remain to be dissected. We previously showed that binding of fastatin to αvβ3 integrin blocks FAK phosphorylation, leading to the inhibition of the Akt/mTOR and Raf/ERK pathways (Nam *et al.,* 2005). Endostatin, which is an endo-

genous inhibitor of angiogenesis derived from α1 chain of type XVIII collagen, recruit $\alpha5\beta1$ integrin and inhibit the FAK phosphorylation in Raf/ERK, but, no effect on PI3Kinase/ Akt/mTOR signaling pathway (Sudhakar *et al.,* 2003). The antagonist of $\alpha5\beta1$ could activate the cAMP-dependent PKA which then recruits caspase-8, an initiator of apoptotic pathway (Kim *et al.,* 2002b). It will be interesting to know how FIII 9-10 as $\alpha5\beta1$ integrin-recognizing molecule regulate the tumor angiogenesis. Both $\alpha5\beta1$ and $\alpha v\beta3$ integrins will simultaneously be occupied by T-CAM and cross-talk between these two integrins and their intracellular signaling in T-CAM-mediated anti-angiogenic and anti-tumoral activity may help to understand the molecular basis of the dual integrin-targeting therapeutic strategy.

As we have previously reported that T-CAM, as a cell adhesion substrate supports adhesion, migration and proliferation of keratinocyte/fibroblast and promotes wound healing (Jung *et al.,* 2007), T-CAM could help wound healing when it functions as an immobilized cell substrate. As shown in this study, however, when it acts as a soluble form, it could block integrins resulting in the inhibition of angiogenesis.

In conclusion, we provide the example of fusion protein system targeting at least two integrins, thus, associated with more effective anti-angiogenic and anti-tumoral property. In the context of the rising trends of adopting combination therapy for cancer treatment, this study could be important in designing improved anticancer agents of therapeutic value.

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